# THE EFFECTS OF ANTENATAL GLUCOCORTICOID TREATMENT ON LACTOGENESIS II IN EWES AND WOMEN

**Jennifer Henderson**

BSc, RN, RM, Grad Dip Adv Nursing, MPH

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# ABSTRACT


**Background**

There is a large body of evidence describing the benefits and risks, to the human fetus, of antenatal glucocorticoid treatment, but no published research on the effects on lactation. The withdrawal of progesterone, in the presence of high levels of endogenous glucocorticoids and prolactin, triggers the onset of copious milk secretion (lactogenesis II) at the end of pregnancy. The alteration of lactogenesis II by exogenous glucocorticoids could potentially have adverse impacts on postnatal nutrition in both term and preterm infants.

I aimed to determine the effects of maternal antenatal glucocorticoid treatment on lactogenesis II in both ewes and women. I found profound adverse effects on lactation in ewes, and similar but more subtle effects on lactation in women.

1. **Antenatal Glucocorticoid Treatment Causes Premature Lactogenesis in Pregnancy and Disrupts Normal Lactogenesis II after Parturition at Term in Ewes**

The treatment of ewes with betamethasone at 125 days of pregnancy caused a marked surge in plasma lactose concentration, indicating premature onset of lactogenesis. Median (range) lactose concentration in plasma increased from 3.5 (1.2-8.9) μM before treatment to 42.4 (11.7-74.0) μM on day five. Increased lactose concentration was accompanied by marked reduction in the concentration of progesterone (from 6.90 (2.78-18.71) ng/mL to 1.36 (0.67-3.72) ng/mL), and transient increase in the concentration of prolactin in maternal blood. This event was independent of medroxyprogesterone acetate (MPA) treatment required to prevent premature abortion and did not occur in saline placebo-treated sheep. Milk secretion resolved approximately nine days after treatment. After parturition, reduced milk lactose

concentration and milk volume, and restricted lamb growth indicated delays in lactogenesis II at term in betamethasone-treated ewes.

2. **Preterm Birth is Associated with Delayed Lactogenesis II**

Women who delivered before 34 weeks' gestational age (N = 50) measured expressed milk volume and gave samples of breastmilk from days one to ten postpartum. Gestational age at delivery strongly predicted both milk volume ($P = 0.017$) and milk lactose concentration ($P < 0.001$). Shorter gestational age at birth was significantly associated with reduced milk volume and decreased milk lactose levels, indicating delays in lactogenesis II.

3. **Effects of Antenatal Glucocorticoid Treatment on Postnatal Lactogenesis II in Mothers of Preterm Infants are Modified by Gestational Age and the Time Interval Between Treatment and Delivery**

Subtle effects of antenatal glucocorticoid treatment on lactogenesis II were found in women who delivered before 34 weeks' gestational age, although this effect was modified by gestational age.

Average milk volume per expression was significantly associated with the time interval between glucocorticoid treatment and delivery ($P = 0.036$). At 28 – 33 weeks' gestational age, women who delivered between three and nine days after treatment obtained reduced milk volumes compared with either less than three or greater than nine days after treatment. This suggests that lactogenesis II was delayed when preterm delivery did not occur immediately after betamethasone treatment. Women who delivered at extremely preterm gestational ages (<28 weeks' gestational age) had delayed lactogenesis II but no effect of glucocorticoid treatment.

Antenatal glucocorticoid treatment was not associated with changes in the concentration in milk of either lactose or citrate (markers of lactogenesis II).

### 4. Antenatal Glucocorticoid Treatment in Women is Associated with a Transient Lactogenic Stimulation in Pregnancy

Lactogenesis was assessed in pregnant women (N = 92) by measuring the urinary output of lactose and pregnanediol glucuronide (PdG). After adjustment for gestational age, there was a significant transient surge in antenatal daily urine lactose output ($P < 0.001$) following betamethasone treatment, accompanied by a slight reduction in PdG output ($P = 0.007$). Median (range) excretion of lactose in urine increased from 0.713 (0.050-3.217) mmol/24 hours on treatment day to 1.088 (0.267-6.676) mmol/24 hours on day 3 and PdG decreased from 1.445 (0.162-3.495) mmonl/24 hours to1.151 (0.139-2.401) mmol/24 hours on day 4 after treatment. This suggests that there was a precocious lactogenic stimulation that resolved if delivery did not occur soon after treatment. In contrast to sheep, precocious lactogenesis was not associated with changes in either postnatal milk volume or milk lactose concentration in women who delivered at term.

**Conclusions**

This thesis represents the first investigation of the effects of antenatal glucocorticoid treatment on lactogenesis II in both ewes and women. I found that, in ewes, antenatal glucocorticoid treatment stimulated premature lactogenesis II, and this was caused by disruptions to hormonal regulation during pregnancy. This event was followed by profound delays in lactogenesis II after term parturition. More subtle effects in women suggest that antenatal glucocorticoid treatment did not have a major, prolonged impact on postnatal lactogenesis II. Very preterm gestational age strongly predicted delays in lactogenesis II stressing the importance of assistance for these mothers when they are establishing lactation.

## STATEMENT OF CANDIDATE CONTRIBUTION

This thesis comprises original work, no part of which has been submitted for examination for any other degree or diploma at The University of Western Australia or any other institution. Except where due acknowledgement has been made, the work contained within this thesis has been performed by myself.

Jennifer Henderson

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# PUBLICATIONS ARISING FROM THIS THESIS

***Manuscripts***

1. **Henderson JJ**, Hartmann PE, Moss TJM, Doherty DA, Newnham JP, 2006. *Disrupted lactogenesis II after antenatal glucocorticoid treatment in sheep* under review.
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***Abstracts***

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This thesis is dedicated to you.

# ABBREVIATIONS

| | |
|---|---|
| APH | Antepartum haemorrhage |
| BETA | The group of sheep treated with betamethasone and medroxyprogesterone acetate |
| BF | Breastfeeding |
| BMI | Body mass index |
| CBG | Corticosteroid binding globulin |
| d | Days of pregnancy |
| EBM | Expressed breastmilk |
| ECM | Extracellular matrix |
| GH | Growth hormone |
| GR | Glucocorticoid receptor |
| h | hours |
| HPA | Hypothalamus-pituitary-adrenal axis |
| IDDM | Insulin dependent diabetes mellitis |
| IgG | Immunoglobulin G |
| IM | intramuscular |
| IQR | Interquartile range |
| IUGR | Intrauterine growth restriction |
| IV | intravenous |
| JAK2 | Janus Kinase 2 |
| MPA | Medroxyprogesterone acetate; also the group of sheep treated only with medroxyprogesterone acetate |
| PdG | Pregnanediol glucuronide |
| PPROM | Prolonged preterm rupture of membranes |

| | |
|---|---|
| PR | Progesterone receptor |
| PrlR | Prolactin receptor |
| RDS | Respiratory distress syndrome |
| RIA | radioimmunoassay |
| SALINE | The group of sheep treated with saline (control group) |
| sd | Standard deviation |
| SGA | Small for gestational age |
| STAT (3 or 5) | Signal transducer and activator of transcription |
| VLBW | Very low birthweight |

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# CHAPTER 1 THE EFFECTS OF PRETERM BIRTH AND ANTENATAL GLUCOCORTICOID TREATMENT ON INITIATION OF LACTATION: A LITERATURE REVIEW

*"Development is a continuous cascade that does not stop at the moment of birth but progresses along a continuum." (Wagner 2002)*

*"At the end of pregnancy the task of feeding the young passes from the placenta to the mammary gland."(Kuhn 1983)*

The period of lactation following birth can be likened to the "fourth trimester" – a vital period in the development of the young; yet sometimes lactation is unsuccessful and failure to provide mother's own milk can lead to lifelong problems. For preterm infants, who are at risk of a wide range of morbidities and potential lifetime disability, human milk is the optimum form of nutrition. Many mothers of preterm infants, however, have lactation problems leading to early, unplanned cessation of breastfeeding. The initiation of copious milk secretion (lactogenesis II) is important for subsequent lactation and there is greater potential for mothers of preterm infants, compared with mothers of term infants, to have problems at this stage, possibly due to stress, maternal illness, operative delivery or antenatal pharmacological therapies. The effect of antenatal glucocorticoid treatment in particular, on lactogenesis II has not been examined to date.

The endocrine system plays an important role in not only the maintenance of pregnancy and regulation of the birth process but also is vital for the development of the mammary gland during pregnancy. Experimental studies of preterm birth, focusing principally on the sheep, have established that the fetus initiates parturition through activation of the

hypothalamus-pituitary-adrenal axis (HPA) leading to withdrawal of progesterone production in the placenta (Challis, Matthews *et al.* 2000). The surge of cortisol before parturition is vital not only for the development of fetal organs such as the lungs, kidney and brain but also for the differentiation of the mammary gland, and lactogenesis II can only commence once there are adequate maternal levels of glucocorticoids and prolactin, and progesterone is withdrawn.

Exogenous glucocorticoid treatment before preterm birth accelerates the development of fetal lungs, reducing neonatal morbidity due to respiratory problems (Liggins & Howie 1972). A large body of evidence has established the benefits of antenatal glucocorticoid therapy for the preterm neonate (Crowley 2003) but no research has been undertaken to investigate the effects of antenatal glucocorticoids on lactogenesis II. The addition of glucocorticoids at levels sufficient to mature fetal organs such as the lung prematurely suggests a similar precocious maturation of the mammary gland may occur. On the basis of these effects, I propose that antenatal glucocorticoid treatment has the potential to adversely affect early lactation and because of the importance of mother's own milk for the preterm neonate, an investigation into the effects of antenatal glucocorticoid treatment on lactation is timely.

## 1.1 Lactation

Lactation is critical for the survival of mammals and can be seen as "… the final phase of the complete reproductive cycle of mammals" (Cowie, Forsyth *et al.* 1980). Mammalian species are dependant on maternal milk during the neonatal period and milk contains all the nutrients necessary for the growth and development of the neonate. Thus problems with lactation, and specifically the initiation of lactation, may be equally as important as either failure to ovulate or other problems of reproduction.

In humans, breastfeeding is recommended as the optimal method of neonatal nutrition and currently exclusive breastfeeding is promoted for the first six months after birth with breastfeeding as the major nutrient source for at least the first 12 months of life (WHO 1998; Binns & Davidson 2003; Gartner, Morton *et al.* 2005). Australian guidelines currently aim for an initiation rate of 90% and for 80% of infants to be receiving breastmilk at 6 months of age (Binns & Davidson 2003).

Successful lactation requires a fully-developed mammary system and sufficient circulating levels of a number of hormones including glucocorticoids and prolactin.

### 1.1.1 Mammary Gland Development

The development of the adult mammary system during pregnancy and lactation is a continual cyclical process of growth and involution from the undifferentiated pre-pregnancy state to full functional differentiation shortly after parturition, to involution and loss of differentiation at weaning. The endocrine system has a major role in synchronizing this process either by acting directly on the mammary gland or indirectly by coordinating metabolic changes to facilitate the additional energy demands of providing milk for the offspring. Lactogenesis is defined as the initiation of milk secretion and is generally accepted to occur in two stages, lactogenesis I (mammary development) and lactogenesis II, which is the onset of copious mammary secretion (Hartmann, P E 1973).

#### *1.1.1.1 Lactogenesis I*

Lactogenesis I, which occurs during pregnancy, involves the development of the mammary ducto-alveolar tissues and differentiation of the alveolar epithelial cells to lactocytes (alveolar secretory epithelial cells) that are able to produce small quantities of mammary secretion containing milk specific compounds. This early milk secretion, known as colostrum, is detected in the second half of gestation in most species (Cowie,

Forsyth *et al.* 1980; Arthur, Kent *et al.* 1991). The composition of colostrum is similar in most species such as sheep and women in that it contains high concentrations of sodium, chloride and proteins including the immunoprotective proteins secretory IgA and lactoferrin. Lower concentrations of lactose and potassium are found in colostrum compared with mature milk (Cowie, Forsyth *et al.* 1980; Neville, M C, Morton *et al.* 2001).

The hormones required for lactogenesis I are depicted in Figure 1.1. In all species, the reproductive hormones estrogen, progesterone and prolactin are required for alveolar growth and development (Neville, M C, McFaddin *et al.* 2002). Increasing concentrations of progesterone during pregnancy also act to inhibit the onset of copious milk secretion until parturition (Kuhn 1969; Hartmann, P E, Trevethan *et al.* 1973). Glucocorticoids also have an essential action on the developing mammary system. This has been demonstrated by mammary tissue culture experiments where the addition of cortisone is mandatory for the survival of cells *in vitro*. (Cowie, Forsyth et al. 1980)

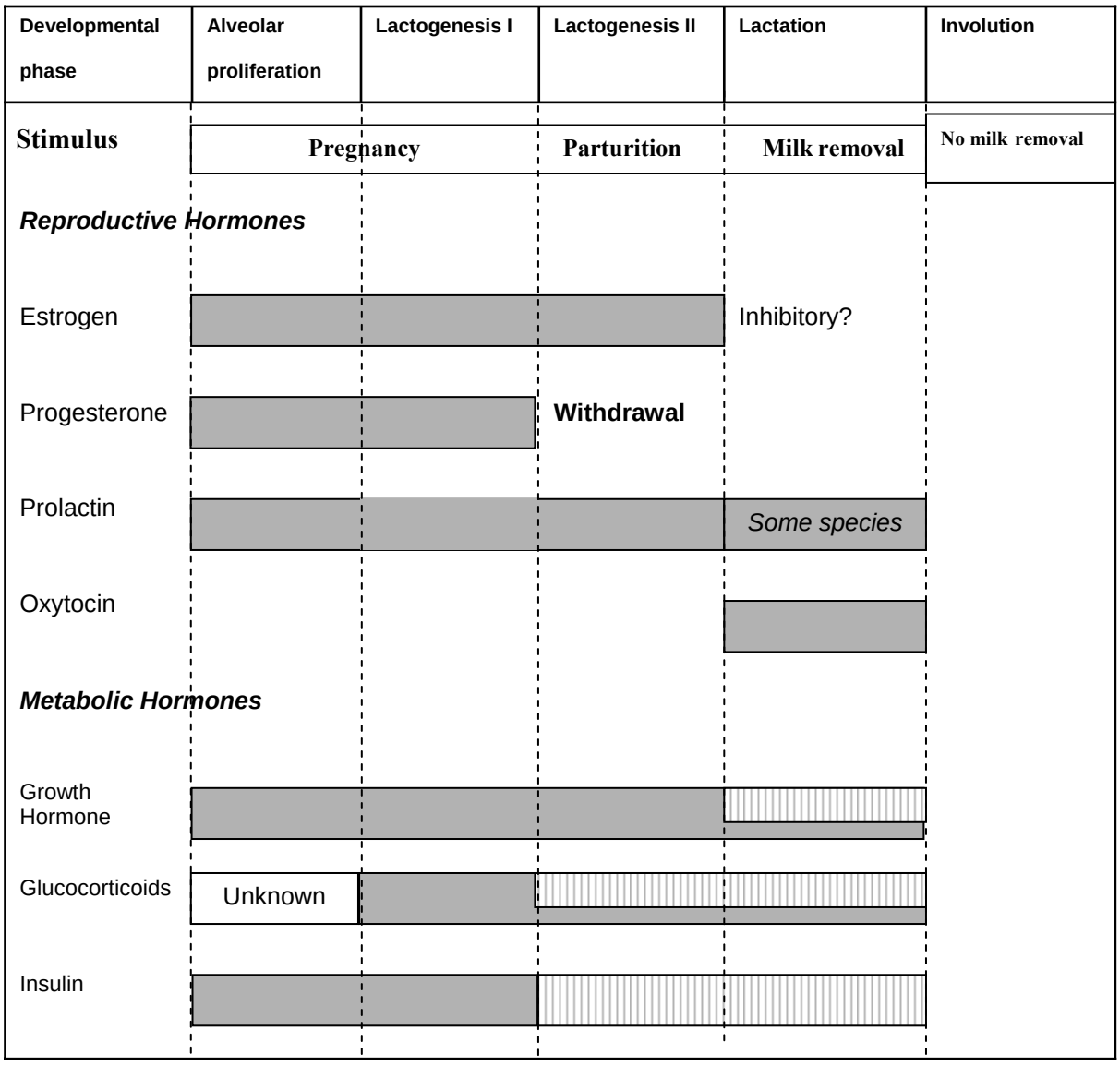
#### *1.1.1.2 Lactogenesis II*

Whereas lactogenesis I indicates the readiness of the lactocytes for milk synthesis, the commencement of secretion of quantities of milk sufficient for nurturing the young only occurs after parturition. The onset of copious milk secretion is defined as lactogenesis II (Hartmann, P E 1973). This process is accompanied by rapid increases in milk volume and profound changes in milk composition. At this time women perceive breast fullness, heat and tenderness as well as large increases in milk transfer and the milk is said to be "coming in". Lactogenesis II occurs after parturition regardless of whether milk removal occurs (Kulski & Hartmann 1981; Neville, M C, Morton *et al.* 2001).

While some hormones act directly on the mammary gland, other metabolic hormones are necessary to coordinate the body's response to the metabolic changes of lactation

(Figure 1.1). In all species lactogenesis II is triggered by the withdrawal of progesterone in the presence of high circulating concentrations of prolactin (Kuhn 1969; Turkington & Hill 1969; Hartmann, P E, Trevethan *et al.* 1973).

**Figure 1.1 Hormonal Action Necessary for Phases of the Lactation Cycle**




Note: Adapted from (Neville, M C, McFaddin *et al.* 2002)

Hormone has direct action on mammary gland

Hormone has indirect action on mammary phases by co-ordinating metabolism

The timing of lactogenesis II is species specific. In women, where the sudden drop in progesterone concentration occurs with the removal of the placenta, the onset of copious milk secretion does not occur until about 48 hours after the birth (Kulski, Smith *et al.* 1977). Studies have shown that lactogenesis II is delayed in women who have retained placental products after the birth (Neifert, McDonough *et al.* 1981). The capacity of the remaining placental fragments to secrete progesterone prolongs the inhibition of milk secretion in these women.

In other species, such as sheep, secretion of progesterone by the placenta decreases sharply just before parturition and lactogenesis II occurs within the first day after parturition (Hartmann, P E, Trevethan *et al.* 1973). In contrast, in species such as the rat where the ovary maintains secretion of progesterone during pregnancy, lactogenesis II commences before parturition (Neville, M C, McFaddin *et al.* 2002). In rats, luteal secretion of progesterone decreases due to the increased production of 20α-OH progesterone before parturition associated with a surge of prolactin, leading to lactogenesis II (Kuhn 1969).

A decrease in permeability of the tight junctions between lactocytes accompanies the increase in milk secretion rate at the time of lactogenesis II (Linzell & Peaker 1974). Before lactogenesis II there is leakage of interstitial fluid between the secretory cells and milk products are also able to move out of the alveoli through paracellular pathways. The tight junctions close with lactogenesis II under the influence of glucocorticoids and the withdrawal of progesterone at parturition (Nguyen, Parlow *et al.* 2001).

Studies of milk transfer in women breastfeeding their term infants have demonstrated rapid increases in milk volume from 36 to 96 hours postpartum after which volumes stabilize at an average volume of 600ml per day (Saint, Smith *et al.* 1984; Neville, M C, Allen *et al.* 1991). Changes in milk composition commence before increases in volume

are noted. Chiefly these changes include decreases in the concentration of sodium, chloride and protein and rapid increases in the concentration of lactose, citrate and potassium (Kulski & Hartmann 1981; Neville, M C, Allen *et al.* 1991). The early changes in sodium and lactose concentration are attributed to closure of tight junctions between lactocytes, which prevent movement of constituents through the paracellular pathway between lactocytes (Nguyen & Neville 1998; Nguyen, Parlow *et al.* 2001).

*Methods of Determination of Lactogenesis II*

Measurement of milk volume is the most obvious method for determining the onset of copious milk secretion but accurate measurement is often difficult to determine. Milk volume can be easily measured when mothers are expressing for infants who are sick or preterm. However the effect of frequency of expression on milk production during initiation of lactation is not known (Neville, M. C. & Morton 2001; Hartmann, P. E., Cregan *et al.* 2003). In mothers who are breastfeeding their term infants, test weighing of the infant before and after all feeds is the most reliable method of determining milk transfer from mother to infant (Arthur, Hartmann *et al.* 1987; Scanlon, Alexander *et al.* 2002). However this method is a demanding procedure for new mothers and may not accurately determine synthesis if milk production exceeds demand for milk by the infant.

The synthesis by lactocytes of most milk constituents such as lactose increases rapidly at the onset of copious milk secretion. As lactose is the principal osmotic component in the mammary secretion, measurement of its concentration in milk provides an accurate estimate of lactogenesis II when measurement of milk volume is not feasible (Arthur, Smith *et al.* 1989). In addition, lactose concentration in either blood or urine is a useful indicator of increased mammary secretory activity before parturition and the early days after parturition when little milk is being removed (Arthur, Kent *et al.* 1991; McNeill, Murphy *et al.* 1998; Cox, Kent *et al.* 1999). Before closure of the paracellular pathways,

lactose is able to leak more readily from the alveolar lumen of the mammary gland into the circulatory system (Linzell & Peaker 1974; Hartmann, P E, Whitely *et al.* 1984). As very little lactose is normally present in the non-pregnant circulation, it is presumed that lactose in the blood has originated from the mammary gland. In women, plasma lactose concentration has been shown to increase during the second half of pregnancy peaking three to five days after birth and then decreasing once lactation is established (Arthur, Kent et al. 1991). As very little lactose in the maternal circulation is metabolized, plasma lactose is excreted unchanged in the urine. Thus, the rate of lactose excretion in urine also accurately reflects the plasma concentration of lactose and is therefore a useful indicator of lactogenesis II (Cox, Kent et al. 1999).

The concentration of sodium in mammary secretion is also a useful marker of lactogenesis II. Rapid decreases in $Na^+$ concentration in milk occur with the closure of tight junctions.(Neville, M C, Allen *et al.* 1991; Neville, M C, Morton *et al.* 2001) Maternal perception of breast fullness is frequently used to quantify timing of the onset of lactogenesis II in studies of women (Chapman & Pérez-Escamilla 2000b; Pérez-Escamilla & Chapman 2001). This marker is less reliable than either biochemical markers or milk volume, however, because of its subjective nature.

#### *1.1.1.3 Milk Synthesis and Secretion*

Although the rapid onset of copious secretion soon after parturition is independent of milk removal, sustained lactation requires regular emptying of the mammary gland (Figure 1.2) (Neville, M C, McFaddin *et al.* 2002). Once lactation is established, milk secretion is regulated by either the demands of the suckling offspring or by frequent expression and the associated hormone release. This is achieved by a feedback mechanism in which stimulation of the nipple and emptying of the mammary alveoli lead to the release of prolactin by the anterior pituitary gland. Suckling also stimulates

release from the posterior pituitary gland of the hormone oxytocin which acts on the myoepithelial cells of the mammary gland to cause milk ejection (Neville, M C 2001). The changes that occur in the lactocytes during one lactation cycle and between milk removals are shown in Figure 1.2. After a breastfeed or expression the empty alveolus rapidly fills under the influence of prolactin increasing expression of milk proteins such as α-lactalbumin. As the alveoli become more distended, the milk becomes more fatty, expression of α-lactalbumin is down-regulated and lactoferrin is up-regulated. At the next feed or expression oxytocin aids milk ejection and the emptying of the lactocyte again.

#### *1.1.1.4 Involution*

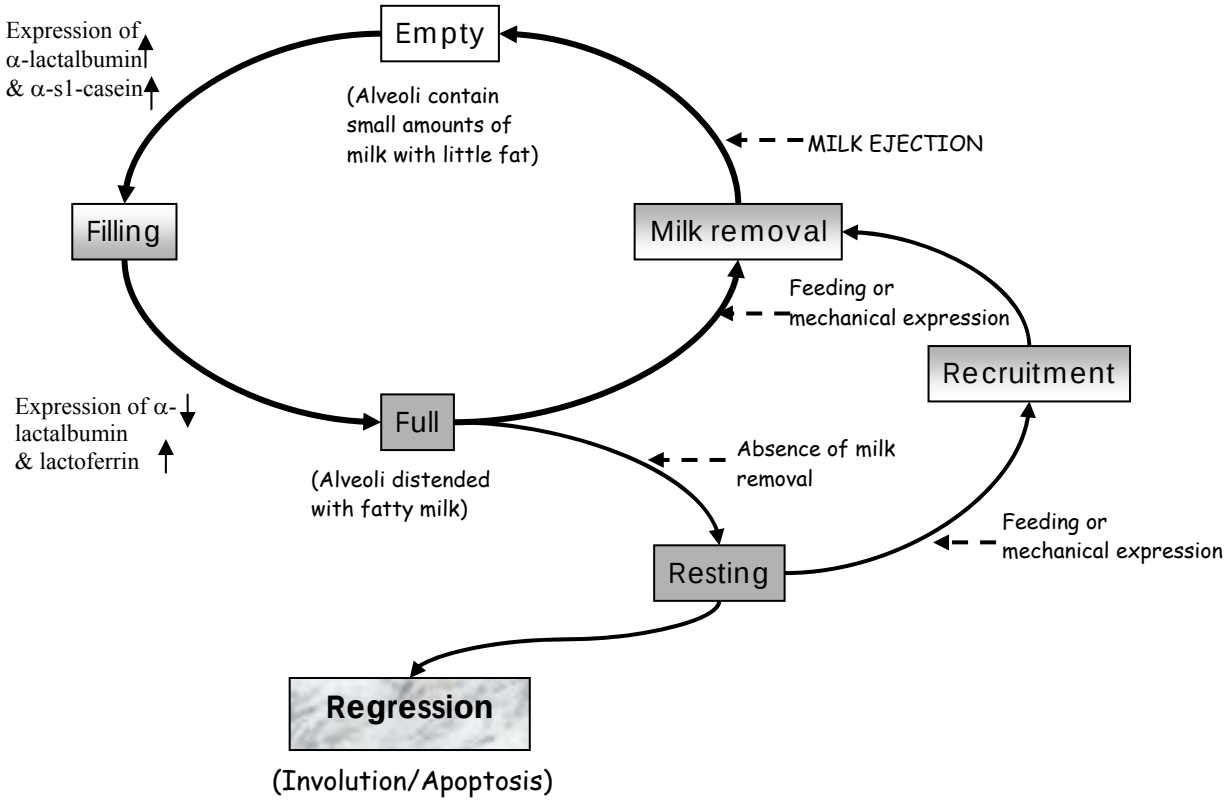
Involution of the mammary gland commences when emptying of the gland by either suckling or expression ceases. The trigger for involution is mechanical pressure from the distended gland coupled with the cessation of stimulation of the gland (Marti, Feng *et al.* 1977; Akers 2002). Milk stasis in the distended gland initiates a cascade of signals leading to apoptosis of the lactocytes and, in mice, restructuring of the gland. Li *et al.* demonstrated that local signals of milk stasis are sufficient to induce apoptosis in mice even in the presence of systemic lactogenic hormones including exogenous glucocorticoids (Li, Liu *et al.* 1997). A number of studies have shown that the initiation of apoptosis at involution is multifactorial and requires both the activation of a wide number of signalling triggers including milk stasis, α-lactalbumin and STAT3; as well as the inactivation of a wide number of survival factors including glucocorticoids, integrins, laminin, and STAT5 (Green & Streuli 2004).

In rodents, apoptosis is followed by proteolytic degradation of the epithelial basement membrane leading to complete remodelling of the mammary gland (Talhouk, Chin *et al.* 1991; Lund, Rømer *et al.* 1996; Akers 2002). Lund *et al*. found that treatment with

systemic glucocorticoids significantly inhibited this tissue remodelling but did not affect the initial apoptosis (Lund, Rømer et al. 1996). In contrast, ewe mammary glands regress solely by apoptosis of the lactocytes without any tissue remodelling (Tatarczuch, Philip *et al.* 1997). In one study, apoptosis was shown to reach a peak four days after lambs ceased suckling, followed by a gradual regression leading to a complete involution after 30 days. A "dry" period during pregnancy between lactations is normally practised with dairy ruminants to allow the mammary gland to rest after involution in order to maximize milk production in the subsequent lactation (Tatarczuch, Philip *et al.* 1997; Wilde, Addey *et al.* 1997; Akers 2002).

The lack of stimulation via suckling or mechanical expression is hypothesised to lead to a drop in the lactogenic hormones prolactin and glucocorticoids (Baik, Lee *et al.* 1998), which in turn leads to a decrease in the synthesis of milk proteins. In ewes the expression of α-lactalbumin mRNA, a milk protein which is required for the synthesis of the milk sugar, lactose, is markedly reduced in alveoli distended with fatty milk whereas lactoferrin, a milk protein accepted to be a marker for involution, is highly expressed in secretory cells in distended alveoli (Molenaar, Davis *et al.* 1992; Molenaar, Kuys *et al.* 1996). This process is depicted in Figure 1.2.

**Figure 1.2 Cyclic Changes In Lactocytes During Lactation**




Note: Adapted from (Molenaar, Davis et al. 1992)

### 1.1.2 Milk composition

Milk is a complex biological fluid containing a wide range of components required for the growth and development of the offspring (Mepham 1983; Jensen 1995). There are marked differences between species but all contain sugars, proteins, lipids, and other components specific to the needs of the neonate. Nevertheless the milk of eutherian (placental) mammals such as sheep, cows and humans contains a number of common constituents including lactose, the protein casein and whey protein α-lactalbumin, and lipids. A comparison of the macronutrients in milk from different species is shown in Table 1 and demonstrates the different proportions of components between species. For

example mature human milk has a higher concentration of lactose and a lower concentration of casein than other species such as the sheep.

**Table 1.1 Comparative Components of Milk from Different Species**

| Component | Human | Sheep | Cow | Rat |
|---|---|---|---|---|
| Lactose (mM) | 192 | 133 | 133 | 90 |
| Casein (g/L) | 4 | 46 | 28 | 64 |
| Whey Proteins (g/L) | 6 | 9 | 6 | 20 |
| Fat (g/L) | 38 | 74 | 37 | 103 |

Note: Data adapted from Mepham, TB *ed*, (Davies, Holt *et al.* 1983).

#### *1.1.2.1 Lactose*

Lactose is the principal sugar in milk in most species and the rapid increase in lactose concentration is an early indicator of lactogenesis II. Lactose is synthesized in the lactocytes from glucose in the presence of the enzyme lactose synthase of which α-lactalbumin is a co-factor (Kuhn 1983). During pregnancy, paracellular pathways allow the leakage of lactose into plasma. The decrease in permeability of the mammary epithelium at the time of lactogenesis II leads to a decrease in plasma lactose concentration associated with a rapid increase in the concentration of lactose in milk (Linzell & Peaker 1974; Hartmann, P E, Whitely *et al.* 1984). Conversely, on cessation of milk removal, the synthesis of milk lactose decreases and permeability increases. Thus the concentration of lactose decreases in milk, and increases in plasma when involution of the mammary gland occurs.

### *1.1.2.2 Proteins*

In humans and many other species the concentration of proteins is very high in colostrum but decreases to relatively low levels after lactogenesis II (Lonnerdahl & Atkinson 1995). During early lactation the principal proteins are concerned with transfer of immunity from the mother to the offspring and consist of immunoglobulins and lactoferrin. In species such as sheep, IgG is the main immunoglobulin transferred in colostrum (Cowie, Forsyth et al. 1980) and hence colostrum is crucial for the neonatal lamb which obtains almost all its systemic IgG within 24-hours of birth. In humans, who obtain most of their IgG *in utero* in the last trimester of pregnancy, secretory IgA (sIgA) is the main immunoglobulin in colostrum and milk. Secretory IgA is not readily absorbed through the neonate's gut and exerts its main protective function within the lumen of the intestines and respiratory tract. These immunoglobulins are crucially important for preterm infants for whom delivery occurs before placental transfer of IgG. An interesting finding in the milk of mothers of preterm infants shows that protein levels are relatively high compared with mothers of term infants although it is not known whether sIgA concentration is associated with gestational age at birth (Atkinson, S A, Radde *et al.* 1980).

Lactoferrin is present in high concentrations in colostrum (Kulski & Hartmann 1981) but is down-regulated during established lactation and then increases markedly during either inflammation of the mammary gland or involution (Hartmann, P E & Kulski 1978; Neville, M C, Chatfield *et al.* 1998; Wang & Hurley 1998). Lactoferrin is an iron-binding milk protein that is a major host defence agent for the offspring as well as protection against mastitis during involution (Wang & Hurley 1998).

Mature milk has relatively higher concentrations of α-lactalbumin and β-casein depending on species. The milk protein α-lactalbumin is an essential component of the enzyme lactose synthase required for regulation of the synthesis of lactose (Akers

2002). Activation of α-lactalbumin is closely associated with lactogenesis II and its synthesis is influenced by prolactin and glucocorticoids. The synthesis of milk proteins requires the coordination of a large number of mRNAs and this process is regulated synergistically by prolactin and glucocorticoids and inhibited during pregnancy by progesterone (Figure 1.3 ) (Mercier & Gaye 1983; Groner 2002).

### 1.1.3 Endocrinology of lactation

The hormones regulating lactation are well conserved between species (Figure 1.1). Prolactin, estrogen, placental lactogen and glucocorticoids promote mammary growth and differentiation during pregnancy and are essential for lactogenesis II and maintenance of lactation. Oxytocin regulates milk ejection by stimulating contraction of the myoepithelial cells of the mammary gland. Progesterone is active in the development of the mammary gland during pregnancy but its withdrawal triggers the onset of lactogenesis II.

#### *1.1.3.1 Prolactin*

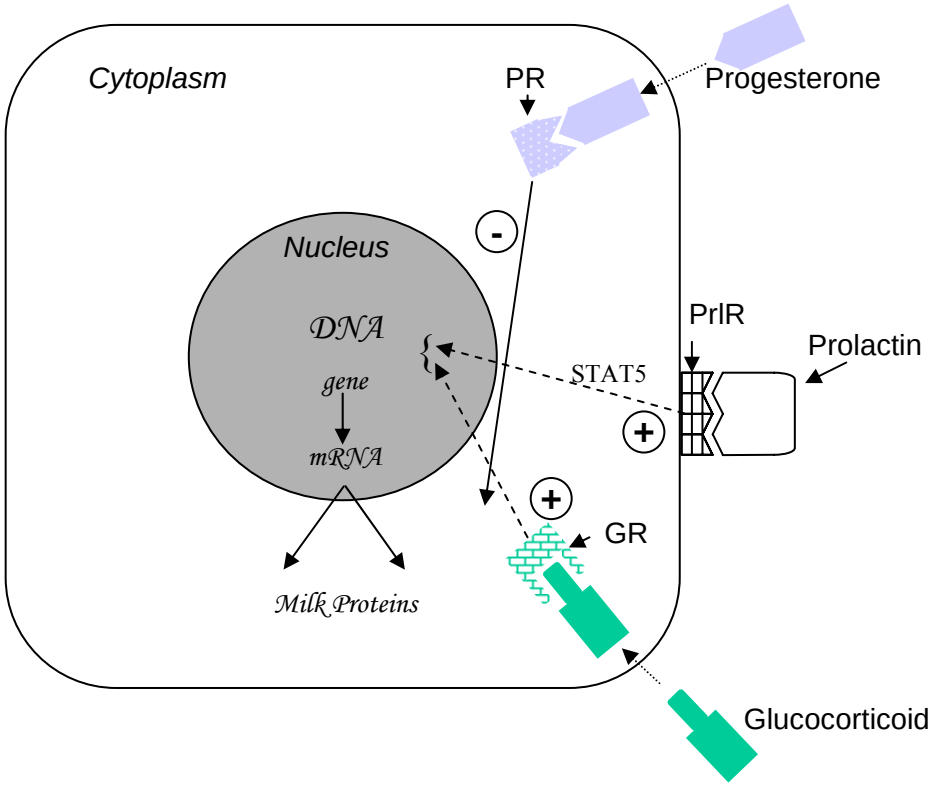
Prolactin is essential for mammary growth and differentiation during pregnancy and increasing plasma concentrations at the end of pregnancy are critical for lactogenesis II to commence (Kulski, Hartmann *et al.* 1978; Tucker 2000; Neville, M C, McFaddin *et al.* 2002). Once lactation is established, however, high basal levels are not required in most species.

Secretion of prolactin by the anterior pituitary gland for ongoing lactogenic activity is dependent on a positive feedback (Cowie, Forsyth et al. 1980). The suckling stimulus in addition to regular emptying of the gland is essential to promote prolactin secretion. Conversely prolactin release is inhibited by alveolar distension.

*Prolactin Receptor*

Prolactin binds to specific prolactin receptors (PrlR) on the surface of lactocytes, inducing a lactogenic signalling pathway that leads to the switching on of the transcription of genes that regulate the secretion of milk proteins such as casein and α-lactalbumin (Figure 1.3) (Rosen, Wyszomierski *et al.* 1999; Tucker 2000). PrlR is a member of the cytokine receptor family, which is up-regulated in the lactocytes at the time of parturition accompanying lactogenesis II (Liu, Robinson *et al.* 1996).

**Figure 1.3 Intracellular Hormonal Signaling In The Lactocyte During Lactation**




Note: Adapted from (Mercier & Gaye 1983).

Prolactin and glucocorticoids up-regulate milk protein gene expression (+)

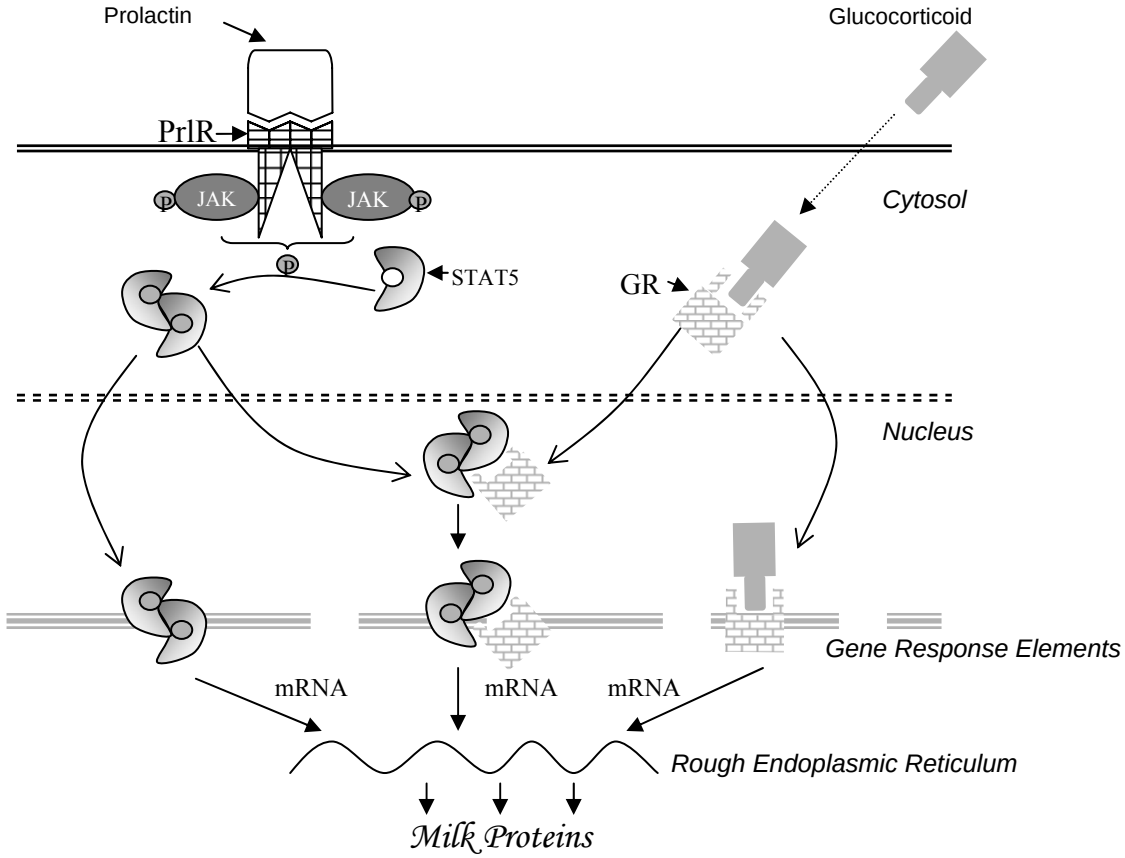
Progesterone inhibits milk protein expression during pregnancy (-)

PR = Progesterone Receptor, PrlR = Prolactin Receptor, GR = Glucocorticoid Receptor

The coordination of the large number of mRNAs promoting synthesis of milk proteins is regulated by PrlR and amplified by the glucocorticoid receptor at a transcriptional level (Mepham 1983). This process is depicted in Figure 1.4. In brief, binding of prolactin to PrlR induces dimerisation of the receptor, which activates Janus Kinase 2 (JAK2). JAK2 then induces phosphorylation and activation of transcription factors STAT5a and STAT5b. STAT5s are members of the signal transducer and activator of transcription (STAT) protein family and are major transducers in cytokine receptor signalling. Phosphorylated STAT5s then dissociate from the PrlR, dimerise with other STAT molecules, and then translocate to the nucleus. STAT5s then activate specific STAT DNA-binding motifs in the promoter of a target gene (GAS (γ-interferon activated sequence)), thereby promoting expression of specific genes regulating milk protein synthesis (Freeman, Kanyickska *et al.* 2000; Tucker 2000) (Figure 1.4). In addition, STAT5 synergizes with activated glucocorticoid receptor in the transcription of these genes (Stocklin, Wissler *et al.* 1997; Rosen, Wyszomierski *et al.* 1999). Milk protein genes promote synthesis of milk proteins from amino acids on the rough endoplasmic reticulum (Cowie 1984).

Integrity of the extracellular matrix is also required for continued activity of PrlR in the lactocyte (Edwards, Wilford *et al.* 1998; Zoubiane, Valentijn *et al.* 2003). Cellular interaction with extracellular matrix proteins (chiefly laminin), transduced via β1 integrins, controls the ability of prolactin to activate PrlR, thereby initiating the JAK/STAT pathway. This pathway is therefore interrupted by milk stasis, which leads to apoptosis of lactocytes (Section 1.1.1.4).

**Figure 1.4 Signal Transduction Pathways In The Lactocyte During Lactation**




Note: modified and simplified from (Stoecklin, Wissler *et al.* 1997; Freeman, Kanyickska *et al.* 2000).

PrlR = Prolactin Receptor,

JAK = Janus Kinase 2 (JAK2, a tyrosine kinase),

STAT5 is a member of the Signal transducer and activator of transcription (STAT) family,

GR = Glucocorticoid Receptor.

#### *1.1.3.2 Glucocorticoids*

Glucocorticoids are essential for mammary alveolar development during pregnancy and for maintenance of lactation as well as being critical for the onset of lactogenesis II (Tucker 2000; Neville, M C, McFaddin *et al.* 2002). In the dairy industry it has long been known that administration of exogenous glucocorticoids, prolactin and estrogen can initiate lactation when the lobulo-alveolar system of mammary glands is well-developed. Glucocorticoids also play an indirect role in lactation through their roles in energy homeostasis, immune system regulation, organ development, stress adaptation and behaviour modification (Reichardt, Horsch *et al.* 2001).

In ruminants and humans, cortisol is the main endogenous glucocorticoid whereas corticosterone is predominant in species such as mice and rats. In sheep, mice and rats maternal plasma concentrations of glucocorticoids remain relatively low during pregnancy (around 10 ng/mL) but rise to a peak just before parturition (Tucker 2000; Young 2001; Neville, M C, McFaddin *et al.* 2002). In sheep, the source of the rise of cortisol has been attributed to a surge in secretion of fetal cortisol in late gestation, which increases maternal levels, inducing labour and stimulating lactogenesis II in conjunction with a withdrawal of progesterone (Whittle, Patel *et al.* 2001; Young 2001). The basal, maternal concentration of cortisol in plasma is higher in humans and increases by a factor of five during pregnancy (above 200 ng/mL, (Neville, M C, McFaddin *et al.* 2002)). In maternal circulation, cortisol is bound to corticosteroid binding globulin (CBG) thereby inactivating its lactogenic effects. Levels of CBG reduce during the peri-parturient period, increasing the availability of cortisol for uptake and binding in mammary tissue during lactogenesis II (Tucker 2000; Akers 2002).

In dairy species such as sheep, maternal glucocorticoid levels often increase during mature lactation as they are required to maintain blood glucose in a time of relative

nutrient starvation (Neville, M C, McFaddin *et al.* 2002). In contrast, cortisol levels in women are reduced in mature lactation compared with pregnancy levels (urine 24 hour cortisol 270 ± 67 nmol/day at 3 months postpartum compared with 747 ± 213 nmol/day in pregnancy, (Butte, Hopkinson *et al.* 1999)).

One mechanism for the lactogenic effects of glucocorticoids on mammary development and lactation is indirectly via body metabolism. Glucocorticoids are essential for the maintenance of glucose homeostasis. Direct actions of glucocorticoids on lactation include the regulation of tight junction permeability as well as prevention of involution and apoptosis of mammary tissue during established lactation (Feng, Marti *et al.* 1995; Nguyen & Neville 1998; Berg, Dharmarajan *et al.* 2002). Glucocorticoids are also required to assist the synthesis of milk proteins such as casein and α-lactalbumin.

*Glucocorticoid Receptor*

Glucocorticoid receptors (GR) were first identified in the cytoplasm of mammary cells in the 1970s (Gardner & Wittliff 1973). After binding with glucocorticoids, activated receptors translocate to the nucleus where they act synergistically with prolactin-activated transcription factors to enable synthesis of milk proteins such as casein and α-lactalbumin (Figures 1.3 and 1.4) (Stöcklin, Wissler *et al.* 1996; Stocklin, Wissler *et al.* 1997; Rosen, Wyszomierski *et al.* 1999).

Progesterone also binds to GR in the mammary cytoplasm, possibly explaining the inhibitory action of progesterone during pregnancy (Collier & Tucker 1978; Maki, Hirose *et al.* 1980). Progesterone does not translocate to the nucleus after binding, thus preventing GR from reaching sites where transcription of milk protein genes occurs (Forsyth 1983). Although the affinity of progesterone to GR is lower than that of glucocorticoids, the concentration of progesterone is much higher during pregnancy thus displacing glucocorticoid binding until GC concentrations increase dramatically in the peri-parturient period (Mesiano 2001). It is hypothesised that exogenous

glucocorticoid treatment induces premature labour in sheep by prematurely displacing progesterone from GR in the placenta (Liggins 1969; Young 2001). This raises the question of whether exogenous glucocorticoid treatment in pregnancy, for example when preterm birth is anticipated, has a similar effect on the mammary gland.

#### *1.1.3.3 Growth Hormone*

As discussed previously (Section 1.1.3.1), in many species, high levels of prolactin are not essential to maintain lactation after lactogenesis II. In ruminants such as sheep, growth hormone (GH) plays a more important role in the maintenance of lactation (Forsyth 1986). Sheep demonstrate increased yields of milk and lactose when treated with bovine GH (Min, Mackenzie *et al.* 1997) or GH releasing factor (Kann 1997). The principal action of GH in lactation is by regulation of metabolism. It has been shown that GH partitions glucose away from body tissues and towards milk secretion (Forsyth 1986). Treatment of lactating ruminants with GH has marked coordinated effects on glucose metabolism in a number of tissues including liver, adipose tissue and the mammary gland (Bell & Bauman 1997).

#### *1.1.3.4 Placental Lactogen*

Placental lactogen is a hormone produced in the placenta in many species including sheep and women, which has prolactin-like activity (Forsyth 1986). During pregnancy it stimulates growth and development of the mammary gland. The lactogenic activity of placental lactogen occurs only during pregnancy and exogenous treatment during lactation has not been shown to have any effect on milk yield in sheep (Min, Mackenzie et al. 1997). Moreover, a role for human placental lactogen either in lactogenesis I or II has not been found in women (Neville, M C, McFaddin *et al.* 2002)

#### *1.1.3.5 Oxytocin*

The hormone oxytocin is released from the posterior pituitary during stimulation of the nipple by either the suckling infant or by expression or milking (Zinamen, Hughes *et al.* 1992; Neville, M C 2001). Oxytocin stimulates the contraction of mammary myoepithelial cells, known as milk ejection or "let down", thereby aiding removal of milk from the mammary gland. Milk ejection is a neuroendocrine reflex and as such is a conditioned reflex that can be affected by psychological stimuli. In some women milk ejection can be stimulated merely by the sight, sound or thought of their infant. Conversely, stress such as that occurring after either a traumatic or preterm delivery is known to inhibit oxytocin release and may therefore adversely influence milk ejection (Nissen, Gustavsson *et al.* 1998; Chatterton, Hill *et al.* 2000; Dewey 2001; Lau 2001).

#### *1.1.3.6 Progesterone*

It has long been known that administration of progesterone in pregnancy prevents normal initiation of synthesis of α-lactalbumin, lactose and casein. In the rat, lactation was shown to be initiated when progesterone is removed by ovariectomy (Kuhn 1969; Turkington & Hill 1969). It was later shown that artificially maintaining high levels of progesterone after parturition blocks lactogenesis II in the ewe (Hartmann, P E, Trevethan *et al.* 1973).

In species such as mice and rats, the corpus luteum in the ovary secretes progesterone throughout pregnancy whereas the placenta is the principal source of progesterone in women and sheep (Forsyth 1983). In the ewe the concentration of progesterone increases steadily throughout pregnancy to a peak of 3 – 10 ng/mL at about day 120 to 140 of pregnancy (Cowie, Forsyth et al. 1980). There is a wide variation of values reported by different investigators and within individual ewes although early studies used questionable methodology to determine progesterone concentration. Most studies

show declining levels in the last two weeks of gestation although this may only occur on the day of lambing.

The concentration of plasma progesterone in pregnant women is much higher than in sheep, increasing 100-fold above non-pregnant levels and nearing 200 ng/mL before parturition (Heap & Flint 1984). In many species such as humans, progesterone is rapidly metabolized and removed from the blood stream (Heap & Flint 1984). Measurement of the concentration of the urinary metabolite pregnanediol glucuronide (PdG) by immunoassay is therefore a good indicator of plasma progesterone levels with high sensitivity and specificity observed in several studies (Brown, Blackwell *et al.* 1988; Brown, Blackwell *et al.* 1989; Sauer & Paulson 1991). In the non-pregnant woman daily urinary PdG levels rise from below 1 mg/24 hours pre-ovulation to up to 6 mg/24 hours in the second half of the menstrual cycle (Brown, Blackwell et al. 1989). These levels are vastly increased during both ovarian hyper stimulation for fertility treatments and also pregnancy.

The presence of progesterone is required to maintain uterine quiescence during pregnancy (Challis, Matthews et al. 2000). Progesterone withdrawal at the end of pregnancy triggers the onset of parturition in many species including sheep, but not women (Heap, Galil *et al.* 1977; Challis, Matthews *et al.* 2000). In contrast with most other species, progesterone is maintained during parturition in women (Challis, Matthews et al. 2000) and is withdrawn only after birth with the delivery of the placenta. In all species, however, progesterone withdrawal is essential for the initiation of lactation.

Increasing concentrations of progesterone during pregnancy inhibit lactogenesis II by suppressing the up-regulation of prolactin receptors in the lactocytes (Djiane & Durand 1977). In addition progesterone competes with glucocorticoids for binding to glucocorticoid receptors in epithelial cells, thus suppressing the lactogenic activity of

endogenous glucocorticoids (Collier & Tucker 1978). Once lactation is established, however, exogenous progesterone has been shown to have little or no effect on milk supply, allowing women to use either the progesterone-only mini pill or depo provera (medroxyprogesterone acetate) as effective contraceptive agents during lactation (Danli, Qingxiang *et al.* 2000; Hale 2006). Furthermore, milk production is not suppressed when menstrual cycles return.

It is of interest that it has been shown to be necessary to supplement pregnant sheep with medroxyprogesterone acetate (MPA, a progesterone analogue) to prevent premature parturition after treatment with synthetic glucocorticoids (Nathanielsz, Buster *et al.* 1988). This is not the case in women although transient increases in uterine activity have been documented after betamethasone treatment particularly in multiple pregnancies (Yeshaya, Orvieto *et al.* 1996). These interesting differences between species suggest the need for detailed investigation of the responses of progesterone to antenatal glucocorticoid treatment and the impact on lactogenesis II in the two species.

### 1.1.4 Factors Associated with Lactation Problems and Failure

In mothers of term infants, the timing of lactogenesis II has been shown to influence the success of breastfeeding. Women who experience delayed lactogenesis II are more likely to have shorter durations of breastfeeding regardless of their original intentions (Chapman & Pérez-Escamilla 1999a). Moreover, subsequent milk transfer is strongly influenced by maternal milk yield in the first weeks of lactation (Neville, M C, Keller *et al.* 1988). Hence lactogenesis II is a critical event in the breastfeeding experience in mothers of term infants. The evidence is less clear for mothers who deliver before term. Although the necessary conditions for lactogenesis II to occur (fully-developed lactocytes, raised plasma concentration of prolactin and a sharp decrease in

progesterone concentration) are present in most women after birth, a number of factors can cause delay of lactogenesis II (Neville, M C, Morton *et al.* 2001).

#### *1.1.4.1 Parity*

Parity influences the timing of lactogenesis II with studies showing that postnatal increases in milk volume occur later in primiparous women compared with multiparous women (Chen, Nommsen-Rivers *et al.* 1998; Dewey, Nommsen-Rivers *et al.* 2003). A study which used maternal report of increased breast fullness to define onset of lactation found that primiparous women were more likely to perceive delayed onset (Dewey, Nommsen-Rivers et al. 2003). In this study of middle-class American mothers, primiparas were also more likely to experience early breastfeeding failure.

#### *1.1.4.2 Mode of Delivery*

The literature is inconsistent about the effects of mode of delivery on timing of lactogenesis II in women who deliver at term. However, studies that examined the effect of emergency (non-elective) caesarean section were more likely to find adverse effects on onset of lactation than those which investigated elective caesarean section alone. Kulski and associates found no effect of elective caesarean section on changes in milk composition indicating lactogenesis II in a sample of women who were highly motivated to breastfeed (Kulski, Smith *et al.* 1981). Chapman and Pérez-Escamilla found no effect of elective caesarean section on maternal perception of timing of onset of lactation compared with vaginal delivery (Chapman & Pérez-Escamilla 1999b). In contrast the authors found that unplanned caesarean section was significantly associated with perceived delay of onset of lactation.

Dewey *et al* found that maternal report of breast fullness was delayed in mothers having a caesarean section (Dewey, Nommsen-Rivers et al. 2003). This study, which did not specify whether the operative delivery was elective or emergency, suggested a delay in

lactogenesis II in women delivering by caesarean section for any reason. In another study that did not discriminate between elective and non-elective caesarean section, milk transfer was reduced in women who delivered by caesarean section on days two to five compared with vaginal delivery but no difference was observed after that time (Evans, Evans *et al.* 2003). Healthy women who delivered a term infant weighed their infants before and after each feed (test weighing) for up to six days postpartum in that study of breastmilk transfer. However, test weighing has limitations when used as a marker of lactogenesis as it only measures the infant's capacity and hence may underestimate potential milk production in women who are able to produce more than the infant's needs.

A study which sampled blood for hormonal analysis during breastfeeding on day two postpartum found different patterns of hormone release according to mode of delivery suggesting a delay in onset of lactation after emergency caesarean section (Nissen, Uvnäs-Moberg *et al.* 1996). Compared with normal vaginal delivery, women who delivered by emergency caesarean section demonstrated fewer pulses in oxytocin and lower prolactin concentration in blood samples taken while they were breastfeeding their term infants suggesting less effective lactation in the early postnatal period.

The findings of the potential for non-elective caesarean section to adversely affect lactogenesis II and the fact that operative delivery is frequently required in the case preterm labour suggest that any study of the effects of preterm delivery on lactogenesis II should consider mode of delivery.

#### *1.1.4.3 Maternal Diabetes*

Several studies have shown that lactogenesis II is delayed in mothers with insulin dependent diabetes mellitus (IDDM) (Ferris, Dalidowitz *et al.* 1988; Arthur, Smith *et al.* 1989; Ferris, Neubauer *et al.* 1993; Neubauer, Ferris *et al.* 1993). Arthur *et al* used the

milk constituents, lactose and citrate, as markers of lactation to demonstrate a delay of 15 to 28 hours in women with IDDM (Arthur, Smith et al. 1989). These findings were confirmed by Neubauer *et al* (Neubauer, Ferris et al. 1993). This study of 33 women with IDDM and 33 matched controls found significantly lower lactose and higher nitrogen concentrations in milk on postnatal days 2, 3 and 7 and lower milk transfer on day 7 suggesting less effective lactation in the first postnatal week. This delay was more marked in women with less stable plasma glucose. The cause of this delay is unclear. The issue of metabolic control during lactation has not been defined, but episodes of hypoglycaemia may influence the ability to successfully initiate lactation.

IDDM also generally results in a high risk pregnancy with a higher risk of caesarean section delivery. Delivery, postnatal and neonatal management protocols tend to delay early breastfeeding events with fewer opportunities to breastfeed in the first postpartum day than non-diabetic women. Neonates of diabetic mothers also frequently have hypoglycaemia and are more likely to be offered supplementary feeds. Ferris *et al* demonstrated that women with IDDM spent less time with their term infant, the first breastfeed was later and frequency of breastfeeding was reduced in the first week regardless of mode of delivery (Ferris, Neubauer et al. 1993). The majority of infants of IDDM mothers were admitted to the neonatal nursery within the first day and this early separation was correlated significantly with the time the mothers perceived their breasts commencing to fill.

Compared with IDDM, there is a paucity of research about the effects of either Type 2 diabetes or gestational diabetes on lactation. A retrospective New Zealand study found that mothers with Type 2 diabetes were less likely to breastfeed than mothers with gestational diabetes (Simmons, Conroy *et al.* 2005). In this study mothers were more likely to be breastfeeding on discharge from hospital when the first feed was a breastfeed or if delivery was not by caesarean section.

It is interesting that even a short period of lactation has a beneficial effect on glucose metabolism in women who have gestational diabetes (Kjos, Henry *et al.* 1993; McManus, Cunningham *et al.* 2001). Moreover, longer duration of breastfeeding has been found reduce the incidence of Type 2 diabetes later in life in women who had gestational diabetes during pregnancy (Stuebe, Rich-Edwards *et al.* 2005).

#### *1.1.4.4 Maternal Obesity*

Several studies have suggested an association between maternal obesity and reduced initiation and duration of lactation (Hilson, Rasmussen *et al.* 1997; Rasmussen, Hilson *et al.* 2001; Lovelady 2005). In a study of 40 mothers where blood samples were taken before and after breastfeeds, mothers who were obese before the pregnancy were less likely to have increased prolactin concentration after breastfeeds at two and seven days postpartum than those of normal weight (Rasmussen & Kjolhede 2004). These results suggest a hormonal cause of the potential negative impact of maternal body mass index on lactogenesis II and breastfeeding duration. Other studies have shown sub-optimal breastfeeding behaviours and delayed lactogenesis II in mothers with raised BMI (Hilson, Rasmussen et al. 1997; Dewey, Nommsen-Rivers et al. 2003).

#### *1.1.4.5 Retained Products of Conception*

Delayed lactogenesis II resulting from placental fragments retained after birth was first reported in1981 (Neifert, McDonough et al. 1981). In this case series, breast engorgement indicating onset of lactation only commenced after surgical removal of the retained products of conception. The cause of this delay is hypothesised to be the continued secretion of progesterone by the placental fragments, which inhibits the onset of copious milk secretion.

#### *1.1.4.6 Stress in Labour and Delivery*

High levels of stress during labour and delivery have been found to be associated with delayed onset of lactation in some women (Chen, Nommsen-Rivers *et al.* 1998; Grajeda & Pérez-Escamilla 2002). In a study that measured salivary cortisol during labour and defined lactogenesis II according to women's perception, multiparous women with high salivary cortisol level had significantly delayed onset compared with multiparous women without high levels. In this study, primiparous women were more likely to have high antenatal and postnatal cortisol levels than multiparas. Furthermore compared to multiparas, primiparas who had an emergency caesarean delivery had later onset of lactation (Grajeda & Pérez-Escamilla 2002). Dewey and associates have also demonstrated an association between stress in the postnatal period and delayed perception of onset of lactation (Dewey 2001), however the authors qualified their findings by speculating about the causation of the postnatal stress and suggesting that delayed lactogenesis II could in fact be a causative factor.

#### *1.1.4.7 Delay in Commencement of Breast Emptying*

Early milk removal either by breastfeeding or expression is hypothesised to promote early onset of lactation although the evidence for this is inconsistent. A randomised controlled trial of women at risk of delayed lactogenesis II after caesarean section delivery found that early breast pumping was not effective in improving the volume of breastmilk produced in the first 72 hours postpartum (Chapman, Young *et al.* 2001). In addition early milk expression was thought to have a potential negative impact on breastfeeding duration in this study.

#### *1.1.4.8 Delayed Lactogenesis II and Lactation Failure*

A number of studies have shown a strong association between delayed onset of lactogenesis II or low supply in the first postnatal week and early unplanned cessation

of breastfeeding in term infants (Houston, Howie *et al.* 1983; Neville, M C, Keller *et al.* 1988; Chapman & Pérez-Escamilla 1999a; Ingram, Woolridge *et al.* 1999; Hruschka, Sellen *et al.* 2003; Sievers, Haase *et al.* 2003). These findings were unrelated to mothers' motivation to successfully breastfeed. The evidence is less clear for mothers of preterm infants.

## 1.2 Preterm birth

Up to 12% of pregnancies result in a preterm birth, defined as delivery before 37 completed weeks of pregnancy in women (Lye & Challis 2001; Iams 2003). Preterm birth is associated with up to 70% of all neonatal deaths and 75% of morbidity in the neonatal period (Lye & Challis 2001). Preterm infants are at significantly greater risk of respiratory disease, infections, necrotizing enterocolitis (NEC) and other complications that occur in the neonatal intensive care unit as well as neurological conditions leading to lifetime disabilities such as cerebral palsy, blindness and deafness, and learning and behavioural difficulties. Mother's own milk is the optimal nutrition for these vulnerable infants (Gartner, Morton et al. 2005). In addition to the health advantages that make human milk far superior to formulae for healthy term infants, mother's own milk offers specific advantages for preterm infants and is protective against many health problems of prematurity. Authorities now recommend feeding the preterm infant with mother's own breastmilk for as long as possible in order to reduce the impact of neonatal morbidities (Gartner, Morton et al. 2005). The rate of human milk feeding, however, remains significantly lower in neonatal intensive care units compared with term infants (Meier, Brown *et al.* 1998) and mothers of these vulnerable infants are highly likely to experience barriers to successful lactation including operative delivery, maternal-infant separation and delayed commencement of breast expression.

### 1.2.1 Specific benefits of human milk for preterm infants

#### *1.2.1.1 Gastrointestinal System*

The wide range of bioactive factors present in human milk modulates growth, development and function in the immature gastrointestinal system (Meier, Brown et al. 1998). Preterm infants tolerate human milk more readily than formulae and they have been shown to progress more rapidly to full enteral feeding when fed human milk (Simmer, Metcalf *et al.* 1997). Early feeding with human milk has been shown to be beneficial for preterm infants (Schulman, Schanler *et al.* 1998; Hirai, Ichiba *et al.* 2002). Digestion and absorption of nutrients may be compromised in preterm infants because of either immaturity or disease (King & Jones 2005). Studies of fetal intestinal cell cultures have shown that human milk contains multiple growth factors similar to those found in amniotic fluid and both fluids elicit a strong trophic effect on the immature cells (Hirai, Ichiba *et al.* 2002; Dvorak, Fituch *et al.* 2003). Human milk feeding has also been demonstrated to reduce the risk and severity of NEC (Lucas & Cole 1990; Schulman, Schanler *et al.* 1998).

#### *1.2.1.2 Protection from Infection*

Because of their immature immune systems and greater exposure to opportunistic organisms due to invasive procedures in the neonatal unit, preterm infants have a high risk of infection. For example, because of their premature birth, they receive very little maternal IgG, which is transferred across the placenta in the last trimester of pregnancy (Cowie, Forsyth et al. 1980). Human milk has long been known to have a protective effect against most infections (Howie, Forsyth *et al.* 1990; Raisler, Alexander *et al.* 1999; Schanler 2001; Gartner, Morton *et al.* 2005). Human milk contains a number of bioactive factors such as secretory IgA, lactoferrin, lysozyme, antioxidants, oligosaccharides and white cells, which promote the host defence of preterm infants

(Schanler 2001). These factors are either absent or present in very low concentrations in cow's milk-based formulae (King & Jones 2005) because they are host specific and/or inactivated by pasteurisation. Secretory IgA in particular resists digestion and therefore conveys immense local immunological protection in the gut (King & Jones 2005). In addition, when a mother has skin-to-skin contact with her infant in the neonatal unit, the enteromammary immune system produces antibodies targeted at the specific bacteria colonizing her own infant (King & Jones 2005).

#### *1.2.1.3 Neurodevelopmental Advantages*

Preterm infants are at risk of suboptimal brain growth and poor long-term neurological outcome. A number of studies have demonstrated that human milk feeding improves long-term cognitive development in very preterm infants, even after accounting for confounding due to maternal education and social status (Lucas, Morley *et al.* 1992; Horwood, Darlow *et al.* 2001; Quinn, O'Callaghan *et al.* 2001; Reynolds 2001). In addition, visual acuity is improved by reducing the risk and severity of retinopathy of prematurity [ROP, (Hylander, Strobino *et al.* 2001)].

#### *1.2.1.4 Maternal Attachment*

The birth of a preterm baby is a very stressful time for parents and often leads to considerable psychological distress (Singer, Salvator *et al.* 1999). Maternal anxiety and depression and long periods of separation can result in dysfunctional maternal-infant attachment. Expression of milk provides significant psychological benefits for mothers of preterm infants. Mothers who are expressing have the satisfaction of knowing their babies are receiving the best possible nutrition and they are more likely to feel that they are contributing significantly to the care of their infant in the neonatal unit (Kavanaugh, Meier *et al.* 1997). Moreover, the neuroendocrine hormone oxytocin, conveys potent physiological effects to the mother when it is released in a pulsatile fashion resulting in

milk ejection during either breastfeeds or breastmilk expression in women (Uvnäs-Moberg 1998). Oxytocin induces both short- and long-term "anti-stress" effects including reduced blood pressure and cortisol levels. Breastfeeding mothers are calmer and more interactive than mothers who are not breastfeeding, aiding maternal behaviour (Uvnäs-Moberg 1998). Similar calming effects and reduced corticosterone were found when female rats were infused with oxytocin (Windle, Shanks *et al.* 1997). This suggests that the release of oxytocin during breastmilk expression can potentially assist new mothers of preterm infants to cope with what is often a very stressful and distressing event.

#### *1.2.1.5 Nutritional Programming (Developmental Origins of Health and Adult Disease)*

It is now well established that fetal under-nutrition has the potential to cause life-long health problems by adversely programming systems such as the endocrine system and the hypothalamus-pituitary-adrenal (HPA) axis (Barker 1992; Gluckman 1997; Moss, T. J., Sloboda *et al.* 2001). In addition, postnatal catch-up growth has been implicated in the development of metabolic and cardiovascular diseases of adulthood (Eriksson, Forsén *et al.* 1999).

The effects of nutrition during early postnatal life have been less well studied although this is a time of rapid growth and development, and hence lactation is likely to be very important (de Moura & Passos 2005). We do not yet know if preterm delivery affects mammary development and secretion or how this may affect the relatively growth-restricted preterm infant.

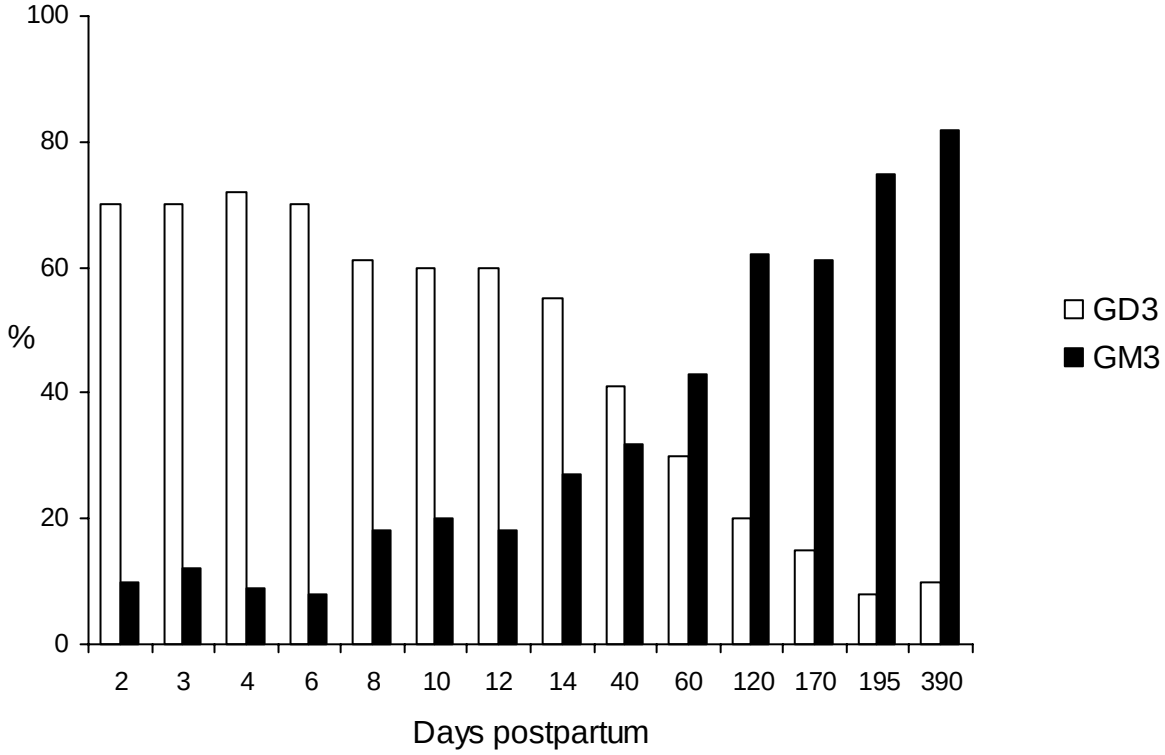
Although further study is indicated, breastfeeding has been found in some epidemiological studies to modify the risk of developing adult onset metabolic diseases. For example, feeding with mother's milk is hypothesised to reduce the risk of

predisposed development of cardiovascular disease (Jackson 2000), and non-insulin-dependent diabetes mellitus (Pettitt, Forman *et al.* 1997). Animal studies have demonstrated that maternal nutrition during lactation can influence heavier body weight in their offspring in adulthood (de Moura & Passos 2005). To date, however, the evidence in humans is less clear. Breastfeeding was shown to be associated with a reduced risk of being overweight in children aged 12 months in a large Australian prospective study (Burke, Beilin *et al.* 2005). That study also found being breastfed for less than four months conveyed the greatest risk for being overweight up to eight years of age but this was modified by other maternal risk factors for childhood obesity suggesting that familial factors had a stronger effect on the weight of children than early nutrition (Burke, Beilin et al. 2005). A recent meta-analysis of observational studies of the effects of breastfeeding on body mass index (BMI) in adulthood has shown a small reduction in mean BMI, but suggested that publication bias and confounding factors strongly influenced these findings (Owen, Martin *et al.* 2005). The precise variance in BMI that can be explained by breastfeeding is still to be determined.

Mammary development plays an important role in the growth and development of the neonatal offspring. An elegant study cross-fostered both offspring of control rats onto spontaneously hypertensive rat (SHR) dams as well as *in utero* growth-restricted rat pups on to control rats and measured pup weights six days after parturition (Wlodek, Westcott *et al.* 2003). The study found that the weight of the growth restricted pups suckling on control dams was increased in comparison with growth restricted suckling on SHR dams. Conversely the weight of normally developed pups was decreased when suckling on the SHR dam suggesting that mammary development is impaired and this is responsible for significant decreases in appropriate nutrients for the growth-restricted neonate.

In some mammals the stage of lactation is crucial and there are potential "developmental clock" factors present in milk which switch on stages of development (Nicholas 1988; Trott, Simpson *et al.* 2003). In the tammar wallaby it has been shown that milk composition and development of pouch young is regulated by the mother and not by the suckling young (Trott, Simpson et al. 2003). In this study, in which 60-day joeys were back-fostered repeatedly onto one group of host mothers to allow the lactational stage to progress 42 days ahead of the age of the pouch young, the rate of growth and development of the fostered pouch young was significantly increased compared with control pouch young. The fostered joeys in this study ingested milk with higher energy content and different composition than normal pouch young at their age (Trott, Simpson et al. 2003). These findings demonstrate that, in marsupials, the time interval after parturition and not the developmental age of the young determines the composition of milk. If this is the case in humans this suggests that preterm young may not receive the appropriate nutrients for their developmental stage in their mothers' milk. Although macronutrients in species such as either humans or sheep do not change as dramatically as marsupials, there are major changes in some micronutrients (Hurrel, Berrocal *et al.* 1989; Minda, Kovács *et al.* 2004). For example it has been demonstrated that the proportions of two major lipids important in brain development (gangliosides $GD_3$, $GM_3$) change dramatically with stage of lactation (Figure 1.5 (Hurrel, Berrocal et al. 1989)). It should be noted, however, that the fatty acid composition of most infant formulae is vastly different from that of human milk at any stage of lactation (Minda, Kovács et al. 2004).

**Figure 1.5 Concentration Of GD3 And GM3 Gangliosides In Human Milk As % Of Total Lipid-Bound Sialic Acid At Various Days Of Lactation**




Note: Adapted from (Hurrel, Berrocal et al. 1989).

There is evidence that there are variations in human milk levels of leptin, which is a protein that regulates body functions such as body weight, food intake and satiety, relating to birth weight (Dundar, Anal *et al.* 2005). In this study, infants who were born small for gestational age (SGA) had significantly lower leptin levels in their mothers' milk in the first fortnight of life than infants born either average or large for gestational age. Moreover SGA infants gained more weight in the first fortnight. These findings suggest that leptin in breastmilk plays a role in appetite, regulation of nutrition and growth in the early lactation period and that production of milk leptin is regulated according to the infant's state. It has also been suggested that high plasma leptin concentration during critical periods of growth results, in adult life, in leptin resistance

in conjunction with higher food intake and body weight (de Moura & Passos 2005; Desai, Gayle *et al.* 2005).

### 1.2.2 Preterm milk composition

A large number of studies have demonstrated that milk from mothers delivering preterm has a different nutrient composition when compared with milk from term mothers at similar stages of lactation (Atkinson, S 1995). Preterm milk has greater concentrations of proteins including immune proteins, lipids, energy and some vitamins and minerals. However, there is a greater variability in milk composition between individual preterm mothers (Cregan, De Mello *et al.* 2002), and the considerable heterogeneity between individual studies suggests further research is needed (Atkinson, S 1995). Atkinson posited a number of possible reasons for differences observed in nutrient composition of preterm milk including immature development of mammary glandular tissue; different hormonal environment associated with preterm delivery; artificial expression involving different patterns of nipple stimulation compared with breastfeeding such as pumping frequency and suction; and greater maternal anxiety disrupting oxytocin secretion and milk ejection; and perinatal drugs such as glucocorticoids (Atkinson, S 1995).

### 1.2.3 Barriers to successful lactation in mothers of preterm babies

Despite the undoubted advantages of human milk, a range of studies in different countries and cultures has shown that fewer mothers of preterm infants initiate breastfeeding and that duration of breastfeeding is shorter compared with mothers of healthy term infants (Meier, Brown *et al.* 1998; Flacking, Nyqvist *et al.* 2003). A Swedish study of low birth weight infants found that while 93% were breastfeeding on discharge from hospital only 36% were still breastfeeding at six months compared with 75% of term infants (Flacking, Nyqvist et al. 2003). In an American prospective study of 87 mothers who intended to breastfeed their infants with gestational age < 33 weeks,

only 34% were still providing breastmilk when their infants reached the equivalent of 40 weeks' gestational age (Furman, Minich *et al.* 1998). A recent American study of preterm infants (≤34 weeks' gestational age) born in a rural hospital found only half had received human milk at some stage in their hospital admission (Espy & Senn 2003). Earlier Australian studies have also found low rates, only 64%, 44% and 23% were receiving some human milk on discharge, at three months and six months respectively (Yu, Jamieson *et al.* 1981; Yip, Lee *et al.* 1996). A further US study found that the median duration of breastmilk feeding was only 36 days in mothers of very low birthweight (VLBW, <1500g) infants compared with 112 days in mothers of term infants (Killersreiter, Grimmer *et al.* 2001). Elsewhere, the evidence is contradictory, showing that mothers of low-birth-weight infants have a similar potential to breastfeed as mothers of term infants (Lefebvre & Ducharme 1989). This study, however, included breastfeeding patterns of preterm infants with term infants of low birth weight who were more likely to be breastfed from birth.

Either actual breastmilk production problems or maternal perception of inadequate supply is the most frequent concern expressed by mothers of preterm infants (Hill, Hanson *et al.* 1994; Kavanaugh, Mead *et al.* 1995). Hill *et al.* showed that mothers expressing for their preterm infants were almost 3 times as likely to have insufficient supply to meet their infants' needs in the first 6 weeks postpartum compared with mothers of term infants (Hill, Aldag *et al.* 2005). These findings were consistent with an Australian study, which found that mothers expressing for their preterm infants produced lower volumes of milk at day 5 postpartum than mothers of term infants (Cregan, De Mello et al. 2002). An earlier American study of mothers who delivered between 28 to 30 weeks' gestation found that mothers who delayed the onset of pumping milk after birth were more likely to have inadequate milk production (Hopkinson, Schanler *et al.* 1988). Although early initiation of breastfeeding within a

few hours of birth is now recommended universally for mothers of term infants (WHO 1998), preterm mothers frequently delay initiation of pumping by up to 24 hours after the birth (Jaeger, Lawson *et al.* 1997). Hopkinson *et al.* also found that the frequency of pumping less than 5 times per day was associated with reduced milk supply (Hopkinson, Schanler et al. 1988). The importance of maintaining a pumping frequency greater than 5 times per day to enable adequate milk supply has been confirmed in several small studies (de Carvalho, Anderson *et al.* 1985; Hill, Aldag *et al.* 2001).

## 1.3 Obstetric Management of suspected preterm birth

Current obstetric management is unable to prevent preterm delivery with most therapy options aiming only to delay delivery with tocolytic therapy until conditions allow the least risk of preterm delivery, for example transfer to a tertiary referral hospital with neonatal intensive care facilities and maternal treatment with glucocorticoids (Creasy 1991; Lye & Challis 2001).

### 1.3.1 Glucocorticoids and Parturition

In normal pregnancy endogenous glucocorticoid levels rise to a peak close to the onset of parturition. There is strong evidence that, in most species including sheep and humans, the fetal HPA axis is the central mechanism driving this increase (Challis, Matthews et al. 2000). Infusion of glucocorticoids to fetal sheep results in subsequent premature parturition and, conversely, reduction in fetal glucocorticoid concentration caused by ablation of either the fetal pituitary gland or the fetal adrenal gland results in prolonged gestation (Challis, Matthews et al. 2000). Furthermore, the fetal HPA axis is also sufficient to support lactogenesis II in the absence of maternal glucocorticoids. Rats, which were delivered by caesarean section, and maternal ovariectomy and adrenalectomy was performed, continued to secrete the same level of lactose in mammary tissue as rats which were not adrenalectomized suggesting that lactogenesis II

is not reduced in the absence of maternal glucocorticoids, but is supported by fetal glucocorticoid release (Nicholas & Hartmann 1981).

It has long been known that administration of exogenous glucocorticoids such as either dexamethasone or betamethasone to pregnant sheep results in premature parturition but this effect is reduced when the progesterone analogue medroxyprogesterone acetate (MPA) is given concurrently (Nathanielsz, Buster et al. 1988). In women, however, the administration of exogenous glucocorticoids does not lead to premature labour and MPA is not required.

The surge of cortisol at the end of pregnancy is vital for the maturation of fetal organ systems such as the lungs and kidneys as well as for brain development and programming. In addition raised cortisol levels activate altered steroid genesis in the placenta, leading in sheep to a fall in progesterone secretion and subsequent increase in prostaglandin synthesis which drives the onset of parturition (Challis, Matthews et al. 2000). In humans, the withdrawal of progesterone does not occur until after birth; however, raised cortisol at the end of pregnancy also triggers prostaglandin mediated parturition through other mechanisms (Challis, Matthews et al. 2000).

### 1.3.2 Antenatal Glucocorticoid Treatment

Since Liggins' first report in 1969 of effective respiration in lambs born prematurely after maternal administration of glucocorticoids (also known as corticosteroids) (Liggins 1969), at least 18 randomised controlled trials have been conducted supporting the current clinical practice of administering a single course of either betamethasone or dexamethasone to women at risk of preterm delivery (Liggins & Howie 1972; Crowley 2003).

In Australia women are treated with a course of two maternal intramuscular injections of betamethasone 11.4mg 24 hours apart when preterm delivery is anticipated. There is

strong evidence that antenatal glucocorticoid treatment accelerates fetal lung maturation leading to reduced neonatal mortality and morbidity due to respiratory distress syndrome (RDS) and intraventricular haemorrhage (IVH), providing the interval from treatment to delivery is between 24 hours and 7 days (NIH Consensus Conference 1995; Crowley 2003). The Cochrane Review of 18 trials of prophylactic glucocorticoids for preterm birth found a significant reduction of mortality (OR 0.60, 95% CI 0.48-0.63) and RDS (0.53, 0.44-0.63) in neonates born before 34 weeks' gestation; and no adverse consequences were detected (Crowley 2003). Although the initial dosage regimens for current practice were determined arbitrarily, studies have shown that the treatment protocol results in concentrations of maximum benefit to the fetus (NIH Consensus Conference 1995).

There is less evidence, however, that the neonatal benefits of glucocorticoid treatment remain present when the interval between treatment and delivery is greater than 7 days (Crowley 2003; McLaughlin, K J, Crowther *et al.* 2003). Moreover, a systematic review of seven trials in which delivery occurred more than seven days after treatment found not only no reduction in risk of RDS but also a doubling in the risk of perinatal mortality (McLaughlin, K J, Crowther *et al.* 2003).

There is accumulating evidence from animal and human trials that multiple antenatal courses of glucocorticoids lead to increased morbidity and mortality (National Institutes of Health Consensus Development Panel 2001). At least 20% of women who receive antenatal glucocorticoids do not eventually deliver early but, for many, the risk of preterm delivery either remains or recurs (Quinlivan, Evans *et al.* 1998; McLaughlin, K J, Crowther *et al.* 2002; Moss, T J M, Harding *et al.* 2002). As a consequence many practitioners prescribe repeat courses of glucocorticoids to these women despite recent concerns about potential adverse fetal effects (National Institutes of Health Consensus Development Panel 2001; McLaughlin, Kristin J & Crowther 2003) Animal studies

have demonstrated a wide range of deleterious effects of multiple courses on fetal growth and organogenesis (Ikegami, Jobe *et al.* 1997; Quinlivan, Archer *et al.* 1998; Huang, W. L., Beazley *et al.* 1999; Quinlivan, Archer *et al.* 2000; Sloboda, Newnham *et al.* 2000; Huang, W. L., Harper *et al.* 2001), and this has been supported in humans by retrospective studies of neonates after multiple courses (French, N. P., Hagan *et al.* 1999; French, N P, Hagan *et al.* 2004). Although adverse effects of multiple courses on humans have yet to be confirmed, a number of randomised controlled trials are currently underway to examine this question (Crowther, Haslam *et al.* 2006). In the largest trial to be published to date, Crowther *et al.* found a lowering of risk of both respiratory distress syndrome and severe lung disease. Infant weight and head circumference were reduced at birth in infants whose mothers received more than one course of betamethasone but this difference did not persist until hospital discharge (Crowther, Haslam et al. 2006). In contrast a recent small trial, which was not randomised, found altered cardiovascular status in the first week after birth in infants exposed to multiple courses of antenatal glucocorticoids (Mildenhall, Battin *et al.* 2006). Meanwhile current best practice is for a single course only of glucocorticoids for all women for whom delivery is anticipated before 34 weeks of pregnancy (National Institutes of Health Consensus Development Panel 2001).

### 1.3.3 Impact of Antenatal Glucocorticoid Treatment on Lactation

Despite the large body of evidence concerning the risks and benefits to the fetus of antenatal glucocorticoid treatment no published studies have investigated possible effects on maternal lactation. Pilot data from our laboratory have indicated that administration of antenatal steroids to the pregnant ewe may influence the timing of lactogenesis II. A study of pregnant ewes administered betamethasone or saline placebo at 104, 111 and 118 days' gestation showed a significant increase in plasma lactose

levels at 125 days in the betamethasone-treated ewes but not in control sheep. In that study, mammary glands were observed to be full and leaking shortly after administration of betamethasone (Personal communication T. Moss). These findings indicate that a precocious initiation of lactation in the betamethasone-treated group may have occurred. This pilot study also found a high rate of neonatal mortality in the repeated betamethasone-treated sheep after subsequent delivery at term (Moss, T. J., Sloboda *et al.* 2001). The finding of increased postnatal loss was attributed to a reduction in maternal milk supply suggesting that multiple antenatal glucocorticoid treatment may have an adverse effect on initiation of lactation after parturition at term. Whereas glucocorticoids play an important role in the development and differentiation of mammary tissues, the lactogenic effect of exogenous glucocorticoid treatment in pregnancy before parturition, for example when preterm birth is anticipated, is unknown. The onset of lactogenesis II requires well-developed lactocytes as well as sufficient circulating levels of prolactin and glucocorticoids and a withdrawal of progesterone. In view of the reduction in progesterone concentration following the peak in endogenous glucocorticoids at the end of normal pregnancy, I hypothesised that the conditions for onset of lactogenesis II could be met artificially after maternal administration of glucocorticoids in the second half of pregnancy and before parturition. The current high frequency of antenatal glucocorticoid treatment, the potential for this treatment to influence lactogenesis II and the importance of mother's own milk for the growth and development of the preterm infant all suggested to me that it is timely that an examination of the impact of antenatal glucocorticoid treatment on lactation be undertaken.

## 1.4 Study Aims

I aimed to examine the effects of antenatal glucocorticoid treatment on lactation during pregnancy and also after parturition in both sheep and women. I examined glucocorticoid treatment in sheep because the vast majority of research of the effects of glucocorticoids on the fetus has been performed using the sheep model. In light of the differences in progesterone between sheep and women it was essential to compare the response to glucocorticoid treatment of both species in order to determine the relevance of any sheep findings to women who received antenatal glucocorticoid prophylaxis.

### 1.4.1 Study Hypotheses

Specific hypotheses were as follows:

1. That maternal administration of glucocorticoids interrupts the progesterone inhibition of lactation during pregnancy.
2. That antenatal glucocorticoids cause a precocious onset of lactogenesis II prior to parturition in the event that preterm birth does not ensue.
3. That the normal initiation of lactation after parturition is compromised.
4. That this response occurs at all stages of gestation within the second half of pregnancy.

# CHAPTER 2 MATERIAL AND METHODS

## 2.1 Materials

### 2.1.1 Enzymes

All enzymes required for laboratory research methods used throughout this thesis are listed in Table 2.1. Enzymes supplied lyophilised were solubilised with buffer prior to use. Enzymes supplied as suspensions were used without further treatment.

**Table 2.1 Source of enzymes used for metabolite analysis**

| Enzyme | Source | Abbreviation | E.C. Number |
|---|---|---|---|
| **Roche Diagnostics, Mannheim, Germany** | | | |
| β-Galactosidase | Escherichia Coli | | 3.2.1.23 |
| β-Galactose dehydrogenase | Escherichia Coli | | |
| Citrate lyase | Aerobacter aerogenes | | 4.1.3.6 |
| Malate dehydrogenase | Pig heart | MDH | 1.1.1.37 |
| **Sigma Chemical Co, St Louis, Missouri, USA** | | | |
| Bacterial Luciferase | Vibrio harveyi | | 1.14.14.3 |
| Glucose Oxidase | Aspergillus niger | GOD | 1.1.3.4 |
| Peroxidase | Horseradish | POD | 1.11.1.7 |

### 2.1.2 Chemicals

All chemicals required for the laboratory research methods are listed in Table 2.2. The chemicals used were of analytical grade requiring no further purification. Solutions were prepared in double deionised water (DDI).

**Table 2.2 Chemicals used for laboratory research methods**

| Chemical | Abbreviation |
|---|---|
| **Merck Pty Ltd, Melbourne, Vic, Australia** | |
| Ammonium sulphate | $(NH_4)_2SO_4$ |
| 2,2'-azino-di-(3-ethyl-benzthiazolinsulphonate-6-sulphonate | ABTS |
| Bromo-cresol purple | |
| di-potassium hydrogen orthophosphate 3-hydrate | $K_2HPO_4$ |
| Perchloric acid (70% pure) | $HClO_4$ |
| Potassium dihydrogen orthophosphate | $KH_2PO_4$ |
| Potassium hydroxide | KOH |
| Sodium azide | $NaN_3$ |
| Sodium hydroxide | NaOH |
| Zinc Sulphate | $ZnSO_4$ |
| **Boehringer Mannheim Australia, North Ryde, NSW, Australia** | |
| 2,2'-azino-di-(3-ethyl-benzthiazolinsulphonate)-6-sulphonate | ABTS |
| **Sigma Chemical Co, St Louis, Missouri, USA** | |
| Bovine Serum albumin | BSA |
| Lactose | |
| Magnesium Chloride | $MgCl_2$ |
| β-Nicotinamide adenine dinucleotide | $NAD^+$ |
| Reduced β-Nicotinamide adenine dinucleotide (disodium salt) | NADH |
| Triethanolamine hydrochloride | Tri.HCl |

## 2.2 Sample collection

### 2.2.1 Ovine plasma

Blood samples (10 mL) were collected into heparinised tubes by venipuncture of the jugular vein. The blood was centrifuged for 10 minutes at 3,000 rpm (Beckman Allegra™ 21R centrifuge), and plasma was separated and kept on ice for a maximum of 2 hours before transportation and storage at -20°C.

### 2.2.2 Ovine milk

Samples of mammary secretions (5 mL) were collected by manual expression daily for 5 days after parturition. Samples were collected in the morning (09:00) and the time between suckling of the lamb and milk expression was random. The milk was collected in polypropylene vials (Disposable Products Pty Ltd, Adelaide, SA, Australia), kept cool for a maximum of 2 hours during transport before storing at -20°C. Whole milk samples were thawed at 37°C, mixed and pipetted into microcentrifuge tubes (200 μL) then centrifuged for 5 minutes at 10,000 *g* (Beckman Microcentrifuge II, Beckman, Woodville, SA, Australia). Fat was removed by slicing the tube below the level of the fat plug. The defatted milk was either assayed immediately or stored at -20°C until analysis.

### 2.2.3 Human urine

Urine samples were collected over a 24-hour period. At the commencement of the collection period the participant voided her bladder and recorded the time. The volume of each subsequent void was recorded and a 5mL aliquot was collected in a polypropylene tube and stored at -20°C. A 24-hour collection was completed at the same time the next day as the commencement of the collection period on the previous day. Samples collected in the hospital were kept on ice on the ward for a maximum of

12 hours until collection and storage at -20°C. Samples collected at home were initially stored in a household freezer and then transported to the laboratory on ice where they were stored at -20°C for analysis.

A 24-hour composite void was constructed by using relative proportions of the volume of each void to construct a sample representative of the total urinary output. If the volume was not recorded for one void, the average volume in that 24-hour period was used as the missing volume. If one urine sample was missed a representative sample was taken from the void before the missing void, using the average volume to determine the required sample volume if the volume was not recorded as well. If more than one urine sample was missed or volume was not recorded in a 24-hour period then all samples for that day were excluded.

### 2.2.4 Human milk

Milk samples (≤ 1 mL) were collected daily into 5 mL polypropylene vials. Women who were expressing for preterm infants collected mixed milk samples using a commercial electric breast pump (Medela Lactina or Medela Symphony, Medela AG, Baar, Switzerland). Women were advised to collect a sample at the first expression of the morning but this did not always occur at this time. Breastfeeding women collected milk by hand expression of one breast directly into the vial after the first feed of the day. Samples collected in the hospital were refrigerated for a maximum of 5 hours until collection and storage at -20°C. Samples collected at home were initially stored in a household freezer and then transported to the laboratory on ice where they were stored at -20°C for analysis.

## 2.3 Metabolites in Plasma

### 2.3.1 Progesterone

The concentration of progesterone in sheep plasma was measured in duplicate using a double-antibody radioimmunoassay based on the method of Gales *et al*. (Gales, Williamson *et al.* 1997). The progesterone radioimmunoassay was undertaken by me with technical assistance by Margaret Blackberry, School of Animal Science, The University of Western Australia, using the method described below.

Duplicates (100μL) of plasma samples were extracted in 12mm x 75mm glass tubes by adding 2 mL distilled hexane and vortexing for 2 minutes. The aqueous phase was frozen in an acetone-dry ice bath and the solvent layer was removed to clean tubes and dried under a stream of compressed air. Triplicates of standards (0.125 to 32.0 nM) were diluted with hexane and dried using the same process. The samples and standards were reconstituted in 0.1M phosphate-buffered saline containing 0.1% (w/v) bovine serum albumin (BSA) and the antibody (200μL, diluted 1:12,000) was added, the tubes mixed and incubated at 4°C overnight. The tracer (100μL, Amersham Pharmacia Biotech) containing 20,000 d.p.m 1,2,6,7-$^{3}$[H]progesterone in buffer (PBS) containing 1:500 Normal Rabbit Serum and 0.1% BSA was added to the tubes, which were then mixed gently by hand and incubated at 4°C overnight. The second antibody (100μL, donkey serum diluted 1:7 in PBS) was then added to the tubes which were then mixed and incubated at 4°C for a further 24 hours. Polyethylene Glycol 2% in PBS (1mL) was added to all tubes and the tubes were centrifuged at 3,000 *g* for 25 minutes at 4°C. The supernatant was aspirated and the pellet was redissolved in 0.5mL 0.05M HCl. The solution was then dispensed into counting vials with 4mL toluene-based scintillant and counted in a liquid scintillation counter.

The antibody was raised in a rabbit against progesterone-11α-carboxymethyloxime-human serum albumin. The major cross-reactions were with deoxycorticosterone (2%w/v) and 20α-OH progesterone, 17β-OH progesterone, 17α-OH progesterone and allopregnenolone (all < 1%). All samples were processed in a single assay and three pooled sheep plasma samples were used as quality controls. The value and intra-assay coefficients of variation of quality controls (CV %) were respectively 3.70ng/mL (5%), 1.55ng/mL (6.9%), and 0.69ng/mL (6.6%). Binding at zero was 33.4%, non-specific binding was 3.5% and the minimum detectable limit was 0.13ng/mL.

### 2.3.2 Cortisol

Plasma cortisol concentration was measured using a GammaCoat™ Cortisol $^{125}$I RIA Kit (DiaSorin, Stillwater, Mn, USA) (Kerzner, Stonestreet *et al.* 2002). Duplicates of standards and samples were added to antibody-coated tubes (Rabbit Anti-Cortisol Serum Coated Tubes). Following incubation with cortisol $^{125}$I tracer at 37°C for 45 minutes, the contents of each tube were decanted and tubes were left to dry overnight. After 24 hours, the tubes were counted on a gamma counter. The anti-serum had 100% cross-reactivity to cortisol, 0.2% to dexamethasone and < 0.1% cross-reactivity to progesterone. The mean intra-assay and inter-assay coefficients of variation were 5.8% and 3.7% respectively.

### 2.3.3 Prolactin

The prolactin radioimmunoassay was undertaken by Margaret Blackberry, School of Animal Science, The University of Western Australia, using the method described below.

Plasma concentrations of prolactin were assayed in duplicate by double-antibody homologous RIA as previously described (Miller, Blache *et al.* 1995). The antiserum was donated by Mr J. A. Avenell (CSIRO Division of Animal Production, Prospect,

Australia). Antiserum (R 160), was raised in rabbits, and was diluted to 1:400,000 in buffer. Cross reactions of less than 1% were found with oFSH (NIDDK-I-1), oLH (NIDDK-23), oTSH (AFP-4017C), oGH (AFP-5285C) and hACTH (L61-14). Prolactin standard provided by NIADDK (oPRL-I-2 reference # AFP-7150B) was used. The stock solution (250 ng/mL) was stored in buffer at -20˚C. On the day of assay, an aliquot was thawed and diluted serially to the following concentrations: 0.49, 0.98, 1.95, 3.9, 7.8, 15.61, 31.25, 62.5 and 125 ng/ml in buffer.

*Iodination*

The hormone was iodinated with $NaI^{125}$ using the Chloramine-T method and purified with a G100 Sephadex column. The following reagents were placed in a reaction vessel:

5 µg prolactin in 10 µl of 0.05M phosphate buffer;

50 µl of 0.5M phosphate buffer;

0.5 mCi $NaI^{125}$ in 5 µl;

5 µg Chloramine-T in 5 µl of 0.05M phosphate buffer.

The reagents were mixed for 30 seconds and the reaction was stopped by adding 2 µg sodium metabisulphite in 10 µl of 0.05M phosphate buffer. The reagents were mixed and 250 µl of KI in 0.05M phosphate buffer and bovine serum albumen (BSA) was added and the mixture was then transferred onto a 30 x 1 cm column of Sephadex G100. The reaction vessel was rinsed with 250 µl KI which was then added to the column. Finally, the column was eluted with 30 ml of buffer, and fractions were collected in 13 x 100 mm glass culture tubes using a fraction collector. The activity of the fractions was determined on a gamma counter, and the fraction after the protein peak was tested for non-specific binding (NSB) and $B_o$. The fraction was diluted with buffer to give a stock solution containing 250 000 cpm/50 µl and stored at -20˚C. On Day 1 of the assay, the tracer was thawed and diluted 1:25 in buffer to give 10 000 cpm/50 µl.

*Assay procedure*

On the first day, plasma samples and standards (10 µl) were diluted to 400 µl with buffer in 12 x 75 mm plastic assay tubes and 50 µl of antiserum was added to all tubes except the NSB's and vortexed for 5 seconds. After 2 hours incubation at room temperature, 50 µl of tracer was added to all tubes and vortexed for 5 seconds. The tubes were left overnight at 4°C to incubate, and on the following day, 100 µl of the second antibody (donkey-anti-rabbit serum, 1:20 in buffer) was added, vortexed and left overnight at 4°C to incubate. On the third day, 1.5 ml of buffer was added to all tubes (except total counts) and centrifuged at 1500g for 30 minutes. The supernatant was decanted off and the activity of the precipitate was determined on a gamma counter.

#### *2.3.3.1 Validation*

The limit of detection was 0.12 ng/ml. Binding at zero was 47.5%. Non-specific binding was 1.6%. The assay included 6 replicates of 3 control samples containing 9.75, 4.95 and 1.11 ng/ml which were used to estimate the intra-assay coefficients of variation – 4%, 2.5% and 4.6%.

### 2.3.4 Ovine Plasma Lactose

The concentration of lactose in ovine plasma is normally very low, necessitating the use of a very sensitive assay. A luciferase-linked reaction which allows substantial signal amplification was selected in order to measure these low lactose levels. The bioluminescence technique of Arthur *et al.* (Arthur, Kent *et al.* 1989) was the method used.

#### *2.3.4.1 Assay Principle*

$$\text{Lactose} + H_2O \xrightarrow{\beta\text{–galactosidase}} \text{galactose} + \text{glucose}$$

$$\text{Galactose} + NAD^+ \xrightarrow{\beta\text{–Galactose dehydrogenase}} \beta\text{-galactonolactone} + NADH + H^+$$

$$2NADH + FMN \xrightarrow{\text{Oxidoreductase}} 2NAD^+ + FMNH_2$$

$$FMNH_2 + \text{R-CHO} + O_2 \xrightarrow{\text{Luciferase}} FMN + \text{R-COOH} + \text{Light}$$

Lactose was hydrolysed with β-galactosidase to galactose and glucose and the galactose was assayed by producing NADH with the aid of the enzyme β-galactose dehydrogenase. NaOH solution was then used to stop the reaction and stabilize NADH, which was then measured on a plate luminometer using a luciferase reagent.

#### *2.3.4.2 Reagents*

1. *Lactase reagent:*

   1.9 mM $MgCl_2$

   85 mM Potassium phosphate buffer ($KH_2PO_4$, pH 7.2)

   6.3 U/mL β-galactosidase

2. *Galactose reagent:*

   1.9 mM $MgCl_2$

   85 mM Potassium phosphate buffer ($KH_2PO_4$, pH 7.2)

   $NAD^+$ 2.9mM

   1.2 U/mL β-galactose dehydrogenase

3. *Luciferase reagent (prepared in advance and stored at -80°C:*

   100 mM $KH_2PO_4$

   4 mM EDTA.Na

   1.6% w/v BSA

   300 mM TES

   8 μM FMN

   400 μg/mL Luciferase

   0.1% w/v Tetradecanal

#### *2.3.4.3 Procedure*

Plasma samples (20 μL) were deproteinised by perchloric acid (1.0 M, 80 μL) and neutralised by a KOH (2.0 M)/phosphate buffer (0.5 M $K_2HPO_4.3H_2O/KH_2PO_4$, pH 7.2) solution (17.5 μL). Lactose standards (10 μM to 125 μM) were diluted to the same levels. Duplicates of samples and standards (30 μL) were plated on a 96 well plate, lactase reagent (60 μL) added to each well and incubated for 60 minutes at 37°C. The samples and standards were incubated for a further 60 minutes at 37°C with 30 μL of the galactose assay reagent. Aliquots (20 μL) were transferred to a Microlite plate and 100 μL of NaOH solution (10mM) was added to each well. Luciferase reagent, (previously prepared and frozen at -80°C) was thawed in ice and 15 μL was added before the light output was measured on a plate luminometer (Dynatech Laboratories,

Microlite ML2250 Microtiter Plate Luminometer). I did not correct for any galactose present in the plasma as the concentration was consistently below 2 μM.

#### *2.3.4.4 Validation*

Intra- and inter-assay coefficients of variation were 6% and 10% and the detection limit was 1.62 μM.

## 2.4 Metabolites in urine

### 2.4.1 Progesterone

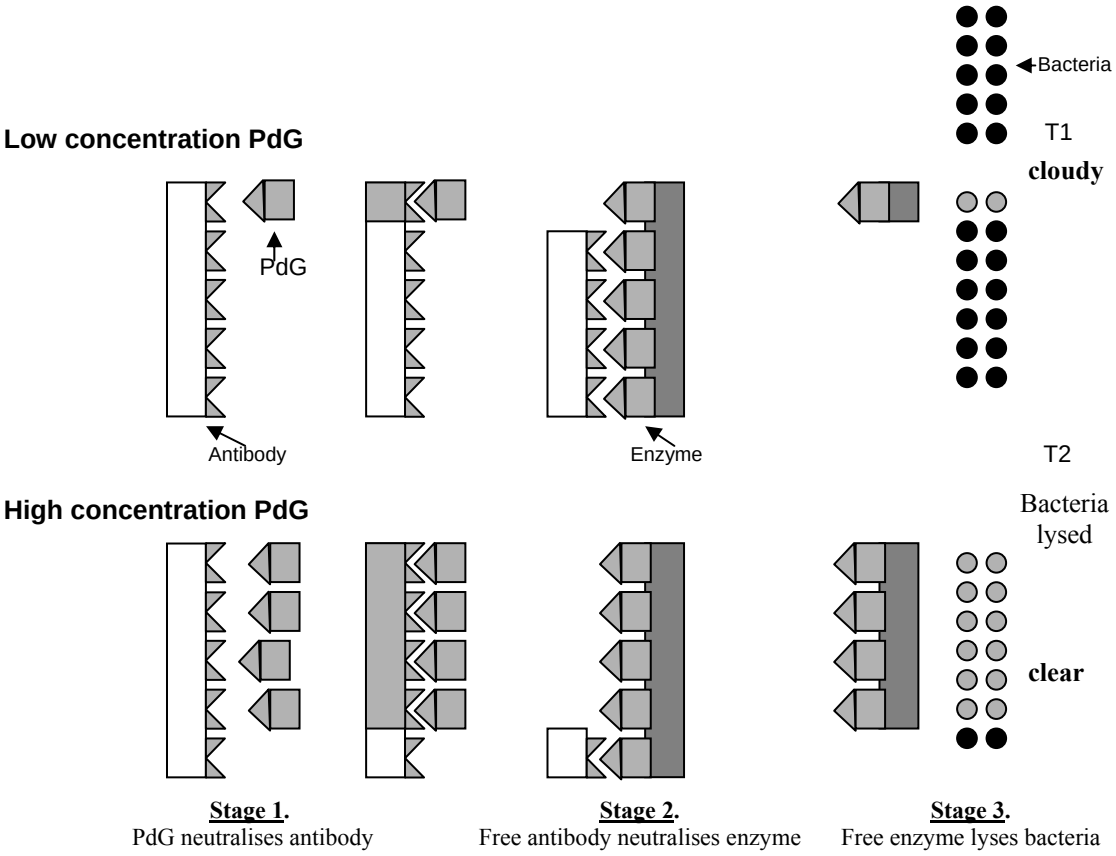
Pregnanediol-glucuronide (PdG) is the principal metabolite of progesterone present in human urine and PdG concentration has been shown to correlate well with plasma progesterone concentration when used either for monitoring treatment for infertility (Sauer & Paulson 1991; Stanczyk, Gentzschein *et al.* 1997) or for avoidance of pregnancy (Brown, Blackwell *et al.* 1989; Brown, Holmes *et al.* 1991). The concentration of PdG in urine was measured by an enzyme linked immunoassay using the Home Ovarian Monitor (St Michael NFP Services, Macleod, Victoria, Australia) and the method of Brown *et al.* (Brown, Blackwell *et al.* 1988; Blackwell, Brown *et al.* 2003). The assay was adapted for multiple samples using the method of Hughes (Hughes 1996).

Manufactured assay tubes were purchased from St Michael NFP Services; each containing a known amount of antibody to PdG in the bottom of the tube, a PdG enzyme-conjugate on the side of the tube, and a micrococcus spot at the top of the tube. Each sample or standard was assayed in the one tube in 3 stages (Figure 2.1):

1. PdG in the urine neutralised some of the antibody.
2. The remaining antibody neutralised some of the PdG-enzyme-conjugate.

3. The remaining PdG-enzyme-conjugate caused lysis of the cloudy micrococcus suspension. The concentration of PdG in the urine is directly proportional to the rate of clearing of the bacteria, which was measured as a change in transmittance on the Home Ovarian Monitor.

**Figure 2.1 Principle of Ovarian Monitor Enzyme-linked Assay**




### *2.4.1.1 Procedure*

Assay tubes were stored at room temperature in a desiccator. Before use the tubes were checked to ensure integrity of the micrococcus spot, the enzyme and the mixing bead; and the monitor and heating block were preheated to 40°C. Samples were first diluted

so that the volume was equivalent to 150 mL/hr. Then samples were further diluted to 1:50 in order for the transmittance to be within the range that could be read on the monitor.

After allowing the assay tubes to warm on the heating block, standards and diluted samples (50μL) were added and the assay tubes were gently mixed for 20 seconds so that the mixing bead was free. Double deionised water (DDI) (300μL) was then added to each tube; tubes were gently shaken for 30 seconds to release the conjugate spot and then incubated on the heating block for 10 to 15 minutes. Assay tubes were then laterally shaken to release the micrococci and initial transmittance was measured. Tubes were then incubated at 40°C for exactly 5 minutes before the final transmittance was measured. The change in transmittance (ΔT) was then converted to the PdG concentration by log transforming ΔT and transforming against a standard quadratic curve with appropriate adjustment for dilution.

#### *2.4.1.2 Validation*

Intra- and inter-assay coefficients of variation were 6.6% and 9.5% respectively. The recovery of a known amount of PdG added to urine samples was 102%.

### 2.4.2 Lactose

(Method modified from (Kuhn & Lowenstein 1967) and (Arthur, Kent et al. 1989)).

#### *2.4.2.1 Assay principle*

$$\text{Lactose} + H_2O \xrightarrow{\beta\text{-galactosidase}} \text{galactose} + \text{glucose}$$

$$\text{Galactose} + NAD^+ \xrightarrow{\beta\text{-Galactose dehydrogenase}} \beta\text{-galactonolactone} + NADH + H^+$$

#### *2.4.2.2 Reagents*

72 μM $NAD^+$

0.1 M Potassium phosphate buffer ($K_2HPO_4.3H_2O/KH_2PO_4$, pH 7.2)

β-galactosidase (22.5U/mL)

β-galactose dehydrogenase (220mU/mL)

#### *2.4.2.3 Procedure*

Composite 24 hour samples (Section 2.2.3, 200 μL) were deproteinised with 1.0 M perchloric acid (200 μL) and neutralised with KOH in phosphate buffer (pH 7.2), making the final dilution 1 in 2.5. Lactose standards (0.1 mM to 1.5 mM) were diluted to the same levels with 0.1 M phosphate buffer. The deproteinised sample/standard (20 μL) was plated onto a 96 well flat-bottom plate in quadruplicate and freshly prepared assay reagent (200 μL) was added to two of the wells and reagent without β-galactosidase was added to the other two wells. After incubating at room temperature for 120 minutes, the 340 nm absorbance peaks were measured using a plate spectrometer (Titertek Multiskan® MCC/340, Flow Laboratories, McLean, Va, USA). The wells without β-galactosidase were measured to control for the expected absorbance, at 340 nm, of components of urine other than galactose. In addition, if any background galactose was present, this would also be detected in the wells without β-galactosidase thus allowing absolute measurement of the concentration of lactose. This assay presented difficulties for measurement of lactose concentration greater than 1.5 mM due to flattening of the standard curve. Hence appropriate dilution of samples was performed when higher values were likely.

#### *2.4.2.4 Validation*

The intra- and inter-assay coefficients of variation were 5.3% and 14.1% and the minimum detection limit was 0.086 mM. Recovery ± sd of a known amount of lactose added to samples after deproteinisation was 102.8 ± 12.3%

## 2.5 Metabolites in milk

### 2.5.1 Lactose

Method modified from (Kuhn & Lowenstein 1967) and (Arthur, Kent et al. 1989)

#### *2.5.1.1 Assay principle*

$$\text{Lactose} + H_2O \xrightarrow{\beta\text{-galactosidase}} \text{galactose} + \text{glucose}$$

$$\text{Glucose} + O_2 \xrightarrow{GOD} \text{gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{ABTS}_{(\text{reduced})} \xrightarrow{POD} \text{ABTS}_{(\text{oxidised})} + H_2O$$

#### *2.5.1.2 Reagents*

1. *Lactase reagent:*

   0.1 mM $MgCl_2$

   0.01 M Potassium phosphate buffer ($K_2HPO_4.3H_2O/KH_2PO_4$, pH 7.2)

   8 U/mL β-galactosidase

2. *Glucose reagent:*

   0.1 M Potassium phosphate buffer (as above)

   9.6 U/mL Glucose oxidase (GOD)

   2.5 U/mL Peroxidase (POD)

   500µg/mL $\text{ABTS}_{(\text{reduced})}$

#### *2.5.1.3 Procedure*

Defatted milk samples (Section 2.2.2) and standards (30 mM to 300 mM) were diluted 5 μL in 750 μL distilled deionised water (DDI). Duplicates of diluted samples and standards (5 μL) were plated onto a flat-bottom 96 well microtitre plate and the lactose was hydrolysed with β-galactosidase using a modification of the method for plasma and urine described above. Lactase reagent (50 μL) was added to each well and the plate was mixed and incubated at 37°C for 60 minutes. The glucose reagent (200 μL) was then added to each well and incubated at room temperature for 15 minutes. The resulting absorbance was read at 405 nm on a plate spectrometer (Titertek Multiskan® MCC/340, Flow Laboratories, McLean, Va, USA) at 5 minute intervals until a peak absorbance was reached (approximately 30 minutes).

#### *2.5.1.4 Validation*

In ovine milk, intra- and inter-assay coefficients of variation were 2% and 6% and the minimum detection limit was 3.17 mM. Recovery ± sd of a known amount of lactose added to ovine milk samples was 99.5 ± 6.1. In human milk, intra- and inter-assay coefficients of variation were 5% and 2% and the detection limit was 3.02 mM. Recovery ± sd of a known amount of lactose added to human milk samples was 101.9 ± 3.5%.

## 2.5.2 Citrate

Adapted from (Dagley 1974), modified by (Arthur, Smith et al. 1989).

### *2.5.2.1 Assay principle*

$$\text{Citrate} + H_2O \xrightarrow{\textit{Citrate lyase}} \text{Acetate} + \text{Oxaloacetate}$$

$$\text{Oxaloacetate} + \text{NADH} \xrightarrow{\textit{MDH}} \text{Malate} + NAD^+$$

### *2.5.2.2 Reagents*

1. *Enzyme reagent 1:*

   0.1 M Tri.HCl/KOH buffer (pH 7.8)

   0.34 mM NADH

   10 µL Malate Dehydrogenase (1200 U/mg)

2. *Enzyme reagent 2 (Citrate lyase reagent) made up just before use:*

   1 mL Buffer 0.2 mM (10 mL 0.1 M Tri.HCl/KOH buffer as above (pH 7.8), 4 g $(NH_4)_2SO_4$, 130 mg $ZnSO_4$, 100 mg $NaN_3$)

   120U/350mg Citrate lyase 16 mg

### *2.5.2.3 Procedure*

Defatted milk samples were deproteinised using the a modification of the $HClO_4$/KOH method described previously for plasma (Section 2.3.4.3) and urine, adapted for milk by Arthur *et al*. (Arthur, Smith et al. 1989). Citrate standards (0.25 mM to 10.00 mM) were diluted with DDI to the same dilution as samples. Diluted standards and samples (40µL) were plated on to a 96 well flat bottom plate. Enzyme reagent 1 (200 µL) was added to each well. After incubation (covered) at room temperature for 10 minutes the absorbance was measured at 340 nm using the Titertek Multiskan® MCC/340 plate spectrometer (Flow Laboratories, McLean, Va, USA). The citrate lyase reagent was

made up just before use and 10 µL was added to each well before incubating, covered, at room temperature for a further 10 minutes. The decrease in absorbance was then measured.

#### *2.5.2.4 Validation*

Intra- and inter-assay coefficients of variation were 3.8% and 14.7% respectively. The detection limit was 0.78 mM. The recovery of a known amount of citrate added to deproteinised milk samples was 103 ± 10%.

# CHAPTER 3 THE EFFECTS OF ANTENATAL GLUCOCORTICOID TREATMENT ON LACTOGENESIS II IN SHEEP

*"Lactation cannot, of course, be considered in isolation from development and growth of the young in utero since the two processes are sequential and part of the whole reproductive strategy of eutherian mammals." (Peaker 2002)*

*"… more than the calf wishes to suck does the cow yearn to suckle."(Mepham 1983)*

## 3.1 Introduction

Liggins' serendipitous discovery of the benefits of maternal glucocorticoid treatment in prevention of respiratory morbidity in preterm neonatal lambs revolutionised the antenatal management of women with anticipated preterm delivery (Liggins 1969). At the current time, the majority of women are treated with either antenatal betamethasone or dexamethasone if delivery is judged to be imminent before 34 weeks' gestational age. A large body of research has supported the advantages, and lack of adverse outcomes, on the fetus of a single course of these steroids (Crowley 2003). However, studies conducted by our group of the long-term postnatal consequences in sheep were complicated by a high rate of neonatal mortality which was attributed at the time to a reduction in milk supply in ewes treated with multiple courses of betamethasone (Moss, T. J., Sloboda *et al.* 2001). Udders of betamethasone-treated ewes in that study were observed to be distended with milk during pregnancy and this was not present in control ewes (Unpublished data). However, there has been no other research conducted on the effects of maternal glucocorticoid treatment during pregnancy on lactation in ewes or women.

Lactogenesis II, defined as the onset of copious milk secretion, occurs normally at the time of parturition in sheep and is accompanied by rapid increases in the concentration of lactose in milk and plasma (Hartmann, P E, Trevethan *et al.* 1973; McNeill, Murphy *et al.* 1998). Milk production is inhibited during pregnancy by a high circulating concentration of progesterone and the withdrawal of progesterone, just before parturition, in the presence of high circulating concentrations of prolactin and cortisol, triggers the onset of lactogenesis II (Hartmann, P E, Trevethan *et al.* 1973; Cowie, Forsyth *et al.* 1980).

Antenatal glucocorticoid treatment in ewes results in premature parturition and pregnancy loss unless ewes are supplemented with medroxyprogesterone acetate (MPA, a long-acting progesterone analogue). This is thought to occur because exogenous glucocorticoids compete with and displace progesterone from binding with glucocorticoid receptor in the placenta (Challis, Matthews et al. 2000). In light of the prior observation of disrupted milk production after multiple doses of glucocorticoids, I proposed that a similar effect of antenatal glucocorticoid treatment occurs in the mammary gland of pregnant sheep leading to a precocious stimulation of mammary secretory function. My hypotheses were that a single maternal course of antenatal glucocorticoid treatment in sheep leads to precocious stimulation of lactation during pregnancy. Furthermore, in the absence of milk removal, I hypothesised an involution of the mammary gland followed by reduced mammary secretion after parturition at term.

My aims were to investigate in sheep, by means of two randomised placebo-controlled experiments, the effects of a single dose of antenatal betamethasone on lactation during pregnancy and after parturition. Specific objectives were as follows:

1. To investigate the effects, in sheep, of a single injection of betamethasone at 125 days of pregnancy on plasma lactose concentration during pregnancy.

2. To investigate the effects of a single injection of betamethasone at 125 days of pregnancy on the secretion of hormones responsible for lactogenesis II, namely progesterone, cortisol and prolactin, during pregnancy.
3. To investigate the effects, in sheep, of a single injection of betamethasone at 125 days of pregnancy on milk production after parturition at term.
4. To investigate the effects of medroxyprogesterone acetate (MPA) on lactogenesis II during pregnancy and after parturition.
5. To investigate the effects on plasma lactose concentration of betamethasone treatment either one, two or seven days before delivery at 124 days of pregnancy

## 3.2 Methods

Pregnant Merino ewes carrying single fetuses of known gestational age were maintained in open pasture and allowed to feed *ad libitum* in the following experiments unless stated otherwise. Both experiments were conducted in accordance with Western Australian Animal Welfare Regulations (2003) and were approved by the Animal Ethics Committee of the Western Australia Department of Agriculture.

### 3.2.1 Experiment One

Ewes (N = 36) were randomised to receive one of three treatments at 118 and 125 days (d) of pregnancy administered as a single, maternal, intramuscular injection as follows:

i) BETA (N = 12): 118 d - medroxyprogesterone acetate (MPA, Pharmacia & Upjohn, 150 mg), and 125 d – betamethasone (Celestone Chronodose, Schering Plough, 0.5 mg/kg).

ii) MPA (N = 12): 118 d – MPA, and 125 d – saline.

iii) SALINE (N = 12): 118 d – saline and 125 d – saline.

The intramuscular injection of MPA, which is a progesterone derivative, at 118 days was necessary to prevent subsequent glucocorticoid-induced pregnancy loss in the

BETA ewes (Nathanielsz, Buster *et al.* 1988; Jobe, Newnham *et al.* 2003). Because the effects of MPA on lactation are unknown, the MPA group (group ii) was included to control for the potential of the progestational activity of MPA to independently disrupt the timing of initiation of lactation (Jeppsson 1981).

Blood samples (10 ml) were collected into heparinised tubes by venipuncture of the jugular vein before treatment and at 125, 126, 127, 128, 132, 134, 136 and 143 days of pregnancy and 1, 2 and 5 days after parturition. Plasma was separated and stored at -20°C before analysis of lactose, progesterone, cortisol, and prolactin concentration as described in Chapter 2.

Ewes were weighed before treatment at 118 days of pregnancy and at 132 days of pregnancy. The ewes were transferred to individual pens at 143 days of pregnancy where they had unlimited access to water and chaff; pellets (Econosheep, Milne Feeds, Perth, Western Australia) were fed daily. The normal length of pregnancy in Western Australian merino sheep, used in our studies, is approximately 150 days.

After spontaneous delivery, lambs were housed with their mothers and were able to suckle *ad libitum*. Samples (5 mL) of mammary secretions were collected by hand expression daily from birth until day 5 and stored at -20 °C. Lamb weights were measured at birth and on postnatal day 5. On day five, after a 4-hour separation of ewes from lambs, milk volume was measured by hand milking until all milk was removed. The hand milking method assumed that the technique would provide an accurate measure of milk production although formal studies confirming the effectiveness are not available in the literature. Ewes were then euthanased by lethal injection of pentobarbitone (75-100mg/kg, IV) and mammary glands were removed and weighed. Lambs were also euthanased by lethal injection and sampled for other research studies in our laboratory (data not included in the present thesis).

### 3.2.2 Experiment Two

Blood and milk samples and mammary glands were taken from ewes participating in a study of the effects of maternal betamethasone on fetal immune cells (Kramer, Ikegami *et al.* 2004). Ewes were administered maternal injections of either betamethasone (0.5 mg/kg) or saline placebo at one of three different gestational ages and all were delivered at 124 days' gestational age. The ewes were randomised to receive treatment either at one (betamethasone N=5, saline N=3), two (betamethasone N=10, saline N=6) or seven (betamethasone N=8, saline N=8) days before delivery. Sheep randomised to receive treatment either two or seven days before delivery (either betamethasone or saline) were given MPA injections at 113 days of pregnancy. Sheep randomised to receive treatment one day before delivery were not given MPA because they were participating in a trial of betamethasone over short periods of time and MPA protection against pregnancy loss was not required.

Blood samples were obtained from the jugular vein before either betamethasone or saline treatment and from the femoral artery at delivery. At 124 days of pregnancy, ewes were sedated with an intramuscular injection of ketamine (20mg/kg) and xylazine (0.2 mg/kg), and were given a spinal anaesthetic of 2% lignocaine (4 mL), and lambs were delivered by caesarean section. Milk samples were obtained by hand expression at delivery if there was sufficient milk. Ewes were then euthanased by lethal intravascular injection of pentobarbitone (75-100mg/kg) and mammary glands were removed and weighed.

The author's participation in this experiment comprised maternal blood sampling prior to administration of study treatment, the administration of maternal injections, collection of milk samples, and the removal and analysis of mammary glands. Assistance was provided for the maternal femoral blood sampling at delivery. All biochemical analysis was performed by the author.

### 3.2.3 Statistical analysis

The primary outcome in both experiments was concentration of lactose in plasma. Based on an estimated plasma concentration of 6 μM during pregnancy (McNeill, Murphy et al. 1998), a sample size of 12 sheep per group was selected, for the first experiment, to detect a difference between group means of plasma lactose concentration of one standard deviation with a power of 80% and a Type 1 error rate of 5%. Secondary outcomes were antenatal plasma progesterone, cortisol and prolactin concentrations as well as postnatal milk lactose concentration, milk yield at day five postpartum and lamb weight gain. Plasma lactose and hormone concentrations, lamb weight gain, milk volume and concentration of milk lactose were summarised using non-parametric methods (median, interquartile range (IQR)). Plasma lactose, progesterone, cortisol and prolactin concentrations were $\log_e$-transformed and analysis of variance for repeated measures was performed. Postnatal outcomes were $\log_e$-transformed and compared after adjustment for pregnancy length, birth weight and day of measurement where appropriate. All analyses were performed with the SAS statistical software package (Statistical Analysis System Version 8.2, Cary, NC).

## 3.3 Results

### 3.3.1 Experiment One

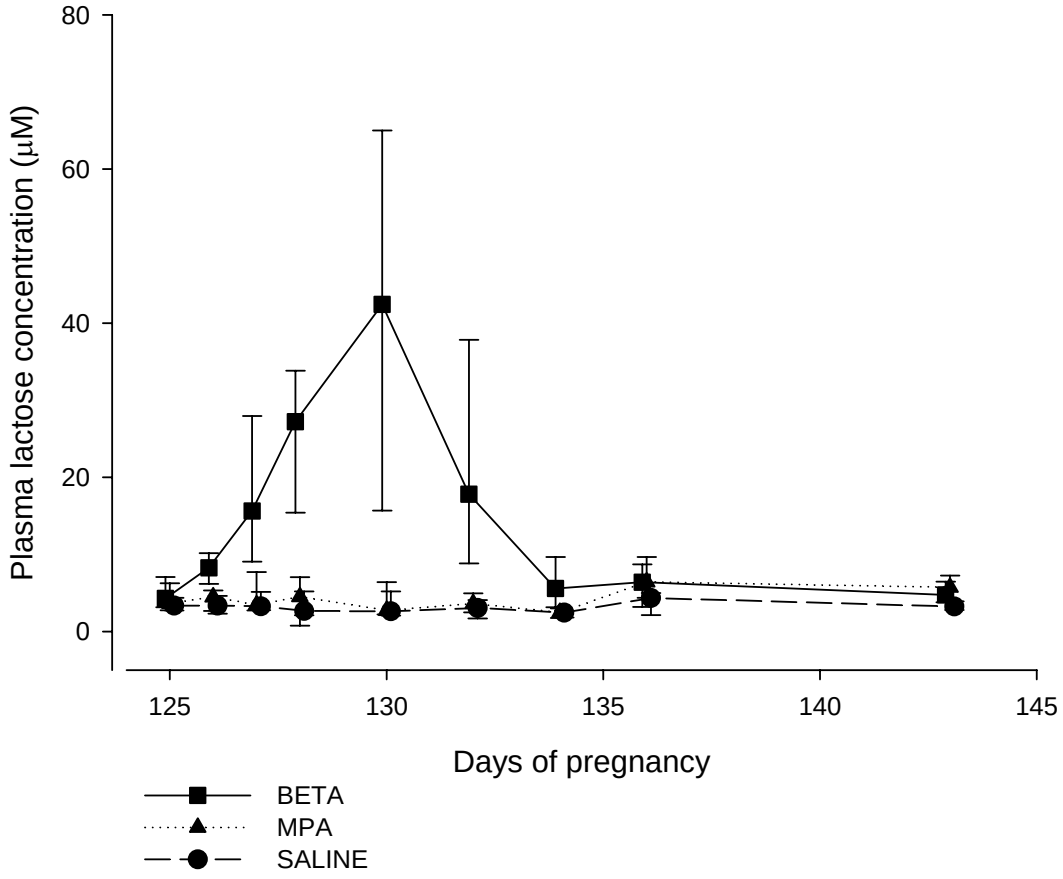
#### *3.3.1.1 Antenatal Outcomes*

*Concentration of lactose in maternal plasma*

The median (range) concentration of lactose in maternal plasma was 4.49 (0-98.30) μM. There was no difference in concentration of lactose in maternal plasma between groups before treatment at 125 days of pregnancy (3. 5 (1.2-8.9) μM). After treatment, there was a significant association between betamethasone treatment and concentration of

lactose in maternal plasma (Figure 3.1). Plasma lactose concentration increased within two days of treatment in BETA ewes, peaking at day five (130 d, median (range) 42.4 (11.7-74.0 μM)) and decreasing to baseline levels by nine days after treatment ($P < 0.001$). Plasma lactose concentration did not change significantly during this time in any of the MPA and SALINE sheep.

**Figure 3.1 Concentration of Lactose in Maternal Plasma after Treatment at 125 days of Pregnancy**




Note: Symbols represent median (IQR) concentration of lactose in maternal plasma (μM) after treatment at 118 and 125 days' (d) of pregnancy in ewes treated with betamethasone (BETA: closed square, solid line), medroxyprogesterone acetate alone (MPA: closed triangle, dotted line), or saline (SALINE: closed circle, dashed line) groups. BETA ewes received MPA at 118 d and betamethasone at 125 d. MPA ewes received MPA at 118 d and saline at 125 d. SALINE ewes received saline at 118 and 125 d.

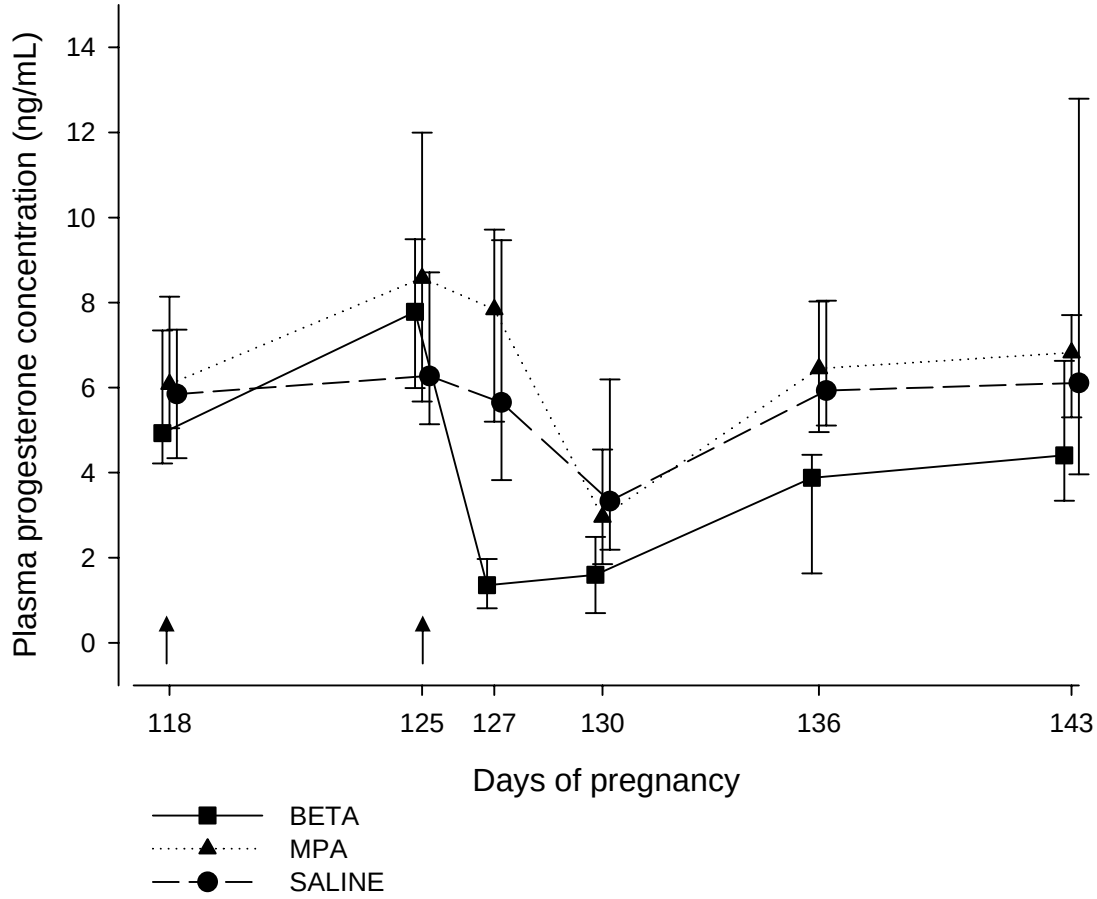
There was a significant effect of betamethasone treatment on plasma lactose during pregnancy ($P < 0.001$) but not MPA when compared with SALINE.

*Concentration of progesterone in maternal plasma*

The median (range) concentration of progesterone in maternal plasma was 5.30 (0.18-18.71) ng/mL but values were significantly different over time in each group (Figure 3.2, $P<0.001$). There was no difference in plasma progesterone concentration between groups when MPA or saline was given at 118 d (5.40 (2.32-11.20) ng/mL) or when betamethasone or saline was given at 125 d (6.90 (2.78-18.71) ng/mL). Concentration of progesterone in maternal plasma was significantly reduced in the BETA group compared with either MPA or SALINE ewes at all other time-points with the lowest value occurring two days after betamethasone treatment (1.36 (0.67-3.72) ng/mL, $P<0.001$). Progesterone concentration was also reduced temporarily in MPA and SALINE ewes at 130 d but this difference failed to reach significance.

The concentration of progesterone in maternal plasma was also significantly inversely associated with the concentration of lactose in plasma ($P < 0.001$) although there was wide variability due to outliers from the BETA group with very high lactose concentration compared with other groups (Figure 3.3).

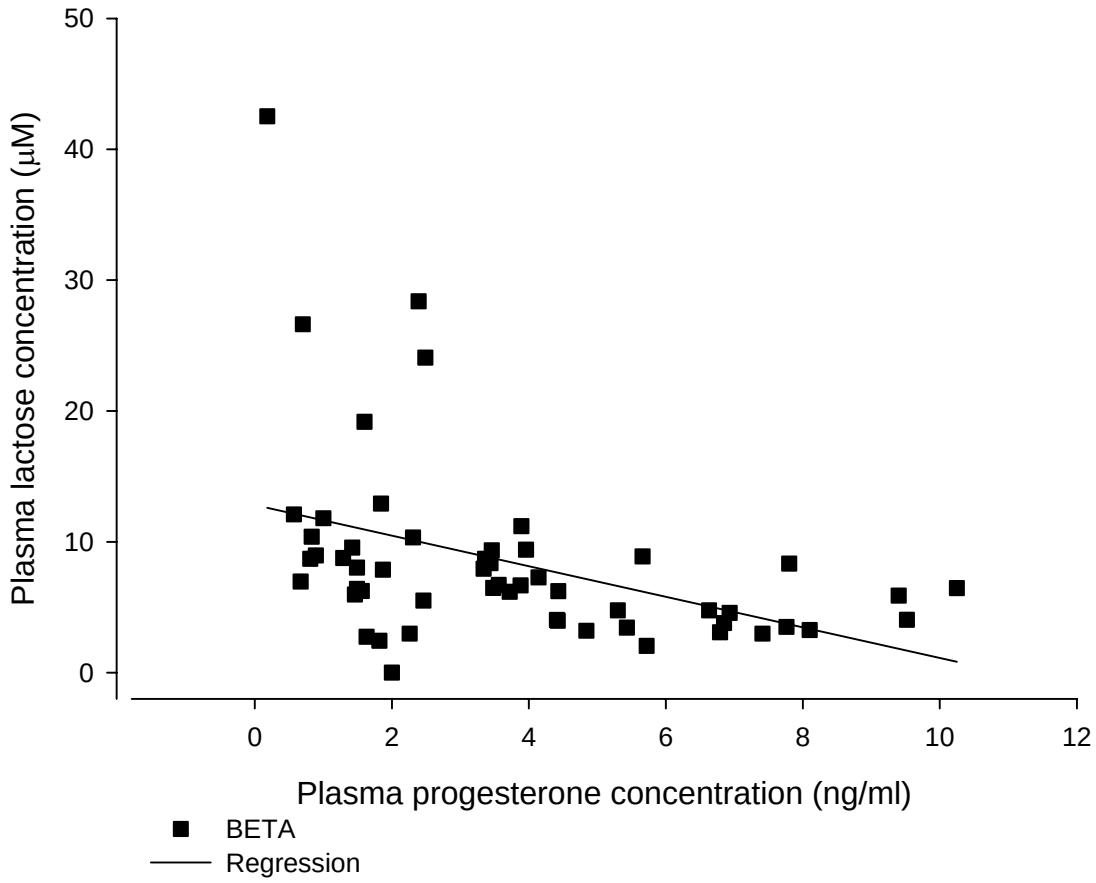
**Figure 3.2 Concentration of Progesterone in Maternal Plasma after MPA/Saline at 118 d and Betamethasone/Saline at 125 days of Pregnancy**




Note: Symbols represent median (IQR) concentration of progesterone in maternal plasma (ng/mL) in ewes treated with betamethasone (BETA: closed square, solid line), medroxyprogesterone acetate alone (MPA: closed triangle, dotted line), or saline (SALINE: closed circle, dashed line) groups. BETA ewes received MPA at 118 d and betamethasone at 125 d. MPA ewes received MPA at 118 d and saline at 125 d. SALINE ewes received saline at 118 and 125 d.

There was a significant reduction in plasma progesterone concentration on days 127 to 143 after betamethasone treatment during pregnancy ($P < 0.001$) but not MPA when compared with SALINE.

**Figure 3.3 Relationship between Concentration of Progesterone and Lactose in Plasma in Sheep Treated with Betamethasone**



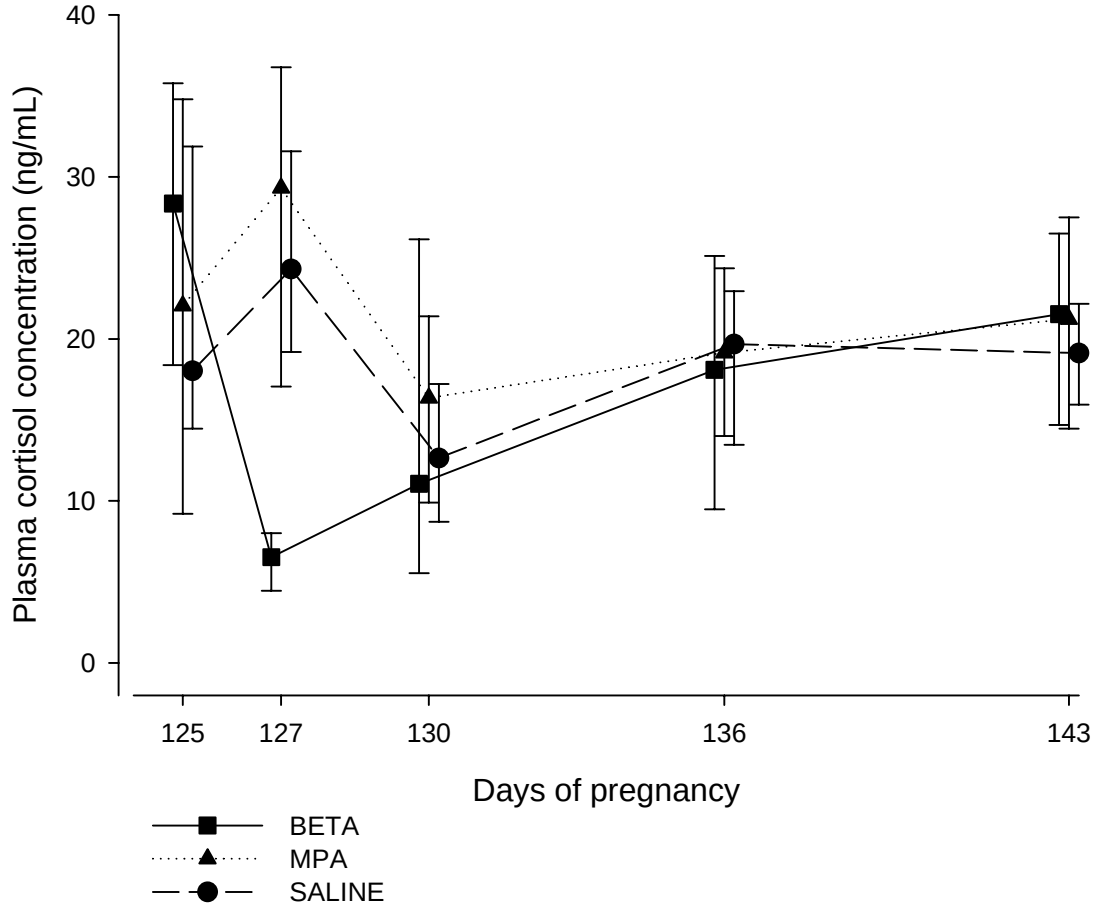

Note: Symbols represent plasma lactose concentration (μM) plotted against plasma progesterone concentration (ng/ml) in sheep treated with betamethasone (N = 12). Samples were analysed on days 125, 127, 130, 136, and 143 of pregnancy.

Increasing plasma lactose concentration was significantly associated with decreasing plasma progesterone concentration ($P < 0.001$, $R^2 = 0.17$). Outliers from the BETA group with exceptionally high lactose concentration were responsible for the wide variability.

*Concentration of cortisol in maternal plasma*

The median (range) concentration of cortisol in maternal plasma was 19.0 (1.9-72.0) ng/ml (Figure 3.4). There was no difference in concentration of cortisol in maternal plasma between groups before treatment (median (range) 27.2 (5.5-61.7) ng/ml). Overall there were no changes in plasma cortisol concentration in either the MPA or SALINE groups but there was a significant transient reduction in cortisol concentration in the BETA group two days after treatment with the lowest value of 6.53 (2.63-10.42) (Figure 3.4. $P < 0.001$). Concentration of cortisol in maternal plasma was not significantly associated with concentration of lactose in plasma ($P = 0.050$) but was significantly associated with concentration of progesterone in plasma ($P = 0.005$). Decreasing plasma cortisol concentration was associated with decreasing progesterone concentration.

**Figure 3.4 Concentration of Cortisol in Maternal Plasma after MPA/Saline at 118 d and Betamethasone/Saline at 125 days of Pregnancy**




Note: Symbols represent median (IQR) concentration of cortisol in maternal plasma (ng/mL) in ewes treated with betamethasone (BETA: closed square, solid line), medroxyprogesterone acetate alone (MPA: closed triangle, dotted line), or saline (SALINE: closed circle, dashed line) groups. BETA ewes received MPA at 118 d and betamethasone at 125 d. MPA ewes received MPA at 118 d and saline at 125 d. SALINE ewes received saline at 118 and 125 d.

There was a significant effect of betamethasone treatment on plasma cortisol concentration during pregnancy ($P < 0.001$) but not MPA when compared with SALINE. Plasma cortisol concentration was significantly decreased at 127 days of pregnancy in the BETA group.

*Concentration of prolactin in maternal plasma*

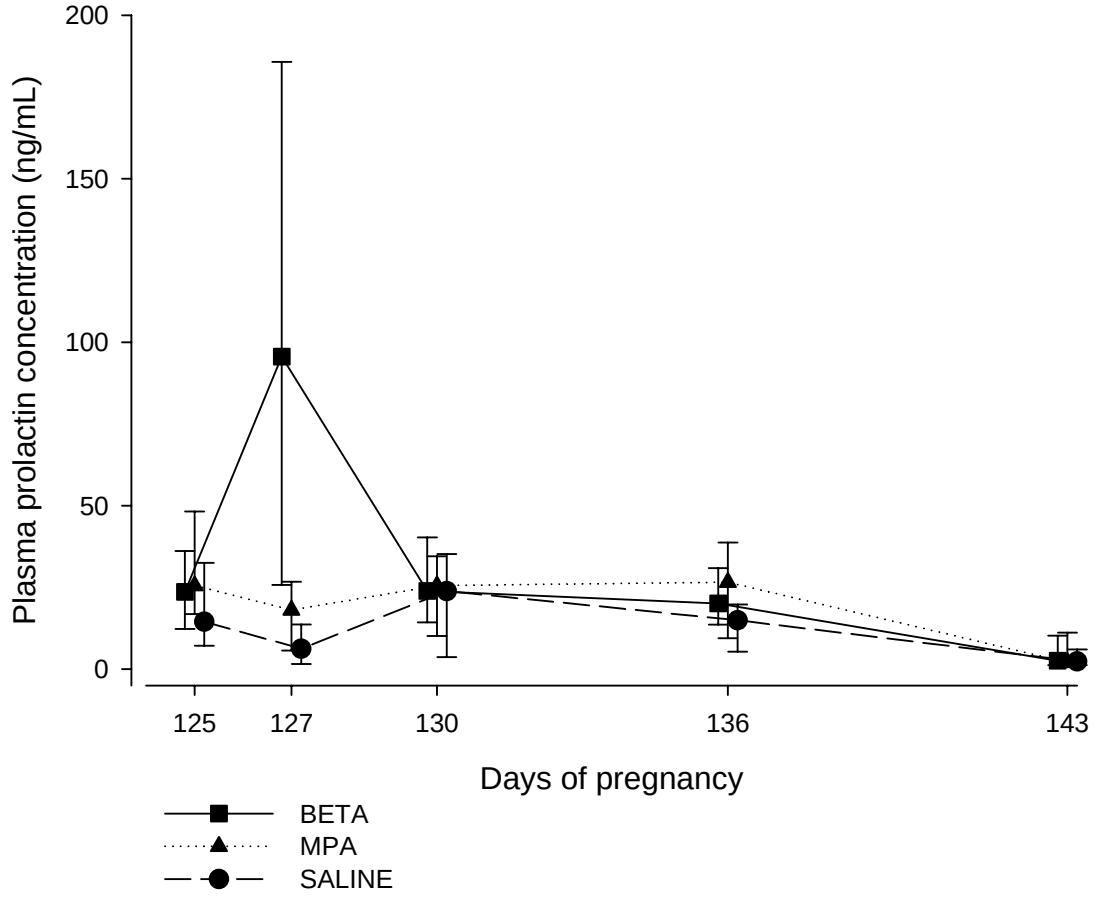
The median (range) concentration of prolactin in maternal plasma was 4.49 (0-98.30) ng/mL. There was no difference in concentration of prolactin in maternal plasma between groups before treatment (median (range) 23.6 (2.5-290.8) ng/ml). Overall there were no changes in plasma prolactin concentration in either the MPA or SAL groups but there was a significant transient increase in the BETA group two days after treatment with a maximum median (range) prolactin concentration of 95.6 (7.4-381.4) ng/ml, (Figure 3.5 $P < 0.001$).

Concentration of prolactin in maternal plasma was significantly reduced at gestational age 143 days in all groups (2.6 (1.2-26.1) ng/ml, $P < 0.001$). Increasing concentration of prolactin in maternal plasma was significantly associated with increasing plasma concentration of lactose ($P < 0.001$), decreasing concentration of progesterone ($P = 0.029$) and decreasing concentration of cortisol ($P < 0.001$) although there was wide variability in all three relationships.

*Antenatal weight gain in ewes*

The mean (sd) change in ewe weight between 118 and 132 days of pregnancy was -1 (3.35) in the BETA group, 0.54 (1.51) in the MPA group, and 0.71 (1.81) in the SALINE group. Although more sheep in the BETA group lost weight compared with either the MPA or SALINE groups, these differences did not reach statistical significance ($P = 0.174$).

**Figure 3.5 Concentration of Prolactin in Maternal Plasma after MPA/Saline at 118 d and Betamethasone/Saline at 125 days of Pregnancy**




Note: Symbols represent median (IQR) concentration of prolactin in maternal plasma (ng/mL) in ewes treated with betamethasone (BETA: closed square, solid line), medroxyprogesterone acetate alone (MPA: closed triangle, dotted line), or saline (SALINE: closed circle, dashed line) groups. BETA ewes received MPA at 118 d and betamethasone at 125 d. MPA ewes received MPA at 118 d and saline at 125 d. SALINE ewes received saline at 118 and 125 d.

There was a significant effect of betamethasone treatment on plasma prolactin concentration during pregnancy ($P < 0.001$) but not MPA when compared with SALINE.

#### *3.3.1.2 Postpartum Outcomes*

Delivery and postpartum characteristics of the ewes and lambs are presented in Table 3.1. There were more fetal and early neonatal lamb losses in the BETA group than in both the MPA and SALINE groups. Two BETA fetuses died *in utero* and 3 lambs died at or soon after birth. There was one neonatal death in the SALINE group and no losses in the MPA group.

The median pregnancy length was longer in BETA sheep (154 days) than in the MPA group (151 days) and the SALINE group (150 days). After adjustment for pregnancy length, lamb birth weights were significantly reduced in the BETA group ($P = 0.011$) but not the MPA group ($P = 0.329$) when compared with SALINE lambs. There were marked effects of betamethasone treatment on milk production after parturition (Table 3.1). Milk volume on day five was significantly lower in the BETA group than in the MPA and SALINE groups ($P < 0.001$, adjusted for pregnancy length). Lamb weight gain between birth and the fifth postnatal day reflected this observation, with significantly reduced weight gains in the BETA group ($P < 0.001$, adjusted for pregnancy length). Three surviving BETA lambs and one MPA lamb lost weight after birth. On day five postpartum, mammary gland weights after hand milking until empty were not different between the groups ($P = 0.702$).

**Table 3.1 Delivery and postpartum characteristics of sheep randomised to receive either betamethasone, MPA or saline at 125 days of pregnancy**

| | BETA | | MPA | | SALINE | | |
|---|---|---|---|---|---|---|---|
| | Median | Range | Median | Range | Median | Range | P value |
| Pregnancy length (d) | 154 | 129 -157 | 151 | 149 -156 | 150 | 148 -155 | 0.065 |
| Lamb survival[1] | 7 | 58% | 12 | 100% | 11 | 91.7% | 0.031 |
| Lamb sex male[1] | 4 | 44% | 7 | 58.3% | 5 | 41.7% | 0.844 |
| Lamb birth weight (g) | 4550 | 3441 – 5600 | 4680 | 4050 – 5250 | 4675 | 4000 – 6230 | 0.037[2] |
| Lamb weight gain birth – d5 (g) | 150 | -320 – 1200 | 1125 | -1200 – 1650 | 1285 | 900 – 1650 | <0.001[2] |
| 4 hour milk volume d5 (mL)[3] | 60 | 0 – 130 | 92.5 | 10 – 180 | 117.5 | 16 – 425 | <0.001[2] |
| Mammary gland weight (g)[3] | 1066.7 | 274 – 1480 | 1132.3 | 761 – 1670 | 1193 | 733.4 – 2110.6 | 0.702 |

[1] N (%), [2] P value after adjustment for pregnancy length,

[3] Mammary glands were hand-milked until empty, four hours after separation from lambs. Ewes were then sacrificed and mammary glands removed and weighed.

*Concentration of lactose in milk*

Milk samples were taken only from ewes with living lambs. There were no significant differences in concentration of lactose in milk over time in the SALINE group (Table 3.2). Concentration of lactose in milk on the first and second postnatal days were lower in BETA and MPA groups than in the SALINE group and a wide range was found in the BETA group on all five postnatal days. After adjustment for pregnancy length and postnatal day this difference neared statistical significance (P = 0.076).

**Table 3.2 Concentration of lactose in milk samples on days 1 to 5 postpartum in sheep randomised to receive either betamethasone, MPA, or saline at 125 days of pregnancy**

| Postnatal Day | BETA (N = 7) | | MPA (N = 12) | | SALINE (N = 11) | |
|---|---|---|---|---|---|---|
| | Median | Range | Median | Range | Median | Range |
| 1 | 113.1 | (30.2-129.6) | 103.2 | (40.8-138.6) | 127.1 | (105.8-138.1) |
| 2 | 120.1 | (35.4-152.1) | 127.2 | (79.6-146.2) | 136.4 | (104.7-161.5) |
| 3 | 144.1 | (38.3-161.3) | 145.0 | (68.1-159.6) | 142.4 | (100.1-163.3) |
| 4 | 139.6 | (40.4-155.8) | 141.3 | (74.7-155.4) | 144.3 | (117.3-156.4) |
| 5 | 147.6 | (88.4-178.7) | 141.6 | (98.3-168.7) | 142.7 | (110.8-170.9) |

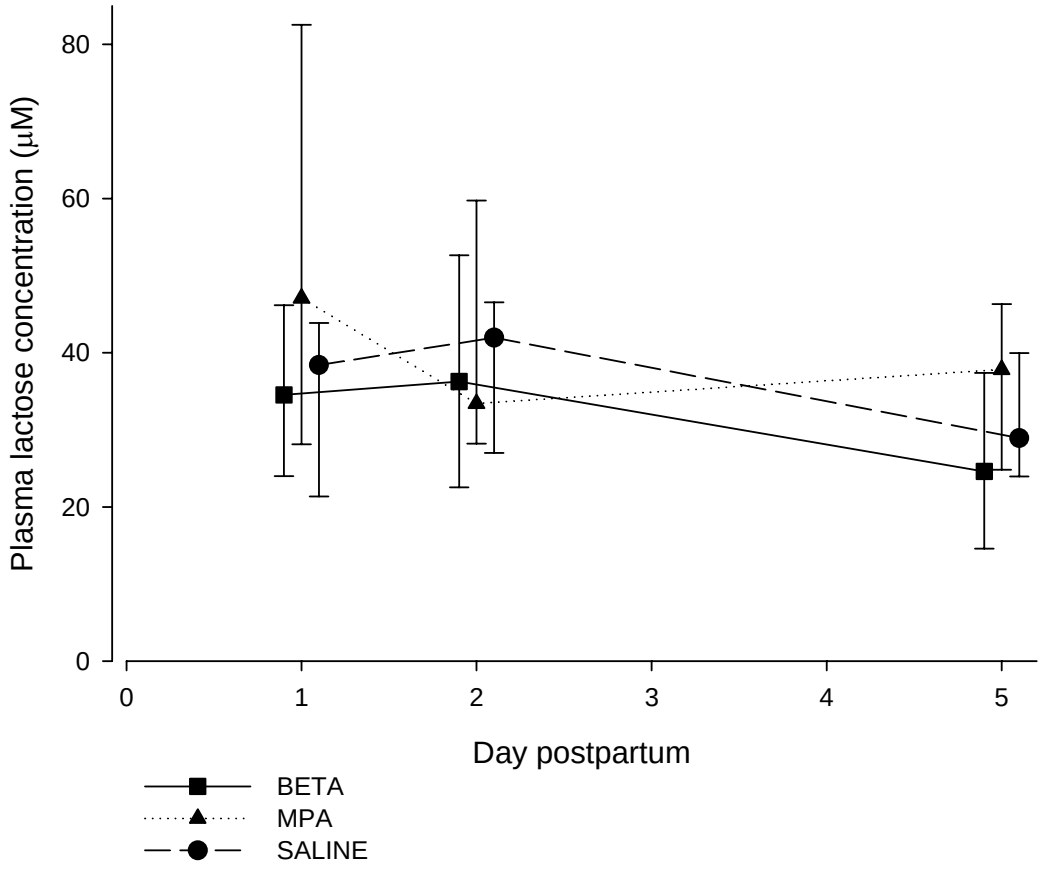
Note: data are median (range) milk lactose concentration (mM). After adjustment for length of pregnancy and day of measurement, milk lactose concentration was not significantly associated with treatment group (P = 0.076).

*Concentration of lactose in postnatal plasma*

The concentration of lactose in maternal postnatal plasma was significantly associated with day postpartum ($P = 0.008$), pregnancy length ($P < 0.001$), and treatment group ($P = 0.001$). After statistical adjustment for pregnancy length and postnatal day, plasma lactose concentration was significantly reduced in the BETA group ($P = 0.001$) but not the MPA group ($P = 0.973$) when compared with the SALINE group (Figure 3.6). This occurred at all postnatal days sampled.

Neither lamb sex ($P = 0.595$), birth weight ($P = 0.122$), nor maximum concentration of lactose in antenatal plasma ($P = 0.065$), were associated with concentration of lactose in postnatal plasma.

**Figure 3.6 Concentration Of Lactose In Maternal Plasma On Days 1, 2 And 5 Postpartum After Treatment At 125 Days Of Pregnancy**




Note: Symbols represent median (IQR) concentration of lactose in maternal plasma (μM) on days 1, 2 and 5 postpartum in BETA (closed square, solid line), MPA (closed triangle, dotted line), or SALINE (closed circle, dashed line) groups. BETA ewes received MPA at 118 d and betamethasone at 125 d. MPA ewes received MPA at 118 d and saline at 125 d. SALINE ewes received saline at 118 and 125 d. The range of pregnancy length was 148-157 days. Blood was sampled only if lambs were alive.

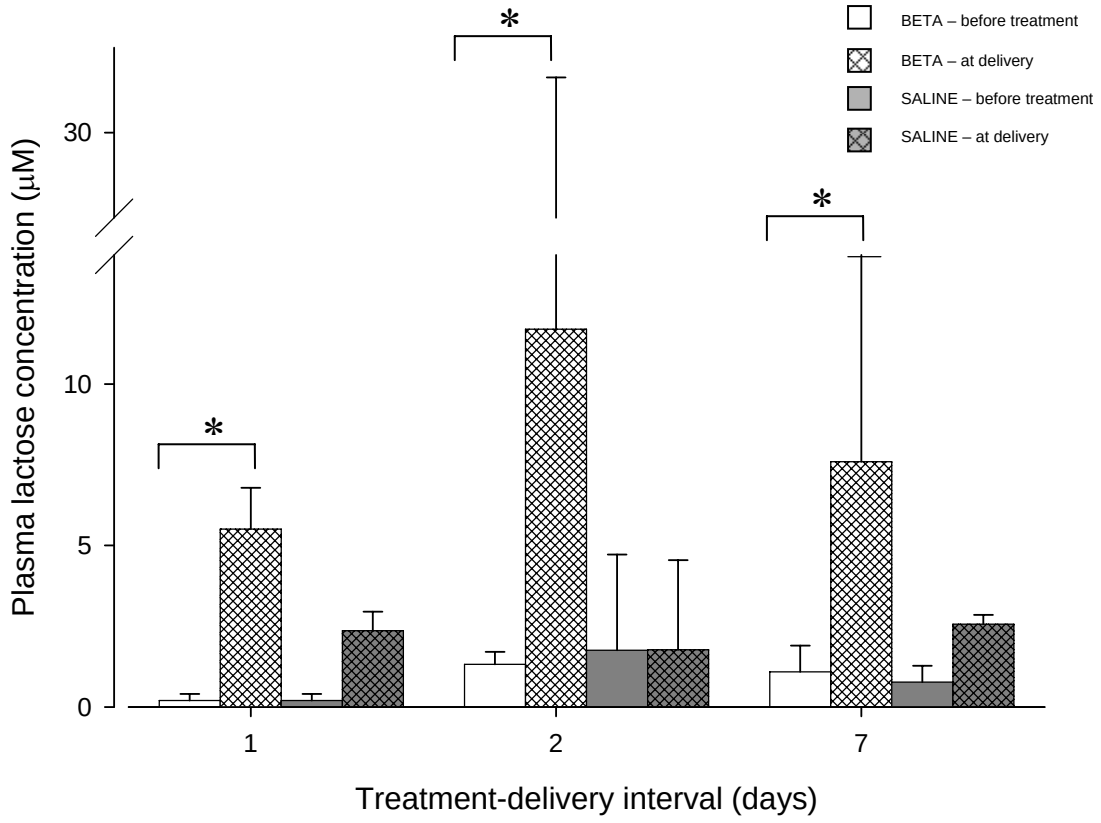
After adjustment for length of pregnancy and day of measurement, plasma lactose concentration was significantly reduced in the BETA group compared with the SALINE group ($P < 0.001$).

### 3.3.2 Experiment Two.

Maternal injections were either betamethasone (N=5, 10, 8) or saline (N=3, 6, 8) at one, two or seven days respectively before delivery at 124 days' gestational age.

The concentration of lactose in maternal plasma before treatment and at delivery is presented in Figure 3.7. There was no difference between groups before treatment ($P = 0.540$; median (range): 0.88 (0-8.34) μM). Plasma lactose concentration was significantly increased at delivery at 124 days of pregnancy after betamethasone treatment ($P < 0.001$) but not after saline treatment ($P = 0.732$). In addition, compared with delivery at one day after treatment, plasma lactose concentration was significantly higher when delivery occurred two days after treatment ($P = 0.034$), but not when delivery occurred seven days after treatment ($P = 0.629$), after adjustment for treatment group.

**Figure 3.7 Concentration Of Lactose In Maternal Plasma Before And After Treatment With Either Betamethasone Or Saline At 1, 2 Or 7 Days Before Delivery At 124 Days**




Note: Histograms represent median (IQR) plasma lactose concentration where delivery occurred 1, 2 or 7 days after treatment with either betamethasone or saline. Delivery was by caesarean section at 124 days of pregnancy. The white histograms represent sheep treated with betamethasone and the grey histograms represent sheep treated with saline. The solid histograms represent plasma lactose concentration before treatment and the hatched histograms represent plasma lactose concentration at delivery.

Plasma lactose concentration was significantly greater than day 1 when delivery occurred 2 days after delivery ($P = 0.034$) but not 7 days after betamethasone treatment ($P = 0.629$).

* Plasma lactose concentration was significantly increased at delivery after betamethasone treatment at all treatment-delivery intervals ($P < 0.001$) but not in any of the saline-treated sheep.

Delivery characteristics are presented in Table 3.3. There was no difference in lamb birth weight either between betamethasone and saline treated sheep or at different intervals between treatment and delivery (P = 0.609 overall and P = 0.732 in ewes that received betamethasone). Nor was there any difference in sex of the lamb. There were significant differences between betamethasone and saline treated sheep in composition of mammary secretions when delivery occurred at either one, two or seven days after treatment. Colostrum was present in ewes treated with betamethasone that delivered one day after treatment. Milk was present only in betamethasone treated ewes that were delivered two or seven days after treatment. Saline treated ewes at all time intervals had either no mammary secretion or small amounts of colostrum only.

Milk lactose concentration was unable to be ascertained in saline treated ewes because of insufficient volumes. In betamethasone-treated ewes, milk lactose concentration was significantly associated with interval between treatment and delivery (P < 0.001). Compared with sheep delivered one day after betamethasone treatment, milk lactose concentration was significantly greater in ewes delivered either two days (P < 0.001) or seven days after treatment (P = 0.026). Concentration of lactose in milk was also significantly greater in ewes delivered two days after treatment compared with ewes delivered seven days after treatment (P = 0.042). Concentration of lactose in milk was significantly associated with plasma lactose concentration at delivery but not plasma lactose concentration before treatment, fetus birth weight (P = 0.486) or sex (P = 0.826). Increasing plasma lactose concentration was significantly associated with increasing milk lactose concentration at delivery (P = 0.027).

Because of constraints caused by the protocol of the main study from which the mammary samples were obtained, I was not able to remove mammary secretions from glands before weighing. Consequently there was a wide range of mammary gland weights. Mammary gland weights were significantly associated with treatment group

(P = 0.008) but not interval between treatment and delivery (P = 0.178). Glands were significantly heavier in ewes treated with betamethasone compared with control ewes (Figure 3.8). Mammary gland weight was not associated with plasma lactose concentration at either treatment (P = 0.362) or delivery (P = 0.056). Nor was mammary gland weight significantly associated with visual milk secretion (P = 0.523) or milk lactose concentration (P = 0.349). There were no interactions between treatment and treatment interval (P = 0.540). Mammary gland weight was significantly associated with fetal weight at delivery (P = 0.015) but not fetal sex (P = 0.806). Increasing fetal weight was associated with increasing mammary weight.

**Table 3.3 Experiment Two. Delivery characteristics at 124 days after treatment with either betamethasone or saline at 1, 2 or 7 days before delivery**

| $R_x$ interval | 1 day | | | | | 2 days | | | | | 7 days | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Beta (N=5) | | Saline (N=3) | | ***P value*[1]** | Beta (N=10) | | Saline (N=6) | | ***P value*[1]** | Beta (N=8) | | Saline (N=8) | | ***P value*[1]** |
| | Median | Range | Median | Range | | Median | Range | Median | Range | | Median | Range | Median | Range | |
| Lamb birth weight (kg) | 2.5 | 2.3-2.75 | 2.6 | 2.3-2.85 | ***0.549*** | 2.53 | 2.2-3.2 | 2.53 | 2.45-2.85 | ***0.586*** | 2.58 | 2.2-2.9 | 2.75 | 1.3-2.9 | ***0.562*** |
| Lamb sex M[2] | 5 | 100% | 2 | 67% | ***0.375*** | 6 | 60% | 3 | 50% | ***0.999*** | 3 | 37.5% | 3 | 37.5% | ***0.999*** |
| Mammary secretion[2,3] | | | | | | | | | | | | | | | |
| Milk | 0 | 0% | 0 | 0% | | 9 | 90% | 0 | 0% | | 7 | 88% | 0 | 0% | |
| Colostrum | 5 | 100% | 0 | 0% | | 1 | 10% | 4 | 67% | | 0 | 0% | 6 | 75% | |
| None | 0 | 0% | 3 | 100% | ***0.018*** | 0 | 0% | 2 | 33% | ***0.001*** | 1[4] | 12% | 2 | 25% | ***<0.001*** |
| Milk lactose conc. (mM)[5] | 8.9 | 0.8-15.3 | -- | -- | | 76.2 | 32.4-104.1 | -- | -- | | 29.6 | 7.1-58.2 | -- | -- | ***<0.001*[6]** |
| Mammary gland wt (g)[7] | 331 | 253-414 | 291 | 282-305 | ***0.180*** | 519.5 | 270-982 | 286 | 228-515 | ***0.051*** | 376 | 316-597 | 277 | 134-406 | ***0.018*** |

Table 3.3 Notes.

Delivery was at 124 days' of pregnancy. Data are grouped according to interval between treatment and delivery (1, 2 or 7 days).

[1] P value is for differences between betamethasone- and saline-treated ewes within treatment-delivery interval groups. P values are determined either by Kruskal-Wallis test for non-parametric data or Fishers exact test or Chi Square test where appropriate for categorical data;

[2] N (%);

[3] Mammary secretions were determined by visual appearance. Colostrum was characterized by small amounts of thick, viscous, yellow secretion. Milk was present in greater volumes, was easier to express, and was white, and less viscous than colostrum;

[4] One mammary gland was necrotic and no weight or milk was obtained;

[5] Insufficient mammary secretions to determine lactose concentration in saline-treated ewes and when only colostrum was secreted.

[6] P value for difference between treatment-interval groups in sheep that received betamethasone, adjusted for plasma lactose concentration.

[7] Mammary glands were weighed without removal of existing milk.

**Figure 3.8 Sheep Mammary Glands at 124 Days of Pregnancy after Treatment at 117 Days of Pregnancy with either Saline or Betamethasone**

Figure 3.8a. Sheep mammary gland at 124 days of pregnancy seven days after maternal *saline* treatment

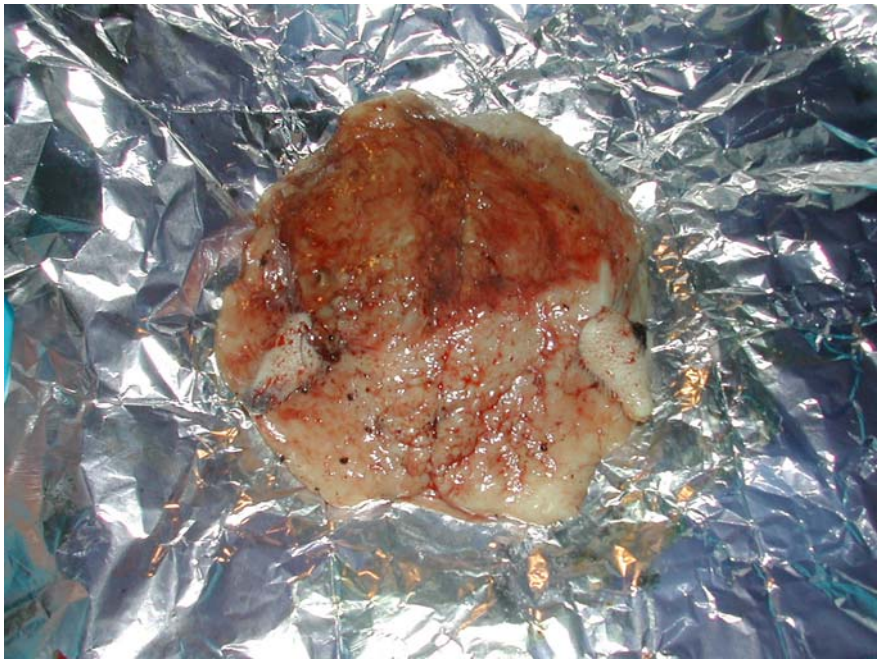
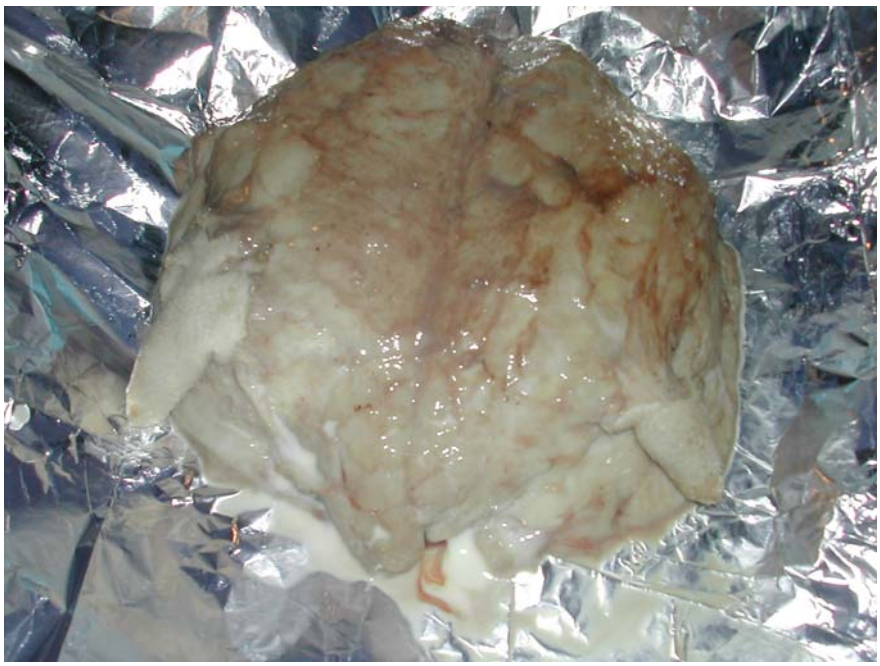


Figure 3.8b. Sheep mammary gland at 124 days of pregnancy seven days after maternal *betamethasone* treatment, showing active secretion of milk and increased size



## 3.4 Discussion

The present studies show that, in pregnant sheep, treatment with betamethasone resulted in elevations in the concentration of lactose in milk and plasma while the sheep were still pregnant, indicating a precocious stimulation of mammary secretion. The rise in plasma lactose concentration was accompanied by a sudden decrease in progesterone concentration, suggesting that the precocious onset of secretion was caused by the temporary removal of the normal progesterone inhibition of lactation during pregnancy. The precocious onset of milk secretion resolved in the absence of milk removal but, after parturition, milk production was significantly reduced, compromising neonatal lamb growth. This process occurred independently of the progesterone-like inhibitory effects of medroxyprogesterone acetate (MPA).

### 3.4.1 Antenatal Outcomes

#### *3.4.1.1 Concentration of lactose in maternal plasma*

The onset of copious milk secretion is inhibited by high levels of progesterone during pregnancy and normally occurs after the withdrawal of progesterone close to parturition (Hartmann, P E, Trevethan *et al.* 1973; Cowie, Forsyth *et al.* 1980). In the present studies there were rapid increases in plasma lactose levels, indicating the onset of mammary secretion (Arthur, Kent et al. 1991; McNeill, Murphy et al. 1998), soon after treatment with betamethasone, but not in either saline controls or sheep treated only with MPA. Nine days after treatment at 125 days of pregnancy, plasma lactose levels in betamethasone-treated sheep had decreased to levels similar to those in the other groups suggesting that involution occurred in the prematurely stimulated mammary glands in the absence of milking (Tatarczuch, Philip *et al.* 1997; Neville, M. C. & Morton 2001). The changes in milk secretion were not associated with changes in ewe weight as there

was no significant difference found between groups for ewe weight change during pregnancy.

#### *3.4.1.2 Experiment Two-Treatment at Earlier Gestational Age*

This finding of a premature initiation of mammary secretion after betamethasone treatment at 125 days of pregnancy was supported by similar findings, at 124 days of pregnancy, when sheep were treated with betamethasone at earlier gestational ages (117 and 122 days of pregnancy). Mammary glands were visibly distended with milk at both two and seven days after betamethasone treatment.

Sheep delivered two days after betamethasone treatment at 122 days of pregnancy had the greatest increases in plasma lactose concentration and this finding was supported by significantly greater milk lactose concentration. Both plasma and milk lactose concentration were still elevated in sheep delivered seven days after betamethasone treatment at 117 days of pregnancy, although values were reduced compared with treatment-delivery interval of two days suggesting that glands had commenced to involute in the absence of milk removal.

#### *3.4.1.3 The Effect of Betamethasone Treatment on the Endocrinology of Lactogenesis II*

Successful lactogenesis II requires a complex interplay of a number of lactogenic hormones, chiefly prolactin, glucocorticoids, insulin, and progesterone. The present study showed that administration of synthetic glucocorticoids to pregnant sheep profoundly disrupted the concentration and function of these hormones. Decreasing levels of progesterone were found to significantly predict increased lactose concentration (Figure 3.3), consistent with the premise that the withdrawal of progesterone is an essential trigger of the onset of lactogenesis II (Kuhn 1969; Hartmann, P E, Trevethan *et al.* 1973). Increasing lactose concentration was also

significantly associated with increasing prolactin concentration, consistent with the understanding that high prolactin concentration is a prerequisite for onset of lactogenesis II (Kulski, Hartmann *et al.* 1978; Tucker 2000; Neville, M C, McFaddin *et al.* 2002).

The administration of glucocorticoids to pregnant ewes is known to induce premature parturition (Nathanielsz, Buster et al. 1988; Jobe, Newnham et al. 2003). This is prevented by the prior administration of the progestagen MPA which is assumed to compensate for the rapid withdrawal of progesterone that follows glucocortiocoid administration in sheep (Liggins, Grieves *et al.* 1972). The present study of pregnant sheep found also a rapid decrease in progesterone concentration after betamethasone treatment. Although there was a recovery, progesterone concentration remained depressed for the rest of gestation compared with either MPA or SALINE ewes. Progesterone concentration was not affected by MPA treatment alone.

The decrease in progesterone concentration at 130 days of pregnancy in all groups cannot be readily explained, although this was not significantly significant. However, the fall in the MPA and SALINE groups was not sufficient to induce a lactogenic effect in the mammary gland (Figure 3.1). Furthermore, ewes treated with betamethasone had a significantly greater and sustained depression of progesterone up until term.

Elevated levels of endogenous glucocorticoids are required for the onset of lactogenesis II after parturition (Tucker 2000; Neville, M C, McFaddin *et al.* 2002). The finding of a transient suppression of maternal plasma cortisol concentration immediately after betamethasone administration is consistent with previous reports (Kutzler, Coksaygan *et al.* 2004; Quaedackers, Roelfsema *et al.* 2005). This reduction was not sufficient to inhibit the onset of lactogenesis II probably due to the presence of high concentrations of synthetic glucocorticoids.

Plasma prolactin concentration was generally low at all gestational ages in the SALINE and MPA groups and the decrease in all groups at 143 days was consistent with previous findings (Cowie, Forsyth et al. 1980). Prolactin normally has a seasonal variation in pregnant sheep and tends to be present in very low levels until a few days before parturition (Cowie, Forsyth et al. 1980). The present experiment protected against potential confounding effects of seasonal variation by the randomisation process.

Prolactin has been shown to be essential for the expression of milk protein genes and has a synergistic action with activated glucocorticoid receptor (Figure 1.4) (Rosen, Wyszomierski et al. 1999). Glucocorticoids have been reported to stimulate prolactin up-regulation *in vitro* and the glucocorticoid receptor is involved in this process (Fu & Porter 2004). The finding of a transient surge in prolactin soon after betamethasone administration *in vivo* supports this finding and suggests that betamethasone treatment may cause a temporary withdrawal of the progesterone inhibition of lactogenesis II, and that this response allows other lactogenic hormones to up-regulate temporarily. Alternatively raised levels of prolactin may be caused by stress induced by the glucocorticoid treatment as has been shown previously (Cooke, Holsberger *et al.* 2004).

#### *3.4.1.4 Effects of Medroxyprogesterone Acetate*

Medroxyprogesterone acetate treatment alone had no effect on plasma progesterone, cortisol or prolactin concentrations. Moreover, plasma lactose concentration was unchanged after MPA treatment suggesting that MPA has negligible impact on mammary function during pregnancy. The finding that MPA pre-treatment did not prevent premature stimulation of the mammary gland following progesterone withdrawal, while preventing the onset of premature parturition, suggests differences between the responses of the uterus and the mammary gland to the progestagens

progesterone and MPA. Of interest, other authors have found that premature parturition in rats (induced by inflammation) is prevented by MPA but not exogenous progesterone administration (Elovitz & Wang 2004), suggesting that MPA has a potent protective effect on the gravid uterus that does not occur after progesterone administration. As the present study showed that MPA did not have a protective effect on the mammary gland, this finding further emphasizes the difference in response to MPA between the uterus and the mammary gland.

Despite the absence of an inhibition of onset of lactogenesis II after glucocorticoid treatment during pregnancy, lower milk lactose concentration on the first postpartum day in ewes that received MPA but not betamethasone was evidence of a 24 hour delay in initiation of lactogenesis II after parturition. This finding suggests that MPA, which is a long-acting progesterone analogue, given at 118 days of pregnancy, may have had some residual inhibitory effect on lactation after parturition at term. However, compared with the postpartum effects of betamethasone, this effect did not have any medium term adverse outcomes on either subsequent lactation or lamb growth.

#### *3.4.1.5 Involution of the Mammary Gland*

Involution of the mammary gland results in rapid reductions in synthesis of major milk components such as lactose (Hartmann, P E & Kulski 1978; Neville, M C, Allen *et al.* 1991). The first experiment showed that, in the absence of milk removal after betamethasone-induced premature stimulation, plasma lactose concentration in the BETA group decreased to that of the SALINE and MPA groups suggesting a loss of function of the gland. In experiment two, reduced milk and plasma lactose concentration seven days after treatment compared with two days after treatment, confirmed at earlier gestational ages that secretory function decreased in the absence of milk removal. As no attempt was made to milk the sheep during pregnancy in either experiment, both the

conditions of milk stasis and the lack of teat stimulation, which trigger involution (Li, Liu et al. 1997; Tatarczuch, Philip et al. 1997), were present in betamethasone-treated sheep after premature initiation of milk secretion. This condition is comparable to involution in mothers of term infants who elect not to breastfeed and thus do not stimulate their breasts after lactogenesis II (Hartmann, P E & Kulski 1978).

### 3.4.2 Postnatal outcomes

The first experiment showed that postpartum onset of lactogenesis II after normal parturition at term was severely disrupted by betamethasone treatment at 125 days of pregnancy. After adjustment for pregnancy length and postpartum day, plasma lactose concentration was significantly reduced in BETA but not MPA ewes when compared with SALINE ewes (Figure 3.6). Moreover, the concentration of lactose in milk was reduced in both BETA and MPA ewes compared with SALINE ewes on the first two days postpartum, although there was wide variability and the association failed to reach statistical significance. Altered secretion of lactose in milk and plasma suggests that the onset of lactogenesis II was delayed in some ewes that had received MPA and betamethasone treatment.

Increased rates of pregnancy loss and longer lengths of pregnancy in the BETA group were consistent with previous reports (Moss, T. J., Sloboda *et al.* 2001; Jobe, Newnham *et al.* 2003), and fetal growth restriction has been reported after betamethasone treatment (Newnham, Evans *et al.* 1999; Moss, T. J., Sloboda *et al.* 2001; Jobe, Newnham *et al.* 2003). This study is the first to show an adverse effect of maternal glucocorticoid treatment on postnatal weight gain although high neonatal lamb mortality rates after antenatal treatment with repeated doses of betamethasone have been reported previously (Moss, T. J., Sloboda *et al.* 2001).

These findings do not exclude the possibility that the effect of betamethasone on postnatal initiation of lactogenesis II was moderated by indirect effects on the lambs. The relatively low birth weights and high neonatal morbidity in BETA lambs suggests subnormal suckling attempts, possibly influencing supply (Dwyer 2003). Moreover, since maternal behaviour in ewes has been shown to be adversely affected by antenatal insults such as undernutrition (Dwyer, Lawrence *et al.* 2003), it is feasible that antenatal betamethasone treatment may also restrict normal postnatal mothering behaviour. Unlike studies with rodents, cross-fostering to eliminate this potential confounding was not possible in the present study design as it is difficult to achieve in ruminants such as sheep. Moreover, in contrast to mature lactation, the onset of copious milk secretion occurs independently of milk removal in all species (Cowie, Forsyth et al. 1980). The findings of reduced concentration of lactose in milk and plasma in the first two postpartum days in the BETA group is consistent with the suggestion that altered physiology in the ewes rather than restricted development in the lamb was responsible for postpartum delays in lactogenesis II.

No difference was found between groups in mammary gland weights after milk was removed on day five postpartum suggesting that the structural development of the gland may be approaching completion by 125 days' of pregnancy when betamethasone was administered and that the changes that were observed during pregnancy and after parturition were possibly due to functional but not structural changes. Interestingly, in the second experiment, mammary weight was found to be significantly associated with fetal weight, but neither milk nor plasma lactose concentration. This was despite weights being measured without removal of the milk secretions present in betamethasone-treated sheep but not saline-treated controls. This finding from the second experiment is consistent with the supposition that, despite changes in function, growth of the mammary gland is resistant to betamethasone treatment at late stages of

pregnancy and suggests that fetal weight is a more robust predictor of mammary weight after 117 days of pregnancy at least.

In the present studies it was not possible to account fully for environmental factors that may influence the secretion of galactopoietic hormones postpartum (Tucker 1988). As lactation is a time of relative under-nutrition in ewes, thereby causing increases in the secretion of endogenous glucocorticoids, adequate maternal feed intake was critical. Moreover, differences in environmental temperatures and photoperiod also influence secretion of hormones such as prolactin (Tucker 1988). Although all sheep were housed in individual pens with the same amount of natural light and sufficient quantities of food, the relatively poor condition of some ewes had the potential to confound the findings in the present study. However, ewe weight gain/loss was distributed evenly between treatment groups. Moreover, both randomisation of treatment and subsequent blinding to treatment group reduced the potential for biased assessment of maternal nutritional status.

### 3.4.3 Implications and Further Research

Whereas the use of an ovine model of fetal growth is well established, (Moss, T. J., Sloboda *et al.* 2001; Jobe, Newnham *et al.* 2003) a generalisation of this model to initiation of lactation in women is more problematic. Further research is indicated to determine whether the effect observed in sheep of exogenous glucocorticoids on progesterone and lactose concentration also occurs in women. Moreover, in the present experiments, ewes received betamethasone treatment at approximately 80% of pregnancy whereas anticipated preterm delivery in women is treated clinically with glucocorticoids as early as 24 weeks' gestational age (60% of pregnancy). It remains to be investigated whether the effects observed in sheep would be as great after treatment at earlier gestations.

# CHAPTER 4 THE EFFECT OF PRETERM BIRTH AND ANTENATAL GLUCOCORTICOIDS ON LACTOGENESIS II IN WOMEN

*" "La fixité du mileu intérieur est la condition de la vie libre" – to permit maximal survival and growth of the young, a constancy (is required) that enables the supply of appropriate nutrients at appropriate intervals to develop and maintain the constancy of the internal environment of the offspring." Claude Bernard cited in (Peaker 2002)*

## 4.1 Introduction

Mothers' own milk is the optimum form of nutrition for preterm infants, when it is fortified. The nutritional, immunological and other advantages of human milk have been shown to either prevent or reduce a wide range of morbidities associated with preterm birth (Gartner, Morton et al. 2005). Despite the advantages of human milk, fewer mothers of preterm infants initiate lactation and the duration of breastfeeding is shorter compared with mothers of healthy term infants (Yip, Lee et al. 1996; Furman, Minich et al. 1998; Meier, Brown et al. 1998; Killersreiter, Grimmer et al. 2001; Flacking, Nyqvist et al. 2003).

Factors such as stress, maternal illness, operative delivery or antenatal pharmacological therapies as well as insufficient breast tissue development potentially increase the risk of disruption to lactogenesis II after preterm delivery, compared with term delivery. Although problems with insufficient milk supply have been shown in some mothers of preterm infants during the early weeks postpartum (Cregan, De Mello et al. 2002; Hill, Aldag et al. 2005), there is a paucity of research about the impact of premature delivery on lactogenesis II.

Furthermore, despite the large body of evidence about the advantages and risks for the preterm neonate of antenatal glucocorticoid treatment (NIH Consensus Conference 1995), no research has reported the effects of antenatal glucocortiocoid treatment on lactogenesis II in mothers of preterm infants. The findings in the previous chapter of profound adverse effects of antenatal glucocorticoid administration on lactogenesis II in sheep suggest the importance of determining if these findings can be applied in women. The research comprising the present chapter was designed to investigate the effects, in women, of both preterm birth and antenatal glucocorticoid treatment on the timing of lactogenesis II after birth. The study was a prospective study of women who received antenatal betamethasone and progressed to deliver a very preterm infant less than 34 weeks' gestational age. As very little research has been published on the most appropriate methods to determine lactogenesis II after preterm delivery, a secondary aim was to evaluate the effectiveness of using the concentration in milk of either lactose or citrate compared with expressed milk volume as indicators of lactogenesis II in mothers of preterm infants.

## 4.2 Methods

The study was a prospective cohort trial of women who received antenatal glucocorticoid treatment for anticipated preterm delivery. Women were approached to participate in the study when they presented to King Edward Memorial Hospital for Woman, Subiaco, Western Australia with a singleton or twin pregnancy at risk of preterm delivery before 34 completed weeks of pregnancy. Women were recruited within 24 hours of receiving their first glucocorticoid injection. Exclusion criteria were age younger than 18 years, women who did not intend to breastfeed, likely poor perinatal outcome and women with multiple pregnancies of triplets or greater order. All women received a single course of two intramuscular injections 24 hours apart of

betamethasone (Celestone Chronodose, Schering Plough) 11.4 mg as per institutional policy unless delivery occurred before the second dose could be given.

This chapter comprises an analysis of the lactation outcomes from birth to day 10 postpartum in the subgroup of women who proceeded to deliver before 34 weeks' gestational age and who were expressing for their infants' entire nutritional requirements because their infants had not commenced suckling at the breast (N=50). Obstetric and neonatal data were abstracted from patient records. Before ten days' postpartum the women completed a questionnaire which included a social and breastfeeding history and a record of their current breastfeeding experience (Appendix 3).

Informed consent was obtained from all participants before recruitment to the study (Appendices 1 and 2). Institutional ethics approval was obtained from The University of Western Australia and the Women's and Children's Health Service Ethics Committees.

### 4.2.1 Milk Volume Measurement

Women were encouraged to express at least six times per day including once overnight as per institutional policy. The women expressed using a commercial electric breast pump (either Medela 'Lactina' or Medela 'Symphony', Medela AG, Baar, Switzerland). Using digital scales accurate to 0.1 g (Tanita Digital Scale, Model 1212, Tanita Corporation, Japan), they measured the weight of milk obtained at every expression on days one to ten postpartum. For the purposes of this analysis the day of delivery was counted as day one. Before commencing expressing, the weight of the empty collection bottle was measured and recorded ($w_1$) and, at the end of the expression, the weight was measured and recorded again ($w_2$). Milk volume (mL) was estimated to equal the difference ($w_2 - w_1$ g) without adjustment for density of milk. If the volume was not recorded for one expression session within a 24-hour period, the average volume for the

other sessions in the day was used for the missing volume. If more than one volume was missing then the remaining data for that day were excluded. Milk samples were collected as described (Section 2.2.4), once daily in the morning on days one to ten, and analysed for concentration of lactose and citrate as described (Section 2.5)

### 4.2.2 Statistical Analysis

The primary endpoints were differences in milk volume as well as the concentration of biochemical markers of lactogenesis II in milk (lactose and citrate) with principal independent variables being gestational age at delivery and time interval between antenatal betamethasone treatment and delivery. Maternal, obstetric and neonatal factors likely to influence outcome variables were summarised by either frequency tables or by non-parametric (median (range)) or parametric (mean (sd)) methods where appropriate. Milk volume was $\log_e$-transformed to obtain normality. The concentrations in milk of both lactose and citrate were normally distributed. Analysis of variance with repeated measures was used.

Significance levels were set at 0.01 for testing interactions (to attain an overall 0.05 significance level for all contrasts) and at 0.05 for testing of main effects. With a sample size of 50, a repeated measures design could achieve almost 100% power to test for a reduction in group means of 25% for milk lactose concentration, assuming an interaction between treatment and postnatal day. The SAS statistical package version 8.2 was used for all statistical analysis.

Because of low volumes on the first postpartum day and the concern that their infants received most if not all of their colostrum, very few women collected milk samples on day one. Milk samples were provided by only four of the women who delivered before 34 weeks' gestation and ten in the overall cohort, which included women who delivered at term. In all day one milk samples obtained, concentrations of both lactose and citrate

were low with small ranges. Therefore baseline data were imputed for each missing day one sample. To allocate baseline data for each missing sample, the lactose and citrate concentration of the woman's day two samples were compared with the percentiles for day two for all samples obtained. Then the value from day one (from the overall cohort, N = 10) corresponding to the same percentile was allocated as that woman's Day One lactose or citrate concentration. If the day two value was also missing, then the value from the first day on which a sample was collected was used.

## 4.3 Results

A total of 100 women were recruited and 44 women were excluded from the present analysis because they delivered at or greater than 34 weeks' gestational age. A further six women failed to provide either milk volume measurements or milk samples leaving a sample size of 50 women. A total of 37 women provided both milk volume measurements and milk samples. A further four women provided milk samples only, and nine women measured milk volume without providing samples. Milk volume data were excluded from six women on the days when they partially breastfed their infants, but milk samples were available from all these women and milk volume was measured on days when no breastfeeding occurred, hence these women were included in the following analysis.

### 4.3.1 Descriptive statistics

Maternal demographics, and breastfeeding and smoking history are shown in Table 4.1. Data were missing for two women because the maternal questionnaire was not completed. The mean age was 32 years with the majority of women in a stable relationship but only 54% having completed secondary schooling. Almost 30% admitted to smoking during their pregnancy. A total of 58% had breastfed previously and 56% of women intended to breastfeed this infant for longer than six months.

Pre-pregnant body mass index (BMI) was estimated from maternal height and weight measured on admission to delivery suite, by subtracting the expected weight gain for the woman's gestational age from her weight when betamethasone was given. Obesity was classed as BMI greater than or equal to 30 kg/m$^2$ (N = 9).

**Table 4.1 Maternal characteristics of women who delivered before 34 weeks' gestational age and provided milk samples and/or measured milk volume**

| N = 50 | N/Mean | %/(sd) |
|---|---|---|
| Age (years) [1] | 32.2 | (5.4) |
| Maternal education – completed secondary school [2] | 26 | 54.2 |
| Maternal tertiary education [2] | 15 | 31.3 |
| Marital status – married or defacto [2] | 45 | 93.8 |
| Maternal Body Mass Index (BMI) [1,3] | 24.3 | (5.3) |
| Obese pre-pregnancy[3] | 9 | 18.0 |
| Intended breastfeeding duration [2] | | |
| Short term only | 1 | 2.1 |
| ≤ 6 months | 20 | 41.7 |
| > 6 months | 27 | 56.3 |
| Previous breastfeeding experience | 29 | 58.0 |
| Smoking [2] | | |
| During pregnancy | 14 | 29.2 |
| In postnatal period | 9 | 18.8 |

[1] Mean (sd)
[2] N = 48. Demographic data missing for 2 women
[3] Body mass index (kg/cm$^2$) imputed for pre-pregnancy. Obesity classed as BMI ≥ 30.

The obstetric characteristics are presented in Table 4.2. The index pregnancy was the first pregnancy for 40% of women with 32% in their second pregnancy and 28% in their third or greater pregnancy. A total of seven women had a twin pregnancy and seven women had used Assisted Reproductive Technology to achieve this pregnancy. The majority of women experienced preterm labour (80%) with 48% having prolonged preterm rupture of membranes, 12% having chorioamnionitis and 26% having antepartum haemorrhage. A further 12% received antenatal betamethasone treatment for high risk of preterm delivery for severe pre-eclampsia. The median duration of first stage of labour was 3.1 hours but there was a wide range of durations. Epidural analgesia was required by 70% and the mode of delivery was caesarean section for 52% of women. Two women did not receive betamethasone treatment because of insufficient time before their deliveries.

The median (range) gestational age at first betamethasone treatment was 29.4 (23.7-33.1) weeks and the median (range) interval between first betamethasone treatment and delivery was 3 (0-44) days.

**Table 4.2 Pregnancy and delivery characteristics of women who delivered before 34 weeks' gestational age and provided milk samples and/or measured milk volume**

| N = 50 | N/median | %/(range) |
|---|---|---|
| Parity | | |
| 0 | 20 | 40.0 |
| 1 | 16 | 32.0 |
| ≥ 2 | 14 | 28.0 |
| Assisted Reproductive Technology | 7 | 14.0 |
| Twin pregnancy | 7 | 14.0 |
| Obstetric complications | | |
| Pre-eclampsia/Hypertension | 6 | 12.0 |
| Preterm Labour | 40 | 80.0 |
| Prolonged Preterm Rupture of Membranes | 24 | 48.0 |
| Chorioamnionitis | 6 | 12.0 |
| Antepartum Haemorrhage (APH) | 13 | 26.0 |
| Gestational Diabetes/IDDM | 5 | 10.0 |
| Intrauterine Growth Restriction (IUGR) | 4 | 8.0 |
| Analgesia during labour and delivery | | |
| None ($NO_2$ gas) | 9 | 18.0 |
| IM/IV narcotic | 13 | 26.0 |
| Epidural Anaesthesia | 35 | 70.0 |
| General Anaesthetic | 5 | 10.0 |
| Duration of labour – 1st Stage (h) [1,2] | 3.1 | (0.1–36.8) |
| Duration of labour – 2nd Stage (h) [1,3] | 0.2 | (0.02–3.32) |

| N = 50 | N/median | %/(range) |
|---|---|---|
| Mode of delivery | | |
| Spontaneous vaginal delivery | 22 | 44.0 |
| Assisted vaginal delivery | 2 | 4.0 |
| Caesarean Section | 26 | 52.0 |
| Blood loss (ml)[1,4] | 450 | (50–5100) |
| Gestational age at first betamethasone treatment (weeks) [1,5] | 29.4 | (23.7 – 33.1) |
| Interval between first betamethasone treatment and birth (days) N = 48 [1,5] | 3 | (0 – 44) |
| 0 – 2 days | 20 | 41.7 |
| 3 – 9 days | 16 | 33.3 |
| ≥10 days | 12 | 25.0 |

[1] median (range)
[2] 13 women did not labour
[3] 24 women had no 2nd stage
[4] One woman with placenta accreta had a life-threatening postpartum haemorrhage
[5] Two women did not receive betamethasone

The neonatal characteristics are shown in Table 4.3. There were seven sets of twins. The median (range) gestational age at delivery was 30.8 (24.2 – 33.7) weeks and median (range) birth weight was 1465 (640 – 2580) g. The median (range) length of stay was 4 (0 – 113) days in the Level 3 intensive care nursery and 31 (0 – 62) days in the Level 2 special care nursery. A total of 70% infants had some respiratory distress requiring respiratory support. The method of feeding on the infants' discharge from the nursery was full breastfeeding in 29%, breastfeeding with expressed breastmilk top-ups in 47%, breastfeeding with formula top-ups in 6% infants. A total of nine mothers (18%) had ceased either breast expression or breastfeeding and were giving their infants formula only on discharge. The median (range) duration of expressing of women who had ceased breastfeeding or expressing by the time their infants were discharged from the nursery was 49 (7 – 94) days.

**Table 4.3 Characteristics of neonates who delivered before 34 weeks' gestational age and whose mothers provided milk samples and/or measured milk volume**

| N = 57 [1] | N/median | %/(range) |
|---|---|---|
| Gestational age at birth (weeks) [2] | 30.8 | (24.2–33.7) |
| < 28 weeks | 13 | 26.0 |
| 28-33 weeks | 37 | 74.0 |
| Birthweight (g) [2] | 1465 | (640–2580) |
| Male infants [1] | 40 | 70.2 |
| Nursery Admission (days) | | |
| Level 3 (Intensive Care) [2] | 4 | (0 – 113) |
| Level 2 (Special Care) [2] | 31 | (0 – 62) |
| Neonatal Complications [1] | | |
| Respiratory distress | 40 | 70.2 |
| Patent Ductus Ateriosus (PDA) requiring treatment | 11 | 19.3 |
| Sepsis | 7 | 12.3 |
| Feeding method on discharge | | |
| Fully breastfeeding | 14 | 28.6 |
| Breastfeeding + EBM | 26 | 46.9 |
| Breastfeeding + formula | 3 | 6.1 |
| Formula only | 9 | 18.4 |
| Duration of breastfeeding/expression (days) [2,3] | 49 | (7 – 94) |

[1] Includes 7 sets of twins
[2] Median (Range)
[3] Duration of breastfeeding or expression in women who ceased prior to their infants' discharge from hospital (N=9)

### 4.3.2 Milk Expression Volumes

The volume of milk expressed, and frequency and duration of expressing for each postpartum day are shown in Table 4.4. A total of 46 women recorded the volume of milk expressed in 320 days. There was a wide range of volumes obtained by individual women on each day. Milk volumes were low on the first two days but tended to increase from the first to the seventh postpartum day, stabilizing at a median of at least 450 mL. The median expression frequency was only three on the first postpartum day increasing to five or six times per day in following days and total duration of expression each day reflected this observation.

**Table 4.4 Volume, Frequency and Duration of Milk Expressions per Day (N=320 expression days from N=46 women)**

| | Volume (mL) | | Frequency of expressions (N/day) | | Duration of expressing (minutes) | |
|---|---|---|---|---|---|---|
| Postnatal day | Median | Range | Median | Range | Median | Range |
| 1 | 5.5 | 0-105.0 | 3 | 1-6 | 45 | 0-100 |
| 2 | 19.0 | 0-213.1 | 5 | 1-9 | 121 | 20-234 |
| 3 | 76.5 | 0-455.7 | 5 | 1-10 | 110 | 20-280 |
| 4 | 229.5 | 25.6-736.0 | 6 | 4-8 | 140 | 62-280 |
| 5 | 322.9 | 56.0-760.1 | 5 | 3-8 | 144 | 70-245 |
| 6 | 442.5 | 50.0-860.2 | 6 | 3-8 | 140 | 50-345 |
| 7 | 540.0 | 133.0-1607.8 | 6 | 2-8 | 125 | 68-265 |
| 8 | 459.0 | 112.0-1104.8 | 6 | 4-8 | 125 | 60-340 |
| 9 | 571.4 | 133.0-1164.2 | 6 | 4-8 | 145 | 58-225 |
| 10 | 531.0 | 135.0-1141.0 | 5 | 3-8 | 130 | 60-275 |

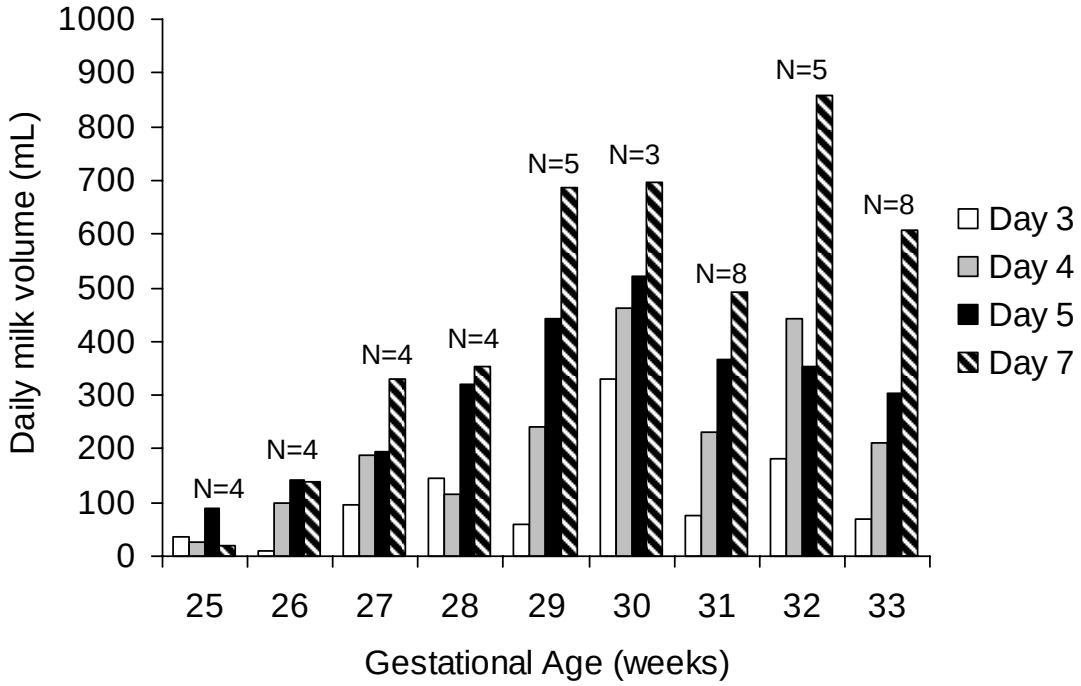
#### *4.3.2.1 Effect of Gestational Age at Delivery on Milk Volume*

When the daily volume of milk expressed was stratified by gestational age at delivery (in weeks), there was a wide range of milk volumes obtained. Median values for postpartum days three, four, five and seven are shown in Figure 4.1. Overall, milk volume increased from day one to day seven but volumes obtained by women who delivered at earlier gestational ages (< 28 weeks' gestation) were reduced compared with volumes obtained by women who delivered at later gestational ages (Figure 4.1).

For ease of interpretation Figures 4.2 to 4.8 depict gestational age at delivery in two groups: < 28 weeks' and ≥ 28 weeks' gestational age. This *post hoc* division is arbitrary and is designed purely for ready interpretation of the following results and graphs. The cut-off gestational age of 28 weeks was used for convenience but it also allowed sufficient numbers in each group and reflected the general distinction, by many authors, between very preterm and extremely preterm infants (Field, Petersen *et al.* 2002). The statistics for the following analyses, however, refer to milk volume obtained when gestational age is expressed as a continuous variable. At all times, the analyses are adjusted for postpartum day.

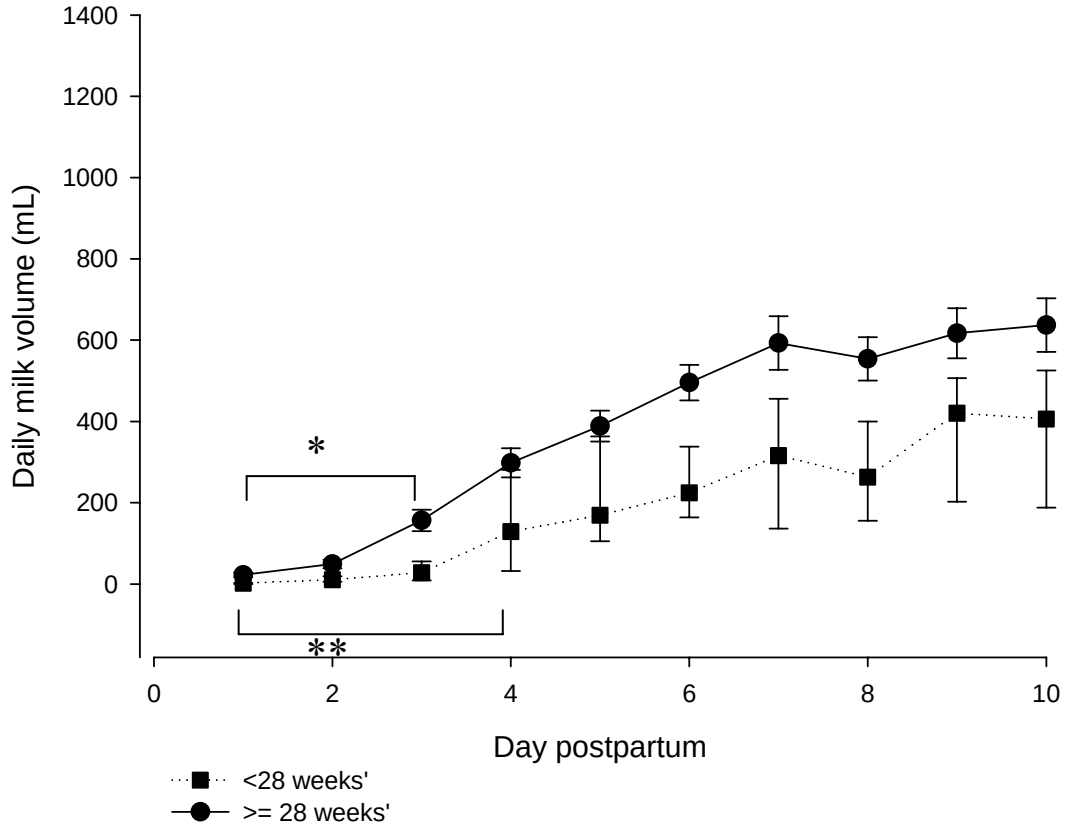
Milk volume was significantly associated with gestational age and day postpartum when gestational age at delivery was expressed as a continuous variable (P = 0.017). Positive significant associations were found between increasing milk volumes and advancing gestational age at delivery as well as advancing day postpartum. Daily volume stratified by gestational age groups: <28 weeks' and ≥ 28 weeks' gestation is shown in Figure 4.2. Milk volume was first significantly increased from day one postpartum on day three in women whose gestational age at delivery was 28-33 weeks, but not until day four postpartum in women who delivered before 28 weeks.

**Figure 4.1 Total Volume of Milk Expressed on Postpartum Days 3, 4, 5, and 7 Stratified by Gestational Age Week at delivery**




Note: histograms represent median 24-hour milk volume on day 3 (white histograms), 4 (grey histograms), 5 (black histograms) and 7 (diagonal black/white histograms) by women who delivered between 25 – 33 weeks' gestational age on the x-axis. Error bars are not shown as there were low numbers (N = 3 to 8) and variable range in many gestational age weeks. Milk volume increased significantly with postnatal day ($P < 0.001$) and gestational age ($P = 0.017$).

**Figure 4.2 Total Volume of Milk Expressed on Days 1 to 10 Postpartum Stratified by Gestational Age at Delivery**




Note: symbols represent median (IQR) milk volume expressed per day on days 1 to 10 postpartum in gestational age groups <28 weeks (black squares, dotted line; N = 13) and ≥28 weeks (black circles, solid line; N = 37) at delivery. There were significantly lower volumes obtained at earlier gestational ages at delivery (P = 0.017).

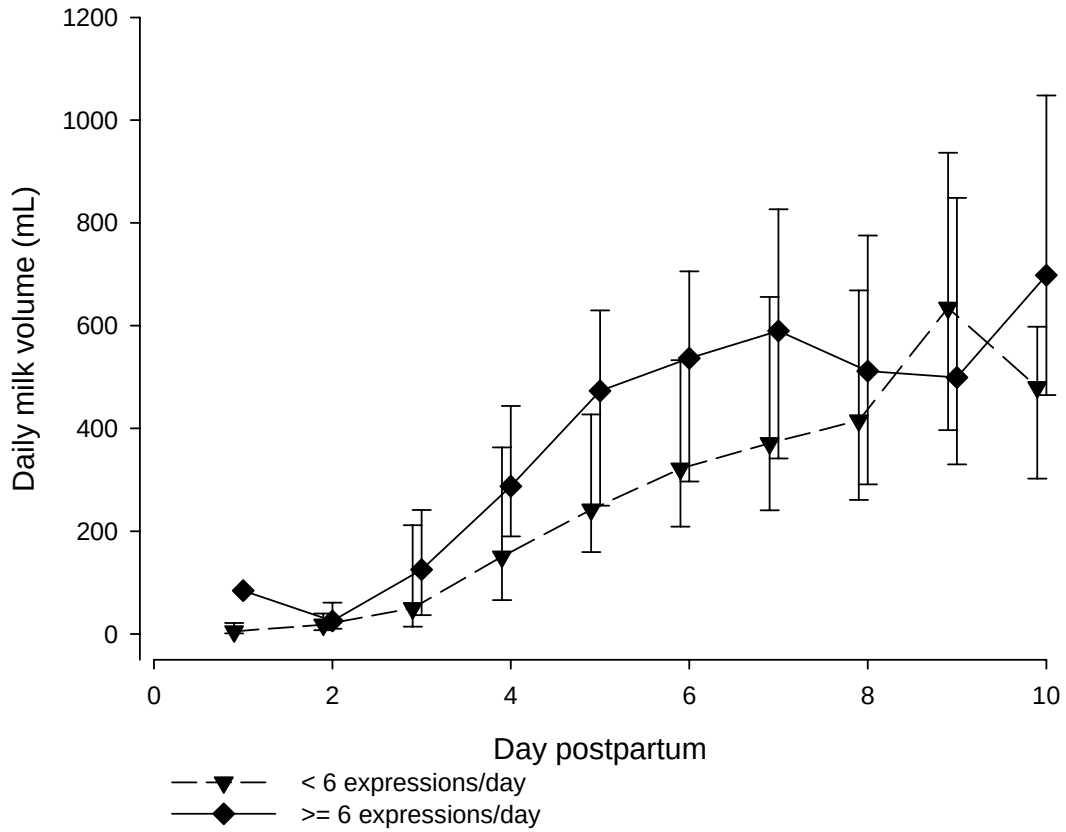
* Gestational age ≥ 28 weeks: Significant increases in volume from day 1 first occurred on day 3 postpartum (P < 0.001).

** Gestational age < 28 weeks: Significant increases in volume from day 1 first occurred on day 4 postpartum (P < 0.001).

#### *4.3.2.2 Effect of Frequency of Breast Expression on Milk Volume*

There was a significant effect of frequency of expressing on daily volume ($P < 0.001$). Milk volume increased with increasing frequency of expressing on nearly all postpartum days. This is depicted in Figure 4.3 where women who expressed six or more times per day are shown to obtain significantly greater milk volumes than women who expressed less frequently. Note there was no significant effect of gestational age on frequency of expressing ($P = 0.650$).

**Figure 4.3 Total Volume of Milk Expressed on Days 1 to 10 Postpartum Stratified by Number of Expressions per Day**




Note: symbols represent median (IQR) milk volume expressed per day stratified by the number of expressions per day. There were significantly higher volumes obtained when women expressed 6 or more times per day ($P < 0.001$).

#### *4.3.2.3 Effect of Betamethasone Treatment on Milk Volume*

There was no effect shown of time interval between antenatal betamethasone treatment and delivery on milk volume when the interval was expressed as a continuous variable. However on observation of data patterns it appeared that the relationship between betamethasone interval and milk volume was not linear. After experimenting with categorizing the steroid interval into different categories, it appeared that the effect of betamethasone-delivery interval on milk volume was U-shaped, that is there was greater volume when delivery occurred either soon after betamethasone treatment (within 48 hours of first dose) or ten days or greater after treatment (Figure 4.4b). Volumes were reduced when the interval between treatment and birth was three to nine days and there was little difference in effect between different categories within the 3 – 9 day interval. The interval between treatment and delivery was between zero and two days in 42%, between three and nine days in 33%, and ten days or greater in 25% of women (Table 4.2).

When the interval between betamethasone treatment and birth was expressed as a categorical variable with categories 0 – 2 days, 3 – 9 days and ≥10 days, there was a trend towards a significant interaction between gestational age at delivery and betamethasone treatment interval after adjustment for postnatal day (P= 0.088). This effect is shown in Figure 4.4. Overall there was a trend for women who delivered 0 – 2 days after treatment to obtain greater volumes than women who delivered 3 – 9 days after treatment (P = 0.086). Women who delivered ≥10 days after betamethasone treatment obtained daily volumes that were not significantly different from the other treatment-delivery intervals (compared with women who delivered 0 – 2 days after treatment, P = 0.732; compared with women who delivered 3 – 9 days after treatment, P = 0.446).

When delivery occurred before 28 weeks' gestation there was no difference in milk volume at different intervals between betamethasone treatment and delivery although there were small numbers in each treatment interval group at earlier gestational ages (Figure 4.4a). In contrast, when delivery occurred at more advanced gestational ages (e.g. between 28 and 33 weeks' gestation) there were differences between treatment interval groups (Figure 4.4b). Women who delivered 3 – 9 days after treatment obtained significantly reduced volumes compared with women who delivered 0 – 2 days after treatment.

**Figure 4.4 Total Volume of Milk Expressed on Days 1 to 10 Stratified by Interval between Betamethasone Treatment and Delivery by Women whose Gestational Age at Delivery was either < 28 weeks or ≥ 28 weeks**

Figure 4.4a Gestational age at delivery < 28 weeks

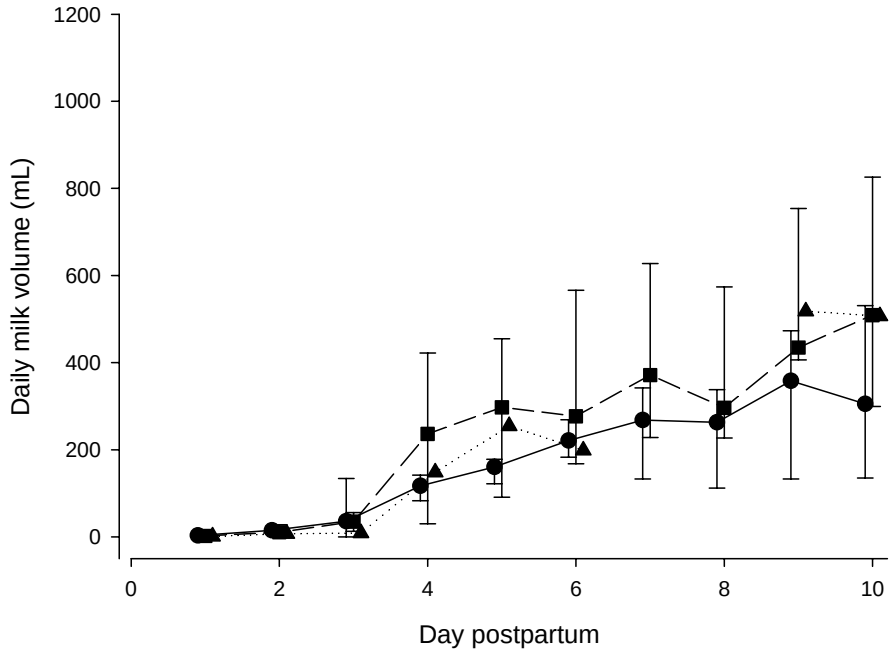



Figure 4.4b Gestational age at delivery 28-33 weeks

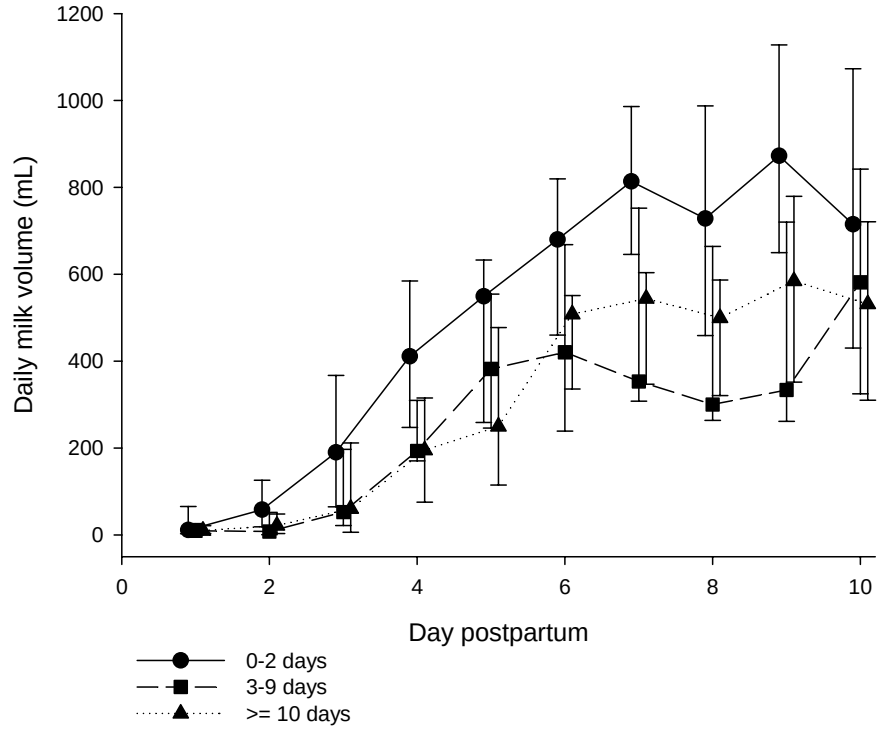


Figure 4.4 Notes

Symbols represent median (IQR) milk volume expressed per day when delivery occurred 0 – 2 days (black circles, solid line), 3 – 9 days (black squares, dashed line), and ≥ 10 days (black triangles, dotted line) after betamethasone treatment. Gestational age at birth modified the effect of betamethasone treatment-delivery interval on milk volume (P value for interaction = 0.088).

Figure 4.4a represents gestational age < 28 weeks: there was no difference in milk volume between different intervals between betamethasone treatment and delivery

Figure 4.4b represents gestational age ≥ 28 weeks: women who delivered 3 – 9 days after betamethasone treatment expressed the least milk volume and women who delivered 0 – 2 days after betamethasone treatment expressed the greatest volume.

#### *4.3.2.4 Effect of Betamethasone Treatment on Average Milk Volume per Expression*

Because of the significant effect of expression frequency, milk volume was analysed as average volume per expression. Using the interval between betamethasone treatment and birth expressed as a categorical variable, there was a significant interaction between gestational age at delivery and betamethasone treatment interval and a significant effect of treatment interval after adjustment for day postpartum (P = 0.036). This effect is shown in Figure 4.5. At more advanced gestational ages (28 to 33 weeks) the average volume was significantly reduced when the betamethasone-delivery interval was 3 – 9 days compared with 0 – 2 days (Figure 4.5b). This difference did not persist at either earlier gestational ages or at earlier postnatal days.

**Figure 4.5 Average Milk Volume per Expression on Days 1 to 10 Stratified by Interval between Betamethasone Treatment and Delivery by Women whose Gestational Age at Delivery was either < 28 weeks or ≥ 28 weeks**

Figure 4.5a Gestational age at delivery < 28 weeks

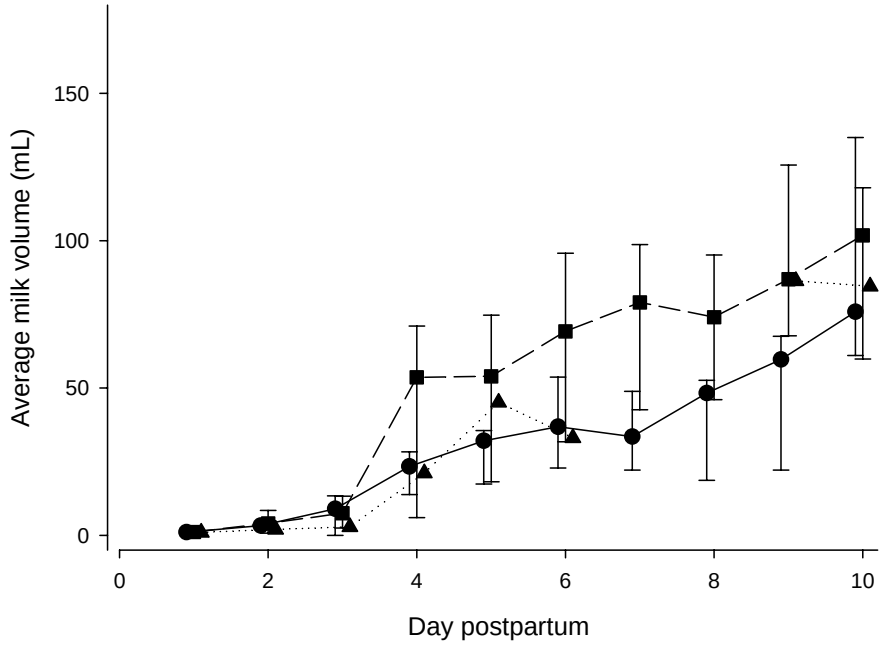



Figure 4.5b Gestational age at delivery 28-33 weeks

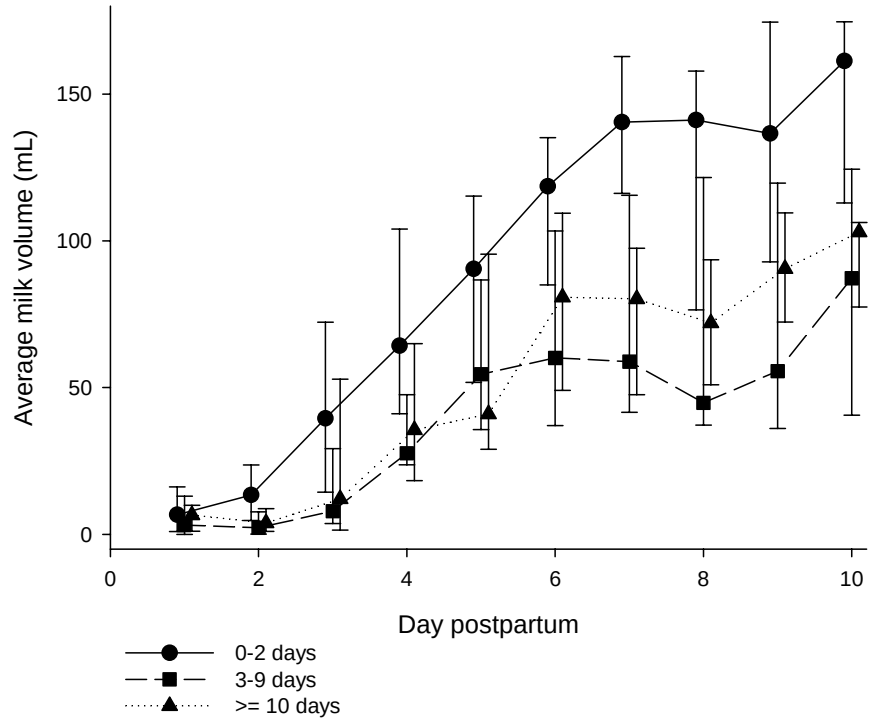


Figure 4.5 Notes

Symbols represent median (IQR) average milk volume expressed per day when delivery occurred 0 – 2 days (black circles, solid line), 3 – 9 days (black squares, dashed line), and ≥ 10 days (black triangles, dotted line) after betamethasone treatment.

Figure 4.5a represents gestational age < 28 weeks: there was no difference in average milk volume between different intervals between betamethasone treatment and delivery

Figure 4.5b represents gestational age ≥ 28 weeks: women who delivered 3 – 9 days after betamethasone treatment expressed the least average milk volume per day and women who delivered 0 – 2 days after betamethasone treatment expressed the greatest average volume per day.

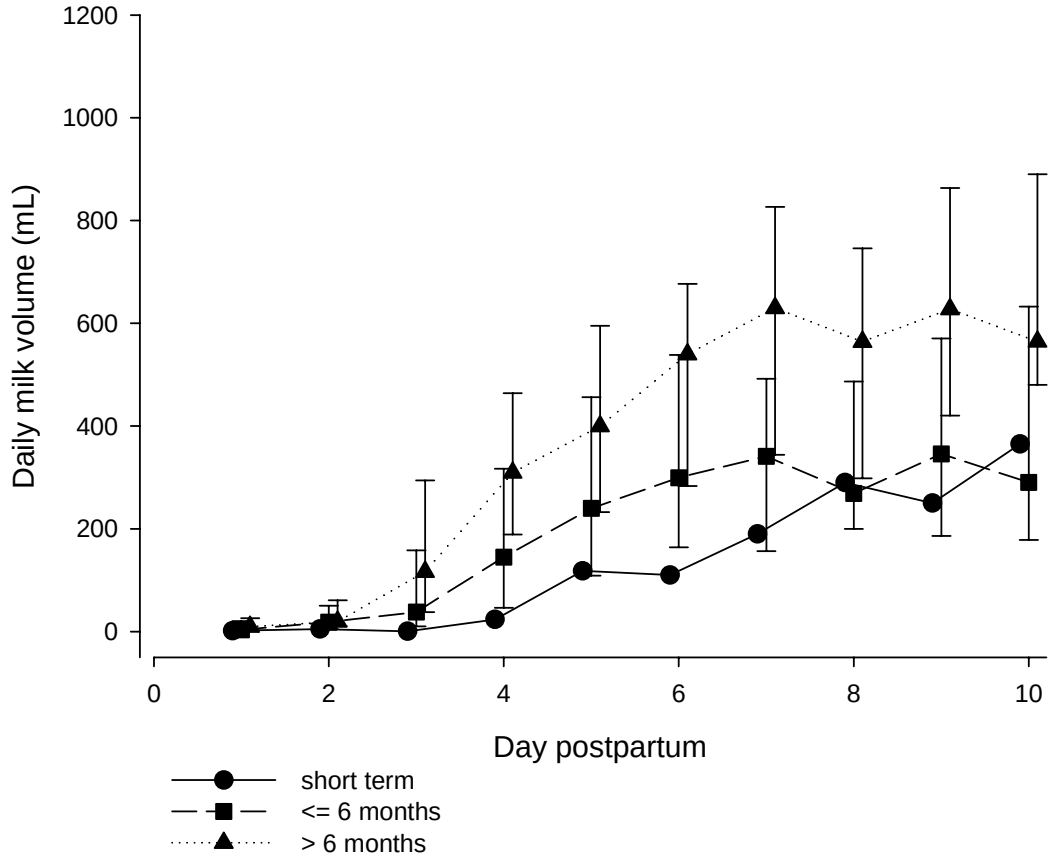
#### *4.3.2.5 Effect of other characteristics on milk volume*

No effect was found on milk volume for maternal age, maternal education, smoking during pregnancy or postpartum, parity, twins, any antenatal complication including maternal diabetes, epidural, mode of delivery, duration of labour, time when narcotics given, blood loss, birth weight, previous breastfeeding experience, gestational age when betamethasone was given, or any interactions between these factors and gestational age at birth. After adjustment for day postpartum, pre-pregnant obesity was significantly associated with a reduced milk volume ($P = 0.019$). On day three postpartum, expressed milk volume was significantly reduced in obese women compared with non obese women ($P < 0.001$) suggesting a delayed initiation of lactation.

There was a significant effect of maternal intention for breastfeeding duration as stated in the questionnaire completed by the mothers in the first week postpartum (Figure 4.6; $P = 0.002$). If women stated they intended to breastfeed for longer than six months, significant increases in volume were observed on day three, whereas if the intended breastfeeding duration was less than 6 months increases in volume occurred on day four. Gestational age at delivery did not change this effect but parity modified the association. Among primiparas, intended breastfeeding duration was significantly associated with milk volume ($P = 0.018$), whereas there was only a trend to significance in multiparas ($P = 0.075$).

Note no relationship was found between intended duration of breastfeeding and frequency of expressing ($P=0.780$).

**Figure 4.6 Total Volume of Milk Expressed on Days 1 to 10 Postpartum Stratified by Intended Duration of Breastfeeding**




Note: symbols represent median (IQR) milk volume expressed per day stratified by intended duration of breastfeeding: short term only (black circles, solid line), ≤ 6 months (black squares, dashed line) or > 6 months (black triangles, dotted line). There were significantly higher volumes obtained when women intended to breastfeed for longer than 6 months ($P < 0.001$).

#### *4.3.2.6 Summary of Milk Volume Results*

There was a wide range of milk volumes obtained between individual women across different postpartum days. Milk volumes increased from 5.5 mL on the first postpartum day to 531 mL on day 10 with the largest increases occurring between days three and four. There was also a wide range of frequency of expressing – from as low as one to a maximum of ten expressions per day. There was a significant positive association between milk volume and frequency of expressing. Increased volumes were obtained with greater frequency of expressing on each postpartum day.

Milk volume was significantly associated with gestational age at birth with milk volume increasing with more advanced gestational ages. There was a significant interaction between gestational age at delivery and the interval between betamethasone treatment and delivery with a trend to significance for the effect of treatment-interval on milk volume and the greatest differences occurring at more advanced gestational ages. This was more clearly seen when average volume per expression was used. The association between interval between betamethasone treatment and delivery and average milk volume, adjusted for gestational age at delivery and postnatal day was significant (P=0.036). At more advanced gestational ages, women who delivered between three and nine days after treatment were significantly more likely to have reduced average milk volumes compared both with women who delivered two days or less after treatment and women who delivered ten days or more after treatment. In contrast, among women who delivered at earlier gestational ages, those women who delivered less than three days after betamethasone treatment were more likely to have lower milk volumes per expression.

There was a significant positive association between intended duration of breastfeeding and milk volume. Women who intended to breastfeed for long periods had significantly

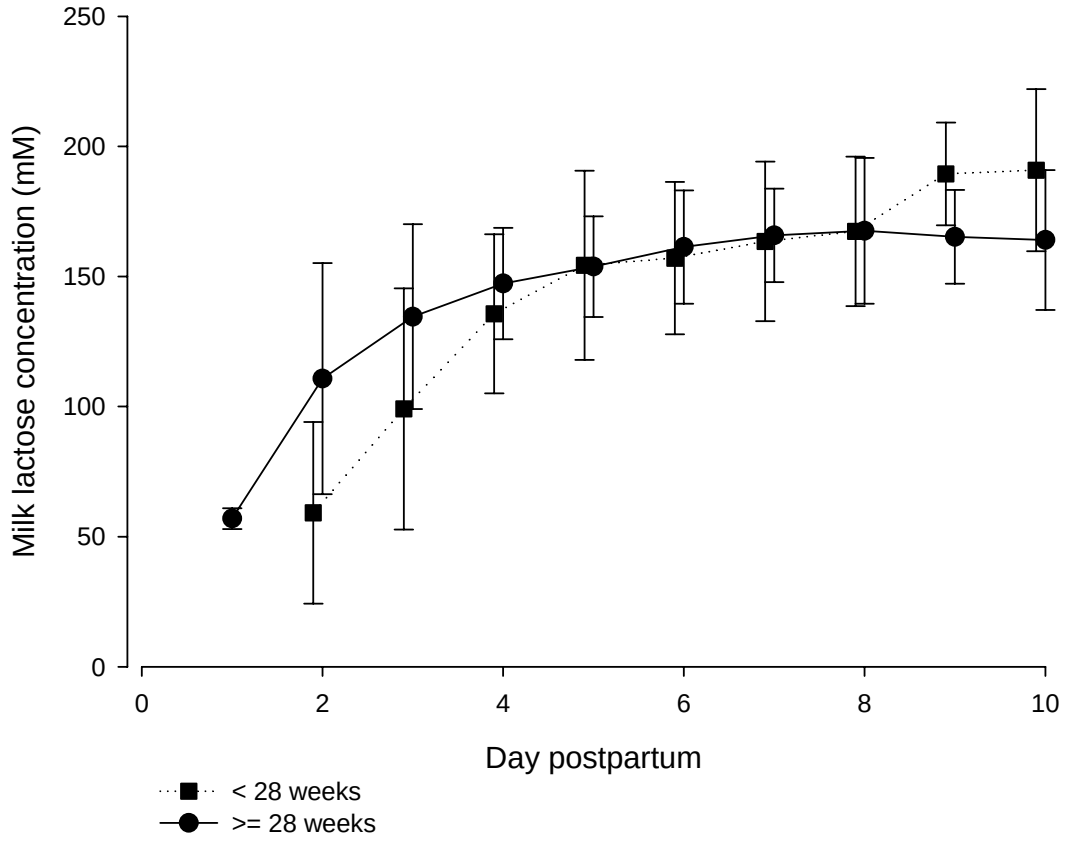
higher daily volumes. This finding was unrelated to frequency of expression. No other demographic, obstetric or neonatal factor was associated with milk volume except for maternal obesity.

### 4.3.3 Concentration of Lactose in Milk

A total 68 women from the whole cohort (including women who delivered at term) provided 516 milk samples for analysis. The following analysis comprises 324 samples from 41 mothers of infants born less than 34 weeks' gestational age. Lactose concentration data were weighted for women who gave more than one sample per day and were normally distributed. The mean ± sd lactose concentration was 156.800 ± 36.217 mM.

There was a significant effect of postnatal day ($P < 0.001$) and gestational age on milk lactose concentration (Figure 4.7, $P < 0.001$). Milk lactose concentration increased with increasing days postpartum. Lower values were obtained with earlier gestational ages at delivery. With increasing gestational age at delivery milk lactose concentration was increased.

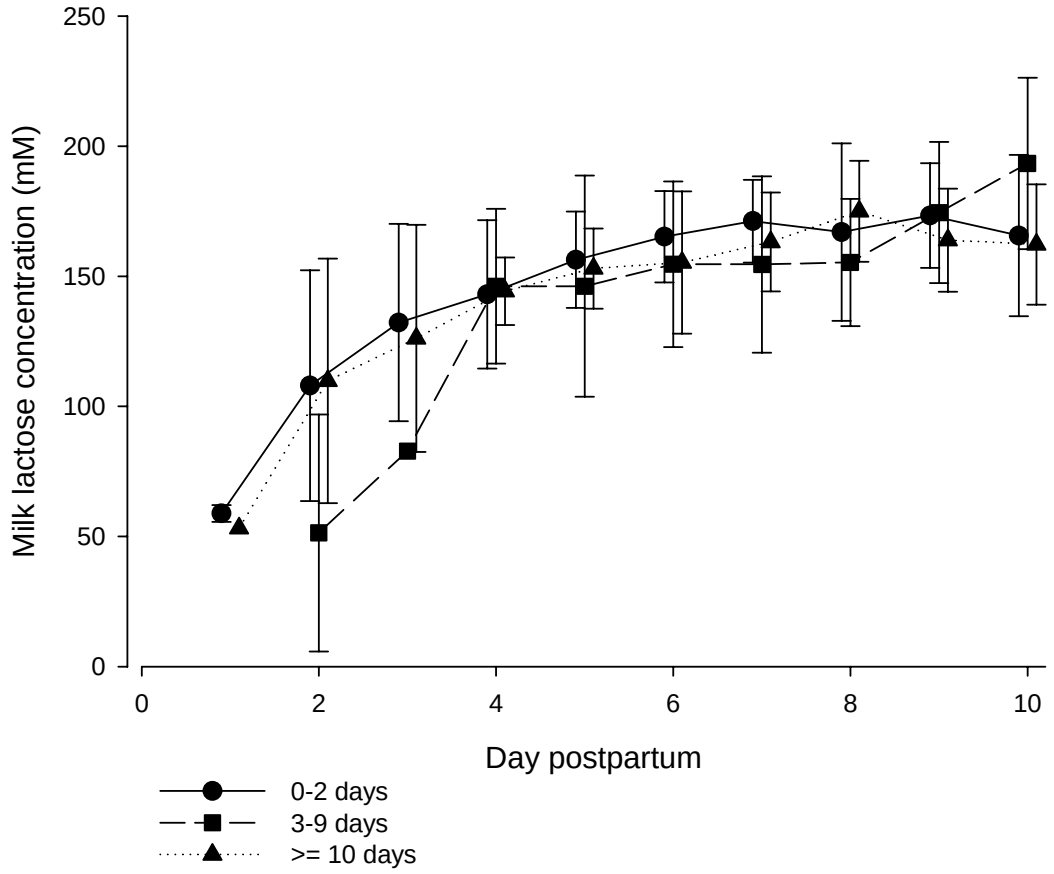
**Figure 4.7 Concentration of Lactose in Milk on Days 1 to 10 Postpartum Stratified by Gestational age at Delivery**




Note: Symbols represent mean ± sd concentration of lactose in milk (mM) stratified by gestational age at delivery. For the first 3 days postpartum, milk expressed by women who delivered earlier than 28 weeks' gestational age (squares, dotted line) had significantly lower concentration of lactose than those who delivered at 28 weeks or greater (circles, solid line) ($P < 0.001$).

There was a significant effect of frequency of breast expression on milk lactose concentration (P = 0.010). This was most marked on day two postpartum where women who expressed six to eight times a day had significantly higher milk lactose concentration compared with women who only expressed two to five times per day. No other maternal, obstetric or neonatal factor had any significant association with milk lactose concentration, including maternal obesity, intended duration of breastfeeding or the infant taking any breastfeeds. After adjustment for postpartum day, the interval between antenatal betamethasone treatment and delivery was not associated with milk lactose concentration (Figure 4.8; P = 0.857), nor was there any interaction with gestational age at delivery (P = 0.548).

**Figure 4.8 Concentration of Lactose in Milk on Days 1 to 10 Postpartum Stratified by Interval between Betamethasone Treatment and Delivery**




Note: Symbols represent mean ± sd milk lactose concentration (mM) stratified by interval between betamethasone treatment and delivery; with women who delivered two days or less after betamethasone treatment (circles, solid line), those who delivered between three and nine days after betamethasone (squares, dashed line), and those who delivered ten days or greater after betamethasone (triangles, dotted line). Note one woman in the three to nine day treatment interval group had consistently low lactose concentration on all postpartum days. There was no significant difference between groups (P = 0.857).

### 4.3.4 Concentration of Citrate in Milk

A total of 484 milk samples were available for analysis of citrate concentration and 322 samples from 41 mothers of infants born less than 34 weeks' gestational age were used in this analysis. Citrate concentration data were weighted for women who gave more than one sample per day and data were normally distributed. The mean ± sd citrate concentration was 3.458 ± 1.442 mM.

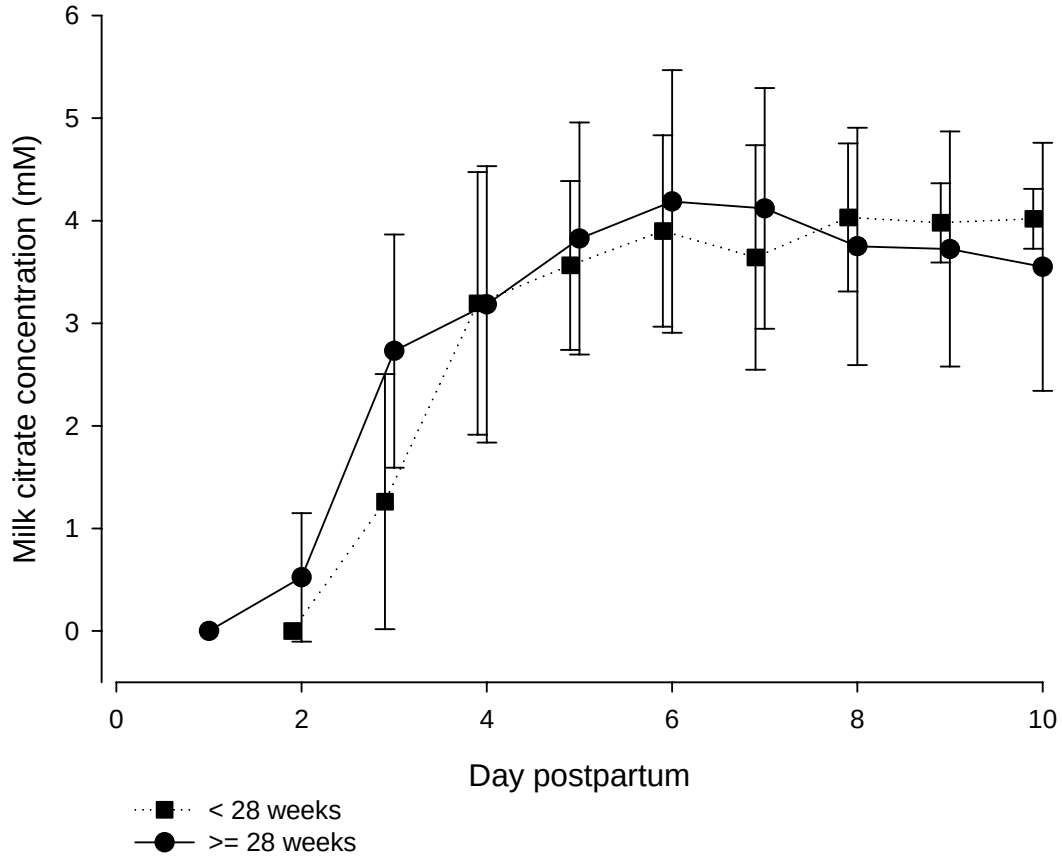
Concentration of citrate in milk was significantly associated with postpartum day ($P = 0.003$), and there was a trend to significance for the effect of gestational age (Figure 4.9, $P = 0.082$). When the gestational age at birth was less than 28 weeks there were significant increases in milk citrate concentration by day three postpartum. When the gestational age at delivery was more advanced, significant increases occurred by day two. In addition, citrate concentration values peaked on day 6 in women who delivered at gestational ages < 30 weeks and then started to reduce. This occurred on day 5 at gestational ages ≥ 30 weeks.

Frequency of expressing was significantly associated with milk citrate concentration ($P = 0.010$). Women who expressed five or fewer times per day had lower concentrations of citrate in their milk. Parity was also significantly associated with milk citrate concentration ($P = 0.004$). Primiparas had significantly higher milk citrate concentration than women who had given birth to more than one child. Women who delivered by non-elective caesarean section also had significantly higher milk citrate levels than other modes of delivery ($P = 0.022$). No other maternal, obstetric or neonatal factor had any significant association with milk citrate levels, including maternal obesity, intended duration of breastfeeding or the infant taking any breastfeeds.

There was also no association between milk citrate concentration and interval between betamethasone treatment and delivery ($P = 0.312$) although there was a non-significant

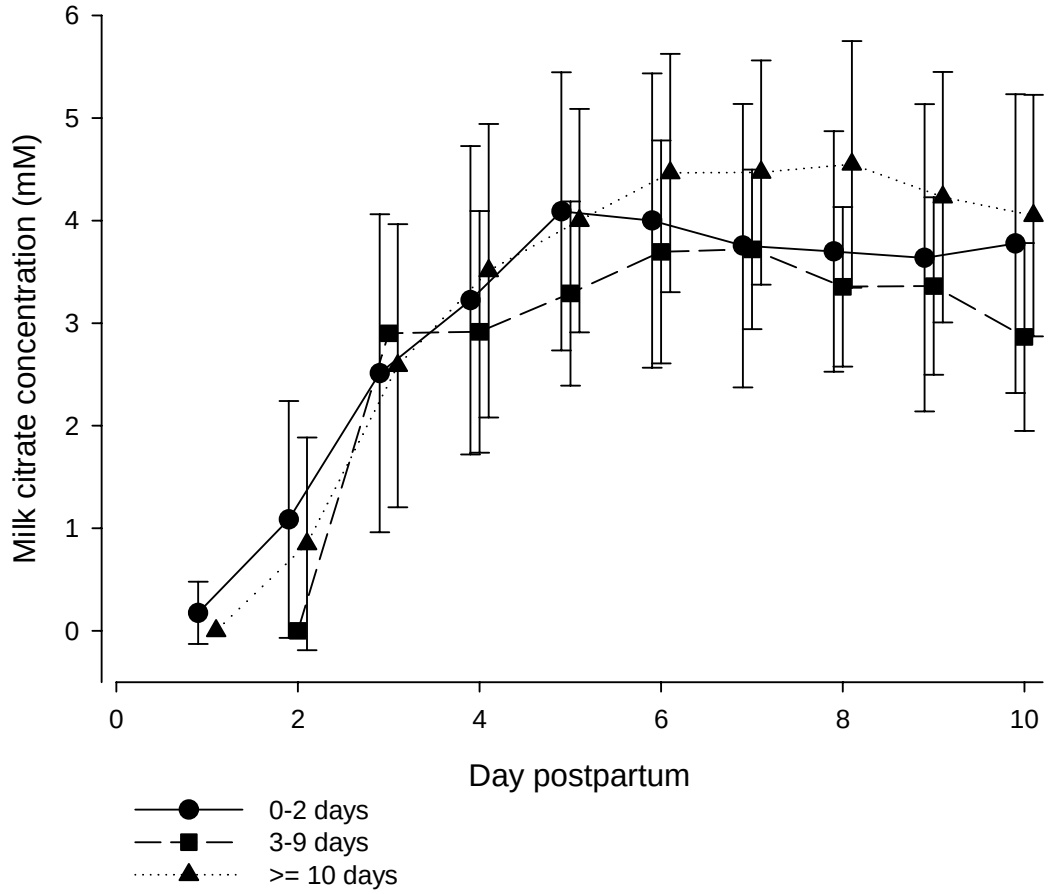
reduction in citrate concentration when the interval between treatment and delivery was 3 to 9 days compared with shorter and longer intervals at all gestational ages at delivery less than 33 weeks (Figure 4.10).

**Figure 4.9 Concentration of Citrate in Milk on Days 1 to 10 Postpartum Stratified by Gestational age at Delivery**




Note: Symbols represent mean ± sd concentration of citrate in milk (mM) stratified by gestational age at delivery. Before day 4 postpartum, women who delivered earlier than 28 weeks' gestational age (squares, dotted line) tended to have lower concentrations of citrate in milk than those who delivered at 28 weeks or greater (circles, solid line) (P = 0.082).

**Figure 4.10 Concentration of Citrate in Milk on Days 1 to 10 Postpartum Stratified by Interval between Betamethasone Treatment and Delivery**



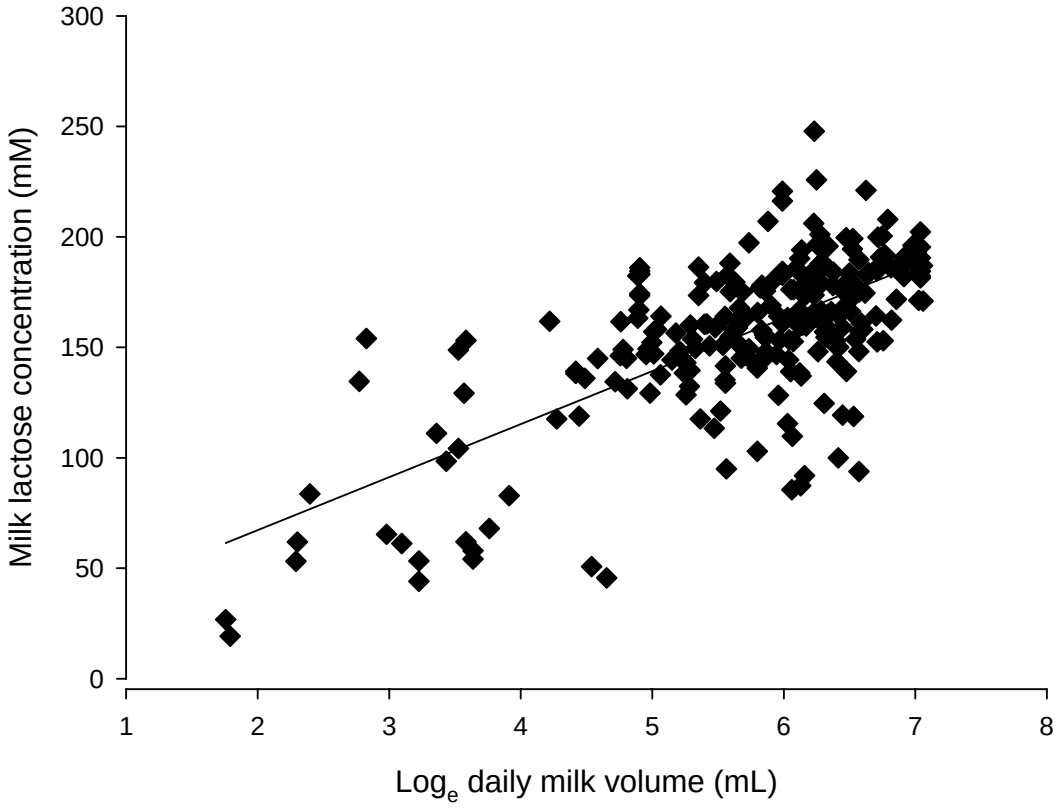

Note: Symbols represent mean ± sd milk citrate concentration (mM) stratified by interval between betamethasone treatment and delivery; with women who delivered two days or less after betamethasone treatment (circles, solid line), those who delivered between three and nine days after betamethasone (squares, dashed line), and those who delivered ten days or greater after betamethasone (triangles, dotted line). There was no significant difference between groups (P = 0.312).

### 4.3.5 Relationship between Milk Volume and Concentration of Lactose and Citrate in Milk

The relationships between milk volume and concentrations of both lactose and citrate in milk are shown in Figures 4.11, 4.12 and 4.13 respectively. The relationships with milk volume were not linear for either milk component, therefore milk volume was $\log_e$ transformed for this analysis. In the subgroup of women who delivered before 34 weeks' gestational age there were significant associations between log milk volume and the concentrations of lactose (Figure 4.11, $P < 0.001$) and citrate (Figure 4.12, $P < 0.001$) in milk. Increasing log volume was significantly associated with both increasing lactose concentration and increasing citrate concentration. In addition, milk lactose concentration was significantly associated with milk citrate concentration (Figure 4.13, $P = 0.008$).

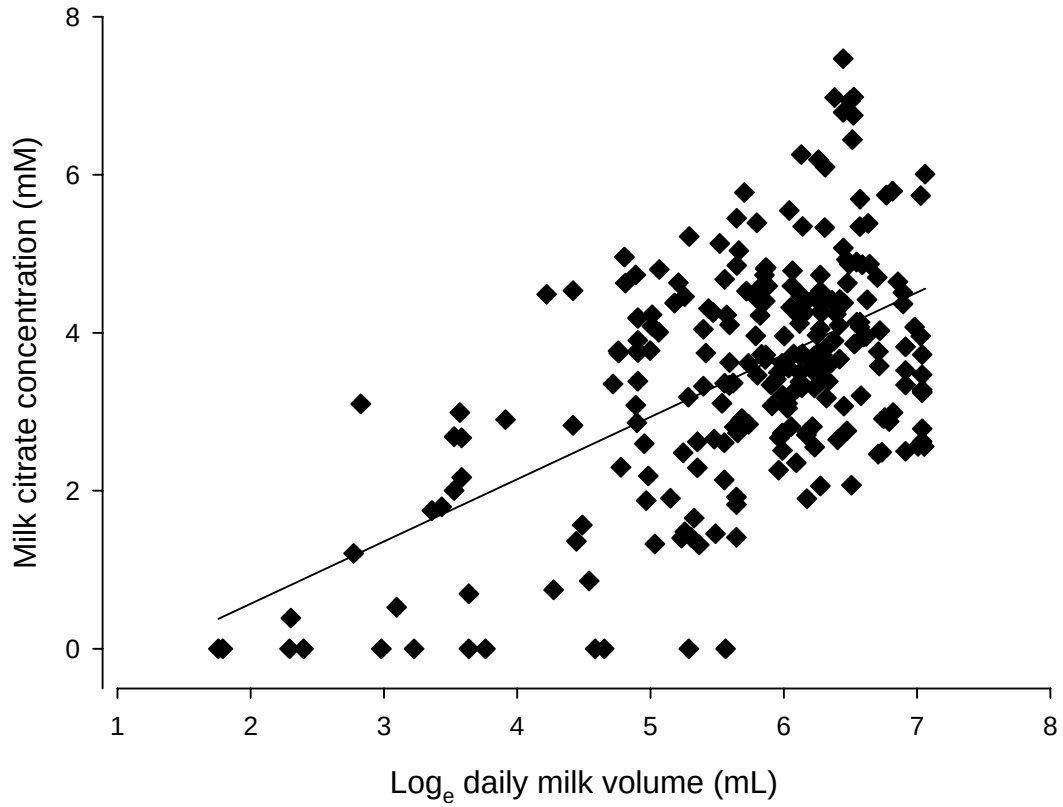
The relationship between milk lactose concentration and log milk volume was compared between different postnatal days (Figure 4.14). The positive association was found only on days one to three ($P = 0.036$), but not on days four to seven ($P = 0.175$) or days eight to ten ($P = 0.350$).

**Figure 4.11 Relationship between Milk Lactose Concentration and Log Milk Volume on Days 1 to 10 Postpartum**



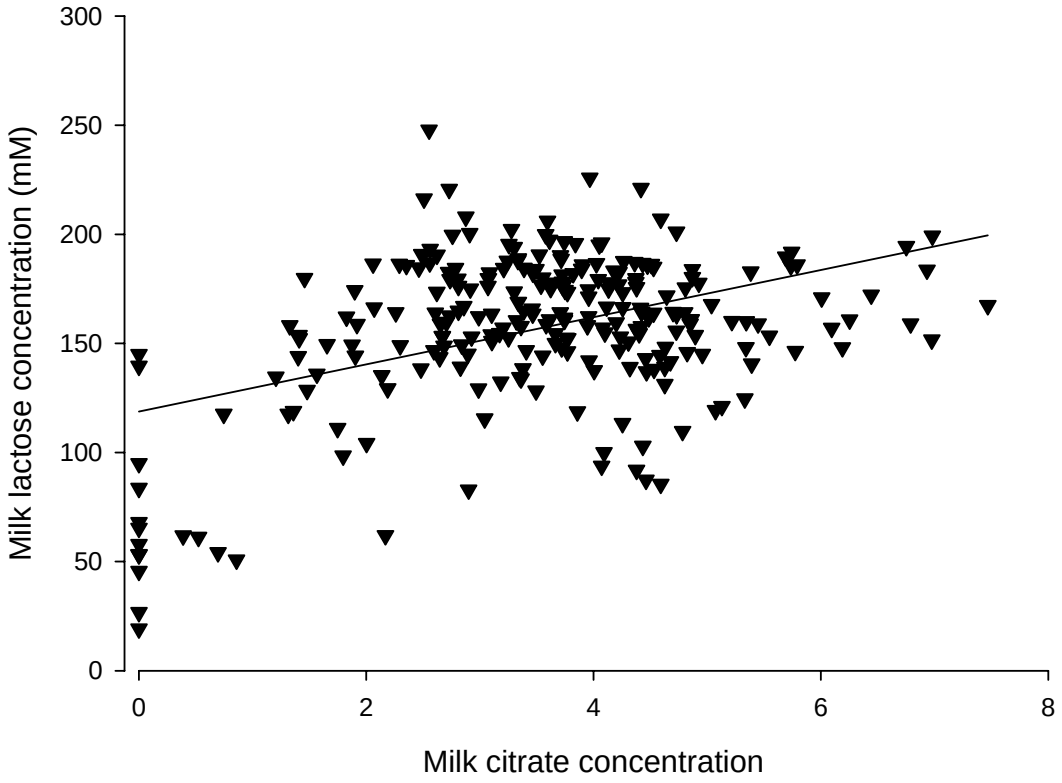

Note: Symbols represent $\log_e$ total daily milk volume plotted against milk lactose concentration on days 1 to 10 postpartum. There was a significant positive association between milk volume and milk lactose concentration, $R^2 = 0.464$, $P < 0.001$

**Figure 4.12 Relationship between Milk Citrate Concentration and Log Milk Volume on Days 1 to 10 Postpartum**




Note: Symbols represent $\log_e$ total daily milk volume plotted against milk citrate concentration. There was a significant positive association, $R^2 = 0.310$, $P < 0.001$. There were 11 samples in which concentration of citrate in milk was too low to detect.

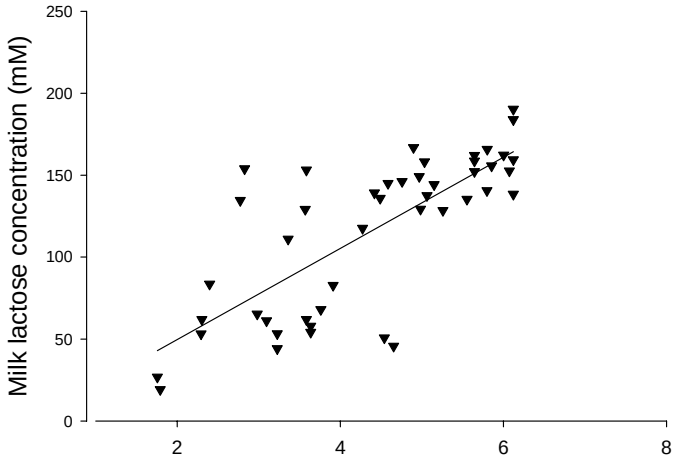
**Figure 4.13 Relationship between Milk Lactose Concentration and Milk Citrate Concentration on Days 1 to 10 Postpartum**



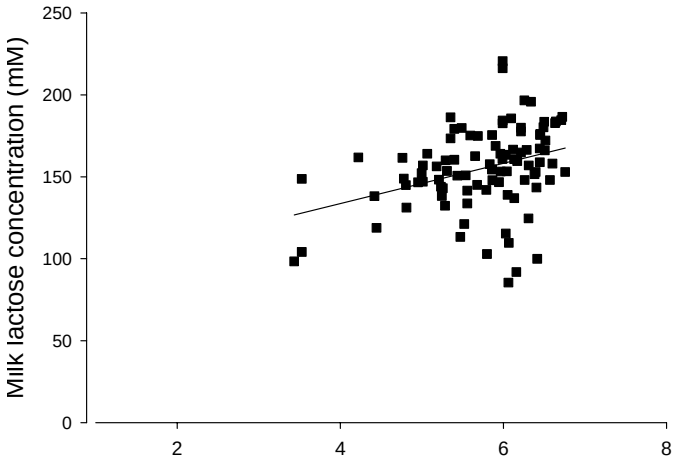

Note: Symbols represent milk lactose concentration plotted against milk citrate concentration. There was a significant positive association between milk lactose concentration and milk citrate concentration, $R^2 = 0.191$, $P = 0.008$. There were 11 samples in which concentration of citrate in milk was too low to detect.

**Figure 4.14 Relationships between Milk Lactose Concentration and Log Total Daily Milk Volume Stratified by Day Postpartum**

**4.14a. Days 1-3 postpartum**




**4.14b. Days 4-7 postpartum**




**4.14c. Days 7-10 postpartum**

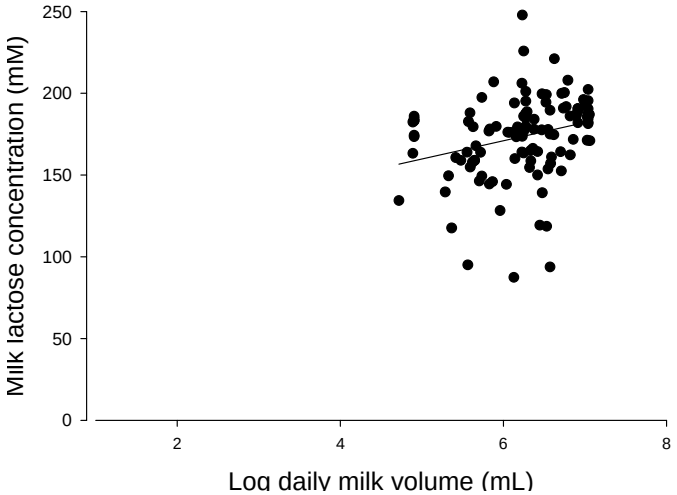


Figure 4.14 Notes: Symbols represent $\log_e$ 24-hour milk volume plotted against milk lactose concentration on either days 1-3, 4-7 or 7-10 postpartum.

Figure 4.14a: Days 1-3 postpartum (black triangles). Log milk volume was significantly associated with milk lactose concentration ($P = 0.036$, $R^2 = 0.574$).

Figure 4.14b: Days 4-7 postpartum (black squares). Log milk volume was not significantly associated with milk lactose concentration ($P = 0.118$, $R^2 = 0.175$).

Figure 4.14c: Days 7-10 postpartum (black circles). Log milk volume was not significantly associated with milk lactose concentration ($P = 0.350$, $R^2 = 0.072$).

## 4.4 Discussion

These data represent the first attempt to describe the effects of antenatal glucocorticoid treatment on lactogenesis II in women who deliver preterm. These findings suggest that the strongest association is with gestational age at birth and that, if antenatal glucocorticoids have an impact on lactogenesis II in women, then this effect is mild and dependent on gestational age and the time interval between glucocorticoid treatment and delivery. At more advanced preterm gestational ages, women who delivered between three and nine days after being treated with betamethasone obtained average milk volumes per expression session that were significantly reduced compared with women who delivered immediately after the betamethasone treatment. On the other hand among women who delivered at extremely preterm gestational ages, there was no difference, suggesting either insufficient power due to low numbers or possibly that lactocytes may not be sufficiently developed at this early gestational age for synthetic glucocorticoids to have a significant lactogenic effect. The significant association with antenatal betamethasone treatment did not persist when either milk lactose or citrate levels were used as markers of lactogenesis II.

These findings were less conclusive than those discovered in sheep (Chapter 3), which are known to be very sensitive to glucocorticoids compared with women. For example, antenatal glucocorticoid treatment in sheep is associated with a high risk of abortion and requires treatment with a progesterone analogue to reduce this risk (Nathanielsz, Buster et al. 1988; Jobe, Newnham et al. 2003). There was, however, a wide range of responses to antenatal glucocorticoids by individual women. I speculate that a lactogenic effect of betamethasone treatment may only occur in some individual women who are more sensitive than others, particularly in light of the somewhat arbitrary dose of betamethasone that is currently used in humans. However, analysis of either maternal or

obstetric factors that may be expected to modify individual responses, such as maternal age, parity and previous breastfeeding, twins, antenatal complications, mode of delivery and analgesia, blood loss and infant birthweight did not find any co-factor other than gestational age which predicted milk volume.

### 4.4.1 Effect of Gestational age at Delivery on Lactogenesis II

In the present study, gestational age at delivery strongly predicted time of onset of lactogenesis II when measured either by change in milk volume or by increase in lactose concentration. In the women who delivered at extremely premature gestational ages (< 28 weeks), significant increases in milk volume from day one did not occur until the fourth day postpartum. In comparison, women who delivered at more advanced, but still very preterm gestational ages had significant increases in volume on the third day postpartum. Delays in the time taken for significant increases in milk volume and concentration of lactose and citrate in milk to occur suggest significant delays in lactogenesis II at extremely early gestational ages. These findings compare adversely with studies of mothers of term infants which showed the onset of lactogenesis II generally occurs within two days of the birth (Kulski, Smith *et al.* 1977; Neville, M C, Allen *et al.* 1991) but are consistent with another study which suggested a delay in lactogenesis II in mothers of preterm infants (Cregan, De Mello et al. 2002).

Most importantly for clinical consideration, women who delivered at extremely premature gestational ages continued to express significantly lower volumes at day ten postpartum, compared with women who delivered at more advanced gestational ages, suggesting a prolonged effect of extreme prematurity on milk production. This may have significant impact on their ability to maintain a sufficient milk supply when the infant is older and may lead to early unplanned cessation of breastfeeding. This has been shown in studies of mothers breastfeeding their term infants, where low milk

production in the first week postpartum has adverse implications for breastfeeding duration (Houston, Howie *et al.* 1983; Neville, M C, Keller *et al.* 1988; Chapman & Pérez-Escamilla 1999a). Moreover, in mothers of preterm infants, milk output in the first week postpartum significantly predicts milk production for at least six weeks postpartum (Hill, Aldag et al. 2005).

### 4.4.2 Effect of Other Factors on Lactogenesis II

#### *4.4.2.1 Expression Frequency*

Frequency of expressing strongly predicted both the volume of milk expressed and the concentration of lactose and citrate in milk. This confirms earlier studies which found increased milk volume with increasing frequency (de Carvalho, Anderson *et al.* 1985; Hopkinson, Schanler *et al.* 1988; Hill, Aldag *et al.* 1999, 2001) and supports current guidelines which encourage women to express at least six times per day to establish and maintain supply. It was disappointing that so many women did not attain the recommended frequency, particularly on postpartum days one to three when the median frequency was only three to five expressions per day respectively. Although this low frequency could be the result of either the stress of having given birth to a preterm infant or because many women were still medically unwell on these days, this finding suggests that obstetric, midwifery and nursery staff need to place greater emphasis on the importance of frequent pumping from the first postnatal day.

#### *4.4.2.2 Intended Duration of Breastfeeding*

It was interesting that intended duration of breastfeeding had a strong impact on expressed milk volume. It has long been known that intention to breastfeed for prolonged durations is a strong predictor of breastfeeding duration in mothers of term infants (Bloom, Goldbloom *et al.* 1982; Henderson, Evans *et al.* 2003). A strong

motivation to succeed at breastfeeding is believed to overcome most problems of early lactation. The mechanism by which intended duration affects milk volume in the first ten days in mothers of preterm infants remains to be determined. One possible explanation is that highly motivated women may express more frequently. In the present study, however, expression patterns were similar between women who intended to breastfeed for long periods and those who intended to breastfeed for less than six months. The effect of intended duration of breastfeeding on milk volume was stronger in primiparas, and not significant in multiparas, most of whom had breastfed previously. This finding suggests that previous lactation outweighs the impact of motivation in mothers of preterm infants.

#### *4.4.2.3 Maternal Factors*

There were surprisingly few additional factors found to influence lactogenesis II. No obstetric, labour and delivery or neonatal factor was found to affect either milk volume or milk lactose concentration in the first ten days postpartum in these mothers of preterm infants. Management of high risk cases, such as comprised this cohort, dictated that 50% of study participants had caesarean section deliveries, most of which were emergency deliveries. In contrast with much of the evidence for women who have caesarean sections at term (Dewey, Nommsen-Rivers et al. 2003; Evans, Evans et al. 2003), the present study found that caesarean section delivery did not delay the onset of lactogenesis II.

The present study also did not find a delay in women with diabetes. In contrast, findings from studies of women with insulin dependent diabetes mellitus (IDDM) delivering at term found delayed onset of lactogenesis II (Arthur, Smith et al. 1989; Neubauer, Ferris et al. 1993), although this has not been established in women with gestational diabetes. Numbers were low in the present cohort with only six women having either gestational

diabetes or IDDM suggesting there was insufficient power to explore associations with diabetes.

Other studies of women at term have found that onset of lactogenesis II occurs earlier in multiparas and delayed onset is more likely to be perceived by primiparous women (Dewey, Nommsen-Rivers et al. 2003). In contrast with those findings, the present study did not find an effect of parity on lactogenesis II in mothers of preterm infants. Smokers had reduced milk supply compared with non-smokers at four weeks in an American study (Hopkinson, Schanler et al. 1988). This was not confirmed in the present study and, despite a large proportion of the cohort admitting to having smoked during the pregnancy (30%) and 17% still smoking in the postpartum period, smoking was not found to affect milk volume in the first ten days.

The present study of preterm mothers found a significant association between pre-pregnancy obesity and reduced milk volume, suggesting a delay in lactogenesis II, consistent with findings of reduced milk yield in obese mothers of term infants (Rasmussen, Hilson *et al.* 2001; Rasmussen & Kjolhede 2004). However the present study did not find an effect of maternal obesity on either milk lactose or citrate concentrations. As BMI is not a valid measure of obesity during pregnancy, pre-pregnancy BMI was estimated from available anthropometric data extracted from the patient notes. The estimation of pre-pregnancy BMI from maternal weight and height on admission to delivery suite, because of a lack of available data regarding actual pre-pregnancy weight and height, was unable to account for actual weight gain during pregnancy and hence limited the reliability of the analysis of BMI in the present study.

Unexpected positive associations were found between increased milk citrate concentration and both primiparity and non elective caesarean section delivery. The reasons for these observations are unknown and, in light of the lack of associations with

either milk volume or lactose concentration, are likely to be unimportant in the explanation of lactogenesis II in this group of women.

It is interesting that a large number of the factors known to influence the timing of lactogenesis II after term delivery were not found to have a relationship with lactogenesis II after preterm delivery. This suggests that gestational age has a very potent effect on timing of lactogenesis II and overwhelms the influence of other maternal and obstetric factors.

### 4.4.3 Validity of different methods for determining onset of lactogenesis II in mothers of preterm infants

In this cohort of women, who were not breastfeeding but were solely expressing for all their infants' requirements, it was possible to estimate fairly accurately the exact timing of lactogenesis II by measurement of volume of milk expressed. Rapid increases in measured volume indicated the onset of copious milk secretion in these women. This approach is limited only by the frequency of expressing of individual women and the ability of pumping to effectively empty the breast at each expression.

For women who were already breastfeeding or those who were unwilling to weigh their expressed milk at all expressions, many provided small samples of milk each day for analysis of milk composition (N = 41). A total of 37 women measured milk volume on at least one to ten days postpartum as well as providing milk samples on these days. Many studies have used the changes in breastmilk composition, principally lactose and citrate concentration, as markers for the onset of lactogenesis II in mothers of term infants (Hartmann, P E, Trevethan *et al.* 1973; Arthur, Smith *et al.* 1989; Neville, M C, Allen *et al.* 1991; McNeill, Murphy *et al.* 1998). To my knowledge the present research presents the largest published series of data in which milk volume is compared with the concentration of lactose and citrate in milk coinciding with lactogenesis II.

The present study demonstrated that increasing milk volume was significantly associated with increases in concentration of both lactose and citrate in milk. The correlations, however, were not strong due to variations in individual milk volumes and concentration of milk components. As expected, the strongest correlations between volume and milk lactose concentration occurred in the early postpartum days (days one to three). Once lactocyte tight junctions close and regular expression is established, milk production is less closely related to the concentration of macro-components in milk. These findings suggest that, while measurement of expressed milk volume is the gold standard for determination of the time of lactogenesis II (by definition the initiation of copious milk secretion), measurement of the concentration of milk markers is reasonable approximation in the early days postpartum.

### 4.4.4 Breastfeeding Rates of Preterm Infants

Authorities including the World Health Organization (WHO), the American College of Pediatrics and the Australian National Health and Medical Research Council (NHMRC) recommend exclusive breastfeeding for six months and for a further six months breastmilk is recommended as the chief nutrition source supplemented with complementary foods (WHO 1998; Binns & Davidson 2003; Gartner, Morton *et al.* 2005). Many authorities have enacted breastfeeding goals such as the Australian government's target of 90% breastfeeding at two months and 80% at six months postpartum with 50% fully breastfeeding (Nutbeam, Wise *et al.* 1993; Binns & Davidson 2003).

The breastfeeding rate of 82% on discharge from the nursery in the present study compares favourably with other reports but still falls short of recommended rates and the breastfeeding rates of term infants on discharge from hospital. A study of mothers who delivered term infants in the same hospital that provided the sampling frame for the

present study found 96% to be breastfeeding on discharge (Henderson, Evans et al. 2003). However, mothers of very preterm infants will have effectively been expressing and/or breastfeeding for at least two months before discharge of the infant from hospital, and at two months, only 79% of the mothers of term infants in the study by Henderson *et al.* (2003) continued to breastfeed. Comparative national figures were lower with the 2001 Australian National Health Survey finding 83% of term infants were breastfed on discharge from hospital (Australian Bureau of Statistics 2003).

The relatively high rate of breastfeeding on discharge found in this study may be due to selection bias as intention to breastfeed was a requirement for recruitment to the study. Other comparable studies of preterm infants found the proportion of infants taking breastmilk on discharge from hospital to range from 87% (Jones, Dimmock *et al.* 2001), 64% in a Canadian study (Pinelli, Atkinson *et al.* 2001), 62% in an Australian study (Simmer, Metcalf et al. 1997), to two American studies which found 57% and 49% respectively (Hill, Hanson et al. 1994; Furman, Minich et al. 1998). A German study of mothers of very low birthweight infants (VLBW, <1500 g) who elected to express milk from birth found the median duration of breastmilk feeding was only 46 days compared with 154 days for term infants (Killersreiter, Grimmer et al. 2001). This finding was consistent with a Canadian study, which found the median duration of breastmilk feeding was 54 days in mothers of preterm VLBW infants (Kaufman & Hall 1989). Strong levels of social support, particularly by the partner but also support from nursery staff was shown to be predictive of breastfeeding success in several studies (Kaufman & Hall 1989; Furman, Minich *et al.* 1998; Killersreiter, Grimmer *et al.* 2001) although randomised controlled trials of breastfeeding support have failed to find positive effects of long-term breastfeeding counselling for mothers of preterm infants (Pinelli, Atkinson et al. 2001).

### 4.4.5 Milk production in mothers of preterm infants

The present study found that daily milk production rose to a median of 323 mL on day five and 530 mL by day ten postpartum although there was an extremely wide range on all days. Few studies have reported milk volume in mothers of preterm infants during the first postpartum week but other studies of production in mothers of preterm infants within the first month have found similar ranges. Hill *et al*. have found milk yields of 433 mL/day (Hill, Aldag et al. 1999), and in a separate study comparing frequency of pumping in weeks two to five postpartum they found high frequency pumping yielded a mean of 632 mL per day compared with 319 mL with low frequency pumping (Hill, Aldag et al. 2001). An American study found non-smokers were producing 639 ± 344 mL/day at four weeks and that smokers had a significantly reduced supply (Hopkinson, Schanler et al. 1988). Smoking was not found to affect milk volume in the first ten days in my study despite a large proportion of the cohort admitting to having smoked during the pregnancy. Not only do preterm mothers have a significantly higher risk of failing to produce adequate milk supply for the infants than mothers of term infants but also poor early milk output is significantly associated with continued poor milk supply (Hill, Aldag et al. 2005).

It is less easy to measure milk production in mothers whose infants are feeding at the breast. Most studies have weighed either the infant or the mother before and after feeds. This method is subject to error as it only measures the infant's capacity and does not account for women who produce more than the infant's requirements – a common event in the first postpartum week. Neville *et al.* found a mean milk transfer of about 498 mL/day at five days postpartum (Neville, M C, Keller *et al.* 1988). Other authors have found milk yield of mothers of term infants at three days postpartum to be 408 mL/day (Saint, Smith et al. 1984), and at six days postpartum to be 556 mL/day (Arthur, Smith et al. 1989) up to as much as 1.2 kg/day in the first month (Hartmann, P E 1987). These

findings exceed those found in the present study suggesting that milk volumes in the first week to ten days postpartum are reduced in mothers of preterm mothers compared with term mothers.

### 4.4.6 Biochemical Markers of Lactogenesis II

Several studies have shown differences in the macro-components of milk expressed by mothers of preterm infants compared with those of term mothers (Atkinson, S 1995). Major findings are that preterm milk has greater concentrations of nitrogen, immune proteins, lipids, and median chain fatty acids. Less has been recorded of the differences in markers of lactogenesis II such as lactose and citrate concentrations.

The mean ± sd concentrations of lactose (156.8 ± 36.2 mM) and citrate (3.5 ± 1.4 mM) in milk in the present study are within the ranges of those in published studies. Cregan *et al*. found a wide range in mothers of preterm infants on day five postpartum with mean ± sd concentrations of lactose and citrate in milk being 147 ± 10 mM and 4.3 ± 0.7 mM respectively (Cregan, De Mello et al. 2002). One early small study found lower concentrations of lactose in milk of preterm mothers compared with term mothers (Gross, Geller *et al.* 1981). In contrast, Neville *et al*. found lactose and citrate concentrations in mothers of term infants to be similar to my study findings at 160 mM and 4.0 mM respectively (Neville, M C, Keller *et al.* 1988).

Rapid increases in concentrations of lactose and citrate in milk signifying lactogenesis II occurred between one and three days after delivery in the present study, although this was delayed by at least one day in mothers of extremely preterm infants (< 28 weeks' gestational age). This contrasts with published findings for mothers of term infants where increases usually occur between days one and two postpartum (Saint, Smith et al. 1984; Arthur, Smith et al. 1989).

### 4.4.7 Limitations of the present study

Generalisation from these findings is limited by being an observational study only. It is obviously not feasible, with our current knowledge, to conduct a randomised controlled trial of glucocorticoid treatment in women because of the established advantages for the preterm neonate. Nor is it possible to predetermine time to delivery after treatment. However, in the absence of the ability to conduct a randomised trial of a single course of glucocorticoids, a prospective cohort study such as the present study represents the next acceptable level of evidence and the prospective recruitment of subjects and collection of data minimizes most possible bias in the results.

Furthermore, although the power calculation had predicted that the sample size was sufficient to find a significant effect, there were small numbers in some treatment-delivery intervals at some gestational ages, particularly in the earlier gestational ages, suggesting the need for further investigation with larger sample size, perhaps weighted to recruit women at extremely early gestational ages, in order to determine the strength of any effect.

The absence of total milk volume measurements in the few women who were already giving some breastfeeds over the time interval when lactogenesis II was expected to occur (N = 6) limited the findings to mothers who were expressing only. Although weighing the infant before and after feeds is the next best indicator of milk volume (Meier, Engstrom *et al.* 1996), I felt this was too much of an imposition on these mothers so soon after the presumably stressful event of a preterm birth and when they were already undertaking a reasonably heavy workload for my study. Hence total daily milk volume was not ascertained on days in which the infant breastfed. It is possible that having the infant feeding directly on the breast may stimulate earlier lactogenesis II than that occurring when milk removal is only obtained by pumping the breast, although this was not found in concentrations of either lactose or citrate in milk. In refutation of

this argument, it has been established that lactogenesis II always occurs after birth regardless of whether or not milk is removed (Kulski & Hartmann 1981; Neville, M C, Morton *et al.* 2001). A parallel analysis was done including women who were only giving token feeds (data not shown) and there was little change to the results.

### 4.4.8 Further investigation

In light of the previous chapter that showed, in sheep, a premature initiation of lactation before parturition, which lead to profound disruption of subsequent lactogenesis II at term, and the suggestion in this chapter that betamethasone treatment may disrupt lactogenesis II in women who deliver preterm, further investigation in women, of the putative consequences of betamethasone on antenatal indicators of lactation is warranted. Moreover a study of the effects on women who do not deliver preterm but progress to term after earlier antenatal treatment is also justified.

# CHAPTER 5 ANTENATAL AND POSTNATAL EFFECTS OF ANTENATAL GLUCOCORTICOIDS ON LACTOGENESIS IN WOMEN WHO DELIVER BOTH TERM AND PRETERM

*"By this means (placental source of mammogenic hormones) pre-partum influences on subsequent milk yield may be brought about." (Forsyth 1983)*

*"Most of us become acquainted with the mammary gland at a very early stage of our existence and develop a relatively enduring interest" (Cowie 1974)*

## 5.1 Introduction

In the previous chapter I demonstrated subtle effects on lactogenesis II after antenatal glucorticoid administration in women who delivered preterm, and showed that delays in onset of lactogenesis II may be dependent on the interval between treatment and delivery. What is not known is what happens to women who do not subsequently deliver prematurely. There are no data on whether these adverse effects on lactation persist in women who eventually progress to deliver at term. In this context it is of interest that I demonstrated a profound disruption of postnatal lactogenesis II after term delivery in sheep (Chapter 3).

Recent studies have attempted to characterise women who will eventually deliver preterm (Goepfert, Goldenberg *et al.* 2000; McLaughlin, K J, Crowther *et al.* 2002; Romero, Espinoza *et al.* 2002; Iams 2003). Despite our improved understanding of the causes of preterm birth, we continue to have a poor rate of success at predicting who will eventually progress to term. Therefore a number of women with high risk pregnancies receive unnecessary and potentially risky treatment with antenatal glucocorticoids. For example, studies have shown that at least 20% of women who

receive antenatal glucocorticoids do not eventually deliver early (Quinlivan, Evans *et al.* 1998; McLaughlin, K J, Crowther *et al.* 2002; Moss, T J M, Harding *et al.* 2002).

In Chapter 3 I demonstrated that antenatal glucocorticoid administration caused a marked precocious initiation of lactogenesis II during pregnancy in sheep and that this was associated with alterations in the secretion of lactogenic hormones, principally progesterone. No study has attempted to characterize the changes in the mammary gland in pregnant women after synthetic glucocorticoid administration, although plasma progesterone concentration is thought to be unchanged (Ohrlander, Gennser *et al.* 1977; Ylikorkala, Dawood *et al.* 1978; Ogueh, Jones *et al.* 1999).

The study in this chapter was designed to answer two questions in women: what happens to lactogenesis during pregnancy after antenatal glucocorticoid administration and whether there is any impact of antenatal glucocorticoid administration on lactogenesis II after term delivery. In line with my findings in sheep (Chapter 3), my hypothesis for the present research was that antenatal glucocorticoid treatment would cause a premature initiation of lactogenesis in pregnant women and that this would lead to disrupted lactogenesis II after term delivery.

The principal objectives of this study were as follows:

1. To investigate the effects of antenatal betamethasone treatment for anticipated preterm delivery on progesterone concentration during pregnancy by examining changes in the excretion of pregnanediol glucuronide in urine.
2. To investigate the effects of antenatal betamethasone treatment for anticipated preterm delivery on lactogenesis during pregnancy by examining changes in excretion of lactose in urine.
3. To investigate the effects of antenatal betamethasone treatment and gestational age at delivery on postnatal onset of lactogenesis II.

## 5.2 Methods

Participants in this study were recruited into the prospective trial of women who received antenatal prophylactic glucocorticoid treatment for anticipated preterm delivery (Section 4.2). All participants in that trial including not only those who delivered preterm but also those who delivered at later gestational ages were included in this analysis. Participants were given a single course of two intramuscular injections 24-hours apart of betamethasone (Celestone Chronodose, Schering Plough) 11.4 mg unless delivery occurred before the second dose could be given. In addition, a small number of women with normal pregnancies and normal term deliveries were recruited as controls (N = 6).

### 5.2.1 Urine Samples

Urine samples were collected and 24-hour samples were reconstituted using the method described in the Methods Chapter 2 (Section 2.2.3). Women commenced collecting urine samples as soon as possible after the first betamethasone dose was given and continued on days 1,2,3,5,7, and 14 after treatment providing they had not given birth. For the purposes of this analysis the day of treatment was counted as day one. Controls each collected 24-hour urine samples on 3 random days between 24 and 33 weeks of pregnancy.

Women were also asked to collect 24-hour urine samples after delivery for up to 5 days but many participants were unwilling to undertake urine collection in the puerperium. Consequently, only 35 women collected postnatal urine samples over 63 24-hour periods.

Reconstituted 24-hour urine samples were assayed for pregnanediol glucuronide (PdG; the main progesterone metabolite in urine; Section 2.4.1) and lactose (Section 2.4.2).

### 5.2.2 Milk Volume Measurement in Mothers of Term Infants

Women who were expressing for their preterm infants measured the amount of expressed breastmilk using the methods recorded in the previous chapter (section 4.2.1). Mothers of infants who were breastfeeding recorded the volume of milk transferred to the infant during all breastfeeds in at least one 24-hour period using the method of Arthur *et al.* (Arthur, Hartmann et al. 1987). Women were instructed to feed their baby on demand and to record the amount of milk taken for all feeds in a 24-hour period. They weighed their fully clothed baby on electronic scales (Medela BabyWeigh™ Scale, Il, USA) and recorded the time and weight immediately before and after each feed. All clothing from the initial weight was included in the after-feed weight measurement.

### 5.2.3 Statistical Analysis

The primary endpoints for this analysis were differences in excretion of PdG and lactose in antenatal urine samples. Secondary endpoints included excretion of lactose in postnatal urine and concentration of lactose in milk. Milk volume, both expressed for preterm infants and transferred to breastfeeding infants, was also a secondary endpoint, as well as maternal perceptions of lactation. Results of the analysis of the concentration of citrate in milk are not reported here because they followed a similar pattern to those of the concentration of lactose in milk. I demonstrated in Chapter 4 that the concentration of lactose in milk was well correlated with milk volume and milk citrate concentration, and was a good surrogate measure of postnatal lactogenesis II. Therefore analysis of citrate concentration was deemed to be redundant in the present analysis. Data were summarised using the Wilcoxon rank sum test for non-parametric data such as gestational age at betamethasone treatment and birth. Categorical and ordinal data were compared using either Fisher's Exact Test or the chi-squared test as appropriate.

No formal demographics were performed comparing betamethasone-treated women with controls because of sample size limitations (N = 6 controls). To obtain normality, $\log_e$ transformation of urine lactose and PdG values was conducted and analysis of variance with repeated measures was used. Significance levels were set at 0.01 for testing interactions (to attain an overall 0.05 significance level for all contrasts) and at 0.05 for testing of main effects. The SAS statistical package version 8.2 was used for all statistical analysis.

## 5.3 Results

A total of 92 women (including 5 controls) provided complete 24-hour urine samples on at least one antenatal day (N = 330 samples). Maternal characteristics for the women treated with betamethasone that provided antenatal urine samples are presented in Table 5.1. The median age was 32 years and less than half had completed 12 years of schooling. Half of the antenatal responders intended to breastfeed for longer than six months.

Pregnancy characteristics are presented in Table 5.2. There were 11 sets of twins and 44% were in their first pregnancy. The median (range) gestational age at betamethasone treatment was 28.8 (23.6 – 33.6) weeks.

**Table 5.1 Maternal Characteristics of Women Who Received Antenatal Betamethasone and Who Collected At Least One Complete 24-Hour Urine Sample[1]**

| N = 76[2] | N/mean | %/(sd) |
|---|---|---|
| Age (years) [3] | 31.6 | (5.7) |
| Maternal education – completed secondary school | 38 | 50 |
| Maternal tertiary education | 18 | 23.7 |
| Marital status – married or defacto | 71 | 92.2 |
| Maternal Body Mass Index [3,4] | 25.4 | (6.2) |
| Obese pre-pregnancy | 14 | 16.3 |
| Intended breastfeeding duration | | |
| Short term only | 2 | 2.4 |
| ≤ 6 months | 39 | 47.6 |
| > 6 months | 41 | 50 |
| Previous breastfeeding experience | 46 | 52.9 |
| Smoked during pregnancy | 24 | 29.3 |

[1] Excludes controls
[2] Demographic data missing for N=11 women
[3] mean (sd)
[4] Body mass index (kg/cm$^2$) imputed for pre-pregnancy from weight on admission. Obesity classed as BMI ≥ 30

**Table 5.2 Pregnancy Characteristics of Women Who Received Antenatal Betamethasone and Who Collected At Least One Complete 24-Hour Urine Sample[1]**

| N = 87 | N/median | %/(range) |
|---|---|---|
| Parity | | |
| 0 | 37 | 42.5 |
| 1 | 25 | 28.7 |
| ≥ 2 | 25 | 28.7 |
| Assisted Reproductive Technology | 13 | 14.9 |
| Twin pregnancy | 11 | 12.6 |
| Obstetric complications | | |
| Pre-eclampsia/Hypertension | 10 | 11.5 |
| Preterm Labour | 68 | 78.2 |
| Prolonged Preterm Rupture of Membranes | 28 | 32.2 |
| Antepartum Haemorrhage (APH) | 28 | 32.2 |
| Gestational Diabetes/IDDM | 5 | 5.8 |
| Intrauterine Growth Retardation (IUGR) | 12 | 13.8 |
| Gestational age at first betamethasone treatment (weeks) [2] | 28.9 | (23.6 – 33.6) |

[1] N = 87, excluding controls
[2] median (range)

### 5.3.1 24 Hour Excretion of Progesterone in Urine

A total of 330 24-hour urine samples were collected by participants during pregnancy. The median (range) PdG excretion overall in antenatal samples was 1.355 (0.139 – 5.069) mmol/24 hours. PdG concentration was not measured in postnatal or control urine samples because of a limited number of assay tubes available. Median (range) PdG 24-hour excretion on days one to seven after betamethasone treatment are shown in Table 5.3.

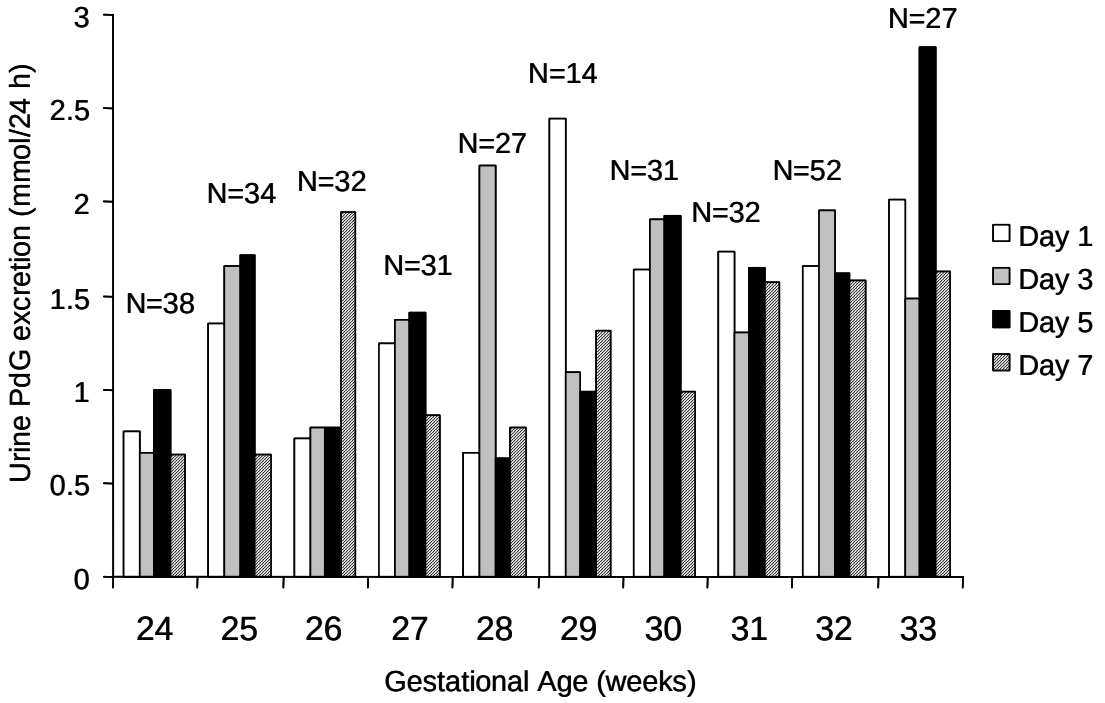
**Table 5.3 24 Hour Urinary Excretion of PdG and Lactose at Intervals after Betamethasone Treatment**

| Day after betamethasone treatment | N | 24 hour PdG excretion (mmol/24 hour) | | 24 hour Lactose excretion (mmol/24 hour) | |
|---|---|---|---|---|---|
| | | Median | Range | Median | Range |
| 1 | 57 | 1.445 | 0.162-3.495 | 0.713 | 0.050-3.217 |
| 2 | 62 | 1.486 | 0.231-3.827 | 0.888 | 0.214-2.545 |
| 3 | 59 | 1.225 | 0.218-4.544 | 1.088 | 0.267-6.676 |
| 4 | 13 | 1.151 | 0.139-2.401 | 0.981 | 0.239-2.358 |
| 5 | 32 | 1.445 | 0.286-3.977 | 0.831 | 0.178-2.031 |
| 7 | 59 | 1.267 | 0.199-5.069 | 0.694 | 0.035-2.457 |
| 14 | 35 | ** | ** | 0.826 | 0.310-3.031 |
| Controls | 13 | ** | ** | 0.881 | 0.273-1.643 |

Total N = 330
** PdG was not assayed on day 14 or control samples.

The relationship between 24 hour urine PdG excretion and gestational age on days 1, 3, 5 and 7 after betamethasone treatment is presented in Figure 5.1. Overall, urine PdG excretion increased significantly with increasing gestational age ($P < 0.001$). There was no significant interaction between gestational age at treatment and time after treatment ($P=0.435$). In both Figures 5.2 and 5.4, gestational age at sampling is depicted in two groups < 28 weeks and ≥ 28 weeks. This *post hoc* division is arbitrary and is designed purely for ready interpretation of the results and graphs and to continue the characterization of gestational age groups depicted in Chapter 4. In the repeated measures ANOVA, gestational age was always analysed as a continual variable. After adjustment for gestational age at treatment, there was a significant effect of betamethasone on urine PdG excretion (trend $P = 0.007$). Compared with day two after treatment, PdG excretion was significantly reduced on day seven (adjusted $P = 0.010$). Urine PdG excretion was also significantly associated with plurality (adjusted $P = 0.007$). Twin pregnancies had significantly higher PdG excretion than singleton pregnancies. There was a trend for lower PdG excretion in women with threatened preterm labour (adjusted $P=0.053$). In addition PdG excretion was significantly increased with increasing BMI (adjusted $P = 0.039$)

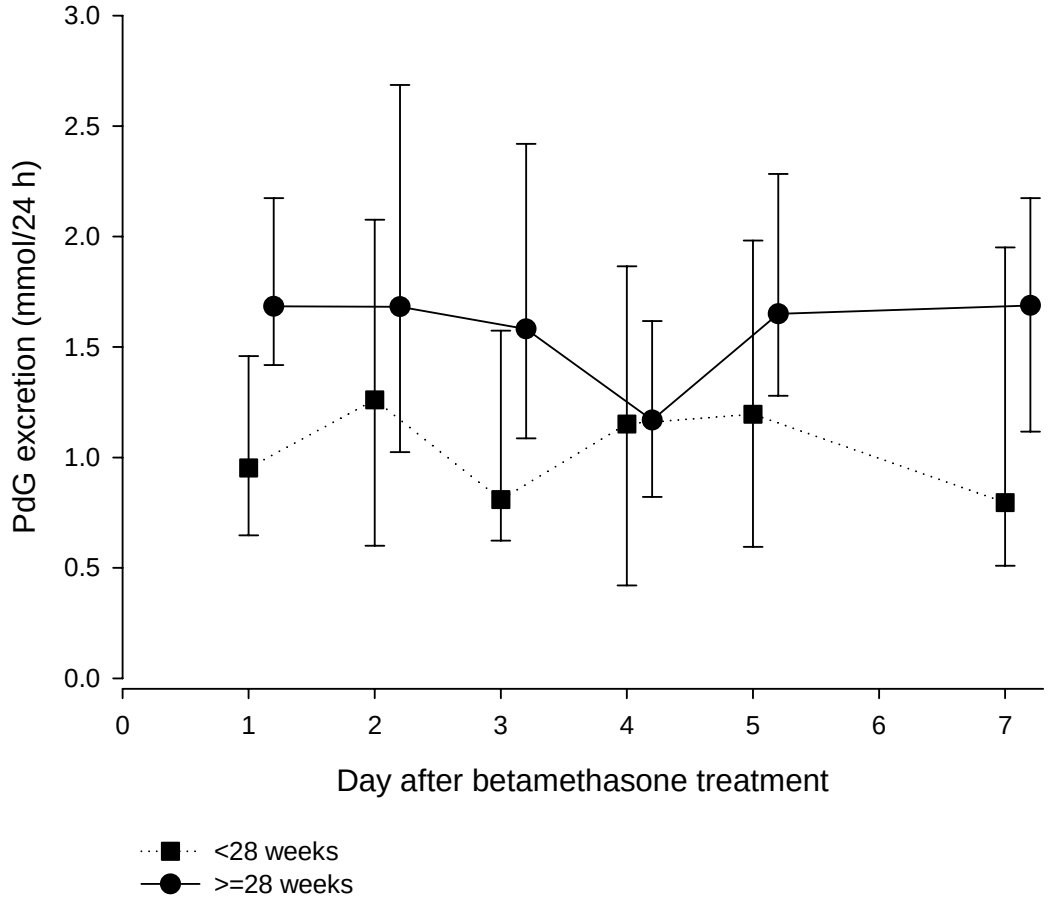
**Figure 5.1 Relationship between 24 hour PdG Excretion in Urine and Gestational age Stratified by Interval after Betamethasone Treatment**




Note: Histograms represent median 24 hour urinary PdG excretion (mmol/24 hours) at intervals after betamethasone treatment – white histograms represent day of treatment, grey day 3 after treatment, black 5 days after treatment and diagonal black/white 7 days after treatment. Numbers above each gestational age week represent the total number of samples at the gestational age on the x-axis.

Baseline (Day 1) PdG excretion increased with increasing gestational age (trend $P < 0.001$). Urine PdG excretion decreased on day 7 compared with day 2 after betamethasone treatment ($p=0.010$).

**Figure 5.2 Relationship between 24 hour PdG Excretion in Urine and Gestational age Stratified by Interval after Betamethasone Treatment**




Symbols represent median (IQR) excretion of PdG in urine at daily intervals after betamethasone treatment in two gestational age groups < 28 weeks or 28-34 weeks. PdG excretion was greater at more advanced gestational ages (trend P < 0.001). After adjustment for gestational age, PdG excretion decreased over time after betamethasone treatment (P = 0.007).

### 5.3.2 Twenty-four Hour Excretion of Lactose in Urine before Delivery

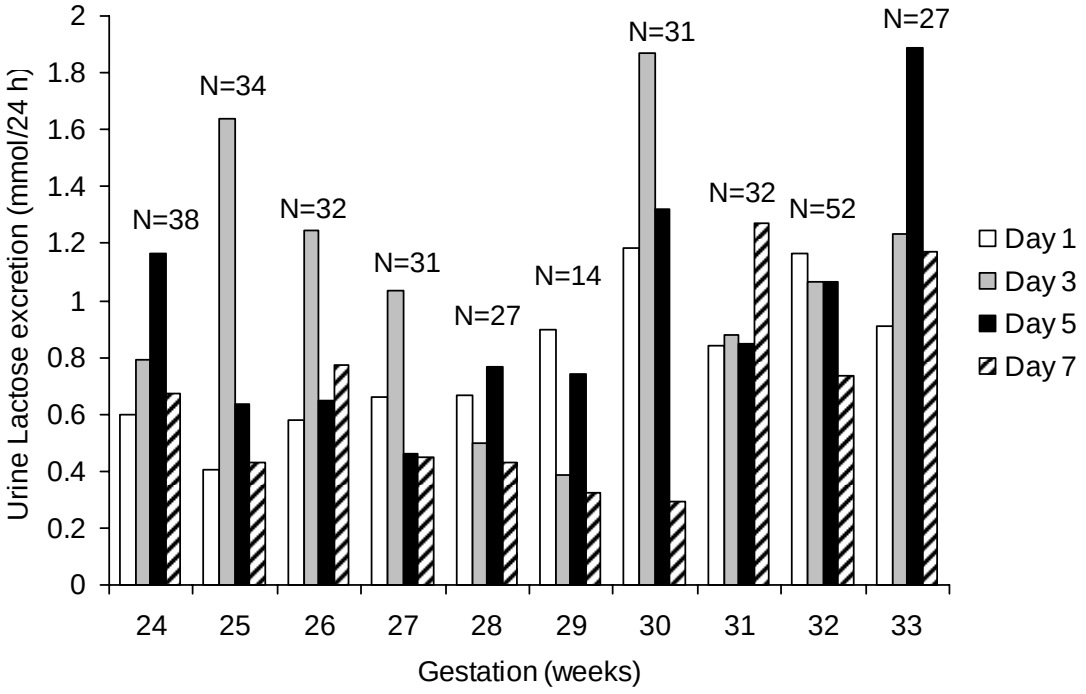
The median (range) 24 hour urinary lactose excretion in antenatal samples was 0.823 (0.035 – 6.676) mmol/24 hours. Median urine lactose excretions for each antenatal day after the first betamethasone injection from days 1 to 14 are shown in Table 5.3. Urine lactose excretion increased significantly with increasing gestational age (Figure 5.3, $P = 0.001$). There was also a significant effect of betamethasone treatment after adjustment for gestational age (Figure 5.4, $P < 0.001$).

Compared with the treatment day, log lactose excretion was significantly increased on day 2 ($P = 0.033$), day 3 ($P < 0.001$), and day 4 ($P = 0.025$) after treatment. There was no interaction between gestation at treatment and time after treatment ($P= 0.166$).

The magnitude of the increase differed between different gestational ages. Earlier gestational age was associated with a greater absolute increase in urine lactose concentration than more advanced gestational ages at treatment, although the range was wide (Figure 5.4). Values returned to the baseline between five and seven days after treatment.

After adjustment for gestational age, there was no statistically significant difference between lactose excretion in urine of control women and in urine collected by women treated with betamethasone at any day after treatment ($P = 0.943$).

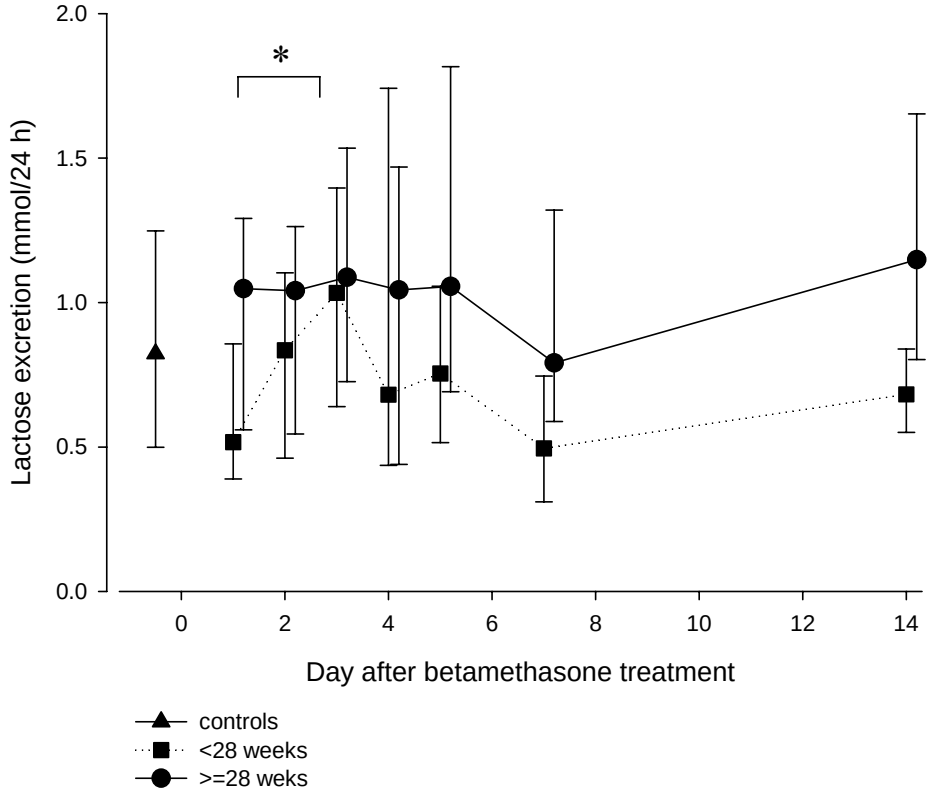
**Figure 5.3 Relationship between 24 hour Lactose Excretion in Urine and Gestational age Stratified by Interval after Betamethasone Treatment**




Note: Histograms represent median 24 hour urinary lactose excretion at intervals after betamethasone treatment – white histograms represent day of treatment, grey day 3 after treatment, black 5 days after treatment and diagonal black/white 7 days after treatment. Numbers above each gestational age week represent the total number of samples at the gestational age on the x-axis.

There was a small general increase in baseline (Day 1) values with increasing gestational age ($P = 0.001$). There also tended to be increases in urine lactose excretion on days 3 to 5 after betamethasone treatment at most gestational age weeks. Compared with the treatment day, log lactose excretion was significantly increased on day 2 ($P = 0.033$), day 3 ($P < 0.001$), and day 4 ($P = 0.025$) after treatment.

**Figure 5.4 Relationship between 24 hour Lactose Excretion in Urine and Gestational age Stratified by Interval after Betamethasone Treatment During Pregnancy**




Symbols represent median (IQR) urinary excretion of lactose at daily intervals after betamethasone treatment in two gestational age groups < 28 weeks (squares, dotted line) or 28-34 weeks (circles, solid line). Controls are represented by the triangle symbol and error bar on the left of the chart. After adjustment for gestational age, there was no difference between controls and women who received betamethasone (adjusted P = 0.943). In women who received betamethasone treatment, antenatal urine lactose excretion was significantly associated with interval after betamethasone treatment (adjusted P < 0.001). * Lactose excretion increased to a peak at approximately day four after treatment (P < 0.001) and then reduced to levels equal to day one. These differences were more marked at earlier gestational ages (< 28 weeks).

The urinary excretion of PdG was significantly positively associated with the excretion of lactose (data not shown, $P < 0.001$), although the range of values was wide. Increasing PdG excretion was significantly associated with increasing lactose excretion, although the wide variation suggests that PdG only contributed a small amount to the variation in lactose. There were no significant interactions explaining lactose excretion between PdG and either days after treatment ($P = 0.392$) or gestational age ($P = 0.500$). After adjustment for gestational age, nulliparas excreted more lactose than multiparas (adjusted $P = 0.0247$) and this was consistent with an increased lactose excretion in women who had not previously breastfed (includes some multiparas, adjusted $P = 0.023$). There was a trend for increased lactose excretion in twin pregnancy ($P = 0.057$) and IVF pregnancy ($P = 0.046$).

### 5.3.3 Postpartum – Comparison between Women who Delivered at Term and those who Delivered Preterm

A total of 78 women contributed at least one of the postnatal protocol samples (excretion of lactose in urine and/or concentration of lactose in milk), or milk volume. Milk volume was determined from total expressed volume or volume transferred to the breastfeeding infant. There were 50 women who delivered before 34 weeks' gestational age, eight who delivered between 34 and 37 weeks and 20 who delivered at term including six controls. A comparison of the delivery and postnatal characteristics between women who delivered either term, preterm or very preterm is presented in Table 5.4. Women who delivered between 34 and 36 weeks' gestational age were more likely to be in their first pregnancy than to be multigravid. As expected, rates of preterm labour, prolonged preterm rupture of the membranes (PPROM) and chorioamnionitis were higher in women who delivered very preterm infants. In addition, both the time

interval between betamethasone treatment and delivery as well as birth weight were significantly lower in mothers who delivered earlier than 34 weeks' gestational age and highest in women who delivered at term. The mode of delivery was significantly associated with gestational age group (P = 0.004). Sixty percent of women delivering at term required a caesarean section delivery. Although high proportions of women delivering at earlier gestational ages had caesarean section deliveries, significantly fewer women delivering between 34 and 36 weeks delivered by caesarean section. In addition the proportion of women delivering between 34 and 36 weeks with a growth restricted infant was significantly higher than at earlier or later gestational ages.

**Table 5.4 Delivery and Postnatal Characteristics of Women who provided Postnatal Samples**

| N = 78 | < 34 weeks (N = 50) | | 34 – 36 weeks (N = 8) | | ≥ 37 weeks (N = 20) | | |
|---|---|---|---|---|---|---|---|
| | N/median | %/(range) | N/median | %/(range) | N/median | %/(range) | P value |
| Parity 0 | 20 | 40.0 | 7 | 87.5 | 8 | 40.0 | 0.038 |
| Twins | 7 | 14.0 | 2 | 25.0 | 1 | 5.0 | 0.330 |
| Obstetric complications | | | | | | | |
| Hypertension | 6 | 12.0 | 2 | 25.0 | 1 | 5.0 | 0.322 |
| Preterm labour | 40 | 80.0 | 5 | 62.5 | 10 | 50.0 | 0.040 |
| PPROM | 24 | 48.0 | 1 | 12.5 | 2 | 10.0 | 0.004 |
| Chorioamnionitis | 6 | 12.0 | 0 | 0 | 0 | 0 | -- |
| APH | 13 | 26.0 | 2 | 25.0 | 6 | 30.0 | 0.936 |
| Diabetes | 5 | 10.0 | 0 | 0 | 1 | 5.0 | 0.536 |
| IUGR | 4 | 8.0 | 4 | 50.0 | 2 | 10.0 | 0.004 |
| Duration of labour – 1st stage (hr)[1,2] | 3.1 | (0.1-36.8) | 6.9 | (4.9-11.7) | 6.0 | (1.0-11.0) | 0.108 |
| Duration of labour – 2nd stage (hr) [1,3] | 0.20 | (0.02-3.32) | 0.53 | (0.32-2.67) | 0.38 | (0.13-0.88) | 0.077 |

| N = 78 | < 34 weeks (N = 50) | | 34 – 36 weeks (N = 8) | | ≥ 37 weeks (N = 20) | | |
|---|---|---|---|---|---|---|---|
| | N/median | %/(range) | N/median | %/(range) | N/median | %/(range) | P value |
| Analgesia during labour & delivery | | | | | | | |
| IM/IV narcotic | 13 | 26.0 | 3 | 37.5 | 4 | 20.0 | 0.629 |
| Epidural anaesthesia | 35 | 70.0 | 7 | 87.5 | 12 | 60.0 | 0.356 |
| General anaesthesia | 5 | 10.0 | 0 | 0 | 1 | 5.0 | 0.536 |
| Mode of delivery | | | | | | | |
| Spontaneous vaginal delivery | 22 | 44.0 | 3 | 37.5 | 8 | 40.0 | 0.004 |
| Assisted vaginal delivery | 2 | 4.0 | 1 | 12.5 | 0 | 0 | |
| Caesarean section | 26 | 52.0 | 4 | 40.0 | 12 | 60.0 | |
| Blood loss[1] | 450 | (50-5100) | 500 | (150-1000) | 400 | (50-1500) | 0.969 |
| Gestational age at first betamethasone (weeks)[1,4] | 29.4 | (23.7-33.1) | 30.4 | (30.1-33.7) | 31.3 | (23.6-33.6) | 0.238 |
| Interval between first betamethasone treatment and birth (days) [1,4] | 3 | (0-44) | 31.5 | (2-45) | 62.5 | (33-109) | <0.001 |
| Gestational age at birth (weeks) [1] | 30.8 | (24.3-33.7) | 35.7 | (34.1-36.6) | 38.8 | (37.0-41.7) | <0.001 |
| Birthweight (g) [1] | 1465 | (640-2580) | 2230 | (1790-2420) | 3180 | (2160-4275) | <0.001 |

| N = 78 | < 34 weeks (N = 50) | | 34 – 36 weeks (N = 8) | | ≥ 37 weeks (N = 20) | | |
|---|---|---|---|---|---|---|---|
| | N/median | %/(range) | N/median | %/(range) | N/median | %/(range) | P value |
| Male infants[5] | 40 | 70.2 | 3 | 30.0 | 16 | 80.0 | 0.019 |
| Infant feeding on discharge [6] | | | | | | | |
| Exclusive BF | 14 | 28.6 | 4 | 50.0 | 16 | 80.0 | 0.012 |
| BF & EBM | 23 | 46.9 | 3 | 37.5 | 2 | 10.0 | |
| BF & formula | 3 | 6.1 | 0 | 0 | 0 | 0 | |
| Formula | 9 | 18.4 | 1 | 12.5 | 2 | 10.0 | |

[1] Median (range)

[2] 25 women did not labour,

[3] 39 women did not have 2nd stage of labour

[4] (N = 70) Excludes 6 controls and 2 mothers of preterm infants who did not have betamethasone,

[5] Includes 10 sets of twins,

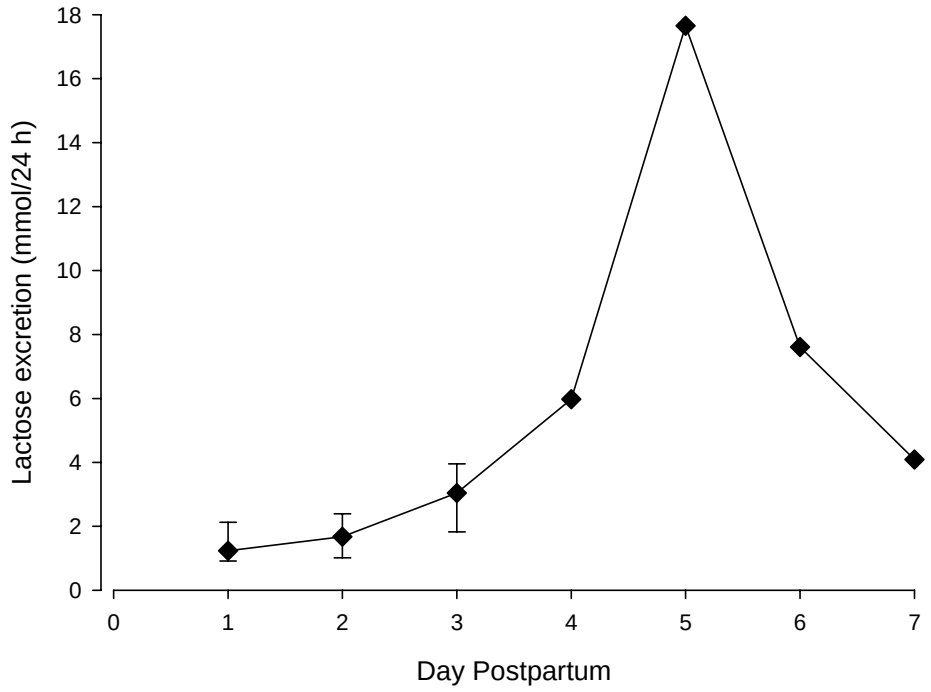
[6] BF = breastfeeding, EBM = expressed breastmilk

### 5.3.4 Excretion of Lactose in Urine after Delivery

Postnatal urine samples were collected for a total of 60 24-hour periods with the majority collected on days one and two postpartum (Figure 5.5). The median (range) postnatal excretion of lactose was 1.785 (0.452-17.651) mmol/24 hours. Gestational age at delivery had no significant effect on postnatal lactose excretion ($P = 0.932$). Nor was the interval between betamethasone treatment and delivery significantly associated with the lactose excretion ($P = 0.080$).

The only factor to have a marked significant effect on lactose excretion in postnatal urine was interval after delivery ($P < 0.001$). Lactose excretion increased with days after delivery until about day five or six postpartum. There were low numbers after the third postpartum day. No other maternal, pregnancy, delivery or postnatal factor was associated with postnatal urinary lactose excretion.

**Figure 5.5 Excretion of Lactose in Postnatal Urine**




Note: Symbols represent median (IQR) urinary lactose excretion (mmol/24 hours) after delivery. There was a significant increase with day after delivery ($P < 0.001$). There were low numbers on days four (N=2), five (N=1), six (N=2) and seven (N=1).

There was a similar lack of significant associations when excretion of lactose in postnatal urine was compared with either the concentration of lactose in milk or the volume of milk. No association was found between lactose excretion in postnatal urine and the concentration of lactose in milk ($P=0.453$). After adjustment for postnatal day, there was a trend for an association between postnatal lactose excretion and milk volume ($P = 0.061$). Gestational age at birth did not change the association between urinary and milk markers of lactogenesis II. It should be noted that the numbers of women days in which urine, milk lactose and milk volume were all collected was low ($N = 26$).

The relationships between postnatal milk volume, urinary lactose excretion, and the concentration of lactose in milk are described in a series of case studies in Figure 5.6. These figures demonstrate the wide variability between individual cases. Most women had low milk volume on the day of delivery with matching low milk lactose concentration. Milk volumes and lactose concentration increased on the second day for most women. Although in the majority of cases lactose excretion in urine increased between postnatal days 1 and 2, this was variable and in one case did not appear to reflect milk lactose or milk volume.

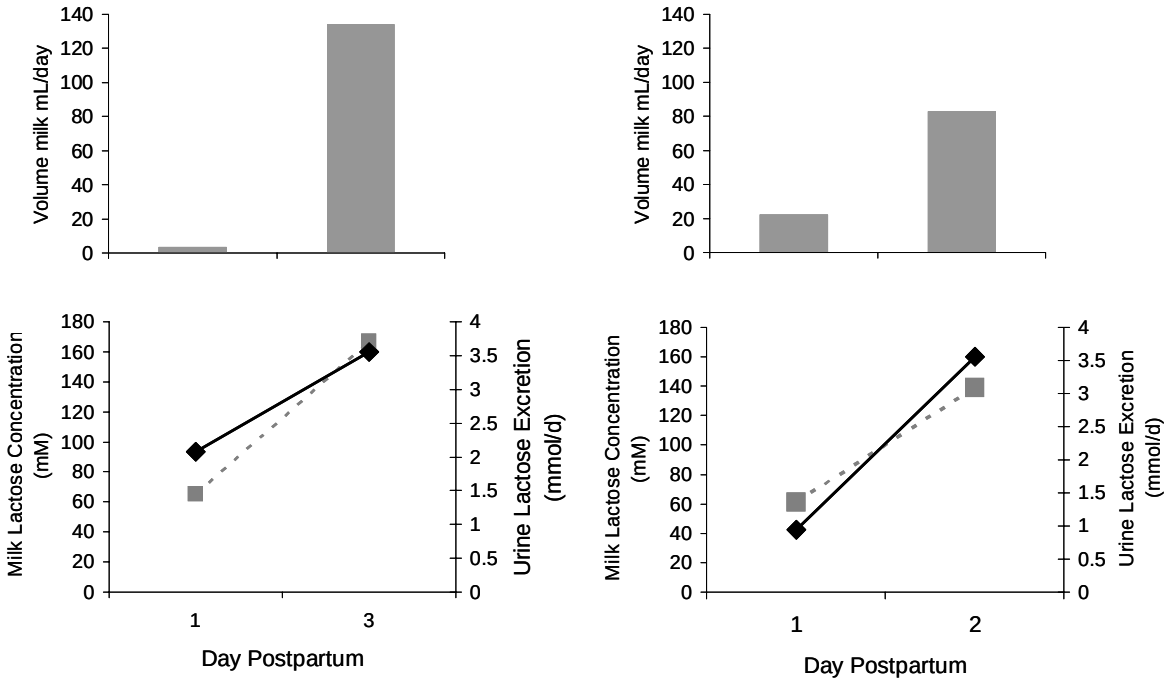
**Figure 5.6 Relationships between Postnatal Milk Volume, Milk Lactose Concentration and Urinary Lactose Excretion – Case Studies**

i) ID 136, Gestational age 27 weeks

Delivery 1 day after betamethasone

ii) ID 129, Gestational age 30 weeks

Delivery on day of betamethasone

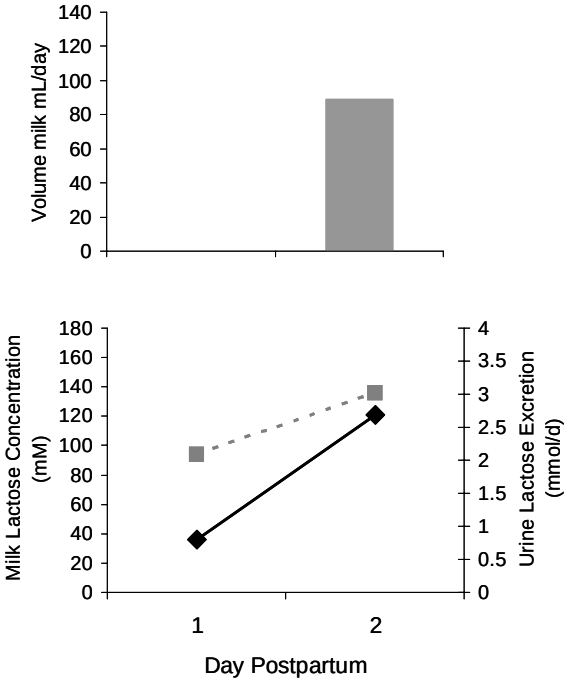



Note: The upper graphs show total milk volume expressed on postnatal day on lower x-axis. The lower graphs show milk lactose concentration on the left y-axis (grey squares on dotted line) and urine lactose excretion on the right y-axis (black diamonds on solid line). This figure is not a representative sample of the study cohort but merely represents the findings for the few women who were willing to undertake all sample collections.

**Figure 5.6 Relationships between Postnatal Milk Volume, Milk Lactose Concentration and Urinary Lactose Excretion – Case Studies Continued**

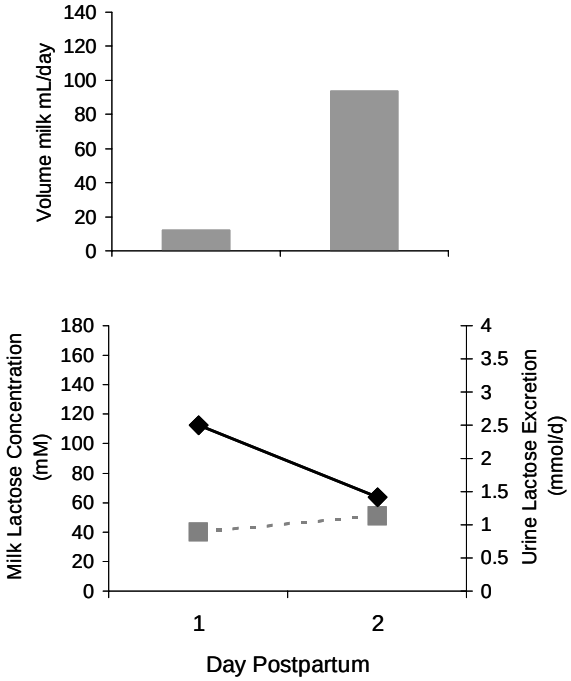
iii) ID 188, Gestational age 31 weeks

Delivery 7 days after betamethasone



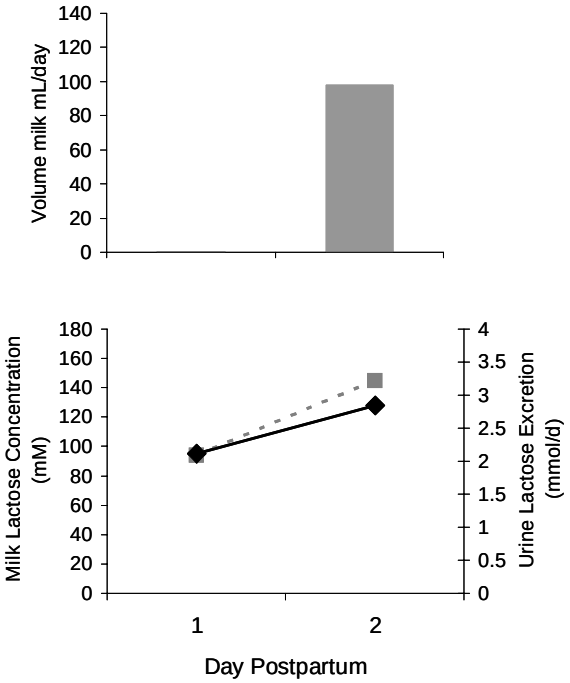

iv) ID 156, Gestational age 31 weeks

Delivery 1 day after betamethasone



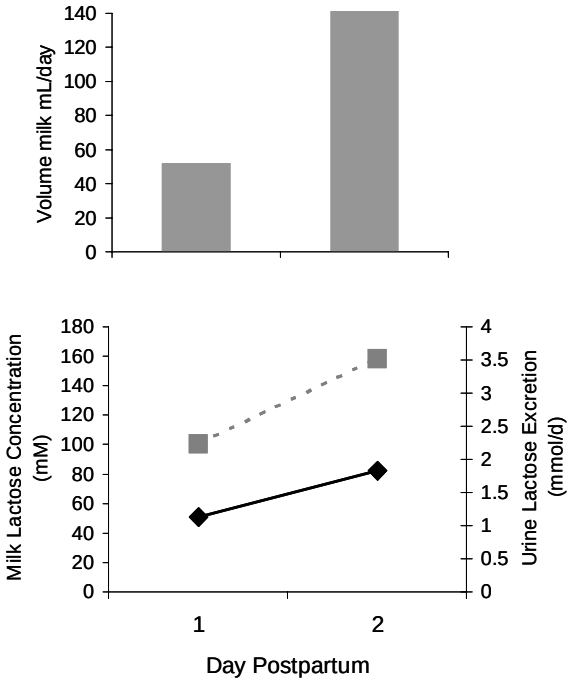

v) ID 191, Gestational age 31 weeks

Delivery 1 day after betamethasone




vi) ID 205, Gestational age 33 weeks

Delivery 2 days after betamethasone

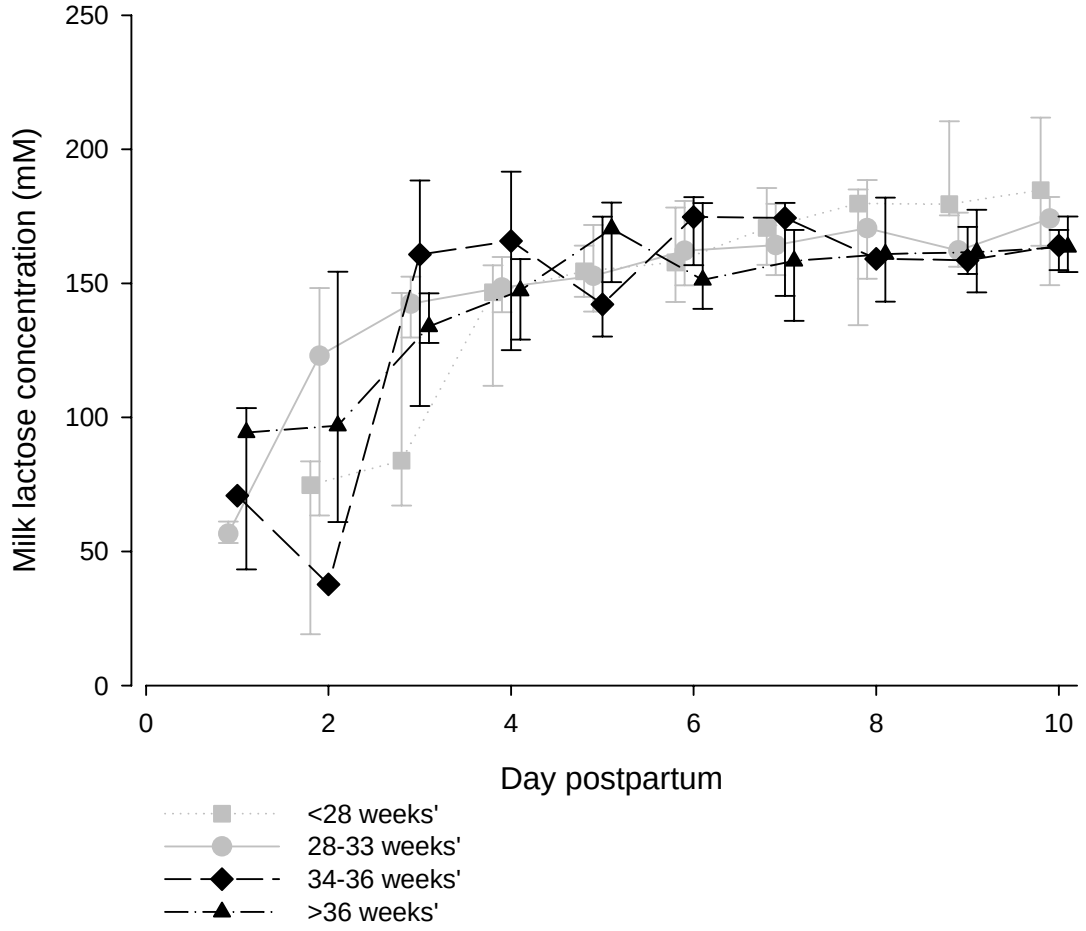


### 5.3.5 Relationship between Preterm and Term Delivery and the Effect on Concentration of Lactose in Milk in the first Ten Days

The concentration of lactose in milk on days one to ten postpartum was measured in 516 samples from 68 women including 22 women who delivered at term, 12 women who delivered between 34 and 36 weeks' gestational age and 34 who delivered very preterm. When the concentration of lactose in milk was compared between mothers of term infants and mothers of preterm infants, gestational age at birth was significantly associated with milk lactose concentration (Figure 5.7, P=0.003). Milk lactose concentration significantly increased with increasing gestational age, although this was more evident in the early days postpartum, suggesting that postnatal increases in milk lactose concentration were delayed at earlier gestational ages. On the second day postnatal, mothers of term infants had significantly higher lactose concentration in milk than both mothers of infants with gestational age less than 28 weeks and also mothers of infants with gestational age between 34 and 36 weeks. Mothers of term infants continued to have significantly higher milk lactose concentration than mothers of infants with gestational age less than 28 weeks on postpartum day three. There were no significant differences in milk lactose concentration between gestational ages 36 to 42 weeks (P = 0.135).

There was no significant association between milk lactose concentration and the duration of the interval between betamethasone treatment and delivery when this was assessed on the full cohort (including term and preterm mothers, P = 0.231), or only on women with gestational age at birth greater than 34 weeks (P = 0.842).

**Figure 5.7 Concentration of Lactose in Milk– Mothers of Term Infants Compared with Mothers of Preterm Infants**



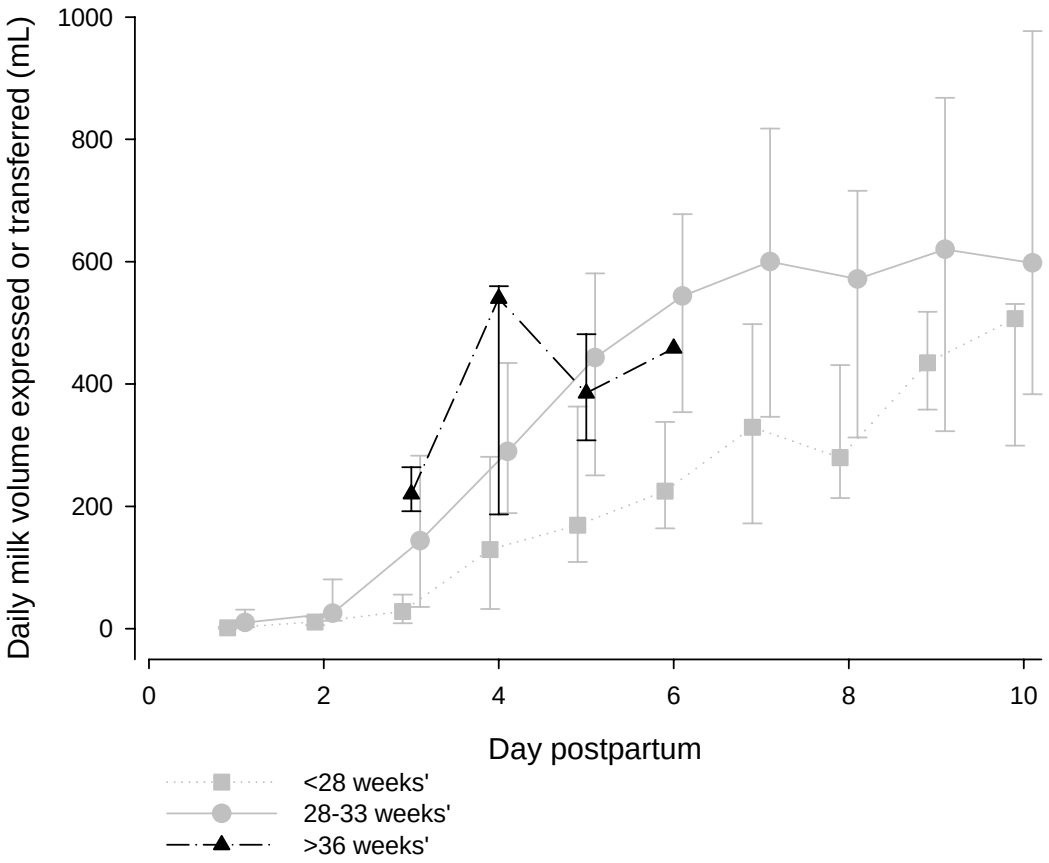

Note: Values represent median (IQR) milk lactose concentration (mM). Grey symbols represent preterm values as shown in the previous chapter (Figure 4.7). Milk lactose concentration from women who delivered between 34 and 36 weeks' gestational age (black diamonds, dashed line) and women who delivered later than 36 weeks' gestational age (black triangles, dash/dotted line) are superimposed on that previous figure. After adjustment for postnatal day, milk from women with gestational age greater than 36 weeks had significantly higher concentration of lactose in milk than women who delivered earlier than 28 weeks. There was no difference in milk lactose concentration between women who delivered 28 to 33 weeks and women who delivered at term. The low value on day two postpartum in the 34-36 week gestational age group was influenced by only two women providing milk samples and one of those women had a very low milk lactose concentration (20.01 mM).

There was a trend to significance for the effect of maternal body mass index (BMI) on milk lactose concentration with women with higher BMI tending to have lower milk lactose concentration (P = 0.055). After adjustment for postnatal day and gestational age, no other maternal, obstetric, intrapartum, postpartum or neonatal factor was associated with milk lactose concentration in the present combined cohort. The only other factor to have a significant effect on the concentration of lactose in milk was intended duration of breastfeeding (P = 0.016). After adjustment for gestational age at birth and day postpartum, women who intended to breastfeed for greater than six months had significantly higher concentrations of lactose in milk compared with women who elected to breastfeed for shorter durations. Parity did not alter the association of intended duration of breastfeeding with milk lactose concentration.

### 5.3.6 Relationship between Preterm and Term Delivery and the Effect on Volume of Milk Expressed or Transferred in the first Ten Days

A total of 11 mothers of term infants measured milk volume transferred during breastfeeding for a total of 14 days. Six women had received antenatal betamethasone at an earlier gestational age and subsequently progressed to deliver at term and the remaining five women were volunteer controls. After adjustment for day postpartum, gestational age was significantly associated with volume of milk expressed or transferred although the difference was most marked on early postpartum days (Figure 5.8, P = 0.005). For instance, on days three and four postpartum, milk transfer volume was significantly greater in mothers of term infants compared with mothers of extremely preterm infants (< 28 weeks, P < 0.001). There was no significant effect of interval after steroid treatment on volume of milk transferred when mothers of term infants were examined (P = 0.231).

**Figure 5.8 Volume of Milk Transferred or Expressed Each Day – Mothers of Term Infants Compared with Mothers of Preterm Infants**




Values represent median (IQR) daily milk volume either transferred by mothers who were breastfeeding their term infants (black triangles) or expressed by mothers of preterm infants (grey squares < 28 weeks' or grey circles 28 to 34 weeks' gestational age). Milk volume was not measured by any mother who delivered between 34 and 26 weeks' gestational age. Daily milk volume was significantly associated with gestational age at delivery (P = 0.005).

There was a significant association between maternal intended duration of breastfeeding and milk volume (P = 0.002). No other maternal, obstetric, delivery or neonatal factor was associated with milk volume in this combined group of preterm and term mothers after adjustment for gestational age at birth and postpartum day.

### 5.3.7 Maternal Symptoms of Lactogenesis II

Study participants were asked to record their symptoms of the onset of lactation, both before and after delivery, on the postnatal questionnaire (Appendix 3). Maternal perceptions of lactogenesis II stratified by gestational age at delivery group are listed in Table 5.4. There was no statistically significant difference between gestational age groups for intended duration of breastfeeding although women who delivered between 34 and 36 weeks' gestational age were less likely to intend to breastfeed for longer than six months.

The majority of women did not feel any breast changes while still pregnant after the betamethasone injection, although there were small numbers who experienced increased fullness of the breast (N = 6), increased milk leakage (N = 7) and other changes (N = 5) before delivery occurred. There was no significant association between excretion of lactose in antenatal urine and either breast fullness (P = 0.781), milk leakage (P = 0.148), or other breast changes (P = 0.099).

After delivery, most women perceived the onset of lactation to occur on the third postnatal day although there was a wide range of days on which women first perceived symptoms occurring. There was no difference in timing of perceived onset of lactation between different gestational ages at delivery (fullness P = 0.508, warmth P = 0.395, leaking P = 0.665). Nor was there any difference in timing of perceived onset of lactation between different intervals between betamethasone treatment and delivery

(data not shown; fullness P = 0.592, warmth P = 0.569, leaking P = 0.354). Less than 50% of women were able to detect milk ejection occurring and this was not affected by gestational age at delivery.

When compared with other measures of lactogenesis II there were no significant associations between maternal perception of either onset of breast fullness or milk leakage and postnatal urinary excretion of lactose (fullness P = 0.470, leaking P = 0.683). Maternal perceptions of breast changes were also not significantly associated with either milk lactose concentration (fullness P = 0.122, warmth P = 0.583), or milk volume (fullness P = 0.304, warmth P = 0.270). In contrast delayed onset of leaking was associated with reduced milk lactose concentration ($P < 0.001$) and there was a trend for significance with reduced milk volume (P = 0.056).

Problems with lactation in the first ten days differed significantly according to gestational age at delivery. Mothers of term infants were more likely to experience engorgement and cracked nipples whereas mothers of infants born before 34 weeks' gestational age were significantly more likely to perceive that they had problems with low breastmilk supply.

**Table 5.4 Maternal Perceptions of Lactogenesis II**

| N = 74[1] | < 34 weeks (N = 48) | | 34 – 36 weeks (N = 8) | | ≥ 37 weeks (N = 18) | | |
|---|---|---|---|---|---|---|---|
| | N | % | N | % | N | % | P value |
| Intended duration of breastfeeding | | | | | | | |
| Short term only | 1 | 2.1 | 0 | 0 | 0 | 0 | 0.423 |
| ≤ 6 months | 20 | 41.7 | 6 | 75.0 | 10 | 55.6 | |
| > 6 months | 27 | 56.3 | 2 | 25.0 | 8 | 44.4 | |
| Breast changes after betamethasone injection | | | | | | | |
| None | 39 | 81.3 | 6 | 75.0 | 39 | 81.3 | 0.881 |
| Fullness | 2 | 4.1 | 1 | 12.5 | 3 | 16.7 | 0.061 |
| Leaking | 5 | 10.4 | 1 | 12.5 | 1 | 5.6 | 0.801 |
| Other[2] | 4 | 8.3 | 0 | 0 | 1 | 5.6 | 0.492 |
| Onset of symptoms of lactogenesis II (days postpartum)[3] | | | | | | | |
| Breast fullness | 3 | (1-9) | 3 | (2-6) | 3 | (2-5) | 0.508 |
| Breast warmth | 3 | (0-8) | 3 | (2-10) | 3.5 | (2-4) | 0.395 |
| Tingling | 3 | (0-9) | 3 | (2-10) | 3 | (1-5) | 0.586 |
| Leaking | 3.5 | (0-10) | 3 | (2-7) | 4.5 | (3-7) | 0.665 |
| Feels milk ejection | 16 | 33.3 | 4 | 50.0 | 6 | 33.3 | 0.647 |
| Lactation problems | | | | | | | |
| Engorgement | 10 | 20.8 | 1 | 12.5 | 7 | 38.9 | 0.223 |
| Mastitis | 1 | 2.1 | 0 | 0 | 0 | 0 | |
| Cracked nipples | 1 | 2.1 | 0 | 0 | 7 | 38.9 | < 0.001 |
| Low milk supply | 12 | 25.0 | 1 | 12.5 | 0 | 0 | 0.055 |
| Feeding difficulty | 0 | 0 | 2 | 25.0 | 4 | 22.2 | 0.002 |

[1] maternal questionnaire was not completed by 4 women.
[2] includes nipple change, nipple tingling, veins more obvious,
[3] median (range)

### 5.3.8 Non-Responders

A total of 108 women were originally approached to participate in the study. Seven women withdrew soon after betamethasone treatment, without providing any antenatal or postnatal samples. A further 23 women withdrew before delivery after initially providing antenatal 24-hour urine samples. Therefore 78 women provided at least one of 24-hour postnatal urine samples, milk samples, or milk volume measurement. In order to determine whether the sample of 78 women was representative of all women, the characteristics of non-responders was compared with those of women who participated in the postnatal phase of the present study (Table 5.5).

There was no difference in age between non-responders and participants in the postnatal phase of the study. Non-responders were less likely to have completed tertiary education (P = 0.048). There was no difference in intended breastfeeding duration or previous experience of breastfeeding. As occurred in study participants, around 40% of non-responders were in their first pregnancy and there was no significant difference in the timing of the first betamethasone injection. The gestational age at delivery was significantly longer in non-responders with a median of 37 weeks compared with 32 weeks in the participants (P = 0.002) and there were corresponding significant increases in interval between treatment and delivery as well as infant parameters such as birth weight and admission to the neonatal nursery. Non-responders were also less likely to have a male infant than study participants (P = 0.012).

**Table 5.5 Characteristics of Non-Responders and Responders who participated in Postnatal Sample Collection**

| | Non-responders (N=23) | | Responders (N=78) | | |
|---|---|---|---|---|---|
| | N/mean/median | %/sd/range | N/mean/median | %/sd/range | P value |
| Age (years)[1] | 30.1 | (6.5) | 32.1 | (5.3) | 0.187 |
| Tertiary education | 2 | 11.1 | 27 | 36.5 | 0.048 |
| Intended breastfeeding duration | | | | | |
| Short term only | 2 | 10.5 | 1 | 1.4 | 0.110 |
| ≤ 6 months | 7 | 36.8 | 36 | 48.7 | |
| > 6 months | 10 | 52.6 | 37 | 50.0 | |
| Previous breastfeeding experience | 11 | 47.8 | 40 | 52.6 | 0.813 |
| Smoked during pregnancy | 8 | 42.1 | 19 | 25.7 | 0.169 |
| Parity 0 | 11 | 40.7 | 35 | 44.9 | 0.823 |
| Gestational age at first betamethasone treatment (weeks)[2] | 27.9 | (23.7-33.3) | 30.3 | (23.6-33.7) | 0.158 |
| Caesarean section delivery | 8 | 32.0 | 42 | 53.9 | 0.068 |
| Interval between first betamethasone treatment and birth (days)[2] | 61 | (1-118) | 8 | (0-109) | <0.001 |
| Gestational age at delivery (weeks)[2] | 37.0 | (24.6-41.1) | 32.4 | (24.3-41.7) | 0.002 |
| Birth weight (g)[2] | 2728 | (495-4200) | 1807 | (640-4275) | 0.001 |
| Male infant[3] | 13 | 43.3 | 59 | 67.0 | 0.012 |

[1] Mean (SD),
[2] Median (range),
[3] Includes 13 sets of twins

## 5.4 Discussion

Antenatal glucocorticoid administration was associated with transient increases in the excretion of lactose in antenatal urine indicating a premature onset of lactogenesis in women who remained pregnant. In contrast to women who delivered preterm (Chapter 4), this precocious onset of lactogenesis had no impact on timing of lactogenesis II after birth in women who remained pregnant and progressed to deliver at either term or near-term.

Betamethasone-induced increases in lactose excretion in antenatal urine were not accompanied by clinically significant changes in the excretion in urine of the progesterone metabolite pregnanediol glucuronide (PdG). There was only a mild, transient decrease in PdG, suggesting that the increase in lactose secretion may not have been caused by a removal of the progesterone-inhibition of the onset of secretion from the mammary lactocytes in women.

While these findings are interesting they are not necessarily of major clinical importance in mothers who remain pregnant and who eventually deliver at term as the changes were only transient and did not lead to long-standing adverse effects on postnatal lactogenesis II. Clinically significant postnatal effects of betamethasone treatment on either milk lactose concentration or milk production were observed only in women who delivered preterm (Chapter 4). Moreover gestational age at delivery was shown to be the most important indicator of the success of lactation.

### 5.4.1 Pregnanediol Glucuronide Excretion in Urine

The present study used the excretion of the progesterone metabolite pregnanediol glucuronide (PdG) in antenatal urine samples as a marker of plasma progesterone after antenatal glucocorticoid treatment. This represents the first recorded examination of which I am aware of the excretion of PdG in urine samples of pregnant women although

the procedure has been validated in women who are aiming to avoid pregnancy and in those who are undergoing super-ovulation procedures for assisted reproduction technology (Sauer & Paulson 1991; Hughes 1996; Stanczyk, Gentzschein *et al.* 1997). The present study found that PdG excretion in urine increased with advancing gestational age. Unlike the findings in sheep (Chapter 3), the present study did not find a sustained decrease in PdG concentration in urine after administration of betamethasone. This finding is consistent with those of other published studies that have found no changes in circulating progesterone levels after glucocorticoid administration in women (Ohrlander, Gennser et al. 1977; Ylikorkala, Dawood et al. 1978; Ogueh, Jones et al. 1999). Antenatal dexamethasone treatment has been found in one relatively recent study to precede a transient decrease in estrogen concentration in the 24 hours following treatment and a prolonged decrease in plasma human chorionic gonadotrophin (hCG), but no change in plasma progesterone up to 48 hours following treatment (Ogueh, Jones et al. 1999). In that reported study all hormones were measured by ELISA. Two earlier small studies used radioimmunoassay (RIA) to measure plasma concentration of estrone, estradiol-17β and progesterone in maternal plasma and amniotic fluid before and for up to ten days after antenatal betamethasone treatment (Ohrlander, Gennser et al. 1977; Ylikorkala, Dawood et al. 1978). Neither study found any changes in either hCG concentration or plasma progesterone concentration. Ohrlander *et al*. found significant reductions in estrone and estradiol-17β but this finding was not confirmed in the other study.

The magnitude of decrease in PdG concentration after betamethasone treatment was small. However, in light of the overall increase in PdG concentration with increasing gestational age, any putative betamethasone-induced decrease in PdG must also take account of a concomitant increase with time, suggesting an effect of betamethasone treatment which was greater than that observed in the unadjusted data.

There is strong existing evidence that treatment with synthetic glucocorticoids in pregnant women is associated with a transient increase in uterine activity associated with a marked decrease in plasma concentration of cortisol (Elliott & Radin 1995; Yeshaya, Orvieto *et al.* 1996; Challis, Matthews *et al.* 2000). The reason why the small decrease in PdG excretion was observed after betamethasone treatment in the present study is not readily explained as Challis *et al*. and other authors have found no withdrawal of progesterone accompanying the reported reduction in cortisol concentration in women (Challis, Matthews et al. 2000), but is consistent with my findings in sheep (Chapter 4). I speculate that the differences observed between the current study and previous research into the effects of glucocorticoid treatment on plasma progesterone may be explained by a reduction in the rate of breakdown of plasma progesterone into its metabolites after betamethasone treatment in order to maintain adequate circulating levels of progesterone.

The suggestion of a reduction in PdG excretion in women with threatened preterm labour also challenges the conventional belief that progesterone levels stay high until after labour and delivery (Challis, Matthews et al. 2000; Mesiano 2001). Other authors have found that decreases in the progesterone/17β-estradiol ratio, both systemically and also locally in amniotic fluid, accompany preterm birth in humans but these changes are due to increases in estrogen rather than decreases in progesterone (Mazor, Hershlkovitz *et al.* 1994). On the other hand, prophylactic administration of progesterone has been shown to be effective in reducing the frequency of preterm labour and birth in at-risk women suggesting that high concentrations of progesterone play a role in maintaining uterine quiescence in pregnancy (da Fonseca, Bittar *et al.* 2003).

My speculation of a reduction in the metabolism of circulating progesterone following glucocorticoid treatment may also explain the differences observed between findings in the present study and the published literature, and suggests that measurement of urinary

PdG excretion may not be the most appropriate marker of circulating progesterone concentration during pregnancies complicated by preterm labour. The present study was limited by the failure to take maternal plasma samples in order that plasma and urine concentrations of progesterone and its metabolites could be compared. It was decided *a priori* that serial blood sampling was overly intrusive and that urine sampling would be more accepted by study participants. However, I suggest that future research should include measurements of plasma progesterone concentration in order to determine if progesterone undergoes the same changes that were observed in the current study in PdG.

### 5.4.2 Effect of Antenatal Glucocorticoids on the Excretion of Lactose in Antenatal Urine

After onset of lactogenesis II, tight junctions between lactocytes become closed, and permeability only increases again during either mastitis or at involution (Hartmann, P E & Kulski 1978; Nguyen & Neville 1998). Before and during onset of lactogenesis II, permeable tight junctions between lactocytes allow the transfer of milk products, chiefly lactose, back into the circulation leading to increased concentrations of lactose in plasma. As little lactose is absorbed intact through the adult gastrointestinal tract, the mammary gland is virtually the only source of lactose in the circulation, and so increases in plasma lactose levels are presumed to indicate increases in secretion in the lactocytes (Arthur, Kent et al. 1991). Lactose is not metabolised and hence is excreted unchanged in urine. Therefore, the 24-hour excretion of lactose in urine is a relatively convenient and accurate indicator of mammary secretion during pregnancy when other methods such as concentration of lactose in either plasma or breast secretion are difficult to determine (Cox, Kent et al. 1999).

The present study found the excretion of lactose in urine increased with advancing gestational age and also increased significantly after betamethasone treatment suggesting precocious secretion by lactocytes occurred following glucocorticoid administration but before delivery. Significant positive associations between plasma lactose concentration and advancing gestational age have been shown previously (Arthur, Kent et al. 1991) but no other studies of which I am aware have examined the effects of synthetic glucocorticoids on plasma lactose concentration.

It was interesting that women who were treated with betamethasone at earlier gestational ages had a greater lactogenic response than at later gestational ages. This may be explained by the fact that they had a lower baseline concentration of lactose in urine; hence any increase in urine lactose concentration had a significant effect. Women at more advanced gestational ages were already starting to have lactose spilling over into their urine and so there was not so great a relative difference observed.

In addition, the present study finding that women as early as 24 weeks' gestational age were able to stimulate a lactogenic response suggests both that the lactocytes were sufficiently developed and that there were sufficient levels of the other hormones that stimulate lactogenesis to commence secretion even at very early stages of the third trimester. Secretion of lactose has been observed as early as 21 weeks' gestational age in the circulation (Arthur, Kent et al. 1991) and at 22 weeks' gestational age in urine (Cox, Kent et al. 1999) confirming that the breast is capable of synthesizing lactose by the third trimester of pregnancy.

The lack of a direct association between decreased excretion of progesterone and increased excretion of lactose in urine after betamethasone treatment contrasted with my findings in sheep (Chapter 3). Increasing concentrations of PdG in urine were positively associated with increasing lactose concentration as occurred with increasing gestational age. These findings suggest that advancing gestational age had a greater impact on the

relationship between the concentrations of PdG and lactose in urine than the effect of betamethasone treatment in women.

A potential confounding factor is the risk of increased plasma and urine concentrations of glucose after treatment with glucocorticoids. This is unlikely to impact on the concentration of lactose that was measured in urine, however, as the assay measured galactose rather than glucose with the enzyme β-galactose dehydrogenase used to reduce $NAD^+$ to NADH for measurement of absorbance. This assay also accounted for background galactose to give an absolute measure of lactose in urine.

### 5.4.3 Postnatal Excretion of Lactose in Urine

After birth, the extent of escape of lactose to the general circulation is strongly influenced by both the timing of closure of the tight junctions between lactocytes (Hartmann, P E & Kulski 1978; Nguyen & Neville 1998), and the extent of milk removal, either by frequent breastfeeding or by effective emptying of the breasts using mechanical expression (Arthur, Kent et al. 1991).

My research represents the largest study of which I am aware to compare postnatal concentration of lactose in urine with other more conventional measures of lactogenesis II. No relationship was found between excretion of lactose in postnatal urine and concentration of lactose milk in the first seven days postpartum. In contrast to the present study, positive correlations between urine lactose concentration and either milk lactose concentration or milk production were found in several small studies although methodological problems occurred in all studies (Strand & Johnston 1992; Murtaugh, Kerver *et al.* 1996; Cox, Kent *et al.* 1999). While Cox *et al.* found a strong association between increases in urine and milk lactose concentration in a small study of women in the first three days after birth (Cox, Kent et al. 1999) no other studies have examined urine lactose concentration within two weeks of delivery. Kalkwarf *et al.* found a

positive association between urine lactose concentration and milk transfer measured by infant test-weighing with infants over six weeks of age, although the relationship was not strong and the authors concluded that urine lactose excretion best measures only gross changes in milk production (Kalkwarf & Kalis 1997). Murtaugh *et al.,* who studied mothers of preterm infants at least four weeks of age, and used expressed milk volume as the marker of milk production, found a positive relationship between milk production and urine lactose:creatinine ratio in first morning spot urine samples (Murtaugh, Kerver et al. 1996). However the small sample size and broad heterogeneity between subjects is a major limitation of that study. In contrast another small study found no relationship between urinary lactose excretion in 24-hour samples and milk output determined by infant test-weighing (Yoon, Fung *et al.* 1996).

The failure to find an association between postnatal urinary lactose excretion and milk lactose concentration in the present study may also be due to low numbers with only 37 observations where both milk and urine lactose concentration were measured on the same day, 41 observations where both urine samples were collected and milk volume was measured, and only six observations where all of milk and urine lactose concentration and milk volume were measured on the same day between days four and seven postpartum. The effective sample size in the present study, however, vastly exceeds that of any other published study to my knowledge.

It is also possible that maternal hydration status, postpartum diuresis, or contamination from vaginal loss may have reduced the effectiveness of urine lactose concentration as a marker for lactogenesis II in the present study of puerperal women. However, 24-hour urinary excretion, as used in the present study, is a more valid measure of lactose concentration in urine than other methods of analysis of urine. For example studies, which used a single first morning void and measured urinary creatinine to correct for differences in hydration and urine volumes found that urine lactose concentration in 24-

hour samples better predicted milk production than spot urine samples with measurement of creatinine clearance ratios (Kalkwarf & Kalis 1997).

The urine collection and storage protocol in the present study was adequate to maintain stability of lactose concentration over time (Section 2.2.3) The protocol for collection, which required that urine samples were kept on ice at all times for a maximum of 12 hours and then transferred to storage at -20°C, was comparable with, or more rigorous than, those used by previous authors (Strand & Johnston 1992; Murtaugh, Kerver *et al.* 1996; Kalkwarf & Kalis 1997; Cox, Kent *et al.* 1999). For example no loss of lactose concentration has been observed when urine was left at room temperature up to 24 hours when compared with urine that had been immediately frozen (Murtaugh, Kerver et al. 1996; Yoon, Fung et al. 1996).

#### *5.4.3.1 Effect of Gestational Age at Birth and Antenatal Glucocorticoids on Postnatal Urine Lactose Excretion*

The excretion of lactose in postnatal urine was significantly associated with days after delivery with increases occurring with increasing postnatal age up to day five postpartum. However, antenatal synthetic glucocorticoid treatment had no impact on the excretion of lactose in urine of postpartum women. In addition, there was no association found between excretion of lactose in postnatal urine and gestational age at delivery. These findings were in contrast with the significant effect of glucocorticoids found in the excretion of lactose in antenatal urine and suggest that, in women, transient, antenatal stimulation of premature lactogenesis may not result in changes to postnatal onset of lactogenesis II as determined by urinary excretion of lactose. However, as the urine lactose assay is less sensitive than other measures of lactogenesis II in the postnatal period (milk volume and/or concentration of lactose in milk), we cannot

automatically infer an absence of an effect of gestational age or betamethasone treatment on postnatal lactogenesis II from these findings.

### 5.4.4 Effect of Gestational Age at Birth, Glucocorticoid Therapy and Other Factors on Milk Lactose Concentration

When adjusted for postnatal age, the concentration of lactose in milk was significantly associated with gestational age at delivery. Higher values were observed at more advanced gestational age. This difference was more evident in the early days postpartum, suggesting that lactogenesis II was delayed with more premature gestational age at delivery. This finding confirms that of the previous chapter, which found an effect of gestational age at delivery on milk lactose concentration in mothers of preterm infants.

Similarly (see Chapter 4), there was no significant effect observed of betamethasone treatment on the concentration of lactose in milk. As was found with the excretion of lactose in postnatal urine, these findings support the assumption that if lactogenesis is prematurely stimulated by glucocorticoids during pregnancy, there is no adverse impact on the concentration of lactose in milk postpartum and thus no effect on the onset of lactogenesis II postpartum at term. Furthermore there were no significant differences in milk lactose concentration between women treated with betamethasone who subsequently delivered at term and the healthy control women. However, no inference can be made from this finding because of the small numbers of controls.

An interesting finding of the present analysis was the significant association between intended prolonged duration of breastfeeding and increased concentration of lactose in milk. This confirms the finding in the previous analysis of mothers who delivered preterm of a strong association between maternal intention and expressed milk volume. The precise biological mechanism by which the association between maternal intention

and concentration of lactose in milk is unclear but may be related to increased frequency of breastfeeding.

### 5.4.5 Effect of Gestational Age at Birth, Glucocorticoid Therapy and Other Factors on Milk Volume

In the present analysis, the inclusion of milk volume transferred by term mothers confirmed the finding in the previous chapter that gestational age at delivery strongly predicts milk volume in the first week. There were only small numbers at term but these women measured significantly higher milk transfer on days two to four postpartum than mothers who were expressing for preterm infants. However, the use of milk transfer has limited accuracy for measuring milk production in mothers of breastfeeding infants. Milk transfer, determined by weighing the infant before and after all feeds, is at best a measure of the infant's capacity to consume milk and does not account for either the remaining milk, if mothers had an over-supply, or insensible loss by mother and infant. Thus the secretory capacity of the lactocytes is likely to be under-estimated in mothers who were breastfeeding their term infants in this study indicating that, at worst, the effect of gestational age on milk production was under-estimated in the present study. There was no significant effect observed of betamethasone treatment on subsequent postpartum milk transfer when mothers who delivered at term were included in the analysis. This is consistent with the findings when the concentration of lactose in either postnatal urine or milk were used as the outcome marker for lactogenesis II in the present study and reinforces the conclusion that glucocorticoid treatment during pregnancy has no long-lasting effect on postnatal lactogenesis II in women. Maternal intended duration of breastfeeding strongly predicted milk volume transfer in this analysis with women intending to breastfeed for a prolonged duration measuring greater volumes of milk than those who intended to breastfeed for a shorter duration.

This confirms similar findings with milk lactose concentration and suggests that maternal intended duration of breastfeeding is a robust predictor of lactogenesis II regardless of gestational age at birth. This finding for lactogenesis II is consistent with the large body of research which shows that maternal intention is a strong predictor of either breastmilk volume or duration of breastfeeding (Bloom, Goldbloom et al. 1982; Henderson, Evans et al. 2003).

### 5.4.6 Maternal Perceptions of Onset of Lactogenesis II

Maternal report of the sensation of the "milk coming in" is frequently used by researchers as a convenient marker for the onset of lactogenesis II (Chapman & Pérez-Escamilla 2000a; Pérez-Escamilla & Chapman 2001). Chapman and Pérez-Escamilla deduced that maternal perception is a valid indicator of delayed onset of lactogenesis II after finding that the sensitivity and specificity of breast symptoms such as fullness and leakage were 71% and 79%, respectively, when compared with test weighing in mothers of term breastfeeding infants (Chapman & Pérez-Escamilla 2000a).

In order to assess the effectiveness of maternal perception of lactogenesis II in the present cohort, which was heavily weighted towards preterm delivery, women were asked to record the day after birth in which they first felt the sensations of breast fullness, warmth, tingling or leaking. Perceived onset of lactogenesis II was reported at a median of 3 days postpartum, although there was wide variability. There was no difference in timing of maternal perception between gestational age groups suggesting maternal perception may be only a crude marker of lactogenesis II in populations similar to the present cohort and less sensitive than measures of either milk volume or biochemical markers of lactogenesis II such as milk lactose. Moreover, maternal perception of onset of breast fullness or warmth did not predict any of the other markers

of lactogenesis II in the present study. Initiation of breast leaking was the only symptom to have a relationship with lactogenesis II markers.

These findings of limited efficacy of maternal perception of onset of lactation in determining the actual onset of lactogenesis II suggest that a requirement of any future study of precursors to lactogenesis II is that more rigorous methods of detection of onset of milk production should be used, at least in populations of women who deliver prematurely.

#### *5.4.6.1 Maternal Perceptions of Precocious Onset of Lactogenesis II after Betamethasone Treatment*

The present study also failed to find any association between gestational age at delivery and maternal perceptions of breast changes after betamethasone treatment but before delivery. To avoid the possibility of biasing their responses, the study participants were not coached in what breast symptoms to expect after treatment (See Appendix 3 - Maternal Questionnaire, question 35). Consequently it was surprising to find that as many as 20% of women perceived some breast changes occurring as a result of the betamethasone treatment. There was no correlation between the reported breast changes and changes in antenatal urine lactose concentration. A possible Hawthorne effect in the reports of antenatal breast changes cannot be excluded (Burton 2006). Despite my attempts to avoid leading their responses, participants in this study may have been more alert to any breast changes than the general population of women being treated with antenatal glucocorticoids.

### 5.4.7 Further Limitations of the Study

The present study represents the only research of which I am aware to investigate the effects of antenatal glucocorticoid treatment on precocious lactogenesis using changes in the excretion of lactose and progesterone in urine. Whereas the sample size was

adequate for antenatal outcomes, a moderate drop-out rate (28%) after delivery may restrict the possibility of generalizing postnatal results to other populations. Moreover the drop-out rate was not homogeneous with more women who eventually delivered at term or near term being lost to follow-up than women who delivered a preterm infant. Few women in the present study delivered between 34 and 36 weeks' gestational age (N = 8). In addition a lower proportion of the women who delivered between 34 and 36 weeks' gestational age provided samples and no women in this gestational age group measured their milk volumes. This is because it was difficult for these women, who were attempting to establish breastfeeding in trying conditions with relatively small, immature infants, to undertake the demanding study protocol of weighing the infant as well as measuring any expressed breastmilk. The group of women who delivered between 34 and 36 weeks' gestational age was different in obstetric characteristics compared with women who delivered either earlier or at term. In this gestational age group there were significantly fewer male infants and fewer women who delivered by caesarean section. There were also significantly more women having their first pregnancy and half of the infants were growth restricted *in utero* compared with lower proportions of mothers of either very preterm or term infants.

Differences between the study participants and those women who either chose not to take part or failed to complete the study protocol may also limit the ability to generalise these findings. Women who dropped out early and provided no or very few samples were not only less likely to be well educated, but also were more likely to deliver at term and with a lengthy interval after betamethasone treatment (Table 5.5). The median gestational age at delivery for non-responders was 37 weeks compared with 32 weeks for women who completed the study. The study protocol was very demanding for many women and they required frequent contact with me to remain motivated to continue. In particular, women who were no longer at risk of a preterm delivery were likely to

eventually deliver in hospitals other than the tertiary referral hospital in which they were recruited, and were, therefore, less likely to have maintained the rapport with myself. Therefore women who eventually delivered a term infant were less likely to be motivated to undertake the postnatal sample collection or the milk transfer measurement. Consequently the women who had term or near-term deliveries are under-represented in these results suggesting that there is a lower likelihood that the findings could be able to generalise to other populations.

Other factors which may affect the ability to generalise the present study findings are reflected in the high rates of obstetric complications and obstetric interventions. The caesarean section rates were very high, up to 60% in the women who delivered at term. These differences, however, can be explained by the high risk population from which the study sample was recruited.

Another potential difference from the general population was the relatively high proportion of mothers who were still breastfeeding on discharge of the infant from hospital. Previous studies of breastfeeding rates show that not only are non-responders more likely to have low social class and to be smokers but they are less likely to breastfeed their infants (Shepherd, Power *et al.* 1998). The present study also found that non-responders were less likely to be well-educated, an indication of social class, suggesting that women who dropped out may be more likely to eventually artificially feed that women who participated in the postnatal phase of the study. A motivation to breastfeed was an important selection criterion in this study and breastfeeding rates on discharge from hospital were comparable or better than previous research in preterm infants (Yip, Lee et al. 1996; Furman, Minich et al. 1998) and comparable to studies in mothers of term infants (Donath & Amir 2000; Henderson, Evans *et al.* 2003) (Table 5.4).

#### *5.4.7.1 Control Group*

The small numbers in the control group (N = 6) posed limitations in the ability to compare data from betamethasone-treated women with that of women with normal pregnancies. Initially it was planned to recruit two control groups, one with women with normal pregnancies and one with women who had preterm deliveries without receiving antenatal betamethasone treatment. Because of evolving management of suspected preterm delivery in Western Australia, in the study time frame there were very few women delivered early without receiving any antenatal betamethasone treatment, available for recruitment.

Similarly, due to time constraints accompanied by general lack of interest among women with normal pregnancies, recruitment of adequate numbers for the normal control group was very difficult. Consequently, the small control group was recruited from a convenience sample of women employed by the institution. These women differed from the study population in their high levels of education and professional employment. For these reasons, it was not possible to make statistical comparisons between study participants and controls, and hence control urine data are presented out of interest only.

### 5.4.8 Summary

This present study was a comprehensive attempt to investigate the effects of antenatal glucocorticoid administration on lactogenesis II in women. I found mild, transient, precocious stimulation of lactogenesis occurred in women who were still pregnant and this stimulation was associated with small decreases in pregnanediol glucuronide (PdG) excretion in urine. Decreases in PdG excretion may suggest a withdrawal of circulating progesterone as I found in sheep, but could also reflect differences in the metabolism of progesterone after glucocorticoid treatment.

Despite finding a significant impact on antenatal lactogenesis, the current study did not find any long-lasting effects on onset of lactogenesis II after term delivery, suggesting that postnatal onset of lactogenesis II is more likely to be affected by gestational age at delivery than antenatal glucocorticoid administration.

# CHAPTER 6 EFFECTS OF ANTENATAL GLUCOCORTICOID TREATMENT ON LACTOGENESIS IN BOTH SHEEP AND WOMEN: GENERAL DISCUSSION

*"It is obvious to the whole world that a service is better than an injury, that gentleness is preferable to anger. It only remains, therefore, to use our reason to discern the shades of goodness and badness."* (*Voltaire, 1826, Dictionnaire Philosophique)*

There are no previous reports on the effects of the antenatal administration of glucocorticoids for anticipated preterm delivery on lactation. I found that, in sheep, antenatal glucocorticoid administration caused a precocious onset of lactogenesis II-like milk secretion and subsequently to profound disruption to normal lactogenesis II after parturition. The findings in women were less clear-cut than those in sheep with only subtle effects observed on the synthesis of lactose at the time of glucocorticoid administration and on lactogenesis II after birth. Indeed of all the factors examined, early gestational age at delivery was the strongest predictor of delayed onset of lactogenesis II in women.

## 6.1 Proposed mechanisms of the effect of antenatal exogenous glucocorticoids on lactogenesis

The mechanism explaining the effect of antenatal glucocorticoid treatment on lactogenesis is likely to be multifactorial. Potential factors that triggered the premature onset of mammary secretion in sheep include either premature withdrawal of progesterone, or the displacement of progesterone from glucocorticoid receptors in the presence of high levels of prolactin and exogenous glucocorticoids. This event may

have been followed by the involution of lactocytes in the absence of milk removal and failure of subsequent lactogenesis II after parturition.

### 6.1.1 Progesterone withdrawal

In sheep, administration of synthetic glucocorticoids resulted in a marked suppression of progesterone which was sustained for a prolonged period. This finding is consistent with a large body of research, which shows that rising cortisol levels in pregnant sheep cause changes in placental steroid output resulting in reduced levels of progesterone, thereby stimulating the onset of parturition (Challis, Matthews et al. 2000).

The placenta is the source of progesterone in advanced pregnancy in both ewes and women, but patterns of progesterone secretion differ between species. In sheep, the sharp decline in the synthesis of progesterone by the placenta is brought about by rapid increases in circulating glucocorticoids either by the surge of fetal cortisol secretion, which causes maturation of fetal organ systems at the end of pregnancy (Challis, Matthews et al. 2000; Mesiano 2001), or by exogenous glucocorticoid administration (Liggins 1969; Nathanielsz, Buster et al. 1988). Raised glucocorticoid levels induce the up-regulation in the placenta of 17α-hydroxylase, which shifts placental steroidogenesis in favour of increased estradiol secretion at the expense of progesterone secretion (Challis, Matthews et al. 2000). The fall in progesterone and increase in estrogen concentrations stimulate increased prostaglandin output in the uterus which leads to increased contractility and subsequent parturition (Challis, Matthews et al. 2000).

#### *6.1.1.1 Medroxyprogesterone acetate*

Pre-treatment with the progesterone analogue medroxyprogesterone acetate (MPA) inhibits premature parturition caused by exogenous glucocorticoid administration in sheep (Nathanielsz, Buster et al. 1988). However, in Chapter 3 I showed that MPA treatment was not effective in preventing the betamethasone-induced premature onset of

lactogenesis. Moreover, sheep treated with MPA alone (without betamethasone) showed no differences from saline controls in antenatal concentrations in plasma of either lactose or the lactogenic hormones (prolactin, cortisol and progesterone). The only difference was a small delay in initiation of copious milk secretion after parturition (Section 3.3.1.2). The finding that MPA treatment did not prevent the premature stimulation of lactogenesis II, despite protecting against premature parturition, suggests that, in sheep, there are differences in function of progesterone receptors between the placenta and mammary gland. It is unclear whether or not these differences in response to progesterone also occur in women. As there is no ready explanation for these putative differences, further research into differential effects of glucocorticoids on hormone receptors in mammary glands and the placenta is indicated.

#### *6.1.1.2 Progesterone withdrawal triggers the postnatal onset of lactogenesis II*

While there are differences in the origin and function of progesterone during pregnancy and parturition between rats, ewes and women, progesterone withdrawal is a common trigger for lactogenesis II in all three species (Kuhn 1969; Turkington & Hill 1969; Hartmann, P E, Trevethan *et al.* 1973). I propose that, in sheep, the premature withdrawal of progesterone after exogenous glucocorticoid treatment was sufficient to trigger a premature onset of mammary secretion in the presence of high levels of exogenous glucocorticoids and a surge of prolactin, despite the presence of MPA.

#### *6.1.1.3 Effects of antenatal glucocorticoids on progesterone in women*

In primates such as women, where 17α-hydroxylase is not present in the placenta, glucocorticoids do not exert the same function on steroid synthesis as in sheep (Challis, Matthews et al. 2000). In women progesterone levels remain high throughout pregnancy and a tangible withdrawal only occurs after delivery; hence lactogenesis II is normally delayed until around two days after birth.

The role of progesterone in the onset of parturition in women is poorly understood (Mesiano 2001). There is evidence in primates, however, of a functional withdrawal of progesterone prior to parturition caused by a change in the dominance of specific progesterone receptor isoforms in the myometrium and fetal membrane tissues (Haluska, Wells *et al.* 2002). Despite the existence of similar progesterone receptor isoforms in the mammary gland (Shymala 1999; Shymala, Louie *et al.* 1999), it is unknown whether a similar process stimulates the onset of lactogenesis II, but it seems unlikely.

Furthermore, compared with ewes, women do not require progestagen cover (MPA) to prevent preterm delivery after glucocorticoid treatment in pregnancy, although increases in uterine irritability have been reported after antenatal betamethasone treatment (Elliott & Radin 1995; Yeshaya, Orvieto *et al.* 1996; Challis, Matthews *et al.* 2000).

This absence of a major effect on progesterone in women may explain why only weak responses on lactogenesis to betamethasone were observed either in pregnancy (Chapter 5) or after preterm delivery (Chapter 4). Although progesterone withdrawal is unlikely to explain the observed minor effects, antenatal betamethasone treatment resulted in a transient arrested increase in excretion of the progesterone metabolite, pregnanediol glucuronide (PdG) in urine (Section 5.3.1). This was a measurable change, but was not of a magnitude consistent with the triggering of lactogenesis II. This finding suggests that the progesterone withdrawal mechanism in women is more complex than previously considered and that there may be may be more than one mechanism existing for triggering lactogenesis in pregnancy.

An alternative explanation for the mild suppression of PdG after antenatal betamethasone may be a decreased catabolism of progesterone in order to maintain adequate levels of circulating progesterone. This suggests that future studies of

glucocorticoid treatment should compare blood progesterone levels with urinary PdG output.

### 6.1.2 Competitive Binding with Glucocorticoid Receptor

An alternative potential mechanism by which antenatal glucocorticoid administration may cause a premature onset of lactogenesis II is via changes in glucocorticoid receptor binding. Attachment of synthetic glucocorticoids to glucocorticoid receptors, which competitively bind with progesterone during pregnancy, may lead to premature displacement of progesterone in the lactocytes. Progesterone binding to glucocorticoid receptors in the mammary gland inhibits up-regulation of milk protein genes because, unlike glucocorticoids, progesterone does not translocate to the nucleus of lactocytes (Collier & Tucker 1978; Lee & Oka 1992). Similarly, progesterone has been shown to inhibit the activation of pathways leading to the onset of labour in women (Karalis, Goodwin *et al.* 1996).

Thus, progesterone displacement from glucocorticoid receptors may potentially cause a functional withdrawal of the progesterone-inhibition of lactogenesis (Maki, Hirose *et al.* 1980; Lindenbaum & Chatterton 1981), allowing the onset of increased milk secretion. In contrast, the studies in this thesis demonstrated an actual reduction in circulating progesterone, as evidenced by mild transient reductions of PdG in urine in women, and by profound effects on plasma progesterone concentration in sheep. These contradictions reinforce the suggestion of multi-factorial mechanisms for the effect of antenatal glucocorticoid treatment on premature lactogenesis.

### 6.1.3 Involution of Mammary Gland after Precocious Stimulation

I found reductions in the concentration of lactose in sheep plasma after premature glucocorticoid-stimulated onset of lactogenesis, suggesting that involution had commenced in mammary glands in the absence of milk removal before parturition.

Local signals, triggered by milk stasis in the distended mammary gland, are known to initiate involution soon after cessation of milk removal during mature lactation (Figure 1.2) (Li, Liu et al. 1997; Tatarczuch, Philip et al. 1997). The significance of this finding is that involution involves the apoptosis of lactocytes, which takes approximately 30 days in sheep after a peak of programmed cell death at four days after cessation of milk removal (Tatarczuch, Philip et al. 1997). I propose that incomplete involution after premature stimulation of lactogenesis II may adversely impact on the successful up-regulation of milk proteins during the normal onset of lactogenesis II at parturition.

In rodents, involution consists of apoptosis of the lactocytes followed by complete tissue remodelling (Strange, Li *et al.* 1992) but remodelling does not occur in ruminant species such as sheep, which maintain general structural integrity of the mammary gland during involution (Tatarczuch, Philip et al. 1997). However, completion of the functional changes occurring during involution is necessary to optimise milk production in the subsequent lactation period. Dairy animal husbandry generally aims for a nonlactating ("dry") period before the subsequent lactogenesis II (Hurley 1989).

This suggests to me that incomplete involution may have been responsible at least in part to the delayed and inadequate increases in the secretion of milk lactose that were observed in betamethasone-treated sheep after parturition. This suggestion is supported by studies using mice, which have shown that premature initiation of lactation disrupts subsequent milk secretion after parturition (Gorska, Jensen *et al.* 2003).

A similar process is unlikely in women after the observed premature stimulation of the mammary gland, which was mediated by antenatal glucocorticoid treatment. Women who did not deliver prematurely after receiving antenatal glucocorticoids did not show any adverse effects on lactogenesis II after term delivery. This difference in species effect may be explained by the mild, transient, and not universal, lactogenic stimulation in women, which may not have been of sufficient magnitude to cause distension of the

lactocytes, thereby failing to stimulate the onset of an apoptotic cascade, leading to involution. Moreover, there is evidence, in women who continue lactation during a second pregnancy and subsequently tandem feed the older child as well as the newborn infant, that subsequent milk production is not affected by continuing feeding during pregnancy (Prosser, Saint *et al.* 1984). The initiation of copious milk secretion after the birth of the second infant occurred normally, despite a gradual reduction in milk production during pregnancy. This suggests that women may not be subject to the putative "partial involution" and subsequent delay in lactogenesis II after birth that I have hypothesised occurs in sheep.

### 6.1.4 Gestational Age at Treatment with Antenatal Glucocorticoids

In women, antenatal betamethasone treatment stimulated a lactogenic response during pregnancy in the form of elevated excretion of lactose in urine but this was only transient and minor. No major adverse effects were found on subsequent lactogenesis II after delivery. There was, however, a suggestion that the effects of glucocorticoids differed with gestational age at treatment. At more advanced but still very preterm gestational ages (28-33 weeks), milk production was reduced in women who delivered three to nine days after antenatal betamethasone treatment compared with women who delivered immediately after treatment (Figure 4.4). No adverse effect was observed after delivery at extremely preterm gestational ages (< 28 weeks). This may have been related to the finding that the degree of prematurity caused a considerable delay in lactogenesis II, obscuring any subtle effects associated with antenatal betamethasone treatment after delivery at early gestational ages.

It is interesting that increases in lactose excretion in urine after glucocorticoid treatment were more marked at extremely early gestational ages (23-27 weeks; Figure 5.4) compared with more advanced preterm gestational ages. This finding suggests that

lactocytes were sufficiently developed at early stages in the second half of pregnancy to enable a small lactogenic response to maternal betamethasone during pregnancy.

## 6.2 Utility of the Sheep Model in Predicting Lactogenesis II in Women

The difference in response to antenatal glucocorticoid treatment between women and sheep raises the question of the suitability of using a sheep model to predict breast function in women. Whereas sheep have long been used to model effects of antenatal insults such as exogenous glucocorticoids on the fetus [Table 6.1; (Liggins 1969; Nathanielsz, Buster *et al.* 1988; Quinlivan, Archer *et al.* 1998; Huang, W. L., Beazley *et al.* 1999; Sloboda, Newnham *et al.* 2000; Moss, T. J., Sloboda *et al.* 2001; Moss, T J M, Harding *et al.* 2002)], the effects on lactation have not been explored previously. The differences between species in endogenous hormones such as glucocorticoids and progesterone, which influence the timing of lactogenesis II as well as parturition, emphasise the importance of confirming that the phenomena observed in pregnant sheep are also relevant to pregnant women. A comparison of current knowledge, and the validity of sheep models of the impact of antenatal glucocorticoids on fetal and postnatal development, is presented in Table 6.1.

**Table 6.1 Validity of Sheep Models to Explain the Effect of Exogenous Glucocorticoids on Human Fetal Development**

| System | Model Validity | Conditions | References |
|---|---|---|---|
| Respiratory | ✓ ✓ | *Betamethasone treatment induces early lung maturation* | (Ikegami, Jobe et al. 1997; Jobe, Newnham et al. 2003) |
| Cardiovascular | ✓ ✓ | *Betamethasone treatment alters postnatal blood pressure* | (Moss, T. J., Sloboda *et al.* 2001) |
| Kidney | ✓ ✓ | *Nephrogenesis is complete before birth in sheep and women* | (Berry, Polk *et al.* 1997) |
| Glucose metabolism | ✓ ✓ | *Postnatal insulin insensitivity to glucose challenge test* | (Moss, T. J., Sloboda *et al.* 2001) |
| Fetal programming of adult disease | ✓ ✓ | *Multiple maternal betamethasone & pregnancy undernutrition induce intrauterine growth restriction & predispose to alterations in adult cardiovascular & renal function* | (Dodic, Baird *et al.* 2001; Moss, T. J., Sloboda *et al.* 2001; Newnham 2001) |
| Brain | ✓ | *Developmental stages similar but more advanced at birth in sheep* | (Huang, W. L., Beazley *et al.* 1999) |
| HPA axis | ✓ | *Fetal up-regulation of HPA axis stimulates onset of parturition in sheep but not humans* | (Sloboda, Moss *et al.* 2002) |
| Parturition | ✓ | *Systemic withdrawal of progesterone in late pregnancy in sheep but not women* | (Challis, Matthews et al. 2000; Whittle, Patel et al. 2001) |
| ***Lactogenesis II*** | ? | *Occurs earlier in sheep because of earlier withdrawal of progesterone. The response to betamethasone is muted in women* | |

In the present studies, the lactogenic response to betamethasone was found to be dramatic in sheep and subtle in women, suggesting that human lactation is less sensitive to exogenous glucocorticoids than ovine lactation. This proposal is consistent with observations of premature parturition that occur in sheep but not in women after glucocorticoid treatment (Liggins 1969; Nathanielsz, Buster et al. 1988).

This may be explained by differences in the dosage of synthetic glucocorticoids given to each species. The dose of betamethasone used in the sheep experiments (a single intramuscular injection of 0.5 mg/kg ewe weight) is the lowest dose that induces a maturation of the fetal respiratory system (Rebello, Ikegami *et al.* 1996; Berry, Polk *et al.* 1997; Moss, T. J., Sloboda *et al.* 2001). This dosage exceeds that given clinically to women at risk of preterm delivery.

Based on the groundbreaking work of Liggins *et al.*, women are routinely given a single course of either two intramuscular injections of 11.4mg of betamethasone or a similar total dose of dexamethasone, regardless of maternal body weight (Liggins & Howie 1972). The broad range of responses found among women for milk volume as well as concentration of lactose and citrate in milk and concentration of lactose in antenatal urine, suggests a heterogeneous sensitivity to exogenous glucocorticoid stimulation. This is supported by similar variation in the pulmonary response of neonates whose mothers had been treated with betamethasone (Table 4.3). In the present study, only 30% of preterm infants whose mothers received antenatal betamethasone had no respiratory distress in the neonatal period. I was unable to detect, however, any maternal or obstetric factors, which may explain individual differences in lactogenic response to betamethasone, either during pregnancy or after birth. For instance, factors such as maternal age, body mass index or parity had no effect on precocious lactose increases during pregnancy after betamethasone treatment.

Despite differences in the magnitude of effect of glucocorticoid treatment and timing of lactogenesis II, both the endocrinology and the process of lactogenesis II in sheep follow the same pattern as in women. However, although the novel use of a sheep model to investigate lactogenesis II may be as plausible as it is to investigate the development of systems and organs in the fetus, this is conditional upon a better

understanding of the role and timing of progesterone withdrawal after glucocorticoid treatment.

## 6.3 Long-term implications

### 6.3.1 Initiation of lactation in mothers of preterm infants

The present thesis has clearly demonstrated the difficulties that mothers face when attempting to establish lactation after a very preterm birth. Compared with mothers of healthy term infants, the onset of copious milk secretion was delayed with the greatest delays occurring with the earliest gestational ages at delivery. Moreover, in many mothers of extremely preterm infants, milk production remained low by day ten postpartum. This delay has profound implications for the successful establishment of lactation and continuation of breastfeeding for prolonged periods. Either delayed lactogenesis II or reduced milk supply in the first week postpartum is strongly linked to premature cessation of breastfeeding in mothers of term infants (Houston, Howie *et al.* 1983; Chapman & Pérez-Escamilla 1999a; Ingram, Woolridge *et al.* 1999; Hruschka, Sellen *et al.* 2003) and these findings have been confirmed in mothers expressing for preterm infants (Hill & Aldag 2005). Low milk production on day four postpartum significantly predicted inadequate milk production at six weeks postpartum in mothers who were expressing for their infants less than 31 weeks gestational age (Hill & Aldag 2005).

These findings emphasize the importance of education, support and encouragement for mothers of preterm infants who are commencing to express and establish their milk supply (Meier, Engstrom *et al.* 1993; Pinelli, Atkinson *et al.* 2001).

Of particular importance is the low frequency of milk pumping by many of the mothers in the present study. The finding that frequency of pumping was strongly associated with milk volume is consistent with other studies of mothers of preterm infants (de

Carvalho, Anderson et al. 1985; Hopkinson, Schanler et al. 1988; Hill, Aldag et al. 2001), suggesting that education and encouragement to express at least six times per day is vital from the first day postpartum.

#### *6.3.1.1 Maternal Motivation to Succeed with Breastfeeding*

The psychological aspects associated with onset of lactation should not be discounted when evaluating the effects of gestational age at birth and antenatal interventions such as glucocorticoid treatment. Maternal motivation to breastfeed for long periods emerged as a strong predictive factor of the timing of lactogenesis II (Chapter 4, Figure 4.6). This association was robust between most measures used to describe lactogenesis II, including both expressed milk volume in mothers of preterm infants (Section 4.3.2.5) and milk lactose levels when all gestational ages were included (Section 5.3.5), although the precise biological mechanism of these associations is not clear.

Mothers of preterm infants are susceptible to increased rates of stress, anxiety and other psychological disorders (Singer, Salvator et al. 1999). Furthermore, stress is thought to adversely impact on timing of lactogenesis II (Chen, Nommsen-Rivers *et al.* 1998; Dewey 2001; Grajeda & Pérez-Escamilla 2002), as well as duration of breastfeeding (Henderson, Evans et al. 2003). It is highly likely that a strong motivation to succeed with lactation may assist mothers to overcome the potential barriers to breastfeeding that are prevalent in mothers of preterm infants. These findings confirm that psychological support of mothers of preterm infants is a vital element in measures that may be designed to increase rates of breastfeeding success in the neonatal nursery (Meier, Engstrom *et al.* 1993; Pinelli, Atkinson *et al.* 2001; Sisk, Lovelady *et al.* 2006).

### 6.3.2 Multiple Courses of Antenatal Glucocorticoids

The research described in this thesis addresses the effects of a single course of antenatal glucocorticoids on lactation. Many medical practitioners prescribe repeated courses in

the event that women remain at risk of preterm delivery after the first course (Quinlivan, Evans *et al.* 1998; McLaughlin, Kristin J & Crowther 2003). However, recent animal research has demonstrated risks of multiple courses to fetal growth and development and indicates that there may be a cumulative effect of repeat courses on the magnitude of disruption (Ikegami, Jobe *et al.* 1997; French, N. P., Hagan *et al.* 1999; Moss, T. J., Sloboda *et al.* 2001),

The recent controversy about the risks and benefits to the neonate of multiple courses of glucocorticoids has resulted in a number of randomised controlled trials investigating the effects of multiple courses (Abbasi, Hirsch *et al.* 2000; National Institutes of Health Consensus Development Panel 2001; Caughey & Parer 2002; Guinn 2004). A recent large randomised placebo-controlled trial found reduced incidence of both neonatal respiratory distress syndrome and chronic lung disease (Crowther, Haslam et al. 2006). Although birth weight and head circumference were lower in infants exposed to multiple courses of betamethasone, there was no effect on short-term growth (Crowther, Haslam et al. 2006). In contrast a small un-randomised cohort study found significantly greater blood pressure in the first week postpartum in infants exposed to multiple courses of antenatal betamethasone (Mildenhall, Battin et al. 2006), suggesting that neonatal effects of repeat courses of antenatal glucocorticoids have yet to be fully elucidated.

Furthermore, the effects of multiple courses of glucocorticoids on lactogenesis II have yet to be established although adverse consequences on postnatal growth have been suggested (Moss, T. J., Sloboda *et al.* 2001). Adverse findings on lactation in sheep and potential disruption of lactogenesis II in women suggest that lactation outcomes need to be included as an important outcome measure in randomised controlled trials of repeated antenatal glucocorticoid treatment that are currently in progress to investigate

if the findings observed in this thesis translate into clinical practice (Caughey & Parer 2002).

### 6.3.3 Nutritional Programming

There is recent evidence that neonatal benefits of antenatal glucocorticoid therapy may not persist if delivery occurs more than seven days after treatment (McLaughlin, K J, Crowther *et al.* 2003). An increase in perinatal mortality has been reported in studies that investigated the effects of betamethasone after prolonged intervals between treatment and delivery in preterm infants (Liggins & Howie 1972; McLaughlin, K J, Crowther *et al.* 2003) and in neonatal sheep (Moss, T. J., Sloboda *et al.* 2001). This is consistent with the findings, that lactation is also compromised when delivery is delayed after glucocorticoid treatment in sheep (Section 3.3.1.2). Studies in sheep have suggested that restricted intrauterine growth, as occurs after antenatal glucocorticoid treatment (Moss, T. J., Sloboda *et al.* 2001), may adversely influence mammary development. A similar process has also been observed in rats, where postnatal lactation was shown to be impaired in dams of pups with spontaneous *in utero* growth restriction (Wlodek, Westcott et al. 2003).

As I also identified potential adverse implications of glucocorticoid treatment on lactogenesis II in women (Section 4.3.2.3), it is important to confirm whether similar processes to that observed in sheep occur in women. More urgently, I showed that preterm gestational age at delivery strongly impacted on the onset of lactogenesis II (Chapter 4), potentially leading to early failure of lactation and substitution of artificial infant feeding. Moreover, evidence is emerging that preterm infants are at risk of metabolic programming as a consequence of their early birth (Gluckman, Cutfield *et al.* 2005).

Sub-optimal nutrition *in utero* is now known to play a crucial role in the genesis of many adult diseases (Barker 1992) and antenatal exposure to glucocorticoid treatment is believed to contribute to an increased risk of adult-onset metabolic and cardiovascular disorders. An increased risk of insulin resistance was found in 30-year old adult offspring who were exposed to antenatal betamethasone in the original trial by Liggins *et al.* but there was no difference in other cardiovascular risk factors (Dalziel, Walker *et al.* 2005). Moreover, over-nutrition in the subsequent neonatal period has recently been shown to modulate obesity and adult-onset metabolic diseases programmed by fetal exposure to under-nutrition (Eriksson, Forsén *et al.* 1999; Burke, Beilin *et al.* 2005; Desai, Gayle *et al.* 2005; Stanner & Smith 2005; Huang, R C, Burke *et al.* 2006), rendering those antenatal and early neonatal factors that impact on lactation pivotal in the development of human health and disease.

Several studies have demonstrated the protective effect of breastfeeding against adult-onset diseases in vulnerable preterm infants (Singhal, Fewtrell *et al.* 2003; Singhal, Cole *et al.* 2004; Martin, Gunnell *et al.* 2005). Furthermore, breastfeeding has been shown to modify the risk of cardiovascular disease in adults who were subjected to under-nutrition during pregnancy (Ravelli, van der Meulen *et al.* 2000). In contrast, artificial feeding, particularly when nutrient enriched, can be viewed as sub-optimal neonatal nutrition which leads to growth acceleration in infancy and subsequent adverse metabolic consequences (Osmond & Barker 2000; Singhal & Lucas 2004). It is imperative we confirm whether the phenomena described in this thesis translate to long-term disruptions to lactation in women receiving glucocorticoids in pregnancy as such an effect in women could result in life-long consequences for the child.

## 6.4 Future research

I have shown profound effects on lactation when sheep were treated with antenatal glucocorticoids at 117 to 125 days of pregnancy (Chapter 4). I propose that future research projects should establish the earliest gestational age at which a lactogenic response to exogenous glucocorticoid treatment occurs in sheep. Gestational ages were selected for treatment by convenience to take advantage of other studies running concurrently in our unit on the fetal effects of glucocorticoid treatment and there were no studies being conducted at earlier gestational ages at the time I was examining the effects on lactation. The earliest gestational age determined in these studies to demonstrate an effect of betamethasone was 117 days which is 78% of gestation (approximately equivalent to 31 weeks of pregnancy in the human pregnancy). As women with anticipated preterm delivery currently receive glucocorticoid treatment as early as 23 to 24 weeks of pregnancy, it remains to be shown if a similar lactogenic response would occur at such early gestational ages in sheep.

Future animal research should also examine the histology of the mammary gland in order to determine whether exogenous glucocorticoid treatment during pregnancy leads to an impairment of mammary development. The finding of prolonged suppression of plasma progesterone concentration following glucocorticoid treatment has the potential to adversely impact on duct and lobuloalveolar development (Neville, M C, McFaddin *et al.* 2002).

### 6.4.1 Cellular effects of antenatal glucocorticoid treatment in the mammary gland

The present research has established the impact of glucocorticoid treatment on mammary secretion in sheep at an organ and endocrine level, but little is known of the biomolecular sequelae that bring about the identified changes. For example, lactation

induced by high levels of synthetic glucocorticoids has been shown, in mice, to produce different effects at a cellular level than suckling, which stimulated lactation with physiological levels of endogenous glucocorticoids (Li, Liu et al. 1997). Future research needs to focus on changes in gene expression in mammary parenchyma in order to identify in more detail the lactational phases following glucocorticoid treatment, for example milk protein genes, cellular markers of involution, extracellular matrix and steroid hormone receptors.

Marked changes occur in expression of milk protein genes such as α-lactalbumin, $\alpha_{s1}$-casein and lactoferrin at different phases of lactation. During lactogenesis II, α-lactalbumin and $\alpha_{s1}$-casein are highly expressed by actively secreting lactocytes and, conversely, during involution, α-lactalbumin and $\alpha_{s1}$-casein are down-regulated and lactoferrin mRNA is highly expressed (Figure 1.2) (Molenaar, Kuys et al. 1996).

In addition, in order to verify the hypothesised process of involution following glucocorticoid-induced premature lactogenesis II, analysis of the activity of cellular markers of involution is recommended. The expression of a wide range of genes is up-regulated during involution of the mammary gland, including sulphated glycoprotein-2, tissue inhibitor of metalloproteinases, bax and cell cycle control proteins (Baik, Lee *et al.* 1998; Colitti, Stefanon *et al.* 1999). Two members of the signal transducer and activators of transcription (STAT) factors, STAT5 (the primary transcription factor responsible for prolactin signalling in the mammary gland (Rosen, Wyszomierski et al. 1999)) and STAT3 should be examined as studies in mice have shown that STAT5 is down-regulated and STAT3 is up-regulated during involution (Humphreys, Bierie *et al.* 2002).

The action of exogenous glucocorticoids on the extracellular matrix (ECM) of the mammary gland is another area that needs to be addressed. Interaction between lactocytes and ECM proteins, such as laminin, is required in order for prolactin to

activate its receptor to initiate the JAK/STAT pathway for milk protein gene signalling (Zoubiane, Valentijn et al. 2003). Glucocorticoid receptor amplifies this process. Activated glucocorticoid receptor is a crucial factor in the cascade of transcription factors resulting in milk protein gene expression (Figure 1.4) (Stöcklin, Wissler *et al.* 1996; Cella, Groner *et al.* 1998) and future research should also focus on the effects of glucocorticoid treatment on glucocorticoid receptors in mammary tissue. One possible explanation for the onset of premature parturition after exogenous glucocorticoid treatment in sheep is displacement, by artificially high concentrations of glucocorticoids, of progesterone from glucocorticoid receptors in the placenta (Liggins 1969; Challis, Matthews et al. 2000). This suggests to me that a similar process may be happening in the mammary gland and hence study of glucocorticoid receptor activity in the mammary gland is warranted.

## 6.5 Conclusions and Speculations for Future Practice

The excessive, indiscriminate use of synthetic glucorticoids in patients with the potential for preterm delivery is concerning (Challis, Matthews et al. 2000). The findings in this thesis support the necessity of developing tests that more accurately determine the specific patient for whom the diagnosis of preterm labour is correct in order to reduce the frequency of glucocorticoid therapy and to target it solely to those women for whom a preterm delivery is inevitable (Goepfert, Goldenberg *et al.* 2000; Honest, Bachmann *et al.* 2002).

The effects of premature delivery on lactogenesis II and the possible adverse impact of antenatal glucocorticoid therapy identified in this thesis imply potential adverse impacts on postnatal nutrition for vulnerable preterm infants. Because of the undoubted value of mother's own milk, these findings strongly suggest the importance of intensive clinical

encouragement and assistance in the early weeks after preterm birth so that all mothers of preterm infants have the opportunity to successfully establish lactation.

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# APPENDICES

## Appendix 1: Information sheet given to potential participants

THE UNIVERSITY OF WESTERN AUSTRALIA


**THE EFFECT OF STEROIDS ON THE INITIATION OF LACTATION**
**INFORMATION SHEET**

**Aims**

This study aims to investigate the effects of steroids (given in pregnancy for possible preterm birth) on the initiation of breastmilk production (lactation). We also aim to find out how milk composition changes during the establishment of lactation in mothers who are expressing milk for their preterm infants.

**Experimental methods**

You are being asked to take part in this study because you have been given steroids in pregnancy OR because your baby has been born preterm (before 35 weeks' gestation).

The initiation of lactation will be determined by collecting small samples of your urine and breastmilk to measure the concentration of milk sugar (lactose). Daily milk production will be determined by measuring the volume of milk you express or by weighing your baby.

**Before your baby is born, you will be asked to:**

1. Give us urine samples every time you pass urine over 24 hour periods by collecting your urine in a beaker, measuring the volume, noting the time and keeping a small sample (3 ml) for analysis. You will be asked to collect these urine samples daily for 3 days then on days 5 and 7 then once the following week if your baby has not been born.

**After your baby is born, you will also be asked to:**

2. Collect urine samples *as before for 10 days*.
3. Express and collect small samples of breastmilk (less than 1 ml) from each breast the first time you express or feed your baby *each day for 10 days*.
4. Measure the volume of milk you make by:
    a. *If your baby is born too early to be able to breastfeed* you will be shown how to express your breasts using an electric breast pump. You will be asked to express at least 6 times a day and measure the milk volume using small portable scales *for 10 days.*
    b. *OR if your baby is able to breastfeed* you will be asked to weigh your baby before and after each breastfeed for 24 hours when your baby is 3 and 5 days old. We will provide electric scales for you to do this at home when you take your baby home from hospital.
5. Complete a short questionnaire including some background information about you and your feelings about breastfeeding and expressing.

It should only take a few minutes each day to collect the samples. We will pick the samples up from you while you are in hospital or when you are visiting your baby in the

nursery. If you and your baby go home before your baby is 10 days old you we will collect the samples from your home.

If you have any difficulties with expressing or breastfeeding, you will be able to be referred to the lactation consultant in the neonatal unit at King Edward Memorial Hospital.

All information collected will be strictly confidential. You will be free to withdraw from taking part in the study at any time without affecting your care or that of your baby. Your participation in this study does not prejudice any right to compensation, which you may have under law. If you take part, you or your baby might not have any direct benefits, but there will be no discomfort or risk to you.

**Benefits of this study**

Breastmilk is the best food for all babies but it is even more important for babies who are born early (preterm) because it protects them from infections and other problems that preterm babies are more likely to develop and helps their brain development. The early production of breastmilk (initiation of lactation) is sometimes delayed in mothers of preterm babies. Many mothers stop breastfeeding because they are worried about low milk supply. This study will help us to determine whether steroids (given to pregnant women to help prevent breathing difficulties and other problems that may occur if their babies are born preterm) affect the initiation of lactation after the baby is born. We will also have a better understanding about supply and nutrient content of breastmilk in mothers who are expressing after a preterm delivery. These findings will allow us to develop methods to help breastmilk production in mothers who need to express breastmilk for their preterm babies.

**Further information**

If you have any further questions about the study please contact one of the study investigators:

| | | |
|---|---|---|
| **Jenni Henderson** | **phone 9340 1389** | **or 0421 568 149** |
| Bronwyn Davis | phone 9340 2050 | or phone 9340 2222 and page 1640 |
| Professor Karen Simmer | phone 9340 2050 | |
| Professor Peter Hartmann | phone 9380 3327. | |

If you have any concerns about the study, please contact the Executive Director, Medical Services, phone 9340 2222

## Appendix 2: Consent Form

THE UNIVERSITY OF WESTERN AUSTRALIA


**FORM OF CONSENT**

I ..... have read
Given Names Surname

the information explaining the study entitled The effect of steroids given to women with anticipated preterm delivery on the initiation of lactation

I have read and understood the information given to me. Any questions I have asked have been answered to my satisfaction.

I understand I may withdraw from the study at any stage and withdrawal will not interfere with routine care of myself or my baby.

I agree that research data gathered from the results of this study may be published, provided that
names are not used.

Dated ..... day of ..... 20 .....

Signature .....

I, ..... have explained the above to the
(Investigator's full name)
signatory who stated that she understood the same.

Signature

.....

## Appendix 3: Maternal Questionnaire

THE UNIVERSITY OF WESTERN AUSTRALIA

Women's & Children's Health Service

**THE EFFECT OF STEROIDS ON THE INITIATION OF LACTATION STUDY**

Thank you for taking part in this study.

This questionnaire asks some questions about yourself, your breastfeeding history and your experience of breastfeeding or expressing for this baby.

Please read each question carefully. The questions can be answered by putting a circle around the number for the appropriate response or by filling in the space provided.

All answers will be strictly confidential.

When you have finished, please return this questionnaire to the research midwife.
If you have any questions or concerns about the questionnaire, please contact Jenni Henderson, phone 9340 1389 or 0421 568 149, or Bronwyn Davies, phone 9340 1640 or 9340 222 and page 1640.

*[Please circle the appropriate number or fill in the space provided]*

ID : _____

*First, some questions about yourself*

1. What is today's date? ……/……/……
2. How old is your baby today? …….. days

3. What is <u>your</u> date of birth? ……/……/……

4. What was the last year at school that you completed? (eg Year 10)

   Year …… or Equivalent ……

5. Since leaving school, what further education have you completed?
   a. none
   b. trade certificate or apprenticeship
   c. professional registration (non-degree) eg Police
   d. college diploma (eg TAFE)
   e. university degree
   f. other *(please specify)* …….

6. What job/s have you had in the past two years?

   ……

   ……

7. What is your current employment status?
   a. No paid employment
   b. Maternity leave
   c. Working part-time
   d. Working full-time
   e. Studying full or part-time

8. If you are on maternity leave, how old will your baby be when you intend going back to work? …… months

*[Please circle the appropriate number or fill in the space provided]*

9. What is your marital status?
    a. married or defacto
    b. never married
    c. divorced or separated
    d. widowed

10. What is your partner's current employment status?
    a. No paid employment
    b. Paternity leave
    c. Working part-time
    d. Working full-time
    e. Studying full or part-time
    f. Not applicable

11. What is your height? …….
12. What is your weight? ……. kg

13. Are you taking any medications?
    a. No
    *b.* Yes *Please list* ……

14. Did you smoke during your pregnancy?
    a. No
    b. Yes

15. If you gave up smoking during pregnancy, how many months pregnant were you at the time you gave up? …… months

16. If you smoked at all during your pregnancy, how many cigarettes per day did you usually smoke?
    a. 1-10 daily
    b. 11-20 daily
    c. 21 or more daily

*[Please circle the appropriate number or fill in the space provided]*

17. How many cigarettes per day are you smoking now?

a. None
b. 1-10 daily
c. 11-20 daily
d. 21 or more daily

*Here are some questions about your past breastfeeding experiences*

18. How many other children do you have at home (not including this baby)?

…….

19. If you have other children, did you breastfeed any of them?

a. No other children *(please go to question 24)*
b. No, did not breastfeed *(please go to question 24)*
c. Yes

20. For how long did you breastfeed each child?

a. First child …… months
b. Second child …… months
c. Third child …… months
d. Subsequent children …… months

21. What were your reasons for stopping breastfeeding the last time?

*(Circle all appropriate answers)*

1) inadequate milk supply
2) baby unsettled after feeds
3) breastfeeding too tiring
4) painful or cracked nipples
5) problems attaching the baby to the breast
6) baby refused the breast
7) too time consuming
8) you were ill
9) breast infection
10) breast abscess
11) you returned to work
12) your baby was ill
13) you disliked breastfeeding
14) natural weaning
15) baby biting
16) baby demanding feeds too frequently
17) inconvenient
18) own choice
19) others wanting to feed baby
20) partner not keen on you breastfeeding
21) baby not gaining enough weight
22) other *(please specify)*

……

For your <u>main reason</u> for stopping breastfeeding, please circle the number below that corresponds to the reason from the list above.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

*[Please circle the appropriate number or fill in the space provided]*

22. When you breastfed previously did you ever experience any of the following?

| | | |
|---|---|---|
| a. engorgement | No | Yes |
| b. mastitis | No | Yes |
| c. cracked nipples | No | Yes |
| d. other | No | Yes |

*please specify*

…….

23. Did you sense your milk let down? No Yes

*Now, here are some questions about your breastfeeding experience for this baby*

24. How long do you intend to breastfeed your baby for? ……months

25. Has your baby been admitted to the Special Care Nursery?

a. No

b. Yes

26. What food is your baby receiving now?

a. Nothing by mouth (IV only)

b. Breastmilk only

c. Formula only

d. Breastmilk and formula

27. How is your baby being fed now?

a. breast fed

b. bottle fed

c. tube fed

d. breast and tube fed

e. bottle and tube fed

f. breast and bottle fed

g. breast, bottle and tube

h. other ……..

*[Please circle the appropriate number or fill in the space provided]*

28. How many times a day are you expressing for your baby? ……

29. How many times a day are you breastfeeding your baby now? …….

30. How old was your baby when you first experienced the following?

| | | | |
|---|---|---|---|
| a. | breast fullness | …… days | NA |
| b. | breast warmth | …… days | NA |
| c. | tingling of the nipples | …… days | NA |
| d. | leaking from the breasts | …… days | NA |

31. Have you sensed let-downs since your baby was born? No Yes

If yes, please describe them eg. Sensation, start time, duration, triggers etc:

……
……
……
…

32. Have you sensed multiple let-downs? No Yes

If yes, please describe them

……
……

33. Have you used any breast pumps since your baby was born? No Yes

If yes, please describe the breast pumps that you have used. eg. brand, manual/electric, settings etc:

……
……
……

34. Have you been satisfied with your breast pumps? No Yes

……
……
……

*[Please circle the appropriate number or fill in the space provided]*

35. Did you notice any breast changes <u>after you were admitted to hospital and before your baby was born</u>? No Yes (*please specify)*

……

……

Did this occur after you had your steroid injections?

N/A No Yes (*please specify when this occurred)* ……days later

36. Have you experienced any of the following problems <u>since this baby was born</u>?

*(Circle all appropriate answers)*

a. High blood pressure
b. Retained placenta
c. Postpartum haemorrhage
d. Depression
e. Breast Engorgement
f. Mastitis
g. Cracked nipples
h. Low milk supply
i. Feeding difficulties
j. Other *(please specify)* …….

37. Do you have any comments you would like to make about your early experience of breastfeeding or expressing for your new baby?