"EVALUATION OF MARKERS OF PRETERM BIRTH IN EARLY PREGNANCY AMONG RURAL PREGNANT WOMEN OF BELAGAVI DISTRICT; NESTED CASE CONTROL STUDY"

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In the Faculty of

Medicine

By

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Under the Guidance of

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KAHER, J. N. MEDICAL COLLEGE, BELAGAVI.

2024

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Date : 6 may 2024

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Ph.D. Research Scholar,

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*I am truly grateful to my parents****,*** *my brother* ***Mr.Suresh R Araganji,*** *and all my family members who helped me realize my dreams and made me what I am today by always being there for me.*

I bow my head in respect before ***God Almighty****.*

Date :

Place: Belagavi ***Dr.Ramesh R Araganji.***

ABBREVATIONS

- ACTH – ADRENOCORTICOTROPHIC HORMONE
- ALP – ALKALINE PHOSPHATASE
- AMH – ANTIMULLERIAN HORMONE
- APO – ADVERSE PREGNANCY OUTCOME
- ASPIRIN – ASPIRIN SUPPLEMENTATION FOR PREGNANCY FOR RISK REDUCTION IN NULLIPARAS
- BMI – BODY MASS INDEX
- CI – CONFIDENCE INTERVAL
- CL – CORPOUS LUTEUM
- CRH – CORTICOTROPHIN RELEASING HORMONE
- DRC – DEMOCRATIC REPUBLIC OF CONGO
- GA – GESTATIONAL AGE
- GDP – GROSS DOMESTIC PRODUCT
- GN – GLOBAL NETWORK
- Hb – HAEMOGLOBIN
- hCG – HUMAN CHORIONIC GONADOTROPHIN
- HDP – HYPERTENSIVE DISORDER OF PREGNANCY
- HPG – HYPOTHALAMO PITUITARY GONADAL AXIS
- HR – HEART RATE
- hsCRP – HIGH SENSITIVITY C – REACTIVE PROTEIN
- IL – INTERLEUKIN
- IUGR – INTRAUTERINE GROWTH RETARDATION
- JNMC – JAWAHARLAL NEHRU MEDICAL COLLEGE

- LDA – LOW DOSE ASPIRIN
- LMP – LAST MENSTRUAL PERIOD
- LR – LIKELIHOOD RATIO
- MoM – MULTIPLE OF MEDIAN
- ms AFP – MATERNAL SERUM ALPHA FETO PROTEIN
- NICHD – NATIONAL INSTITUTE OF CHILD HEALTH AND HUMAN DEVELOPMENT
- PLGF – PLACENTAL GROWTH FACTOR
- PPROM – PRETERM PRE-LABOUR RUPTURE OF MEMBRANE
- Pre E – PREECLEMPSIA
- PTB – PRE TERM BIRTH
- PTL – PRETERM LABOUR
- ROC – RECEIVER OPERATING CHARACTERISTIC CURVE
- SGA – SMALL FOR GESTATIONAL AGE
- sPTB – SPONTANEOUS PRETERM BIRTH
- TGF – TUMOR GROWTH FACTOR
- USA – UNITED STATES OF AMERICA
- WHO – WORLD HEALTH ORGANISATION

ABSTRACT

Introduction and Background: Adverse pregnancy outcomes (APO's) like preterm birth (PTB), foetal growth restriction and hypertensive disorders of pregnancy (HDP)) are the primary drivers of perinatal mortality. C-Reactive Protein (hsCRP), Anti-Mullerian Hormone (AMH) and Alpha-Feto Protein (AFP) have shown promise in predicting these APO's.

Aims and Objectives: To determine the impact of low dose aspirin (81mg) on markers of maternal inflammation and placental function.

Material and Methods: This study was conducted during 2016 to 2018 in a rural setting of Southern India. Population for the study included Nulliparous women with a singleton pregnancy dated by ultrasound who were enrolled in the ASPIRIN (Aspirin Supplementation for Pregnancy Indicated risk Reduction In Nulliparas) Trial. A nested case control study was performed to elucidate the impact of low dose aspirin (LDA) on markers of placental function and maternal inflammation among women who delivered prematurely compared to term controls in women enrolled in the ASPIRIN trial. Women were prospectively enrolled in an ancillary observational trial wherein maternal serum was collected and measured between 10 to 13 weeks and 17 to 21 weeks of gestation after initiation of aspirin or an identical placebo.

Results: A total of 666 women were enrolled in this ancillary trial of whom 269 were selected for analyte analysis. Women who received LDA had lower levels of Alpha Feto-Protein (AFP) at 10 to 13 weeks than women who received placebo (Placebo) (LDA 18.3 ng/mL vs 21.4 ng/mL -P 0.001). AFP was similar between the two groups at 17 to 21 weeks. No other differences were seen in in C-Reactive protein or Anti-Mullerian Hormone.

Discussion: The study demonstrated that low AFP was associated with early PTB before 34 weeks. Similarly, we saw a trend with CRP but this was not statistically significant and directionally opposite of what we would have clinically anticipated (higher CRP being associated with lower rates of PTB before 34 weeks). AFP has been shown to be associated with bad obstetrical outcomes including both PTB and placental malperfusion disorders. However, limited data in this study did not yield a correlation of first trimester AFP with other obstetric outcome parameters. This difference did not persist when the group of women randomized to ASPIRIN were uniquely examined.

Conclusion: These results suggest measurement of AFP during first trimester may be a useful as a biomarker of LDA efficacy in pregnancy; nevertheless, larger studies for validation are required.

Keywords: maternal serum alpha-fetoprotein (MSAFP), preterm birth, aspirin

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INTRODUCTION:

Majority of women and associated families go through an important phase in their life. This phase called "**Pregnancy**" is a nine-month period of complex physiological interactions involving mother, fetus and placenta, and the goal is to create and sustain an ideal intrauterine setting for growth and development of the fetus. Creating and managing this balance is a composite event and it is definitely not simpler one. Slight alterations in this complex physiological process can result in profound consequences; most importantly preterm birth (**PTB**) and Hypertensive disorders of pregnancy. PTB and pre-eclampsia are believed to originate in early gestation and major causes for morbidity and mortality in perinatal period [1-3].

Preterm Birth (PTB) is a multi-factorial and results in notable effects on medical, economic and human health. As per the World Health Organization (WHO) report published in the year 2012, about 15 million preterm deliveries occur in a year and, 1 million of premature babies unfortunately are dying even though interventions are available to prevent mortality[4].

Across the 184 countries, preterm birth rate varies from 5% to 18%. PTB remains a major driver of neonatal mortality throughout the world[5-7]. Worldwide the PTB rate is seen increased over the past few decades in spite of efforts with advanced medical progress. PTB accounts for about 3/4th of all neonatal deaths and leads to long-term neurological disabilities in almost 50% of children.[8] Preterm infants will have higher risk than term infant with respect to mortality and also leads to a variety of health and developmental problems. Complications of PTB occur across systems including respiratory, gastrointestinal, immunologic, central nervous system, hearing,

and vision problems, and additionally, longer-term complications related to central nervous system such as motor, cognitive, behavioural, social-emotional, health, and growth problems.[9]

The PTB additionally results in considerable emotional and economic costs to societies and affects public-sector services, health insurance, education, and social support systems. Although many risk factors have been identified in previous studies, PTB is still hard to predict. Over half of the PTBs are spontaneous in nature and molecular pathways responsible have not been sufficiently understood. It has been recognized that PTB is an outcome of several biological pathways including; inflammation, myometrium capacitance, abruption and activation of the maternal hypothalamo-pituitary axis[10]. This level of complexity has hindered the effective diagnostics as well as treatment strategies. To disentangle this biological Gordian knot, the collection and assessment of markers from those undergoing preterm birth and the potential impact of interventions must be considered. The candidate maternal serum protein markers will be assessed for interval changes over the duration of pregnancy. In multiple meta-analyses of the preeclampsia (PreE) prevention trials, administration of Low-dose aspirin (LDA) has been noted to reduce the PTB rate. LDA is associated with a substantial decrease in sPTB<34wks in low-risk nulliparous women. These findings endorse a new therapeutic option for PTB prevention[11].

The Global Network study (GN); Aspirin Supplementation for Pregnancy Indicated Risk Reduction In Nulliparas (ASPIRIN) trial that was funded by the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD) is the largest intervention trial undertaken for prevention of preterm birth. This multicentric trial was conducted in six countries including India, Belgaum

(Global Network site 08; Women's and Children's Health Research Unit, J N Medical College, Belagavi).

Primary hypothesis of the study was that nulliparous women with less than three prior pregnancy losses supplemented daily with Low Dose Aspirin (LDA) started between six weeks and 13 weeks six days of gestation until 36 weeks gestational age will result in reduction preterm birth incidence from all causes as well as reduction in perinatal mortality and morbidity.

With the currently available cost-effective interventions[12], 75% of these deaths could be prevented. Currently, a third or more PTBs are iatrogenic[13]. The greatest causes of iatrogenic PTB are preeclampsia and severe intrauterine growth restriction[14]. Over the last three decades, prophylactic use of LDA has been studied to prevent preeclampsia. As early as 1979, Crandon and Isherwood observed lesser preeclampsia incidence in women who consumed aspirin regularly during pregnancy among nulliparous women. Beaufils et al in 1985 published the first randomized study which showed that the benefit of using 150 mg aspirin and 300 mg dipyridamole in high-risk women in decreasing the risk of stillbirth, fetal growth restriction and preeclampsia. Prior studies suggested that early intervention with low-dose aspirin may profoundly reduce preeclampsia[15]. However, biomarkers are needed to screen the high-risk women and provide preventive treatment as part of antenatal care. In addition to low dose aspirin therapy, other prophylactic strategies such as progesterone, cervical length screening and care management have been suggested approaches to prevent preterm birth (PTB); however, the ability to accurately identify at risk women to meaningfully direct resources has been elusive. Several candidate biomarkers for PTB have been suggested including anti-mullerian hormone (AMH),

alpha feto-protein and c-reactive protein (CRP). Nonetheless, these studies have conflicting results in predicting preterm birth.

The purpose of this study is to examine potential protein markers (AMH, msAFP & hs CRP) serially in early pregnancy among preterm and term labour and also to test efficacy of two serum based potential protein marker tests among women who receive LDA and those who do not receive LDA in ASPIRIN Trial.

The Global Network site No 8 ASPIRIN study gave us a unique opportunity to collect and assess the potential protein markers (AMH, msAFP & hs CRP) among rural nulliparous pregnant woman for understanding the biology of preterm birth.

NEED FOR THE STUDY:

Across the world it is observed that, PTB is one of the greatest direct and definitive reason for neonatal deaths. The complications of preterm birth are responsible for the world's 31 lakhs i.e. 35% of deaths in a year (with an annual increasing trend)[16]. It is unfortunate that under-developed and developing countries spanning across Africa and South Asia contribute more than 60% of preterm births. Though higher percentage deaths are observed in these geographical areas, preterm birth is remarkably a global problem[4]. PTB is also the leading cause of child deaths in almost all low and, low-middle income countries of the world[17].

PTB is the second most common cause of death under the age of 5 years next to pneumonia. The preterm birth phenomenon has two major blows namely, a) significantly contributing to the mortality, b) unmanageable effect amongst some surviving babies. Preterm survivors in their life go through impaired development of neuro-developmental functions. This increases risk of cerebral palsy, learning

impairment and milestone achievements, visual disorders and affects longer-term physical health problems specifically, non- communicable disease [18]. The impact of preterm birth leads to considerable burden on families, society and health systems in terms of economical implications.[19-, 20]. According to the Global Burden of Disease analysis, PTB is one of the largest and most definitive condition for higher mortality risk and poses a substantial risk of lifelong impairment[21.]

India accounts for the highest number of preterm births in the world, about 24% of the global burden with approximately one in eight babies being born preterm[4]. As India emerges globally from "a developing" to a developed country, current PTB rate has significant impediment in its growth. The medical resources and infrastructure in the rural areas are in their nascent state and mostly they lack in addressing the challenges imposed by preterm birth. Unfortunately, the current neonatal care is most expensive compared to the GDP of a rural citizens. To proactively identify preterm labour, the rural health systems need to be equipped with mandatory facilities like ultrasound assessments, measurement of biochemical markers[22] and human capital. Unless the state and central government addresses these core issues, we can expect the rural India to face the ongoing challenges. Compared to last few decades, country has started realizing these basic needs and they are getting addressed in pockets. However, more focus needs to be given to containing and reducing the mortality percentage related to preterm birth.

The present study is a step towards identifying set of opportunities to evaluate biomarkers of preterm birth in rural population. It is evident that prevention and effective management of preterm labor should improve neonatal outcomes as well as a positive impact on societal and reduces longer term public health care costs. There

is a recommendation from several national professional bodies to treat high-risk pregnancies with low dose aspirin. ASPIRIN study is uniquely poised to examine the impact of LDA among nulliparous pregnant women in reducing preterm birth across seven low-middle income countries. It is important to develop low-cost interventions having higher impact as the financial costs on public health system in treating illness related to preterm birth is very high[23]. The LDA has potency to reduce preeclampsia and SGA. The nulliparous pregnant women who are on LDA and off LDA are included and assessed for potential protein markers.

AIM AND OBJECTIVES:

Aim of the project:

Overarching goal of this study is to validate the predictive ability of the three biomarkers in identifying women at risk for preterm birth in early pregnancy and also to test the effect of LDA on inflammatory and placental function markers.

Primary Objective:

Examine serum markers (Antimullerian hormone, Alpha-fetoprotein and C-reactive protein) of Preterm Birth sequentially in early pregnancy (2 visits – 1st and 2nd trimesters) among women, who go on to deliver prematurely against term delivery.

Secondary Objectives:

Assess the efficacy of two serum-based tests for preterm labor among women who receive LDA and those who do not.

`

REVIEW OF LITERATURE:

After identifying the problem statement, the project team gathered necessary data related to the stated objectives. The primary focus of this data gathering exercise was to refer the information with verified data points, which are produced earlier by various pioneers and experts in this space. The data referred in this section and beyond adopted existing material in condensed form with the adequate guided curation.

The Source of References:

The reference data has been sourced from:

- The project guide (Dr. S.S. Goudar, MD MHPE, Professor of Physiology, Director and PI women and child health research, KAHER JNMC, Belagavi.
- Leading experts with whom this subject was discussed several times:
    - Dr Mathew Hoffman M.D, MPH FACOG Obstetrics and Gynecology for Christiana Care Health System.
    - Dr Robert L Goldenberg M.D. Professor of OBGYN, Columbia University School of Medicine, USA
- Medical journals
- Articles / reports found on the Internet
- University books / library
- Studies carried out by previous research fellows and scholars

While the reference material was voluminous and vast, project team has made sincere attempt to curate the required information to create big impact on this study. The reference material provided necessary direction and guidance to this project.

WHO definition of Preterm Birth:

Definition: Birth before completion of 37 weeks of gestational age (GA) or before 259 days from the first day of last menstrual period (LMP)[24].

Based on the gestational age preterm birth is classified into three sub-categories:

- Extremely preterm (less than 28 weeks)
- Very preterm (28 to 32 weeks)
- Moderate to late preterm (32 to 37 weeks).

Table 1: Classification of PTB: Morken et al,, 2005

SUBGROUP (%)	<28 Weeks	28-31 Weeks	32-33 Weeks	34-36 Weeks	<37 Weeks
SPONTANEOUS PTB	**49.5**	**35.6**	**42.6**	**60.6**	**55.2**
IATROGENIC PTB	**17.4**	**26.7**	**23.9**	**18.7**	**20.2**
INTRAUTERINE FETAL DEATH	**2.3**	**9.0**	**4.6**	**1.4**	**2.7**
MALFORMATIONS	**4.7**	**5.5**	**5.6**	**4.3**	**4.6**
MULTIPLE BIRTH	**16.0**	**14.7**	**16.0**	**10.1**	**11.6**
UNKNOWN ONSET OF DELIVERY	**10.1**	**8.5**	**7.3**	**4.9**	**5.7**
TOTAL	**100**	**100**	**100**	**100**	**100**

PTB based on Clinical Classification:

Clinical classification for the preterm birth is one of the most widely used and practiced method. This method has thrown wide variety of datasets for further study and research in the field of PTB.

PTB based on Clinical Classification Groups:

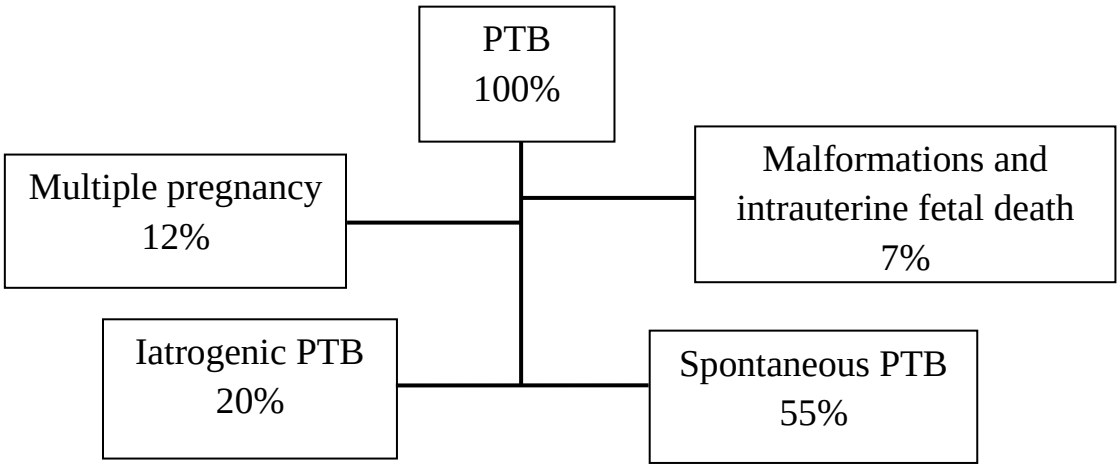
This classification approach can also be presented based on clinical presentation (Figure) [25-29]

- **Spontaneous**: This classification is significant contributor to the clinical classification. This category represents slightly more than 50% of PTB and it encompasses preterm labour with intact membrane (PTL) as well as preterm pre-labour rupture of membrane (PPROM)[30]

- **Iatrogenic:** The second most contributors in clinical classification adopt medically indicated method and it approximately contributes 20% of PTB. This is heterogeneous category based on aetiology and encompasses induction of labour or maternal or fetal complications indicated cesarean section by before 37 weeks of gestation[26 -29]. Such indications include hypertensive disorders of pregnancy/ preeclampsia, antepartum haemorrhage, intrauterine growth restriction, non-reassuring tests of fetal wellbeing, small for gestational age (SGA) [27-29, 31-33]

- **Other subgroups**: Apart from the previous two methods, there are many sub classifications and they are generally classified under "Other Subgroups". This generalised group attributes to twins or triplet pregnancy (12%), fetal malformations as well as antepartum fetal death (7%) [30]

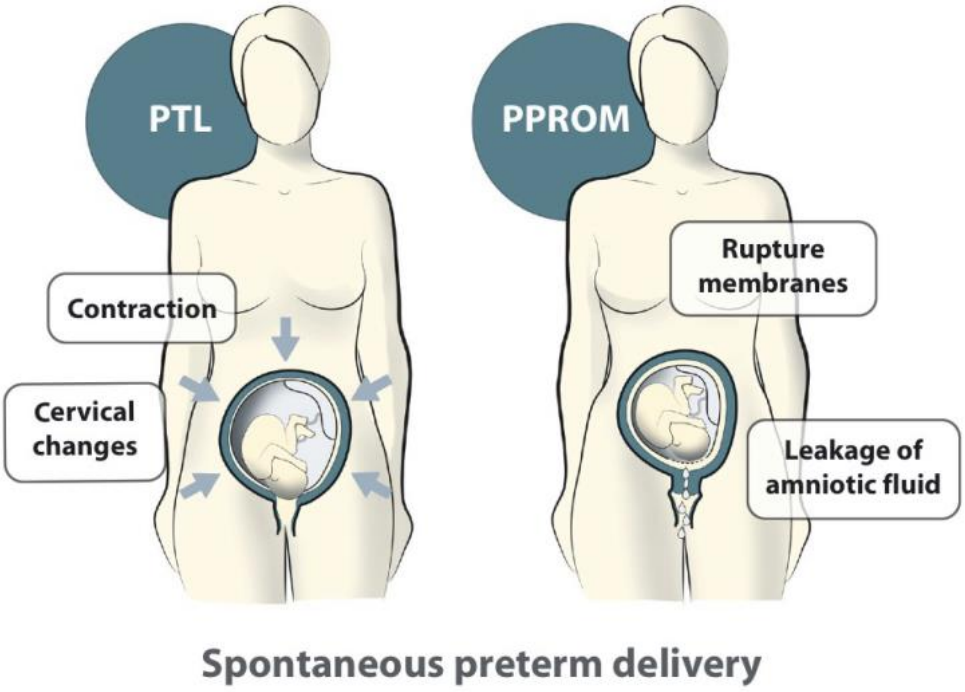
Figure 1: PTB and Clinical presentation (Morken et al,,2005)



Subtypes of spontaneous PTB:

Since spontaneous PTB contributes majorly, studying spontaneous PTB and its subtypes is very important. This study has considered key two subtypes, namely preterm labour (PTL) and preterm pre-rupture of membrane (PPROM).

Figure 2: PTB and PPROM. Illustration Jan Funke



Preterm Labour (PTL):

Preterm labour (PTL) involves regular contractions of uterus that are proceeded by ripening of cervix, and it accounts for about half of all PTBs[25,28,30, 31, 34]. Causes for PTL comprises many factors originating due to behavior, environment, or due to genetic influence. The intra-amniotic inflammation is the major subset of PTL which is associated with cytokine overproduction, resulting in local prostaglandin production that consequently increasing uterine contractions[35, 36].

Acceleration of physiological events is not the only reason for cervical remodeling that is associated with PTL. Several animal models have demonstrated more than one mechanism for cervical ripening. Preterm Labor induced due to progesterone withdrawal leading to decreased hyaluronan synthase-2 expression. Hyaluronan a glycosaminoglycan increases considerably during cervical ripening. Additionally, PGS-2, IL-6 and MMP-8 enzyme levels were elevated in a mouse model. Specifically, MMP-8 expression is mostly associated with degradation of collagen matrix at the time of cervical ripening[37-39]. Inhibiting or decreasing contraction of uterus itself may not result in reduction of preterm delivery as well as it may not lead to neonatal outcome improvement based on existing evidence. However, shorter term prolongation of pregnancy may be accomplished by tocolytic administration rather than treating the cause of preterm labor process. Tocolytic administration would be useful in such situations so that there is ample time for administration of corticosteroid and referral of patients to higher/tertiary centers[40].

Preterm Premature Rupture of Membrane (PPROM):

In some cases, disease pertaining to fetal membranes forces mechanical and functional disruption to fetal membrane. Hence, P-PROM is generally well-defined as spontaneous rupture of foetal membrane before the ideal period of gestation (37 weeks) and it contributes in the range of 25-30% of all PTB cases[25,28,31,33,34]. Underlying mechanism of P-PROM is through proteolysis of extra cellular matrix that leads to weakening of fetal membranes[41-43].

PPROM can be classified in 3 major groups[44].

Category 1: with a longer latency period to delivery in the absence of cervical changes

Category 2: with a shorter latency period to delivery that is associated changes in the cervix

Category 3: This invlolves Coagulopathies or Disorders of bleeding which may be associated to baruption of placenta

Clinical Classification Limitations:

Classification based on clinical picture alone has many limitations as multiple heterogeneous conditions being classified in a single group. For e.g., iatrogenic PTB group includes deliveries secondary to preeclampsia, abnormalities in growth and maternal hemorrhage. But, such conditions might be related to other risk factors and pathophysiological mechanism[27]. The phenotypic classification is better off to overcome these limitations compared to classification based on clinical picture and GA. This phenotypic classification highlight clinical characteristics as well as reduce influence of underlying etiopathologies. However, limitation of such classification is

that each of the phenotype may include several underlying etiopathologic pathways rather than considering each presentation as a single phenotype[27,45,46,47].

A large, multicentric cohort study utilized this phenotypic classification and 12 different PTB phenotypes were recognized. In this prospective cohort, 11 out of 12 phenotypes were grouped by one single condition. Spontaneous contraction nearly accounted for 1/3rd and/or P-PROM in more than 70% of patients[47,48].

Based on current literature in the etiopathogenesis of PTB, another classification was proposed in 2015 which incorporated nine potential phenotypic groups and applied as a part of prospective cohort study among spontaneous PTBs. Major phenotype was maternal stress (~60%) in African-American women, however, white women had more of placental dysfunction and decidual hemorrhage categories. Additionally, decidual hemorrhage and infection/inflammation conditions were higher among less than 28 week gestation births. Subsequent study looked at groups of comparable phenotypic profiles. In this study, women were grouped within 5 different phenotypes, i.e. Maternal stress, P-PROM, Co-morbidities, infection/decidual hemorrhage/placental dysfunction and familial factors, wherein such conditions had overlapping/related pathophysiological mechanism[46]. It is to be noted that, such phenotypic organization provides a unique context for evaluation of PTB and of course need to be validated in different settings.

Etiological risk factors in PTB

The PTB risk factors can be grouped into static or dynamic and there may be more than one risk factor that is related to PTB, signifying that multiple etiologies are responsible for preterm birth[49].

Static risk factors

These are non-modifiable risk factors.

- **Heredity Risk**: Heritability has important role in PTB. The genetic study of heritability suggests PTB is closely related to other members of the related family members[50]. "Twin studies" reports the 17-40% heritability for PTB [51,51]. The study reveals that, preterm born women herself have an increased risk of PTB [53].

- **Pregnancy Loss**: The recurrence risk of PTB in the range of 15% to 50+% is most common in a women those have experience spontaneous PTB and second trimester pregnancy loss[28,31,54]

- It is unfortunate that, there are no clear reasons for uterine anomalies. With these anomalies risk of PTB are more[55-57]

- Ethnicity has its own role to play in higher risk of spontaneous PTB. Black women are three times more prone to have PTB compared to other races. These women also experience an early PTB. Such racial discrepancy in PTB is still poorly understood by many[28]

- Smoking is definitely injurious to health. Women who are maternal smoking habits carry higher risk of spontaneous PTB and other poor pregnancy outcomes[58].

- Like smoking, consumption of alcohol and use of drugs in abused manner increases risk Spontaneous PTB[28]

- Stress is universally affecting human kind across men and women. The incidence of sPTB seen increased in maternal stress and low socioeconomic group[59-63].

- Long hours of work, and hard work under stressful situations are associated with sPTB

- Maternal age remains the most significant risk factor, with younger and older mothers having a higher chance of spontaneous PTB. Women under the age of 20 have a higher incidence of spontaneous PTB, which tends to increase with parity[65].

- High as well as low BMI during pre-pregnant period have both been correlated with increased incidence of spontaneous PTB. [66-68]

- Maternal diet also influences risk of sPTB. The consumption of artificial sweetened beverages is linked to an higher incidence of sPTB.[69] Consumption of probiotics and dairy products improves vaginal flora, hence, associated with a decreased risk of sPTB.[70]

- The shorter inter pregnancy interval has been linked to increased risk of sPTB [71-73] Although mechanism is not yet delineated, it is postulated that latent period of time is required for restoration of affected uterus because of inflammation.

Dynamic risk factors

These refer to clinical menaces or pathological things connected with bad obstetric outcomes that independently or in combination with static factors, might predispose to or lead to PTB. Multifaceted interplay among different risk factors may lead to changes in epigenetics and thus leading to gene expression alterations which add to dynamic clinical risks.[46] Following are the resultant phenotypes due to these interactions:

- Infection is the key pathological route which is directly linked to sPTB[28] which is especially more important in the early gestation. Frequency of such infection related inflammation in sPTB is more common in earlier period of gestation[28,74,75]

- Maternal anti fetal cellular and antibody facilitated processes contribute a subset of sPTB.[76-77]

- There will be higher manifestation of gap junctions due to myometrial cells stretching. Uterine contractions require spread and harmonisation of gap junctions.[78,79]

- Uterine contractions increase secondary to placental abruption and resultant bleeding. This is associated with sPTB in more than 50% of such cases[80,81]. Etiopathologies implicated in such conditions is related to alteration at placental vascular bed and bleeding in chorio decidual zone[82].

- Shorter the length of cervix higher is the risk for sPTB.[83]

Pathophysiology of PTB

PTB complexity increases with multiple pathways like inflammation and oxidative stress. There is a need to look at the maternal and fetal signals and subsequently, causal pathways.[84]

Inflammation and spontaneous PTB

Following inflammatory changes have direct correlation with spontaneous PTB:[35, 85,86]

- Activation of leukocytes
- Elevation of the cytokines (inflammatory) and chemokines
- Metalloproteinases degrading the myometrial matrix as well as extracellular matrix of cervical and fetal membrane

Above listed events lead to membrane structural integrity loss, activation of myometrium as well as ripening of cervix. In this context, PTB and P-PROM can be considered to occur secondary to immune responses triggering the delivery event[87].

Infection-associated inflammation

With the available data, it is estimated 25-40% of spontaneous PTB is directly linked with infection[85]. The amniotic cavity can be considered as sterile as per physiological environments. Isolation of any microorganisms in the amniotic fluid indicates pathology.

Preventing deaths and complications from PTB relies on adopting healthier pregnancy procedures. Quality care provided during pre-conception, antenatal and interpregnancy periods would ensure positive outcomes of pregnancy and child birth.

The World Health Organization recommends following interventions in the antenatal care period to prevent PTB; healthy diet & nutrition counseling, avoiding tobacco; Using USG in determining GA and detection of high risk pregnancies; and a minimum of eight ANC visits from health care providers during pregnancy to categorize and treat risk factors.

Gravett, Vadillo-Ortega, Elovitz and Mrinalini have spent considerable amount of time in studying and presenting effect of infection & inflammation in PTB from animal models. These animal models have helped to understand the mechanisms and effect of PTB as well as to aid developing rational & effective curative/prevention strategies and use of antibiotics and immune modulators positively.

PTB occurs due to a variety of reasons. Most PTBs occur spontaneously, however, few happen because of induction of labour or caesarean section before labor sets in. Hypertensive Disorders of Pregnancy, Infections, Multiple gestation and repeated pregnancies, and other chronic/medical conditions notably, Diabetes Mellitus and Hypertension are common causes of preterm birth. However, many a times, cause is not identified. Additionally, genetic influence may also be contributing. It is important to understand the causes of PTB and underlying mechanisms so as to improve the development of solutions in preventing PTB.

The ultimate goal of identifying and treating PTB is to prolong the pregnancy adequate to reduce the incidence of neonatal mortality/morbidity connected to prematurity, and to minimize risk to mother and baby. For preterm labor, health provider may give medicinal treatment like **antenatal corticosteroids, antibiotics and tocolytics including** these medical regimes there are other kind of treatment like progesterone, cerclage and bed rest. Progesterone, a steroid hormone is essential for

maintaining pregnancy in mammals. In early pregnancy, progesterone is a viable treatment option for high-risk women, however, its mechanism of action is not completely understood. The source of progesterone in humans is 2-staged, for first 9 weeks of gestation it comes from corpus luteum, and for the remaining period of gestation it is produced from trophoblasts. As there are two varieties of cell types that are situated at two tissues, it is difficult to study underlying processes of progesterone synthesis. In humans, corpus luteum of the ovary is responsible for major chunk of progesterone synthesis to maintain early period of pregnancy, and hence, pregnancies fail in absence of this ovary derived progesterone[88,89]. Still, progesterone is not a useful biomarker as serum levels of this hormone do not accurately represent levels of placental production[90]. Hence, we need to determine CL-derived progesterone with other methods at the time of switchover in production of progesterone from ovary to placenta. Anti-Mullerian hormone (AMH), is an ovarian hormone that may be useful in providing evidence regarding ovarian activity in the early pregnancy period.

Disruption of placentation during early pregnancy due to inflammation and/or infection is one of important pathway for PTB. Late first and early second trimesters are the critical periods for placentation. This is the time trophoblast invasion occurs and myometrial junctional zone is remodeled. Utero placental ischemia and up-regulation of the pro inflammatory pathways occur secondary to disruption of this process and alteration in the integrity of the maternal placental barrier.[10] This leads to crossing the maternal circulation by fetal proteins for e.g. Alpha fetoprotein and this may either induce or suppress placental hormone production.[91] Pregnancy complications appear in later half of pregnancy but underlying pathophysiology take place in early pregnancy therefore, it seems possible that hsCRP determination may have help prediction of adverse pregnancy outcome[92]. Currently, it is getting more

clearer that causes of PTB is multi factorial and may vary as per gestational age of pregnancy[93].

Global prevalence of preterm births

As per WHO estimates[23]; 15 million babies were born preterm worldwide in 2010 among 135 million live births, amounting to a PTB rate of just over 11%. South-eastern and South Asia regions contribute to largest. Higher PTB rates in these countries is mainly attributed to larger number of births, high infection rate, poor nutritional status, heavy physical work and unavailability of medicines/basic obstetric and neonatal care. The ten countries; India, Bangladesh Pakistan, Indonesia, Philippines, China (South Asia), Nigeria, USA, DRC and Brazil, have largest number of estimated PTBs and account for majority of the PTBs (~60%) globally. Mortality rates usually increase with decrease in GA, additionally, babies born preterm and who are small for gestational age as well are at higher risk[95].

Figure 3: Global prevalence of preterm births[95]

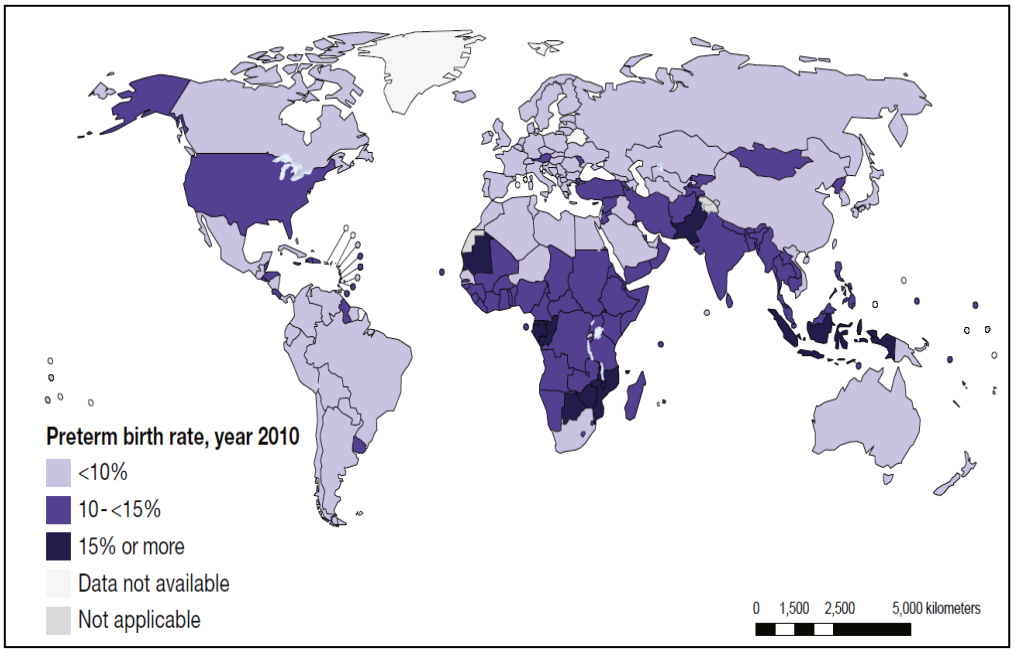


Table 2: Indian prevalence of preterm birth

GN Country	All Live Births	PTB Rate(%)	Total PTB	PTB Deaths
Democratic Republic of Congo	2,872,600	10.7%	341,400	42,200
Guatemala	466,600	7.7%	35,900	2,400
India	27,200,000	13.0%	3,519,100	308,600
Kenya	1,529,300	12.3%	188,100	14,700
Pakistan	4,741,500	15.8%	748,100	72,100
Zambia	599,600	12.9%	77,600	6,800
Totals	**37,409,600**	**13.1%**	**4,910,200**	**446,800**
United States of America	3,930,000	11.4%	450,000	5,800

(Born Too Soon, WHO 2012)

Preterm birth rate is constantly rising across the world over a period of time. In India, incidence of preterm labor is around 22% and that of preterm birth is 20.9%[96]. As stated earlier, rate of PTB is increasing because of adoption of assisted reproduction, frequent, multiple pregnancies, working mothers, psychological stress and medically induced prematurity.

Biomarkers of PTB

Biomarkers: are those parameters that can be estimated in biological samples, and which reflects an exposure, or provides valuable information on actual or potential effect of\ exposure in a sample population or group[97].

Predicting and preventing PTB is challenge for a modern obstetrics. The primary reason for this challenge is PTB syndrome associated with multiple origins. Identification of multiple biomarkers which can assess clinical and other risk factors looks a better strategy[98].

Also, identification of high-risk patients/conditions with a suitable and effective risk-predicting system using biomarkers is very important in avoiding treatment/overtreatment of low risk patients. In association with technically sound tool will definitely add a value in predicting risk in early pregnancy. Since the evident risk factors may not be always seen and about half of deliveries are of sPTBs that happen in low risk pregnancies, it is difficult to identify in early pregnancy. Additionally, initial presenting signs and symptoms are usually milder, it further complicates early detection[99].

There are two broad categories of biomarkers to predict spontaneous PTB:

- Women with all variety of PTB types
- Obtaining the biological samples that are measurable with minimal maternal and fetal risk
- Recommendations to develop ideal biomarker test:
- Inexpensive so that, every women of different socioeconomic group can afford
- Reproducible so that countries across the world adopt easily

- Easy to perform early in pregnancy with minimal invasive techniques
- Higher positive likelihood ratio (LR+): increases probability;
  women with a + test result → at risk of sPTB
- Lower negative likelihood ratio (LR-): confidently rules out disorder/ disease/ condition if the test result is negative
- Wider availability of technological platform is essential for analysis of samples[100,101]

As per current data, single biomarkers are neither sensitive nor can act as predictors of sPTB risk. Frequently reported/studied biomarkers for sPTB are as follows: AFB, IL-6, ferritin , CRH, ACTH, beta-HCG, IL-8, IL-2, CRP, IL-1β, TNF-α, cortisol, estriol, MMP-9, and relaxin. This list is derived from over 100 parameters that were investigated over the period of 43 years (1965-2008), however, there were several limitations in these studies: using improper definitions, heterogeneous assay methodologies, study designs, study populations as well as sampling procedures etc[102]. The future roadmap for essential and combinable biomarker studies must encompass the standardized approach with research oriented guidelines[102, 103]

Key potential biomarkers for PTB in early pregnancy

Following three key three potential protein biomarkers have been considered for this study. Additionally, efficacy of low dose aspirin on these potential biomarkers is point of interest.

Anti-Mullerian hormone

It is an ovarian hormone which provides crucial information on overall activity of ovary in early gestation. It is homo-dimeric glyco-protein consisting of two

subunits, belonging to the family of Tumor Growth Factors (TGF-beta). Gene that encodes AMH is present on the short arm of chromosome 19.

AMH acts via trans-membrane receptors, namely type I (AMHRI) and type II (AMHRII). Regression of Mullerian ducts during embryonal development in males occurs because of AMH action in males and it leads further phenotype development as male. The hypothalamo-pituitary-gonadal (HPG) axis brings about the changes in AMH expression and follows male reproductive system development[104]. AMH hormone is criticial in allowing Wolffian system to predominate and in formation of internal male genitalia. AMH in males is produced in sertoli cells of testes from 5th week of embryonal development period and then continues whole life. It is synthesized in females within ovaries since 36 week of gestation. Maternal AMH produced in a dimorphic pattern during pregnancy could predict fetal gender and also adverse outcome for.eg. PTB. Study results have shown low AMH among preterm fetuses compared to term infants. It was also observed high level of maternal AMH in women delivering preterm females.[105].

AMH is produced in granulosa cells of small growing follicles; primary and preantral, within ovary until menopause. However, AMH is not produced in FSH-dependent i.e. antral as well as in atretic follicles. Subsequently, AMH levels can be measured in blood as the hormone enters systemic circulation. AMH remains high throughout the first trimester of pregnancy, however, drops suddenly at 13 weeks of gestation. The AMH continues to remain at low levels up to birth and they come back to non-pregnant levels after few days of delivery. In earlier studies serum AMH increased with other biomarkers like MSAFP. Although, more recent studies suggested that lower levels of AMH is associated with adverse outcomes such as pre-

eclampsia, literature on association of AMH with preterm birth is not evident[106]. Hence, further studies are required to analyze patterns of AMH in pregnancies, especially in early gestation and complicated by adverse pregnancy outcomes. In a study, AMH levels varied substantially with gestational age at the time of sample collection in early gestation, and showed decrease trend in first trimester and further throughout pregnancy[107].

Maternal serum Alpha - fetoprotein

Alpha fetoprotein (AFP): glycoprotein containing 590 amino acids. Biological function of AFP is not completely understood, however, it postulated to represent as a fetal type of albumin that stimulate growth**,** acts as a carrier for several factors (such as bilirubin and estrogen), as well as acts as an immuno- suppressant. Fetal liver and yolk sac are the organs of production and enterohepatic circulation of AFP occurs in fetus as well. As Fetal AFP gets eliminated leading to increase in the levels in amniotic fluid as well as in maternal blood. Additionally, placental clearance occurs through fetal side to maternal part. AFP levels in the amniotic fluid decrease as pregnancy advances (80,000 ng/ml at ten weeks of GA to about 200-3000ng/ml at full term gestation).

AFP levels in the fetal serum are found to be highest at around 14 weeks of gestation; maternal blood AFP levels are found to be highest at 32 weeks of gestation. Infant blood AFP rapidly declines after birth. AFP measurements in Maternal blood and amniotic fluid has clinical relevance during screening. Raised levels of AFP are linked to several conditions; neural tube defect, twin/triplet pregnancies, Diabetes mellitus, Threatened abortion etc. Maternal AFP levels are found to be lower in

conditions of intrauterine death, missed abortion, Down's syndrome, and hydatidiform mole.

Small amounts of alpha-fetoprotein crosses placenta and can be measured in maternal serum. Majority of fetal production occurs in yolk sac until three months of gestational age. AFP level increases due to elevated amniotic fluid concentration in conditions such as fetal defects (for e.g. exomphalos, spina bifida); fetal to maternal circulation increases secondary to placental damage; increased synthesis in mother occurring in germ cell tumors, hepatocellular carcinoma and hepatic metastatic cancers[90]. Elevated serum AFP levels were associated with higher risk for adverse pregnancy outcomes (i.e. stillbirth, pre-eclampsia, PTB and fetal growth restriction)[108,109]. Elevated levels of ALP with other marker like ferritin, and AFP are associated with preterm labor and it is suggested that PTB could be predicted by measuring the amniotic levels and even serum levels of these biomarkers[110].

Unexplained mid trimester AFP elevation has been linked to increased risk for low birthweight, growth restriction, preterm birth, abruptio placenta & pre-eclampsia. High levels of AFP, and inhibin A in second trimester found in 51% to 86% of PTB in all pregnancies[111]. Pregnant women with AFP values ≥ 2.5 multiple of the median (MoM) during 16 to 20 weeks of gestation have a higher risk for adverse outcomes such as PTB, preeclampsia, & eclampsia, small for gestational age (SGA), fetal and neonatal deaths[112]. Serum AFP during 19 to 24 weeks gestation may be beneficial in combination with other markers in recognizing pregnancies at higher risk for early pre-eclampsia who require delivery less than 32 weeks of gestation[113].

Study results showed that significant association between unexplained second trimester MSAFP elevation and poor maternal/fetal outcome, where the fetus is structurally normal, elevated MSAFP indicates a defect in the placentation[114]. In a case control study conducted by Beta et al. showed a significantly higher AFP in the 1st trimester (1.33 multiple of Median vs 0.97, p = 0.006) in women delivering preterm[115]. Similarly, couple of other studies also observed higher AFP levels linked to preterm birth and, they were strongly trending with values of serum AFP[116-118]. Serum AFP levels in mother during 11–13 weeks of gestation was seen elevated among pregnancies that resulted in spontaneous early preterm birth. Hence, serum AFP measurement may improve prediction of pre-term delivery. Serum AFP gets elevated with increasing gestation and decreases with higher maternal weight[119]. This study also showed assessment of Placental Growth Factor (PLGF) and AFP may be helpful for screening; trisomy 18 & 13[120]. The study established a reference distribution for maternal serum AFP during 11–13 weeks of gestational age and demonstrated the impact of maternal characteristics on assessed values. These normal ranges may be used in further investigations for screening of pathological pregnancies[121.]

Cervical Interleukin-6 (IL) and AFP, along with fetal fibronectin are promising biomarkers, however, information about sensitivity, specificity, and positive predictive values is not completely known at this time so as to assess clinical usefulness of these biomarkers[122]. Decreased utero-placental blood flow is more commonly seen in women with higher levels of hCG and / or AFP during mid trimester and, they might be helpful as biomarker for such sub-set of women having higher risk for pregnancy related complications; fetal loss in later pregnancy, Pregnancy Induced Hypertension, pre-eclampsia, Fetal growth restriction (IUGR),

preterm birth[123-127] AFP along with other serum biomarkers, especially hCG, in combination with uterine artery Doppler increases identification of pregnancy complications [114]. One of the randomised clinical trial showed reduction in early-onset birth and related adverse pregnancy outcome on aspirin administration among women having higher mid trimester AFP[128]

C-Reactive protein (CRP):

It plays an important role in inflammatory processes. CRP is an annular (ring-shaped), penta-meric plasma protein , whose levels increase in response to inflammatory conditions. It removes pathogen by binding to the surface antigen and opsonizing them for uptake by phagocytes and additionally activating the classic complement pathway.

CRP acts by inducing cytokines and tissue factor of monocytes. But, major function of CRP is to lower inflammation by decreasing neutrophil migration to site, prevent adhesion of neutrophil to cells of endothelium, and it also helps in removing nuclear antigens that are secreted from necrotic and apoptotic cells[129]. CRP is one of the acute-phase protein originating from liver and levels of CRP increase due to secretion of interleukin-6 from macrophages and T- cells.

G.P. Sacks & colleagues carried out a well-controlled study to understand association of systemic maternal inflammatory response in early pregnancy period and showed that maternal CRP levels were increased as early as 4 weeks of gestation and concluded that inflammatory response in pregnancy might be established during earliest phases of implantation[130]. Tjoa M L and colleagues conducted the study and

observed that first trimester CRP levels in women were associated with pre-eclampsia or Intrauterine Growth Restriction[131].

Elevated CRP levels during pregnancy, as a marker of low grade inflammation, were associated with increased risk of fetal growth restriction and neonatal complications, such as PTB, low birthweight, and small for gestational age (SGA)[132] Low grade inflammation is associated with dysfunction of endothelium that leads to vascular dysfunction & suboptimal placental development. Syncytiotrophoblast immune reactivity may be due to premature formation of intervillous blood circulation early in the first trimester of pregnancy[133]. Also, maternal systemic inflammation may also occur in response to placental ischemia, leading to sub-optimal placentation.[134] Above mentioned risk factors of preterm birth are more likely to be associated with elevated CRP during early pregnancy; increased CRP levels (more than 6mg/dL) in the absence of medical, surgical or obstetric complications may predict higher incidence of preterm birth. Conditions such as oligo-hydramnios, growth restriction and pre-mature rupture of membranes are correlated with increased levels of CRP[135]. Elevated serum CRP level in second trimester primi-gravidae was strongly associated with adverse pregnancy outcomes such as preterm birth and Gestational Hypertension. Raised CRP levels in mother was linked to higher risk of preterm birth as compared to pregnancies with normal levels of CRP. In women with CRP values greater than 8 mg/L, risk of preterm delivery was more than two times. Hence, CRP could potentially be utilized to predict preterm birth and gestational hypertension in early pregnancy so that maternal and fetal complications can be minimized.[136] One of the study demonstrated that in pre-eclamptic and primigravidae women, CRP level was higher than multigravida patients[137]. Significantly high levels of CRP were found in third trimester in

pregnancies that are complicated by pre-eclampsia as compared to normo-tensive pregnant women. The higher levels of CRP were seen negatively correlated with fetal birthweight in the pre-eclamptic group[138].

Evidence indicates stronger association between the higher level of CRP "a sensitive biomarker" and pre-mature contractions of uterus. Estimation of C-reactive protein levels during pregnancy can be used to predict and used as biomarker for screening/detection of subclinical infections that may result in preterm contraction of uterus and, hence, early intervention in the form of intensive ante-natal care to decrease the perinatal morbidity and mortality.[139] Non-surgical supportive periodontal treatment may reduce preterm birth risk in pregnancies affected with periodontitis by reducing CRP values[140].

Predicting preterm birth may be done using statistical combination of positive fetal fibronectin, length of cervix (<21.5 mm), CRP levels in serum more than 6.1mg/L, increased levels of intrlukin-6 in cervico-vaginal secretions[141]. **CRP levels from** 1.5–2.5 mg/L is considered as **normal range of pregnancy** based on observations from several studies. Reference range of CRP goes higher in the second-half of pregnancy. Low-dose-Aspirin (LDA) increases chance of clinical recognition of pregnancy and livebirth among women with prior pregnancy loss and having moderately increased levels of CRP[142]. Since the function of cyclo-oxygenase-2, which is directly inhibited by aspirin, is inter-related with many of the inflammatory mediators involved in reproductive processes, LDA may prevent down-stream effect of inflammatory pathways that may have been chronically up-regulated [143].

MATERIAL AND METHODS:

At Jawaharlal Nehru Medical College, Belagavi Karnataka, India, we conducted a nested case-control study on participants enrolled in ASPIRIN trial implemented by the JNMC Women's and Children's Health Research Unit as part of the GN common protocol. This research work was carried out over a period of two years (2016 to 2018) by covering 666 nulliparous pregnant women.

The data collection instrument (case record forms) for the study was organized and details of all the participants were documented after appropriate assessment & scrutiny. Nulliparous pregnant woman in first trimester (10 to 13 weeks) and second trimesters (17 to 21 weeks) were included. The participants/enrolled women a) who might have received LDA and b) those who might not have receive LDA in ASPIRIN trial. Goal was established to recruit about 50 preterm delivery (cases) and 150 women delivering at term (controls) in 1:3 case control ratio:

a) Cases: Study participants delivering less than 37 weeks of gestation and,
b) Controls: Study participants delivering at or more than 37 weeks completed gestation.

Study Design

Study Design: Nested case-control study within ASPIRIN trial.

Source of Data: Research participants (Rural Nulliparous pregnant women) enrolled in ASPIRIN trial of Belagavi district. Ethical approval is obtained from J.N.M.C Institutional ethics committee Belagavi. The voluntary informed consent was obtained from the participants before enrolling into the study.

Inclusion and exclusion criteria

Inclusion criteria-

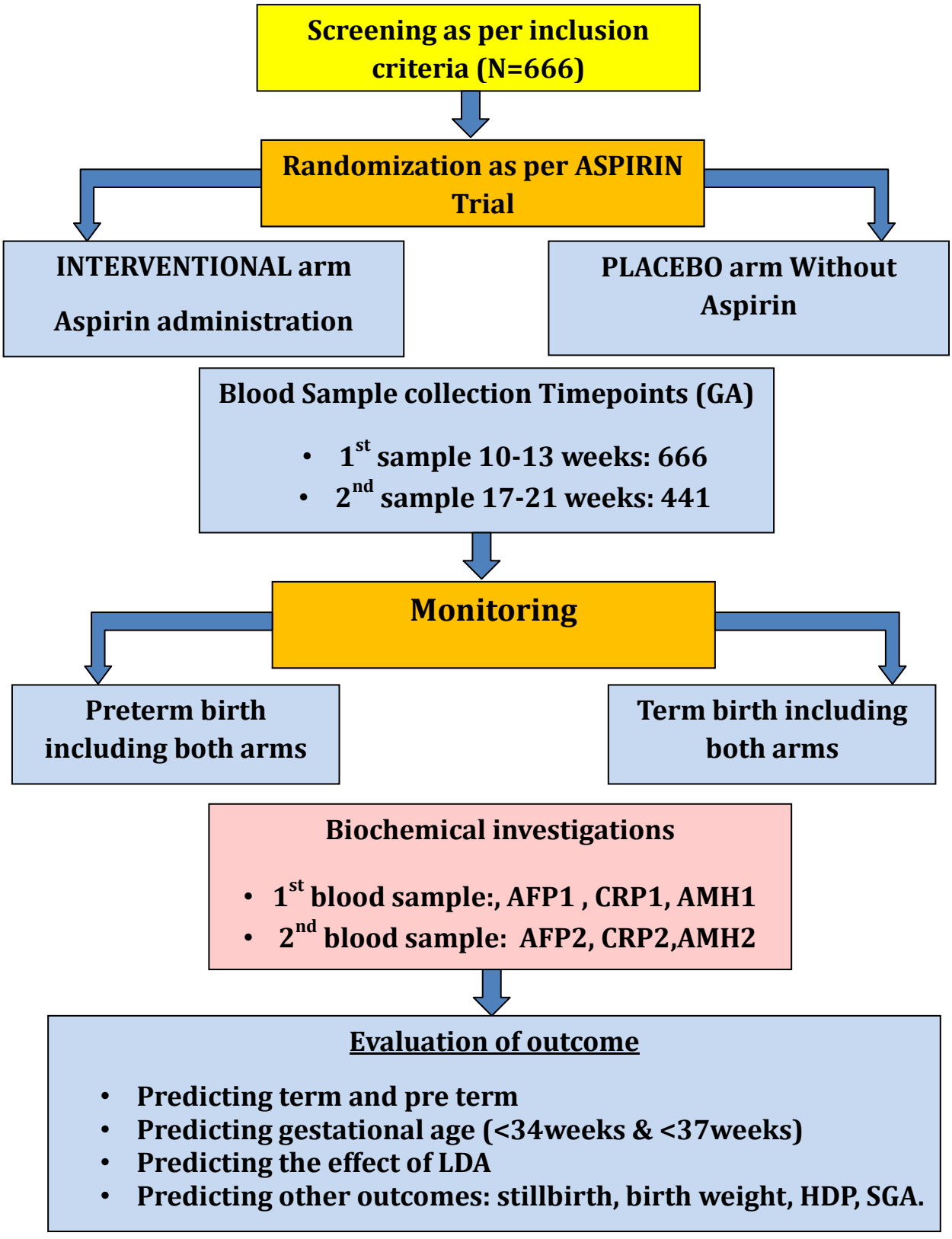
- Nulliparous pregnant women between 18-40 years of age
- Less than two prior first trimester pregnancy losses.
- Absence of medical contraindication to aspirin.
- Single, live, and intrauterine pregnancy; gestational age from 6 weeks to 13 weeks. (confirmed by dating scan in early pregnancy and with HR >110 beats /min)

Exclusion criteria-

- On medication with aspirin for > one week.
- Twin/Triplet pregnancies.
- Hemoglobin < 7 gm% at screening.
- Other medical condition like hypertension, diabetes and other known significant diseases.

Sample Size: Research participants aged 18-40 years who are eligible as per ASPIRIN trial inclusion & exclusion criteria & screened will be enrolled at the time of data collection from the rural areas of Belagavi District.

Overall study plan



Key Parameters

1. **Anthropometric parameters**
    a. Height (cm) by Commercial stadiometer.
    b. Weight (kg) by Digital Weighing Scale (sec) with an accuracy of +100gm
2. **Physiological parameter**
    a. Blood pressure - Using sphygmomanometer, INCO, Ambala (as per JNC VII criteria)
3. **Biochemical parameters–**
    a. Antimullerian Hormone – Enzyme Immuno Assay
    b. Alpha fetoprotein - Enzyme Immuno Assay
    c. High sensitivity C reactive protein– reagent method using ERBA biochemical analyzer.
    d. Hb % -- by using Hemocue method.

Protocol Execution Stages

Stage 1: Sample Collection

Venous blood sample (about 4 to 5 mL) was collected from the participants under aseptic precautions. Serum was then separated after 30 minutes of collection by centrifugation. Serum stored in 2 ml cryovials and transported under cold chain to Dr Prabhakar Kore Basic Science Research Centre of KLE Academy of Higher Education and Research, Belagavi and stored at -80^0C until further analysis.

Stage 2: Analysis of Samples

Assay of AFP, AMH and CRP were done by standard immuno-assays as recommend in kit literature utilizing the following

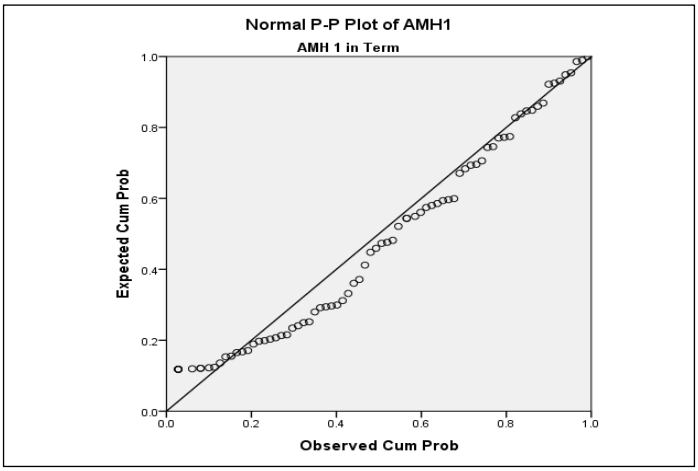
1. Alpha-fetoprotein: R and D Systems; Catalog number DAFP00
2. Anti-Mullerian Hormone: R and D Systems; Kit number; DY1737
3. C-Reactive Protein: Immuno-turbidimetric method (Roche systems)

Stage 3: Normalization of Analytes:

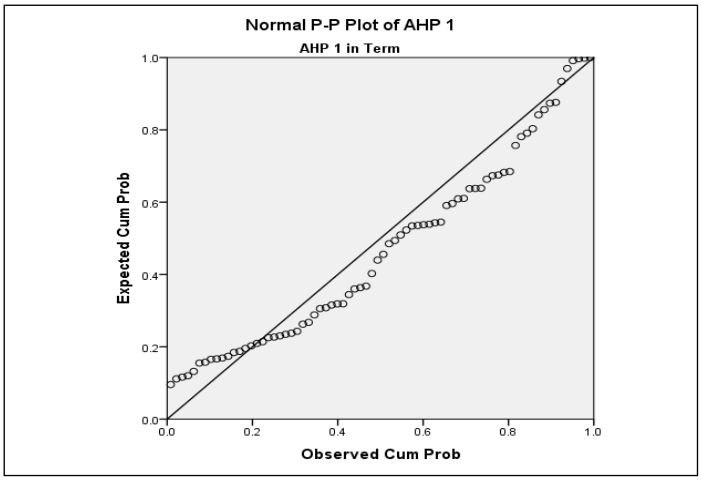
After analyzing the samples, we found that, we had wide variety of data spread alongside normal curve. We took exercise to exclude outliers to bring required focus. We adopted the following normalization methods.

Figure 4:

Method 1: Normality of AMH 1 in term



Method 2: Normality of AFP 1 in term



Method 3: Normality of CRP 1 in term

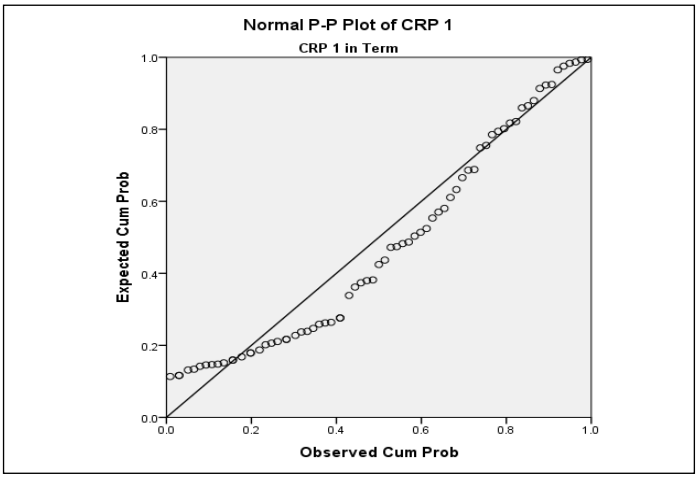
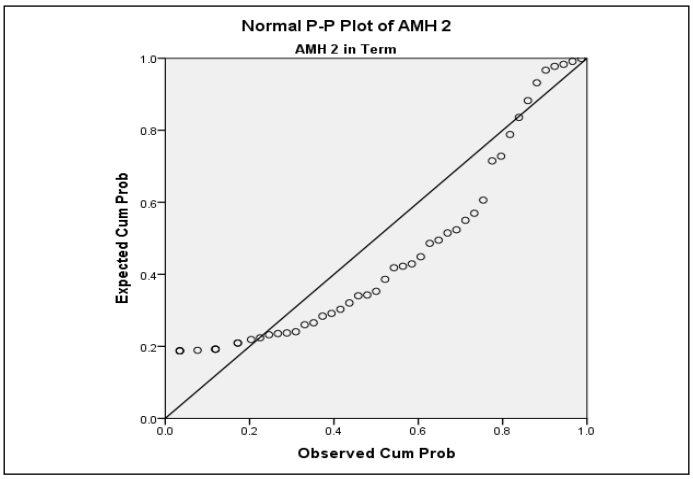
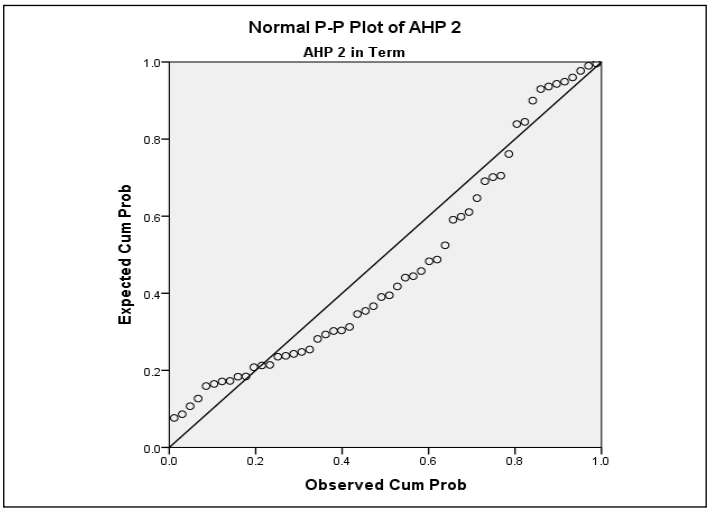


Figure 5:

Method 4: Normality of AMH 2 in term



Method 5: Normality of AFP 2 in term



Method 6: Normality of CRP 2 in term

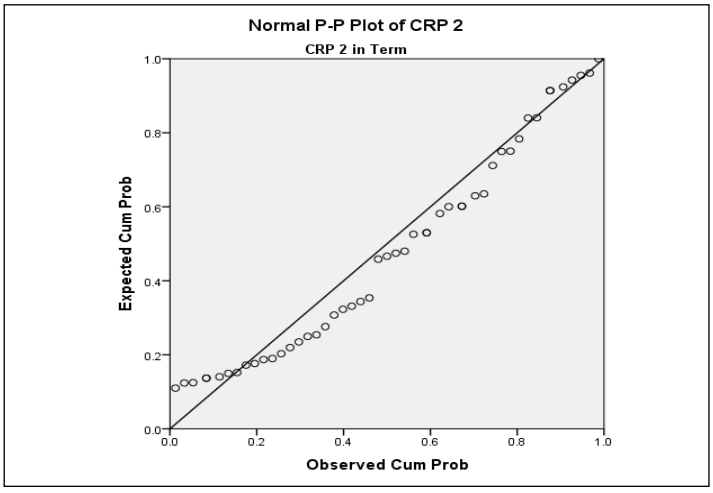
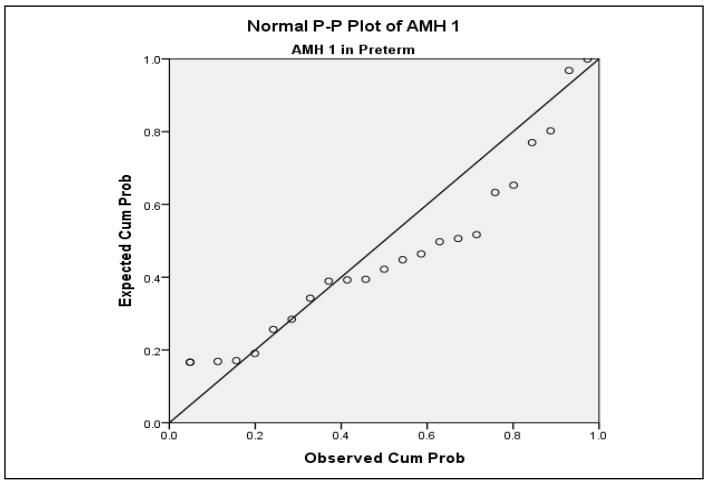
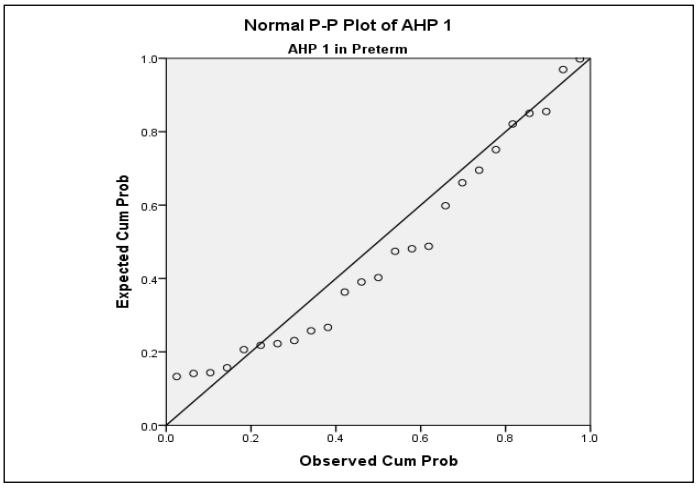


Figure 6:

Method 7: Normality of AMH 1 in preterm



Method 8: Normality of AFP 1 in preterm



Method 9: Normality of CRP 1 in preterm

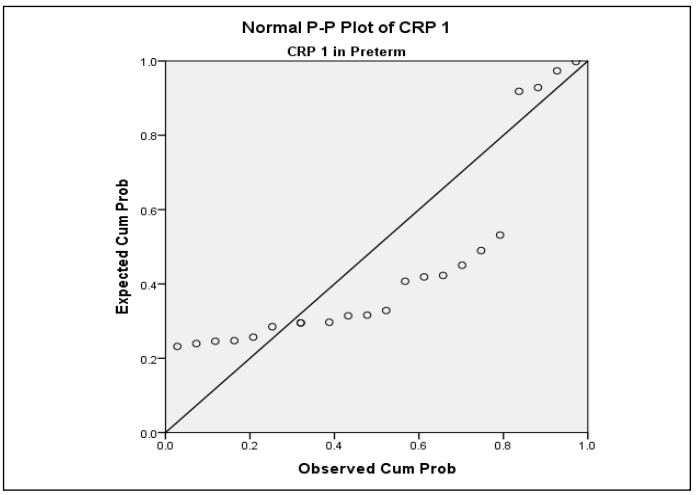
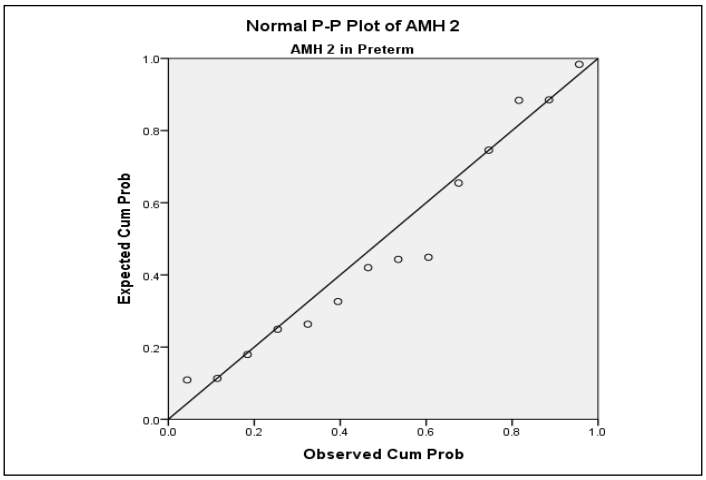
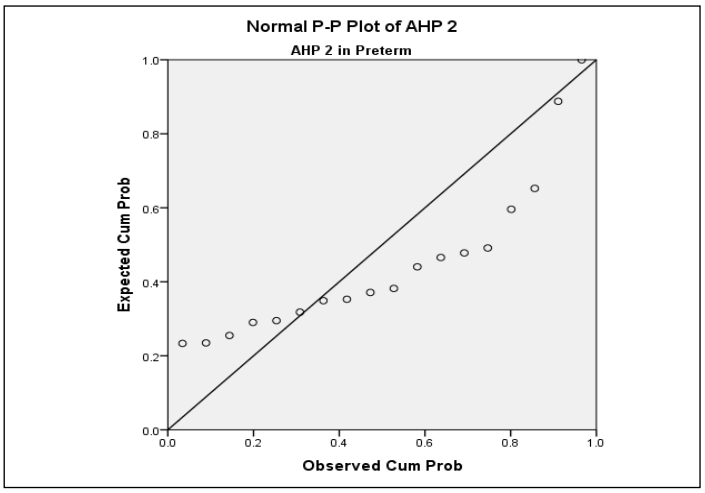


Figure 7:

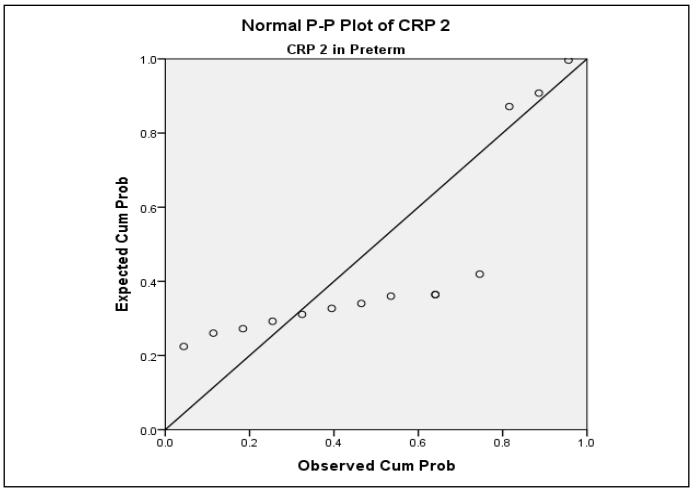
Method 10: Normality of AMH 2 in preterm



Method 11: Normality of AFP 2 in preterm



Method 12: Normality of CRP 2 in preterm



Stage 4: Statistical Analysis

- The data was analyzed by Mann-Whitney U test (Wilcoxon Rank-Sum tests), Z-test and Correlation analysis.
- The data computed for the % of nulliparous women with change in levels of biomarkers of Preterm and Term delivery was compared using Mann-Whitney U test (Wilcoxon Rank-Sum test); Z-test.
- Correlation analysis was used for biomarker levels within the preterm group.
- The comparison of serum biomarkers between LDA treated and non LDA treated group analyzed by using Z-test.

RESULTS

A total of 666 nulliparous pregnant women enrolled in this study / research work. Total of 6 women had abortion within the first 16 weeks gestation. We were able to gather and analyze samples of 275 women. Out of these, 42 women (Group 1) experienced preterm delivery and 233 women (Group 2) experienced term delivery.

The study data analysis has been provided below (refer tables TR1 to TR25). Each of these analysis sections provided us insight on how the PTB is correlated with multiple key considerations during the delivery. Summary of these multiple analysis is given here.

Analysis of Demographic Profiles

Demographic profile of respondents: Demographic information for cohort is displayed in Table R1. We focused on getting their details from “age group” to “birth weight of the baby”. This fundamental information has helped us to establish the correlation between preterm/term delivery results with profile data. Maternal demographics were found comparable across both groups. The data represented in demographic table is consistently representing the data of ASPRIN trial participants. Women who had intervention of aspirin exhibited lower rate of preterm birth, however, this did not build much of a statistical correlation from the smaller cohort’s data covered as part of this study.

Table 3: Demographic profile of respondents:

Profile	Preterm			Term		
	Placebo	**Aspirin**	**Total**	**Placebo**	**Aspirin**	**Total**
Maternal age						
18-20 yrs	15	12	27	184	199	383
21-25 yrs	8	2	10	89	97	186
3>=26yrs	3	2	5	26	18	44
Maternal education						
No formal education	1	0	1	16	4	20
Primary	1	1	2	19	16	35
Secondary	19	14	33	221	247	468
University+	5	1	6	43	47	90
BMI						
Underweight	8	4	12	108	120	228
Normal weight	16	9	25	172	169	341
Overweight and Obese	2	3	5	19	25	44
No of previous abortions						
None	21	16	37	266	272	538
One	4	0	4	31	37	68
Two	1	0	1	2	5	7

Hb1						
<=7	0	0	0	0	0	0
7.1-9.0	1	0	1	22	22	44
9.0-11.0	8	4	12	92	94	186
>11	17	12	29	185	198	383
Anaemia						
Yes	9	4	13	114	116	230
No	17	12	29	185	198	383
Sex of baby						
Male	19	9	28	149	166	315
Female	7	7	14	150	148	298
Birth weight						
<2500grams	18	7	25	54	46	100
>=2500grams	8	9	17	245	268	513
Total	26	16	42	299	314	613

Demographic profile of respondents – Placebo and Aspirin: This analysis may sound similar to the previous analysis (Table R1), however in this analysis; the pivotal elements are Placebo and Aspirin. This provided another view of, the fundamental information in comparison with these two primary data points.

Table 4: Demographic profile of respondents:

Profile	Placebo			Aspirin		
	Preterm	**Term**	**Total**	**Preterm**	**Term**	**Total**
Maternal age						
18-20 yrs	15	184	199	12	199	211
21-25 yrs	8	89	97	2	97	99
>=26yrs	3	26	29	2	18	20
Maternal education						
No formal education	1	16	17	0	4	4
Primary	1	19	20	1	16	17
Secondary	19	221	240	14	247	261
University+	5	43	48	1	47	48
BMI						
Underweight	8	108	116	4	120	124
Normal weight	16	172	188	9	169	178
Overweight and Obese	2	19	21	3	25	28
No of previous abortions						
None	21	266	287	16	272	288
One	4	31	35	0	37	37
Two	1	2	3	0	5	5

Hb1						
<=7	0	0	0	0	0	0
7.1-9.0	1	22	23	0	22	22
9.0-11.0	8	92	100	4	94	98
>11	17	185	202	12	198	210
Anaemia						
Yes	9	114	123	4	116	120
No	17	185	202	12	198	210
Sex of baby						
Male	19	149	168	9	166	175
Female	7	150	157	7	148	155
Birth weight						
<2500grams	18	54	72	7	46	53
>=2500grams	8	245	253	9	268	277
Total	26	299	325	16	314	330

Maternal Demographics & Birth Outcomes: Table R3 elaborates the details of demographic history, maternal anthropometric parameters, past obstretic history and the details of present birth outcomesin both aspirin and placebo groups.

Table 5: Maternal Demographics & Birth Outcomes

	Aspirin (N= 129)	**Placebo (N=146)**	**Total (N=275)**
Demographics			
Age	20.6+/-3.0	21.0 +/- 3.2	20.8 +/- 3.1
Education			
None	0.80%	5.50%	3.27%
Primary	3.10%	4.10%	3.64%
Secondary	81.40%	77.40%	79.27%
University+	14.70%	13.00%	13.82%
Height (Cm)	151.8 +/- 5.5	151.7 +/- 5.4	151.7 +/- 5.4
Weight (Kg)	47.1 +/- 8.2	47.1 +/- 7.5	47.1+/- 7.8
BMI	20.4 +/- 3.4	20.4 +/- 3.0	20.4 +/- 3.2
Prior Abortion	0.11 +/- 0.34	0.10 +/- 0.32	0.11+/-0.35
Hb on enrollment (G/dL)	11.6 +/- 1.4	11.4 +/- 1.4	11.5+/- 1.3
Gestational Age at sample 1 (weeks)	11.6 +/-2.6	11.7 +/-5.1	11.7+/-4.1
Gestational Age at sample 2 (weeks)	18.7 +/-2.2	19.6 +/-3.8	19.1+/-3.3
Birth Outcomes			
Gestational Age (Weeks)	37.7 +/- 5.2	37.6 +/- 5.4	37.6+/-5.3
Birth Weight (gm)	2759+/- 40.1	2588+/-43.4	2669+/-49.0
PTB <37 weeks	12.10%	18.40%	15.50%

PTB <34 weeks	7.80%	7.50%	7.64%
Stillbirth	3.20%	5.70%	4.53%
Hypertensive Disorders of Pregnancy (HDP)	11.30%	10.60%	10.94%
<2500 gm	15.70%	34.80%	26.92%
<1500 gm	1.70%	4.40%	3.13%

Comparison of Serum Analytes by Mann-Whitney U Test;Wilcoxon Rank Sum Test

The non-parametric test was used here as outcome was not normally distributed between independent samples and sample size was small. Mann Whitney U test was utilized as it is most popular one to relate outcomes between the two independent groups.

Comparison of placebo and Aspirin with AMH, AFP and CRP by Mann-Whitney U Test (Wilcoxon Rank Sum Test) (Preterm): The process of completing this study has referred previously carried out multiple research work. We found that, "**Mann-Whitney U Test**" is one of the important exercises to complete with our participant data. Hence, we fed preterm participants data to "Mann-Whitney U Test" and found that, comparison of placebo and Aspirin with Serum Analytes (AMH, AFP and CRP) by Mann-Whitney U Test in preterm births has no significant statistical difference.

Table 6: Comparison of placebo and Aspirin with AMH, AFP and CRP (Preterm):

Variables	Placebo				Aspirin				U value	Z value	P value
	n	Mean	SD	Mean Rank	n	Mean	SD	Mean Rank			
AMH 1	23	2.19	2.25	19.72	14	1.64	1.01	17.82	144.50	-0.5167	0.6054
AFP1	25	18.33	11.14	19.48	13	17.28	7.93	19.54	162.00	-0.0154	0.9877
CRP 1	22	1.96	2.01	18.75	15	1.88	1.63	19.37	159.50	-0.1701	0.8649
AMH 2	14	1.71	1.37	11.75	8	1.54	1.03	11.06	52.50	-0.2389	0.8112
AFP2	18	72.62	75.58	14.78	9	47.68	31.05	12.44	67.00	-0.7201	0.4715
CRP 2	14	2.76	3.55	10.21	9	4.17	2.72	14.78	38.00	-1.5749	0.1153

Comparison between placebo and Aspirin with serum analytes AMH, AFP and CRP (Term): While previous analysis (table 4) was focused on preterm data, in this analysis, we fed term data to Mann-Whitney U Test and found that, only AFP 1 values shows some relevance to this test. AFP 1 values were statistically significant with the confidence interval of 95% ($p<0.05$).

Table 7: Comparison between placebo and Aspirin with serum analytes AMH, AFP and CRP (Term):

Variables	Placebo				Aspirin				U-value	Z-value	p-value
	n	Mean	SD	Mean Rank	n	Mean	SD	Mean Rank			
AMH 1	76	1.68	1.41	69.13	76	2.13	1.47	83.88	2327.50	-2.0654	0.0389*
AFP1	74	25.72	17.08	89.16	77	17.34	13.14	63.36	1875.50	-3.6236	0.0003*
CRP 1	71	2.38	1.91	70.11	66	2.24	1.81	67.81	2264.50	-0.3382	0.7352
AMH 2	47	1.61	1.81	44.87	44	1.39	1.06	47.20	981.00	-0.4209	0.6738
AFP2	54	69.48	40.39	53.70	48	61.97	34.84	49.02	1177.00	-0.7978	0.4250
CRP 2	49	3.75	2.96	48.28	39	2.86	2.51	39.76	770.50	-1.5539	0.1202

Comparison of preterm and term groups with AMH, AFP and CRP by Mann-Whitney U test (Wilcoxon Rank Sum Test) (Placebo): In this analysis, we shifted our focus to Placebo as pivotal point. We compared both preterm and term groups data with serum analytes (AMH, AFP and CRP) by "Mann-Whitney U test". The results showed statistical significance in AFP 1 analyte that indicates change in the maternal serum alfafeto protein level in early pregnancy.

Table 8: Comparison of preterm and term groups with AMH, AFP and CRP by Mann-Whitney U test (Wilcoxon Rank Sum Test) (Placebo):

Variables	Preterm				Term				U-value	Z-value	p-value
	n	Mean	SD	Mean Rank	n	Mean	SD	Mean Rank			
AMH 1	23	2.19	2.25	52.91	76	1.68	1.41	49.12	807.00	-0.5551	0.5788
AFP1	25	18.33	11.14	40.08	74	25.72	17.08	53.35	677.00	-1.9974	0.0458*
CRP 1	22	1.96	2.01	41.64	71	2.38	1.91	48.66	663.00	-1.0668	0.2861
AMH 2	14	1.71	1.37	34.04	47	1.61	1.81	30.10	286.50	-0.7289	0.4661
AFP2	18	72.62	75.58	33.44	54	69.48	40.39	37.52	431.00	-0.7153	0.4745
CRP 2	14	2.76	3.55	24.93	49	3.75	2.96	34.02	244.00	-1.6367	0.1017

*$p<0.05$

Comparison of preterm and term groups with AMH, AFP and CRP (Aspirin): In this analysis, we shifted our focus to Aspirin as pivotal point. We compared both preterm and term groups data with serum analytes (AMH, AFP and CRP) by "Mann-Whitney U test". The results showed no statistical significance in any of the serum analytes.

Table 9: Comparison of preterm and term groups with AMH, AFP and CRP (Aspirin)

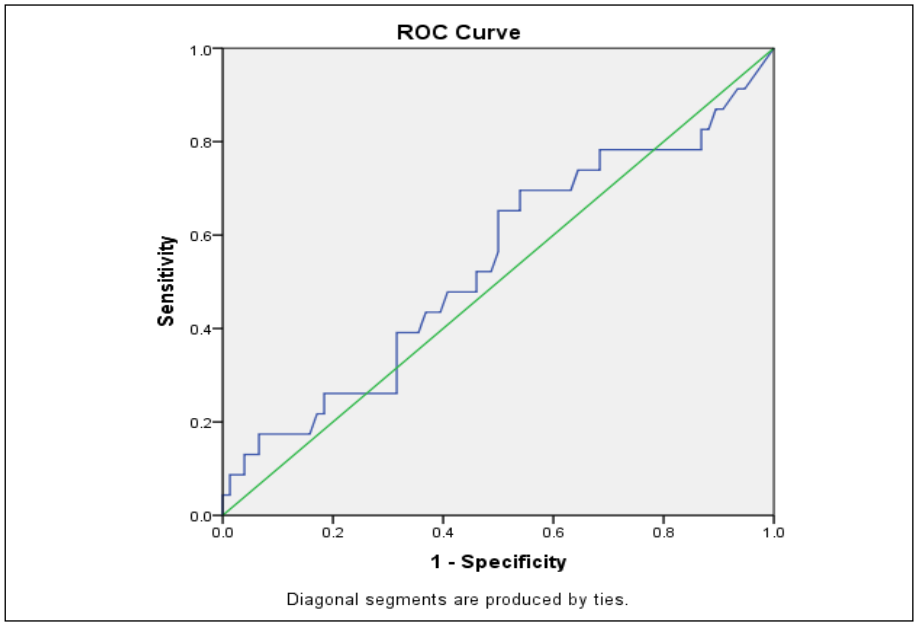
Variables	Preterm				Term				U value	Z value	P value
	n	Mean	SD	Mean Rank	n	Mean	SD	Mean Rank			
AMH 1	14	1.64	1.01	38.36	76	2.13	1.47	46.82	432.00	-1.1133	0.2656
AFP1	13	17.28	7.93	50.62	77	17.34	13.14	44.64	434.00	-0.7633	0.4453
CRP 1	15	1.88	1.63	37.20	66	2.24	1.81	41.86	438.00	-0.6930	0.4883
AMH 2	8	1.54	1.03	29.31	44	1.39	1.06	25.99	153.50	-0.5706	0.5682
AFP2	9	47.68	31.05	22.22	48	61.97	34.84	30.27	155.00	-1.3349	0.1819
CRP 2	9	4.17	2.72	31.33	39	2.86	2.51	22.92	114.00	-1.6245	0.1043

Models using Anti Mullerian Hormone (AMH1)

We developed first trimester model using AMH1. This model predominately concentrated on analyzing the early changes occurring in early pregnancy.

ROC AMH 1: We developed ROC curve chart to analyze a) AMH1 with preterm and term delivery insights, b) Sensitivity & Specificity impact on AMH1 and c) Positive & Predictive values of AMH1

Figure 8: ROC AMH 1:



Comparison of preterm and term delivery with AMH1 analyte and its Sensitivity and Specificity data: Statistical comparison of preterm and term data was made keeping AMH 1 as single analyte. With the available participants data, we took ROC curve value of AMH1 in the range of >= 1.61 and <=1.60 and found out that the result proportionally varies in accordance with participant values for both preterm and term.

With help of ROC curve we calculated the Sensitivity and Specificity of AMH 1 levels and also the positive and negative predictive values.

Table 10: Comparison of preterm and term delivery with AMH1 Sensitivity and specificity

AMH 1	Preterm	Term	Total	%
>=1.61	12	36	48	48.48
<=1.60	11	40	51	51.52
Total	**23**	**76**	**99**	**100.00**
%	23.23	76.77	100.00	

Table 11: Sensitivity and Specificity

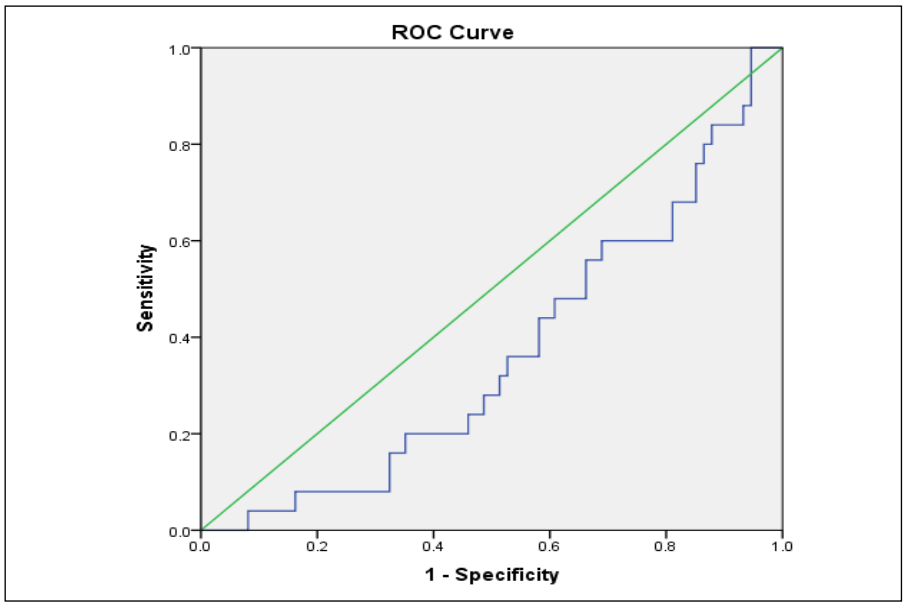
Statistic	Value	95% CI
Sensitivity	52.17%	30.59% to 73.18%
Specificity	52.63%	40.84% to 64.21%
Positive Predictive Value	25.00%	17.42% to 34.50%
Negative Predictive Value	78.43%	69.29% to 85.42%
Accuracy	52.53%	42.24% to 62.66%

Models using Alfa Feto Protein (AFP1)

Alpha-fetoprotein levels were altered in the first trimester with various factors. We studied AFP1 as a single analyte.

ROC AFP1: We developed ROC curve chart to analyze a) AFP1 with preterm and term delivery insights, b) Sensitivity & Specificity impact on AFP1 and c) Positive & Predictive values of AFP1

Figure 9: ROC AFP 1



Comparison of preterm and term delivery with AFP1 analyte and its Sensitivity and Specificity data: Statistical comparison of preterm and term data was made keeping AFP1 as single analyte. With the available participants data, we took ROC curve value of AFP1 in the range of >= 18.43 and <=18.43 and found out that the result Consistently differs in accordance with values for both preterm and term research subjects.

With help of ROC curve, we calculated the Sensitivity and Specificity of AFP 1 levels and also the positive and negative predictive values.

Table 12: Comparison of preterm and term delivery with AFP1

AFP 1	Preterm	Term	Total	%
<=18.43	16	31	47	47.47
>18.43	9	43	52	52.53
Total	**25**	**74**	**99**	**100.00**
%	25.25	74.75	100.00	

Table 13: Sensitivity and specificity

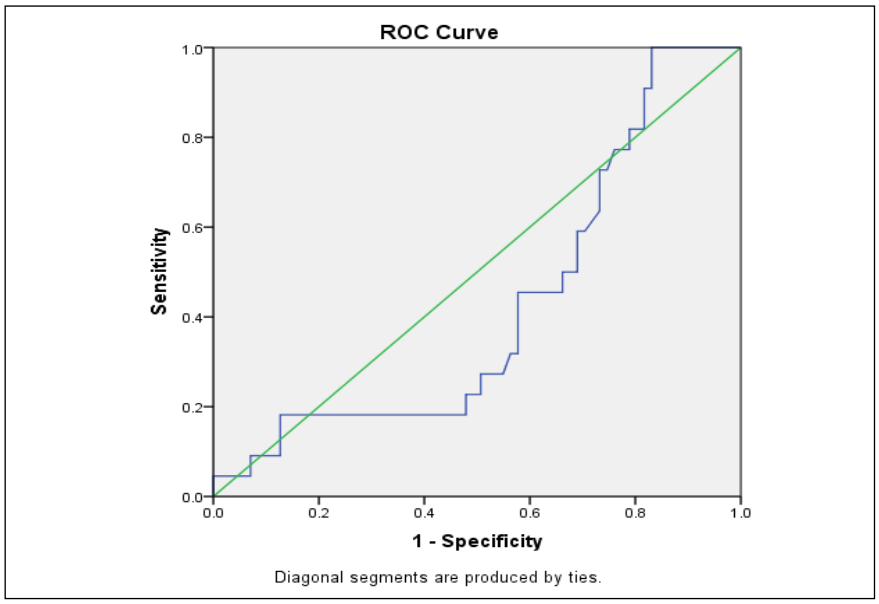
Statistic	Value	95% CI
Sensitivity	64.00%	42.52% to 82.03%
Specificity	58.11%	46.06% to 69.49%
Positive Predictive Value	34.04%	25.74% to 43.45%
Negative Predictive Value	82.69%	73.24% to 89.30%
Accuracy	59.60%	49.26% to 69.34%

Models using C Reactive Protein (CRP1)

We studied that, C Reactive protein levels altered in first trimester of pregnancy with various factors. We spent considerable amount of time studying CRP1 as a single analyte.

ROC CRP1: We developed ROC curve chart to analyze a) CRP1 with preterm and term delivery insights, b) Sensitivity & Specificity impact on CRP1 and c) Positive & Predictive values of CRP1

Figure 10: ROC CRP 1:



Comparison of preterm and term delivery with C Reactive Protein and its Sensitivity and Specificity data: Statistical comparison of preterm and term data was made keeping CRP1 as single analyte. With the available participants data, we took ROC curve value of CRP1 in the range of >= 1.52 and <=1.52 and found out that the result for both preterm and term subjects are comparable in values.

With help of ROC curve, we calculated the Sensitivity and Specificity of CRP1 levels and also the positive and negative predictive values.

Table 14: Comparison of preterm and term delivery with CRP1

CRP 1	Preterm	Term	Total	%
<=1.52	13	30	43	46.24
>1.52	9	41	50	53.76
Total	**22**	**71**	**93**	**100.00**
%	23.66	76.34	100.00	

Table 15: Sensitivity and specificity

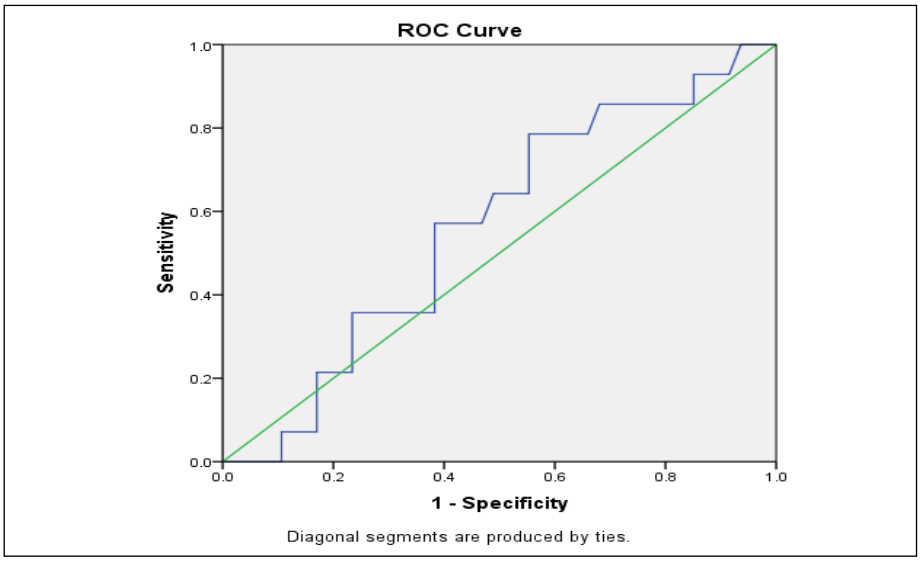
Statistic	**Value**	**95% CI**
Sensitivity	59.09%	36.35% to 79.29%
Specificity	57.75%	45.44% to 69.39%
Positive Predictive Value	30.23%	21.80% to 40.26%
Negative Predictive Value	82.00%	72.63% to 88.66%
Accuracy	58.06%	47.38% to 68.22%

Models using Anti Mullerian Hormone (AMH2)

After completing AMH1 for first trimester, we developed second trimester model using AMH2. This model predominately concentrated on analyzing the changes occurring during the most important phase of pregnancy.

ROC AMH2: We developed ROC curve chart to analyze a) AMH2 with preterm and term delivery insights, b) Sensitivity & Specificity impact on AMH2 and c) Positive & Predictive values of AMH2.

Figure 11: ROC AMH 2



Comparison of preterm and term delivery with AMH2 analyte and its Sensitivity and Specificity data: Statistical comparison of preterm and term data was made keeping AMH2 as single analyte. With the available participants data, we took ROC curve value of AMH2 in the range of >= 1.41 and <=1.40 and found out that the result proportionally varies in accordance with participant values for both preterm and term.

With help of ROC curve we calculated the Sensitivity and Specificity of AMH2 levels and also the positive and negative predictive values.

Table 16: Comparison of preterm and term delivery with AMH2

AMH2	Preterm	Term	Total	%
>=1.41	18	8	26	42.62
<1.40	29	6	35	57.38
Total	**47**	**14**	**61**	**100.00**
%	77.05	22.95	100.00	

Table 17: Sensitivity and specificity

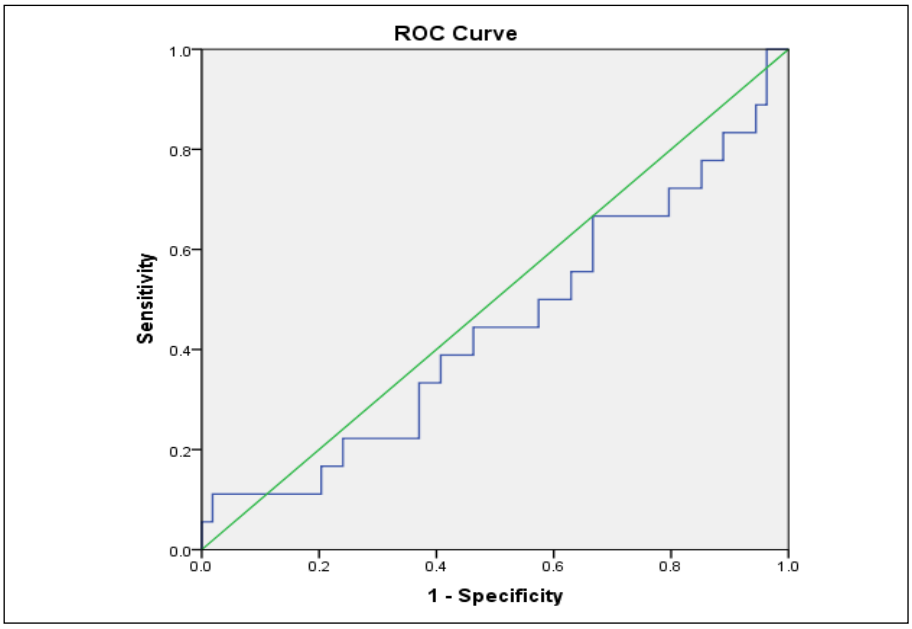
Statistic	Value	95% CI
Sensitivity	38.30%	24.51% to 53.62%
Specificity	42.86%	17.66% to 71.14%
Positive Predictive Value	69.23%	55.72% to 80.09%
Negative Predictive Value	17.14%	9.79% to 28.29%
Accuracy	39.34%	27.07% to 52.69%

Models using Anti Mullerian Hormone (AFP2)

After completing AFP1 for first trimester, we developed second trimester model using AFP2. AFP levels during the second trimester of pregnancy are altered in pregnancies with various factors. We studied AFP2 as a single analyte.

ROC AFP2: We developed ROC curve chart to analyze a) AFP2 with preterm and term delivery insights, b) Sensitivity & Specificity impact on AFP2 and c) Positive & Predictive values of AFP2

Figure 12: ROC AFP 2



Comparison of preterm and term delivery with AFP2 analyte and its Sensitivity and Specificity data: Statistical comparison of preterm and term data was made keeping AFP2 as single analyte. With the available participants data, we took ROC curve value of AFP2 in the range of >= 51.71 and <= 51.71 and found out that the result proportionally varies in accordance with participant values for both preterm and term.

With help of ROC curve we calculated the Sensitivity and Specificity of AFP2 levels and also the positive and negative predictive values.

Table 18: Comparison of preterm and term delivery with AFP2

AFP2	Preterm	Term	Total	%
<=51.71	23	10	33	45.83
>51.71	31	8	39	54.17
Total	**54**	**18**	**72**	**100.00**
%	75.00	25.00	100.00	

Table 19: Sensitivity and specificity

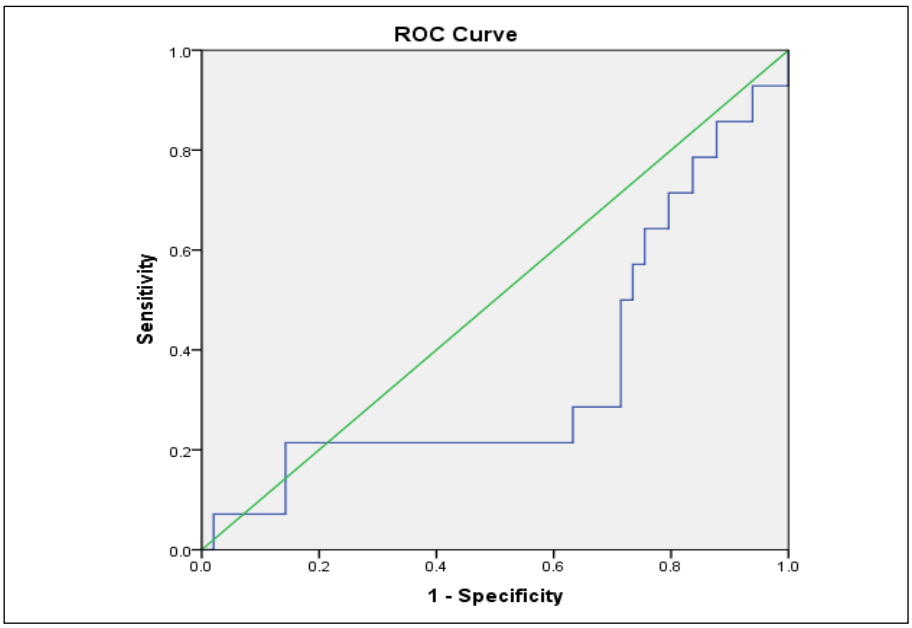
Statistic	Value	95% CI
Sensitivity	42.59%	29.23% to 56.79%
Specificity	44.44%	21.53% to 69.24%
Positive Predictive Value	69.70%	57.85% to 79.40%
Negative Predictive Value	20.51%	12.79% to 31.23%
Accuracy	43.06%	31.43% to 55.27%

Models using C Reactive Protein (CRP2)

We studied that, C Reactive protein levels during the first trimester of pregnancy are altered in pregnancies with various factors. We spent considerable amount of time studying CRP2 as a single analyte.

ROC CRP2: We developed ROC curve chart to analyze a) CRP2 with preterm and term delivery insights, b) Sensitivity & Specificity impact on CRP2 and c) Positive & Predictive values of CRP2

Figure 13: ROC CRP 2



Comparison of preterm and term delivery with C Reactive Protein and its Sensitivity and Specificity data: Statistical comparison of preterm and term data was made keeping CRP2 as single analyte. With the available participants data, we took ROC curve value of CRP2 in the range of >= 1.51 and <=1.51 and found out that the result proportionally varies in accordance with participant values for both preterm and term.

With help of ROC curve we calculated Sensitivity and Specificity of CRP2 levels and also positive and negative predictive values.

Table 20: Comparison of preterm and term delivery with CRP2

CRP2	Preterm	Term	Total	%
<=1.51	14	8	22	34.92
>1.51	35	6	41	65.08
Total	**49**	**14**	**63**	**100.00**
%	77.78	22.22	100.00	

Table 21: Sensitivity and specificity

Statistic	Value	95% CI
Sensitivity	28.57%	16.58% to 43.26%
Specificity	42.86%	17.66% to 71.14%
Positive Predictive Value	63.64%	48.14% to 76.74%
Negative Predictive Value	14.63%	8.36% to 24.35%
Accuracy	31.75%	20.58% to 44.69%

DISCUSSION

PTB, global incidence of higher than 15 million neonates being born prematurely annually, has been considered to be a key health issue during perinatal period, both in respect to related mortality and morbidities and, overall effect on economy [144]. Worldwide, effort towards decreasing the Preterm Birth (PTB) rates has not seen much progress even with substantial developments at the care during perinatal period.[25] Forecasting and prevention of PTB is challenging due to fact that spontaneous PTB being a complex process and numerous pathways are responsible for its pathogenesis. Hence, it becomes difficult to predict the PTB and it poses an ongoing and substantial encounter in the field of maternal and fetal medicine.

There has been Key efforts made to identify biomarkers for inflammation in predicting PTB among women with and without symptoms as well as to advance our understanding of underlying mechanism and paths that lead to PTB. Although, numerous biomarkers are studied to predict PTB, limited number of them have been demonstrated as valuable in clinical settings.[28, 145]

To advance our knowledge in predicting PTB, we should consider and note that it is a multifactorial condition and does not have a one answer or treatment, but, it occurs due to many coinciding patho-physiologic pathways. Undeniably, one single biomarker would not be useful in precisely predicting the sPTB[145-147]. Since, the pathophysiology of PTB is multi factorial, where several pathways and protein bio-markers are tangled, immuno-assays that are capable of analysing multiple protein markers is presented during the last decade, which has been useful in concurrent assay

of numerous markers representing diverse and distinctive biological pathways in predicting the risk of PTB in a better way[148]

Our study demonstrates that low AFP was associated with early PTB before 34 weeks. Similarly, we saw a trend with CRP but this was not statistically significant and directionally opposite of what we would of clinically anticipated (higher CRP being associated with lower rates of PTB before 34 weeks). Evidence indicates that bad pregnancy outcomes such as PTB and placental ischemic diseases are associated with AFP levels in mothers[108,109,149-152]. While our results did not find a association between first trimester AFP and other obstetric outcomes, this finding may be due to the limitations in the sample size. Not surprisingly, this difference did not persist when the group of women randomized to ASPIRIN were uniquely examined. Hence, results submit that AFP assay during the first trimester may be used as biomarker in understanding the LDA effectiveness; still, larger studies to validate these findings are required.

In compared to AFP, AMH and CRP levels did not differ at first or second trimester time points. AMH has been found previously to be related to PTB; however, this has been looked in different settings; for .eg. among women having infertility problems that interconnect with other medical conditions[153, 154].

Finally, CRP being marker of inflammation, we found a trend with preterm birth before 34 weeks but no other obstetrical outcomes.

This study had numerous strengths as well as limitations. First of all, ours is an ancillary study nested within a well-defined prospective randomized controlled trial. Additional advantage of blinding/masking to treatment groups was preserved

over the entire study duration. Secondly, GA was confirmed by using early dating ultrasound. Limitations of our study includes; issues with availability of specimens for assay due to technical challenges encountered during the analysis. Furthermore, we had a limited sample size and hence, study may be lacking acceptable power for detecting the significant differences.

CONCLUSION

The present study aimed to assess potential protein markers (AMH, msAFP & hs CRP) serially in early pregnancy among nulliparous rural pregnant women at 10-13 weeks and 17-21 weeks of gestational age and also the efficacy of two serum based potential protein marker tests among women who receive LDA and those who do not receive LDA in ASPIRIN Trial .

Our study results suggest that AFP levels during the early pregnancy may predict preterm birth prior to 34 weeks; however, is not predictive of other obstetrical outcomes. Unfortunately, possibly because of limited sample size, we were not able to show AMH, AFP and CRP levels as predicting parameters of the other key outcomes of perinatal period.

SUMMARY

Majority of women and associated families go through an important phase in their life. This phase called "**Pregnancy**" is a nine-month period of complex physiological interactions involving mother, fetus and placenta, and the goal is to create and sustain an ideal intrauterine setting for growth and development of the fetus. Creating and managing this balance is a composite event and it is definitely not simpler one. Slight alterations in this complex physiological process can result in profound consequences; most importantly preterm birth (**PTB**) and Hypertensive disorders of pregnancy. PTB and pre-eclampsia are believed to originate in early gestation and major causes for morbidity and mortality in perinatal period.

It has been recognized that PTB is an outcome of several biological pathways including; inflammation, myometrium capacitance, abruption and activation of the maternal hypothalamo-pituitary axis. This level of complexity has hindered the effective diagnostics as well as treatment strategies. To disentangle this biological Gordian knot, the collection and assessment of markers from who undergo preterm birth and those who receive an intervention must be considered. The ASPIRIN study at Jawaharlal Nehru Medical College, Belagavi Karnataka India, Global Network site number 08 gave us a unique opportunity to collect and assess the potential protein markers (AMH, msAFP & hs CRP) among rural nulliparous pregnant woman for understanding the biology of preterm birth. In our study, we examined potential protein markers (AMH, msAFP & hs CRP) serially in early pregnancy among pre-term and term labour and also tested efficacy of two serum based potential protein markers among women who receive LDA and those who do not receive LDA in ASPIRIN Trial.

Ethical approval was granted from J.N.M.C Institutional ethics committee, Belagavi. The written informed consent was taken from the participants before enrolling into the study.

This was designed as a nested case-control study on women who enrolled for ASPIRIN trial implemented by the JNMC Women's and Children's Health Research Unit as part of the NICHD Global Network for Women's and Children's Health Research common protocol. This research work was carried out over a period of two years (2016 to 2018) by enrolling 666 nulliparous pregnant women.

The data collection instrument for the study was organized and details of all the participants were documented after appropriate assessment & scrutiny. Nulliparous pregnant woman in the first trimester (10 to 13 weeks) and second trimesters (17 to 21 weeks) were included. The participants/enrolled women a) who might have received LDA and b) those who might not have received LDA in ASPIRIN trial. Goal was established to recruit about 50 preterm delivery (cases) and 150 women delivering at term (controls) in 1:3 case control ratio

Venous blood sample (about 4 to 5 mL) was collected from the participants under aseptic precautions. Serum was then separated after 30 minutes of collection by centrifugation. Serum stored in 2 ml cryovials and transported under cold chain to Dr Prabhakar Kore Basic Science Research Centre of KLE Academy of Higher Education and Research, Belagavi and stored at -80^0C until further analysis.

Assay of AFP, AMH and CRP were done by standard immuno-assays as recommend in kit literature. AFP and AMH were done by Immuno-metric assays (R and D systems: DAFP00 and DY1737 respectively) and CRP was done by Immuno-turbidimetric methods

After analyzing the samples, we took exercise to exclude outliers as wide variety of data spread alongside normal curve.. We adopted the normalization methods. The data was analyzed by using Mann-Whitney U test (Wilcoxon Rank-Sum tests), Z-test and Correlation analysis.

Total of 666 nulliparous pregnant women participated in this study / research work. Total of 6 women had abortion within the first 16 weeks of gestation. We were able to gather and analyze samples of 275 women. Out of these, 42 women (Group 1) experienced preterm delivery and 233 women (Group 2) experienced term delivery.

Our study demonstrates that low AFP was associated with early PTB before 34 weeks. Similarly, we saw a trend with CRP but this was not statistically significant and directionally opposite of what we would of clinically anticipated (higher CRP being associated with lower rates of PTB before 34 weeks). Evidence indicates that bad pregnancy outcomes such as PTB and placental ischemic diseases are associated with AFP levels in mothers. While our results did not find a association between first trimester AFP and other obstetric outcomes, this finding may be due to the limitations in the sample size. Not surprisingly, this difference did not persist when the group of women randomized to ASPIRIN were uniquely examined. Hence, results submit that AFP assay during the first trimester may be used as biomarker in understanding the LDA effectiveness; still, larger studies to validate these findings are required. In compared to AFP, AMH and CRP levels did not differ at first or second trimester time points.

This study had numerous strengths as well as limitations. First of all, ours was an ancillary study nested within a well-defined prospective randomized controlled trial. Additional advantage of blinding/masking to treatment groups was preserved

over the entire study duration. Secondly, GA was confirmed by using early dating ultrasound.

Limitations of our study includes; issues with availability of specimens for assay due to technical challenges encountered during the analysis. Furthermore, we had a limited sample size and hence, study may be lacking acceptable power for detecting the significant differences.

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ANNEXURES

ANNEXURE – I ETHICAL CLEARANCE LETTER

KLE UNIVERSITY
(Formerly known as KLE Academy of Higher Education & Research, Belagavi)
[Declared as Deemed-to-be-University u/s 3 of the UGC Act, 1956 vide Government of India Notification No.F.9-19/2008-U.3(A)]
'Accredited 'A' Grade by NAAC Placed in Category 'A' by MHRD (GoI)
Director, Academic Affairs
JNMC Campus, Nehru Nagar, Belagavi-590 010, Karnataka State, India
☎: 0831-2444444/2493779 FAX: 0831-2493777 Web: http://www.kleuniversity.edu.in E-mail: info@kleuniversity.edu.in

Ref.No.KLEU/Ethic/2016-17/D225 27th August 2016

To,
Dr. Ramesh R Araganji
Ph.D. Part-Time Research Scholar,
2015-16 Batch,
KLE University,
Belagavi

Dear Research Scholar,

Sub:- Regarding Ethical Clearance.

The KLE University **Ethics Committee on Human Subjects** for Ph. D Research Project met on **Wednesday, 29th June 2016** to consider your application for approval of the research project **"Evaluation of markers of preterm birth in early pregnancy among rural pregnant women of Belagavi District – Nested case control study".**

As there are no ethical issues involved in your proposed research project, the Committee has provided approval for this research project.

You are requested to report to Ethical Committee in case of the following:

1. Any deviation from or change of the protocol.
2. All serious adverse events.
3. Any changes in study documents.

(Dr. Anita Dalal)
Member Secretary,
Ph.D. Ethical Committee(Human),
K.L.E. University,
Belagavi.

(Dr. Anil Hogade)
Chairman
Ph.D. Ethical Committee(Human),
K.L.E. University,
Belagavi.

CC to: - The Director Academic Affairs, KLE University, Belagavi.
- The Director Research Foundation, KLE University, Belagavi.
- The Registrar, KLE University, Belagavi

ANNEXURE-II

INFORMED CONSENT FORM

Study Title: "**Evaluation of markers of preterm birth in early pregnancy among rural pregnant women of Belagavi District. Nested case control study.**"

Investigators:

Dr. Ramesh Araganji,

Research Scholar

Dept. of Physiology

KLE University's J N Medical College, Belagavi

Dr. Shivaprasad .S. Goudar,

Professor of Physiology,

Dept. of Physiology

KLE University's J N Medical College, Belagavi

Invitation for participation

You have been requested to participate in the research because you are selected into the study group/comparative group. A member of the research team will describe the study to you and answer all of your questions. Please read the information below and ask questions about anything you don't understand before deciding whether or not to take part. You may also request that the research staff read the form to you.

Purpose of the study

The purpose of this study is to evaluate the markers of preterm birth in early pregnancy among rural pregnant women of Belagavi District.

Who will be in study?

Research participants aged 18-40 years who are eligible as per ASPIRIN trial inclusion & exclusion criteria & screened will be enrolled at the time of data collection from the rural areas of Belagavi District.

Procedure

If you are agreed to participate in this study, you will be given a set of Questionnaire to which you are supposed to answer to the best of your knowledge. Socio demographic history pertinent to the study will be taken and tests mentioned in the study, which is collection of blood sample will be done first during 10-13 weeks and second during 17-21 weeks of pregnancy after explaining to you the nature and procedure of those tests and after taking ethics into consideration and other physiological parameters like blood pressure, height, weight and haemoglobin levels also will be recorded. Sample collection will done in Primary Health Centres by trained Lab Technicians and collected samples will be stored at -80^{o} C in KLE's BSRC Belagavi. The samples of the pregnant ladies who go into preterm labor only will be analysed. The following serum markers AMH, MSAFP and HSCRP will be analysed.

Possible benefits/ risk:

The inconveniences include visiting the local health centre or community centre for measurements of blood pressure, height and weight. The discomforts are limited to the collection of blood from a vein in your arm. At 2 times during the study, about 5ml of blood will be removed from you by putting a needle into a vein in your arm. This is the standard method used to obtain blood for tests. You may feel pain when the needle goes into the vein. A bruise may form at the site. 10ml of blood will be taken for research purposes over the course of this study.

Voluntary participation / withdrawal

Taking part in this study is voluntary. You have the right to refuse to participate or to withdraw your participation at any time. If you refuse or decide to withdraw, you will not lose any benefits or rights to which you are entitled. These actions will not have any negative effect on the health care you receive from your local health providers. You will still receive your normal medical care.

Compensation:

In the event that you become injured as a result of taking part in this study, treatment will be offered to you or you will be provided with information about where to receive medical care. No reimbursement, compensation or free medical care is offered by JNMC, Belgaum.

Confidentiality / Privacy

All the information collected about you during the course of this study will be kept confidential to the extent permitted by the law. You will be identified in research records by a coded number. The results from the research may be in published articles. Your name will be kept private when information is presented.

What should you do if you have additional questions?

If you have any questions regarding the study, you may contact the investigators Dr Shivaprasad S Goudar, Professor, Department of Physiology, KLE University's Jawaharlal Nehru Medical College, Belgaum at +91 94481 26371 or Dr. Ramesh Araganji, Research Scholar, Department of Physiology, KLE University's Jawaharlal Nehru Medical College, Belgaum at +91 95908 56885. If you have questions about your rights as a project participant, please contact Dr Subarna Roy, Chairman, JNMC Institutional Ethics Committee for Human Subjects Research, Belgaum 590010

Institutional / sponsor policy

In case of any research related injury the researcher will be liable to pay for the treatment at KLES Dr. Prabhakar Kore Hospital & Medical Research Centre, Belgaum

Financial incentives for participation

Your participation is voluntary and you will not be paid any remuneration for your participation in the study or for your expenses.

Agreement to be in this study

I have read this paper about the study or it was read to me. I understand the possible risks and benefits of this study. I know that being in this study is voluntary. I choose to be in this study: I will get a copy of this consent form.

Signature:_____ Date:_____

(Participant)

Print Name:_____

Consent form explained by:_____ Date:_____

Print Name: _____

(witness)

Signature:_____ Date:_____

Relationship with participant:_____

Investigator:_____ Date:_____

ANNEXURE – III

PROFORMA

Title: "Evaluation of markers of preterm birth in early pregnancy among rural pregnant women of Belagavi District. Nested case control study"

Personal detail:

Serial Number	
ID Number	
Name:	
Address:	
Contact Number:	
Age:	
Sex:	Female
Education:	
Occupation	: Employed/ self-employed/laborer/agriculture/others
Habits:	

Examination:

Parameters	Between 10 to 13 Weeks of Pregnancy	Between 17 to 21 Weeks Pregnancy
Height(cm)		
Weight(Kg)		
BP(mmHg)		
Hb(gm%)		
Markers Anti mullerian Hormone		
Maternal .Serum. Alpha feto Protein		
High Sensitivity C Reactive Protein		

ANNEXURE – IV

PUBLICATIONS

The Journal of Maternal-Fetal & Neonatal Medicine

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The impact of low-dose aspirin on markers of inflammation and placental function: an ancillary study of the ASPIRIN trial

Ramesh Araganji, Manjunath S. Somannavar, Sunil S. Vernekar, Avinash Kavi, Matthew K. Hoffman & Shivaprasad S. Goudar

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ORIGINAL ARTICLE

Check for updates

The impact of low-dose aspirin on markers of inflammation and placental function: an ancillary study of the ASPIRIN trial

Ramesh Araganji[a], Manjunath S. Somannavar[b], Sunil S. Vernekar[a], Avinash Kavi[c], Matthew K. Hoffman[d] and Shivaprasad S. Goudar[a]

[a]Department of Physiology, J N Medical College, KLE Academy of Higher Education and Research, Belagavi, India; [b]Department of Biochemistry, J N Medical College, KLE Academy of Higher Education and Research, Belagavi, India; [c]Department of Community Medicine, J N Medical College, KLE Academy of Higher Education and Research, Belagavi, India; [d]Christiana Care, Newark, DE, USA

ABSTRACT

Objective: To determine the impact of low-dose aspirin (81 mg) on markers of maternal inflammation and placental function.
Setting: Rural Southern India
Population: Nulliparous women with a singleton pregnancy dated by ultrasound who were enrolled in the ASPIRIN (Aspirin Supplementation for Pregnancy Indicated risk Reduction In Nulliparas) Trial.
Methods: We performed a case-control study to elucidate the impact of low dose aspirin (LDA) on markers of placental function and maternal inflammation among women who delivered prematurely compared to term controls in women enrolled in the ASPIRIN trial. Women were prospectively enrolled in an ancillary observational trial wherein maternal serum was collected and measured between 10 to 13 weeks and 17 to 21 weeks of gestation after initiation of aspirin or an identical placebo.
Results: From 2016-18 with a total of 666 n women enrolled in this ancillary trial of whom 269 were selected for analyte analysis. Women who received LDA had lower levels of Alpha Feto-Protein (AFP) at 10 to 13 weeks than women who received placebo (Placebo) (LDA 18.3 ng/mL vs 21.4 ng/mL -P 0.001). AFP was similar between the two groups at 17 to 21 weeks. No other differences were seen in C-Reactive protein or Anti-Mullerian Hormone.
Conclusion: Low-dose aspirin administration lowers AFP early in pregnancy.

ARTICLE HISTORY
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KEYWORDS
Maternal serum alpha-fetoprotein (MSAFP); preterm birth; aspirin; preclampsia; fetal growth restriction

Introduction

Background

The World Health Organization estimates that 15 million children are born prematurely each year resulting in the second leading cause of neonatal mortality throughout the world and in particular low-middle income countries [1,2]. In addition to its significant contribution to mortality, the impact of preterm birth amongst children born prematurely often continues throughout their lifespan. Compared to children born at term, children born prematurely have higher rates of cerebral palsy, milestone delay, impairment in learning, visual disorders and higher rates of long-term physical health problems [3–5].

Recently, the Aspirin Supplementation for Pregnancy Indicated Risk Reduction In Nulliparas (ASPIRIN) trial demonstrated that nulliparous women with no more than two previous first-trimester pregnancy losses treated with Low Dose Aspirin(LDA) 81 mg daily beginning between 6 0/7 weeks and 13 6/7 weeks through 36 0/7 weeks gestational age have lower rates of preterm birth from all causes and a decrease in perinatal mortality [6]. Though the ASPIRIN trial holds the promise of a therapeutic option for the prevention of preterm birth, the mechanism by which it works has not been fully elucidated. LDA has been well chronicled to affect both the COX-1 (thrombosis pathway) and the COX-2 (inflammatory pathway); however, the exact pathway through which it exerts its effect remains unclear [7,8]. We thus sought to examine the impact of LDA on both inflammatory markers and established markers of placental function amongst women who received either LDA or placebo in the ASPIRIN trial.

CONTACT Manjunath S. Somannavar manjunathsomannavar@gmail.com Department of Biochemistry, J N Medical College, KLE Academy of Higher Education and Research, Belagavi, India

Material and methods

We performed a case-control study of women enrolled within the study within the ASPIRIN Trial conducted at the J N Medical College, Belagavi, Karnataka, India. (JNMC Women's and Children's Health Research Unit; Global Network for Women's and Children's Health Research). Specifically, we identified women who delivered before 37 weeks (Cases) and women who delivered at term (controls). As this analysis was exploratory in nature with no prior data documenting differences due to aspirin treatment in pregnancy, we were not able to calculate the power analysis. We thus established a goal of including approximately 50 preterm birth cases and 150 term controls. Either sample limitations or technical challenges in laboratory measurement intermittently precluded achieving the full 50/150 ratio. Due to sample limits, a separate either preterm or control case was chosen rather than having a fixed cohort of patients used for all specimens. The study protocol was approved by the ethics committee of the institution and women were individually consented for participation. As part of the primary study, women were included if they were nulliparous with no more than 2 prior pregnancy losses, pregnant with a singleton pregnancy between 6 weeks and 13 weeks and 6 days as verified by ultrasound. Women with fetal anomalies or medical conditions contraindicated to receive LDA were excluded. About two weeks after randomization to either LDA or placebo, serum was obtained and stored at -80^0C degree until analysis. A second sample was obtained at 17-21 weeks gestation and similarly stored. Women who experienced an abortion prior to 16 weeks were excluded.

We chose markers AFP and AMH, either because in prior publications they were associated with poor outcomes that low dose aspirin has been shown to positively affect (preterm birth, preeclampsia and fetal growth restriction) or in the case of C reactive protein a well-established marker of chronic inflammation.

Measurements of the various analytes were performed using a standardized immunometric immunoassay (Alphafetoprotein [AFP]- R&D Systems, kit# DAFP00; Anti-Mullerian Hormone [AMH]-Catalog Number: DY1737 R&D Systems) and Immunoturbidimetric (C-Reactive Protein [CRP]- Roche Diagnostics) methods in accordance with the kit manufacturers protocol.

Statistical analysis

Our primary outcome was preterm birth defined as delivery before 37 weeks. Secondary outcomes of interest were PTB <34 weeks and hypertensive disorders of pregnancy. All continuous variables were assessed for normality using the Shapiro Wilk test. Bivariate analysis was performed using the Wilcoxon Rank Sum test where appropriate. Additionally, we compared the impact of LDA on these biomarkers on these analytes using a Wilcoxon Rank-Sum Test. All analyses were complete using STATA v15.1 (Colleges Station, TX).

Results

A total of 666 women participated in this investigation of which a total of 7 experienced an abortion prior to 16 weeks. A total of 269 women had sample results that were able to be included (45 preterm and 224 term). Demographic data of the total cohort are displayed in Table 1. Maternal demographics were noted to be similar between the two groups. Consistent with the results of the parent trial, women who received Aspirin had lower rates of preterm birth but this did not meet statistical significance in this much smaller cohort enriched with preterm birth cases. Similarly, birthweight tended to be greater in the Aspirin group and conversely the rate of small for gestational age (SGA) and birthweight <2500gm were lower in the Aspirin group. Of note the rate of birthweight <1500gm was noted to be similar.

Results of the analytes are displayed in Table 2. Variable numbers of samples are noted. This is noted to reflect participants declining a second blood draw, hemolysis encountered with certain samples and insufficient quantities of available blood. Notably, none of the values were normally distributed by the Sharipo–Wilk test and therefore only Wilcoxon Rank-Sum tests are reported. LDA use was associated with markedly lower levels of AFP at the first time point that blood was drawn. No difference was seen in the values of any of the analytes as they related to either preterm birth or hypertensive disorders of pregnancy; excepting that AFP at the time of the first blood draw was found to be lower. Neither of these differences persisted at the second time point.

The relationship of the varied measured analytes on gestational age is displayed in Table 3. Only C reactive protein at the first visit was shown to correlate with gestational age. This result was noted to be positive suggesting that higher-reactive protein is associated with longer gestational ages.

Table 1. Maternal demographics and birth outcomes.

	Aspirin ($N = 129$)	Placebo($N = 146$)	p Value
Demographics			
Age	20.6 ± 3.0	21.0 ± 3.2	.38
Education			
None	0.8%	5.5%	
Primary	3.1%	4.1%	
Secondary	81.4%	77.4%	
University+	14.7%	13.0%	.16*
Height Cm	151.8 ± 5.5	151.7 ± 5.4	.90
Weight Kg	47.1 ± 8.2	47.1 ± 7.5	1.0
BMI	20.4 ± 3.4	20.4 ± 3.0	.98
Prior Abortion	0.11 ± 0.34	0.10 ± 0.32	.78
Hb on enrollment	11.6 ± 1.4	11.4 ± 1.4	.28
Gestational Age at sample 1	11.6 ± 2.6	11.7 ± 5.1	.80
Gestational Age at sample 2	18.7 ± 2.2	19.6 ± 3.8	.16
Birth Outcomes			
Gestational Age Weeks	37.7 ± 5.2	37.6 ± 5.4	.90
Birth Weight (gm)	2759	2588	.005
PTB < 37 weeks	12.1%	18.4%	.18
PTB < 34 weeks	7.8%	7.5%	.94
Stillbirth	3.2%	5.7%	.39*
Hypertensive Disorders of Pregnancy (HDP)	11.3%	10.6%	.87
Small for gestational age	27.9%	45.0%	.006
<2500 gm	15.7%	34.8%	.001
<1500 gm	1.7%	4.4%	.29*

*Fisher's exact test.

Table 2. Serum analytes.

	Preterm (<37 Weeks)	N	Term	N	p Value
AFP1	18.3 ± 9.9	41	21.4 ± 15.7	151	.56
AFP2	64.9 ± 62.5	29	65.9 ± 37.9	102	.31
CRP1	1.9 ± 1.8	39	2.3 ± 1.9	137	.12
CRP2	3.3 ± 3.3	25	3.4 ± 2.9	88	.82
AMH1	2.0 ± 1.8	40	1.9 ± 1.5	152	.96
AMH2	1.7 ± 1.2	24	1.5 ± 1.5	91	.26
	PTB < 34 Weeks		No PTB ≤ 34 Weeks		
AFP1	16.4 ± 6.9	15	21.1	177	.48
AFP2	65.7 ± 41.0	9	66.9 ± 44.6	122	.82
CRP1	1.4 ± 0.8	13	2.3 ± 1.9	163	.21
CRP2	3.7 ± 4.2	8	3.3 ± 2.8	105	.91
AMH1	2.2 ± 1.6	14	1.9 ± 1.5	178	.51
AMH2	1.5 ± 1.1	9	1.55 ± 1.5	106	.69
	HDP		No-HDP		
AFP1	22.3 ± 14.9	25	20.5 ± 14.8	167	.44
AFP2	67.8 ± 47.3	16	65.3 ± 44.4	115	.87
CRP1	2.1 ± 1.8	23	2.3 ± 1.9	153	.65
CRP2	3.4 ± 2.3	13	3.3 ± 2.9	100	.62
AMH1	1.7 ± 1.7	26	2.0±1.5	166	.16
AMH2	1.2 ± 1.2	14	1.6 ± 1.5	101	.34
	Aspirin		Placebo		
AFP1	17.3 ± 12.3	92	23.9 ± 16.0	100	.001
AFP2	60.2 ± 34.1	59	70.3 ± 50.9	72	.41
CRP1	2.2 ± 1.8	82	2.3 ± 1.8	94	.90
CRP2	3.1 ± 2.5	50	3.5 ± 3.1	63	.57
AMH1	2.1 ± 1.4	92	1.8 ± 1.6	100	.10
AMH2	1.4 ± 1.1	54	1.6 ± 1.7	61	.70

NB: All p-values are Wilcoxon Ranks Sums.

Table 3. Analytes and gestational age.

Analyte	Coefficient (weeks/unit)	95% CI	p Value
AFP1	0.054	−0.002 to 0.11	.06
CRP1	0.44	0.14 to 0.88	.043
AMH1	−0.12	−0.64 to 0.40	.65
AFP2	0.002	−0.12 to 0.16	.73
CRP2	−0.02	−0.25 to 0.21	.87
AMH2	−0.033	−0.51 to 0.45	.89

Discussion

The results of our investigation suggest several interesting tenets about how the role in Aspirin in pregnancy. First and foremost, we noted that AFP is decreased at the time of the first blood draw amongst women who took aspirin compared to those who did not take aspirin. This difference was not noted at the second blood draw. AFP has been well documented to be associated with poor obstetrical outcomes including both preterm birth and ischemic placental diseases [9–14]. These are noted to include preterm birth, preeclampsia and fetal growth restriction. All of these outcomes have been shown in randomized controlled trials of aspirin to be positively impacted on maternal LDA consumption. Though our limited data did not show a correlation of first trimester AFP with any obstetric outcomes, this is probably a reflection of sample size. This data is suggestive that aspirin plays an important role in facilitating deep placentation [15,16] and that its early initiation is important as this process occurs largely before 18 weeks. Several meta-analyses have suggested that early initiation of aspirin is key to avoidance of preeclampsia [17–20]. This would suggest that measurement of AFP in the first trimester may be a useful biomarker to understand the efficacy of LDA in pregnancy; however, larger validation studies are necessary.

In contrast to AFP, we saw no differences in the levels of either AMH or CRP at either gestational time points. It should be noted that AMH was lower at both time points among women taking aspirin but

neither was statistically significant. AMH has been shown by others to be associated with preterm birth; albeit it has mostly been evaluated in the setting of women with infertility issues which may intersect with other medical conditions [21,22]. Finally, C-reactive protein is an acute marker of inflammation and may not be predictive of obstetrical conditions.

Our study has several strengths and weaknesses. First, this is an ancillary study on a well-described, prospectively phenotyped group of women and masking to treatment allocation was maintained throughout the study. Second, gestational age was validated using an early ultrasound. Weaknesses of our study include the fact that the overall numbers of specimens were variable based upon technical challenges with the assays. Additionally, our overall sample size is limited and therefore we may lack adequate power to detect meaningful differences.

In conclusion, our study suggests that AFP in the first trimester is reduced by LDA therapy. Unfortunately, most likely due to sample size, we are unable to demonstrate the predictive effects of AMH, AFP and CRP on important obstetrical outcomes.

Author contributions

RA conceived of the manuscript and wrote the first draft with input from MSS, MKH and SSG. AK, RA, SSV, MSS and SSG oversaw study implementation, data collection and quality monitoring. RA, AK, and MSS performed the statistical analyses. All authors reviewed and approved the final manuscript.

Clinical trial registration

ClinicalTrials.gov, NCT02409680, and the Clinical Trial Registry-India, CTRI/2016/05/006970.

Reprints: Reprints will not be provided.

Disclosure statement

The authors report no conflict of interest

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Research article

The utility of biomarkers in the prediction of preterm birth: A nested case control study of the ASPIRIN trial

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ABSTRACT

Introduction and Aim: Adverse pregnancy outcomes (APO's-preterm birth, foetal growth restriction and hypertensive disorders of pregnancy (HDP)) are the primary drivers of perinatal mortality. C-Reactive Protein, Anti-Mullerian Hormone (AMH) and Alpha-FetoProtein (AFP) have shown promise in predicting these APO's. Aim of this present work is to validate the predictive ability of these biomarkers to identify women at risk for preterm birth.

Materials and Methods: A nested observational case-control study was performed drawing nulliparous pregnant women between 10 to 13 weeks and 17 to 21 weeks of gestation in nulliparous women randomized to aspirin or placebo as part of the ASPIRIN (Aspirin Supplementation for Pregnancy Indicated Risk Reduction in Nulliparas) trial.

Results: A total of 126 women with these APO's and 143 controls were selected for analyte analysis. None of the chosen analytes were found to predict preterm birth before 37 weeks or HDP. AFP obtained between 10 to 13 weeks, was able to moderately discriminate against women who had a preterm birth prior to 34 weeks (C-statistic 0.65- 95% CI 0.55 to 0.78). No other analytes were found to be predictive of preterm birth prior to 34 weeks.

Conclusion: Elevated Alpha-FetoProtein early in pregnancy is associated with early preterm birth (PTB) and may be a marker of Aspirin efficacy.

Keywords: Maternal serum alpha-fetoprotein (MSAFP); anti-Mullerian Hormone (AMH); C-reactive protein (CRP); preterm birth; aspirin.

INTRODUCTION

Preterm birth currently affects 15 million children a year; resulting in 1 million neonatal deaths which disproportionately occur in low resource settings (1, 2). Beyond mortality, the impact of preterm birth frequently continues throughout the life of offspring resulting in higher rates of chronic medical conditions and developmental delays (3-5).

Prophylactic strategies such as progesterone (6,7), cervical surveillance (8), care management (9,10) and more recently low dose aspirin (11,12) therapy have been suggested approaches to prevent preterm birth (PTB); however, the ability to accurately identify at risk women to meaningfully direct resources to has been elusive. Several candidate biomarkers for PTB have been suggested including anti-mullerian hormone (AMH) (13-15), alpha fetoprotein (16) and c-reactive protein (CRP) (17). Nonetheless these studies are noted to be conflicting in their ability to predict preterm birth. We have earlier reported the impact of low-dose aspirin on markers of inflammation and placental function (18). This work was sought to validate the predictive ability of these biomarkers to identify women at risk for preterm birth in a single centre ancillary study of women participating in the global network study testing the effectiveness of low dose aspirin in reducing preterm birth (11).

MATERIALS AND METHODS

We performed a case control study of women participating in the ASPIRIN (Aspirin Supplementation for Pregnancy Indicated Risk Reduction in Nulliparas) Trial conducted at the Global Network site, J N Medical College, Belagavi Karnataka India. Main trial randomized nulliparous women with less than three previous first trimester

pregnancy losses and treated with Low Dose Aspirin (LDA) 81mg daily started between 6 0/7 weeks and 13 6/7 weeks continued through 36 0/7 weeks gestational age (GA) for the prevention of preterm birth (11). This ancillary study was approved by JNMC Institutional ethics committee (IRB00001499) and pregnant women were individually consented for participation. (Clinical Trial Registration: ClinicalTrials.gov, NCT02409680, and Clinical Trial Registry of India, CTRI/2016/05/006970).

As part of the study, serum samples were collected and stored in ultralow temperature freezers (-80 ^{0}C degree) until analysis between 10-13 weeks of gestation. Further, a second sample was collected from the same women during the second trimester of pregnancy (at 17-21 weeks' gestation). Women who had an abortion before 16 weeks of pregnancy were excluded from the analysis. Women delivering before 37 weeks of pregnancy were identified as cases and those women delivering at or after 37 weeks' gestation were considered as controls. Power analysis was not performed in this analysis due to the exploratory nature of the study. The goal of the analysis was to include about 50 cases (preterm birth) and about 150 controls (Term deliveries). We choose a separate case or control because of limitations in sample, rather than considering a fixed cohort of participants used for all specimens.

The quantitative estimation of AFP (Catalog Number: R&D Systems, kit# DAFP00) and AMH (Catalog Number: DY1737 R&D Systems) were conducted using standardized immunoassays adopted from the protocols of the manufacturer. CRP was estimated by Immunoturbidimetric assay ((Roche Diagnostics) as per the kit manufacturer's protocol.

The primary outcome of the study was to determine the predictive value of AMH, CRP or AFP in predicting (C-statistic) either alone or in combination for the prediction of PTB defined as delivery before 37 weeks of gestation. Those variables that demonstrated trend (e.g., had a p-value < 0.1) were in stepwise logistic regression to create a multivariable logistic model. Those that retained a p-value of <0.1 in multivariable modelling were retained. Models were then compared using the ROCCOMP command in Stata 15.0 (College Station Tx). Secondary outcomes of this analysis were PTB defined as birth less than 34 weeks of pregnancy and hypertensive disorders of pregnancy (HDP). Further, we also compared the impact of LDA on the predictive value of these biomarkers as it may have impacted the outcome of PTB, PTB before 34 weeks and HDP. An odd's ratio of each individual analyte was performed as well as subgroups of women who took aspirin or placebo as the use of aspirin may have altered these values. All of the analyses were performed using STATA v15.1 (Colleges Station, TX) (19).

RESULTS

A total of 666 women participated in this exploratory analysis of which 269 had results of the analyses and a total of seven participants experienced an abortion. Finally, 262 women were included (45 preterm cases and 217 term controls) in the analysis. Maternal demographics were similar between the women belonging to aspirin and placebo groups as well as the overall cohort of 269 women (table 1).

Table 1: Maternal Demographic parameters & Delivery Outcomes

	Aspirin	Placebo	P-value
	N= 126 (47.0%)	N=143 (53.0%)	
Demographics			
Age in years-IQR	20 (2)	20 (4)	0.57[a]
Education			
None- %	0.8	5.5	0.16[b]
Primary- %	3.1	4.1	
Secondary -%	81.4	77.4	
University & above-%	14.7	13	
Height-cm	151 (8)	152 (7)	0.69 [a]
Weight-Kg	45.9(11.7)	46.1 (10.5)	0.78 [a]
BMI	19.9 (4.8)	20.3 (4.2)	0.85 [a]
Prior Abortion	0 (0)	0 (0)	0.75
Hb on enrollment G/dL (IQR)	11.8(1.6)	11.6 (2)	0.02
Gestational Age at sample 1 in weeks (IQR)	11.4(2.3)	12.3(2.4)	0.16
Gestational Age at sample 2 in weeks (IQR)	17.9(3.3)	19.4 (3.3)	
Birth Outcomes			
Gestational Age in Weeks (IQR)	39.1 (2.1)	39.3 (2.9)	0.80
Birth Weight in gram (IQR)	2750 (480)	2600 (560)	0.0014
PTB <37 weeks	12.1%	18.4%	0.15
PTB <34 weeks	5.6%	7.0%	0.80

Stillbirth	3.2%	5.7%	0.39
Hypertensive Disorders of Pregnancy (HDP)	11.3%	10.6%	0.87
<2500 gm	15.7%	34.8%	<0.001
<1500 gm	1.7%	4.4%	0.29[b]
IQR-Interquartile range; Cm- Centimeter; Kg-Kilogram; BMI-Body Mass Index; PTB-Preterm Birth; gm-gram.			
[a]Wilcoxon Rank-Sum [b] Fisher's Exact			

Table 2 depicts the results of the analytes. Please note that the numbers are not equally distributed by analyte due to availability of specimens in each group or technical issues related to individual assays. The N of each specimen are included in the table. Wilcoxon Rank-Sum tests are reported as values for analytes were not normally distributed by the Shapiro-Wilk test. There was no difference in the analytes relating to either PTB or HDP; excepting that AFP was found to be lower (P=0.02) amongst mothers who delivered before 34 weeks of pregnancy at the first time point of blood collection. Though not statistically significant, CRP trended (P=0.09) lower amongst women who delivered prior to 34 weeks at the first blood draw. Recognizing that LDA therapy may have affected the outcome, the performance of each analyte overall and by their treatment (Aspirin vs. Placebo) using logistic regression is presented in table 3. As anticipated the first draw of AFP in women who received placebo remained statistically significant but not for women who received aspirin.

Table 2: Serum analytes and delivery outcomes

	Preterm (IQR)	N	Term (IQR)	N	P-value
AFP1 in ng/mL	15.4 (14.2)	38	17.2(17.2)	151	0.61
AFP2 (ng/mL)	47.8 (40.1)	27	55.5 (42.8)	102	0.20
CRP1 (mg/L)	1.1 (1.3)	37	1.8 (2.7)	137	0.26
CRP2 (mg/L)	1.5 (4.4)	25	2.6 (3.7)	88	0.78
AMH1 in ng/mL	1.7 (1.4)	37	1.8 (2.1)	152	0.73
AMH2 in ng/ml	1.4 (1.8)	22	1.1 (1.7)	91	0.41
	PTB<34 Weeks		No PTB<34 Weeks		
AFP1 (ng/mL)	17.4 (17.3)	20	11.0 (9.8)	178	0.02
AFP2 in ng/ml	51.1(21.2)	8	54.4(46.3)	123	0.7
CRP1 in mg/L	1.4 (1.4)	16	1.7 (2.6)	164	0.09
CRP2 in mg/L	1.6 (6.6)	7	2.5 (3.7)	106	0.93
AMH1 in ng/ml	1.9 (1.7)	18	1.8 (2.1)	179	0.26
AMH2in ng/ml	1.4 (1.7)	8	1.1 (1.7)	107	0.55
	HDP		No-HDP		
AFP1in ng/ml	18.3 (18.8)	25	17.1 (17.3)	173	0.44
AFP2 in ng/ml	50.1 (37.3)	16	54.4 (43.6)	115	0.87
CRP1 in mg/L	1.4(2.1)	23	1.7 (2.5)	153	0.65
CRP2 in mg/L	3.6 (3.9)	13	2.5 (3.7)	100	0.61
AMH1in ng/ml	1.5 (1.6)	26	1.8 (2.0)	171	0.16
AMH2in ng/ml	1.0 (1.1)	14	1.3 (2.0)	101	0.34
NB: All P-values are Wilcoxon Rank-Sum, Medians (Interquartile ranges) are displayed					
AFP1 and 2: Alpha fetoprotein at first & second trimester time points, CRP1 and 2: C Reactive Protein at first & second trimester time points: AMH1 and 2: Anti Mullerian Hormone at first & second trimester time points					

The table 3 depicts logistic regression analysis of the different analytes on PTB, PTB <34 weeks and HDP. The CRP levels at the time point of first blood draw correlated with GA. This positive association suggested that higher CRP levels are associated with longer GA. The AUCs of 3 different models of prediction of PTB<34 weeks (AFP1, CRP1, AFP1 and CRP1) are displayed in figure 1. AFP at the first blood draw was noted to be mildly predictive of preterm birth before 34 weeks (C-statistic 0.65- 95% CI 0.55 to 0.78). Recognizing CRP at the first blood draw tended to discriminate against women delivering preterm before 34 weeks, three models of prediction using AFP, CRP and AFP and CRP were created to predict preterm birth before 34 weeks. When compared statistically (ROCCOMP function in Stata), none of the models were superior to each other (P-values 0.11, 0.77 and 0.09; Fig. 1).

Table 3: Logistic regression of analytes and outcomes

Analyte	OR	95%CI	
Outcome Preterm<37 weeks			
CRP1	0.90	0.73	1.11
CRP1 Placebo	0.90	0.69	1.18
CRP1 Aspirin	0.90	0.63	1.27
CRP2	1.00	0.85	1.17
CRP2 Placebo	0.89	0.71	1.11
CRP2 Aspirin	1.22	0.92	1.60
AMH1	1.03	0.82	1.29
AMH1 Placebo	1.18	0.91	1.54
AMH1 Aspirin	0.75	0.47	1.20
AMH2	1.06	0.78	1.45
AMH2 Placebo	1.03	0.73	1.46
AMH2 Aspirin	1.11	0.55	2.24
AFP1	0.98	0.96	1.01
AFP1 Placebo	0.97	0.93	1.00
AFP1 Aspirin	1.00	0.96	1.05
AFP2	1.00	99.00	1.01
AFP2 Placebo	1.00	0.99	1.01
AFP2 Aspirin	0.98	0.96	1.01
Outcome Preterm<34 weeks			
CRP1	0.66	0.43	1.01
CRP1 Placebo	0.67	0.38	1.17
CRP1 Aspirin	0.65	0.33	1.18
CRP2	1.08	0.85	1.38
CRP2 Placebo	1.30	0.96	1.75
CRP2 Aspirin	1.22	0.92	1.60
AMH1	1.14	0.87	1.53
AMH1 Placebo	1.21	0.88	1.67
AMH1 Aspirin	1.01	0.59	1.75
AMH2	1.05	0.64	1.70
AMH2 Placebo	0.87	0.43	1.75
AMH2 Aspirin	1.59	0.64	4.00
AFP1	**0.93**	**0.88**	**0.99**
AFP1 Placebo	**0.90**	**0.83**	**0.98**
AFP1 Aspirin	0.97	0.90	1.05
AFP2	0.99	0.97	1.01
AFP2 Placebo	0.98	0.95	1.01
AFP2 Aspirin	1.00	0.97	1.03
Outcome Hypertensive Disorders of Pregnancy			
CRP1	0.95	0.74	1.22
CRP1 Placebo	0.84	0.58	1.20
CRP1 ASPIRIN	1.11	0.79	1.58
CRP2	1.01	0.83	1.24
CRP2 Placebo	0.88	0.64	1.20
CRP2 Aspirin	1.23	0.89	1.68
AMH1	0.86	0.63	1.16
AMH1 Placebo	1.04	0.75	1.45
AMH1 Aspirin	0.57	0.32	1.03
AMH2	0.79	0.49	1.27
AMH2 Placebo	0.87	0.56	1.37
AMH2 Aspirin	0.49	0.19	1.69
AFP1	1.00	0.98	1.04
AFP1 Placebo	1.01	0.98	1.04
AFP1 Aspirin	1.01	0.96	1.06
AFP2	1.01	0.99	1.01
AFP2 Placebo	1.01	0.99	1.02
AFP2 Aspirin	0.97	0.93	1.01
AFP1 and 2: Alpha-fetoprotein at first and second trimester time points, CRP1 and 2: C Reactive Protein levels at first and second trimester time points: AMH1 and 2: Anti Mullerian Hormone levels at first and second trimester time points			

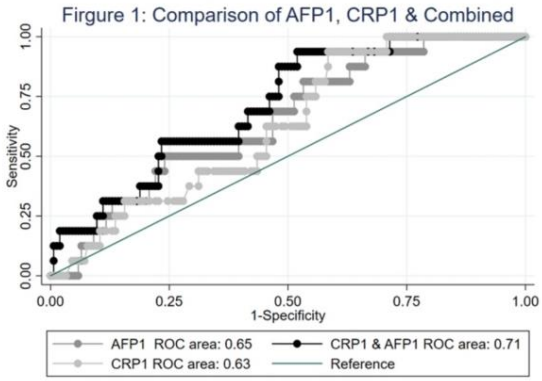

Fig. 1: Comparison of AFP1, CRP1 and Combined. AFP1: Alpha-fetoprotein at first trimester time point, CRP1: C Reactive Protein levels at first trimester time point

DISCUSSION

Our study demonstrates that low AFP was associated with early PTB before 34 weeks. Similarly, we saw a trend with CRP but this was not statistically significant and directionally opposite of what we would have clinically anticipated (higher CRP being associated with lower rates of PTB before 34 weeks). AFP has been shown to be associated with bad obstetrical outcomes including both PTB and placental malperfusion disorders (16, 20-24). However, limited data in this study did not yield a correlation of first trimester AFP with other obstetric outcome parameters, this may be a reflection of smaller sample size. Not surprisingly this difference did not persist when the group of women randomized to ASPIRIN were uniquely examined. These results suggest measurement of AFP during the first trimester may be useful as a biomarker of LDA efficacy in pregnancy; nevertheless, larger studies for validation are required.

The AMH or CRP levels were not different at both the gestational age time points. AMH was associated with PTB in previous studies conducted mostly with infertility issues which may overlap with non-obstetrical conditions (13, 15). Finally, CRP, being an acute marker of inflammation, we found a trend with preterm birth before 34 weeks but no other obstetrical outcomes.

The present study has several strengths and weaknesses as an exploratory design. Firstly, this is a nested case control study embedded with ASPIRIN study with a large cohort of women and maintaining the blinding of treatment allocation throughout the study. Secondly, GA was corroborated using early dating ultrasound. Major limitation of the study is that overall numbers of specimens was variable due to participant refusal for blood draw at second time point, technical challenges with the assays (haemolysis/quantity insufficient) and the lack of available controls. Further, due to limitation of the overall sample size adequate power may be lacking in this study to detect meaningful differences.

CONCLUSION

Results of study suggest that AFP during the first trimester may predict preterm birth before 34 weeks' gestation; however, is not predictive of other obstetrical outcomes. Unfortunately, most probably because of the smaller sample size, we were not able to demonstrate the predictive abilities of AFP, CRP and AMH on other important obstetrical outcomes.

CONFLICT OF INTEREST

Authors report no conflicts of interest.

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