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Anti-Mullerian hormone changes in pregnancy

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ANTI-MULLERIAN HORMONE CHANGES IN PREGNANCY

by

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PH.D. THESIS

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To my family and friends who supported me throughout this journey

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ABSTRACT

When the delicate hormonal balance in early pregnancy is disrupted, the consequences can be significant. We have a poor understanding of the "cross-talk" in the fetal/placental/ovarian axis that is essential for normal fetal development. This lack of knowledge challenges our ability to recognize disruptions in this axis that may be a signal for future disease. As a result, our ability to apply preventive measures against adverse obstetric outcomes, such as preterm birth (PTB), are quite limited.

Attempts to predict PTB using biomarkers of feto-placental health have been largely unsuccessful, but no one has considered the inclusion of ovarian biomarkers in these models. Anti-Mullerian hormone (AMH) is a biomarker of ovarian activity that has recently been found to decline in early pregnancy at a time that corresponds to the involution of the corpus luteum (CL). The signal for CL involution is believed to originate from the placenta; therefore, the AMH levels in pregnancy may reflect the degree of ovarian up or down-regulation based on feto-placental needs. As the major function of the CL in pregnancy is the production of progesterone, which acts as an anti-inflammatory agent in the placental bed, changes in CL-derived progesterone could result in higher or lower degrees of placental inflammation. Therefore, monitoring the changes in AMH levels may provide insight into the inflammatory state of the placenta which could be used as a signal for possible adverse obstetric outcomes resulting from a pro-inflammatory state, such as PTB.

The first aim of this project was to test the hypothesis of an association between AMH levels in early pregnancy and PTB risk. When the differences in AMH levels between the 1st and 2nd trimesters of pregnancy were stratified by the level of maternal serum alpha-fetoprotein (MSAFP) and controlled for maternal weight gain between trimesters, small or absent decreases in AMH levels were associated with a higher probability of PTB. However, when AMH was modeled alone, no significant

associations were found. The need for changes in biomarkers from different endpoints along the fetal/placental/ovarian axis suggests that a change is only significant if it can impact multiple axis points. Therefore, models that included two biomarkers from different part of the axis would find stronger associations than two biomarkers from a single point (e.g. two feto-placental biomarkers), and monitoring these changes may help identify women at risk for PTB.

The strategy of the second aim was to determine if the changes in AMH levels in early pregnancy could be used to predict time to delivery. When the risks of AMH and MSAFP were combined, a significant, dose-dependent relationship was found with time to delivery. Specifically, in women with an MSAFP of >1 multiple of the median (MoM), smaller declines and/or elevations in AMH levels were significantly associated with shorter times to delivery. In fact, 19% of women in the highest risk group delivered prior to 32 weeks gestation compared to 7% in the lowest risk group, and all infants who delivered prior to 24 weeks gestation were in the highest risk category. Thus, the amount of change in the AMH level when MSAFP is elevated may reflect the level of disruption in the fetal/placental/ovarian axis, which can then be used to predict time to delivery.

Finally, the third aim of this study was to determine if AMH levels were associated with a pro-inflammatory placental state other than PTB. The degree of placental inflammation is known to vary by fetal gender, with male placentas having higher levels of inflammation compared to female placentas. When AMH levels were compared between women with male vs. female fetuses in early pregnancy, 1st trimester AMH levels were found to be lower when carrying a male fetus. Further, sexually-dimorphic patterns in AMH levels were seen between genders when stratified by birth outcome (term vs. preterm delivery). The stronger ovarian response seen in women with female fetuses suggests a better survival function and may account for the discrepancies between PTB rates in males and females. This also strengthens our hypothesis that the

dynamic changes in AMH levels reflect the degree of placental inflammation and the need for CL-derived progesterone.

This project demonstrates that the changes in AMH levels may be representative of the cross-talk occurring in the fetal/placental/ovarian axis in early pregnancy. Further, changes in AMH levels may be an indication of the amount of inflammation in the placenta and the physiologic need for higher levels of progesterone to control this inflammatory state, but only when considered along with MSAFP. Therefore, the consideration of AMH levels as a biomarker of ovarian activity along with biomarkers of feto-placental health may provide clinically useful information about the development of future diseases such as preterm birth.

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17-αOHPC	17α-hydroxyprogesterone caproate
AMH	Anti-Mullerian hormone
AUC	Area under the curve
CI	Confidence Interval
CL	Corpus luteum
DNA	Deoxyribonucleic acid
FSH	Follicle stimulating hormone
G-CSF	Granulocyte colony stimulating factor
hCG	Human Chorionic Gonadotropin
HR	Hazard ratio
IGF1	Insulin-like growth factor 1
IL-10	Interleukin-10
IPS	Integrated Prenatal Screen
LH	Luteinizing hormone
LMP	Last menstrual period
LR	Likelihood ratio
MoM	Multiple of the median
MSAFP	Maternal serum alpha-fetoprotein
NICHD	National Institute of Child Health and Human Development
NIH	National Institutes of Health
OR	Odds ratio
PAPP-A	Pregnancy-associated plasma protein-A
PGDH	Prostaglandin dehydrogenase
PP-13	Placental protein-13
PPROM	Premature, preterm rupture of membranes
PTB	Preterm birth

PTGS2	Prostaglandin-endoperoxide synthase 2
ROC	Receiver operating characteristic curve
RR	Risk ratio
SHL	State Hygienic Laboratory
TGF-β	Transforming growth factor β
TLR-4	Toll-like receptor-4
TNF-α	Tumor necrosis factor-α
WHO	World Health Organization
JZ	Junctional Zone

CHAPTER 1.

INTRODUCTION

1.1 Project Overview

Pregnancy is a nine-month period of complex physiologic interactions between the mother and the fetus, mediated by the placenta, with the goal of creating the ideal intrauterine environment for fetal growth and development. Achieving this balance is not easy. Even minor alterations in this interplay can lead to devastating consequences such as preterm birth (PTB) and pre-eclampsia, two major causes of perinatal morbidity and mortality which are believed to originate in early gestation (1-3). Yet our knowledge of the risk factors associated with an abnormal feto-placental environment is limited. A better understanding of the signaling between the mother and the fetus might allow clinicians to identify potential biomarkers of abnormal feto-placental development. Unfortunately, no risk factors have been identified that are useful in clinical risk assessment models. Both demographic factors (4) and serum biomarkers, such as maternal serum alpha-fetoprotein (MSAFP) and inhibin A (5-9), lack the strength of association required to be clinically useful. However, the contribution of the ovary to the maintenance of the feto-placental environment after eight weeks of gestation has been largely ignored. Recently, Baird (10) suggested that the ovary may be an additional source of progesterone in the 2nd trimester if placental progesterone levels are not adequate to control placental inflammation. If true, then the contribution of the ovary must be considered beyond the 1st trimester, where an increase in ovarian activity would indicate a potentially abnormal state. The overarching goal of this project is to determine if anti-Mullerian hormone (AMH), a biomarker of ovarian activity, provides additional insight about the risk of adverse obstetric outcomes (specifically PTB) associated with increased levels of placental inflammation in the 2nd trimester of pregnancy.

1.1.1 Preterm Birth: The Scope of the Problem

Preterm birth (PTB) is a major, worldwide, public health burden (4, 11). As the leading cause of perinatal deaths in the world (12), PTB accounts for over 1 million neonatal deaths each year (11). In 2010, just over 11% (15 million) of all births occurred before completing 37 weeks gestation, meeting the World Health Organization (WHO) definition of PTB (12). Yet, in spite of all efforts aimed at PTB prevention, these rates continue to rise in most of the world (4, 12, 13).

The societal costs of PTB are equally staggering. The United States alone spends over 26 billion dollars annually on PTB and its associated neonatal morbidity (14). As healthcare costs are directly tied to gestational age at delivery (15), the care of extremely preterm infants (those born at <28 weeks gestation) accounts for most of these healthcare expenditures due to the increase long-term health consequences (12, 16). Extremely preterm infants have the highest rates of neonatal death (17), are hospitalized 85 times longer than term infants, and have a mean societal cost for a live birth of $264,412 (15). Thus, reducing the rates of PTB are the major objective for several large international foundations such as the WHO (11), the March of Dimes (11) and the Gates Foundation (18).

Prevention is the most effective method of reducing PTB costs and recently two new therapeutic interventions, 17 α-hydroxyprogesterone caproate and vaginal progesterone, have shown promise in reducing PTB rates. Both interventions are most effective if given early in the disease process and, preferably, prior to the onset of symptoms (19). At present, the use of either form of progesterone is limited to women with a prior preterm delivery (a highly predictive maternal risk factors) (19, 20), or women found to have cervical shortening on a midtrimester ultrasound (2, 19). This limits the usefulness of this therapy to a small subset of pregnant women. A model that could predict PTB early in pregnancy in all women, including those with no previous

obstetric history or ultrasound abnormalities, could have a significant impact on PTB rates worldwide.

1.1.2 Project Goals

The primary goal of this project is to determine if AMH, alone or in combination with other biomarkers of feto-placental health commonly collected in the early pregnancy, is significantly associated with PTB. A secondary goal is to determine if there is an association between AMH and other conditions of increased placental inflammation.

1.1.3 Specific Aims

The specific aims for this project include:

1. To determine if there is an association between 2nd trimester maternal AMH levels and preterm delivery.

Sub-hypothesis 1: 2nd trimester serum AMH levels will be higher in women who deliver at <37 weeks gestation compared to women who deliver at ≥37 weeks gestation, indicating an association between AMH and PTB.

Sub-hypothesis 2: The mathematical difference between the 1st and 2nd trimester AMH levels will be significantly smaller in women who delivery at <37 weeks gestation than in women who deliver at ≥37 weeks gestation.

Sub-hypothesis 3: When AMH level is considered along with other markers of feto-placental health (MSAFP, unconjugated estriol, pregnancy-associated plasma protein A (PAPP-A), human Chorionic Gonadotropin (hCG) and inhibin A), a stronger correlation with preterm delivery will be seen when compared to AMH alone.

2. To determine if there is an association between 2nd trimester maternal AMH levels and time to delivery.

Sub-hypothesis 1: A higher 2nd trimester AMH level will be correlated with a shorter time to delivery.

Sub-hypothesis 2: A smaller mathematical difference between the 1st and 2nd trimester AMH levels will be correlated to shorter time to delivery.

Sub-hypothesis 3: Combining the risk of AMH level with other markers of feto-placental health (MSAFP, unconjugated estriol, PAPP-A, hCG and inhibin A) will strengthen the association with time to delivery.

3. To determine if there is an association between 1st and 2nd trimester maternal AMH levels and fetal gender.

Sub-hypothesis 1: Higher levels of 1st and 2nd trimester AMH levels will be seen in women carrying a female fetus compared to women carrying a male fetus regardless of the gestational age at delivery.

Sub-hypothesis 2: Higher levels of 1st and 2nd trimester AMH levels will be seen in women carrying a fetus who delivers at term vs. a fetus who delivers at <37 weeks gestation within each gender.

Sub-hypothesis 3: The mathematical difference in the 1st and 2nd trimester AMH levels will be smaller for women carrying a female fetus who delivers at <37 weeks gestation compared to women carrying a male fetus who delivers at <37 weeks gestation.

1.1.4 Overview of PTB

1.1.4.1 Defining Preterm and Term Birth

Standard definitions for preterm and term birth do not exist. Classification schemes for PTB range from those which rely solely on time of delivery (e.g. the WHO classification) (21), to those based on clinical presentation (spontaneous preterm labor, maternal or fetal indications, and premature, preterm rupture of the membranes (PPROM)) (4, 22), and finally to those which classify PTB by the inciting pathophysiology (infection/inflammation, vasculopathic, and stress-induced) (23). The most widely used definition is that of the WHO, which defines PTB as a delivery prior to completing 37 weeks gestation (21). This is the definition used in papers 1 and 3 of this project.

Birth can be further defined by time of delivery. Preterm birth categories include: extremely preterm (<28 weeks), early preterm (28-31 weeks), moderately preterm (32-33 weeks), and late preterm (34-36 weeks) (4, 11, 17). Until recently, any birth at ≥ 37 weeks gestation was a "term" birth, but the National Institutes of Child Health and Human Development (NICHD) recently led an initiative challenging this definition, stating that "term" infants born at 37-38 weeks gestation still experienced the negative consequences of an early delivery (24). In December 2012, the NICHD convened a working group of experts and other stakeholders with the goal of redefining term birth and recommended three new "term" birth categories (24). Combining these definitions

with the previously described categories of PTB results in seven categories of birth based on gestational age at delivery: extremely preterm (<28 weeks), early preterm (28-31 weeks), moderately preterm (32-33 weeks), late preterm (34-36 weeks), early term (37-38 weeks), term (39-40 weeks), and late term (≥41 weeks) (4, 11, 17, 21, 24). Categories based on time of delivery are used in the bivariate analysis in paper 2.

1.1.4.2 Demographic Risk Factors for PTB

Multiple demographic factors have been associated with PTB, including black race (4, 22, 25), maternal smoking (7), preexisting maternal diabetes and hypertension (4, 7), urinary tract infections (7), maternal asthma (7), congenital malformations in the fetus (7), multiple gestations (4, 7, 26), vaginal bleeding (4, 7), low weight gain in pregnancy (27) and carrying a male fetus (28). For the majority of these risk factors, the associations with PTB are relatively weak, limiting their usefulness in risk assessment models (5, 29). However, two risk factors demonstrate a very strong association with PTB, namely a shortened cervix on a midtrimester ultrasound (done at about 22 weeks gestation) (7, 30) and a previous history of a PTB (26, 31-33) which carried a reported 22.5% recurrence rate. Unfortunately, the inclusion of these biomarkers in PTB models are limited, the first because the risk factor is not recognized until 22 weeks gestation, about 6 week after the optimal timing for PTB prevention, and the second because it is limited to women who have experienced a previous PTB and thus cannot be used in the majority of women. Therefore, demographic factors alone cannot be used to predict PTB risk.

1.1.4.3 Spontaneous PTB and the Role of Progesterone

Spontaneous PTB account for 40-45% of all preterm deliveries (4, 22), and is the result of abnormal placentation during the second wave of trophoblastic invasion in 2nd trimester of pregnancy (1). In the 2nd trimester, trophoblasts invade the inner third of the myometrium and form the junctional zone (JZ), a highly hormonally-dependent structure

vital to placental health (34). For this to be successful, adequate progesterone levels must be maintained in the placental bed to dampen the inflammatory reaction between the mother and the foreign deoxyribonucleic acid (DNA) of the fetus (35, 36). The anti-inflammatory actions of progesterone are thought to specifically suppress prostaglandin production in the endometrium (1). When placentation is disrupted or shallow, placental production of progesterone is also impaired, resulting in a pro-inflammatory state that is believed to lead to early delivery (2).

The progesterone deficiency theory led to the use of supplemental progesterone in the 2nd trimester to prevent PTB. Intramuscular 17 α-OHPC injections have significantly lowered PTB recurrence rates in women with a previous history of PTB (20), but to be effective, this therapy had to be initiated prior to the onset of symptoms (19). Further, 17 α-OHPC was not successful in reducing PTB rates when given to women with a shortened cervix on mid-trimester ultrasound (30), presumably because it was started too late in the disease process to be effective. These women did respond to vaginal progesterone, presumably due to the local effects of progesterone on the cervix (30). It remains unclear if the cervical shortening would have been prevented with the use of progesterone in any form earlier in the pregnancy. But regardless, the successful use of either IM 17 α-OHPC and vaginal progesterone was limited to a very small subset of women and the majority of women who had a PTB went untreated. Identifying these women is one of the major goals of this project.

1.1.4.4 Role of Fetal Gender in PTB

Finally, male fetuses are carry a higher risk of PTBs and early pregnancy loss compared to female fetuses (28, 37-39). Several theories exist, but there are no definite conclusions about the underlying cause for this disparity between sexes. The leading theory is the existence of a more "pro-inflammatory" placental environment in pregnancies with a male fetus (28, 40, 41). Ghindini et al. (40) found that the placentas

from male fetuses born at <32 weeks gestation had significantly more lesions of chronic inflammation suggestive of a maternal immunologic response than females born at the same gestational age (40). Further, the placental and chorionic trophoblast cells from male fetuses produced more pro-inflammatory tumor necrosis factor-α (TNF-α), more prostaglandin-endoperoxide synthase-2 (PTGS2) and less anti-inflammatory agents such as interleukin 10 (IL-10), granulocyte colony stimulating factor (G-CSF), and prostaglandin dehydrogenase (PGDH) compared to female fetuses (28). These sex-specific responses in the placenta may account for at least part of the increased risk for PTB associated with male fetuses. As of yet, no sex-specific differences in placental progesterone levels have been reported.

1.1.5 The Role of the Ovary in Pregnancy

Prior to eight weeks gestation, the immature placenta is unable to produce sufficient progesterone to maintain the pregnancy. The corpus luteum (CL) of the ovary assumes this role after it is "rescued" at the time of embryo implantation by the rising hCG levels (42, 43). The CL remains the primary source of progesterone until 7-8 weeks gestation when the placenta is sufficiently mature to assume that role and the CL is no longer required. But the placental signal responsible for CL involution is not present until week 13 after which the ovary becomes quiescent (42-45). The reason for the delay in CL involution remained unclear, as do the actual pathways that incite the event (10).

Baird (10) recently proposed that the persistence of the CL may represent a biological need for higher progesterone levels. According to her theory, when the placenta is slow to mature or when placentation is abnormal, the placenta may act to sustain the CL in an effort to boost progesterone levels and salvage a marginal pregnancy. While intriguing, this theory has been difficult to prove (10), particularly because maternal serum progesterone levels do not adequately reflect placental

progesterone levels, and because there are no biomarkers for ovarian activity in pregnancy (46).

1.1.6 Biomarkers Used to Determine Fetal Well-being

Traditional biomarkers of placental function include PAPP-A, MSAFP, hCG, unconjugated serum estriol, and inhibin A. Regardless of whether the biomarker is produced by the fetus or the placenta, maternal levels of these hormones will be increased when placental dysfunction is present. These biomarkers are routinely used to test for aneuploidy in the 1st and 2nd trimesters of pregnancy (47, 48) and are included in the Integrated Prenatal Screen (IPS), one of the most commonly used prenatal screening test.

1.1.6.1 Pregnancy-associated plasma protein-A (PAPP-A)

PAPP-A is produced by the placenta and decidua where it is instrumental in increasing the bioavailability of insulin-like growth factor (IGF1). IGF1 mediates trophoblast invasion of the placenta, which is vital for successful early placentation (49). Elevated levels in the 1st trimester are not believed to have any negative consequences, but low levels are associated with abnormal birth outcomes (14, 49).

Stout et al. (14) found that PAPP-A was significantly associated with PTB in a decreasing dose-response pattern. They reported an area under the receiver operating characteristic curve (AUC) for PAPP-A of 0.91 (95% CI: 0.85-0.96), adjusting for black race, chronic hypertension, diabetes and prior PTB. Model performance was not improved with the addition of other biomarkers (14). However, the major contributors to the risk of PTB in this model were the associated maternal characteristics, which had an AUC of 0.89 (95% CI: 0.84-0.95) when modeled alone.

Maymon et al. (50) were unable to find any significant associations with PAPP-A and PTB, even when considered with other biomarkers. Krantz et al. (51) found a positive association with PAPP-A and adverse obstetric outcomes, but only when PAPP-A reached extremely low levels (<5th percentile) (odds ratio (OR) 2.3, 95% CI: 1.1, 4.7).

1.1.6.2 Maternal Serum Alpha-fetoprotein (MSAFP)

Elevations of this fetal-derived biomarker (levels at >2.0 multiple of the median (MoM)) are associated with chromosomal abnormalities and structural defects in the fetus such as an open neural tube or an abdominal wall defect (49). When fetal levels are extremely elevated, maternal levels simply reflect the high fetal levels. However, when maternal levels are elevated without a known fetal cause, high levels of MSAFP may indicate defective placentation or ischemic placental disease (52). In these cases, an elevated MSAFP has been associated with adverse obstetric outcomes such as PTB (9, 49, 53, 54).

Chandra et al. (9) found that unexplained elevations in MSAFP levels (≥ 2.0 MoM) were associated with spontaneous PTB, independent of any other abnormal feto-placental markers (risk ratio (RR) 3.57; 95% CI: 2.68, 4.78). Yaron et al. (55) reported an OR of 1.7 (95% CI: 1.4, 2.1) with PTB when MSAFP was ≥2.5 MoM and Metcalfe et al. (8) found a positive likelihood ratio (LR) of 7.8 (95% CI: 5.0, 12.2) for PTB when MSAFP levels reached ≥2.5 MoM. However, Duric et al.(56), Androutsopoulus et al. (53) and Yuan et al. (52) found no useful associations between MSAFP and PTB. Jelliffe-Pawlowski et al. (57) found an association between MSAFP and PTB only when PAPP-A was decreased and inhibin A was increased (OR 3.2, 95% CI 2.0, 5.3). The lack of consistency between findings suggests additional pathway leading to PTB exist and are not being assessed with these biomarkers.

1.1.6.3 Human Chorionic Gonadotropin (hCG)

An unexplained low (<0.5 MoM) level of this placenta-derived hormone in the 1st trimester is associated with adverse obstetric outcomes (49). Second trimester elevations (>3.0 MoM) without evidence of a congenital malformation are associated with PTB when MSAFP is also elevated (RR: 3.6, 95% CI 2.14, 6.08) (9, 49, 53). Duric et al. (56) found that levels ≥2.02 MoM were associated with PTB with an OR of 2.5 ($p<0.05$), and

Lepage et al. (47) reported that the rate of PTB was 22.5 % for women with an hCG of >4.0 MoM and a normal MSAFP. Metcalfe et al. (8) reported a positive LR of 4.3 (95% CI: 3.1, 5.9) with an hCG of >3.0 MoM. However, Menon et al. (5) and Androutsopoulos et al. (53) found no useful associations between hCG and PTB.

1.1.6.4 Unconjugated Estriol

A decrease in this placenta-derived hormone is associated with PTB. Women with intrauterine growth restriction and preterm labor had an OR of 2.2 ($p<0.05$) for a low serum estriol level (<0.74 MoM) (56). Metcalfe et al. (8) reported a positive LR of 7.0 (95% CI: 3.1, 16.1) of PTB for women with an estriol level of <0.4. However, both Menon et al. (5) and Yaron et al. (55) found no association between PTB and estriol levels.

1.1.6.5 Inhibin A

Elevations of inhibin A, another placenta-derived hormone, were reported by Metcalfe et al. (8) to have an LR of 7.0 (95% CI: 3.1, 16.1) for PTB when levels were >3.0 MoM (8). However, Muttukrishna et al. (58) and Menon et al. (5) found no associations with PTB. Low levels are not a predictor of poor obstetric outcomes (49).

1.1.6.6 Use of Multiple Biomarkers to Predict PTB

Maternal biomarkers in the 1st and 2nd trimesters of pregnancy predict fetal aneuploidy with a high degree of confidence. Sequential testing of multiple 1st and 2nd trimester biomarkers can detect up to 85% of trisomy 21 pregnancies with a false positive rate of ~1% (50). However, success rates are not as high when these biomarkers are used to predict adverse obstetric outcomes. Metcalfe et al. (8) reported that biomarkers currently used to screen for aneuploidy have detection rates too low to identify women at risk for adverse obstetrical outcomes. A review by Menon et al. (5), looked at 116 difference biomarkers assayed 578 times in 217 studies, and found that no biomarkers

could reliably predict PTB. Jelliffe-Pawlowski et al. (57) developed a model that predicted PTB with an OR of 2.3-3.6, but the model only worked with extreme biomarker levels (PAPP-A in the $\leq 5^{th}$ percentile and/or MSAFP in the $\geq 95^{th}$ percentile, and/or 2^{nd} trimester inhibin A in the $\geq 95^{th}$ percentile. Finally Yuan et al. (52) published a recent meta-analysis on the use of MSAFP in the prediction of PTB and found that MSAFP was strongly related at levels over 2.0 MoM, but only in the context of other markers of abnormal pregnancies.

1.1.7 Anti-Mullerian Hormone (AMH)

Anti-Mullerian hormone (AMH) is a dimeric glycoprotein secreted by the granulosa cells of the growing ovarian follicle (59-62). It is a member of the transforming growth factor-beta (TGF- β) superfamily and is responsible for regulation of oocyte development in the ovarian follicular pool (61-63). Throughout life, AMH levels are a direct reflection of ovarian function (64). In women, AMH levels begin to rise at about age 5, peak around age 27 and then slowly decline until the age of menopause when levels become undetectable (65-67). While AMH levels fluctuate over the span of a woman's reproductive life, in the short-term, AMH levels remain relatively stable (68). Small monthly fluctuations do occur (65), but AMH function is independent of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), so does not change in response to the wide monthly fluctuations of these hormones (68).

1.1.7.1 AMH Levels during Pregnancy

It was originally believed that AMH levels did not change in pregnancy (69), however, more recent data have confirmed a unique and consistent pattern of AMH decline (70-72). AMH levels remain high in the 1^{st} trimester but then drop dramatically around 13 weeks gestation (70, 71, 73). The levels remain low until delivery and return to near pre-pregnancy levels within days of the removal of the placenta (72, 74). This is

the only time in a woman's life that AMH undergoes a natural, reversible decline. The rapidity of the fall and return of AMH to normal levels suggests the presence of a suppressive signal, most probably from the placenta.

1.1.7.2 Fetal Gender and AMH Levels in Pregnancy

The pattern of AMH decline in adult women does not correspond with change in the fetus of either sex. AMH levels in female fetuses are low to non-existent, as the presence of AMH during the development of the female reproductive tract leads to masculinization by suppressing the Mullerian system and allowing dominance of the Wolffian system (75). The opposite is true with a male fetus. AMH must be present at sufficient levels to allow the Wolffian system to predominate and for male genitalia to form (75). In males, the levels rise by week 8 of gestation and remain elevated until the time of puberty, when AMH drops and testosterone rises (75-78). These dissimilarities between fetal and maternal patterns suggest that they are responding to different control pathways.

1.2 Methodology

1.2.1 Development of the Tissue Bank

All women in Iowa can elect to participate in IPS in the 1st and 2nd trimesters of pregnancy. IPS provides a risk calculation for presence of Down Syndrome, Trisomy 18, and open neural tube defects in the fetus that is based on the levels of maternal serum markers. Testing requires two timed serum samples, the first drawn between 10-13.9 weeks gestation and the second drawn between 15-22.9 weeks gestation. Because timing of these blood draws is critical, an ultrasound is required for all pregnancies in the 1st trimester to confirm the gestational age of the fetus.

The State Hygienic Laboratory (SHL) at the University of Iowa performs all testing for the state. After all testing was completed, the remaining serum was frozen at -80˚C and maintained in a serum tissue bank. The samples were linked to birth certificate data after delivery. Thus collected, this serum tissue bank contained over 12,000 maternal 2nd trimester serum samples linked to birth outcome data. Over 3,000 of these were paired with a 1st trimester sample collected as part of IPS, and had maternal biomarker information available (PAPP-A, MSAFP, hCG, unconjugated estriol and inhibin A).

1.2.2 Development of the Dataset

All maternal-infant pairs contained in the serum tissue bank were eligible for inclusion in this dataset. Inclusion criteria included women of any age with delivery of a singleton infant after 20 weeks gestation and with paired serum samples (cases: n=143, controls: n=973). Exclusion criteria were designed to minimize potential confounding by limiting cases to those women with no other identifiable causes for PTB; thus limiting the dataset to women with a spontaneous PTB. Subjects were excluded in the following order: 12 dropped for confirmed chorioamnionitis (0 PTBs, 12 term births), 36 dropped for premature rupture of membranes (possible chorioamnionitis) (19 PTBs and 17 term births), 8 dropped for congenital anomalies (4 preterm and 4 term births), and 19 dropped for pre-eclampsia (3 PTBs and 16 term births) (Figure 1.1). The WHO definition of PTB was used to define cases and controls. At completion of this initial screening, there were a total of 117 eligible cases and 924 eligible controls (Figure 1.1).

1.2.3 Analysis of AMH samples

Because of the requirement to have samples available for retesting, serum samples received from the SHL had been stored for up to eight days at 4˚C prior to freezing. Fleming et al. (79) recently documented the stability of AMH in serum samples stored at 4˚C for up to 7 days so it is unlikely that this had an impact on the AMH levels. Samples

were then frozen and stored at -80˚C for up to 2 years. All samples went through a single freeze/thaw cycle (during which aliquots of the samples were taken), and then remained at -80˚C until the time of testing. All samples were analyzed at a single reference laboratory (Reprosource Inc.,Woburn, MA). The testing procedure was a proprietary laboratory-developed AMH assay using the research-use-only materials and reagents from Beckman Coulter-DSL (Chaska, MN). A unique feature of this laboratory process was the use of multiple controls across each plate, allowing for less variation in AMH levels across the 96 wells. The intra- and interassay coefficients of variation with serum controls were approximately 5%-9% and 7%-12% respectively. When a serum level was below the reported level of detection (0.1ng/mL), it was considered as zero in the analysis (coded as 0.05).

Recent concerns have been raised about possible complement interference with the Beckman Coulter assay. In 2013, Beckman Coulter released a warning to the public confirming that complement interference resulted in lower than expected AMH levels (up to 70%) when samples were analyzed within 1-2 hours of being drawn. They recommended reanalyzing samples using a new assay buffer and comparing the results. Our samples were analyzed before this notice was released, so no samples were available for retesting. Beckman Coulter also recognized that complement interference decreased as the samples aged, regardless of whether or not the samples were frozen. Our samples were frozen for up to 2 years prior to testing, making it less likely that complement interference was an issue.

Finally, we measured blood protein levels in all of our samples, and standardized the AMH levels to these levels. Our analysis was no different using the corrected vs. the uncorrected samples. Because blood protein correction is not routinely performed in the clinical setting, the analyses were run on the uncorrected samples to make them more

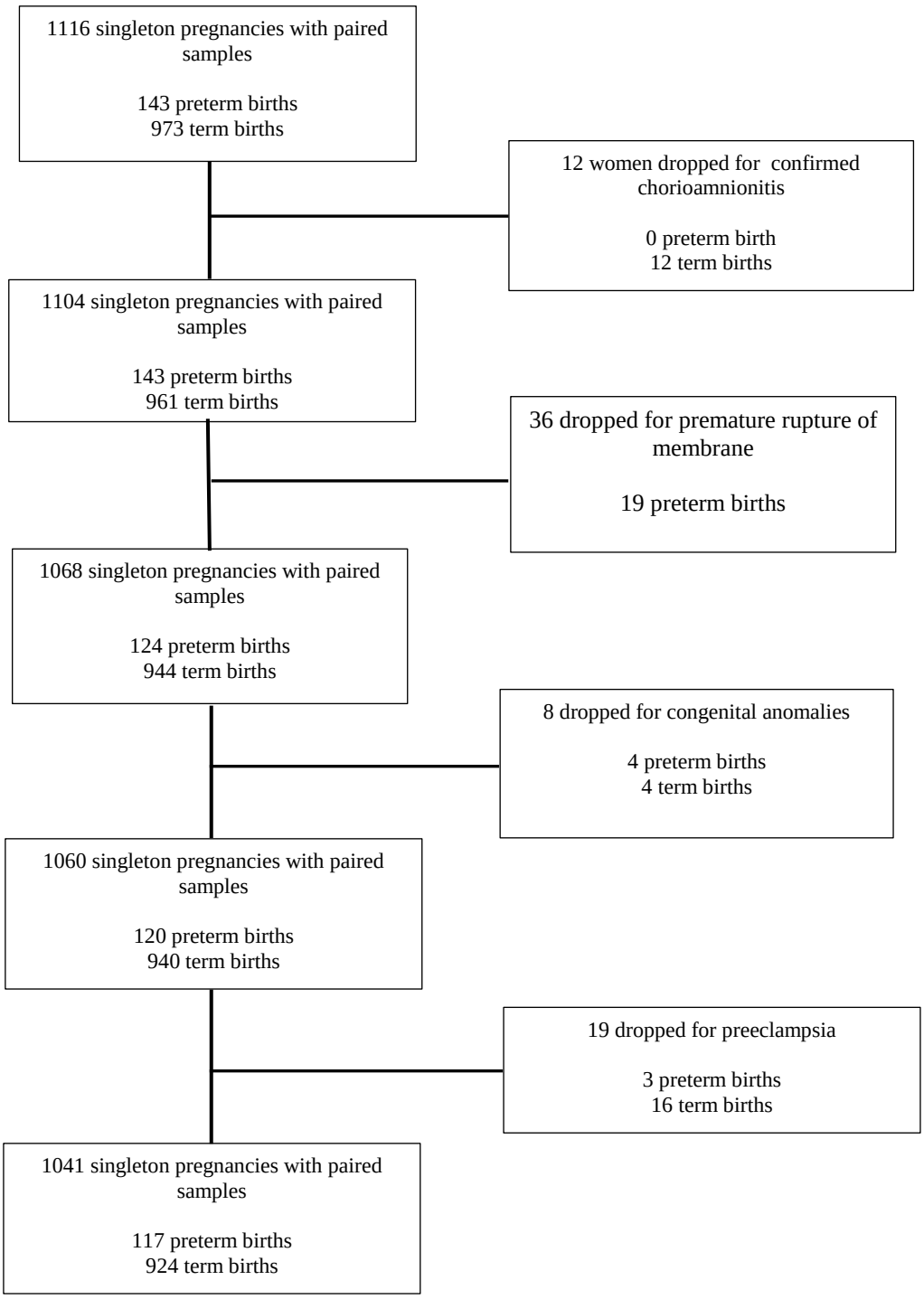


Figure 1.1 Selection of subjects for dataset

clinically relevant. It is also likely that if complement (a blood protein) was present in large quantities, that correcting for protein levels would have significantly changed the results. As this did not happen, we are reasonably reassured that complement interference did not have a great impact on our study results.

CHAPTER 2.
ANTI-MULLERIAN HORMONE LEVELS ARE ASSOCIATED WITH PRETERM BIRTH IN EARLY PREGNANCY

2.1 Summary of findings

Preterm birth (PTB) is a global health concern with major long-term health consequences. Prediction of PTB is challenging and no models exist that successfully predict the risk of PTB early in the pregnancy. As preventive measures are most successful when implemented prior to the onset of disease, the lack of a risk model for use in early pregnancy is limiting. The goal of this study was to determine the association between PTB and anti-Mullerian hormone (AMH) levels, both alone and in combination with other markers of feto-placental health.

This was a retrospective case-control study of white women delivering a child in Iowa between 2009 and 2010 and who had undergone Integrated Prenatal Screening (IPS). Cases delivered at <37 weeks gestation while controls delivered at ≥37 weeks gestation. AMH in the 2nd trimester of pregnancy and the difference between AMH levels in the 1st and 2nd trimesters of pregnancy were the exposures of interest. The main outcome was probability of PTB. Other factors considered in the model were maternal age, maternal weight change between trimesters, tobacco use in pregnancy, previous history of PTB and levels of feto-placental biomarkers included in IPS testing. In addition, each model contained a variable that was used to control for the timing of the blood draw (for the exposure of 2nd trimester AMH level) or to correct for the time difference between blood draws (for the exposure of AMH difference between the 1st and 2nd trimesters).

Second trimester AMH levels were not associated with PTB either independently or after controlling for other markers of feto-placental health. AMH difference was not associated with PTB when modeled alone, but a statistically significant association was

found after adjusting for maternal serum alpha-fetoprotein (MSAFP) and weight change between the 1st and 2nd trimester. After stratifying the model by MSAFP level, most of the risk for PTB was identified in women with an MSAFP >1 MoM in association with stable or rising AMH level.

These findings suggest that a lack of decline in the AMH level in early pregnancy can be used to identify women with a high probability for PTB, especially when MSAFP levels are >1 MoM. Monitoring changes in the AMH level between the 1st and 2nd trimesters of pregnancy may help identify women who would benefit from interventional therapies such as supplemental progesterone. Further studies are required to validate these findings with a more diverse racial mix of subjects and to further elucidate the role the ovary in early pregnancy.

2.2 Introduction

In 2010, approximately 14.9 million (11.1%) (12) of all births worldwide delivered prior to 37 complete weeks of gestation (259 days) after the first day of the last menstrual period preceding the pregnancy, meeting the World Health Organization (WHO) definition of PTB (21). PTB accounts for 75% of perinatal mortality and over half of long-term perinatal morbidity (4), and has an estimated annual cost in the United States of over 26 billion dollars (14). Yet despite efforts to identify and treat women at risk for PTB, rates continue to increase in most of the world (4, 12, 13). Attempts to identify women at risk for PTB using individual risk models based on obstetric and demographic risk factors such as race, smoking during pregnancy, and previous history of a PTB (4, 14) have been largely unsuccessful (7, 8, 29). This lack of success is due, in part, to the multiple pathways that can lead to an early delivery (52) as well as a general lack of understanding about the underlying pathophysiology of the disease.

One of the proposed pathways to PTB includes disruption of placentation early in gestation as the result of inflammation and/or infection (1, 4, 23). One of the critical

periods for placentation is the late 1st and early 2nd trimesters of pregnancy when trophoblasts invade and remodel the myometrial junctional zone (34). Disruption of this process leads to utero-placental ischemia (34) and up-regulation of the pro-inflammatory pathways (1), which alters the integrity of the maternal-placental barrier. This, in turn, allow fetal proteins such as alpha-fetoprotein to cross into the maternal circulation (53), and may induce or suppress production of placental hormones such as human Chorionic Gonadotropin (hCG) and inhibin A (58). Several adverse obstetric outcomes have been associated with abnormal maternal levels of these proteins (9, 52, 53, 55), but unfortunately, the usefulness of feto-placental hormonal biomarkers in identifying women at risk for PTB is limited because statistically significant associations are only seen at very high or very low levels (9, 52, 55).

In early pregnancy, the corpus luteum (CL) of the ovary produces the majority of the progesterone required for pregnancy maintenance, and without this CL-derived progesterone, pregnancies fail (42, 44). Yet, serum progesterone are not useful as biomarkers of feto-placental health because serum progesterone levels do not accurately reflect placental progesterone levels (46). Other methods of determining the need for CL-derived progesterone during the transition of progesterone production from the ovary to the placenta are needed. Anti-Mullerian hormone (AMH) is a candidate ovarian hormone that might provide additional information on overall ovarian activity during pregnancy.

AMH is a dimeric glycoprotein produced in the granulosa cells of the ovary (62, 63, 80). An original report describing changes in AMH levels in pregnancy suggested that this hormone was stable throughout pregnancy (69), but recent studies have shown that AMH levels decline rapidly between the 13th and 15th weeks of gestation (70, 71). This timing corresponds to two key events in pregnancy: the 2nd phase of deep placentation and the involution of the CL (43, 44). The speed of the AMH decline, in addition to the rapid increase to near early-pregnancy levels within days of delivery (72,

74) suggests that AMH is being suppressed (74) as the result of a physiologic cross-talk between the placenta and the ovary. Because of the timing of this decline, it is possible that changes in AMH levels during pregnancy are related to the need for increased progesterone production when placentation is disrupted, making it possible that AMH levels are useful as a biomarker of feto-placental health. The purpose of this study is to determine the association of PTB with AMH levels, both when modeled alone and in combination with other biomarkers of feto-placental health commonly measured in early pregnancy. Specifically we will determine if AMH levels in the 2nd trimester, or alternatively the decline in AMH levels between the 1st and 2nd trimester, are associated with PTB.

2.3 Materials and Methods

We used a case-control study design to predict the odds of PTB in pregnant women undergoing Integrated Prenatal Screening (IPS) in Iowa between 2009 and 2010. Pregnancy information and maternal serum samples were obtained from an existing maternal-fetal serum tissue bank at the University of Iowa. The Human Subjects Office at the University of Iowa approved the study, as did the Iowa Department of Public Health Research and Ethics Review Committee, who granted permission to use birth certificate data. The Congenital and Inherited Disorders Advisory Committee for the state of Iowa granted permission for use of the maternal serum samples.

2.3.1 Development of the Serum Tissue Bank

All women in Iowa can elect to undergo IPS in the 1st and 2nd trimesters of pregnancy. IPS provides risk calculations for the presence of Down Syndrome, Trisomy 18, and for open neural tube defects in the fetus based on levels of maternal serum markers, including MSAFP, hCG, inhibin A, PAPP-A and estriol. Testing requires two timed serum samples, the first drawn between 10-13.9 weeks gestation and the second

drawn between 15-22.9 weeks gestation. Because timing of these blood draws is critical, a fetal ultrasound is required for all pregnancies to confirm gestational age.

The State Hygienic Laboratory (SHL) at the University of Iowa performs all IPS testing for the state. After IPS testing was completed, the remaining serum was stored at -80˚C and maintained in a serum tissue bank at the University of Iowa. When the child delivered, the samples were linked to birth certificate data. This serum tissue bank contained over 12,000 maternal serum samples that were linked to birth certificate data, and yielded over 3,000 paired 1st and 2nd trimester samples from women undergoing IPS. Maternal biomarker information obtained during IPS was also available for these women.

2.3.2 Development of the Dataset

All women with samples in the serum tissue bank were eligible for inclusion in this dataset. Inclusion criteria included women of any age who delivered a singleton after 20 weeks gestation and who had paired serum samples. Exclusion criteria were designed to limit the dataset to women with a spontaneous PTB, which are shown in Figure 2.1. Cases and controls were defined using the WHO definition of PTB. At completion of this initial screening, there were a total of 117 cases and 924 controls available.

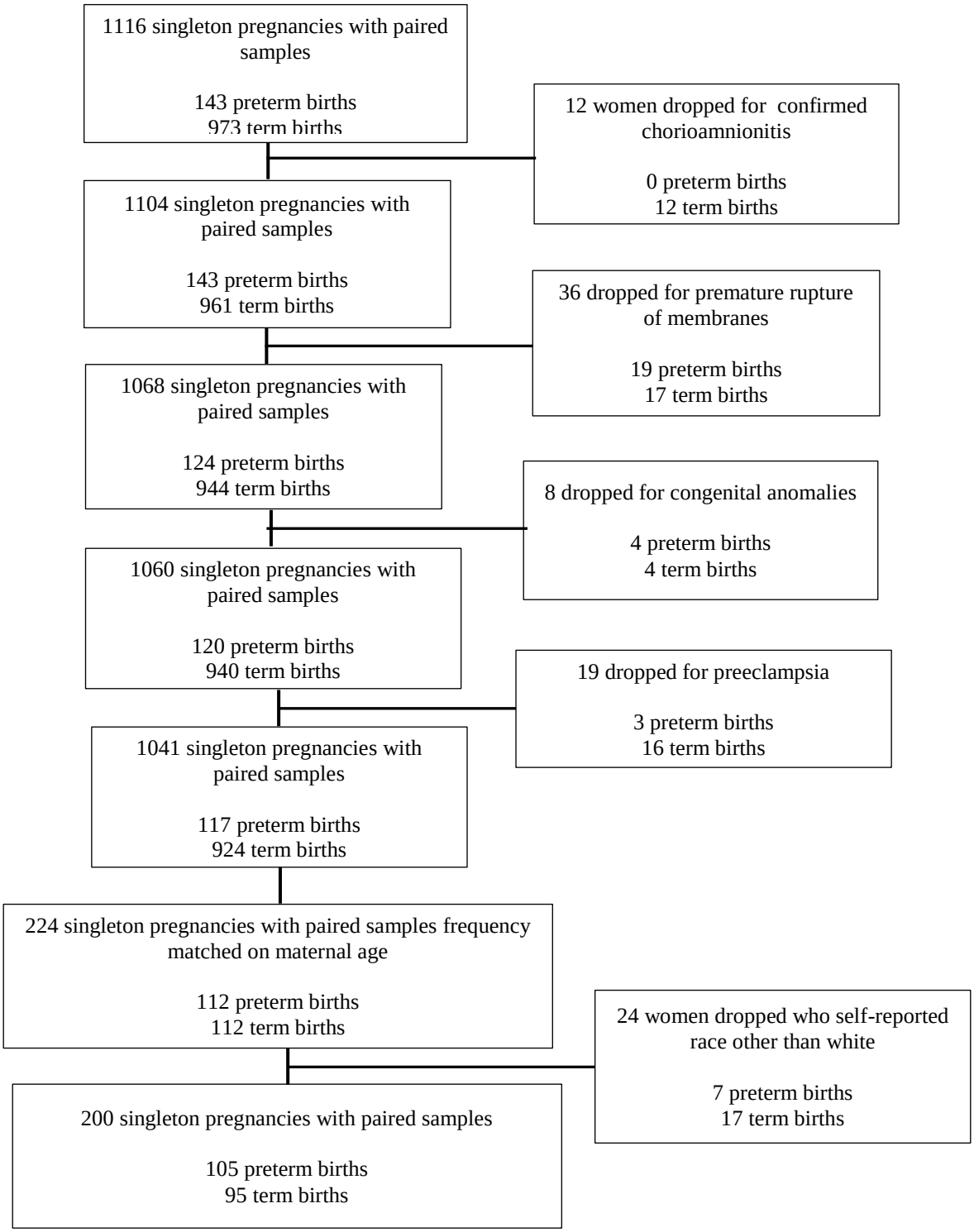


Figure 2.1. Selection of subjects from serum tissue bank for inclusion in the parent dataset

2.3.3 Sample Size

Case-control selections were 1:1 frequency-matched based on the following age groups: <21, 21-22.9, 23-24.9, 25-26.9, 27-28.9, 29-30.0, 31-32.9, 33-34.9, 35-36.9, and ≥37. Sample size analysis indicated a sample size of 100 cases and 100 controls would provide over 80% power to find a 1.0 ng/mL difference in AMH levels with a two-sided alpha of 0.05. Because of concerns over misclassification related to the cause of PTB, a known limitation when working with birth certificate data, the sample size in each arm was increase to 112 women in order to increase the power of the study.

After developing the dataset and the completion of AMH testing, several reports were published noting differences in the AMH levels based on race (4, 28). In reviewing the racial distribution in this dataset, all but 24 women had self-identified as white. As this number was too small to adequately address possible confounding associated with maternal race, the dataset was further restricted to white women. Restriction of the data did not change the bivariate associations observed in the unrestricted dataset. The case-control ratio in the restricted dataset was 105 to 95, which resulted in a slight skew in the number of cases to controls by age group. Specifically, the following age groups had one or two more cases than controls: < 21, 25-<27, 27 to <29, 29 to <31, 31 to <33, 33 to <35, and 35 to < 37. In addition, there was one less case than control in the 21-<23 age group. The imbalance in age was addressed by controlling for maternal age during model development.

2.3.4 Anti-Mullerian Hormone Measurements

Stored samples remained frozen at -80˚C until the time of analysis and had undergone a one freeze-thaw cycle. A single reference laboratory specializing in AMH testing performed all of the analyses using the laboratory-developed ReproSource AMH assay (ReproSource, Inc., Woburn, Massachusetts). This assay is based on research-use-only materials and reagents from Beckman Coulter-DSL (Chaska, MN). Intra- and

interassay coefficients of variation with serum controls were 5%-9% and 7%-12%, respectively. Serum AMH values below 0.1 ng/mL, the reported clinical level of detection, were considered to be zero but were reported as 0.05 in the analysis.

2.3.5 Statistical analysis

Univariate and bivariate analyses (t-test and χ^2 test statistics) were used to determine normality of the data as appropriate. AMH levels in the 1st and 2nd trimesters were not normally distributed. These variables were transformed and the models rerun, but no difference in the results were seen with the transformed data; therefore, the original variables were used in the final models as these more easily translated to the clinical setting.

Models were constructed using two different AMH exposure variables: 2nd trimester AMH level and as the mathematical difference between the 1st and 2nd trimester AMH levels, which was used to identify the magnitude of decline in AMH. Covariates identified as potential confounders and used in model development included: prior history of PTB (4), fetal gender (28), maternal age (81), maternal weight gain between the 1st and 2nd trimesters (27, 82), and smoking during the pregnancy (4). A covariate was also added to each model to control for the differences in the timing of the blood draws. Gestational age (days) at the time of the blood draw was included in models using 2nd trimester AMH level, and the length of time between blood the 1st and 2nd blood draw (days) was used in models using AMH difference between trimesters. The contribution of each covariate was assessed using backward, stepwise logistic regression with a threshold to retain the covariate of a 15% change in the β coefficient of the AMH variable used in the model. Receiver operating characteristic (ROC) curve and Hosmer-Lemeshow testing were used to determine goodness of fit. Conditional (fixed-effects) logistic regression, matched on age group, produced nearly identical results in all models. Therefore, only results from the multivariate logistic regression models are shown.

After completing the models with AMH levels only, MSAFP, inhibin A, hCG, estriol and PAPP-A levels were added to the existing model and the analyses were repeated to determine if model performance improved. Placental biomarkers with missing values had the levels imputed and these were included in the model. All statistical analyses were performed using Stata version 11.2 (College Station, TX).

2.4 Results

Table 2.1 includes demographic and bivariate findings for both the unrestricted and restricted datasets. In the restricted dataset, the average gestational age at delivery was 34.0±2.9 weeks for cases compared to 38.7±1.0 weeks for controls. On average, case fetuses were two days older at the time of 1st trimester screening compared to controls, and were one day older at 2nd trimester screening. The average MSAFP was significantly higher in cases than controls. The difference between the 1st and 2nd trimester AMH level was significantly higher in controls, indicating they experienced a greater drop in AMH levels. However, the average 1st trimester and 2nd trimester AMH levels were not significantly different between groups.

Table 2.1. Demographic and univariate/bivariate data, unrestricted dataset and dataset restricted to white women only

Continuous variables	Unrestricted Dataset			Restricted to white women only		
	Cases Mean (SD) N=112	Controls Mean (SD) N=112	Odds Ratio (95% CI)	Cases Mean (SD) N=105	Controls Mean (SD) N=95	Odds ratio (95% CI)
Maternal age[a]	28.6 (5.4)	28.7 (5.3)	1.0 (0.9, 1.0)	28.8 (5.2)	28.8 (5.5)	1.0 (0.9. 1.1)
Gestational age at delivery[b]	33.7 (3.4)	38.7 (1.0)	NA[k]	34.0 (2.9)	38.7 (1.0)	NA[k]
Gestational age at 1st trimester screen[c]	84.7 (7.0)	82.6 (5.8)	1.1 (1.0, 1.1)	84.6 (6.9)	82.6 (5.8)	1.1 (1.0, 1.1)
Gestational age at 2nd trimester screen[c]	119.0 (9.0)	117.7 (9.2)	1.0 (0.9, 1.0)	119.2 (9.2)	118.0 (9.1)	1.0 (0.9, 1.0)
Maternal weight change between the 1st and 2nd trimester[d]	3.3 (5.9)	1.9 (5.8)	1.1 (1.0, 1.1)	3.5 (5.9)	2.5 (4.1)	1.0 (1.0, 1.1)
MSAFP[e] at 2nd trimester[f]	1.2 (0.5)	1.0 (0.4)	2.5 (1.3, 4.9)	1.2 (0.5)	1.0 (0.4)	2.3 (1.2, 4.6)
Maternal hCG[g] at 2nd trimester[f, h]	1.1 (0.8)	1.1 (0.5)	1.1 (0.7, 1.6)	1.2 (0.8)	1.1 (0.5)	1.1 (0.7, 1.7)
Maternal PAPP-A[i] at 1st trimester[f, h]	1.0 (0.5)	1.1 (0.6)	0.6 (0.4, 1.0)	1.0 (0.5)	1.1 (0.6)	0.7 (0.4, 1.2)
Maternal Estriol at 2nd trimester[f, h]	1.0 (0.3)	1.0 (0.3)	1.1 (0.5, 2.6)	1.0 (0.3)	1.0 (0.3)	0.9 (0.4, 2.1)
Maternal Inhibin A at 2nd trimester[h]	1.3 (1.3)	1.1 (0.6)	1.4 (0.9, 2.1)	1.3 (1.3)	1.0 (0.6)	1.5 (0.9, 2.3)
AMH at 1st trimester screening[j]	3.2 (2.4)	3.7 (2.8)	0.9 (0.8, 1.0)	3.1 (2.4)	3.6 (2.6)	0.9 (0.8, 1.0)
AMH at 2nd trimester screening[h]	2.9 (2.1)	3.1 (2.5)	1.0 (0.9, 1.1)	2.8 (2.1)	3.0 (2.3)	1.0 (0.8, 1.1)

Table 2.1--continued

Difference between 1st and 2nd trimester AMH levels[j]	0.3 (1.4)	0.6 (1.8)	0.7 (0.5, 0.9)	0.4 (1.4)	0.6 (1.6)	0.7 (0.5, 0.9)
Categorical Variables	Cases n (%)	Controls n (%)	Odds ratio (95% CI)	Cases n (%)	Controls n (%)	Odds ratio (95% CI)
Infant Gender						
Male	64 (57.1)	50 (44.6)		60 (57.1)	44 (46.3)	
Female	48 (42.9)	62 (55.4)	0.6 (0.4, 1.0)	45 (42.9)	51 (53.4)	0.7 (0.4, 1.1)
Previous Preterm Birth						
Yes	11 (9.8)	7 (6.3)		10 (9.5)	6 (6.3)	
No	101 (90.2)	105 (93.8)	1.6 (0.6, 4.4)	95 (90.5)	89 (93.7)	1.6 (0.5, 4.5)
Smoked during pregnancy						
Yes	20 (17.9)	15 (13.4)		19 (18.1)	13 (13.7)	
No	92 (82.1)	97 (86.6)	1.4 (0.7, 2.9)	86 (81.9)	82 (86.3)	1.4 0.6, 3.0)
Maternal race						
White	105 (93.8)	95 (84.8)				
Black	3 (2.7)	8 (7.1)				
Asian	1 (1.0)	3 (2.7)				
Other	3 (2.7)	6 (5.4)	0.7 (0.4, 1.1)			

[a]years , [b]weeks of gestation, [c]days of gestation, [d]pounds, [e]Maternal Serum Alfa-fetoprotein, [f]Measures of the Median (MoM)

[g]Maternal human Chorionic Gonadotropin, [h]12 missing values (6 cases and 6 controls), [i]Maternal plasma protein of pregnancy-A, [j]ng/mL,

[k]Odds ratio could not be calculated as this variable used for case-control selection

2.4.1 Models using only AMH

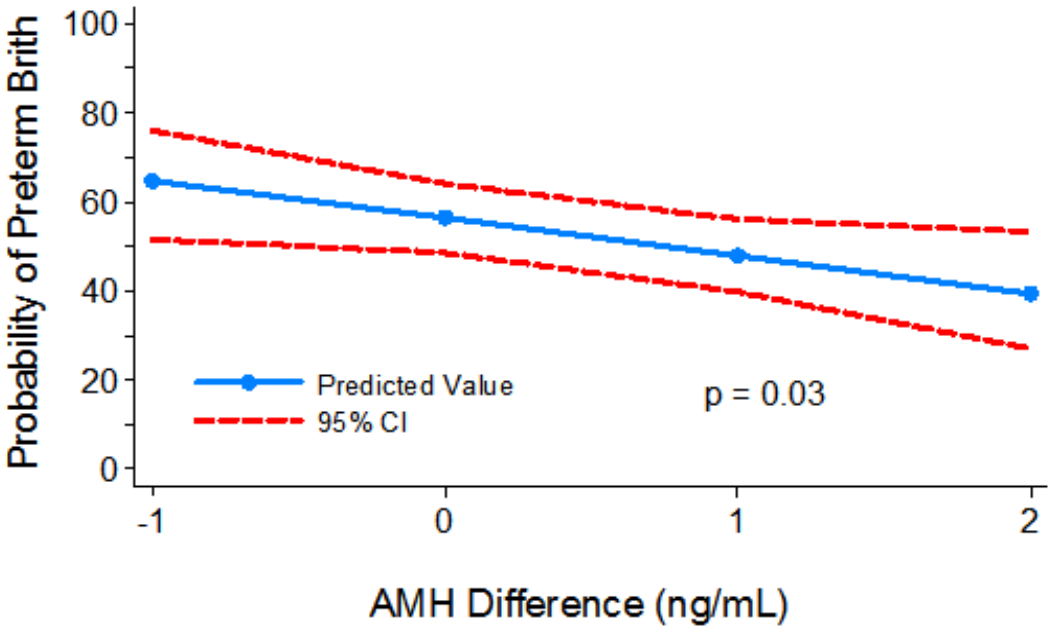
The association between AMH alone and probability of PTB was explored using the exposure variable of 2nd trimester AMH level (Table 2.2). The full model was adjusted for maternal age, fetal gender, maternal weight change between trimesters, previous preterm delivery, smoking status in pregnancy and gestational age at the time of the blood draw. All covariates except fetal gender were dropped in the final model, and no interactions were found. Even after controlling for fetal gender, the OR did not reach statistical significance (Table 2.2). The Hosmer-Lemeshow goodness of fit test for this model was not significant (p=0.68), and the ROC curve was 0.57.

Next, the difference between AMH levels in the 1st and 2nd trimesters was considered as the exposure variable. The same covariates were included in this model except that time difference between blood draws was used instead of the time of the 2nd trimester blood draw. Again, the only covariate that remained in the model was fetal gender. The association between AMH difference and PTB did reach statistical significance after adjusting for fetal gender (Figure 2.2a and Table 2.2), indicating that larger declines in AMH levels decreased the odds for PTB. However, the model was better at predicting women with a low risk for PTB rather than a high risk (OR: 0.74, 95% CI: 0.52, 0.98). The Hosmer-Lemeshow test confirm that the model was a good fit (p=0.24), and the ROC curve for this model was 0.61.

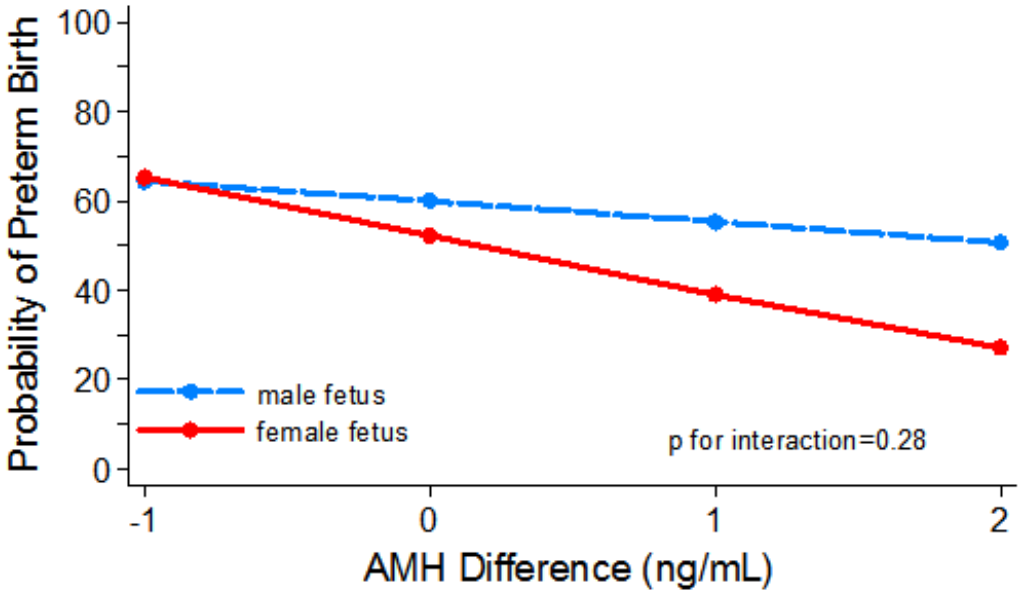
To evaluate a possible interaction between AMH difference and fetal sex, the model was stratified by fetal gender. No significant interactions were seen, although there was a difference in the patterns of AMH decline (Figure 2.2b).

Table 2.2. Odds ratios for preterm birth by 2nd trimester AMH levels and the difference in AMH levels between trimesters

Model	OR	95% CI	p-value
2nd trimester AMH (crude)	1.0	0.9, 1.1	0.6
2nd trimester AMH adjusted for fetal gender			0.3
2nd trimester AMH	1.0	0.9, 1.1	0.7
Fetal gender	0.7	0.4, 1.2	0.1
2nd trimester AMH adjusted for fetal gender			0.4
2nd trimester AMH	1.0	0.9, 1.1	0.7
Fetal gender	0.7	0.4, 1.2	0.2
History of previous PTB	1.5	0.5, 4.3	0.5
2nd trimester AMH adjusted for MSAFP			0.03
2nd trimester AMH	1.0	0.9, 1.1	0.6
MSAFP	2.5	1.3, 5.1	0.01
Weight change between trimesters	1.1	1.0, 1.1	0.1
2nd trimester AMH adjusted for MSAFP			0.046
2nd trimester AMH	1.0	0.9, 1.1	0.6
MSAFP	2.5	1.2, 5.0	0.01
Weight change between trimesters	1.1	1.0, 1.1	0.1
History of previous PTB	1.4	0.5, 4.1	0.6
AMH difference (crude)	0.7	0.5, 1.0	0.04
AMH difference adjusted for fetal gender			0.03
AMH difference	0.7	0.5, 1.0	0.03
Fetal gender	0.6	0.4, 1.1	0.1
AMH difference adjusted for fetal gender			0.06
AMH difference	0.7	0.5, 1.0	0.04
Fetal gender	0.6	0.4, 1.1	0.1
History of previous PTB	1.4	0.5, 4.1	0.5
AMH difference adjusted for MSAFP and weight change			0.006
AMH difference	0.7	0.5, 1.0	0.06
MSAFP	2.4	1.2, 4.9	0.02
Weight change between trimesters	1.1	1.0, 1.1	0.09
AMH difference adjusted for MSAFP and weight change			0.01
AMH difference	0.7	0.5, 1.0	0.07
MSAFP	2.4	1.2, 4.8	0.02
Weight change between trimesters	1.1	1.0, 1.1	0.09
History of previous PTB	1.4	0.5, 4.1	0.6



(a.) Adjusted for fetal gender



(b.) Stratified by fetal gender

Figure 2.2. Predicted probabilities of preterm birth based on AMH difference between 1st and 2nd trimesters.

2.4.2 Models combining AMH with other Markers

The modeling strategy described above was repeated, now with MSAFP, hCG, PAPP-A, estriol, and inhibin A included as covariates in the model. Twelve women did not have values for inhibin A, estriol, and hCG, so the levels were imputed. In the final model using the 2nd trimester AMH level as the exposure variable, all covariates were dropped with the exception of the MSAFP level and maternal weight change between trimesters. However, the association between 2nd trimester AMH and PTB was not significant (Table 2.2). The Hosmer-Lemeshow statistic indicated that the model was a good fit (p=0.25) and the ROC curve was 0.63.

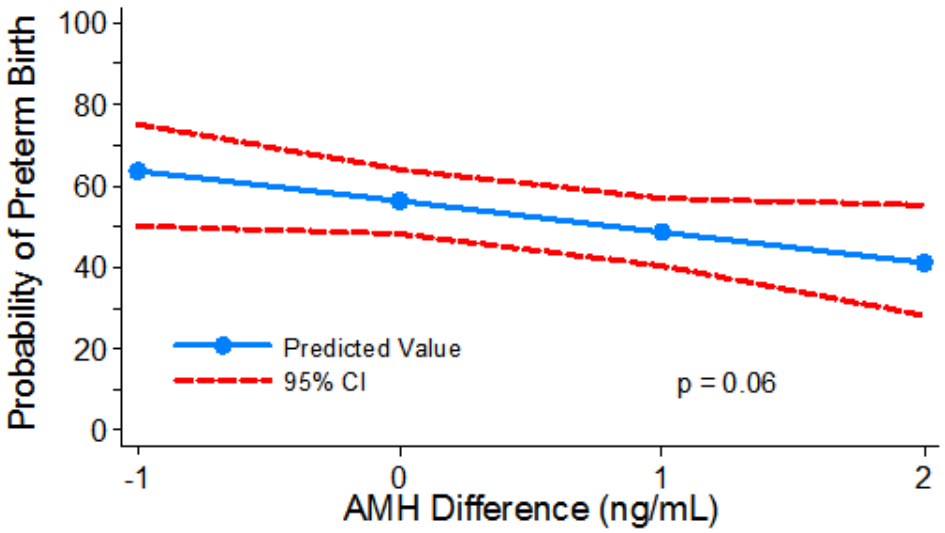
In the second model, the difference between the 1st and 2nd trimesters AMH levels was used as the exposure variable, adjusting for MSAFP, hCG, inhibin A, estriol, and PAPP-A. Again, only MSAFP and weight change between trimesters remained in the final model. The overall model was highly significant (Figure 2.3a and Table 2.2), but the majority of the risk for this model was found in the association between MSAFP and PTB (OR: 2.27, 95% CI: 1.11, 4.64). The Hosmer-Lemeshow statistic for this model was not significant (p=0.11) and the ROC curve indicated a moderate fit at 0.66. As with previous models, this model was better at predicting women at low risk of PTB rather than high risk; thus the clinical usefulness of the model is limited.

In each of the above models, MSAFP was strongly associated with PTB, with an OR over two. To further evaluate the contribution of MSAFP to the PTB model using AMH difference, MSAFP was dichotomized at ≤1 MoM or >1 MoM and evaluated as a class variable (Figure 2.3b). Fifty-three (50.5%) cases vs. 24 (25.3%) controls had an MSAFP of >1 MoM. According to this model, the risk for PTB was greatest with an MSAFP level > 1 MoM (Figure 2.3b). The addition of AMH difference provided further discrimination of the risk in women with an elevated MSAFP so that when the AMH level increased instead of decreasing between the 1st and 2nd trimester, the probability of PTB was as high as 82%.

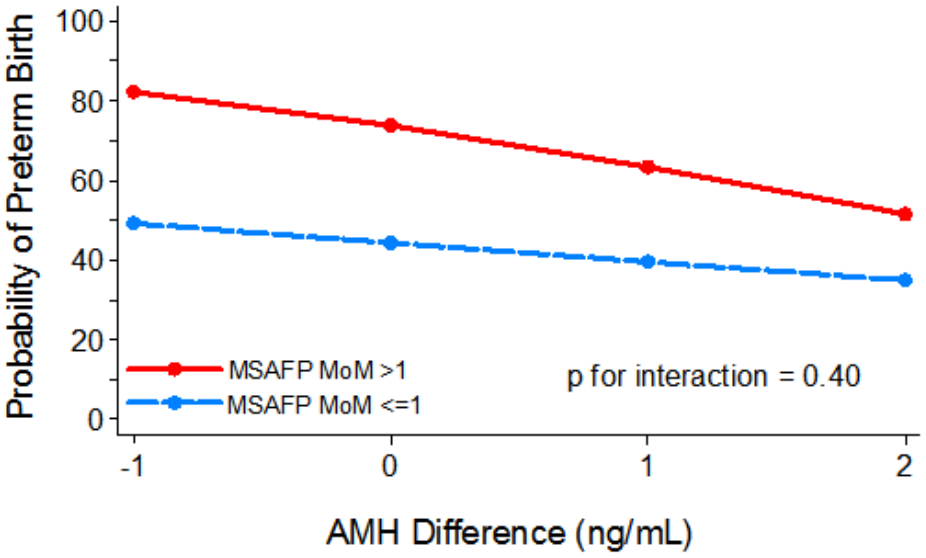
This probability of PTB applies to a case-control population with a 1:1 match so would not apply to the population as a whole. However, the results can be adapted to estimate the probability of PTB in a prospectively collected population if the incidence rate in the population is known. The following formula is used in convert the case-control probability: $P_p = kP_R/(1+ kP_R - P_R)$, where P_p is the incidence probability in the prospective population (12), P_R is the probability based on a fitted regression model from a case-control study, and k is the ratio that depends on your case-control sampling rates (in the case of a 11% incidence, it would be (5/89)/(5/11)). Based on this formula, women with an MSAFP of >1 MoM and an increase in AMH levels between trimesters would have a probability of PTB of 36%, compared to a probability of 6% in women with a decline in AMH of 2.0 ng/mL and an MSAFP of ≤ 1 MoM.

2.4.3 Women with a Previous PTB

Although a history of previous PTB did not have significant influence in these models, we further analyzed the contribution of this variable in our models by stratifying the results by history of previous PTB. In the bivariate analysis, the only variable that was statistically different between these two groups of women was gestational age at delivery, with women with a history of a previous PTB delivering significantly earlier than those without this history. Bivariate comparisons for all variables can be found in Table 2.3. The ORs for each model were recalculated with the previous history of PTB variable in the model, but there were no changes in any of the observed associations. These results can be found in Table 2.2.



(a.) Adjusted for maternal alpha-fetoprotein level and weight change between trimesters



(b.) Stratified by maternal alpha-fetoprotein level (class variable) and adjusted for weight change between trimesters

Figure2.3. Probability of preterm birth based on AMH difference between 1st and 2nd trimesters.

Table 2.3. Demographic and univariate/bivariate data for women with and without a history of preterm birth, restricted to white women only

Continuous variables	History of previous PTB Mean (SD) n=184	No history of PTB Mean (SD) n=16	Odd ratio (95% CI)	p value
Maternal age[a]	28.9 (4.8)	28.8 (5.3)	1.0 (0.9, 1.1)	0.9
Gestational age at delivery[b]	34.3 (4.8)	36.4 (3.0)	0.9 (0.8, 1.0)	0.02
Gestational age at 1st trimester screen[c]	83.1 (6.2)	83.7 (6.5)	1.0 (0.9, 1.1)	0.8
Gestational age at 2nd trimester screen[c]	120.8 (12.5)	118.5 (8.8)	1.0 (1.0, 1.1)	0.3
Maternal weight change between the 1st and 2nd trimester[d]	3.0 (4.2)	3.0 (5.2)	1.0 (0.9, 1.1)	1.0
MSAFP[e] at 2nd trimester[f]	1.2 (0.5)	1.1 (0.4)	1.9 (0.7, 5.4)	0.2
Maternal hCG[g] at 2nd trimester[f, h]	1.4 (0.7)	1.1 (0.7)	1.4 (0.8, 2.5)	0.2
Maternal PAPP-A[i] at 1st trimester[f, h]	0.8 (0.4)	1.0 (0.6)	0.3 (0.1, 1.2)	0.05
Maternal Estriol at 2nd trimester[f, h]	0.9 (0.3)	1.0 (0.3)	0.4 (0.1, 2.7)	0.3
Maternal inhibin A at 2nd trimester[f, h]	1.4 (0.8)	1.2 (1.0)	1.2 (0.8, 1.7)	0.4
AMH at 1st trimester screening[j]	2.8 (2.4)	3.4 (2.5	0.9 (0.7, 1.1)	0.3
AMH at 2nd trimester screening[j]	2.5 (2.1)	2.9 (2.2)	0.9 (0.7, 1.2)	0.4
Difference between AMH readings[j]	0.3 (0.8)	0.5 (0.9)	0.8 (0.4, 1.4)	0.5

Categorical Variables	History of PTB n (%)	No history of PTB n (%)	Odd ratio (95% CI)	p value
Infant Gender				
Male	10 (9.6)	94 (90.4)		
Female	6 (6.3)	90 (93.8)	0.6 (0.2, 1.8)	0.4
Smoked during pregnancy				
Yes	5 (15.6)	27 (84.4)		
No	11 (6.6)	157 (93.4)	2.6 (0.9, 8.2)	0.1

[a]years, [b]weeks of gestation, [c]days of gestation, [d]pounds, [e]Maternal Serum Alpha-fetoprotein,

[f]Measures of the Median (MoM), [g]human Chorionic Gonadotropin

[h]12 missing values (6 cases and 6 controls), [i]Plasma protein of pregnancy-A, [j]ng/mL

2.5 Discussion

A minimal decline or an increase in AMH between the 1st and 2nd trimesters of pregnancy can help identify women with a high probability of PTB, but only when the MSAFP is also elevated. Significant associations were noted with minor changes in either hormone, a finding that is unique to these models. In addition, these models found significant associations in women without a previous history of PTB, making it easier to apply the results to a larger subset of women. Because the decline in AMH between trimesters occurs in early pregnancy and can be observed in the same time periods used for IPS, these findings could be easily incorporated into current clinical practice.

These models improve on previous attempts of PTB prediction using only feto-placental biomarkers. Metcalfe et al. (8) reported that biomarkers currently used to screen for aneuploidy have detection rates too low to identify women at risk for adverse obstetrical outcomes. A review by Menon et al. (5), looked at 116 different biomarkers assayed 578 times in 217 studies, and found that no single biomarker could reliably predict PTB. Jelliffe-Pawlowski et al. (57) developed a model that predicted PTB with an OR of 2.3-3.6, but the model only worked with extreme biomarker levels (PAPPA-A that was $\leq$ the 5th percentile and/or MSAFP that was $\geq$ the 95th percentile, and/or 2nd trimester inhibin A that was $\geq$ the 95th percentile). Finally Yuan et al. (52) published a recent meta-analysis on the use of MSAFP in the prediction of PTB, and found a relationship with MSAFP when levels exceeded 2.0 MoM, but only when other biomarkers are also abnormal.

The improved sensitivity of PTB models with the inclusion of an ovarian biomarker, along with the need to control for fetal gender in some of the models, may indicate that cross-talk is occurring between the ovary and the feto-placental unit during the critical periods of placentation. Specifically, this suggests the existence of a fetal/placental/ovarian axis for progesterone control. The contribution of the ovary, or more specifically the corpus luteum (CL), to the maintenance of a healthy pregnancy

after the first eight weeks of gestation has been largely ignored. While it is recognized that the "rescued" CL is the only source of progesterone prior to the shift of progesterone production to the placenta at 8 weeks gestation (43, 44, 83), the reason for the persistence of the CL until 13-15 weeks gestation remains unclear. Recently, Baird postulated that when the placenta is unable to produce sufficient progesterone, the CL might be recruited for additional progesterone support. She theorized that continued and prolonged stimulation of the CL is an attempt to lessen an existing progesterone deficit and salvage a marginal pregnancy (44). Therefore, an AMH levels that does not decline in the 2nd trimester of pregnancy in the presence of an abnormal feto-placental biomarker may indicate an increased need for progesterone and/or of abnormal placentation. The lack of decline in AMH levels in women with a corresponding increase in MSAFP who were identified to be at high risk for PTB in our model supports this theory.

A major strength of this study was our ability to use banked samples, which allowed us to test 1st and 2nd trimester serum in these women. As all of the samples came from one laboratory (SHL) and were handled according to a single protocol, there was less potential for bias based on how the samples were processed. In addition, all AMH testing was performed by a single reference laboratory with extensive experience in AMH testing and who had strict quality control measures in place (ReproSource), thus the results were less likely to be influenced by variations in the testing procedure.

However, this study is not without weaknesses. Birth certificate data were used to identify risk factors, which might have led to misclassification based on inaccurate reporting of other etiologies for PTB. We recognized this possibility and oversampled in order to address this potential bias. In addition, this misclassification would have likely led to a bias towards the mean and a negative result, which was not the outcome of our study. All women in this study had elected to undergo IPS so these women may be fundamentally different from the rest of the women in Iowa. Further, we only studied white women so results cannot be generalized to the non-white population. But it should

be noted that this restriction to white women could also be considered a strength as it limited the heterogeneity in the data and may have eliminated confounding based on race. Finally, the ELISA kit used for AMH testing has reported abnormal findings due to complement interaction when testing was performed close to the time of the blood draw. However, all of these samples were frozen for a minimum of two years, which would have minimized the amount of complement present in the samples. Therefore, it is unlikely that complement interference was a problem.

2.6 Conclusions

In conclusion, AMH levels in early pregnancy may improve the sensitivity of PTB models. The lack of decline in AMH levels, along with elevation in MSAFP, suggests an increased need for CL-derived progesterone when abnormal placentation has occurred. Therefore, consideration of AMH changes in early pregnancy in PTB risk models may help identify women who would benefit from interventional therapies such as supplemental progesterone (2, 20). However, additional studies are needed to validate these findings and to develop a predictive model for PTB that could be used in the clinical setting.

CHAPTER 3.
DECLINES IN ANTI-MULLERIAN HORMONE LEVELS IN EARLY PREGNANCY ARE DIRECTLY ASSOCIATED WITH TIME TO DELIVERY

3.1 Summary of findings

The long-term consequences of preterm birth (PTB) are closely related to the gestational age at delivery. Predictive models focusing on time to delivery are largely unsuccessful, but their usefulness may be improved by the addition of biomarkers for progesterone production. Anti-Mullerian hormone (AMH), a biomarker of ovarian activity, may be a surrogate biomarker for ovarian progesterone production in pregnancy. The purpose of this study was to test the association between maternal anti-Mullerian hormone (AMH) levels sampled in the 1st and 2nd trimesters with time to delivery, both when AMH is considered as a single biomarker and then in combination with other markers of feto-placental health.

This is a Cox regression analysis of time to delivery using an established case-control dataset of pregnant women residing in Iowa between 2009 and 2010. All women undergoing Integrated Prenatal Screening (IPS) and who then delivered in Iowa were eligible for inclusion. Cox regression models were developed using gestational age at delivery as the outcome along with one of four different exposure variables: 2nd trimester AMH levels, the difference in AMH levels between 1st and 2nd trimesters, the combined risk of 2nd trimester AMH level and maternal serum alpha-fetoprotein levels (MSAFP), and the combined risk of difference in AMH levels between the 1st and 2nd trimesters and MSAFP. Other factors considered in these models were maternal age, maternal weight change between trimesters, tobacco use in pregnancy, previous history of a preterm delivery and levels of the feto-placental biomarkers included in IPS testing. In addition, each model contained a variable that was used to control for the timing of the blood draw

(when 2nd trimester AMH level was used) or to correct for the time difference between blood draws (when AMH difference between the 1st and 2nd trimesters was used)

A significant, direct, dose-dependent association with time to delivery was seen for the AMH difference between the 1st and 2nd trimesters when considered as an independent risk with along with MSAFP and weight change between trimesters. The AMH difference between the 1st and 2nd trimester was not significantly associated with time to delivery when MSAFP risk was controlling for in the model but not included as an independent risk. Second trimester AMH levels were not associated with time to delivery, either in isolation or when considered as an independent risk along with MSAFP.

The lack of decline in AMH between with the 1st and 2nd trimesters of pregnancy may be a useful biomarker to identify women who would benefit from supplemental progesterone use when considered along with the independent risk of MSAFP and controlling for maternal weight change between with 1st and 2nd trimesters. The lack of an AMH decline may reflect the magnitude of the progesterone insufficiency in early pregnancy in women who subsequently go on to a spontaneous preterm delivery.

3.2 Introduction

The long-term consequences of preterm birth (PTB) are well recognized. It is the second leading direct cause of mortality in children under five years of age, and 35% of the 3.1 million neonatal deaths worldwide are directly related to complications from PTB (12). Extremely preterm (<28 weeks gestation) infants have the highest rates of neonatal deaths (17), and over 50% of those deaths occur in infants born prior to 24 weeks gestation (25). Long-term health consequences (12, 16) and healthcare costs (15) are directly tied to gestational age at delivery. Extremely preterm infants are hospitalized 85 times longer than term infants, and the mean societal cost for a live birth for an infant born at <28 weeks is estimated to be $264,412 (15).

While much attention is given to the costs associated with extremely preterm infants, this is only a small subset of infants who face adverse outcomes based on their gestation age at delivery. Only 6% of preterm births occur prior to 28 weeks, while 72% of preterm infants deliver between 34 and 36 weeks gestation (late preterm) (17). Recent evidence of negative health outcomes in infants born as late as 37-38 weeks and in pregnancies continuing past 41 weeks (24) further illustrates the importance of gestational age at delivery as a risk factor for all pregnancies. In December 2012, the Defining "Term" Pregnancy Working Group addressed this concern by introducing several new gestational age birth categories. Specifically, this cross functional group of stakeholders recommended subdividing term pregnancies into early term (37-38 weeks), term (39-40 weeks) and late term (≥41 weeks) (24). In this context, predictive models focusing on gestational age at delivery should be developed, but very few existing models focus on "time to delivery" as the primary outcome (3, 53, 84). The only model that has attempted to predict time to delivery using maternal biomarkers and biochemical data performed poorly as a screening test, which limits its usefulness in clinical practice (85).

The predictive ability of the "time to delivery" models may improve with a better understanding of the underlying pathophysiology of PTB. Inflammation in the placental bed is one factor that has a major impact on time to delivery in preterm infants (1, 34, 86). The anti-inflammatory actions of progesterone at the time of placental implantation are believed to control the degree of inflammation in the placental bed (2, 44). Because serum progesterone levels do not adequately reflect placental progesterone levels (46), the usefulness of a serum progesterone level as a biomarker in pregnancy is limited. However, other maternal biomarkers that signal a need for increased progesterone production may provide useful information.

Ovarian biomarkers are a candidate for this purpose. Prior to eight weeks gestation, progesterone production occurs exclusively in the corpus luteum (CL) of the ovary, while after eight weeks gestation the placenta takes over this role. However,

despite the fact that the CL is not required after eight weeks, it continues to be active until about 13 weeks gestation when the placenta finally signals the CL to involute (2, 44). Baird recently suggested that when the level of placental progesterone was insufficient to control the inflammation in the placental bed, the placenta might "recruit" the CL for additional progesterone support by preventing CL involution (10). Therefore, levels of ovarian activity might identify women with an increased need for progesterone; a highly active ovary in the 2nd trimester of pregnancy would be a signal of a greater progesterone deficit and a shorter time to delivery.

Anti-Mullerian hormone (AMH) is an ovarian biomarker that has been used in non-pregnant women as a marker of ovarian activity (62, 63, 68, 80). Previously, AMH levels were believed to be unchanged in pregnancy (69), but recent work confirms that AMH levels decline sharply between 13-15 weeks gestation and remain low until the placenta is delivered (70, 71, 74). The rapid decline and equally rapid return to prepregnancy levels after delivery suggests an active suppression of AMH in this time-period. We hypothesized that AMH activity in pregnancy directly reflects ovarian progesterone production. We also hypothesized that AMH levels decline when the ovary become quiescent as the physiologic need for ovarian-derived progesterone diminishes. Therefore, little or no decline in AMH levels in early pregnancy would be associated with a shorter time to delivery. The purpose of this study was to test the association between maternal AMH levels sampled in the 1st and 2nd trimesters with time to delivery, both when AMH is considered as a single biomarker and then in combination with other markers of feto-placental health.

3.3 Materials and Methods

This was a secondary data analysis using a previously developed case-control database (parent database) of pregnant women in Iowa. The Human Subjects Office at the University of Iowa approved the study, as did the Iowa Department of Public Health

Research and Ethics Review Committee, who granted us permission to use birth certificate data. The Congenital and Inherited Disorders Advisory Committee for the state of Iowa granted permission for use of the maternal serum samples.

3.3.1 Development of the Serum Tissue Bank

The serum tissue bank at the University of Iowa contains samples from women undergoing 1st and 2nd trimester prenatal screening. When prenatal screening was completed, the remaining serum was stored at -80˚C and then linked to birth certificate data when the child delivered. For women electing to undergo Integrated Prenatal Screening (IPS), two samples were drawn according to protocol: one between 10-13.9 weeks gestation and the second between 15-22.9 weeks gestation. The IPS results were also available in the database, and included maternal serum alpha-feto protein (MSAFP), human chorionic gonadotropin (hCG), inhibin A, pregnancy-associated plasma protein A (PAPP-A) and estriol. Because IPS testing requires accurate gestational dating, a 1st trimester ultrasound was available on all women. In total, over 3,000 women had paired samples that were available for use in the parent study.

3.3.2 Development of the Parent Database

The development of the parent dataset has been previously described in Section 2.3.2**.** In brief, it was a case-control dataset established to examine the association between AMH levels in the 1st and 2nd trimesters and PTB. Inclusion criteria included women of any age with delivery of a singleton infant after 20 weeks gestation who also had paired serum samples. Women were excluded as described in Figure 3.1. These exclusion criteria were designed help restrict the dataset to women with a spontaneous PTB. The dataset was further restricted to women of white race because of concerns that the sample size of non-white women was too small to adequately evaluate the impact of race in the model. The parent dataset used for this analysis included 200 white women who delivered between 20 and 41 weeks gestation with paired serum samples: 105 who delivered

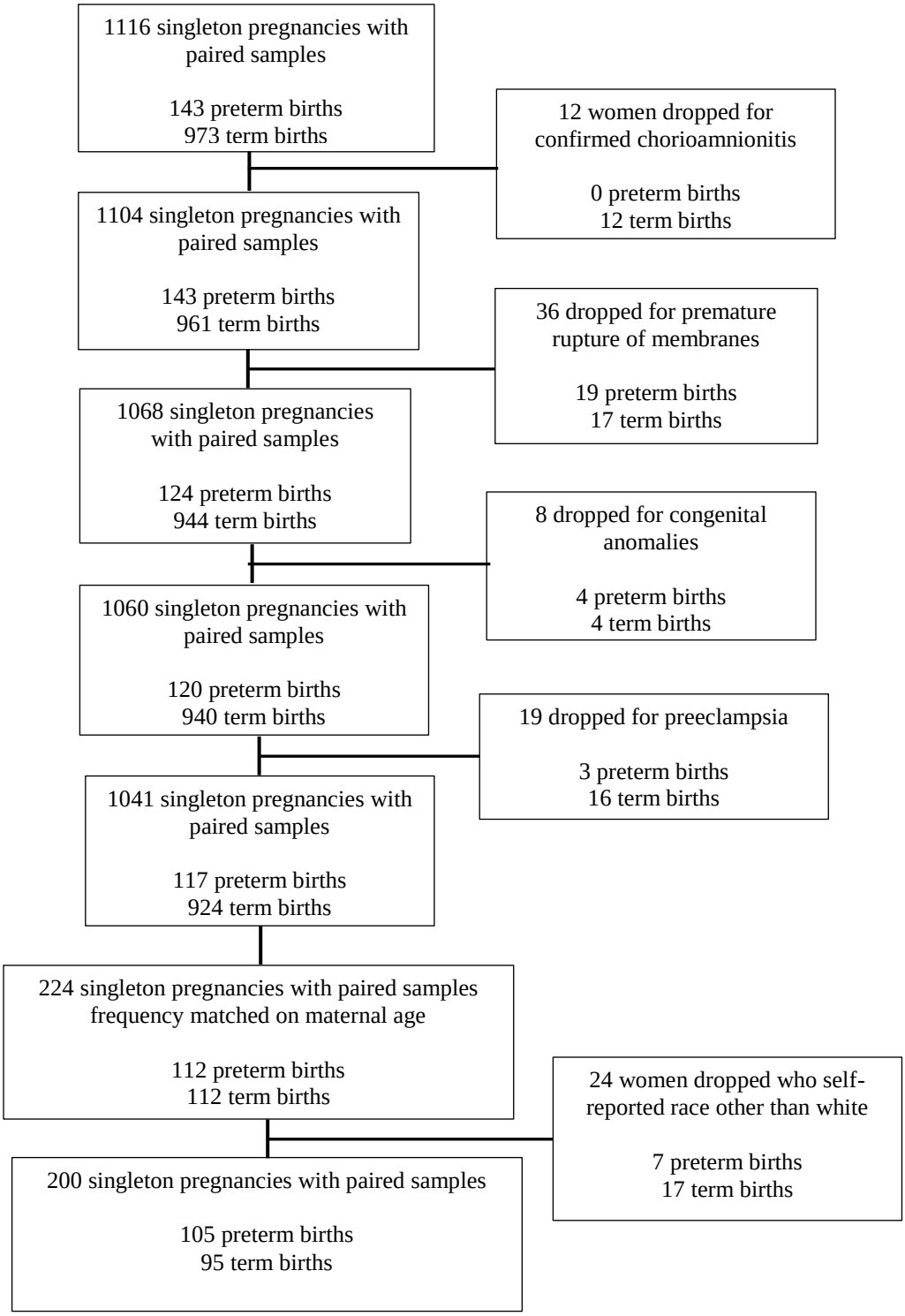


Figure 3.1 Selection of subjects from serum tissue bank for inclusion in the parent dataset

between 24 and < 37 weeks gestation and 95 who delivered at ≥37 weeks gestation. All women had information on MSAFP and PAPP-A, and twelve women were missing information on hCG levels, inhibin A levels and estriol levels. These levels were imputed for the final analysis.

3.3.3 Anti-Mullerian Hormone Measurements

Stored samples remained frozen at -80˚C until the time of analysis and had undergone a single free-thaw cycle. A single reference laboratory (ReproSource, Inc., Woburn, Massachusetts) performed all of the analyses using their laboratory-developed AMH assay. This assay is based on research-use-only materials and reagents from Beckman Coulter-DSL (Chaska, MN). Intra- and interassay coefficients of variation with serum controls were 5%-9% and 7%-12%, respectively. Serum AMH values below 0.1 ng/mL, the reported clinical level of detection, were reported as 0.05 in the statistical analysis.

3.3.5 Statistical Analysis

Univariate and bivariate analyses were used to determine normality of the data. The following covariates were evaluated for inclusion in the models: maternal age, maternal weight change between trimesters, history of a previous preterm delivery, history of smoking in pregnancy, fetal gender, MSAFP, inhibin A, hCG, estriol, and PAPP-A. In order to better describe the data, a categorical variable was created based on the National Institutes of Health (NIH) and WHO definitions of gestational ages for preterm and term infants (11, 21, 24). The categories in this variable included extremely preterm (<28 weeks), very preterm (28-31 weeks), moderately preterm (32-34 weeks), late preterm (34-36 weeks), early term (37-38 weeks), term (39-40 weeks) and late term (≥41 weeks). This variable was used to perform bivariate analyses for all other covariates and to look for trends across continuous variables using a non-parametric test

of trend for the ranks across ordered groups (an extension of the Wilcoxon rank-sum test).

Second trimester AMH level and the difference in AMH levels between trimesters were each transformed to categorical variables: 2nd trimester AMH level was dichotomized based on the median and AMH difference between trimesters was transformed into a three level variable based on tertiles. Time to delivery as a function of each of these variables was tested for violations of the proportional hazards assumption using the log-rank test and Kaplan-Meier plots. Two separate sets of analyses were completed for each AMH exposure variable. The first included only the AMH exposure variable, while the second set included the AMH exposure variable controlled for other biomarkers of feto-placental health (MSAFP, inhibin A, hCG, estriol, and PAPP-A). Multivariable Cox proportional hazards models were fit with each covariate to determine the independent association of each variable to the model as a whole, using the Breslow method for ties. Censor level for gestational age at delivery was set at 40 for all models. Variables that did not appear to contribute to the model (did not alter the β-coefficient by > 15%) were removed and likelihood ratio tests were used to determine the suitability to remain out of the model.

Finally, a variable was constructed for each AMH exposure that was used to control for the difference in gestational age at the time of the blood draw. Gestational age at the time of the 2nd trimester sample was included in the models using 2nd trimester AMH levels, and the time difference between the 1st and 2nd trimester blood samples was included in the using AMH difference. Stata 11.2 (College Station, TX) was used for all analyses, with a two-tailed p-value of 0.05 being considered statistically significant.

3.3.6 Power Calculation

A post-hoc power analysis was calculated based on 200 women. This dataset had a power of >90% to determine a 10% difference in the hazard rates (HR) of a binary

covariate. Significance was set a 0.05 with a two-sided alpha. The methodology used to determine power was based on the theories of Schoenfeld (87) and Hsieh and Lavori (88).

3.4 Results

3.4.1 Dataset Characteristics

This dataset included four extremely preterm, 12 very preterm, 14 moderately preterm, 75 late preterm, 75 early term, 18 term and 2 late term infants (Table 3.1). Extremely and very preterm infants tended to be born to slightly older mothers, and these mothers had a slightly greater weight gain between the 1st and 2nd trimesters of pregnancy, although neither trend was significant (p_{trend}=0.3 and 0.2, respectively). Average 2nd trimester MSAFP and 1st trimester PAPP-A levels declined as gestational age at delivery increased (p_{trend}=0.01 and 0.04, respectively), while estriol did not change. Human Chorionic Gonadotropin and inhibin A levels were higher in women who had an earlier delivery than women who delivered at term (excluding extremely preterm infants), but only inhibin A showed a decreasing trend across birth categories (p_{trend}=0.2 and 0.003, respectively). AMH level in the 1st trimester, AMH level in the 2nd trimester and the difference between these two levels were not significantly different between time to delivery categories. However, the trend was significant for an increase in the difference between AMH level in the 1st and 2nd trimesters of pregnancy with a longer time to delivery (p_{trend}=0.03). There were no significant differences in the gender distribution or in number of women who smoked in pregnancy (χ^2=0.1 and 0.08, respectively). However, none of the women in the extreme preterm or the very preterm categories had a history of smoking. The number of women with a history of PTB was not statistically different between categories (p=0.22). In addition, no variable evaluated for inclusion in these models violated proportional hazards assumption testing.

Table 3.1 Demographic and feto-placental biomarkers by time to delivery

Continuous variables	Extremely preterm (<28 wks) n=4 Mean (SD)	Very preterm (28-31 wks) n=12 Mean (SD)	Moderately preterm (32-33 wks) n=14 Mean (SD)	Late preterm (34-36 wks) n=75 Mean (SD)	Early term (37-38 wks) n=75 Mean (SD)	Term (39-40 wks) n=18 Mean (SD)	Late term (≥41 wks) n=2 Mean (SD)	P_{trend}
Maternal age[a]	31.1 (7.0)	30.2 (4.0)	25.8 (4.5)	29.0 (5.2)	29.3 (5.5)	26.6 (4.9)	25.7 (3.1)	0.3
Gestational age at 1st trimester screen[b]	82.3 (7.9)	85.3 (7.6)	84.0 (6.6)	84.7 (6.9)	82.5 (5.8)	82.1 (5.5)	89.5 (7.8)	0.2
Gestational age at 2nd trimester screen[b]	113.8 (6.8)	123.2 (13.0)	115.8 (7.9)	119.5 (8.7)	118.0 (9.4)	118.8 (8.8)	114.0 (2.8)	0.7
Maternal weight change between 1st and 2nd trimester[c]	6.1 (5.1)	6.4 (8.9)	0.7 (5.3)	3.4 (5.3)	2.7 (4.2)	2.3 (3.3)	-2.4 (5.1)	0.2
MSAFP[d] in 2nd trimester[e]	1.0 (0.3)	1.1 (0.4)	1.6 (0.6)	1.1 (0.4)	1.0 (0.4)	1.0 (0.2)	0.8 (0.007)	0.01
hCG[f] in 2nd trimester[e]	1.3 (0.2)	1.6 (1.1)	1.5 (1.7)	1.1 (0.5)	1.1 (0.5)	1.0 (0.5)	1.2 (0.5)	0.2
PAPP-A[g] in 1st trimester[e]	0.7 (0.6)	1.0 (0.6)	0.9 (0.5)	1.0 (0.5)	1.1 (0.6)	1.2 (0.5)	0.7 (0.06)	0.04
Estriol in 2nd trimester[e]	10. (0.4)	0.9 (0.3)	1.0 (0.3)	1.0 (0.3)	1.0 (0.3)	1.0 (0.5)	0.8 (0.2)	0.7
Inhibin A in 2nd trimester[e]	1.0 (0.4)	1.6 (0.8)	2.2 (2.9)	1.1 (0.6)	1.1 (0.6)	0.9 (0.2)	0.6 (0.07)	0.003
AMH[e] level in 1st trimester[h]	5.5 (1.8)	3.2 (3.1)	3.2 (3.0)	3.0 (2.1)	3.4 (2.0)	4.5 (4.3)	1.8 (1.0)	0.6

Table 3.1 (continued)

AMH level in 2nd trimester[h]	5.4 (2.4)	3.2 (3.0)	3.0 (1.8)	2.5 (1.9)	2.9 (1.9)	3.6 (3.4)	1.5 (1.6)	0.5
Difference in AMH level between 1st and 2nd trimester[h]	0.09 (0.8)	-0.01 (1.2)	0.2 (1.6)	0.5 (1.4)	0.6 (1.6)	0.9 (1.5)	0.3 (0.6)	0.03
Categorical variable	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	X^2
Gender								
Male	0 (0.0)	7 (58.3)	6 (42.9)	47 (62.7)	36 (48.0)	7 (38.9)	1 (50.0)	
Female	4 (100)	5 (41.7)	8 (57.2)	28 (37.3)	39 (52.0)	11 (61.1)	1 (50.0)	0.1
Previous preterm delivery								
Yes	1 (25.0)	3 (25.0)	2 (14.3)	4 (5.3)	5 (6.7)	1 (5.6)	0 (0.0	
No	3 (75.0)	9 (75.0)	12 (85.7)	71 (94.7)	70 (93.3	17 (94.4)	2(100)	0.2
Smoked in pregnancy								
Yes	0 (0.0)	0 (0.0)	6 (42.9)	13 (17.3)	10 (13.3)	3 (15.0)	0 (0.0)	
No	4 (100)	12 (100)	8 (57.1)	62 (82.7)	65 (86.7)	15 (83.3)	2 (100)	0.08

[a] years, [b] days, [c]pounds, [d]Maternal alpha-fetoprotein, [e]Multiple of the Median (MoM), [f]human Chorionic Gonadotropin,

[g]Pregnancy associated plasma protein, [h]ng/mL

3.4.2 Models using only AMH

Two models were developed that included AMH without other biomarkers of feto-placental health; the first used 2nd trimester AMH and the second using AMH difference. After evaluating the contributions of the covariates, fetal gender and weight change were retained in both models. A history of previous PTB also remained in the model based on literature evidence that a history of PTB is strongly associated with PTB in subsequent pregnancies. However, neither model reached statistical significance (2nd trimester AMH: p=0.09; AMH difference between trimesters: p=0.08) (Table 3.2).

3.4.3 Models using AMH and other biomarkers of feto-placental health

The first model in this group evaluated 2nd trimester AMH along with five biomarkers of feto-placental health: MSAFP, inhibin A, hCG, estriol, and PAPP-A. MSAFP, fetal gender and weight change between trimesters had an impact and remained in the model. A history of previous PTB also remained in the model based on literature evidence of a strong effect. The overall model was significant (p=0.01), but AMH did not contribute substantially to the final model (HR: 1.1, 95% CI: 0.9-1.5; p=0.4). (Table 3.2) indicating that 2nd trimester AMH does not have a significant impact on time to delivery.

The second model included AMH difference between trimesters as the exposure variable along with the five biomarkers of feto-placental health. MSAFP and weight change between trimesters remained in the model and again, history of previous PTB was added to the final model. The model was significant (p=0.007), but again, AMH difference did not contribute to the overall significance (HR: 0.9; 95% CI: 0.7, 1.2, p=0.3). Rather, MSAFP and weight change between trimesters accounted for most of the significance in this model (Table 3.2). Although a history of previous PTB did not have an impact on the β-coefficient, the HR for this covariate in the model was significant

Table 3.2 Hazard ratios for time to delivery for model variables

Model	Hazard ratio	95% Confidence interval	p value
2nd trimester AMH with fetal gender and weight change between trimesters			0.09
2nd trimester AMH[a]	1.1	0.8, 1.5	0.6
Fetal gender	0.8	0.6, 1.1	0.2
Weight change between trimesters	1.0	1.0, 1.1	0.05
History of previous preterm birth	1.6	1.0, 2.7	0.06
2nd trimester AMH level controlled for MSAFP[b], fetal gender, and weight change between trimesters			0.01
2nd trimester AMH	1.1	0.9, 1.5	0.4
MSAFP	1.6	1.1, 2.3	0.008
Fetal gender	0.9	0.7, 1.2	0.4
Weight change between trimesters	1.0	1.0, 1.1	0.03
History of previous preterm birth	1.7	1.0, 2.8	0.048
2nd trimester AMH/MSAFP[c] combined risk controlled for weight change between trimesters and inhibin A			0.005
2nd trimester AMH/MSAFP	1.1	1.0, 1.3	0.1
Weight change between trimesters	1.0	1.0, 1.1	0.03
inhibin A level	1.2	1.1, 1.4	0.009
History of previous preterm birth	1.6	1.0, 2.7	0.06
AMH difference between 1st and 2nd trimesters with fetal gender and weight change between trimesters			0.08
AMH difference between 1st and 2nd trimester	0.9	0.7, 1.2	0.3
Fetal gender	0.9	0.7, 1.2	0.3
Weight change between trimesters	1.0	1.0, 1.1	0.05
History of previous preterm birth	1.7	1.0, 2.8	0.05
AMH difference with MSAFP			0.007
AMH difference	0.9	0.7, 1.2	0.4
MSAFP	1.6	1.1, 2.3	0.008
Weight change between trimesters	1.0	1.0, 1.1	0.03
History of previous preterm birth	1.7	1.0, 2.9	0.04
AMH difference/MSAFP[c] combined risk			0.006
AMH/MSAFP	1.1	1.0, 1.2	0.01
Weight change between trimesters	1.0	1.0, 1.1	0.02
History of previous preterm birth	1.7	1.0, 2.9	0.04

[a] AMH—Anti-Mullerian Hormone, [b] MSAFP—Maternal serum alpha-fetoprotein

[c]The lowest risk variable is the (referent)

(HR: 1.7, 95% CI: 1.0, 2.8, p=0.05), confirming that this risk remains an important predictor of PTB. The lack of impact in the overall model was likely related to the low number of women in the study with a history of previous PTB.

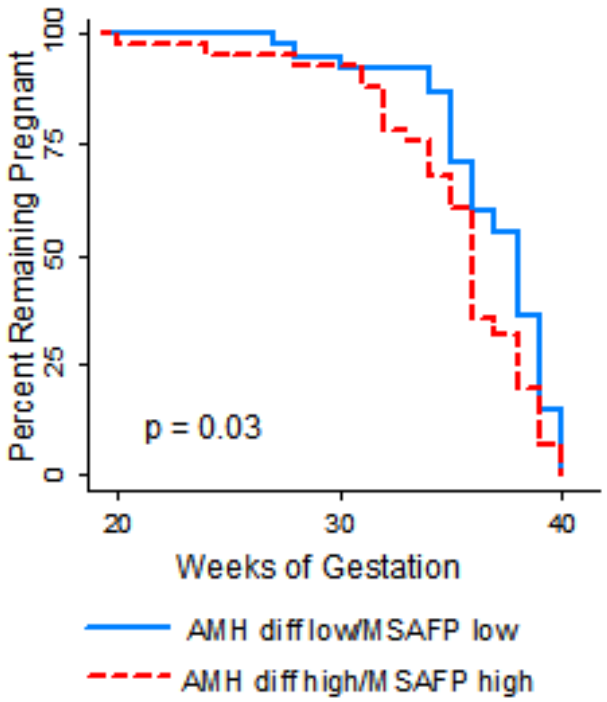
Finally, two new variables were developed that combined the independent risks of AMH and MSAFP to determine if greater discrimination between high and low risk pregnancies could be achieved. For the first new variable, 2nd trimester AMH levels were dichotomized (based on the median) and a four-level variable was generated combining 2nd trimester AMH levels (low risk for AMH ≤0.25 ng/mL, high risk for AMH levels >0.25 ng/mL) and MSAFP (low risk for MoM ≤1, high risk for MoM >1). When the combined risk variable was used as the exposure variable for the final model, controlling for weight change between trimesters and inhibin A, the model was significant (p=0.005). However, the contribution of the four-level variable of 2nd trimester AMH/MSAFP was not significant in the model (p=0.1) (Table 3.2) and the majority of the risk in this model was related to inhibin A and weight change between trimesters. A history of PTB did not reach significance in this model (p=0.06).

For the second new variable, three levels of the AMH difference variable were generated based on tertiles of AMH difference. Tertiles were used as one-third of the women in the dataset had an AMH difference that either did not change or increased between the 1st and 2nd trimester. The new variable included AMH risk (AMH difference risk low >0.73 ng/mL, AMH difference moderate risk >0.01 to ≤0.73 ng/mL and AMH difference high risk ≤0.01 ng/mL) and MSAFP risk (low for MoM ≤ 1, high for MoM of >1). The six levels of risk for this variable were: AMH difference risk low/MSAFP risk low (referent), AMH difference risk moderate/MSAFP risk low, AMH difference risk low/MSAFP risk high, AMH difference risk high/MSAFP risk low, AMH difference risk moderate/MSAFP risk high, AMH difference risk high/MSAFP risk high. The best fitted model, adjusted for weight change between the 1st and 2nd trimesters, was significant (p=0.006) and in this model AMH/MSAFP combined risk had a played a significant role

(p=0.01). As with the previous model of AMH difference controlling for MSAFP, a previous history of PTB did not contribute to the β-coefficient for AMH/MSAFP risk, but was found to be significant in the model.

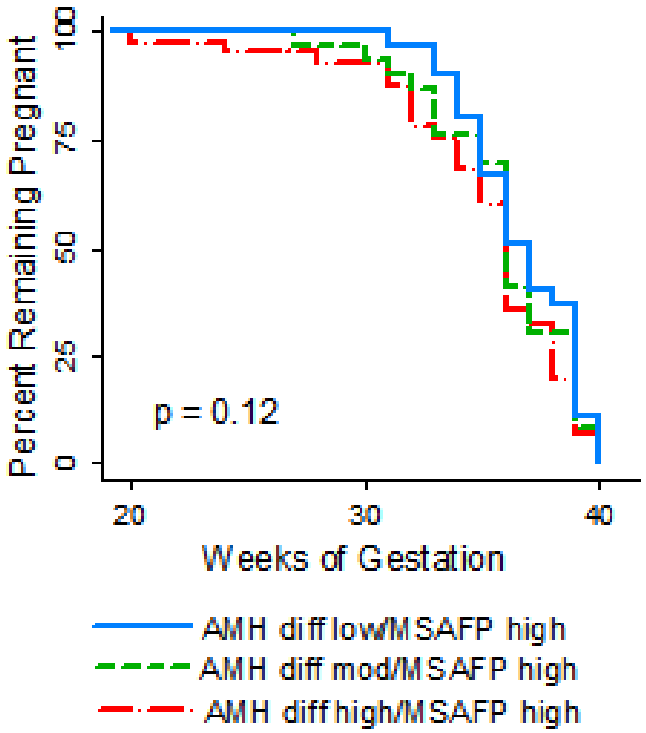
The covariate accounting for the time lapse between the 1st and 2nd trimester blood draws was included in each of the final model but it did not have a impact on the results. The mean time interval was 35 days, the median was 30 days, and the range was 14 to 50 days. The sensitivity analysis based on the time lapse found no impact based in the models. Therefore, this covariate was not included in the final analysis.

To determine if the discrimination provided by this new model was clinical useful, we compared the AMH difference risk high/MSAFP risk high group to the AMH difference risk low/MSAFP risk low group, and found that all infants who delivered prior to 24 weeks gestation were in the high/high risk group (Figure 2a). By 32 weeks of gestation, 19% of the high/high women had delivered compared to 7% of women in the low/low group. Sixty-three percent of the women in the high/high group had delivered by 37 weeks gestation, compared to 44% of women in the low group (Figure 2a). A significant difference was found between these two levels in the model (p=0.03). Figure 2b compared differing levels of AMH difference risk when MSAFP risk is high, and Figure 2c compares differing levels of AMH difference risk when MSAFP risk is low. AMH difference risk provides better differentiation when MSAFP risk is high although the difference between AMH difference groups when MSAFP was high was not significant (p=0.12). Little risk differentiation was seen when MSAFP risk is $\leq$ 1 MoM (p=0.7).



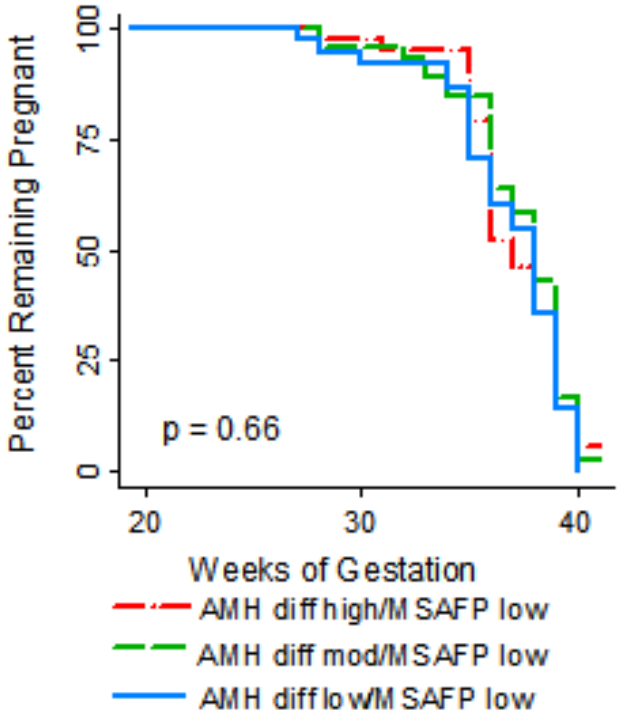
(a.) Comparing low/low risk (AMH difference >0.73 ng/mL) /MSAFP ≤ 1MoM) vs. high/high risk (AMH difference ≤0.01 ng/mL/MSAFP >1 MoM)

Figure 3.2. Time to delivery for the combined risk of AMH difference and MSAFP, adjusted for weight change between trimesters and a previous history of preterm birth



(b.) When MSAFP is >1 MoM and:
-AMH difference risk low (AMH difference >0.73 ng/mL)
-AMH difference risk moderate (AMH difference >0.01 and ≤0.73 ng/mL)
-AMH difference risk high (AMH difference ≤0.01 ng/mL)

Figure 3.2.—continued



(c.) When maternal serum alpha-fetoprotein is ≤1 MoM and
-AMH difference risk low (AMH difference >0.73 ng/mL)
-AMH difference risk moderate (AMH difference >0.01 and ≤0.73 ng/mL)
- AMH difference risk high (AMH difference ≤0.01 ng/mL)

Figure 3.2.—continued

3.5 Discussion

A significant, direct, dose-dependent association with time to delivery was seen for the AMH difference between 1st and 2nd trimesters, with shorter times to delivery being association with an increasing or stable AMH level between the 1st and 2nd trimesters. Assuming that the difference in AMH levels between trimesters is a surrogate for ovarian activity (CL-derived progesterone), these finding support Baird's hypothesis (10) of increased ovarian recruitment when a progesterone deficit leading to an early delivery exists. Further, these findings suggest that ovarian progesterone production may overcome this deficit as some women identified in the high-risk group delivered after 37 weeks gestation. Given that progesterone supplementation in early pregnancy has been shown to decrease the incidence of PTB (2, 20, 30, 89), the combined risks of AMH difference between the 1st and 2nd trimesters and MSAFP could be used to identify the subset of women most likely to respond to progesterone therapy.

This model performed best when an ovarian biomarker (AMH) was combined with biomarkers of feto-placental health, and was able to find significant associations with PTB at minimal to modest alterations in biomarker levels. The use of biomarker information from two separate but interdependent systems may reflect cross-talk occurring between the placenta and the ovary and suggests the existence of a fetal/placental/ovarian axis for progesterone control. In this scenario, a true progesterone deficit would disrupt placentation leading to an increase in feto-placental biomarkers. The placental progesterone deficit would increase recruitment of the CL, which would be reflected in stable or minor declines in AMH between trimesters. Thus, the greatest progesterone deficits would show an increase in the AMH levels between trimesters along with an elevated MSAFP, and would also have the shortest time to delivery. As the contribution of both biomarkers is necessary to evaluate the degree of the deficit, the failure to consider both the ovarian and the placental responses in previous models may be the reason that PTB prediction has been largely unsuccessful.

This lack of an association with biomarker of feto-placental health and PTB when without considering an ovarian biomarker is supported by the findings of many previous studies (5, 7, 8, 14, 29, 53). A review by Menon et al. (5), looked at 116 different biomarkers assayed 578 times in 217 studies, and found that no single biomarker could reliably predict preterm birth. Jelliffe-Pawlowski et al. (57) developed a model that predicted preterm birth with an odds ratio of 2.3-3.6, but the model only worked with extreme biomarker levels which severely limited its clinical usefulness (PAPP-A in the $\leq 5^{th}$ percentile and/or MSAFP in the $\geq 95^{th}$ percentile, and/or 2^{nd} trimester inhibin A in the $\geq 95^{th}$ percentile). Androutsopoulos et al. (53) found that neither mid-trimester hCG nor MSAFP could predict preterm delivery when used alone, and Metcalfe et al. (8) reported that current markers used to screen for aneuploidy have detection rates too low to identify women at risk for adverse obstetrical outcomes, including preterm birth. Lepage et al. (54) reported an association between hCG levels and preterm birth, but only when levels were $\geq$ 4.0 MoM. Stout et al. (14) found that a combination of placental protein 13 (PP-13) levels, PAPP-A and uterine artery pulsatility in the first trimester, along with maternal characteristics gave a good prediction of preterm birth in their small sample, but these results have not been reproduced in other studies (90). Finally Yuan et al. (52) published a recent meta-analysis on the use of MSAFP in the prediction of preterm birth, and found that MSAFP was strongly related at levels over 2.0 MoM, but only in the context of other markers of abnormal pregnancies. None of these studies used ovarian biomarkers and, therefore, were unable to evaluate any possible connections between ovarian and placental progesterone production and PTB.

The performance of this model in patients with term/post-term pregnancies is more difficult to interpret. With only two women delivering after 41 weeks gestation, the sample size was too small to draw any conclusions about the association between AMH differences and post-date pregnancies. In addition, it is likely that the pathways being tested in this model, specifically those of a progesterone deficiency, do not apply to term

pregnancies. The initiation of labor in term pregnancies appears to be related to corticotropin-releasing hormone rather than altered progesterone levels (91, 92). Therefore, it is unlikely that this model would be informative in the setting of term pregnancies.

Inaccuracies in gestational dating may have been a limitation for this study. Much debate exists about the most appropriate measure for gestational age. Last menstrual period (LMP) dating assumes a regular, 28-day menstrual cycle with ovulation occurring on day 14. In reality, 28 days is the average of cycle lengths with a "normal" menstrual cycle length varying by age (93, 94). On the other hand, ultrasound-based estimates are consistently 2-3 days shorter than LMP-based estimates, do not account for differences in fetal growth, and can be operator dependent (23). In this study, we used the same approach to determining gestational age as put forth for IPS. This method uses dating based on the first day of the last menstrual period (LMP), confirmed by ultrasound dating in the first trimester. Use of this recognized and established method of ultrasound dating helped to minimize the bias introduced by using a single (LMP or ultrasound) dating method.

A major strength of this study is the use of time to delivery rather than simple preterm/term birth classification. This method identified women who should be targeted for prophylactic therapies such as progesterone use because they had the shortest time to delivery. This was a population-based study of women across Iowa and was not limited to those women presenting to a tertiary care center, increasing the generalizability of the results. The identified risks did not rely on previous knowledge of pregnancy outcomes (history of previous preterm birth did not alter β-coefficient for the AMH variable in the model and therefore did not impact model performance), therefore, these results are applicable to primiparous as well as multiparous women. Finally, the biomarkers studied are commercially available, in widespread use and are collected simultaneously when women undergo IPS, so they could be easily incorporated into routine prenatal care.

One weakness of this study is the use of birth certificate data, which may not accurately report the cause of a preterm birth. To counter this issue, we oversampled cases and controls to increase the power of the study. Testing was limited to women choosing to have IPS, so this also may limit the generalizability of the model. The sample also excluded all races other than white women because the low number of non-white participants would not allow for accurate evaluation of racial effects in the statistical models. Therefore, these results need to be confirmed in women of other races. However, this may also be a strength of the study as limiting the subjects to those of white race may have eliminated some unrecognized biases in the data and allowed us to identify associations between time to delivery and AMH levels. Because pregnancy outcomes were derived from birth certificate data, there was no information about the use of supplemental progesterone in these women. However, the use of progesterone would have biased the results towards the null, making it less likely that we would find a significant association. Because these results were statistically significant, the use of progesterone was unlikely to be the reason for these associations. Finally, the AMH testing kit used has a known problem with complement interference (95), which may cause a decrease in measured levels when freshly drawn or freshly frozen samples are tested. This effect dissipates as the sample ages and the complement levels decrease (95). As these were aged samples by 24-36 months, the risk of complement interference was less likely to be a problem. In addition, the use of a reference lab with a strict protocol for AMH, including multiple quality control checks across the plate, likely minimized bias in the results.

3.6 Conclusions

In conclusion, differences in the AMH level in pregnant women between the 1st and 2nd trimesters were directly associated with time to delivery when considered along with a simultaneously collected MSAFP levels. The lack of decline in AMH levels

between trimesters may reflect delayed involution of the corpus luteum and continued ovarian progesterone production when placenta progesterone production is insufficient. The use of biomarkers from the two organ systems responsible for progesterone production in pregnancy may have helped identify clinically meaningful progesterone deficiencies that lead to a shorter time to delivery. Finally, these findings suggest the existence of a fetal/placental/ovarian axis responsible for regulation of progesterone production during the critical phases of placentation.

CHAPTER 4.
MATERNAL ANTI-MULLERIAN HORMONE LEVELS DISPLAY A SEX-SPECIFIC RESPONSE PATTERN IN EARLY PREGNANCY

4.1 Summary of Findings

Sexually-dimorphic response patterns in the placenta have been noted in multiple disease states, including preeclampsia and other adverse obstetric outcomes. As of yet, no one has seen similar patterns in ovarian biomarkers in pregnancy. The existence of a sexually-dimorphic pattern in an ovarian biomarker might provide additional information about ovarian-placental crosstalk in early pregnancy and might help explain why male fetuses have a higher rate of preterm birth (PTB) compared to female fetuses. The objective of this study was to determine if AMH levels in the 1st and 2nd trimesters of pregnancy differed by fetal gender and birth outcome.

This was a retrospective data analysis of women in Iowa. Banked serum samples linked to birth certificate data were used to evaluate associations between AMH levels in the 1st and 2nd trimesters and fetal gender alone and then by fetal gender and birth outcome (term vs. preterm).

Maternal AMH levels, adjusted for maternal age and time of blood draw, were lower in the 1st trimester for women carrying a male vs. female fetus, although this difference did not reach statistical significance. When comparing 1st and 2nd trimester maternal AMH levels by both gender and birth outcome (term vs. preterm), maternal AMH levels were lower if carrying a male vs. female fetus who delivered at term, however, levels were nearly identical when carrying a fetus of either gender who delivered preterm. The average percent change in maternal AMH levels between the 1st and 2nd trimesters was slightly higher with a term male fetus (18%) than with a term female fetus (16%). The average percent change with a preterm male was 28% less than

that seen with term males (13% vs. 18%, respectively), while levels with a preterm female were half of the decline of a term females (8% vs. 16%, respectively). Although there was no significant difference in the mean values of the AMH difference between trimesters by gender and birth outcome, there was a significant trend noted across groups from term males, term females, preterm males and finally preterm females with smaller declines seen for women with female fetuses ($P_{trend} = 0.03$).

Maternal AMH levels in pregnancy display a sexually-dimorphic pattern that suggests a stronger placental response with a female fetus. This may represent a self-preservation function and improved female fetal survival.

4.2 Introduction

Anti-Mullerian hormone (AMH) is a direct reflection of ovarian activity in menstruating women because it is produced by the granulosa cells of the growing ovarian follicle (61, 96-99). Until recently, AMH levels were thought to be unchanged in pregnancy, but data now suggest that levels drop sharply between 13-15 weeks gestation (70-72, 74). Further, AMH returns to prepregnancy levels quickly after delivery (72, 74), suggesting that AMH is actively suppressed by signals from the feto-placental unit. The patterns of maternal AMH change in pregnancy are distinct from changes seen in the fetus (100) and cannot be of fetal origin so must reflect maternal ovarian function in early pregnancy.

The main function of the ovary in pregnancy is progesterone production, which is essential for normal placental development (1, 2). Progesterone acts as an anti-inflammatory agent in the placental bed and helps mediate the immune response of the mother to the foreign DNA of the fetus (1, 34, 86). Baird (10) recently theorized that when placental progesterone production was inadequate, the placenta would “recruit” the ovary in an effort to boost progesterone levels and salvage the pregnancy. If true, placental signaling to activate the ovary would also result in alterations of AMH levels.

Thus, the pattern of AMH change in pregnancy could provide useful information about the role of the fetal/placental/ovarian axis in progesterone production.

A pro-inflammatory placental environment (28, 40) is thought to be one of the major pathways to PTB. It is also known that carrying a male fetus results in increased inflammation in the placental bed (40, 41) and a higher risk for PTB. Therefore, if carrying a male fetus results in a greater pro-inflammatory state, and if AMH levels reflect ovarian progesterone production, then sex-specific differences should be seen in the AMH levels in early pregnancy. However, to date, there have been no studies that have examined patterns of AMH change based on fetal gender. The objective of this study is to determine if AMH levels display sex-specific patterns in early pregnancy, and to determine if these patterns in early pregnancy are altered when the fetus goes on to deliver prior to 37 weeks gestation.

4.3 Materials and Methods

Pregnancy information and maternal serum used for this analysis were obtained from an existing maternal-fetal serum tissue bank at the University of Iowa. This bank collected serum samples from pregnant women undergoing prenatal screening in Iowa between 2009 and 2010, which were subsequently linked to birth certificate data after delivery. The Human Subjects Office at the University of Iowa approved the study, as did the Iowa Department of Public Health Research and Ethics Review Committee, who granted permission to use birth certificate data. The Congenital and Inherited Disorders Advisory Committee for the State of Iowa granted permission for use of the maternal serum samples.

4.3.1 Development of the Serum Tissue Bank

The development of the serum tissue bank has been previously described in Section 2.3.1. Briefly, in Iowa all prenatal screening is performed at the State Hygienic Laboratory (SHL). From 2009-2010, the unused portions of the serum samples sent for

prenatal screening were de-identified, stored at -80˚C and then linked to birth certificate data after delivery. As a result, this tissue bank contained over 3,000 paired 1st and 2nd trimester maternal samples linked to birth certificate data. Due to the need to ensure the gestational age of the fetus at the time the samples was taken, all women also had a 1st trimester dating ultrasound. The gestational age ranges for the 1st and 2nd trimester samples were 10-13.9 weeks and 15-22.9 weeks, respectively.

4.3.2 Development of the Dataset

The original dataset was developed to examine the association between AMH levels in the 1st and 2nd trimester and spontaneous PTB. The World Health Organization (WHO) definition of PTB (delivery between 20 and <37 weeks gestation) was used to define case-control status. Women were excluded if birth certificate data suggest that they did not have a spontaneous PTB. The decision tree for the exclusion criteria can be found in Figure 4.1. The final dataset contained a total of 200 white women (105 PTBs and 95 term births) who were frequency-matched based on the following maternal age groups: <21, 21-22.9, 23-24.9, 25-26.9, 27-28.9, 29-30.0, 31-32.9, 33-34.9, 35-36.9, and ≥37.

4.3.3 Anti-Mullerian Hormone Measurements

All samples underwent a single freeze-thaw cycle and remained frozen at -80˚C until the time of analysis. AMH testing was completed by a single reference laboratory (ReproSource, Inc, Woburn, Massachusetts) using their laboratory-developed Reprosource AMH assay. This assay is based on the research-use-only materials and reagents from Beckman Coulter-DSL (Chaska, MN). Intra- and inter-assay coefficients of variation with serum controls were 5%-9% and 7%-12%, respectively. Serum AMH values below the reported clinical detection level of 0.1 ng/mL were considered to be zero but were reported as 0.05 in the analysis.

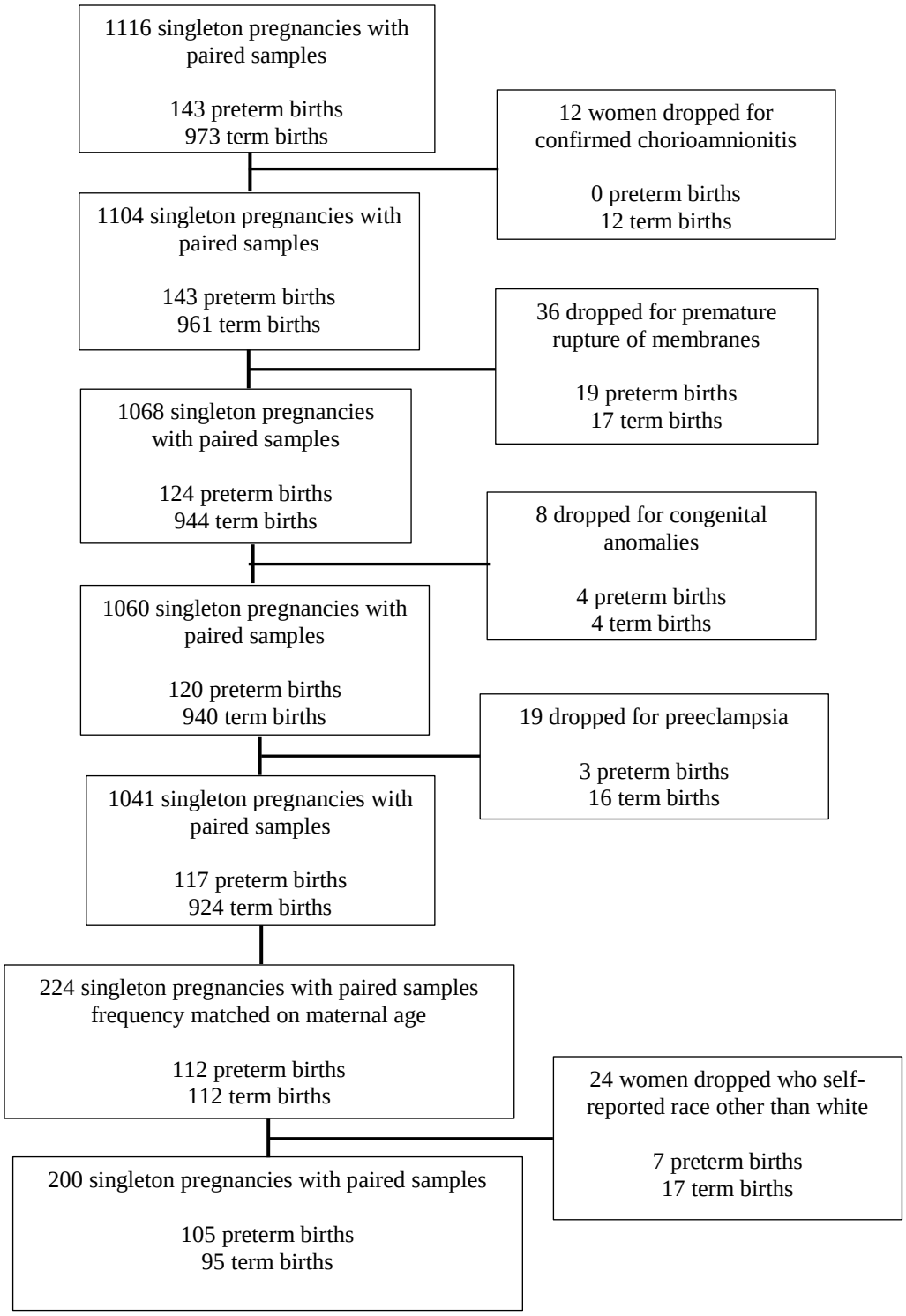


Figure 4.1 Selection of subjects from serum tissue bank for inclusion in the parent dataset

4.3.4 Statistical analysis

The data were examined for normality and for possible confounding using t-tests and chi-squared test statistics as appropriate. Collinearity was assessed using Pearson correlation coefficients. The only variables that were not normally distributed were AMH levels in the 1st and 2nd trimesters. These variables were transformed and the models were repeated with similar results, therefore, the original values were used in the final models as these more easily translated to the clinical setting.

Six separate linear regression (least-squares) models were constructed using two different exposures and three different outcomes. The first exposure was fetal gender and the second exposure was a four-level variable combining gender and birth outcome (term vs. preterm). The following AMH levels were used as outcome variables: 1st trimester AMH level, 2nd trimester AMH level, and difference between the 1st and 2nd trimester AMH levels. Covariates considered for inclusion in the models because of concerns for possible confounding included maternal age (81), maternal weight gain (27, 82), smoking during the pregnancy (4), and either the time of the blood draw (for the 1st and 2nd trimester AMH levels) or the time difference between blood draws (for AMH difference between blood draws). An Analysis of Covariance (ANACOVA) strategy was used to determine the best fit for each model. Variables were retained in the final model if removal resulted in a change of greater than 15% in the β coefficient for the AMH exposure variable. If a covariate was found to be significant in one of the models, it was included in all models with the same exposure variable to permit comparisons of the results. In the models using two exposure variable (fetal gender and birth outcome), a partial F test was used to look for an interaction effect. The difference between AMH levels between the 1st and 2nd trimesters were calculated from the adjusted data using the following formula: % change = 100 X (difference between 1st and 2nd trimester AMH levels)/ 1st trimester AMH level.

Trends across continuous variables were determined using a non-parametric test of trend for the ranks across ordered groups (an extension of the Wilcoxon rank-sum test). Post-hoc power was calculated using the observed means and standard deviations for the dataset with a two-tailed alpha of 0.05. All statistical analyses were performed using Stata version 11.2 (College Station, TX).

4.4 Results

Table 4.1 includes demographic and pregnancy-related characteristics by fetal gender and by fetal gender and birth outcome. There were no significant differences in the variables prior to stratification. When stratified by gender and birth outcome, gestational age at delivery was significantly different between the term and preterm fetuses ($p<0.001$) and timing of the first trimester screen was slightly later in preterm compared to term infants.

4.4.1 AMH levels by gender and birth outcome

Maternal AMH levels were lower in the 1st trimester when carrying a male fetus vs. a female fetus that delivered at term (Table 4.2). However, when the fetus went on to delivery preterm, 1st trimester AMH levels for preterm fetuses of either gender were lower than AMH levels with a term male fetus (3.3, 3.1. and 3.3 ng/mL, term males, preterm males and preterm females, respectively) (Table 4.2 and Figure 4.2). In the 2nd trimester, the decline in maternal AMH levels in term male was slightly greater than the decline with term female fetuses (18% vs. 16%, respectively), and the 2nd trimester AMH levels with a term male fetus were remained lower than what was seen with a female fetus (Table 4.2 and Figure 4.2). In contrast, the decline in AMH levels with a preterm female fetus was only half of what was seen in a term females while the decline with a preterm male was only 28% less that that with a term male. Although there was no significant difference in the mean values of the AMH difference between trimesters by

gender and birth outcome, there was a significant trend noted across groups with a smaller decline in AMH levels for women with female fetuses ($P_{trend} = 0.03$) (Table 4.2).

Table.4.1 Demographic and pregnancy-related characteristics by fetal gender, and by fetal gender and birth outcome

	Fetal Gender			Fetal Gender and Birth Outcome				
Continuous Variables	All Males n=104 Mean (SD)	All Females n=96 Mean (SD)	Partial F test for difference of 2 means	Term Males n=44 Mean (SD)	Term Females n=51 Mean (SD)	Preterm Males n=60 Mean (SD)	Preterm Females n=45 Mean (SD)	Partial F test for difference of 4 means
Maternal age[a]	29.1 (5.4)	28.4 (5.1)	0.4	29.7 (5.9)	28.0 (5.0)	28.7 (5.1)	28.9 (5.4)	0.5
Gestational age at delivery[b]	36.3 (2.6)	36.2 (3.8)	0.90	38.6 (1.1)	38.9 (0.9)	34.6 (2.0)	33.2 (3.6)	0.001
Gestational age at 1st trimester screen[c]	82.9 (6.3)	84.4 (6.6)	0.09	82.2 (5.5)	82.9 (6.2)	83.4 (6.8)	86.1 (6.7)	0.02
Gestational age at 2nd trimester screen[c]	119.8 (9.7)	117.4 (8.4)	0.06	119.0 (10.1)	117.3 (8.2)	120.5 (9.5)	117.5 (8.7)	0.2
Maternal weight change between the 1st and 2nd trimester[d]	3.3 (5.2)	2.7 (5.0)	0.4	2.3 (4.4)	2.7 (3.7)	4.1 (5.6)	2.7 (6.3)	0.3
Categorical variable	n (%)	n (%)	χ^2	n (%)	n (%)	n (%)	n (%)	χ^2
Smoked during pregnancy								
Yes	18 (17.3)	14 (14.6)	0.60	6 (13.5)	7 (13.7)	12 (20.0)	7 (15.6)	0.8
No	86 (82.7)	82 (85.4)		38 (86.4)	44 (86.3)	48 (80.0)	38 (84.4)	

[a]years, [b]weeks, [c]days, [d]pounds

Table 4.2 Models for AMH in 1st trimester, 2nd trimester and for the decline in AMH between trimesters by fetal gender, and by fetal gender and birth outcome

Models using fetal gender	Males (Mean, 95% CI)	Females (Mean, 95% CI)	Partial F test for difference of 2 means
1st trimester AMH by gender adjusted for maternal age and time of blood draw	3.2 (2.7, 3.7)	3.5 (3.0, 4.0)	0.4
2nd trimester AMH by gender adjusted for maternal age	2.7 (2.3, 3.1)	3.1 (2.7, 3.5)	0.2
Difference between 1st and 2nd trimester AMH levels by gender adjusted for maternal age	0.5 (0.3, 0.7)	0.4 (0.3, 0.6)	0.8

Model using fetal gender and birth outcome	Term males (Mean, 95% CI)	Term Females (Mean, 95% CI)	Preterm Males (Mean, 95% CI)	Preterm Females (Mean, 95% CI)	Partial F test for difference of 4 means
1st Trimester AMH by gender and birth outcome adjusted for maternal age and time of blood draw[a]	3.3 (2.6, 4.0)	3.8 (3.1, 4.4)	3.1 (2.6, 3.7)	3.3 (2.5, 4.0)	0.6
2nd trimester AMH by gender and birth outcome adjusted for maternal age[a]	2.7 (2.05, 3.3)	3.2 (2.6, 3.8)	2.7 (2.1, 3.2)	3.0 (2.4, 3.6)	0.5
Difference between 1st and 2nd trimester AMH levels by gender and birth outcome adjusted for maternal age[c]	0.6 (0.3, 0.8)	0.6 (0.4, 0.8)	0.4 (0.2, 0.6)	0.3 (-0.1, 0.5)	0.2
Average percent change between 1st and 2nd trimester AMH levels[b]	18%	16%	13%	8%	

[a]Test for interaction effect was not significant (>0.5)

[b] % change = 100 X [(Difference between 1st and 2nd trimester AMH levels)/1st trimester AMH level]

[c]p(trend) for this variable was significant at 0.03, using a non-parametric test of trend for the ranks across ordered groups (an extension of the Wilcoxon rank-sum test)

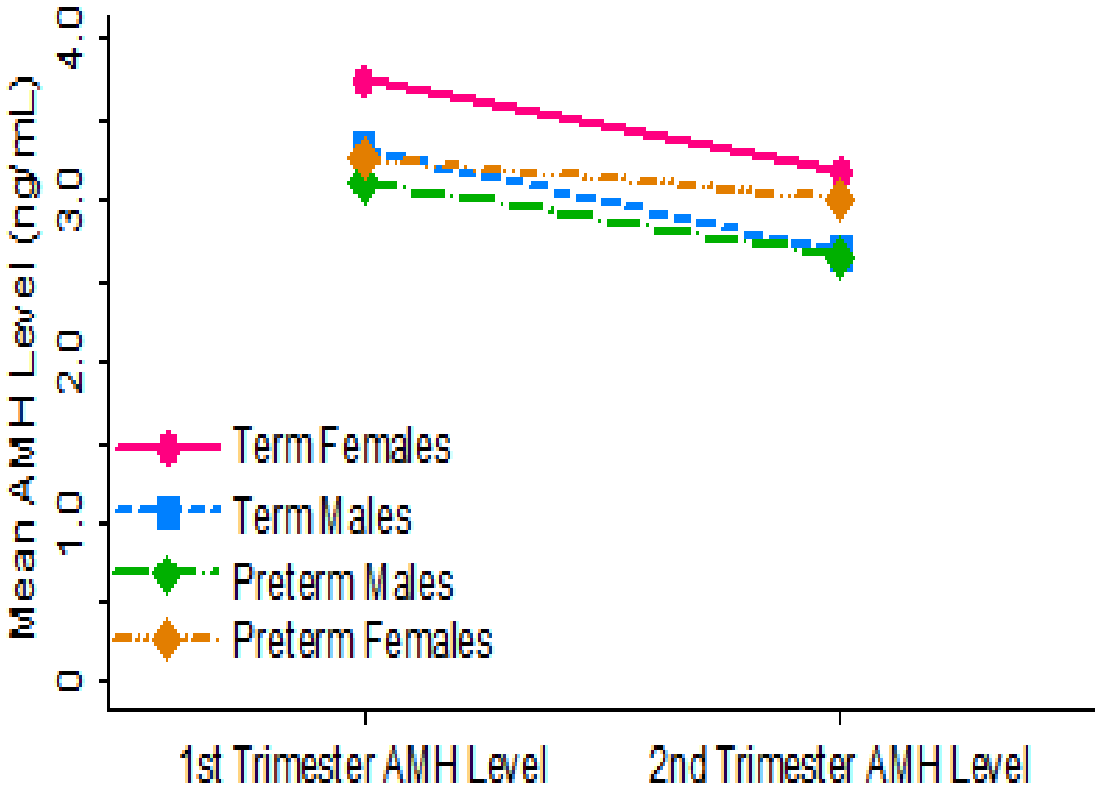


Figure 4.2 The decline in AMH between the 1st and 2nd trimesters by fetal gender and birth outcome, adjusted for maternal age and time of blood draw

4.5 Discussion

This study demonstrates a sex-specific pattern in maternal AMH levels, and thus ovarian actively, in early pregnancy. Different patterns of 1st and 2nd trimester maternal AMH levels based on fetal gender and birth outcome were identified. As the major role of the ovary in early pregnancy is progesterone production, lower AMH levels may reflect lower progesterone levels in early pregnancy when carrying a male fetus, and this could account for the increased incidence of PTB associated in males. Further, the finding of lower AMH levels in preterm infants of either sex is consistent with our assumption that AMH levels may be an indication of progesterone deficiency in pregnancy. Finally, the lack of an AMH decline in preterm female fetuses may indicate a self-preservation response to an adverse environment that is similar to what has been noted in female fetuses with other disease states such as maternal asthma (101, 102).

Maternal AMH levels display a unique pattern of change that is not a simple reflection of AMH levels in the developing fetus. If AMH was able to cross from maternal to fetal circulation, the high maternal AMH levels would result in regression of the Mullerian system and congenital malformations of the female reproductive tract in the developing female fetus (28). Alternatively, AMH does not cross from the fetal compartment to the maternal circulation because male fetuses have very high levels of AMH by seven weeks gestation that remains high throughout pregnancy, which is also not consistent with the maternal AMH pattern. Therefore, the changes in maternal AMH levels observed in early pregnancy must represent alterations in maternal ovarian AMH levels and likely reflect ovarian progesterone production in pregnancy.

This is not the first report of sexually-dimorphic placental signaling and the existence of other sex-specific immune responses in the placenta have been well documented. Differences in expression of toll-like receptor-4 (TLR-4) (103) and tumor necrosis factor-α (TNF-α) (103) in male vs. female placentas support the theory that placental immune function is sex-specific (102). Ghidini and Salafia (40) noted that the

placentas from male fetuses delivered prior to 32 weeks gestation contained lesions suggestive of an increased maternal immune response that were absent in female placentas delivered at the same gestational age. Fetuses of mothers treated with cortisol for asthma in pregnancy display sex-specific responses to maternal cortisol levels, with adaptations occurring exclusively in the female placentas that improve female fetal survival (101). Finally, placentas from pre-eclamptic mothers with male fetuses show sex-specific differences in expression of inflammatory, hypoxic and apoptotic markers (104). With all disease states, female placentas appear to be programmed for better adaptation when exposed to similar adverse environments (102) leading to improved survival in females. Assuming AMH reflects ovarian-derived progesterone, these data suggest that female fetuses can adapt better than male fetuses to a hostile pro-inflammatory placental environment by maintaining ovarian progesterone levels during the critical period of placentation. This may partially account for the differences in male and female PTB rates (4, 28, 40).

This is the first study that has examined sexually-dimorphic changes in maternal AMH levels in early pregnancy. Although these levels did not reach statistical significance, the consistent trend towards higher maternal AMH levels in women with a female fetus suggests that sex-specific differences in AMH levels are present. Higher AMH levels could indicate higher levels of ovarian-derived progesterone early in pregnancy, which could be an advantage to the developing female fetus. The finding that women carrying a male fetus have slightly lower AMH levels, which could indicate lower progesterone levels and a higher baseline risk of PTB, is consistent with epidemiologic data reporting higher rates of PTB in males (4, 28). Finally, when faced with a pro-inflammatory state, the blunting of the AMH decline between the 1st and 2nd trimesters of pregnancy in females suggests that they are better able to adapt to a progesterone deficit by preventing a drop in ovarian-derived progesterone, thus increasing survival potential.

One of the major strengths of this study was the ability to measure AMH levels in both the 1st and 2nd trimesters of pregnancy to evaluate dynamic changes in this hormone. All of the serum samples were obtained from the SHL at the University of Iowa, along with a 1st trimester ultrasound to confirm gestational age at the blood draw. Samples were collected according to a single protocol reducing the bias related to the sample processing. Birth certificate data were available on all subjects to help determine term vs. preterm and to identify comorbidities that could lead to PTB. Finally, AMH levels were measured in a single reference lab (ReproSource, Woburn, Massachusetts) with strict quality control, reducing variations that might be introduced by differences in the testing procedure.

One of the possible limitations of this study is that it did not have sufficient power to find statistically significant differences between AMH levels based on fetal gender. Post hoc power analysis showed that using an alpha of 0.05, this study had limited power (48%) to find a difference in 2nd trimester AMH levels. A sample size of 440 women, 220 with male fetuses and 220 with female fetuses, would have been required to find a statistically significant difference. However, the consistent trends of higher AMH levels in women with female fetuses vs. male fetuses in both the term and preterm groups suggest that an appropriately powered study would have led to significant findings. The use of birth certificate data could have led to recall and reporting bias despite the use of the new standardized birth certificate form (105, 106). Because the study was restricted to white women, the impact of race/ethnicity on AMH changes in early pregnancy was not examined. Thus, the generalizability of the study is limited. Additional studies in women of all races are warranted to determine if similar trends can be found. Finally, the ELISA kit used for AMH testing has reported abnormal findings related to complement interference. All of these samples were frozen for a minimum of two years, which would have minimized the amount of complement present in the samples. Thus, it is unlikely

that complement interference was present. However, the influence of a bias towards the null based on variations in AMH measurements cannot be excluded.

4.6 Conclusions

In conclusion, maternal AMH levels in pregnancy differ in a sex-specific pattern, both in the AMH levels in the 1st and 2nd trimesters, and in the pattern of decline between trimesters. In the setting of PTB, there are distinct patterns associated with female fetuses that suggest a stronger, more adaptive, response from the female placenta to a pro-inflammatory environment. This may help explain some of the differences in PTB rates between male and female fetuses, and suggests that the progesterone deficit may be greater when carrying a male fetus.

CHAPTER 5.

CONCLUSIONS

5.1 AMH as a Biomarker of Ovarian Activity in Pregnancy

These findings present a new paradigm for evaluating the feto-placental environment in early pregnancy. Changes in AMH levels in early pregnancy provide useful information about the probability of PTB when evaluated as part of the overall fetal/placental/ovarian axis. The dynamic changes in AMH levels between trimesters appear to reflect the up- and/or down-regulation of the ovary which may be an indication of the varying requirements for progesterone during placentation. The lack of an association between a single AMH levels and PTB is understandable, as dynamic response would be required to indicate an increasing or decreasing demand. AMH levels that did not decline between the 1st and 2nd trimesters of pregnancy would represent a progesterone-deficient state and a higher risk of PTB. The finding of a dose-dependent response with time to delivery emphasizes the importance of the magnitude of change in discriminating the degree of the progesterone deficit. But most importantly, this information identifies women with a progesterone deficiency who might benefit from supplemental progesterone therapy.

The finding that AMH levels may also be associated with fetal gender may explain the discrepancies between PTB rates in male and female fetuses. The smaller decline in the AMH levels in women with a female fetus suggests that females have a stronger survival function when faced with an adverse placental environment. This finding is similar to the sexually dimorphic responses reported in other disease states (40, 101, 102, 104). This ability to compensate for a possible progesterone deficit by maintaining or increasing ovarian function may account for the lower rates of spontaneous losses and PTBs in female pregnancies.

5.2 Strengths and Limitations in the Tissue Bank Dataset

The major strength of this study was our ability to identify women who had both term and preterm deliveries and then retrospectively measure AMH samples from early pregnancy. This was possible because of the existence of the maternal-fetal serum tissue bank at the University of Iowa, which permitted the use of a case-control study design with sufficient power to identify potential associations between AMH levels and PTB. By using samples obtained during IPS, we also had the advantage of having information on other feto-placental biomarkers that could be used in the modeling process. Further, all of the feto-placental biomarker testing was performed in a single laboratory (SHL) using the same testing procedures, thus minimizing possible bias. Finally, 1st trimester gestational age measurements and maternal weights at each blood draw were available as part of the IPS report, thereby avoiding the need to use birth certificates for this information.

A major limitation for this project is the need to use birth certificate data for fetal outcomes and to determine maternal disease. Underreporting of health conditions in birth certificates is a known concern (105, 107, 108). In 2003, the U.S. Standard Certificate of Live Birth was published in an effort to improve reporting and to limit items on the birth certificate to things which could be collected with "reasonable completeness and accuracy" (109). Recent studies evaluating the effectiveness of this change found reasonable agreement for all categories except for maternal risk factors and comorbidities, both of which were components collected in the conduct of this study (105, 108). Therefore, it is very likely that some of our PTB's were related to unreported maternal comorbidities rather than spontaneous PTBs. However, inclusion of these women would have weakened the observed associations, so the finding of a positive association is encouraging that the noted associations are appropriate.

Finally, the blood samples used in this analysis were collected in the field without regards to the strict requirements for a research protocol. Even though laboratories were asked to follow the published guidelines for IPS blood sample collection, quality control during sample collection may have been lacking. The requirement to have samples available for retesting for up to eight days after the specimens were collected meant led to the need to store the samples at 4˚C for a prolonged period of time, which could have altered the AMH signal. However, it is more likely that this actually improved the accuracy of these samples by allowing the effects of complement to dissipate (95). In addition, Fleming et al. (79) have reported that the AMH signal is stable at 4˚C for up to 7 days after collection, making it less likely that this prolonged storage time impacted the AMH levels in these samples.

5.3 Future directions

These data suggest that the ovarian contribution to the health and maintenance of the pregnancy extends beyond the first eight weeks of gestation, and that the ovary may play a vital role in pregnancies with marginal placental progesterone production. Future research must focus on developing a better understanding of the fetal/placental/ovarian axis to improve our ability to identify women at risk for adverse obstetric outcomes related to progesterone deficiencies, such as PTB. This would include validation of this model using prospective data collection in a larger, more diverse group of women and the development of a useful risk prediction model to identify at-risk women in the clinical setting. Research into the signaling pathways involved in the fetal/placental/ovarian axis may identify additional biomarkers for use in risk prediction models. And finally, while the focus of this work was to identify women at risk for a spontaneous PTB, the usefulness of this model in other diseases association with increased placental inflammation should be explored.

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