Relationships between cord blood leptin and ghrelin levels, milk intake and weight gain in human infants

Rachel J. A. James

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Department of Psychology
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Abstract

Leptin and ghrelin are hormones involved in the regulation of appetite and adiposity. Leptin suppresses appetite and induces weight loss; ghrelin stimulates appetite and promotes weight gain. The study reported in this thesis was designed to examine the relationship of cord blood leptin and ghrelin with milk intake over the first week of life, and with infant growth up to twelve weeks of age. One hundred term formula fed newborns were recruited at birth. Leptin and ghrelin were measured in cord blood by radioimmunoassay. Milk intake was measured by weighing of bottles of formula milk before and after feeding. Measurements of weight, length and head circumference were taken at birth, seven days and at twelve weeks of age. A number of control variables were also measured.

Birthweight was a significant predictor of mean milk intake, which rose significantly from days 1 to 7, with no difference between males and females. Weight gain or loss in the early neonatal period was a direct and significant consequence of milk intake consumed over that period. There was no relationship between cord blood leptin or ghrelin (controlling for birthweight) on the infants' milk intake over the first 24 hours of life or on their mean milk intake over the first week of life. Weight gain was significantly correlated with birthweight, with higher birthweight associated with lower weight gain. There was no relationship between cord leptin and weight gain to three months of age after adjusting for birthweight; but lower cord ghrelin levels were significantly associated with slower weight gain.

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Abbreviations

αMSH	α-melanocyte stimulating hormone
AgRP	Agouti-related protein
ALSPAC	Avon longitudinal study of pregnancy and childhood
AGA	Appropriate for gestational age
BF	Breast fed
BNBAS	Brazelton neonatal behavioural assessment scale
BMI	Body mass index
CART	Cocaine and amphetamine regulated transcript
CCK	Cholecystokinin
CNS	Central nervous system
ENNS	Early neonatal neurobehavioural scale
FF	Formula fed
FFAs	Free fatty acids
GH	Growth hormone
ICV	Intracerebroventricular
IM	Intramuscular
IP	Intraperitoneal
LBA	Leptin binding activity
LEPR	Leptin receptor (reclassified 2002)
LGA	Large for gestational age
LH	Lateral hypothalmus
NPY	Neuro-peptide Y
MC4 receptor	Melanocortin 4
m RNA	Messenger ribonucleic acid
NACS	Neurologic and adaptive capacity score
OB-R	Leptin receptor
POMC	Pro-opiomelanocortin
PWS	Prader-Willi syndrome
SD	Standard deviation
SGA	Small for gestational age
VMH	Ventromedial hypothalamus

Glossary of terms

Ad libitum	Freely, to pleasure
Adipsia	Absence of drinking
Aphagic	Absence of feeding
Endocrine	Secreting internally (used of hormones)
Euglycaemia	A normal level of glucose in the blood
Glucoprivic feeding	Feeding induced by low glucose levels
Hyperglycaemia	An excess of glucose in the blood
Hyperphagic	Excessive eating
Hypoglycaemia	Deficiency of glucose concentration in the blood
Hypovolaemia	Abnormally decreased volume of circulating fluid (plasma) in the body
Labour- first stage	Onset of regular painful contractions and cervical dilatation from 0 to 10 cm
Labour- second stage	From full dilatation of the cervix to expulsion of the fetus
Low birthweight infant	Infant weighing less than 2,500g
Lipogenesis	The formation of fat; the transformation of nonfat food materials into body fat
Macrosomic	Large and overgrown (used of infants of uncontrolled diabetic mothers)
Multiparous	A woman who has had two or more pregnancies resulting in viable fetuses whether or not the offspring were alive at birth
Orexigenic	Appetite stimulating
Primiparous	A woman who has had one pregnancy that resulted in a viable fetus
Term	A birth occurring between 37 and 42 completed weeks of gestation
Thermogenesis	The production of heat, especially within the animal body

Declaration

The research contained in this thesis was carried out by the author between 1999 and 2003 while a postgraduate student in the Department of Psychology at the University of Durham. None of the work contained in this thesis has been submitted in candidature for any other degree.

Statement of Copyright

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Chapter 1

Introduction

1.1 General introduction

1.1.1 Overview of thesis

The main aim of this thesis was to examine the relationship between leptin and ghrelin measured in cord blood and the feeding behaviour and milk intake of infants over the first week of life; and to examine the relationship between these hormones and the growth of infants over the first three months of life. This was achieved by designing a study that recruited infants at birth, when a sample of cord blood was taken for later assay for these hormones. Infant anthropometrical measures of weight, length and head circumference were recorded at birth, at seven days of age and at three months of age. Milk intake was determined from birth to seven days of age by 'direct weighing' of ready to feed bottles of formula milk before and after feeding. It was hoped that this study would be relevant to two important questions. The first was the role of two recently identified hormones, leptin and ghrelin, in the normal control of food intake in humans, a topic of great importance owing to the major health problems that are likely to result from the considerable increase in obesity observed in industrialised societies over the last ten years. The second is the control of weight gain in the first year of life. Slow weight gain (failure to thrive) is an important problem in paediatrics, and its detection is the justification for the time consuming and expensive screening of weight gain in infants in the first year. Its explanation, however, is still obscure, which may be partly because so little is known about the normal control of weight gain in infancy.

The remainder of this chapter reviews previous areas of research relevant to the study reported in the remainder of the thesis.

1.1.2 Regulation of food intake

Energy intake is closely matched to the amount of energy expended. Keeping this energy equation in equilibrium is essential to survival. A loss of appetite either through illness or psychobiological reasons results in a decrease in body weight. An increased appetite which is temporary or permanent but without an increase in energy expenditure results in an increase in body weight and obesity (Kalra, Dube, Pu, Xu, & Horvath, 1999). Food intake is governed by both short and long term mechanisms. Short-term regulation occurs during the meal, when certain stimuli stop the individual

eating; these include the passage of food through the mouth by oral receptors, distention of the stomach and duodenum, and the release of cholecystokinin (CCK) in response to fat. Snowdon (1970) demonstrated the importance of oral cues in terminating feeding; rats fed intragastrically, bypassing the oral sensors, had shorter meal durations (Hoebel, 1971). Janowitz & Grossman established that gastric distention was an important factor in the termination of a meal. They inserted a balloon via a gastric fistula into a dog's stomach (Janowitz & Grossman, 1949). During ad libitum feeding these animals ate less. Likewise free feeding sham fed rats, in which the stomach contents were allowed to drain out through a cannula, instead of accumulating in the stomach, fed for longer, supporting the role of feedback signals from the stomach in satiety (Davis & Campbell, 1973). These experiments suggest that there must be receptors in the alimentary tract which signal satiety. The signaling was proposed to be via the vagus nerve, based on evidence from the behaviour of vagotomised rats, which had an increased gastric emptying rate and shortened time intervals between meals, as they no longer were able to sense stomach distention (Hoebel, 1971). CCK is released by the gut after a meal and stimulates the vagus nerve to inhibit food intake. In rats, peripheral administration of CCK reduces the size of a meal (Riedy, Chavez, Figlewicz, & Woods, 1995), and if CCK is blocked by administration of a CCK antagonist, the amount of food eaten is increased (Wolkowitz et al., 1990). Both these findings suggest a role for CCK in controlling hunger and satiety; whether it has only local or more widespread effects remains to be verified.

The nutritional state of the body is the main factor involved in the long-term regulation of food intake. Ingestion of nutrients whether orally, or directly into the stomach or small intestine have a direct effect upon subsequent food intake. The potential mechanisms which may influence food intake include: changes in the blood glucose levels, the activity of nutritional hormones such as insulin, glucagon-like peptide-1 or amylin and the processes within the liver that metabolize the products of digestion (Feinle, O'Donovan, & Horowitz, 2002). Two main theories relating the nutritional state of the body to food intake were postulated in the 1950s. Kennedy (1953) proposed a lipostatic theory (Woods, Decke, & Vasselli, 1974) and Mayer (1955) proposed a glucostatic theory (Le Magnen, 1971).

Both these theories identified nuclei within the basal hypothalamus, the ventromedial nucleus, the paraventricular nucleus and the dorsomedial nucleus as being the appetite control centers. This was based on earlier studies in rats and other mammals, in which discrete lesions, chemical or electrical stimulation of the hypothalamus resulted in hyperphagic or aphagic responses (Le Magnen, 1971). The 'ventromedial hypothalamic' animal that developed the syndrome of hyperphagia and obesity was first described by Hetherington and Ranson in 1940, and became the model that much feeding research was focused upon (Woods et al., 1974). The hyperphagia was thought to be due to a deficit in the signaling pathways within the ventromedial hypothalamus (VMH), so the animal was unable to satiate to ingested food. However more recent experiments have shown that continuous infusion of a liquid food into the stomach of lesioned hyperphagic rats reduced oral food intake, thus contesting this theory (Le Magnen, 1983). Anand and Brobeck (1951) first described the lateral hypothalamic syndrome in which rats become aphagic following damage to that area (Epstein, 1971). Teitelbaum and Stellar (1954) later found the aphagia to be combined with adipsia to be transient rather than permanent; feeding resumed at 16 days and drinking resumed at 18 days (Epstein, 1971). However, both the ingestive behaviours of feeding and drinking were dysfunctional; the animals had lost their hydrational control of thirst and were unresponsive to hypovolaemia and cell dehydration. Restoration of water balance was by accident by prandial drinking as they had lost the ability to produce saliva and sought water to swallow the dry food (Epstein, 1971). A permanent deficit in intracellular glucose utilization was observed. The animals no longer responded to glucoprivation (Epstein, 1971). Care must be taken with the interpretation of these lesion experiments as it transpired that damage had occurred to the surrounding tissues and pathways of the VMH and LH, so had affected larger areas of brain tissue than first thought (Rolls, 1981).

Mayer and his colleagues proposed and expanded the glucostatic theory based on their research from 1953 to 1966 (Le Magnen, 1971). Intracerebrovascular injection of gold thioglucose into the ventromedial hypothalamic area of mice caused destruction of isolated receptors that switched off the satiety signal, resulting in hyperphagia and obesity. Their conclusion from this observation was that there were certain receptors in the VMH that responded specifically to cell glucose availability and when these receptors had become saturated with glucose, the desire to eat was switched off. Later

research also implicated a role for glucose in the normal physiology of feeding. A small pre-meal decline in plasma glucose was observed in both rats (Campfield, Brandon, & Smith, 1985) and humans (Campfield, Smith, Rosenbaum, & Hirsch, 1995). In rats, this pre-meal decline in glucose could be obliterated by intravenous administration of glucose, which also led to a delay in the onset of feeding (Campfield et al., 1985). Furthermore, intraperitoneal infusion of glucose in dogs (Russek, Rodriguez-Zendejas, & Pina, 1968), caused a substantial inhibition of feeding more than was observed following intravenous injection of glucose. Russek et al. proposed that the glucose was being directly absorbed into the liver via the hepatic portal system thus suggesting the liver had specific glucose receptive cells (hepatic glucosensors). Additionally, intragastric loads of glucose in rats reduced meal size and delayed the onset of the next meal (Booth, 1972). Gluco-receptors have since been identified in the lateral hypothalamus, the nuclei of the solitary tract, and the liver. They detect hypoglycaemia and activate feeding behaviour that restores normo-glycaemia (Timo-Iaria, 1990). Human studies have documented that oral ingestion of 50g of starch disguised within a drink (Booth, 1981) or a glucose pre-load (maltodextrin) (Blundell, Green, & Burley, 1994) administered one hour prior to the mealtime resulted in a reduction in food intake at that mealtime which was equivalent to the energy of the pre-load. Intragastric infusion of dextrose 30 minutes prior to a mealtime resulted in a reduced food intake when compared with an intragastric saline infusion (Shide, Caballero, Reidelberger, & Rolls, 1995). All these studies demonstrate an inhibition of food intake following oral, intragastric and intestinal administration of glucose in both animals and humans, which supports the role of gastrointestinal signals in satiety.

In 1953 Kennedy proposed a lipostatic theory of body weight regulation. He believed that the body had receptors capable of sensing the amount of fat tissue, and an increase or decrease in fat tissue somehow signaled the brain via a feedback mechanism, which then altered feeding behaviour (Woods et al., 1974). His proposal was based on the observation that fat mass remains relatively stable over long periods of time and the evidence from Hervey's (1952) parabiotic experiments (Hoebel, 1971). Hervey's experiments involved joining two rats at their flanks so they shared the same blood supply (Hervey, 1952). One rat was obese as a result of a ventromedial lesion in the hypothalamus and one was not. He observed that the feeding behaviour of the lean rat was severely disturbed; it under ate, almost to the point of starvation and imminent

death. Once the rat was separated from its obese partner it resumed a normal eating pattern. The interpretation of such studies was that a humoral factor in the blood stream of the obese rat had crossed over to the lean rat affecting its feeding behaviour. Woods et al (1974) explored this idea further. They proposed that the humoral factor was the 'ratio' of insulin and growth hormone (GH) and that the interaction of these two hormones controlled body weight and feeding behaviour. Insulin is a hormone produced by the beta cells of the islets of Langherans in the pancreas. It is released following a meal, as the digestive products pass from the blood stream to the pancreas. Insulin promotes glucose utilization or storage as glycogen depending on the body state, lipogenesis (conversion of free fatty acids to triglycerides by the liver) and protein anabolism (conversion of amino acids from liver, muscle and other tissues to stored protein under the influence of GH). GH is continuously released from the anterior pituitary; it decreases following meals and peaks overnight (the longest period without feeding). GH promotes growth of bone and other tissues and under the influence of insulin, protein anabolism. During fasting GH promotes lipolysis (breakdown of stored triglycerides to free fatty acids (FFAs) in adipose tissue), these FFAs are then used as fuel (Woods et al., 1974). Alteration in the ratio of insulin and GH affects body weight. How insulin affects feeding behaviour remains controversial. Intravenous and subcutaneous administration of insulin to rats induced glucoprivic feeding, but insulin administered whilst maintaining euglycaemia by a glucose infusion inhibited their feeding (Orosco, Rouch, & Nicolaidis, 1994). Intracerebroventricular infusion of insulin (Riedy et al., 1995) and continuous 24-hour intravenous infusion of insulin in rats resulted in a decreased food intake and weight loss (Porte & Woods, 1981). Similarly experimental manipulations of both insulin and glucose levels in humans resulted in evidence that insulin was the factor that induced feeding (Rodin, Wack, Ferrannini, & De Fronzo, 1985). Rodin et al. divided individuals into four groups, group 1 were maintained euglycaemic, group 2 were made hyperinsulinaemic and hypoglycaemic, group 3 were made hyperinsulinaemic and hyperglycaemic and group 4 were made euinsulinaemic and hyperglycaemic. Ratings of hunger on a visual analogue scale increased significantly by sixty minutes in groups two and three, with an increase in food intake. Groups one and four showed no change in hunger ratings. This study demonstrated that manipulation of the glucose levels had no effect upon the ratings of hunger and food intake. However groups 2 and 3 who did report increased hunger ratings were kept hyperinsulinaemic throughout the manipulation of glucose

levels, thus suggesting that the high insulin levels were the stimulus to feed. From these findings, Rodin et al. suggested that hypoglycaemia could not be the stimulus to feed, because feeding did not occur simultaneously with the rapid decline in blood glucose. They also suggested that the feeding response was delayed, because the first priority was to initiate a metabolic response to the hyperinsulinaemia, which was to mobilize glucose uptake in the peripheral tissues before stimulating appetite. These findings however should be viewed with caution as they contradict what is known about the physiological actions of insulin in a natural state, that insulin levels rise in response to feeding (Woods et al., 1974). In newborn infants, insulin levels and growth hormone levels were shown to rise following each feed (Ogilvy-Stuart et al., 1998). Rodin et al's findings are also contradictory to the recognized aetiology that insulin deficient individuals have low or absent insulin levels and present with hyperglycaemia and hyperphagia (Schwartz, Woods, Porte, Seeley, & Baskin, 2000; Woods, Schwartz, Baskin, & Seeley, 2000). These findings suggests that insulin levels per se may not be the sole determinant of feeding behaviour, but may stimulate other factors or pathways that determine feeding.

1.1.3 Regulation of energy intake in early infancy

Energy intake in early infancy is characterised by several unique factors. Milk intake is the sole provider of both water and food to the infant and therefore is believed to satisfy both thirst and hunger (Drewett, 1993). Also, the infant's milk intake is dependent upon several complex interactions between the mother and infant (Drewett, 1993; P. Wright, 1993). Firstly, the mother needs to pick up signals from the infant that they wish to feed i.e. hunger/thirst, this can be fussing, crying, rooting or sucking. Secondly, the mother needs to respond appropriately by initiating feeding. Thirdly, the mother needs to identify that the infant is satiated (falls asleep, spits out teat) and lastly the mother needs to recognize these signals and discontinue feeding. This complex series of interactions is subject to both the infant and mother learning. Formula fed infants are more influenced by maternal control than breastfed infants. When coding feeding interruptions (removal of teat/nipple for reasons such as choking, winding, possetting of milk) into maternally driven or infant driven behaviours, mothers of formula fed infants tended to interrupt feeding more often (Wright 1981).

1.1.4 Structure and function of the placenta

As the study for this thesis measures several hormones in cord blood, a brief description of placental structure and function is necessary. In pregnancy, several new tissues are created which develop into the placenta, fetal membranes (amnion and chorion) and the decidua, these provide an interface between the fetus and the mother (Petraglia, Florio, Nappi, & Genazzani, 1996). The placenta, amnion and chorion originate from the process of embryogenesis and the maternal decidua is a result of endometrial differentiation. The placenta is an unique organ that separates fetal and maternal circulation. The placenta is connected to the fetus by the fetal blood vessels within the cord therefore the fetal circulation is a closed one, and does not mix directly with maternal blood. The placenta is supplied by the spiral arterioles in the endometrium from the maternal circulation. The maternal blood is physically separated from fetal blood by a villus membrane.

The four main functions of the placenta are gaseous exchange, provision of nutrients to the fetus, disposal of waste products from the fetus back to the mother and hormone production. The placenta has been shown to produce and metabolise various peptides and hormones. Hormones measured in cord blood are produced by both the placenta and the fetus and may be identical in structure, so estimation of actual contribution of each source to the total amount is not always possible. The three hormones measured in cord blood for the study for this thesis were leptin, ghrelin and insulin. There is evidence that leptin is produced both by the placenta (Butte, Hopkinson, & Nicolson, 1997; Masuzaki et al., 1997) and the fetus (evident in cord blood) (Cetin et al., 2000; Cinaz et al., 1999; Hytinantti, Koistinen, Koivisto, Karonen, & Andersson, 1999; Kirel et al., 2000; Marchini, Fried, Ostlund, & Hagenas, 1998; Matsuda et al., 1997; Schubring et al., 1997; Sivan, Lin, Homko , Albert-Reece, & Boden, 1997; Varvarigou, Mantzoros, & Beratis, 1999; Yang & Kim, 2000) and will be discussed in 1.2.5 and 1.2.6 respectively. Similarly, recent research has shown that ghrelin is produced by both the placenta (Gualillo et al., 2001) and the fetus (Castellino et al., 2002; Chanoine, K, & Wong, 2002; Cortelazzi et al., 2003; Coutant et al., 2002; Farquhar et al., 2003; Kitamura et al., 2003) and will be discussed further in 1.3.4. Finally, insulin is also produced by the placenta (Petraglia et al., 1996) and the fetus (Giudice et al., 1995; Ong et al., 2000) and will be discussed further in 1.6.1.

1.2 Leptin and the control of appetite

1.2.1 The role of leptin in rodent models

Research into the regulation of appetite and bodyweight accelerated in the 1990s with the development of positional cloning techniques and molecular selection experiments in rodent and monkey models. These both advanced the understanding of the molecular interactions involved in feeding and led to a major break through, with the discovery of leptin. Leptin is a hormone produced by adipose tissue which has been shown to be an important regulator of energy balance, appetite and weight (Farooqi et al., 1999; Zhang et al., 1994), and strongly supports the kind of theory Kennedy proposed.

Friedman and his colleagues (Zhang et al., 1994) first discovered leptin in 1994, through positional cloning technology. They showed that the genetic mutation in the ob/ob mouse (now designated the Lep^{ob} mouse) was characterized by the absence of the mature ob gene product. These ob/ob mice fed continuously, leading to early onset obesity, type II diabetes, insulin resistance and infertility. With this information they were then able to sequence the same gene in humans (ob), and identify the gene product. This protein molecule, later to be named leptin (from the Greek word leptos meaning thin) was found to have profound effects upon appetite and energy expenditure in rodents. When leptin was injected into the peritoneum of the ob/ob mouse, plasma leptin levels rose leading to a decrease in food intake, an increase in thermogenesis and energy expenditure, and a reduction in fat mass (Zhang et al., 1994). Further studies found that if leptin was injected into normal and ob/ob mice, they both showed the same reduction in food intake and decreased body weight. However when leptin was injected into db/db mice (now designated Lep^{db} mice), a strain of specifically bred diabetic mice with a leptin receptor defect, they showed no changes in food intake or body weight, probably due to lack of the receptor (Halaas et al., 1995; Stephens et al., 1995). Intracerebroventricular administration of leptin also resulted in an alteration of feeding behaviour in both ob/ob and lean mice suggesting that areas within the brain may be the target for leptin action (Campfield, Smith, Guisez, Devos, & Burn, 1995). In 1995 the site of action was still unknown, but in the same year, Tartaglia et al. (1995) cloned the leptin receptor (Ob-R) which is the product of the Lep db gene (Tartaglia et al., 1995) They found the Ob-R receptor was present in many tissues,

with high levels in the hypothalamus and choroids plexus, where they believed it to be involved in complex neural signaling pathways that control appetite and energy metabolism. The receptor is produced in several alternatively spliced forms and named Ob-Ra (short form), Ob-Rb (long form responsible for signaling), Ob-Rc, Ob-Rd and Ob-Re (Lee et al., 1996). Leptin circulates in a compound with the soluble form of the Ob-R receptor (Ob-Re) (Friedman & Halaas, 1998). The ventromedial hypothalamus (VMH) has for many years been identified as an area involved in the control of appetite, and when rats were directly injected with leptin into the VMH, their food intake decreased and they lost weight (Jacob et al., 1997). This site of action was verified by detection of leptin messenger ribonucleic acid (mRNA) expression in various rat brain tissues of the pituitary gland and hypothalamus (Morash, Li, Murphy, Wilkinson, & Ur, 1999). Fasting suppressed leptin mRNA expression in the hypothalamus, suggesting that central brain leptin has a role in the regulation of appetite. In rodents deprived of food, leptin, insulin and glucose levels decrease and weight is lost (Ahima et al., 1996).

1.2.2 The role of leptin in human physiology

Leptin is the product of the ob gene and is secreted primarily by the adipocyte in white adipose tissue (O'Rahilly, 1999). The amount of leptin secreted by adipocytes depends upon their size. The size of the adipocyte changes with food intake. Leptin and its receptor are essential components of a complex physiological system that links the periphery to the central nervous system (CNS), evolved to regulate fuel stores and energy balance (Friedman & Halaas, 1998). How leptin is transported across the blood brain barrier into the CNS is currently unknown (Friedman & Halaas, 1998), but it is believed to be transported as free leptin in the cerebrospinal fluid, which then binds to its soluble receptor. Restriction of energy intake causes a decrease in leptin gene expression and a decrease in the production of leptin within 18 to 24 hours. This precedes a change in adipocyte size and signals the CNS that energy intake is low, so that adjustments can be made to energy balance. Gene expression is the process of activating the gene to transcribe mRNA, which is then transported to the synthetic apparatus for protein synthesis (translation). Leptin gene expression is increased in human obesity resulting in high serum leptin levels (Considine et al., 1995; Lonnqvist, Arner, Nordfors, & Schalling, 1995), although these high levels of leptin appear to be

ineffective in suppressing appetite. These leptin levels correlate with BMI and percentage body fat (Considine et al., 1996) and are 75% higher in obese women than obese men (Lonnqvist et al., 1995). Leptin's role in human obesity has yet to be defined, though gene sequencing in severely obese individuals has rarely identified a defect in the leptin gene (Considine et al., 1995; Maffei et al., 1996). Although obese people appear to be resistant to their circulating leptin levels, when administered exogenous leptin they lose weight. A trial examining the effect of exogenous administration of leptin (Heymsfield et al., 1999), randomized obese and lean individuals into 5 groups. A placebo group received no leptin and four treated groups received human recombinant leptin in different doses of 0.01, 0.03, 0.10 and 0.30 mg/kg per day. The lean individuals followed a normal diet, but the obese individuals followed a weight reducing diet. All of the participants lost weight by 4 weeks, including those receiving the placebo. The mean weight loss was from -0.4 kg for placebo group to -1.9 kg for the 0.1mg/kg dose of leptin. Weight loss increased with increasing dose of leptin for all subjects up to four weeks. From 4 to 24 weeks only the obese individuals continued with the study, and the mean weight loss was –1.3 kg for the placebo group and -0.7, -1.4, -2.4 and -7.1 kg for the groups receiving human recombinant leptin in doses of 0.01, 0.03, 0.10 and 0.30 mg/kg per day respectively. There was again a dose-response relationship in the treated groups with the greatest weight loss apparent in the highest leptin dosage group. This observation suggests that leptin has an important role in the control of body weight in humans.

In normal weight individuals there is a strong relationship between serum leptin levels and body mass index (BMI), and females have higher leptin levels than males (Blum, Englaro, & Hanitsch, 1997; Saad et al., 1997). There is a great degree of variability in leptin levels in adults of similar body size; the reason for this is not clear (Wiegle et al., 1997), though Maffei et al. suggest it may reflect different rates of fat secretion in fat cells (Maffei et al., 1995). Leptin levels fall with fasting in both normal weight and obese individuals. Wiegle et al. demonstrated a 61.9 % decrease in mean plasma leptin levels with a mean weight loss of 2.6 % in seven normal weight females during a period of fasting (Wiegle et al., 1997). In nine obese males during a period of fasting, they observed a 76.3 % decrease in mean plasma leptin levels with a mean weight loss of 21.4 %. These findings show that the drop in fatmass does not have the same relationship to the drop in leptin levels in the two groups. Leptin levels and leptin gene

expression increase with overfeeding (Levine, Eberhardt, & Jensen, 1999). In this study 16 non-obese subjects were overfed and were instructed to remain sedentary throughout the study period. They put on body fat with a concomitant increase in leptin levels.

Schwartz et al. suggested that the physiological actions of leptin could not be separated from the actions of insulin and proposed a homeostatic model that incorporated both (Schwartz et al., 2000). During fasting the fat cells shrink and insulin and leptin gene expression is inhibited. This in turn inhibits the activity of nerve cells that have leptin and insulin receptors within the hypothalamus. This decrease in neural activity has two main effects upon the other neurons within the hypothalamus. Firstly it stimulates the neuro-peptide Y /agouti related protein neuron (NPY/AgRP) which promotes an increase in NPY and AgRP expression and release of the neurotransmitters that increase appetite. At the same time it inhibits the activity of the propriomelanocortin neuron (POMC) that suppresses α-melanocyte-stimulating hormone (α-MSH) expression and release and interferes with the binding of α-MSH to melanocortin receptors which increase appetite (Schwartz et al., 2000). Further research is required to fully validate this model.

1.2.3 Congenital leptin deficiency in humans

Individuals with the rare presentation of congenital leptin deficiency have provided evidence that the absence of leptin results in hyperphagia and obesity in humans.

Montague et al. (Montague et al., 1997, 1998) identified congenital leptin deficiency in two severely obese children from a family of consanguineous Pakistani origin. Both had leptin levels that were barely detectable and a very high fat mass, greater than the 98th centile. The female child had a normal birth weight of 3.46 kg. Her obesity developed rapidly from the age of four months, by which time she had crossed from the 50th weight centile at birth to above the 98th centile. At the age of eight she weighed 86 kg. Her parents described aberrant behaviour in her quest to obtain food, to the point of trying to eat frozen food from the freezer because the refrigerator was locked (O'Rahilly, 2002). Her three-year-old male cousin showed identical behavior and development. He weighed 3.53 kg at birth and had crossed from the 50th weight centile

to above the 98th centile by three months of age and weighed 29 kg at the age of two. On further genetic investigation, the anomaly was found to be a homozygous frame-shift mutation involving the deletion of a single guanine nucleotide in codon 133 of the gene for leptin (Montague et al., 1997).

Similarly, three members of a Turkish family were found to have leptin deficiency (Strobel, Issad, Camoin, Ozata, & Strosberg, 1998). The first member had a low serum leptin level of 0.9 ng/ml and a body mass index (BMI) of 55.8 kg/m^2, exceeding the 99.6 centile. On genetic analysis this was found to be due to a leptin missense mutation (cytosine to thymine substitution in codon 105 of the leptin gene, LEP, resulting in an arginine to tryptophan replacement in the mature protein). The two other members of the family were also homozygous for the mutation and were very obese. The second, a 34-year-old female, had a serum leptin level of 1.6 ng/ml and a BMI of 46.9 kg/m^2 which was greater than the 99.6 centile, and the third member, a female 6 year old, had a serum leptin level of 1.1 ng/ml and a BMI of 32.5 kg/m^2 which was greater than the 99.6 centile. All the individuals showed excessive food intake, to the point of morbid obesity. The 22-year-old male had failed to go through puberty. He presented clinically with no facial hair, scant axillary and pubic hair, and a small penis and testes, and his endocrine measurements indicated impaired hypothalamic activation of the pituitary gonadotroph axis. The 32-year-old female had failed to menstruate, but no other information on her endocrine status was provided. These case studies provide very important information concerning the role of leptin in appetite and energy regulation.

Further evidence for the role of leptin in the control of appetite became apparent during the treatment of the eight-year-old Pakistani child described above with a daily subcutaneous injection of recombinant methionyl human leptin (Farooqi et al., 1999). There was a noticeable change in her eating behaviour and she no longer sought or demanded food between meals. One year after her treatment she had a marked reduction in fat mass and body weight. This observation suggests that leptin may be an important factor in the control of body weight and appetite regulation in humans.

1.2.4 Leptin resistance in humans

Clement et al. presented further persuasive evidence of the role of leptin in appetite regulation (Clément et al., 1998). They describe three sisters who were leptin resistant due to impaired signalling of the human leptin receptor gene. The family had a homozygous mutation in the human leptin receptor gene that leads to a truncated leptin receptor lacking both the trans-membrane and the intracellular domains. This anomaly prevents circulating leptin from binding to its receptor, and results in high circulating levels of leptin that are ineffective. The three sisters are the children of parents in a consanguineous family of Kabilian origin. They had the same phenotype. They were all of normal birth weight, but developed obesity within the first few months of life, with markedly elevated serum leptin levels. Two of the sisters died at 19 years of age. They also lacked pubertal development and their secretion of growth hormone and thyrotrophin was reduced. The three girls showed marked hyperphagia, even to the point of fighting with other children for food. These cases also suggest that leptin may be an important link between energy stores and feeding behaviour.

1.2.5 Placental leptin

The placenta is the sole organ of communication between the mother and the fetus. The maternal decidua and fetal membranes have the capacity to act as endocrine organs in their own right and have been shown to be the source of brain, pituitary and gut peptides, and of gonodal and adrenocortical steroid hormones. All of these compounds are believed to be the same chemically and functionally as the neuropeptides, peptides and steroids seen in the adult human (Petraglia et al., 1996). Leptin is produced in placental trophoblasts and amnion cells (Henson et al., 1999; Masuzaki et al., 1997) and is synthesised as a single molecular variant identical to human recombinant leptin (Señarís et al., 1997). The placenta secretes leptin into both the maternal and fetal circulation. Evidence for this comes from studies that have measured the maternal serum leptin concentrations throughout pregnancy. Serum leptin levels rise in the mother during pregnancy and fall immediately after birth (Butte et al., 1997; Masuzaki et al., 1997; Schubring et al., 1997). Leptin levels rise within the first trimester of pregnancy before fat mass is laid down. This observed rise in leptin levels was greater than the observed increase in fat mass through out pregnancy. This

suggests that leptin is being produced by an alternative source other than the adipocyte, thus suggesting a major placental contribution (Masuzaki et al., 1997). At term maternal serum leptin levels were much higher (mean ± SD, 20.0 ± 13.2 ng/ml) than amniotic fluid leptin levels (3.6 ± 2.8 ng/ml) and fetal leptin levels (arterial 9.7 ± 9.4 ng/ml, venous 8.9 ± 8.7 ng/ml). This implies that the placenta secreted different amounts of leptin into the different systems. The finding that there was no relationship between maternal serum leptin and fetal leptin levels (Schubring et al., 1997) supports this. These studies suggest that different tissues within the placenta produce differing amounts of leptin; their exact role in pregnancy and fetal development has yet to be determined.

1.2.6 Leptin in cord blood

Cord blood samples taken at delivery provide a brief sampling of the hormonal environment at that time. Several research groups have shown that leptin is present in the cord blood of the human infant (Cetin et al., 2000; Cinaz et al., 1999; Hytinantti et al., 1999; Kirel et al., 2000; Marchini et al., 1998; Matsuda et al., 1997; Schubring et al., 1997; Sivan et al., 1997; Varvarigou et al., 1999; Yang & Kim, 2000). Cord blood leptin levels are independent of maternal levels (Kirel et al., 2000; Schubring et al., 1997). Leptin is present in both arterial and venous cord blood within the fetal circulation and the measurements are significantly related to each other (Schubring et al., 1997). Cord blood leptin levels have a wide and variable range (Matsuda et al., 1997; Ong et al., 1999; Sivan et al., 1997; Tome et al., 1997). Matsuda et al. (Matsuda et al., 1997) reported cord blood leptin levels ranging from 2.0 to 84.5 ng/ml in newborn infants between 36 and 42 weeks gestation. Ong et al. (Ong et al., 1999) reported a range from 1.1 to 19.0 ng/ml in male infants and 2.6 to 42.1 ng/ml in female infants. Sivan et al. (Sivan et al., 1997) reported a range from 0.6 to 55.7 ng/ml. Why there is such variability is unknown.

What the significance of leptin is in cord blood has yet to be fully understood, but leptin levels are related to anthropometric measurements at birth as well as other hormones related to growth. Several studies have documented a positive relationship between cord blood leptin levels and birth-weight (Christou et al., 2001; Mantzoros, Varvarigou, Kaklamani, Beratis, & Flier, 1997; Matsuda et al., 1997; Schubring et al.,

1997; Sivan et al., 1997; Yang & Kim, 2000). Some found a strong relationship between cord blood leptin levels and length at birth (Mantzoros et al., 1997; Yang & Kim, 2000). Some found that females have significantly higher cord blood leptin levels than males (Jaquet, Leger, Tabone, Czernichow, & Levy-Marchal, 1999; Matsuda et al., 1997; Yang & Kim, 2000); others did not (Mantzoros et al., 1997; Schubring et al., 1997). Schubring et al. (1997) reported that leptin levels in cord blood correlated positively with placental weight, and that placental weight correlated positively with birth weight. Overall these studies suggest a relationship between cord blood leptin levels and size at birth.

Matsuda et al. (Matsuda et al., 1999) reported a range of cord leptin levels in 24 infants at birth from 2.4 to 61.9 ng/ml, that decreased to 0 to 4.5 ng/ml at 6 days of age in the infants plasma. This suggested that there was a dramatic decrease in serum leptin levels following delivery. This decrease in leptin levels has been illustrated at 16 hours after delivery (Marchini et al., 1998) and three days after delivery (Hytinantti et al., 1999; Kirel et al., 2000). The significance of this drop has yet to be elucidated. Scubring et al. (1997) propose that it may be a stimulus for appetite and feeding. The difference between cord blood leptin levels and the lower plasma leptin levels recorded in the neonate in the postnatal period raises the question of the source of the fetal leptin levels that are observed in cord blood. Are the cord blood leptin levels observed a product of the adipose cells of the fetus or of the placenta? There is evidence detailed above that both the placenta and the fetus produce leptin, but there is uncertainty as to the level and timing of each of their contributions.

1.2.7 Cord blood leptin and infant feeding

Scubring et al. proposed that the dramatic decrease in leptin levels in the early postnatal period may be the stimulus for feeding in the newborn (Schubring et al., 1997). No studies have directly looked at cord blood leptin and the initial stages of infant feeding, but Cinaz et al. looked at the changes in serum leptin on the second postnatal day in relation to a feeding episode (Cinaz et al., 1999). They found that plasma leptin levels decreased before feeding was initiated and rose after feeding. Using 'Lubencho's intrauterine growth curves' they divided the infants into those large for gestational age infants (LGA, $>90^{th}$ centile), appropriate for gestational age (AGA)

and small for gestational age infants (SGA, <10th centile). They took plasma samples for leptin 2 hours before a feed and half an hour after. All the infants were breast fed for at least 30 minutes. In the SGA infants, leptin levels rose by 23%, in the AGA infants, leptin levels rose by 47% and in the LGA infants leptin levels rose by 136%. This study raised several questions. Why did the plasma leptin levels rise to higher levels in the LGA infants? Did the higher leptin levels inhibit or modify feeding behaviour in any way? Did this affect weight gain? However, this study did not measure milk intake, so these questions were not answered. Several studies have documented rapid weight gain in infancy when the infant is either leptin deficient (Montague et al., 1997, 1998; Strobel et al., 1998) or leptin resistant (Clément et al., 1998). A recent study has also suggested that cord blood leptin levels influence infant growth. In a sub sample of 136 infants from the Avon Longitudinal Study of Pregnancy and Childhood (ALSPAC) cohort, the infants with higher cord levels of leptin had lower weight gain over the period from birth to 4 months (Ong et al., 1999). This effect was said to be independent of birth-weight and sex, and was still apparent at 24 months. Cord leptin levels accounted for 9.4% (6.6% when adjusted for birth weight) of the variance in weight gain from birth to four months (compared with 3.5% for breast/bottle feeding and 1% for early/late introduction of solids). Infants born relatively long tend to gain more weight (Fergusson, Horwood, & Shannon, 1980; Ong et al., 1999); after adjustment for birth-weight, they also have lower leptin levels (Ong et al., 1999). This study divided infants into three groups after converting the weights recorded to z scores. They used a decline in the z scores of −0.67 to identify slow growth and a gain of +0.67 to identify fast growth; this is equivalent to crossing down or up one inter-centile space on the Child Growth Foundation 1996/1 growth chart. They found that the infants showing the faster growth had markedly lower leptin levels. However, if cord blood leptin and growth are related, as suggested by these studies, it would seem reasonable to hypothesise that leptin must be influencing appetite, and hence feeding behaviour.

In summary, leptin appears to have a critical role in the regulation of body weight. This is shown by the extreme early onset obesity described in both rodents and humans with genetic mutations of the leptin gene or receptor. These individuals also show severe hyperphagia. Both these can be reversed by the administration of small doses of leptin. Cord blood leptin levels are related to the size of the infant at birth, suggesting

an intrauterine role in the regulation of body weight, and are related to infant growth over first two years of life. These findings suggest that leptin may be important in the regulation of appetite and body weight.

1.3 Ghrelin and the control of appetite

1.3.1 The discovery of ghrelin

Ghrelin is a peptide that was discovered by Kojima et al. in 1999 (Kojima et al., 1999). They first detected ghrelin in the stomach of the rat and subsequently detected it in the arcuate nucleus of the hypothalamus, an area abundant with NPY neurons important for appetite regulation. Ghrelin is a gut peptide mainly produced by the stomach, though ghrelin gene expression has also been discovered in the intestine, placenta, pituitary gland (Ravussin, Tschop, Morales, Bouchard, & Heiman, 2001) and more recently in the insulin-producing β cells within the pancreas (Volante et al., 2002). Ghrelin is the ligand of the growth hormone secretagogue receptor (GHS-R1a), one of two receptors recently identified. This receptor has been shown to regulate the growth hormone releasing effect of ghrelin, though this role is not fully understood as yet (Gnanapavan et al., 2002; Takaya et al., 2000). Ghrelin is present in the circulation at concentrations of 100-140 pmol/l (Kojima et al., 1999). Its secretion is pulsatile, with higher levels during fasting and lower levels after food intake (Cummings et al., 2001; Cummings et al., 2002).

1.3.2 Rodent studies and ghrelin

Rodent studies have found that intraperitoneal injection and intracerebroventricular (ICV) injection of ghrelin stimulates food intake over the following hour. This effect continued for up to 24 hours in those rats receiving ICV injection (Wren et al., 2000). There was also a surge in plasma growth hormone levels at 15 and 20 minutes after the injection. The findings from this study imply that ghrelin has a direct orexigenic action, i.e. it stimulates appetite. Nakazato et al. similarly reported an increase in food intake following ICV injection of ghrelin in rodents and weight gain following continuous ICV infusion of ghrelin for 12 days (Nakazato et al., 2001). They also identified sites within the nervous system that are stimulated following ICV administration of ghrelin by mapping c-*fos* expression (an early proto-oncogene that

reflects cellular activity following ghrelin binding to its receptor). The areas identified were the piriform cortex, the dentate gyrus and hippocampus, the paraventricular nucleus and the arcuate, dorsomedial and ventromedial hypothalamic nuclei. These are all regions that have been described as being important in the regulation of feeding, particularly the arcuate nucleus which contains the leptin responsive appetite inducing neuropeptides, neuropeptide Y (NPY) and agouti related protein (AGRP) and the leptin responsive appetite inhibiting neuropeptides, propriomelanocortin (POMC) and cocaine and amphetamine regulated transcript (CART). Fos expression was visible in 39% of the NPY neurons. These relationships were investigated further by ICV administration of blocking peptides that work against NPY and ghrelin. When NPY was blocked before injection of the ghrelin, the ghrelin induced feeding effect previously described was cancelled, as it was with administration of a blocking agent to AGRP and when a melanocortin receptor agonist was administered. The reverse was not evident; when ghrelin was blocked, the NPY induced feeding was unaffected. These findings suggest that ghrelin may modulate feeding by interaction with the peptides NPY and AGRP. Leptin appears to reduce food intake by inhibiting the production and release of NPY. ICV administration of leptin to rats that have been fasted for eight hours suppresses ghrelin induced feeding, but when ghrelin is administered following an earlier injection of leptin, the ghrelin overrides the leptin effect. This supports the theory that central ghrelin is an important regulator of feeding and therefore nutritional status.

1.3.3 Human studies and ghrelin

Ariyasu et al. showed that the stomach and duodenum are the main source of circulating ghrelin, by identifiying ghrelin messenger ribonucleic acid (m-RNA) in these tissues as well as the jejunum and the lungs (Ariyasu et al., 2001). The significance of these observations was supported by evidence that ghrelin expression and circulating levels of ghrelin decreased by 65% in patients previously subjected to a gastrectomy. They also showed that ghrelin-like immunoreactivity rose during fasting and decreased within one hour of feeding suggesting that rising ghrelin levels stimulate appetite and initiate feeding.

Similar findings have been documented in human studies looking at the role of ghrelin in feeding. Wren et al. found intravenous injection of ghrelin led to increased ratings of hunger on visual analogue scales, and a 28% increase in the amount of food consumed in comparison to the same subjects receiving a saline infusion on a separate occasion (Wren et al., 2001). They concluded that ghrelin was acting as a peripheral signal to the brain and was responsible for stimulating food intake in man. However care must be taken in interpretation of this finding, as it was an interventional study that administered a dose of ghrelin and then measured appetite and food seeking behaviours. This would have produced pharmacological levels of ghrelin much higher than those observed in normal physiology and may have intensified the effect upon appetite. Cummings et al. found that ghrelin levels rise if food is restricted and during starvation, but that they fall rapidly following ingestion of food (Cummings et al., 2002). They suggest that this pre-meal rise of ghrelin is a trigger to eat. Interestingly in a study comparing obese and lean subjects, the lean subjects showed a sharp decline in plasma ghrelin levels postprandially, where as the obese subjects showed no alteration in plasma ghrelin levels (English, Ghatei, Malik, Bloom, & Wilding, 2002). The lean subjects commenced the study with fasting plasma ghrelin levels much higher than in the obese subjects. This suggests an irregularity of ghrelin secretion in obese subjects; due to a sustained positive energy balance, ghrelin becomes unresponsive to food intake.

High fasting levels of ghrelin have been identified in young adults with a genetic disorder, Prader-Willi Syndrome (PWS) (Delparigi et al., 2002; Haqq et al., 2003). This disorder is characterized by excessive appetite and progressive obesity in childhood, as well as short stature and mental retardation. Comparison of fasting plasma ghrelin levels in a group of adults with PWS and a group of healthy normal adults showed that PWS adults had much higher levels (Delparigi et al., 2002). There was also a strong positive relationship between ghrelin levels and subjective ratings of hunger, measured using a visual analog scale. Similarly a group of children with PWS (Haqq et al., 2003) had much higher fasting ghrelin levels (3 to 4 fold) than BMI matched obese controls. Both these studies suggest that the high ghrelin levels may be causing excessive appetite and weight gain in this disorder.

Caixas et al. examined the effects of food intake on plasma glucose, insulin, ghrelin and leptin levels. All subjects were fasted overnight and then fed with a 790 kcal liquid meal in the morning (Caixas, Bashore, Nash, Pi-Sunyer, & Laferrere, 2002). Following ingestion of the meal, there was a rapid decline in plasma ghrelin levels by 26% at 40 minutes and these lower levels persisted for 120 minutes and plasma glucose and insulin levels rose at 20 minutes. Plasma leptin levels rose significantly at three hours following the liquid meal. This study suggests that ghrelin and leptin levels may have opposing roles in the regulation of feeding.

Ghrelin levels have been related to poor growth in SGA infants (Iniguez et al., 2002). This study measured serum ghrelin levels while fasting and ten minutes after an intravenous glucose load in one-year-old infants born SGA and in a control sample of infants born AGA. The groups had similar ghrelin levels fasting and following the intravenous glucose load. Both groups showed a reduction in ghrelin levels from the fasting level following the glucose load. The study then divided the infants into three groups after converting the weights recorded to z scores as described in section 1.2.7 to identify slow and fast growth from birth to one year of age. They showed that the SGA infants with the poorest growth had a larger decline in serum ghrelin levels following the intravenous glucose load. They also showed that the SGA infants who had the greater weight gain over the first year of life had a lesser decline in serum ghrelin levels following the glucose load. This study suggests that the higher levels of ghrelin in the fast growing infants may have been the stimulus for appetite and consequently for faster growth.

1.3.4 Placental and cord blood ghrelin

Gualillo et al. identified ghrelin messenger ribonucleic acid (mRNA) in the human placenta at the end of the first trimester of pregnancy and at term (Gualillo et al., 2001). Ghrelin has also recently been detected in cord blood (Castellino et al., 2002; Chanoine et al., 2002; Coutant et al., 2002) and is believed to be produced by fetal tissues (Cortelazzi et al., 2003). No sex difference was observed (Chanoine et al., 2002; Coutant et al., 2002). Some studies showed cord ghrelin levels were inversely related to birth weight (Cortelazzi et al., 2003; Farquhar et al., 2003; Kitamura et al., 2003) and birth length (Kitamura et al., 2003), others found no relationship (Castellino et al.,

2002; Coutant et al., 2002). Cord blood ghrelin levels were positively related to cord levels of growth hormone and negatively related to cord levels of insulin (Castellino et al., 2002). There was no relationship between cord ghrelin and leptin levels (Castellino et al., 2002; Chanoine et al., 2002).

To summarize the studies in section 1.3, exogenous ghrelin administered to rats and humans has a specific orexigenic effect, acting as a stimulus to eat. High ghrelin levels lead to weight gain in rodents. Higher ghrelin levels have also been associated with greater postnatal weight gain in SGA infants. These preliminary findings presume a relationship between ghrelin, appetite and weight gain and imply that ghrelin may be part of a complex appetite regulatory system. In the literature, a positive correlation between weight gain and ghrelin levels presumes that ghrelin stimulates appetite, on the other hand a negative relationship between ghrelin levels and weight gain presumes that lower ghrelin levels are associated with a poorer appetite. Ghrelin has recently been found in cord blood, although its role is uncertain at the present time. It is possible that cord blood ghrelin levels in newborn infants may be related to their subsequent milk intake and weight gain.

1.4 Feeding behaviour in infancy

1.4.1 Measurement of milk intake

The three most frequently used methods to measure milk intake in infants are the doubly labelled water method (Lucas, Ewing, Roberts, & Coward, 1987), the test-weighing method (Kohler, Meeuwisse, & Mortensson, 1984), and 'direct measurement', in which milk intake is calculated from the difference in the weight of the bottle before and after feeding (Butte, O'Brian-Smith, & Garza, 1990). The first two can be used in breast and bottle fed infants; the third only in bottle-fed infants.

The doubly labelled water method (Lucas et al., 1987) uses oral administration of water labelled with two naturally occurring stable isotopes, deuterium and oxygen-18. The water is administered to breast-feeding infants during suckling via a nasogastric tube. Formula fed infants are administered the solution mixed with 10 ml of ready to feed formula from a bottle and teat (P. S. W. Davies, Cole, & Lucas, 1989). Urine samples are taken into sterile specimen bags prior to administration of the isotopes and then

every 24 hours for the rest of the study period. Total energy expenditure is estimated from the differential rates of disappearance of the isotopes, after correction for changes in body composition. The main problem with this method is that the isotopes are expensive so it is difficult to conduct large studies (P. S. W. Davies et al., 1989). Furthermore, the procedure may influence the mothers' willingness to participate.

The test weighing method involves weighing the infant on an electronic balance before and after feeding, mothers are instructed to change the infant's nappy prior to feeding and weighing and to keep the same nappy and clothing on the infant until the weighing is complete after the feed. Electronic scales can be programmed to take several weighings within 10 seconds and display the average (Michaelsen, Larsen, Thomsen, & Samuelsom, 1994). The difference in weight of the infant is used to estimate the amount of milk consumed. Researchers that have used this method have reported inaccurate recordings if infants consumed very low milk volumes or were restless (Borschel, Kirksey, & Hannemann, 1986; Michaelsen et al., 1994). Additionally some mothers withdrew from the studies because they found the test weighing to be too difficult (Kohler et al., 1984).

Two procedures have been used to measure formula milk intake directly, weighing 'ready to feed' bottles of milk before and after feeding (Butte et al., 1990) or asking the mothers to make up feeds and record the amount of milk consumed from the bottle by using the measurement marks on the bottles. The former is more accurate, as the latter is subject to error from reconstitution of the feed. Mothers tend to add too much milk powder, so consequently feeds are over concentrated (De Swiet, Fayers, & Cooper, 1977; Dewey, Heinig, Nommsen, & Lonnerdal, 1991).

In an examination of the difference between test weighing of the infant and direct measurement of the bottle, Borschel et al. found the measurements of milk intake from test weighing to be 87 to 93% of those estimated by direct measurement (Borschel et al., 1986). Though these intakes were significantly correlated, they suggest an underestimation from test weighing and could be the reason why intakes compared in breast and formula fed infants tend to show formula fed infants consuming more milk. When Borschel et al. compared breast and bottle fed infants with the same test weighing technique the intake of breast fed infants was 90% of the intake of formula

fed infants. This difference may be due to the evaporative water loss from the infant over the feed. This study highlights the importance of using the same measuring technique for both forms of feeding if comparisons are to be made. A review of seven studies assessing the validity of measuring milk intake in formula fed infants using the doubly labeled water method versus direct measurement of the weight or volume of milk consumed (K. S. Scanlon, Alexander, Serdula, Davis, & Bowman, 2002) ascertained that the mean differences between the measurements were within 15%. Only four of these studies had adjusted for insensible water losses and environmental water influx; nevertheless the reported correlations between the two methods were from 0.93 to 0.98. However, correlations can be high if one method underestimates by a fixed amount.

Several practical problems have been reported when measuring the milk intake of breast-fed infants. It is difficult to calculate the exact energy content of breast-milk an infant consumes because the composition of the milk changes throughout the feed and is different in different mothers (Michaelsen et al., 1994). Accurate recording of the milk intake of breast-fed infants requires the calculation of an exact milk energy density based on 24 hour sampling of the milk and laboratory analysis of protein, fat and lactose (Dewey et al., 1991). Insensible water losses need to be calculated. The most prohibitive factor is the inconvenience to the mother of test weighing the infant before and after every feed and timing each feed accurately (Dewey et al., 1991). Consequently recruitment of breast-feeding mothers in the early postnatal period when they are recovering from the demands of childbirth and coping with the transition to parenthood may be very inconvenient for the mother. Two breast-feeding studies reported high attrition rates. De Carvalho et al. described the reasons for maternal withdrawal from their breastfeeding study to be as follows. Out of 75 mothers recruited, nine said 'there was too much to record', 25 felt the need to supplement the infant feed with formula milk, seven withdrew for no apparent reason and six changed address within the first month after the infants birth. Consequently this study lost 47 of the 75 recruited infants (De Carvalho, Robertson, Friedman, & Klaus, 1983). Cohen et al. reported similar problems; mothers decided to withdraw, returned to work, moved out of the area, introduced formula milk, or came under family pressure for them not to breastfeed. Out of an initial 453 women that expressed an interest in the study, only 141 mothers completed it (Cohen, Brown, Canahuati, Rivera, & Dewey, 1994).

1.4.2 Early feeding behaviour and milk intake in the newborn

The reflexes essential for feeding are the rooting reflex (the infant turns its head if their cheek is stroked), sucking, swallowing and breathing (M. Cole & Cole, 1989). Feeding is initiated soon after delivery and is highly variable across infants (C. E. Casey, Neifert, Seacat, & Neville, 1986). Feed frequency of the newborn breast-fed infant for the first 24 hours of life was (mean number of feeds ± SD) 4.3 ± 2.5 rising to 7.4 ± 3.9 for the second 24 hours of life (Yamauchi & Yamanouchi, 1990).

Four studies have measured milk intake over the first week of life and are presented in Table 1.1. The first study compared the milk intake of breast-fed infants and formula fed infants over the first two days of life (Dollberg, Lahav, & Mimouni, 2001). Milk intake was measured by test weighing breast-feeding infants before and after a feed and by direct weighing of formula in the bottle-fed infants. The researchers implied that the infants were fed *ad libitum*, but then say that they were fed strictly every 4 hours as per hospital protocol. This regime may alter the initiation and production of milk in the breastfeeding mother, and also alter milk intake in the formula fed infants. Comparison of test-weighing of the breastfed infant and direct measurement of the milk in bottle fed infants is not good practice and underestimates the amount of milk consumed in the breastfed infants (Borschel et al., 1986).

Table 1.1 Milk intake measures from four studies that measured milk intake (Mean ± SD) in the first week of life

	Day1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Dollberg (2001)							
Breastfed	9.6±10.3	13.0±11.3					
cc/kg/day	N =15	N = 15					
Formula-fed	18.5±9.6	42.2±14.2					
cc/kg/day	N = 28	N = 28					
Casey (1986)							
Breastfed	37±43		309±143		500±120		
g/day	N=3		N=10		N = 11		
Yamauchi (1990)							
Breastfed							
g/day							
Infants in 2 groups							
Group 1= 0-6			154.7±115.5		287.4±119.2		
feeds per day			N=114		N=114		
Group 2 =7-11			357.9±135.2		433.4±113.1		
feeds per day			N=28		N=28		
Neville (1988)							
Breastfed	44±71	182±86	371±153	451±176	498±129	508±167	573±167
g/day	N = 6	N = 9	N = 10	N =10	N=11	N=9	N=7

The second study measured the milk intake of breast-fed infants over the first five days of life using the test-weighing procedure (C. E. Casey et al., 1986). These results were again based on a small sample (11 infants) and only three infants were weighed on day 1 so their day 1 intakes are poorly estimated. The third study measured the milk intake in breast fed infants on the third and fifth days following birth using the test weighing procedure (Yamauchi & Yamanouchi, 1990). This study had a good sample size. They showed that infants breast fed between 7 and 11 feeds per day had a much higher milk intake than infants fed between 0 and 6 feeds a day. The fourth study recorded the milk intake of 13 breast-fed infants from birth to 12 months of age (Neville et al.,

1988). The infants were born to non smoking, multiparous women with a middle to upper socio-economic status. The infants were test weighed before and after every feed for the first 14 days of life and at different intervals through out their first year. Measurement of milk intake began within 12 hours of birth. This study showed that milk intake in the breast-feeding infants was low during the first two days of life, with a rapid rise on day 3 and 4 and then a more gradual rise to day 7. The sample number varied daily, because data were excluded for a number of reasons. If the mother developed mastitis, this alters the sodium and chloride content of the milk and may have affected the volume suckled. Some mothers fell asleep before the second weighing, so had an incomplete data set. Some mothers experienced problems with the balance and did not get accurate weight recordings. This study was again based on small sample numbers and so the mean and SD are poorly estimated. However these four studies during the early postnatal period, although based on mainly breast-fed infants suggest that milk intake increases noticeably over the first seven days of life.

In normal mother infant dyads, the infant controls maternal milk production by demand feeding (Daly & Hartmann, 1995; Dewey & Lonnerdal, 1986). Dewey & Lonnnerdal recruited 18 breast-fed infants between the age of 6 and 21 weeks. A baseline measurement of milk intake was calculated using the test-weighing procedure, and mothers were then instructed to express milk at each feed above the demand of the infant for two weeks; this extra milk was disposed of. Following this expression phase, milk production had significantly increased in most of the mothers. Infants responded initially by increasing their milk intake, though they did not consume all of the excess milk produced. Once milk expression was discontinued the infants milk intake returned to the baseline levels. This study suggests that infants are in control of their milk intake and regulate their intake even if excess milk is available.

There is evidence that infants can regulate their milk intake as young as seven days of age. Wright observed that breast-fed infants were able to regulate their feed size in relation to the length of the interval from the previous meal (P. Wright, 1981). So if an infant went for a long period without a feed, they consumed more milk at the next feed. Wright showed that by seven days of age breast-fed infants varied their feed size over the course of a twenty-four hour period, which was in contrast to formula fed infants, who showed very little variation in feed size over the same period. On examination it

appeared that the breast-fed infants consumed more milk in the twenty-four period than the formula-fed infants. This probably reflected the fact that the composition of breast milk varies over the course of a feed, where as the composition of the formula milk remains constant. Further evidence that infants are capable of regulating their milk intake as young as eight days of age is provided by Fomon et al. (1975). This study divided thirty infants into two groups to examine the affect of two different formula concentrations on energy intake. One formula had 54 kcal/100ml and the other 100 kcal/100ml. Those infants fed the 54 kcal/100ml formula from eight to forty one days of age drank more milk than the other group, consumed less energy and gained less weight. This suggests that the infants fed the 54 kcal/100ml formula were trying to adjust the volume of milk they consumed in accordance with their energy requirements, so took greater volumes of milk to try and get the required amount of energy. However their gastro-intestinal capacity may have prevented them consuming large enough volumes to consume similar amount of energy (Fomon, Filer, Thomas, Anderson, & Nelson, 1975). Another study by Fomon et al. (Fomon, Thomas, Filer, Anderson, & Nelson, 1976) divided thirty male infants into two groups to examine the effect of different formula content on food intake and growth. One formula had 29% of energy from fat and 62% of energy from carbohydrate; the other formula had 57% energy from fat and 34% of energy from carbohydrate. There was no difference in the amount of milk ingested, weight gain or energy consumed by these infants.

Infants' regulation of energy intake was further supported by a study in Honduras (Cohen et al., 1994), in which infants were randomized into 3 groups. Group 1 were exclusively breastfed (EBF) up to 6 months of age, group 2 were breast-fed but commenced solids at 4 months and had *ad libitum* breast feeding from 4–6 months (SF) and group 3 were breast-fed but commenced solids at 4 months with maintenance of baseline breast feeding from 4–6 months (SF-M). All milk and solid food taken was measured and its energy content calculated. At 4 months of age, the milk intake of the infants from the three groups averaged 797 g per day. Between 4-6 months the breast milk intake remained the same for the EBF group, but decreased significantly in the other two groups. This demonstrated that the infants in the latter two groups adjusted their milk intake in relation to the energy received from the solid food.

1.5 Growth in infancy

1.5.1 Importance of growth in infancy

The first motivation for the study for this thesis was to examine the role of leptin and ghrelin in appetite and the regulation of food intake, but the second was to examine the role of these hormones and appetite in the control of weight gain in early infancy. Growth in infancy is used as an indicator of health (Black, Hutcheson, Dubowitz, & Berenson-Howard, 1994). Poor weight gain in early infancy has been associated with several adverse outcomes, including ischaemic heart disease in adulthood (Barker, Osmond, Winter, & Margetts, 1989; Eriksson, Forsen, Tuomilehto, Osmond, & Barker, 2001) and poor cognitive development (P. H. Casey, Kraemer, Bernbaum, Yogman, & Clifford Sells, 1991; Dowdney, Skuse, Heptinstall, Puckering, & Zur-Szpiro, 1987; Kelleher et al., 1993; Oates, Peacock, & Forrest, 1985). Barker et al. (1989) found that infants that weighed less than 5.5 pounds at birth and that had the lowest weight at one year of age were more likely to die from ischaemic heart disease in adulthood than heavier infants at birth and at one year of age. Similar findings by Eriksson et al. (2001) showed that infants born with a low weight and with a low weight at one year of age were more at risk of heart disease, but the rate of weight gain was the important factor. Low weight gain up to one year of age and fast weight gain in male infants after one year of age were both associated with increased risk of coronary heart disease. Casey et al. (1991) and Kelleher et al. (1993) showed that low birthweight, preterm infants showed slower growth over the first three years of life compared to infants born at term. These infants also showed worse three year cognitive outcomes based on the Stanford-Binet Intelligence Scales. Oates et al. (1985) found that infants with slow weight gain in infancy had lower scores on the verbal intelligence scale of the Wechsler Intelligence Scale for Children, impaired language and reading skills and were more likely to have behavioural problems. Organic causes of slow weight gain can be due to congenital or acquired diseases of childhood, whereas non organic failure to thrive is inadequate growth in childhood without an organic cause (Altemeier, O'Connor, Sherrod, & Vietze, 1985). Failure to thrive often begins within the first few months of life (Altemeier et al., 1985; Drewett, Corbett, & Wright, 1999), so monitoring early weight gain in infancy is important. Much research has been conducted trying to identify the causes of non organic failure to thrive, which has been associated with adverse maternal experiences as a child, problems relating to the

pregnancy and parturition and to maternal discord with the father of the child, which were hypothesized to interfere with maternal infant attachment (Altemeier et al., 1985). One significant finding was that seven out of the 15 mothers of infants demonstrating slower growth had complained of difficulty feeding their infant in the nursery following delivery. This difficulty was thought to be due to poor maternal bonding, as only one of these infants when fed by a nurse was found to be difficult to feed. Poor growth in infancy was more likely in infants of parents classed as 'neglectful, under-controlling with inadequate levels of stimulation' than other parenting styles (nurturant and authoritarian, overcontrolling parents) (Black et al., 1994). However other studies reported poor weight gain in infancy was related to feeding problems and differences in eating behaviour, relating to poor sucking and poor appetite in 'picky eaters' (Dowdney et al., 1987; Drewett et al., 1999; Pollitt & Eichler, 1976). These findings are important and suggest that weight gain in early infancy may be related to nutrition and feeding behaviours, which is the main drive for the study for this thesis.

1.5.2 The measurement of infant growth

For many years measurements of weight, length, head circumference, abdominal girth and chest circumference have been used to monitor the growth, nutritional status and physical development of the infant (Tanner, Whitehouse, & Takaishi, 1966). As these measurements are often the basis of treatments and interventions in clinical practice their accuracy is essential. Likewise in research, if these measurements are to be used as dependent variables their accuracy is important. Johnson et al. evaluated 52 studies assessing their reliability (Johnson, Engstrom, & Gelhar, 1997). They documented whether term or pre-term infants were used, the measuring devices and the number of examiners and calculated the intra-examiner and inter-examiner reliability. Reliability is defined as 'the ability to repeat, reproduce, or consistently obtain the same measurement under identical conditions'. Intra-examiner reliability is 'the ability of the same examiner to obtain similar measurements consistently' and inter-examiner reliability is 'the ability of the different examiners to obtain similar measurements under similar conditions'. They reported that due to problems such as inaccurate documentation of measuring devices, procedures followed and statistics used, many of the studies could not be assessed adequately. They then designed a study to evaluate the intra-examiner and inter-examiner reliability of six anthropometric measurements

in 50 term infants. The anthropometrical indices were weight, head circumference, chest circumference, abdominal circumference, mid-arm circumference and length. Their results showed that intra-examiner differences are smaller than inter-examiner differences, except for weight, which was similar for both using an electronic scale. Weight and head circumference measurements were the most reliable; length and mid arm circumference were the least. One problem is that they used a tape measure to measure length, whereas a proper measuring device such as a Kiddimetre would have been more accurate. A Kiddimetre is a specialist measuring instrument that measures the supine length of the infant; two people are required, one to hold the infants head still and against the headboard and the other to straighten the legs and bring the foot board to the soles of the feet. The reason they used a tape measure was because of the reported practical problems related to using a standard measuring device in a clinical setting. These included the time and effort involved in cleaning instruments between infants, the unwieldy size of such devices, trying to fit it into incubators and cots, the expense of buying such an instrument and the fact that it needed two people to perform the measurement. They do report, however, that measuring devices such as the neonatometer have given more reliable results. Davies and Holding invented the neonatometer in the early seventies and despite it being large and unwieldy, it did give more accurate results than the tape measure alone (D. P. Davies & Holding, 1972). Their standard deviation of differences across examiners was 1.7mm compared to 2.21 and 2.10 mm respectively in the work of Johnson et al.

1.5.3 Growth standards

In view of the importance of being able to assess infant well-being through measurements of growth, several growth charts have been devised based on the collection of data from large normative groups. These are based on infants and children from the general population and are usually referred to as growth standards. The earlier charts that were used to monitor growth were the Tanner charts based on measurements of supine length, height and weight, of which there are several revised versions because of the secular trends towards an increased stature (Tanner et al., 1966). These were originally devised from a British population of children in 1959. Tanner showed that in the 1960s children had got larger over the previous decade, they were growing faster and attained adult size at an earlier age. Over the years health has improved, diet has

changed and maternal practices have changed in relation to feeding. So infants in the 21st Century would not necessarily be the same. Whitehead and colleagues (Paul & Whitehead, 1986; Whitehead, Paul, & Cole, 1989) challenged the Tanner charts in a study conducted in Cambridge which observed differences in growth between breast-fed and bottle-fed infants. They showed that growth in the breast fed infant was faster over the first four months of life and then slowed down in comparison with infants on the standard Tanner growth charts used at that time. Some criticism of the data used to compile the Tanner reference charts was also made. Data collection was in a time when the UK infants were fatter and mainly bottle-fed with a formula of a higher fat concentration than is used now, and the infants commenced solids at seven weeks of age. Early introduction of solids has been related to greater fat deposition (Ferris, Laus, Hosmer, & Beal, 1980); formula fed infants that commenced solids before 2 months of age had higher mean skinfold measurements at three months of age than infants that solely breast fed or formula fed.

More recent growth charts for UK children have been compiled and updated using cross-sectional data for exact age, weight, length and head circumference collected from ten surveys and longitudinal data from one study between the years of 1978 and 1990 (T. J. Cole, 1995; Preece, Freeman, & Cole, 1996). From this information standard deviation (SD) or Z scores can be calculated for each individual. These represent the differences expressed in standard deviations from the median value of the population in SD units (zero). The chart has eighteen centiles, nine for each sex, and the age distribution is from 33 weeks gestation to 23 years. The charts were compiled on 30,535 measurements (T. J. Cole, 1995), but one must bear in mind that the new growth charts were based on relatively small numbers of infants. The number of infants at birth totalled 712 males and 662 females and at three months the numbers declined to 482 males and 421 females. Measurements were only taken intermittently at three month intervals until twelve months of age, and then at 18 months of age and yearly until 23 years of age. Most of the measurements were of different infants at the different time periods with the exception of one study that measured longitudinal growth collected at 15 visits between the age of 4 weeks and two years of age. The method of feeding and the introduction of solids amongst the sample are unknown. In view of the number of surveys collated and presumably the number of people involved in obtaining the data, one has to question the reliability of the measurements. Wright et

al, (1996) discovered a discrepancy with the British 1990 national growth standards. They had not been correctly standardized for sex. They identified within their cohort of 3418 term infants (1591 male, 1580 female) a difference in mean standard deviation scores of 0.42 between males and females after three months of age, which was highly significant when compared with the 1990 standards. This meant that between 6.6 and 7.3 % of females rather than 3 % had SD scores below the 3rd centile over the first 12 months of life when calculated from the British standards. Between 2.0 and 4.7 % of males had an SD score above the 97th centile when calculated from the British standards. It was also interesting that between birth and three months of age the weight SD scores of infants from Newcastle in their study remained below the British 1990 standards (C. M. Wright, Corbett, & Drewett, 1996). This either means that Newcastle infants are different from the rest of the population, or that the standards are not based on a sufficiently large numbers of infants. Following Wright et al's research the British standards were revised (Preece et al., 1996). Following this controversy and in the attempt to engender universal methods of assessment, the Royal College of Paediatrics and Child Health have set up an expert working party called 'the Growth Reference Review Group'. They have reviewed the different growth charts available and came to the conclusion that the UK90 reference charts are the most appropriate for clinical use in assessing weight, length and head circumference for British children up to two years of age (C. M. Wright et al., 2002).

Another important consideration when using growth charts to assess weight gain in infancy is 'regression to the mean' (T. J. Cole, 1995). If an infant is weighed at time point A and then weighed again at time point B, their weight centile on the second weighing on average tends to be nearer the median than their first weight measurement. This means that light infants at birth tend to show relatively faster growth and heavy infants relatively slower growth, as both their weights on the second weighing have moved towards the 50th centile.

1.5.4 *Determinants of fetal and infant growth*

Several factors can affect fetal growth and influence the size of the infant at birth. These can be categorized into genetic, intrauterine, or environmental factors. Genetically, parental size is a major contributor to infant size at birth; both maternal

and paternal height have been related to birthweight (Hindmarsh, Geary, Rodeck, Kingdom, & Cole, 2002; Kramer, 1987; Ounsted, Scott, & Ounsted, 1986; Strauss & Dietz, 1998). Also, sex of the infant is important. Male infants are heavier at birth than female infants (Kramer, 1987); the mean magnitude of the birth weight difference attributable to sex was 126.4g in developed countries and 93.1g in developing countries. Intrauterine influences on birthweight can be related to maternal health during pregnancy and maternal diet; mothers exposed to malnutrition in pregnancy tend to experience slower fetal growth (Godfrey, Barker, Robinson, & Osmond, 1997; Hales & Barker, 2001). Smoking in pregnancy is one of the most significant environmental causes that has been found to affect the size of the fetus. This may be due to the effect smoking has upon maternal appetite (dietary constraint) or the toxic effects of carbon monoxide and cyanide (placental transfer). Kramer (1987) found a significant effect of smoking on birthweight; the sample-sized-weighted birth weight deficit of mothers that smoked was 149.4g. Since 1984, many studies have shown that infants of smokers are born both shorter (Elwood, Sweetnam, Gray, Davies, & Wood, 1987; Godfrey et al., 1997; Hindmarsh et al., 2002), and lighter (Conter, Cortinovis, Rogari, & Riva, 1995; Elwood et al., 1987; Godfrey et al., 1997; Hindmarsh et al., 2002) and have a smaller measured head circumference (Elwood et al., 1987; Hindmarsh et al., 2002) than infants born to mothers that do not smoke.

Factors related to birthweight of the infant are important because birthweight is a significant predictor of infant growth. Birth weight was found to be a significant predictor of growth up to six months of age (Kramer et al., 1985) and 12 months of age (Cohen, Brown, Canahuati, Rivera, & Dewey, 1995), and a major determinant of size in adulthood. Sorensen et al. (Sorensen et al., 1997) examined the records of 4300 Danish military recruits aged between 18 and 26 years and found their adult size (measured by BMI) was significantly related to their weight at birth. Their birth weight and birth length were also significantly related to their adult height (Sorensen et al., 1999). Binkin et al. examined the relationship between birthweight and childhood growth up to five years of age (Binkin, Fleshood, & Trowbridge, 1988). They found that birthweight was a significant predictor of weight and length in early childhood. Even though infants up to one year of age born with a lower birthweight appeared to show a proportionately greater weight gain, they still were more likely to remain lighter

and shorter than infants born of a higher birth weight. In contrast infants born heavier at birth remained heavier and taller.

Sex of the infant was also found to be a significant predictor of infant growth from birth to 4 months of age (Cohen et al., 1995), birth to 6 months of age (Kramer et al., 1985) and birth to 12 months of age (Cohen et al., 1995; Stunkard, Berkowitz, Stallings, & Schoeller, 1999). Boys gained more weight and grew taller than girls.

Feeding behaviour and milk intake have been related to weight gain. Several studies showed that energy intake was related to bodyweight (Cohen et al., 1995; P. S. W. Davies, Wells, & Lucas, 1994; De Swiet et al., 1977; Dewey et al., 1991; Fomon, Owen, & Thomas, 1964). Both Davies et al. and De Swiet et al. showed a significant relationship between milk intake and body weight at six weeks of age in breast fed infants. Fomon et al. (1964) showed that the influence of body weight on milk intake is greatest when body weight varies between 3 and 5 kg, which is the period of most rapid growth. Dewey et al. showed significant correlations between energy intake at 3 months with weight gain from 3 to 6 months, energy intake at 9 months and weight gain from 9 to 12 months. Similarly Cohen et al. showed that weight gain from birth to 4 months of age was related to breast milk energy intake at 4 months. The age at which solid food was introduced was also significantly associated with weight gain at 6 months of age (Kramer et al., 1985) and energy intake and sucking behaviour at 3 months of age was a predictor of weight gain up to 12 months (Stunkard et al., 1999).

Parental height also influences an infant's growth trajectory. In a longitudinal growth study Hallman et al. reported significant correlations between mid-parental height and infant and child length and height from birth to 10 years of age (Hallman, Backstrom, Kantero, & Tiisala, 1971). Gender differences were noted at birth. The length of the male infants was related to maternal, paternal and mid parental height whereas the length of the female infants was only related to mid parental height. At three months of age, the length of both male and female infants demonstrated significant positive relationships with maternal, paternal and mid parental heights. A further study attained similar findings, Smith et al. found boys at birth were an average 0.9cm longer than girls at birth and gained length faster up to 6 months of age, but then length increased similarly in both (Smith et al., 1976). This sample of infants were from middleclass

parents, which reduced the number of potentially confounding variables that could affect growth, such as nutrition and disease and may be more reflective of the genetic influence on growth. Parental height measurements were available for 43 infants; at birth maternal height was significantly related to female infant length ($r = 0.44$). At two years of age, maternal and paternal height was related to female infant length. At one and two years of age, both paternal and maternal height was related to male infant length. Stature is one of the most heritable morphological characteristics, and this study shows that postnatal height eventually became related to mean parental height.

1.5.5 Infant growth and feeding

Some infants lose weight in the early neonatal period (Avoa & Fischer, 1990; Dollberg et al., 2001; Maisels, Gifford, Antle, & Leib, 1988; Yamauchi & Yamanouchi, 1990). Breast-fed infants can lose between 3.8 to 6.9% of their birth weight (Avoa & Fischer, 1990; Dollberg et al., 2001; Maisels et al., 1988; Yamauchi & Yamanouchi, 1990) and formula fed infants can lose up to 4.2 % (Avoa & Fischer, 1990; Dollberg et al., 2001; Maisels et al., 1988; Yamauchi & Yamanouchi, 1990). This weight loss has become accepted as normal and is thought principally to be due to changes in the volume and distribution of water in the body and to feeding practices (Maisels et al., 1988). Bioelectrical resistance studies on a sample of term, healthy breast-fed newborns, have reported a progressive decrease in total body water and body solids from birth to three days of age (Rodriguez et al., 2000). Although infants may lose weight in the early neonatal period, Bishop et al. recorded a gain in infant length (Bishop, King, & Lucas, 1990). They measured length, weight, ulnar length and head circumference on 45 term infants, daily from birth until seven days of age. They reported that despite a weight loss in that period, crown-heel length increased by an average of 11.5mm by day 7 and ulnar length by 2.5mm. Head circumference decreased initially and then also increased after the fourth day. This implies that linear growth proceeds quite rapidly in the early neonatal period, so even though an infant may lose weight in the first week of life a proportion of their energy intake is directed to linear growth.

Feeding practice affects weight gain in the early neonatal period; differences have been observed between infants fed to demand and infants fed to schedule. If infants were allowed to breastfeed frequently and to demand, Jolly et al. found this prevented loss of

weight in the early neonatal period (Jolly, Humphrey, Irons, Campbell-Forrester, & Weiss, 2000). Only two of their 21 infants had lost weight at 3 days of age and by 7 days of age only one infant weighed less than their birth weight. Four significant positive predictors of weight gain were identified up to 24 days of age. These were 1) birth weight, 2) maternal educational level (infants born to mothers with high school or college educations gained more weight up to 24 days of life than infants born to mothers with only a primary education), 3) infants that cried before a feed gained more weight than infants that did not cry and 4) infants fed longer than 15 minute at a feeding episode gained more weight. Feeding practice was the main determinant of weight gain in infants up to 15 days of age (De Carvalho et al., 1983). Frequent feeding to demand as opposed to scheduled feeding 3 to 4 hourly had a significant effect upon weight gain. At 15 days postpartum infants fed to demand had gained more weight and their milk intake on the 15th day was significantly greater than the milk intake of infants fed to schedule.

Evans (1978) showed that the rate of growth is rapid over the first two months of life and then starts to slow down. Table 1.2 is based on his data.

Table 1.2 Growth rate of formula fed infants (mean ± SE), birth to 6 months (Evans, 1978).

	0 –1 month	1 –2 months	2 –3 months	3 –6 months
Weight gain, g/wk	172 ± 11.58	202 ± 11.82	176 ± 8.47	131 ± 4.86
Linear growth velocity, mm/wk	8.51 ± 0.48	8.47 ± 0.54	5.35 ± 0.41	4.16 ± 1.79
Head circumference velocity cm/wk	0.55 ± 0.03	0.41 ± 0.03	0.35 ± 0.02	0.22 ± 0.09

The 'normal pattern of weight gain' in the early infant period proposed by the Committee on Nutrition of the American Academy of Pediatrics are similar to those found by Evans (1978). They recognized that weight gain is rapid over the first three months of life and calculated an expected weight gain of 25 to 30 g per day (infants fed formula milk with an energy density of 67 to 70 kcal/dL), and then a slow down to 10

to 15g/d between six and 12 months of life (Kleinman & Committee on Nutrition, 1998).

Growth rate in infancy is greater in males than females and greater in formula fed than breast-fed infants (Neumann & Alpaugh, 1976). In a longitudinal growth study that followed 357 infants (192 boys, 165 girls, 61% formula-fed, 39% breast-fed) until they had doubled their birth weight, they found that male infants doubled their birth weight sooner than female infants (mean ± SE, 111 ± 3 versus 129 ± 3 days), and that formula-fed infants doubled their birth weight sooner than breast-fed infants (113 ± 4 versus 124 ± 4 days). They also found a significant positive relationship between birth weight and age at birth weight doubling. Infants heavier at birth took longer to double their birth weight. A later study looking at birth weight doubling found no effect of feeding (Jung & Czajka-Narins, 1985), but supported the sex and birth weight differences. One difference between these two studies is that the infants from the latter study commenced solids at a later age, which delayed the mean time to birth weight doubling which was 22 days later. These studies emphasize the importance of feeding practices to weight gain.

There is some debate as to the relative growth rate of breastfed and formula-fed infants. Some studies report no difference in weight or length gain between the two modes of feeding up to six weeks of age (De Swiet et al., 1977), three months of age (Butte, Wong, Hopkinson, Smith, & Ellis, 2000; Hitchcock, Gracey, & Gilmour, 1985), four months of age (Butte et al., 1990) and six months of age (De Swiet et al., 1977; Dewey, Heinig, Nommsen, Peerson, & Lonnerdal, 1992; Ferris et al., 1980; Kohler et al., 1984). Some studies found a difference became apparent only at 3 to 6 months of age, when weight gain was greater in formula fed infants (Butte et al., 2000; Hitchcock et al., 1985) or after 6 months of age (Dewey et al., 1992; Hitchcock et al., 1985). Others found breast-fed infants gained more weight than formula-fed infants over the first 3 months of life (Donma & Donma, 1999; Persson, 1985), but formula fed infants gained more weight between 3 and 6 months (Donma & Donma, 1999). Some studies found differences in body fat distribution. Skinfold thickness were greater in formula fed infants in two studies (Ferris et al., 1980; Oakley, 1977). Others found no difference in skin fold measurements between the two feeding modes (Kohler et al., 1984). One study found that breast-fed infants show slower length velocity from 3 months than

formula fed infants. This effect was still apparent at 9 months of age (Salmenpera, Perheentupa, & Siimes, 1985).

One longitudinal study (Dewey et al., 1992) collected anthropometric data on both breast-fed (BF) and formula fed (FF) infants from birth to 18 months. The two groups were matched for parental socio-economic status, education, ethnic group, birth-weight and sex, and solids were introduced after four months. Table 1.3 is based on their data up to three months of age.

Table 1.3 Growth rate of breast and formula fed infants mean (SD), from birth to three months (Dewey et al. 1992).

	Breast-fed infants		Formula-fed infants	
	Males	Females	Males	Females
Birthweight (g)	3800 (530)	3584 (472)	3556 (542)	3571 (397)
Weight at 1 month (g)	4754 (525)	4434 (491)	4616 (539)	4402 (338)
Weight at 3 months (g)	6605 (714)	5941 (622)	6499 (669)	5952 (487)
Birth length (cm)	51.9 (2.4)	50.8 (2.5)	51.3 (1.8)	50.3 (2.0)
Length at 3 months (cm)	62.6 (2.3)	60.9 (2.1)	62.2 (1.5)	61.1 (1.8)

This study shows that weight gain from birth to three months of age is very fast and similar in males and females, regardless of the mode of feeding. This was apparent until 6 months of age with the female infants and 7 months of age with the male infants, at which point the FF infants became significantly heavier than the BF infants; this difference persisted until 18 months of age. This study suggests that after 6 months of age the growth patterns differ between the infants depending on their mode of feeding. Table 1.3 shows that length similarly showed a pronounced increase in the first three months of life. Analysis showed that the length increments were not significantly different between feeding groups for either males or females. The sample was not necessarily representative of breast and formula fed infants, as both groups of infants were from educated, affluent backgrounds.

1.6 Other factors that might affect feeding in the early neonatal period

1.6.1 The role of insulin in utero and early infancy

Cord blood insulin levels are positively related to birth weight and placental weight (Ong et al., 2000), and are significantly elevated in LGA infants (of non-diabetic mothers) compared with infants with normal or retarded growth (Giudice et al., 1995). Thus suggesting a role for insulin in fetal growth. Furthermore this concept is supported by the fact that infants exposed to hyperinsulinaemia in utero (as in uncontrolled maternal diabetes) are subject to over growth and have macrosomia. Cord blood insulin levels are positively related to cord blood leptin levels (Christou et al., 2001). Cord blood insulin levels are high at birth (range 2.0 to 29.3 mU/l) in term AGA infants, and remain high in the early neonatal period (0-6 days, range <1.0 to 28.5 mU/l). These levels were much higher than those observed in children aged one month to ten years of age (range <1.0 to 7.5 mU/l) (Hawdon, Aynsley-Green, Alberti, & Ward Platt, 1992). The relevance of high insulin levels in utero appears to be its role as a growth factor; what its relevance is in the early neonatal period has yet to be determined. However, Ogilvy-Stuart et al. demonstrated that insulin and growth hormone levels were related to feeding in the newborn infant (Ogilvy-Stuart et al., 1998). Both growth hormone and insulin rose following each feeding episode in the twelve-hour sampling period, suggesting that feeding is the stimulus for anabolic hormone production, which in the long term facilitates growth.

1.6.2 Maternal analgesia and anaesthesia and length of labour

Newborns that have not been exposed to maternal analgesia in labour show spontaneous rooting and sucking movements when placed on the chest of their mothers, move towards the breast and find the nipple and commence suckling at the approximate age of one hour (Widstrom, Ransjo-Arvidson, Christensson, & Mathiesen, 1987). Ransjo-Arvidson et al. examined the effect of maternal analgesia on the infants breast-seeking and breastfeeding behaviors when they were placed on the mothers chest straight after delivery (Ransjo-Arvidson et al., 2001). Video recordings were made of infants, born to mothers categorized into three groups. Group 1 (n = 10) had no analgesia in labour, group 2 (n = 6) had received mepivacaine via pudendal block and group 3 (n = 12) received pethidine (IM) or bupivacaine via epidural or more than

one type of analgesia. In the infant movements of eyes, hands, mouth, tongue, and rooting and sucking were assessed every 30 seconds. One person who did not know which group the infant was from later coded the videos. They found that infants born to mothers that had received analgesia (groups 2 and 3) were less likely to display the hand to mouth movements, made less licking movements and were less likely to suck the breast. Twelve of the infants from the analgesia groups had an interval greater than 150 minutes from birth to time of first feed.

Most mothers have some form of pain relief in labour (Rosenblith, 1992). Any substance that is detectable in maternal blood crosses the placenta to some degree; this can be by simple diffusion, facilitated diffusion, active transport or pinocytosis (Burt, 1971). The way the drug is administered also affects transfer, so that a drug given to the mother intravenously has more of an immediate effect upon the fetus than a drug given intramuscularly which is slowly absorbed into the blood stream and then crosses the placenta. The effects of maternal analgesia and anaesthesia on the fetus have been assessed by measurement of umbilical cord pH, Apgar scores and behavioural testing.

Umbilical cord pH is the measure of the acid-base balance in the blood; pH levels < 7.15 from the umbilical artery and < 7.2 from the umbilical vein are indicative of fetal metabolic acidaemia. Apgar scores are an universal measurement of neonatal well being taken at one and five minutes following delivery of the infant. They come from a scoring system developed by Dr. Virginia Apgar in 1952 to evaluate physical condition at birth (Apgar, 1953). They are an objective assessment of five signs: heart rate, respiratory effort, reflex irritability, muscle tone and colour. A rating of 0, 1 or 2 is given to each sign. A rating of 0 implies the sign is absent, a rating of 1 implies that the sign is there to a degree and a rating of 2 means the sign is present completely. A score of 7 or above implies that neonatal condition is good or excellent. All midwives are trained to use this tool as a method of assessment of the neonate at delivery. Recent re-evaluation of Apgar scoring indicated that it is still relevant for the prediction of neonatal survival today (B. M. Casey, McIntire, & Leveno, 2001). These authors conducted a retrospective analysis of Apgar scoring and umbilical-arterial blood pH values in 145,627 infants to see which was the better predictor of outcome. In 132,228 term infants the mortality rate was 244 per 1000 infants with five-minute Apgar scores of 0 to 3 compared with 0.2 per 1000 for infants with five-minute Apgar scores of 7 to

10. Five-minute Apgar score was a better predictor of neonatal outcome than the measurement of umbilical-artery blood pH, even for infants with severe academia.

Infant behaviour in the early neonatal period has been assessed using three neurobehavioral tests, the Scanlon Early Neonatal Neurobehavioural Scale (ENNS, (J. W. Scanlon, Brown, Weiss, & Alper, 1974)) the Brazelton Neonatal Behavioural Assessment Scale (BNBAS, (Als, Tronick, Lester, & Brazelton, 1977)), and the Neurologic and Adaptive Capacity Score (NACS, (Amiel-Tison et al., 1982)). The ENNS takes 10 minutes to perform and is administered 2 to 8 hours following birth. It places emphasis on tests of muscle tone, reflexes and response to stimulation and gives a general evaluation of the infant, but has been criticized for its use of unpleasant stimuli such as pinpricks and repeated Moro examinations. The BNBAS takes 30-45 minutes to administer and requires extensive training. It has four main areas of behavioural measurement: attention and social responsiveness, muscle tone and motor organization, controlling state of consciousness and physiological response to stress. The NACS was designed to examine central nervous system depression from drugs, it tests neonatal tone by assessing adaptive capacity, passive tone, active tone, primary reflexes and alertness, it takes less than 5 minutes to administer.

There are several types of analgesic and anaesthetic agents used in current obstetric practice; the three main ones used in the Royal Victoria Infirmary, Newcastle-upon-Tyne, where the study reported in this thesis was conducted are Entonox, an inhalational agent, diamorphine hydrochloride, an intramuscular opioid and, anaesthetic agents injected extrathecally into the epidural space (epidural anaesthesia).

Entonox is 50% nitrous oxide and 50% oxygen (Olofsson & Irestedt, 1998). This is self administered by the mother and rapidly crosses the placenta (Stenger, Blechner, & Prystowsky, 1969) but has a low tendency to accumulate in the mother or fetus and is rapidly eliminated in early neonatal life if the infant establishes normal regular respiration. Stefani et al. assessed the neurobehavioural status of infants born to mothers receiving nitrous oxide and oxygen and those who received no inhalation agents (Stefani et al., 1982). The NACS was used at 2 and 24 hours of age and the ENNS at 15 minutes of age. There was no evidence of respiratory depression or of any effect upon muscle tone, reflexes or response to stimulation.

Diamorphine hydrochloride is the opiate that is used for women in labour in Newcastle-upon-Tyne. However, many other obstetric units in the United Kingdom changed to pethidine following research by Way et al. that showed severe respiratory depression of the neonate following administration of morphine (Way, Costley, & Way, 1965). This research changed the use of analgesia in obstetric practice and since 1965 little research data is available on the use of diamorphine in labour, as most of the research has concentrated on the effects of pethidine upon the neonate. Infants born to mothers receiving pethidine in labour have been shown to be drowsier than infants not receiving opioids (Emde, Swedberg, & Suzuki, 1975) and they have a decreased muscle tone, slower reflexes and slower response to stimulation as reflected in ENNS scores (Hodgkinson & Husain, 1982). These affect mother-infant interaction and feeding behaviour during the first few days of life (Belsey et al., 1981; Nissen et al., 1995; Nissen et al., 1997). Similar studies examining the effect of morphine on the neonate have not been carried out. However more recently Gerdin et al. found that morphine has a rapid plasma clearance and a short elimination half-life of less than an hour in pregnant women (Gerdin, Salmoson, Lindberg, & Rane, 1990). The same study showed that morphine was not detectable in the umbilical arterial blood of the infant three hours after maternal administration. Placental transfer of morphine from the mother to infant is quick, but due to rapid elimination by the mother the actual fetal load is low. Additionally Olofsson et al. in a comparison study of the administration of pethidine and morphine in labour, documented infant condition at delivery assessed by Apgar scores (Olofsson, Ekblom, Ekman-Ordeberg, Hjelm, & Irestedt, 1996). They found no relationship between opioid dose and Apgar scores. No infants showed any clinical signs of respiratory depression. Wittels et al. assessed the neurobehavioural status of infants using the BNBAS, in mothers receiving pethidine and morphine using a patient controlled analgesia technique for pain relief following caesarian section (Wittels et al., 1997). Infants exposed to morphine via breast milk from their mother were found to be more alert and oriented more to animate visual and auditory cues than infants exposed to pethidine. The limited research available on morphine use in labour and postnatally via breast milk transfer suggests that it may have less negative effects on the neonate than pethidine. As previously described all infants are routinely assessed using Apgar scores and any signs of respiratory depression are usually treated with a narcotic antagonist, naloxone, which reverses the respiratory depression.

The two most commonly used anesthetic agents used for epidurals in labour are bupivacaine or lignocaine. Research looking at neonatal wellbeing following maternal epidural has been contradictory. Rosenblatt et al. examined the effects of epidural bupivacaine on 53 infants using the BNBAS (Rosenblatt et al., 1981). They reported maximum effects in the first twenty-four hours following delivery of the baby. They reported a decreased motor tone, and poor orienting and alerting skills. Some of these effects were still apparent in the infant at six weeks of age. Abboud et al. using the ENNS and NACS test found no difference in scores between epidural exposed infants and those not exposed to any medication (Abboud, Khoo, Miller, Doan, & Henriksen, 1982). Kangas-Saarela et al. used the ENNS to compare epidural exposed infants and opioid exposed infants (Kangas-Saarela et al., 1987). They reported an enhancement of orientation to auditory cues and better habituation to sound in the epidural exposed infants. Lieberman and O'Donoghue evaluated the data from all randomized control trials and observational studies published on the unintended effects of epidural analgesia from 1980 to date (E. Lieberman & O'Donoghue, 2002). They reported that there was no significant difference in umbilical cord pH of infants born to mothers receiving and not receiving an epidural, and no difference in Apgar scores in 33 out of the 34 studies they reviewed. They analysed 11 studies relating to neonatal behavioural and neurological outcomes, which had used the behavioural tests previously described (the ENNS, BNBAS & NACS), finding contradictory results. In the studies that used the BNBAS to compare epidural exposed infants to infants not exposed to any medication (3 studies), two found motor dysfunction in the epidural exposed groups. In studies which compared epidural exposed infants to opioid exposed infants (5 studies), two found no behavioural differences using the NACS test; the other three reported that epidural exposed infants had better auditory orientation and habituation, but poorer muscle tone. Lieberman et al. compared 59 epidural exposed infants with 51 opioid exposed infants using the BNBAS and found no difference in scores between the two groups (B. A. Lieberman et al., 1979).

Theoretically the length of labor could influence feeding behavior in the newborn, as infants exposed to longer labours may have been under more stress and be more tired. Also if the length of labour is longer, then the mother is more likely to have more analgesia or anaesthesia. This could affect the time to their first feed, the number of

feeds consumed and their milk intake in the first twenty-four hours following delivery, but research examining this possibility has not been conducted.

In summary, breast feeding infants born to mothers that have no form of medication during labour, show early signs of feeding behaviour, display hand to mouth movements, sucking, rooting and licking movements and initiate feeding at about one hour post delivery. These are less obvious in infants born to mothers receiving analgesia in labour, though this study was small (Widstrom et al., 1987) and related to breastfeeding infants only. Evidence based on other neurobehavioural outcomes has been contradictory. Effect of analgesia and anaesthetic agents on feeding in the early neonatal period have not been properly researched.

1.7 Summary and research aims

1.7.1 Summary

There is very good evidence from rodent studies that leptin levels reflect fat stores and influence the control of appetite. Rodents bred with genetic mutation of the Lep^{ob} gene have no circulating leptin, they are hyperphagic and they develop gross obesity (Zhang et al., 1994). Exogenous administration of leptin raises their plasma leptin levels, reduces their food intake and decreases their fat mass and body weight (Campfield, Smith, Guisez et al., 1995; Jacob et al., 1997).

There is also good evidence for the importance of leptin in appetite and weight regulation in humans, from the rare children born with congenital leptin deficiency (Montague et al., 1997, 1998; Strobel et al., 1998) and leptin resistance (Clément et al., 1998). These children were profoundly hyperphagic and developed morbid obesity by three months of age. Treatment of one of the leptin deficient children with subcutaneous injection of leptin has led to a decrease in appetite and a marked reduction in fat mass (Farooqi et al., 1999). These case histories suggest that leptin may have a major role in feeding behaviour and the regulation of body weight.

There is however little clear evidence to date that leptin levels in the normal range affect food intake either in adults or infants. Leptin levels are related to fat mass and the body mass index of normal-weight (Blum et al., 1997; Saad et al., 1997) and obese

individuals (Considine et al., 1996). However, there is a great degree of variability in leptin levels of adults of similar body size (Wiegle et al., 1997). Additionally, obese individuals have high circulating leptin levels that appear to be ineffective in suppressing their appetite (Considine et al., 1996), suggesting that an individual can become resistant to the biological effects of this hormone.

One way to investigate how leptin levels in the normal range influence appetite and weight regulation would be to examine the relationship between cord blood leptin and feeding and weight gain in infants. Cord blood leptin levels are very variable and the leptin is exogenous in origin, so administration of leptin would not be required (and would certainly not be acceptable to an ethics committee). Newborn infants would not be leptin resistant (except in the extremely rare cases in which this is due to a genetic mutation leading to a truncated leptin receptor). Energy intake can be measured very accurately in bottle fed newborn infants, as shown by Fomon in his classic studies (Fomon et al., 1975; Fomon et al., 1964; Fomon et al., 1976; Fomon, Thomas, Filer, Ziegler, & Leonard, 1971).

Several observations from animal studies suggest that the newly identified hormone ghrelin is also involved in energy balance. In rats, both intracerebroventricular and intra-peritoneal injection of ghrelin stimulated food intake (Wren et al., 2000). Mice injected with subcutaneous ghrelin for a period of two weeks showed a significant increase in body weight (Tschop, Smiley, & Heiman, 2000). Both these studies suggest that ghrelin has the capacity to stimulate appetite and regulate body weight.

There is also convincing evidence from human studies that ghrelin influences appetite. Normal healthy adults administered intravenous injection of ghrelin reported increased subjective hunger ratings on a visual analog scale (Arvat et al., 2001). Infusion of ghrelin into a group of normal individuals resulted in an increase of hunger ratings by 45% and an increase in energy consumption by 28% (Wren et al., 2001). Both these studies show that when plasma levels of ghrelin are artificially increased by exogenous administration of ghrelin, appetite is stimulated.

More recently evidence has been gathered from children and young adults with the rare genetic abnormality of Prader-Willi syndrome. These individuals phenotypically

present with hyperphagia and gross obesity as part of their syndrome. The cause of the hyperphagia has not yet been identified, but these individuals have recently been found to have fasting serum ghrelin levels three to four times higher than healthy controls (Delparigi et al., 2002; Haqq et al., 2003). These findings suggest that ghrelin may be the factor stimulating the hyperphagia observed in these individuals. Ghrelin can be studied in infants in the same way as leptin.

A longitudinal observational study was therefore planned to examine the relationship between leptin and ghrelin in cord blood, and milk intake and growth in infancy. The two main outcome measure of this study were milk intake and weight gain.

All observational studies also need to control for relevant covariates that could possibly affect the outcome measures. The first covariate measured was insulin. The role of insulin in feeding is controversial. Rodent studies have shown that intravenous and subcutaneous administration of insulin induced glucoprivic feeding (Orosco et al., 1994), but this effect can be reversed if euglycaemia is upheld. Conversely, intracerebroventricular infusion of insulin or 24 hour intravenous infusion of insulin resulted in a reduction in food intake and a loss in body weight (Porte & Woods, 1981). These findings leave some controversy as to its role in appetite stimulation in rodents. There is also conflicting evidence of the role of insulin in human studies. Insulin deficient individuals are hyperglycaemic and are hyperphagic (Schwartz et al., 2000; Woods et al., 2000), while experimental manipulation of insulin levels to create hyperinsulinaemia in normal adults also resulted in hyperphagia (Rodin et al., 1985). In newborn infants, insulin levels rise following feeding with a concomitant rise in growth hormone (Ogilvy-Stuart et al., 1998), suggesting a role in both feeding and growth. As insulin has been linked to feeding and to both leptin and ghrelin, it seemed prudent to measure insulin as a control variable. Therefore, insulin was also measured in cord blood.

The other covariates measured were related to maternal analgesia and anaesthesia in labour and length of labour; both could potentially affect milk intake in the early neonatal period. Maternal analgesia and anaesthesia has been shown to affect breast seeking and breast-feeding behaviors in the first hour after birth (Ransjo-Arvidson et al., 2001). Therefore maternal analgesia and anaesthesia was documented from the

maternal delivery notes and was related to the milk intake of the infant. The length of labour could potentially affect milk intake in the newborn if prolonged or difficult, so this was also documented from maternal delivery notes, and Apgar scores were documented from the maternal delivery notes. Apgar scores are an indication of fetal condition at birth (Apgar, 1953; B. M. Casey et al., 2001); scores greater than 7 at five minutes are indication of an excellent condition at birth. Only infants with Apgar scores of 7 or above were recruited into the study.

1.7.2 Research aim, procedures and hypotheses

The aim of this study, then, was to examine the role of the cord peptides leptin and ghrelin and milk intake over the first week of life and infant growth over the first twelve weeks of life.

The main hypotheses tested in the study were:

I. Infants with higher cord leptin levels will have a lower milk intake than infants with lower cord leptin levels.

II. Infants with higher cord levels of ghrelin will initiate feeding sooner and have a higher milk intake than infants with lower ghrelin levels.

III. Infants with higher cord leptin levels will gain less weight over the first twelve weeks of life than infants with lower cord leptin levels.

IV. Infants with higher cord levels of ghrelin will gain more weight over the first twelve weeks of life than infants with lower cord ghrelin levels.

The procedures used to achieve these aims were:

a) Accurate documentation of feeding behaviour by measuring the amount of milk consumed at each feed and the number of feeds over the first week of life.

b) Measurement of weight, length and head circumference at birth, seven days of age and twelve weeks of age.

c) Measurement of leptin and ghrelin in cord blood using radioimmunoassay.

d) Measurement and control for relevant covariates.

Chapter 2

Methods

2.1 Design

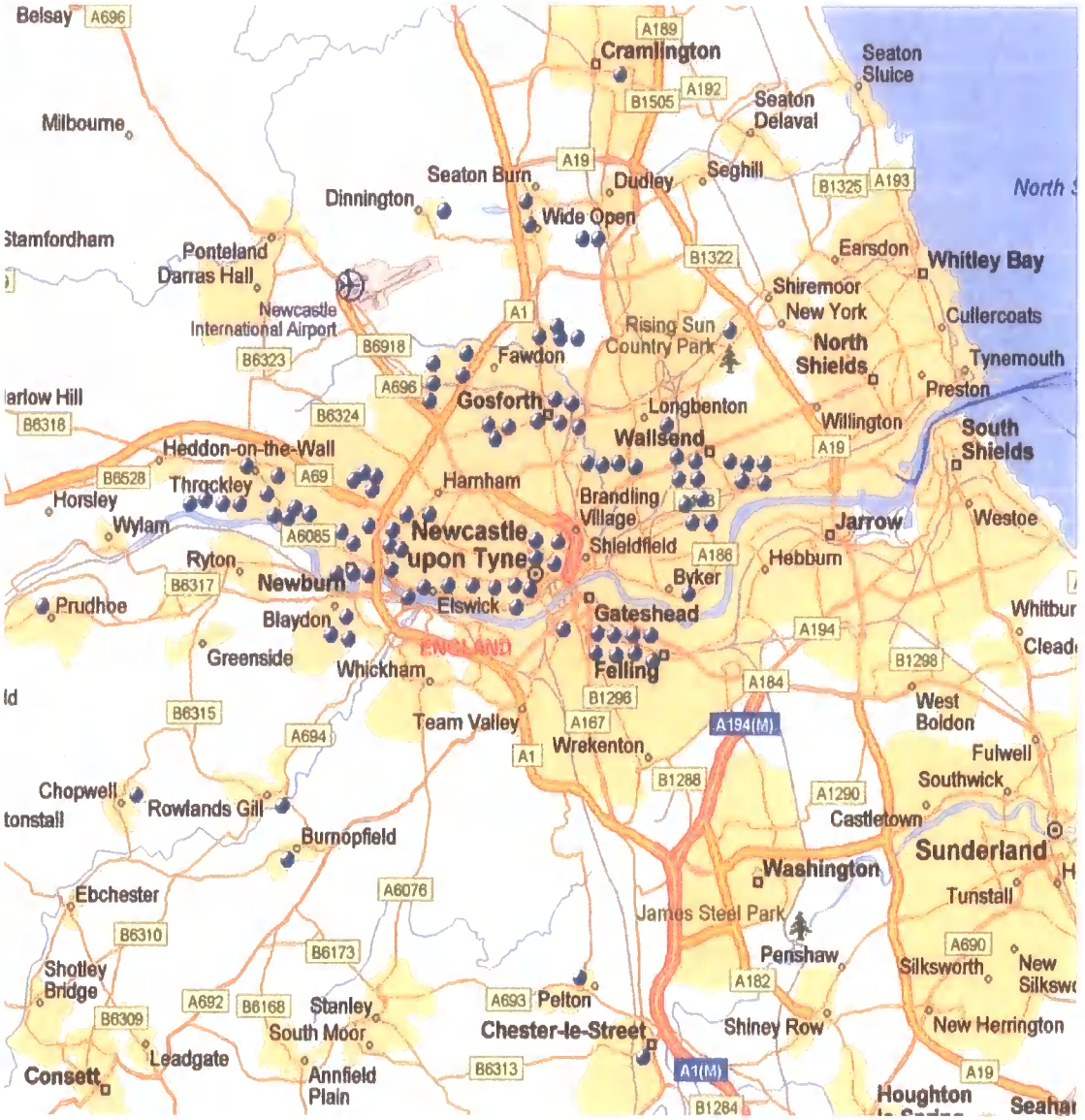
2.1.1 General introduction

Testing the hypotheses outlined at the end of the last chapter required the recruitment of mothers before the birth so that a sample of cord blood could be taken, and then a follow up of the infants to 12 weeks of age to measure growth. This called for a longitudinal study of three months duration. It was not clear at the outset how easy it would be to recruit mothers into the study, and power calculations (presented later) suggested that a sample of 100 women would be required. The decision was therefore taken to base the thesis on a single study so that the numbers required provided a clear test of the hypotheses.

2.1.2 Participants

Recruitment took place between October 2000 and December 2001 on the delivery suite at the Royal Victoria Infirmary, Newcastle-upon-Tyne. During this time period 5805 live births were recorded (personal communication from Information Manager). Figure 2.1 shows a map of the area of recruitment, with each participants home highlighted by a blue dot. One hundred pregnant women between 37 and 42 weeks gestation, carrying a singleton fetus were recruited on the delivery suite for participation in this study. The women were aged between 17 and 43 years of age. The women fulfilled the selection criteria of expecting a term baby (37 to 42 weeks gestation) from a normal pregnancy and with the intention to bottle-feed. The women that were excluded from participation in the study were any women that had diabetes mellitus (gestational or insulin dependent), or hypertension (familial, essential or pregnancy induced) or any other serious medical condition requiring drug treatment, either prior to pregnancy or during pregnancy. The infants were of both sexes and the only infants excluded were those with known congenital abnormalities (physical or metabolic) and those with a weight at birth less than 2500g. Low birth weight infants (<2500g) were a necessary exclusion from the study as their spontaneous feeding behaviour (waking to feed, regulation of meal size and discontinuation of feeding when satiated) are over ruled by interventional procedures. These infants are made to follow a strict feeding regime, they are woken every three hours to feed and if they do not consume the calculated amount of milk expected for their weight they are given the milk via naso-gastric tube.

Figure 2.1 Map showing area of recruitment, with 100 participant's homes highlighted by blue dots



2.1.3 Study plan

The study plan is outlined in Table 2.1; this shows the progression from the first meeting with the participant to the conclusion of the participant's involvement.

Table 2.1	Study plan
Day 1	Before birth Approach to woman in labour by N.H.S midwife/information leaflet Verbal explanation of study to woman and partner by researcher Time for couple to discuss study If woman wishes to participate, consent form signed After delivery Sample of cord blood taken Documentation from delivery notes of analgesia and anaesthesia administered to mother in labour, length of labour and Apgar scores and maternal smoking Birth weight, length and head circumference of infant measured Explanation of use of weighed bottles of infant formula Transfer of infant and mother from delivery suite to postnatal ward
Days 1-7	Mother feeds infant from weighed bottles of infant formula Mother records time of feed, feed number and amount of feed given to infant at each feed on feed chart
Day 7	Home visit Weight, length and head circumference of infant recorded Maternal and paternal height measured Collection of milk bottles
12 week visit	Home visit Weight, length and head circumference of infant recorded Information on whether the infant had commenced solids by this visit was collected

The two main outcome variables of the study were the infants' milk intake over the first seven days of life and infant growth measured by weight, length and head circumference at birth, at seven days and at three months of age. The main predictor variables were leptin and ghrelin measured in cord blood. Cord blood insulin, maternal

smoking and parental height were measured as covariates as they have previously been related to size of the infant at birth. Several control variables were recorded in view of their potential influence upon feeding behaviour (as discussed in Chapter One). These included the length of labour, the infants Apgar score at delivery, maternal anaesthesia and analgesia during labour.

2.1.4 Approval for study

The study was conducted on the delivery suite at the Royal Victoria Infirmary, Newcastle-upon-Tyne. This unit has approximately five thousand deliveries per annum and covers a substantial area of Newcastle-upon-Tyne. Before submission of the research proposal to the local ethical committee, the study was described to the Head of the Obstetric Unit, the Head of the Neonatal Unit, the Delivery Suite Manager and the Senior Midwife for the Community and Antenatal Clinic. They gave permission to access their patients, subject to ethical approval. The Senior Midwife for the Community and Antenatal Clinic would not allow access to the women during pregnancy in case it influenced their choice of feeding towards bottle-feeding (as opposed to breast-feeding) and thought the supply of free milk might be seen as an inducement to participate. However she agreed that the mother would have made her mind up by the time she came in for delivery of her infant, so suggested the women were approached in labour.

Several procedures have to be followed to conduct research on human participants within a hospital environment. Both trust approval and ethical approval must be sought. Trust approval was obtained from the Chief Executive of The Newcastle-upon-Tyne Hospitals NHS Trust to allow the study to take place on trust premises. Ethical approval was obtained from the Joint Ethics Committee of Newcastle and North Tyneside Health Authority, University of Newcastle-upon-Tyne and University of Northumbria at Newcastle. The experimental protocol for this study included recruitment of women in labour, for the reasons noted above. This is not usually permitted, as it requires the obtaining of informed consent when the women may be stressed and under the influence of drugs. This issue was addressed in the application by submitting a covering letter explaining the reasons why the women had to be approached in labour and what measures would be taken by the researcher to safeguard the free consent of the women. All the participants were approached on the delivery

suite whilst they were in labour, but initially by their attending N.H.S. midwife, who briefly explained the study and gave them an information leaflet to read. If they were interested in participation in the study, the researcher explained the study to them and obtained their informed consent to participate. They were assured that at any point they could withdraw from the study. The researcher is a midwife herself and was used to the sensitivity of the situation.

The Award of Clinical Access and Observer Status was also necessary from The Newcastle-upon-Tyne Hospitals NHS Trust. This allowed the researcher access to the infants under the supervision of her clinical supervisor. It requires the researcher to follow the rules and regulations, policies and procedures of the department she is attending. The essential requirements in this case were registration with United Kingdom Central Council for Nursing and Midwifery, medical clearance via the occupational health department and police clearance for security purposes. These were all obtained before commencement of the study.

Prior to commencement of the study several thirty-minute oral presentations of the study protocol were made to as many of the health professionals as were available on the obstetric unit and in the community. Different times were chosen so as to access both day and night staff. This enabled the researcher to explain the reasons for the study, justify the reasons why women would be recruited in labour, make clear why women that intended to bottle-feed were being chosen and emphasize that the study was not promoting bottle-feeding over breast-feeding. The researcher recognized that these would be sensitive issues and subject to some controversy. Via a senior midwife on the delivery suite and the senior midwives on the wards all staff received a printed handout explaining the study, to ensure that they were fully informed of the research protocol (Appendix A).

2.1.5 Power calculations

No previous studies have examined the relationship between cord blood leptin, ghrelin and milk intake, however Ong et al. (1999) demonstrated an inverse correlation of –0.33 between cord blood leptin levels and change in infant weight between birth and four months. A sample size was calculated to allow replication of these results relating leptin to weight gain. To ascertain the required sample size, power was calculated in

respect of the product-moment correlation coefficient used to examine an association between two principle variables in the study, weight gain and cord blood leptin levels. The procedure used is outlined in (Kraemer & Thiemann, 1987). It assumed that the scores came from a bivariate normal distribution but did not make any assumptions about the equality of variance. The power calculation conducted assumed a true ρ of 0.33 and is shown in Table 2.2. One hundred infants would give a power in excess of 90% at the 5% level, 2-tailed, to detect a true correlation of 0.33.

Table 2.2 Power calculations as described in Kraemer & Thiemann (1987)

Δ is the critical effect size

ρ denotes the true product-moment correlation coefficient

ρ_o denotes a null hypothesis of no correlation

$\Delta = (\rho - \rho_o) / (1 - \rho\rho_o)$

$\Delta = (-0.33 - 0) / (1-(- 0.33) (0)) = -0.33$

$\Delta = - 0.33$

Number of subjects required for a two-tailed test at the 5% level with 90% power, where v is tabled against Δ (Kraemer & Thiemann, 1987, page 110)

$N = v + 2$

$N = 91 + 2$

$N = 93$

This shows that 100 subjects would give a power in excess of 90%.

This sample size would also provide a power of 90% at the 5% level, two tailed, for a correlation of 0.33 between leptin and milk intake and ghrelin and milk intake.

2.2 Apparatus and materials

Several measuring devices were required for this study. The bottles of milk were weighed prior to and after feeding on a portable Ohaus strain gauge electronic balance (Model CT1200-S, accurate to 0.1 g). The infants were weighed on electronic weighing scales (Seca, Model 727). This model has a switch that can be set to 2 or 5g resolution, which means that the measured weight is in graduations of 2 or 5 g. The scale used on the home visits was set to 2g resolution, and was accurate to 0.02% on a

weight range from 0 to 10 kg. The scales used on the delivery suite in the hospital were set to 5g resolution, and were accurate to 0.05% on a weight range from 0 to 10 kg.

The Seca scale and all of the scales on the delivery suite were calibrated at 1000g, 2000g, 3000g, 4000g and 5000g using M 3 calibration weights (appendix B), on four separate occasions during the study. The results of the calibration are presented in Chapter 4. The length of the infant was measured in cm (accurate to 0.1cm) using a Kiddimetre (Raven Equipment Limited). The head circumference of the infant was measured in cm (accurate to 0.1cm) using a paediatric tape measure (Perspectives Enterprises). Placental weight was measured by the midwife on the balance on the delivery suite (Model Salter Scale). Inspection of the records collected for placental weight showed that weights were routinely recorded accurate to 50g. Parental height was measured in cm using the Leicester Height Measure (Child Growth Foundation), accurate to 0.1cm.

The blood bottles required for cord blood sampling were prepared in the treatment room on the Programmed Investigation Unit at the Royal Victoria Infirmary. Using a 1ml syringe and needle, 0.2 ml of Trasylol was drawn up and placed into each lithium heparin tube. Trasylol is a protease inhibitor, which protects against protease activity when collecting plasma for the assay of delicate peptides. These were labelled with the preparation and expiration date and placed in the refrigerator until use. Batches of 15 were made up at a time to ensure they did not exceed the expiry date for the Trasylol. The cord blood was taken at the time of delivery of the baby using a needle and 10 ml syringe either by the midwife delivering the infant or by the researcher. The operator wore rubber gloves and a plastic apron for protection. The blood was inserted into the pre-prepared bottles and transported in a container on ice to the Programmed Investigation Unit. The blood was spun using a centrifuge at 4°C for 10 minutes and the plasma supernatant decanted using a pipette and inserted into separate labelled aliquots. The specimens were frozen in labelled sample boxes in two freezers and stored at -40° on the Programmed Investigation Unit and -70° in the Department of Child Health until assayed for leptin, ghrelin and insulin levels. Samples were stored in two separate freezers for safety, in case one of the freezers broke down.

Information leaflets were given to the subjects prior to the start of the study. These explained the study design and what to do if they required any further information.

Following verbal explanation of the study, an informed consent form was signed. During the course of the study several data collection sheets were used to record the data and other measurements and are referred to as and when they were used in the procedure.

Prior to commencement of the study, the maternity unit at the Royal Victoria Infirmary was contacted to find out which formula milks were most popular. The two that were requested the most were SMA gold and Cow and Gate premium. The two companies SMA Nutrition and Cow and Gate were approached by letter and volunteered to supply the formula milk free of charge in ready to feed bottles with sterile individually wrapped teats. If the mother had decided on a brand of formula milk other than the two that were available, they were not recruited into the study. The compositions of the two milks are presented in Table 2.3.

Table 2.3 Nutritional information of the two milks used in the study

Nutritional Information	SMA Gold Per 100ml bottle	Cow & Gate Premium Per 100 ml bottle
Energy	280kj (67 kcal)	280kj (67 kcal)
Protein	1.5 g	1.4 g
Carbohydrate	7.2 g	7.5 g
Fat	3.6 g	3.5 g
Sodium	16 mg	19 mg

Both milks also have the following minor ingredients: vitamins A, D, E, C, B_6, B_{12}, vitamin K, folic acid, biotin, thiamin, riboflavin, niacin, pantothenic acid, choline, taurine, calcium, phosphorous, iron, magnesium, zinc, iodine, chloride, copper, potassium, selenium.

Initially the mothers were given 48 bottles of milk for the seven days of feeding. This number was estimated from the feeding requirements of 5-6 feeds a day proposed by Fewtrell & Lucas (Rennie & Roberton, 1999). However within the first few weeks of the study it became apparent that some infants required more frequent feeding and this number was increased to 56 bottles based on an average of 8 feeds a day. The bottles of milk for each woman were prepared by labelling each one individually with a label indicating a number from 1-56, and a message indicating that the bottle was part of an

infant feeding study and was not to be discarded. Figure 2.2a shows a photograph of the individual labelled bottles of milk. This was to overcome any possible confusion within the hospital. The individual bottles of milk were placed back in their original cardboard boxes. Bottles 1-8 were immediately available at the top of the storage container, bottles 8-32 were in box 1 and bottles 33-56 were placed in box 2. Both boxes were labelled to make things as straight forward as possible for the mother. Each box was labelled with a reminder for the parent and the hospital staff to use each bottle of feed in numerical order, and not to discard any feeds. Sterile packed individual teats were provided for each bottle supplied. All the milk and teats were kept in a plastic storage container. Figure 2.2b shows a photograph of the milk package prepared for each mother. A plastic wallet containing the instruction sheet, contact telephone numbers, feed charts, letter to show health professionals and a pen for documentation of feeds were also placed in the storage container. Plastic items were chosen so they could be easily cleaned between participants.

Figure 2.2a Example of individual labelled bottle

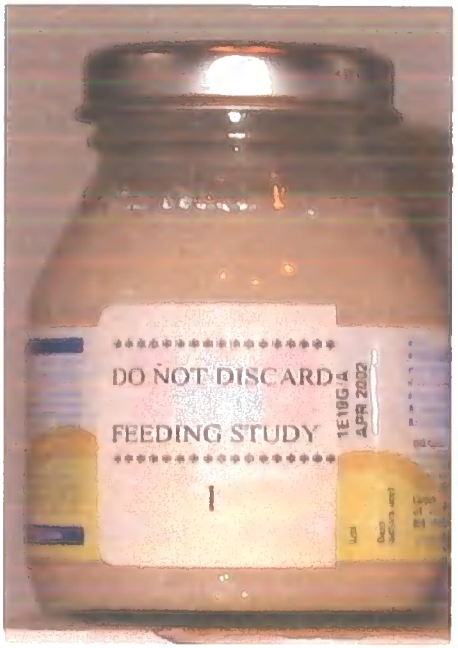


Figure 2.2b Labelled milk package prepared for each mother

2.3 Procedures

2.3.1 Training in the measurement of growth

Prior to commencement of the study the researcher attended several child health clinics at the Royal Victoria Infirmary and was taught how to measure infant length. The technique was taught by a trained auxologist who then supervised the researcher over several clinics.

Finally a small study was conducted to test the researcher's measuring reliability. The length of the infant was measured on a 'Kiddimetre' measuring device. The infant was placed naked in a supine position upon the mattress and the infant's head was positioned between the two head restraints with the head touching the top of the board. The mother was asked to hold the infant's head firmly in place and to keep the infant straight and flat on the mattress, whilst the researcher straightened out the infant's legs and slid the bottom plate against the soles of the infant's feet. A measurement was taken in centimetres accurate to 0.1cm. This procedure was repeated three times. Each time the infant was placed on the 'Kiddimetre' from an upright position and the measurements recorded. Eleven infants participated. The results are presented in Chapter Four.

2.3.2 Study procedure

Recruitment was carried out in accordance with 'Midwifery Guidelines' as approved by the Director of Midwifery Services. These were drawn up acknowledging the possibility of a woman's feelings of vulnerability and obligation to participate if the researcher directly approached them. A neutral intermediate was therefore used to ensure that subjects participated in the study of their own free will and without obligation. Recruitment took place on the delivery suite at the Royal Victoria Infirmary, Newcastle-Upon-Tyne. The researcher attended the delivery suite and asked the midwives if any of the women fulfilled the study's entry criteria. If this was the case and after the attending midwife had ascertained that the woman was in a controlled, calm state, an information leaflet was given to the woman to read (Appendix C). If the woman was interested in the study, the researcher then approached and explained the study in more detail. If the woman wished to participate,

she was asked to sign a consent form (Appendix D). Only women that delivered between 7am and 10pm were included in the study, as strict follow up of the infants was required at 168 hours, and nighttime visits would have been inconvenient to the families. Once the woman had started to participate in the study, a letter was sent to her Community General Practitioner informing them of the study design and patient's participation (Appendix E). As some women have very early discharges from hospital, a similar letter was sent home with the women to explain the study to the other visiting health professionals (Appendix F).

The sample of cord blood was taken following delivery of the baby and prior to delivery of the placenta. The cord was double clamped with forceps and cut. Ten ml of cord blood was taken either by the attending midwife or the researcher from the clamped portion of the cord at the placental end and placed in the prepared blood bottles. These were then processed as outlined in the previous section. Some women had already agreed to participate in another study on delivery suite that was collecting cord blood for stem cells. This did not interfere with recruitment, but did cause a loss of two women to the study as the midwife accidentally drained the cord of blood before taking the required sample.

Immediately following delivery the baby was weighed on Seca scales (model 727). The scale was zeroed with a protective paper towel in place and the naked baby was placed upon it. The reading was checked and recorded in kilograms. Within a few hours of birth the length of the baby was measured on a 'Kiddimetre' measuring device following the procedure outlined in section 2.3.1. The head circumference was measured in centimetres at the largest occipito-frontal diameter of the baby's head while the baby was in a supine position. The tape was placed behind the baby's head at the occipital protruberance and then brought around the head at the level of the supraorbital ridges. The study design incorporated one examiner to conduct all the measurements, with the exception of the length measurement that required a second person to hold the infants head. The second person was usually either the infants mother or father. A length measuring device called a Kiddimetre was used. This was to reduce the previously reported practical problems related to measurement described in chapter one. The Kiddimetre can be used independently of cots and has a mattress that is easily cleaned.

Other information was taken from the booking records and delivery notes. This was: length of the first and second stages of labour, type of delivery, time of birth, Apgar scores and analgesia or anaesthesia that was administered during the labour, family history of diabetes, parity and whether the mother smoked. Labour is the process by which the infant is expelled from the uterus (Beischer & Mackay, 1986). The first stage of labour is characterized by contractions of the uterus which causes dilation and effacement of the cervix. The second stage of labour is the process of expelling the fetus from the birth canal. The type of delivery can be a spontaneous vertex or breech delivery, an assisted delivery by forceps or vacuum extraction or by caesarian section either electively or as an emergency. It is important to note that some women do not complete the first stage of labour if they have a caesarian section. All this information was recorded on data collection sheets (Appendices G to I). For the purpose of this study, any neonates that had Apgar scores at five minutes of less than 7 were excluded on the basis that their physical condition could affect their feeding behaviour. The analgesia and anaesthesia given in labour, Apgar scores and length of labour were used as control variables in examining the relationship between leptin, ghrelin and feeding behaviour.

Milk (of the mother's choice) in ready to feed bottles was provided, labelled and weighed as described in the previous section. These weights were recorded on a data collection sheet (Appendix J). On the delivery suite the researcher explained the procedure that the mother needed to follow for the subsequent seven days. No specific instructions were given to the mothers about how much to feed their infants. General instructions are found on tins of formula powdered milk to prepare 90 mls of feed for an infant weighing 3.5 kg between birth and two weeks of age. Midwives are advised to instruct the mother to feed the infant as much and as often as the infant wants. Therefore the infants were fed to demand using 100 ml bottles of ready-made formula milk. An instruction leaflet for the procedure was also given to the mother to keep (Appendix K), and a contact telephone number should she require any further advice. The mother was asked to feed the baby with the milk, starting with the bottle labelled number one for the first feed and following the numbers in order until the seventh day. On completion of the feed the mother was asked to record on the feed chart provided (Appendix L), the number and time of the feed, the amount taken and whether the baby was sick or if any of the milk was accidentally split. She was then asked to replace the lid on the bottle with the remainder of the milk that was left in it and place it in the

container provided. The mothers and fathers (if available) were given these instructions), however information was not collected on who actually fed the infant during the course of the study. The researcher always waited to transfer the mother and infant pair from the delivery suite to the postnatal ward, so she could inform the ward staff that the infant was part of an infant feeding study and to avoid any extra inconvenience to the midwife. Once the infant was taken to the ward a sign was placed in the cot to indicate that the infant was participating in an infant feeding study with a reminder to use the formula milk provided, to feed the bottles in numeric order, to keep all bottles and with a note of thanks. This was to alert members of staff to the infant's participation in the study should the infant be taken to the nursery over night. Before leaving the postnatal ward the researcher checked that the mother was happy that she knew what procedure she had to follow. A date and time was arranged for the seven-day visit and documented on the instruction sheet for the mother. The time of the seven-day visit was scheduled as near to the time of birth as possible (usually within two hours). The following day, if the mother was still on the ward, the researcher called to ensure everything was running smoothly.

On the seventh day the researcher weighed all the bottles that had been used, following the same procedure as above. The weights were recorded on the data collection sheet 5 (Appendix M). The difference between the pre-feed and post-feed weights of the bottles is a measure of the amount of milk consumed by the baby. This procedure is known as 'direct measurement' and was the method chosen to assess milk intake in this study. The other methods of assessing milk intake 'test-weighing' and the 'doubly labelled water method' were considered inappropriate for the study. Test weighing was considered to be unsuitable for two reasons: provision of electronic scales for several mothers at a time would have been prohibitively expensive and some infants consume very small amounts of milk in the early neonatal period that could be within the margin of error of test weighing. The doubly labelled water method was excluded because the isotopes are expensive making it difficult to conduct large studies and the procedure may influence the mothers' willingness to participate. All unused milk was discarded and the bottles washed and disposed of at a recycling center.

At seven days and at twelve weeks of age, the baby was weighed and its length and head circumference recorded following the procedures outlined above. The twelve-week visit was arranged by telephone at a time and date convenient to the mother. It

was stressed that it was important to try and get it as near to the twelve weeks as possible. Those mothers that did not have a telephone were contacted by letter with a date and a time and were asked to contact the researcher. The same researcher made all the auxological measurements. On the twelve week visit the mother was asked whether the baby had commenced weaning or not and this information was documented.

Maternal and paternal height was measured in cm using 'The Leicester Height Measure' (see appendix N for details on accurate measurement) either on the seven-day visit or the twelve-week visit. The parents were asked to remove their footwear and stand on the measuring device. If the father was unavailable, the mothers were asked to estimate the father's height from their own height standing by the Leicester Height Measure. This completed the study.

To comply with health and safety regulations and to protect the infants, all equipment was cleaned with an anti-bacterial cleansing agent between subjects. The researcher followed hospital policy of strict hand washing between infants. To comply with the data protection act, all data were stored in a locked filing cabinet, within a locked room. Computerized data were coded to maintain anonymity.

2.3.3 Assay Procedures

Leptin and insulin assays were conducted in The Diabetes laboratory in the Department of Diabetes and Metabolism. The leptin assays were carried out using a commercial leptin radioimmunoassay (Linco, St.Charles, MO, USA). Intra-assay and inter-assay coefficients were 8.3% and 6.2% at 4.9 ng/ml and 3.9% and 4.7% at 10.4 ng/ml. The intra-assay coefficient refers to the variation seen between samples within an assay, and the inter-assay coefficient refers to the variation seen between samples put through different assays. Insulin assays were performed using the DAKO insulin assay (DAKO, Denmark). The limit of sensitivity was 0.5 μIU/ml, the intra-assay coefficient was <5% and the inter-assay coefficients < 5.3%. Ghrelin was measured with a commercial radioimmunoassay (Phoenix Pharmaceuticals, Belmont, CA, USA) at the Diagnostic Systems Laboratories, UK Ltd. The limit of sensitivity was 0.14 ng/ml, intra-assay coefficient 3.4% at 1.14 ng/ml, inter-assay coefficient <14%. Because the assays were all carried out at the end of the study, all other measurements were recorded blind to the blood hormone levels.

There are four basic requirements for a radioimmunoassay system, these are a specific antiserum to the antigen being measured, the availability of a radioactive labelled form of the antigen, a method whereby antibody-bound tracer can be separated from the unbound tracer and an instrument to count radioactivity. In the leptin radioimmunoassay (RIA), a fixed concentration of labelled tracer leptin (^{125}I-Human Leptin) is incubated with a constant dilution of antiserum such that the concentration of leptin binding sites on the antibody is limited. For example, only 50% of the total tracer concentration may be bound by antibody. If unlabelled leptin is added to this system, there is competition between labelled tracer and unlabelled leptin for the limited and constant number of binding sites on the antibody. Thus, the amount of tracer bound to antibody will decrease as the concentration of unlabelled leptin increases. This can be measured after separating antibody-bound from free tracer and counting one or the other, or both fractions. A calibration or standard curve is set up with increasing concentrations of standard unlabelled leptin and from this curve the amount of leptin (antigen) in unknown samples can be calculated. The standards used were purified recombinant human leptin (Ma et al., 1996).

Total plasma immunoreactive ghrelin levels were measured by the same methodology in duplicate with a RIA that uses ^{125}I-labelled bioactive ghrelin as a tracer and a rabbit polyclonal antibody raised against full length octanoylated human ghrelin (Tschop et al., 2001).

The assay for insulin is an ELISA (enzyme linked immunosorbant assay) based on two monoclonal antibodies, which measures biologically active insulin with a high degree of specificity. Simultaneous incubation of sample and enzyme labelled antibody in a microplate well coated with a specific anti-insulin antibody forms a complex. A washing step removes unbound enzyme labelled antibody. The remaining bound conjugate is detected by reaction with the substrate 3,3',5,5'-tetramethylbenzidine. The reaction is stopped by adding acid to give a colorimetric endpoint, which is read spectrophotometrically. The inclusion of known insulin concentration in the assay allows a calibration curve to be constructed from which the level of insulin in patient samples can be calculated (Andersen, Dinesen, Jorgensen, Poulsen, & Roder, 1993).

Chapter 3 Results

Infant milk intake and feeding frequency over the first week of life

3.1 Study sample

3.1.1 Study group

One hundred and twenty one women were recruited into the study. Of these 21 were subsequently lost from the study, 16 on the delivery suite and five from the postnatal wards and home.

The 16 that were lost on delivery suite were for the following reasons:

a) Three infants were taken to the special care baby unit following delivery.
b) Two infants were less than 2500g at delivery.
c) Three infants were excluded as it was not possible to get a cord blood sample.
d) Two infants were excluded as the midwife accidentally drained the cord of blood for another study that was in progress before taking the required sample.
e) One infant was excluded because the midwife accidentally put the blood in the wrong blood bottle.
f) Four women were excluded because their delivery time was in the middle of the night.
g) One woman changed her mind after delivery of her infant and decided not to participate in the study.

The five infants that were lost in the early postnatal period were for the following reasons:

a) One infant was taken to the special care baby unit, as it was unable to maintain its body temperature.
b) Two women decided to change the milk to a different brand between recruitment and day seven of the study and so did not use the measured milk.
c) Two women declined access to their homes on the seventh day.

3.1.2 *Maternal characteristics*

One hundred women with a mean age (SD) of 26.2 (6.0) years (median 25, minimum age 17 years, maximum age 43 years) carrying a singleton pregnancy between 37 and 42 weeks gestation completed the study. Of these mothers 44% were primiparous and 56% were multiparous, 45% smoked and 55% did not smoke, 63% had no family history of diabetes, 30% had a family history of non-insulin dependent diabetes and 7% a family history of insulin-dependent diabetes.

3.1.2 Infant characteristics

One hundred infants took part in the study from birth through to 12 weeks of age; of these 50% were male and 50% were female. All infants had Apgar scores of eight or above at five minutes. The infants were delivered by the following means: 70% by spontaneous vertex deliveries, 25% instrumentally (forceps or vacuum extraction) and 5% by caesarian section.

3.2 Milk intake, feeding frequency and birthweight

3.2.1 Principle variables used

Feeding was defined in this study by several variables:

a) 'Time to first feed'. This was the time in minutes from the infant's time of birth to the time the infant commenced its first milk feed.

b) 'Milk intake at first feed'. This was the amount of milk consumed at the infant's first feed.

c) 'Number of feeds day 1'. This was the number of feeds consumed in the first 24 hours from the time of birth of the infant. This measure was obtained from the feed chart that was provided for the mother. She was asked to record the bottle number, the time and the amount of milk consumed by the infant for each separate feed. Once the bottle of formula milk had been opened, the manufacturer's instruction was to discard the milk within one hour of opening (Cow and Gate) or within four hours of opening (SMA). Mothers were advised in this study to discontinue using the bottle within one hour (for both formula preparations) and to use a new bottle of milk for each feed. Each feed was defined by setting a time limit of one hour from opening the bottle, so all milk consumed within that hour was classed as one feed.

If an infant consumed more than one bottle of milk at a feed the mothers documented this on the feed chart and the two bottles were treated as being one feed. 'Number of feeds day 2 to day 7' were calculated using the same method as number of feeds for day 1. Each twenty-four hour period was recorded as a day from the time of birth.

d) 'Milk intake day 1'. This was calculated by accurately weighing the bottles of milk before and after feeding. The difference obtained gave the amount of milk consumed at each feed. The total for the twenty-four hour period was calculated by adding the mass of each feed consumed in that time period. 'Milk intake day 2 to day 7' were calculated using the same method as for milk intake day 1.

The milk data were complete for 79 of the infants, with some missing data for 21. Table 3.1 shows the reasons for the missing milk data.

Table 3.1 Missing milk data for 21 infants

Reason	Number of infants	Infant number
Ran out of measured bottles of milk	9	Eight infants on day seven (infant number 1, 13, 19, 27, 41, 67*, 73, 95) One infant on day six (96)
Mother changed formula milk	2	One on day four (33) One on day six (35*)
Discarded bottles (26 bottles)	12	Each infant had one discarded bottle (10, 67*, 75, 79, 93) {five bottles} Each infant had two discarded bottles (24, 71, 80, 81){eight bottles} Each infant had four discarded bottles (35*, 42) {eight bottles} Infant 12 had five discarded bottles {five bottles}

* Infants 35 and 67 fell in two categories

Nine of the mothers ran out of the measured bottles of milk. Eight mothers ran out on the seventh day and one mother on the sixth day so data are missing for this mother for both day six and day seven. Two mothers changed to another type of formula milk so did not use the measured milk thereafter. One mother changed milk on the sixth day, so data are missing for day six and day seven. The other mother changed milk on the fourth day, so data are missing for the remaining days from four to seven. Twelve mothers accidentally discarded bottles of milk so they could not be weighed. The feeds that could not be measured because the mother ran out of the measured bottles of milk or changed formula were recorded as missing.

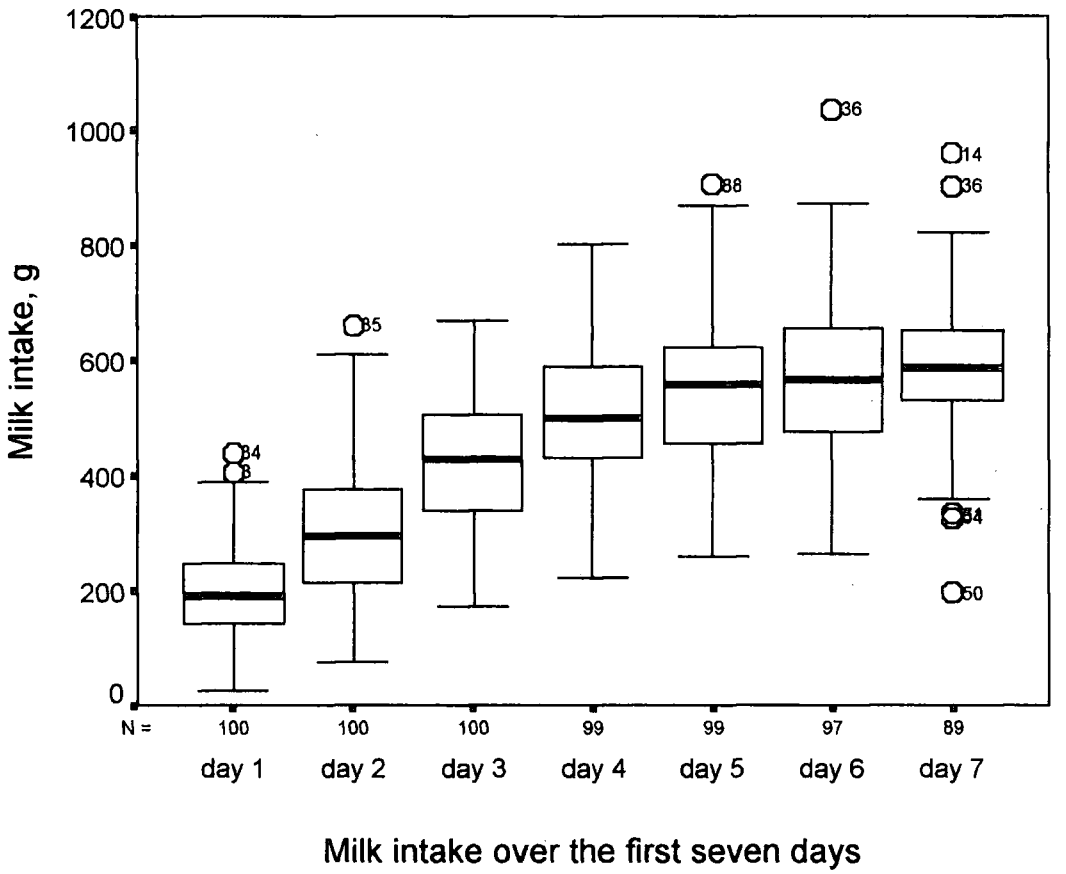
The missing data was handled in the following way. The number of feeds actually measured was 5,181. There were 26 bottles that could not be measured because the bottles had been discarded. The data for the 26 discarded bottles of milk were imputed using the following methods. Inspection of the infant feeding charts showed that even though the mother had discarded the bottles of milk, on 23 occasions the mothers had written down the amount of feed consumed by the infant at that feed. To ensure that the mothers were estimating the amount of milk consumed by the infant accurately, a check was made to see if the other values estimated by the mother were close to the actual measurements recorded by the researcher. The other records estimated by the mothers were very close to the measured records, so in these 23 cases the estimated record value was inserted. The data values for the other three remaining discarded bottles were calculated as follows. 1) The first value was calculated by interpolation from the available existing data. The data value for the feed consumed at the same time as the discarded feed on the day before was 40g. The data value for the feed consumed at the same time of day as the discarded feed on the day after was 50g. The mean of these two values was 45g, so this value was used in the data set. 2) In the case of the second discarded bottle, this bottle was discarded in the first twenty-four hour period following birth. In this case the value for the feed prior to the missing value was 23.1g and the value for the feed following the missing value was 52.6g. The mean of these two values was 37.85g, so this value was used in the data set. 3) In the last case grandma had converted the ml on the bottle to ounces for all feeds, so her record was in oz. The weighed values were available for 55 feeds in grams. The value of the discarded bottle was estimated in grams by a regression of the true values (weighed values) in grams on grandma's record of milk consumed in ounces for all other data points. The predicted value was 38.8g, so this value was used in the data set. The

inaccuracy introduced by these imputations for the 26 discarded bottles would be small, as the imputed values constituted only 0.5 percent of the measured values for the seven-day milk intake.

3.2.2 Milk Intake

Milk intake over the first seven days is shown in Figure 3.1.

Figure 3.1 Box plot showing the milk intake (g/24 hours) over the first seven days of life



The median milk intake increased each day up to day seven. All outliers identified on the boxplots were traced back to the individual records and checked. No errors were identified. The seven infants with high values did have documented higher milk intakes than the other infants, either in the amount taken at a feed, or in the number of feeds per day. The three infants with lower scores on day seven had not consumed as much milk on that day.

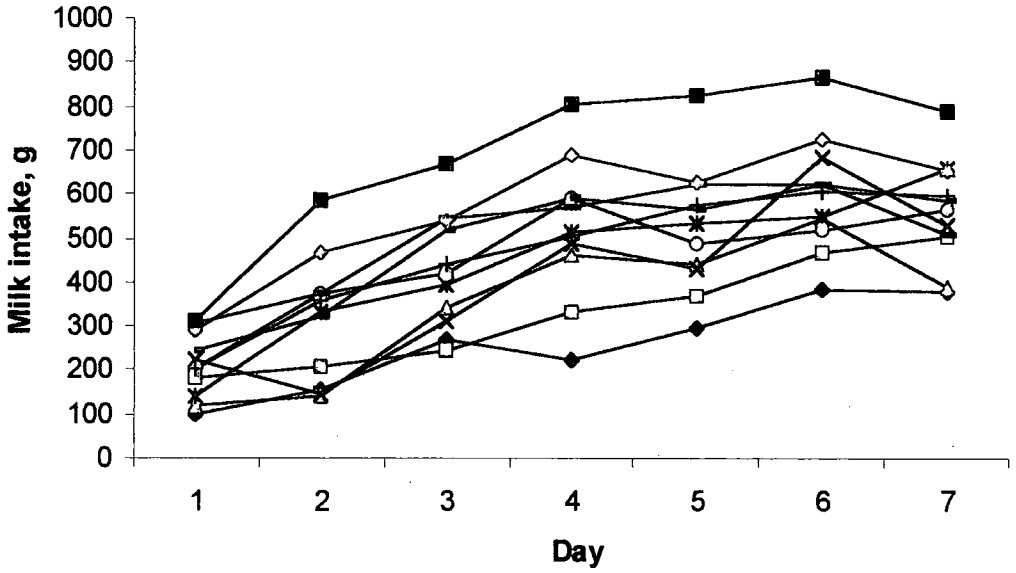
Table 3.2 shows the mean milk intake. The ranges and standard deviations are large, indicating the large variability that exists between infants.

Table 3.2 Descriptive statistics of milk intake from day one to day seven

	Milk intake (g)				
	N	Minimum	Maximum	Mean	S.D.
Day 1	100	25.8	440.1	196.7	83.0
Day 2	100	75.3	662.7	306.6	119.2
Day 3	100	171.0	668.3	423.0	115.5
Day 4	99	221.2	801.6	510.0	120.4
Day 5	99	259.7	907.0	554.5	128.9
Day 6	97	263.4	1037.8	577.3	138.0
Day 7	89	196.1	959.8	585.9	128.4

Plots of cross sectional data of the kind used in Figure 3.1 can be misleading, as a slow rise in the average could result either from slow rises in all individual intakes or from large rises in a smaller number on different days. To distinguish these, longitudinal plots were drawn of milk intakes over the first seven days of life. The milk intake data was ranked by the mean milk intake from the lowest to the highest milk intake and every tenth infant was plotted (Figure 3.2). This is to avoid a plot so dense that individual data cannot be distinguished (Diggle, Liang, & Zeger, 1994).

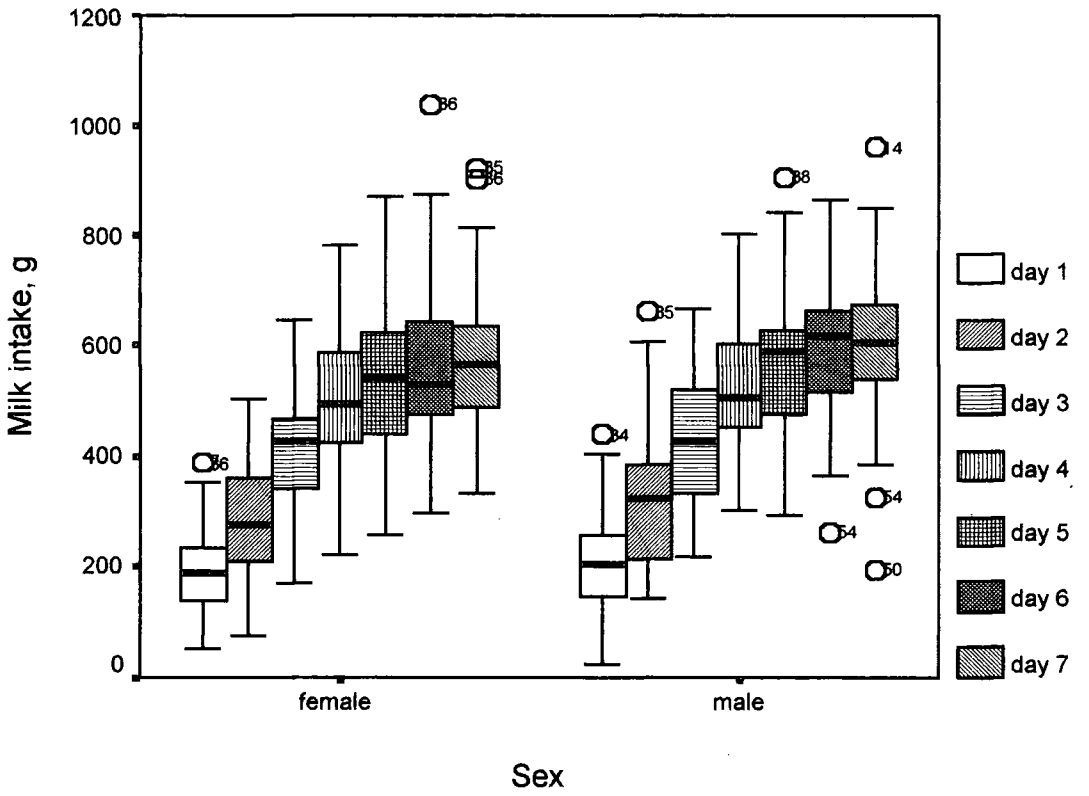
Figure 3.2 Milk intake for the infants with the lowest and highest milk intake and every tenth ranked infant in between



This graph illustrates the individuality and variability for each infant, but also shows similar trends over time. Milk intake rose from day one to four for all eleven infants in the graph, but the change became more variable between day five and seven, where the infants showed different patterns of milk intake, some rising, some dropping and some levelling out.

The study data were next examined for gender differences in the amount of milk consumed. Figure 3.3 plots the median and spread of milk intake for each day for male and female infants. Initial assessment of the graph suggests that the median milk intake is the same for both sexes in the first four days, but higher in males than females subsequently.

Figure 3.3 Box plot showing milk intake for the first seven days by sex



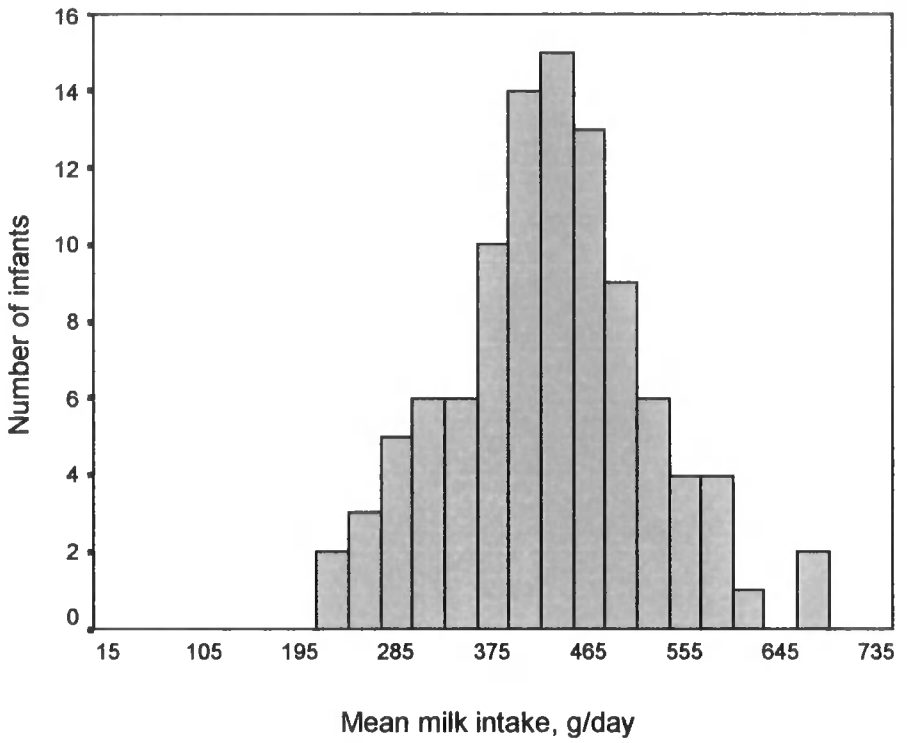
To ascertain whether this observed difference was statistically significant, the data were examined using an analysis of variance (MANOVA) for repeated measures with days as a within subjects factor and sex as a between subjects factor. Mauchly's test of sphericity gave a χ^2 value of 81.18 and a p value of <.001. Because the condition of sphericity was not met, the MANOVA was modified by using the Greenhouse Geisser F test. This is a more conservative test that reduces the degrees of freedom of the numerator and the denominator of the F ratio, which increases the value of F required for significance (Kinnear & Gray, 2001). Table 3.3 shows the results of the MANOVA. This shows that there was a significant effect of days on milk intake, but no effect of sex and no sex by days interaction.

Table 3.3 Multivariate analysis of variance, examining the effect of sex and days on daily milk intake

Source	Type III Sum of squares	df	Mean Square	F	P
Tests of Within Subjects Effects					
Days	13634650.3	4.5	3002282.2	340.7	.000
Sex by days	18006.4	4.5	3964.9	.45	.796
Error (days)	3921738.8	445.1	8811.7		
Tests of Between Subjects Effects					
Intercept	142706007.1	1	142706007.1	2313.0	.000
Sex	121448.5	1	121448.5	1.97	.16
Error	6046363.3	98	61697.6		

Milk intake was one of the main outcome variables for the study. In view of the large amount of raw data, the data were summarized by calculating a mean milk intake for each infant for the first six days of life. This variable was named 'meanmass' and was used in future analyses. The milk data for the seventh day were excluded owing to the missing data for 11 of the infants described earlier. Mean milk intakes are shown in Figure 3.4. The overall mean (mean of means) was 428.0 with SD 93.9.

Figure 3.4 Mean daily milk intake over the first 6 days.



3.2.3 Number of feeds

The number of feeds the infant consumed daily was also an outcome variable for this study. Box plots of the number of feeds consumed showed a daily median of between six and eight feeds per day (Figure 3.5). It is apparent that there is high variability with one infant having only three feeds in their first day and one infant having twelve. Two infants particularly stood out, as frequent feeders on more than one day. Infant 97 consumed more feeds than almost all the other infants on days one, three, five and six. Infant 35 consumed more than the other infants on days four and five. The means and standard deviations and minimum and maximum values (Table 3.4) also show the number of feeds per day to be very variable.

Figure 3.5 Number of feeds over the first seven days

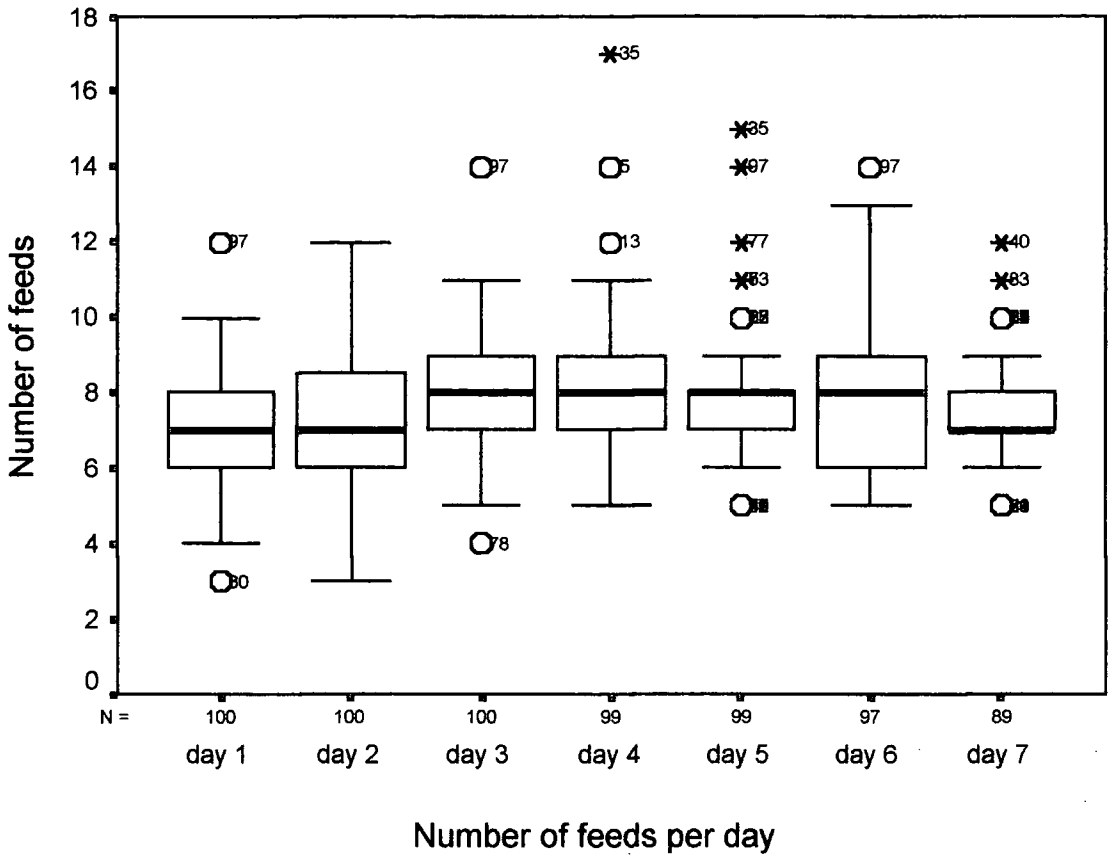


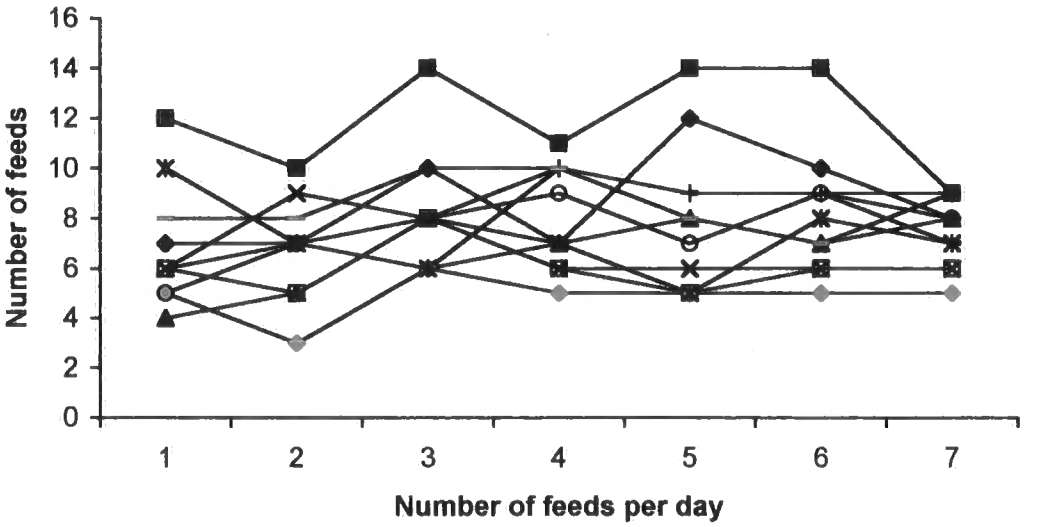
Table 3.4 Mean number of feeds per day

Number of feeds	N	Minimum	Maximum	Mean	S.D.
Day 1	100	3	12	6.7	1.7
Day 2	100	3	12	7.3	1.8
Day 3	100	4	14	7.8	1.7
Day 4	99	5	17	8.0	1.8
Day 5	99	5	15	7.7	1.7
Day 6	97	5	14	7.7	1.7
Day 7	89	5	12	7.6	1.4

In view of the large variability shown between infants in the number of feeds they consumed and again to avoid disguising feeding patterns by averaging the data, some

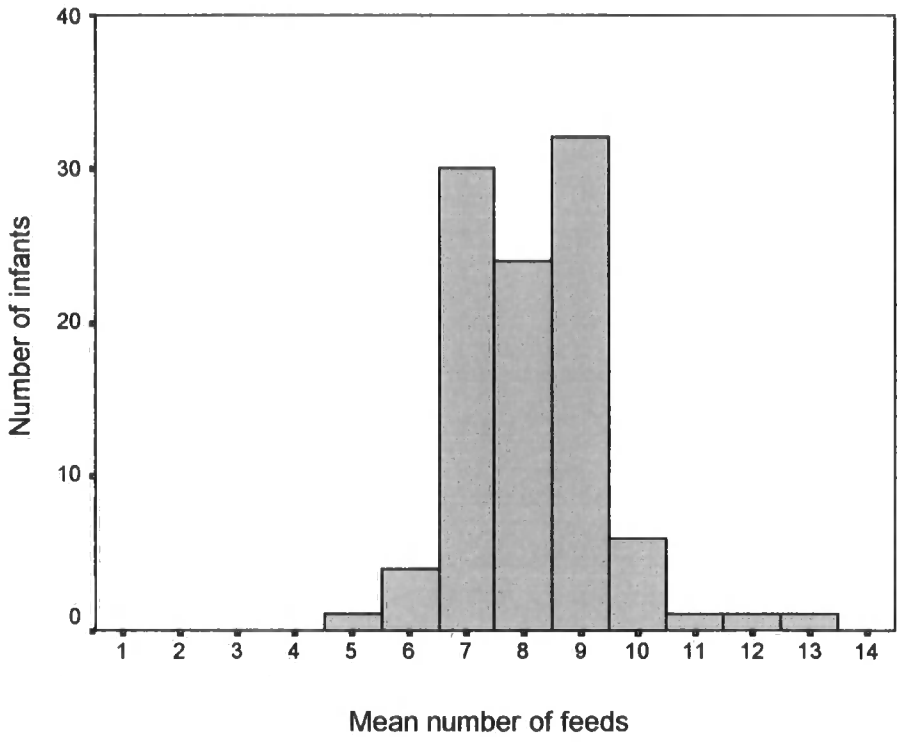
separate individual plots were drawn of feed numbers over the first seven days of life (Figure 3.6). The number of feeds was ranked from the lowest to the highest by the mean number of feeds and every tenth infant was plotted. Feeding patterns were variable between the ten infants shown, but some of the frequent feeders appeared to settle down on the seventh day when feeding became more established.

Figure 3.6 Infants with the lowest and highest mean number of feeds and every tenth ranked infant in between



The data were summarized by calculating the mean number of feeds per day for each infant for the first six days (Figure 3.7). The mean of these means was 7.6 feeds per day, SD 1.2. The individual means were used in future analyses.

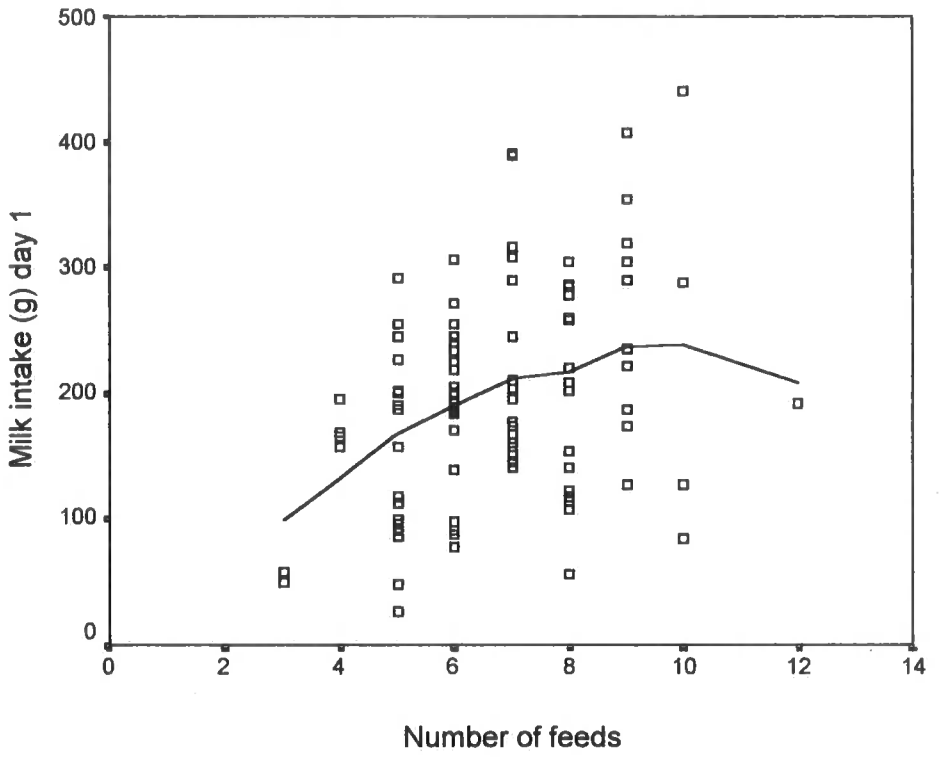
Figure 3.7 Mean number of feeds per day for each infant over the first six days



3.2.4 Relationship between milk intake and the number of feeds

Figure 3.8a shows the relationship between the milk intake and the number of feeds on day one. A lowess line was fitted to the data. The lowess line is a non parametric regression line which connects a local average of the y values, ignoring outliers. Unlike the least squares line, it makes no assumptions about the relationship between the two variables. The lowess line indicates an approximately linear relationship, perhaps with an element of curvature.

Figure 3.8a Scatter plot showing the relationship between milk intake and the number of feeds on day one



Result of a bivariate regression analysis are shown in Table 3.5. The general model is:

Milk intake (g) on day 1 = $\beta_o + \beta_1$ (Number of feeds) + ε

so milk intake (g) on day 1 = 80.2 + 17.2 (Number of feeds) + ε

(ε is the residual for each subject).

In the light of the lowess curve a quadratic term (number of feeds squared) was also tested, but was not statistically significant.

Table 3.5 Regression of milk intake (g) on day 1 on the number of feeds on day 1

		B	SE B	t	p
Constant		80.2	32.5	2.47	.015
Number of feeds		17.3	4.7	3.7	.000
Analysis of Variance					
	df	Sum of squares	Mean square	F	p
Regression	1	83535.5	83535.5	13.7	.000
Residual	98	597854.6	6100.6		
Total	99	681390.1			
R		.350			
R square		.123			
Adjusted R square		.114			
Standard error		78.1			

Outcome variable: milk intake, g, day 1

The value of R square was 0.123 indicating that the number of feeds consumed on day one accounts for 12.3% of the variation in milk intake on day one.

Similar scatter plots with lowess lines fitted to the data were drawn for each of the remaining days from the second day to the seventh day (Figures 3.8b-3.8g). Each scatter plot suggests that there is a linear relationship between milk intake and the number of feeds per day.

Figures 3.8b-3.8g Scatter plots showing the relationship between milk intake and the number of feeds for days two to seven

Figure 3.8b

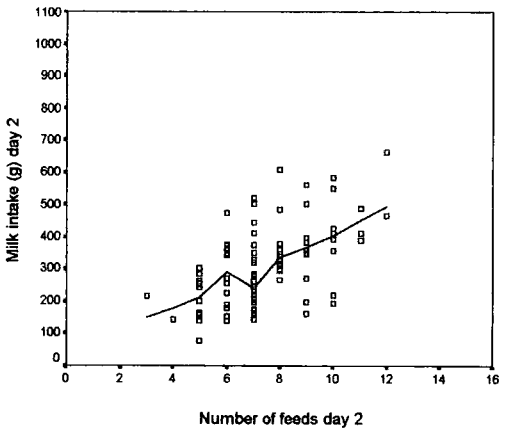


Figure 3.8c

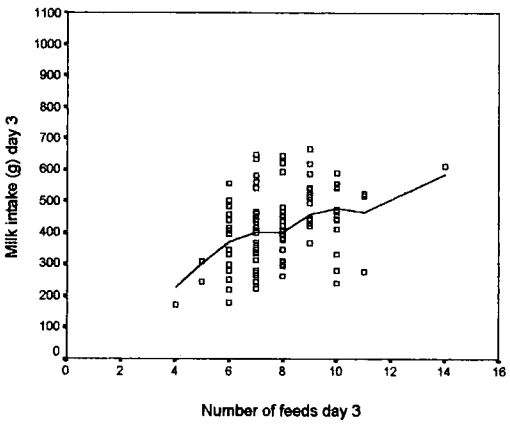


Figure 3.8d

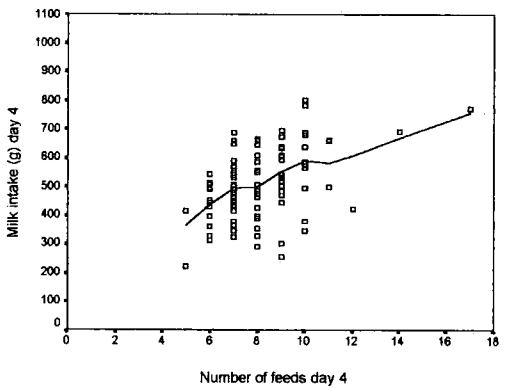


Figure 3.8e

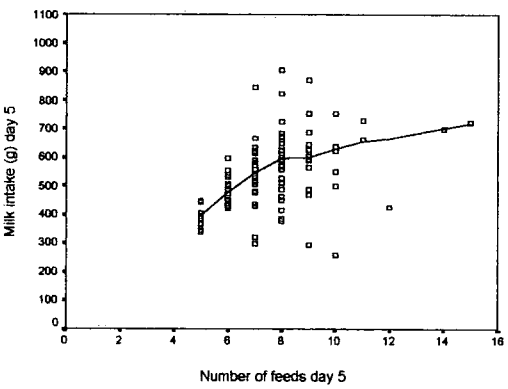


Figure 3.8f

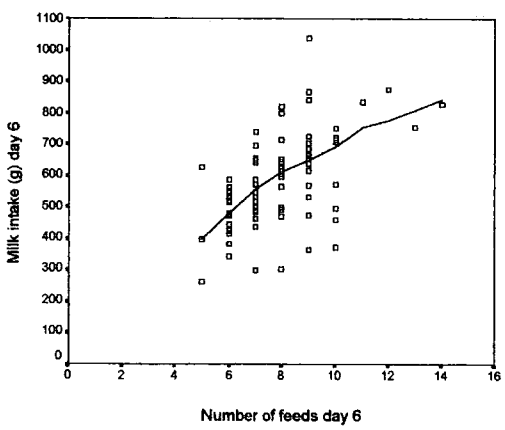
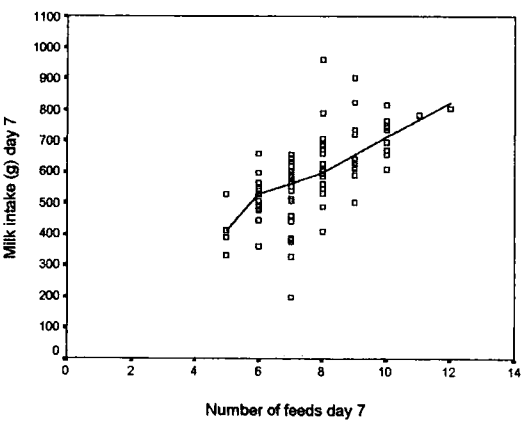


Figure 3.8g



Linear relationships were calculated using bivariate regression and the results and significance values are shown in Table 3.6. In each case the number of feeds consumed on each day is a significant predictor of the milk consumed. The relationship between milk intake and number of feeds per day became stronger over time, as can be seen in the increase of r from .35 to .62.

Table 3.6 Summary of regression analyses of milk intake (g) on number of feeds on day 1 to day 7

Day	β_0 (SE)	β_1 (SE)	t, df	SEE	r	p
1	80.2 (32.5)	17.3 (4.7)	3.7, 99	78.1	.35	p<0.001
2	33.7 (42.8)	37.1 (5.7)	6.5, 99	99.9	.55	p<0.001
3	213.0 (52.1)	26.7 (6.5)	4.1, 99	107.1	.39	p<0.001
4	272.2 (49.6)	29.5 (6.0)	4.9, 98	108.4	.45	p<0.001
5	320.5 (54.2)	30.2 (6.8)	4.4, 98	118.2	.41	p<0.001
6	217.3 (54.5)	46.6 (6.9)	6.8, 96	114.0	.57	p<0.001
7	165.7 (57.4)	55.2 (7.4)	7.5, 88	100.9	.62	p<0.01

Daily milk intake has already been shown to be variable between infants, but are the individual infants consistent in their feeding behaviour? A previous study showed that milk intake in breastfeeding infants on day 5 was significantly related to their milk intake on day 3 (Yamauchi & Yamanouchi, 1990). It also showed that feeding frequency in the second twenty-four hours following birth was significantly related to their feeding frequency in the first twenty-four hours following birth. This finding raises several questions. If an infant has a large milk intake on one day, do they consume more or less milk on the following days? Likewise if an infant feeds frequently on one day do they feed as frequently on the next day? These questions were addressed by producing correlations of milk intake and number of feeds over time. The correlation matrices are shown in Tables 3.7 and 3.8.

Table 3.7 Correlation matrix of milk intake from day 1 to day 7 (Pearson's r)

Milk Intake (g)	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Day 2	0.61					
Day 3	0.50	0.61				
Day 4	0.47	0.65	0.67			
Day 5	0.36	0.54	0.65	0.77		
Day 6	0.27	0.41	0.47	0.67	0.72	
Day 7	0.29	0.48	0.48	0.58	0.67	0.58

All correlations were significant at the 0.01 level (2 tailed)

Table 3.7 shows that milk intake for the succeeding day was quite highly correlated with their previous day's milk intake (r = 0.61 to 0.72). However, on the seventh day this relationship weakened slightly to 0.58. Over the seven days the correlation between day 1 and day 7 is much smaller (0.29). Although it was still significant, this suggests that milk intake on day one is unlikely to be of any predictive value.

Table 3.8 Correlation matrix of number of feeds from day 1 to day 7 (Pearson's r)

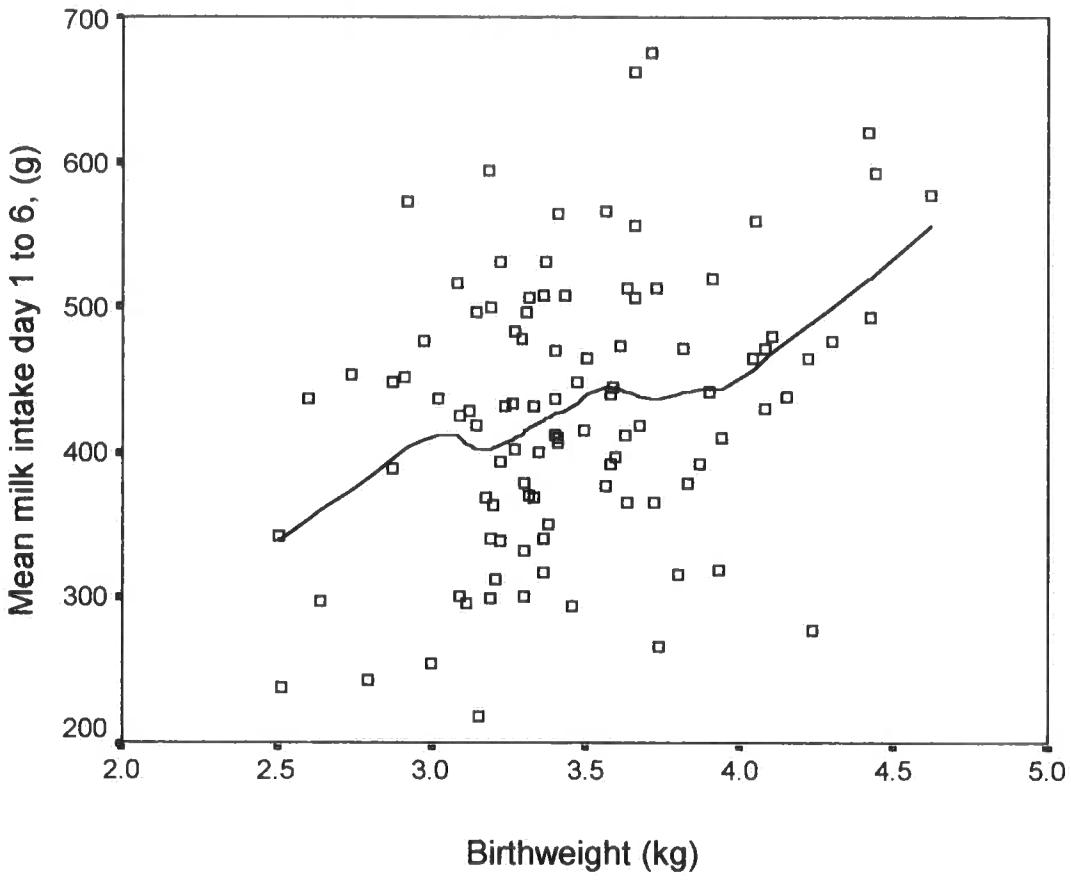
Number of feeds	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Day 2	0.54**					
Day 3	0.31**	0.30**				
Day 4	0.34**	0.37**	0.44**			
Day 5	0.29**	0.37**	0.47**	0.68**		
Day 6	0.13 ns	0.26*	0.33**	0.51**	0.61**	
Day 7	0.15 ns	0.36**	0.22*	0.36**	0.43**	0.26*

** Correlation was significant at the 0.01 level (2 tailed)
* Correlation was significant at the 0.05 level (2 tailed)
ns Correlation not significant

Table 3.8 shows that the number of feeds that an infant consumed each day was significantly related to the number of feeds they consumed on the previous day. However, this relationship weakened by day seven (r =0.26). The infants were not consistent in the number of feeds from day 1 to day 7, as shown by the decreasing strength of the correlation with day 1 to a non-significant level by day 6 and 7 (r = 0.13 & 0.15 respectively).

Several studies have shown that energy intake is related to body weight (Cohen, Brown, Canahuati, Rivera, & Dewey, 1995; Davies, Wells, & Lucas, 1994; De Swiet, Fayers, & Cooper, 1977; Dewey, Heinig, Nommsen, & Lonnerdal, 1991; Fomon, Owen, & Thomas, 1964). So it is likely that birthweight is related to energy intake. Figure 3.9 shows a scatter plot of the relationship between birthweight and mean milk intake. A lowess line was fitted. Visual inspection shows there is a linear relationship between birthweight and mean milk intake.

Figure 3.9 Scatter plot showing the relationship between birthweight and mean milk intake (day 1 to 6)



Results of a bivariate regression analysis are shown in Table 3.9. The relationship can be summarized as:

Mean milk intake (g) = 171 + 74.4 (Birthweight) + ε

Table 3.9 Regression of mean milk intake (g) on birthweight

		B	SE B	t	p
Constant		170.9	71.7	2.4	.019
Birthweight		74.4	20.6	3.6	.000
Analysis of Variance					
	df	Sum of squares	Mean square	F	p
Regression	1	102627.4	102627.4	13.1	.000
Residual	98	769813.2	7855.2		
Total	99	872440.6			
R		.343			
R square		.118			
Adjusted R square		.109			
Standard error		88.6			

Dependent variable: mean milk intake day 1 to day 6

The value of R square was 0.118 indicating that birthweight accounts for 11.8% of the variation in the mean milk intake. This suggests that other factors must also be influencing milk intake in the early neonatal period, but as birthweight was related to milk intake it was used as a control variable in further analyses when assessing the effects of other variables on milk intake.

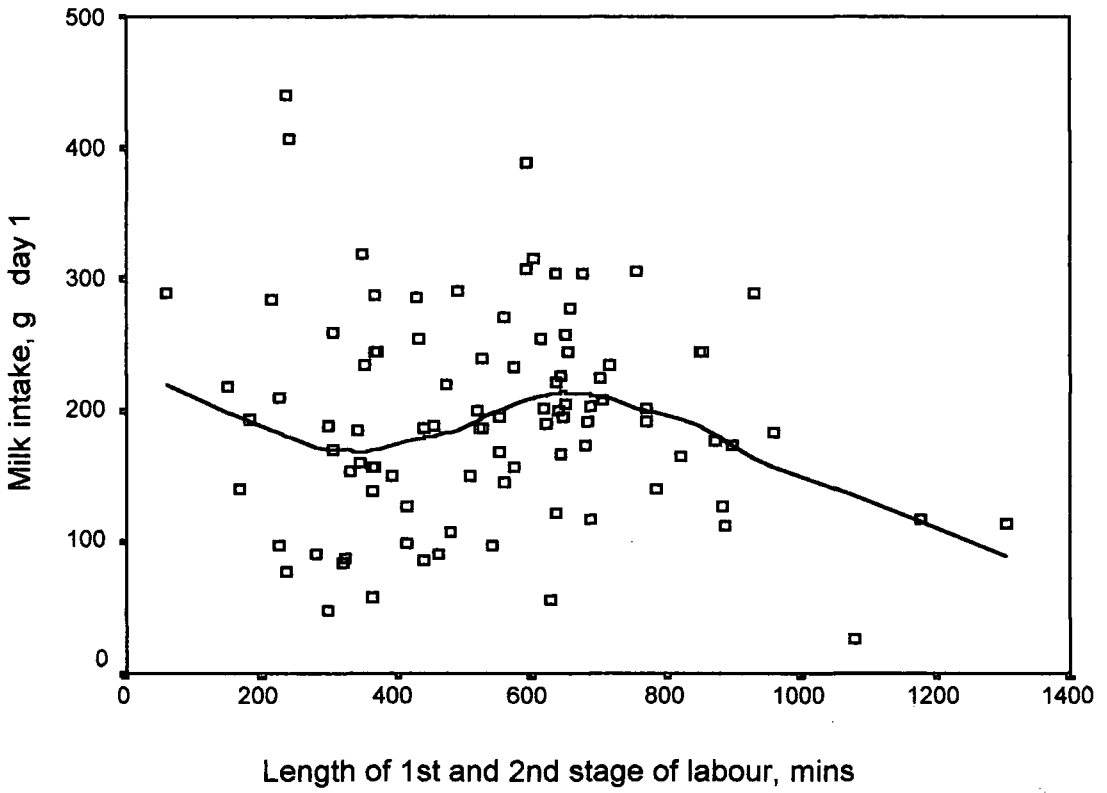
3.3 Other factors that might affect milk intake in the early neonatal period.

3.3.1 Effect of length of labour upon milk intake

The data were examined to see whether the length of the first and second stage of labour was related to the infants milk intake in the first day of life. Theoretically one could expect that those infants that had experienced longer labours could be more tired and this could influence their feeding in the first 24 hours of life, so for the purpose of this analysis milk intake over the first day of life was examined. A scatter plot was drawn with a lowess line fitted to the data. Initial visual inspection suggests that there

may be a relationship between length of labour and amount of milk consumed on the first day of life (Figure 3.10).

Figure 3.10 Length of the first and second stage of labour and milk intake on day 1



To assess in further detail whether the length of the first and second stages of labour had a significant influence upon the amount of milk consumed over the first day of life, a simple regression analysis was carried out (Table 3.10). The variable 'length of labour' was created by combining the time in minutes of the first and second stage of labour. This was used as a predictor variable, whilst controlling for birthweight. The dependent variable was milk intake over the first day of life. Neither birthweight nor the length of labour significantly influenced milk intake on the first day of life.

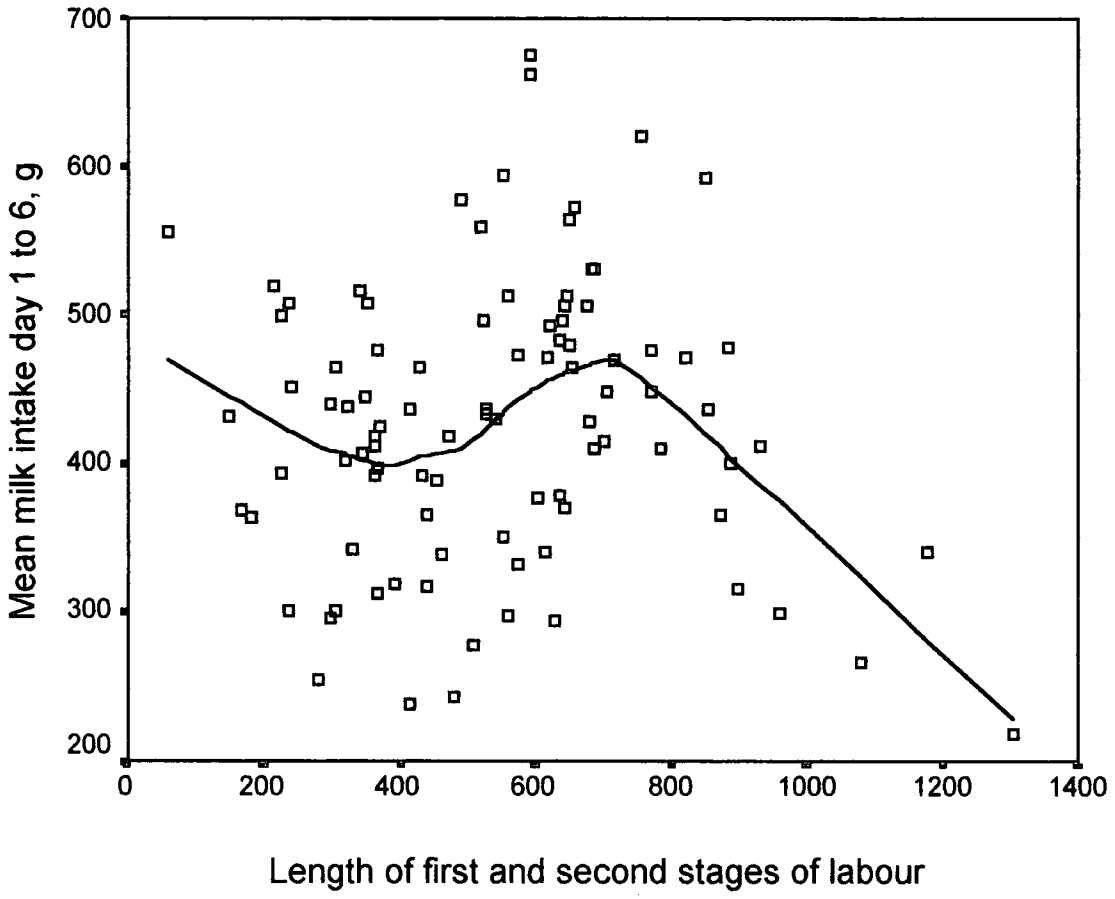
Table 3.10 Regression of milk intake on day 1 on length of first and second stage of labour, controlling for birth weight

		B	SE B	t	p
Constant		102.6	65.5	1.6	.120
Birthweight (kg)		33.1	18.9	1.8	.083
Length of labour (mins)		−0.04	.035	−1.1	.260
Analysis of Variance					
	df	Sum of squares	Mean square	F	p
Regression	2	23095.4	11547.7	1.9	.158
Residual	92	564188.9	6132.5		
Total	94	587284.3			
		Model 1			
R		.198			
R square		.039			
Adjusted R square		.018			
Standard error		78.3			

Outcome variable: milk intake day 1, g

In case the effects of the length of labour affected milk intake of the infant later than the first 24 hours, a similar analysis was carried out using mean milk intake over the first six days. Initial inspection suggests that there may be a relationship between length of labour and mean milk intake day 1 to day 6 (Figure 3.11).

Figure 3.11 Length of the first and second stage of labour and mean milk intake on day 1 to 6



However this does not take into account birthweight, and as shown in Table 3.9, birthweight was a significant predictor of mean milk intake. Therefore a regression analysis was carried out controlling for birthweight, to assess whether this relationship was significant (Table 3.11). Birthweight was significantly related to the mean milk intake day 1 to day 6, but length of labour was not. These findings are important as if the length of labour were related to the amount of milk consumed, then this would need to be taken into account. So from the information obtained in this analysis, the length of labour can be excluded as a possible confounding variable.

Table 3.11 Regression of mean milk intake day 1 to 6 (g) on length of first and second stage of labour, controlling for birth weight

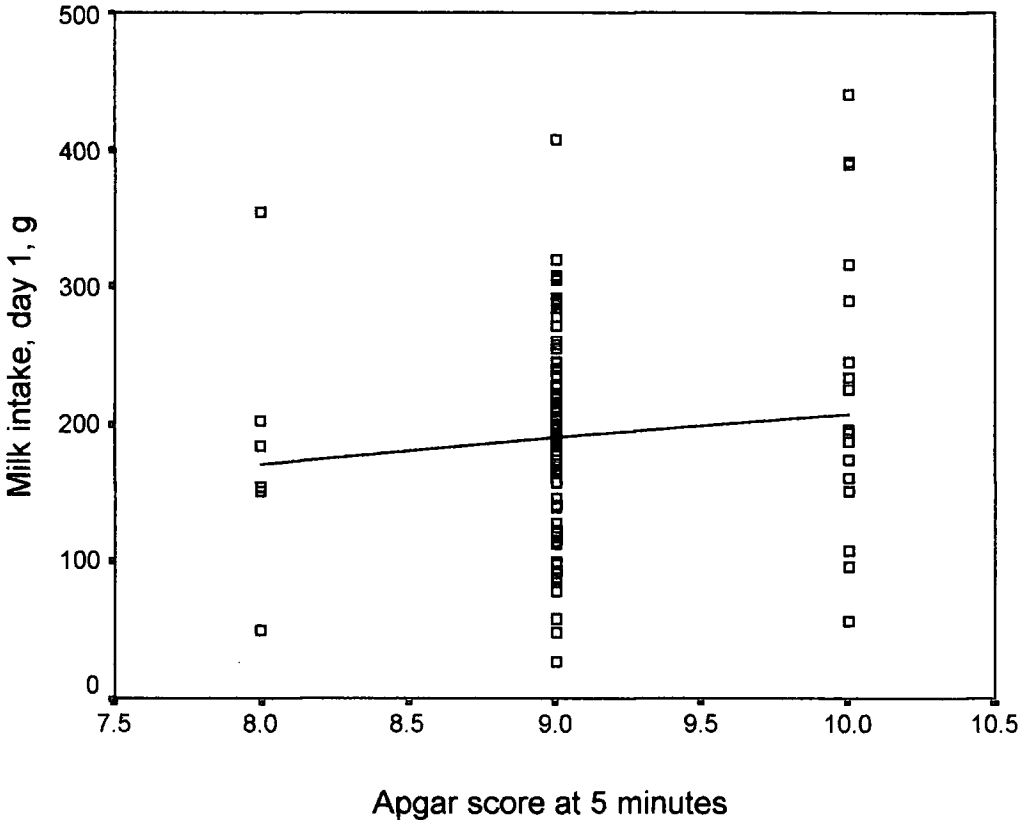
		B	SE B	t	p
Constant		168.3	74.8	2.3	.027
Birthweight (kg)		80.5	21.6	3.7	.000
Length of labour (mins)		–0.04	.040	–.091	.361
Analysis of Variance					
	df	Sum of squares	Mean square	F	p
Regression	2	111454.2	55727.1	6.95	.002
Residual	92	737638.72	8017.8		
Total	94	849092.9			
R		.362			
R square		.131			
Adjusted R square		.112			
Standard error		89.5			

Outcome variable: mean milk intake day 1 to 6, g

3.3.2 Relationship of Apgar scores and milk intake

The data were examined to see whether the infant's condition at birth as measured by Apgar score was related to milk intake in the first day of life. All infants had an Apgar score of 8 or above at five minutes which is indicative of being in a good condition, but visual assessment of the scatter plot and fitting a lowess line to the data suggests a possible relationship (Figure 3.12), in spite of this restriction of range.

Figure 3.12 Scatter plot showing possible relationship between Apgar scores at 5 minutes and milk intake on day one



Further examination of the data was performed by regression of milk intake on day 1 on the Apgar scores at 5 minutes, controlling for birthweight (Table 3.12). Birthweight alone was not statistically significant. The addition of the second predictor, Apgar score at 5 minutes was also not significant. This shows that birth-weight and Apgar scores at 5 minutes of age were not related to milk intake over the first day of life, so Apgar score can also be excluded as a possible confounding variable.

Table 3.12 Regression of milk intake on day 1 on Apgar scores at 5 minutes, controlling for birth weight

		B	SE B	t	p
Constant		–99.7	167.9	–.594	.554
Birthweight (kg)		21.0	19.2	1.1	.277
Apgar at 5 minutes		24.6	17.4	1.4	.161
Analysis of Variance					
	df	Sum of squares	Mean square	F	p
Regression	2	23156.2	11578.1	1.71	.189
Residual	97	658233.9	6785.9		
Total	99	681390.1			
R		.184			
R square		.034			
Adjusted R square		.014			
Standard error		82.4			

Outcome variable: milk intake day 1, g

3.3.3 Effect of analgesia and anaesthesia on milk intake

Most mothers have some form of pain relief in labour (Rosenblith, 1992). In this study, the analgesia and anaesthesia administered to the mother during labour was documented from the delivery notes. Ninety-nine of the 100 mothers had some form of pain relief during labour, some having more than one type. Table 3.13 shows the analgesia and anaesthesia administered to the mothers in labour.

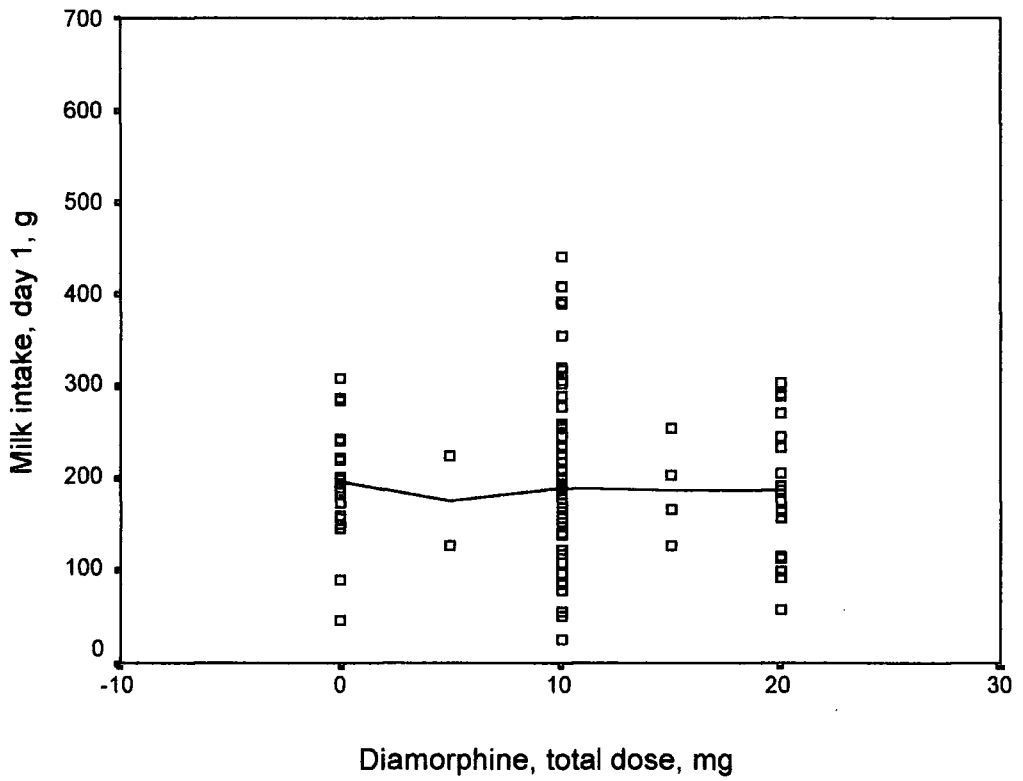
Table 3.13 Analgesia and anaesthesia administered in labour

Type	N of mothers
Diamorphine hydrochloride (analgesic)	76
Epidural (anaesthetic)	50
Entonox (inhalational agent, analgesic)	69
Spinal anaesthetic	4
General anaesthetic	2

Some mothers had more than one form of pain relief in labour

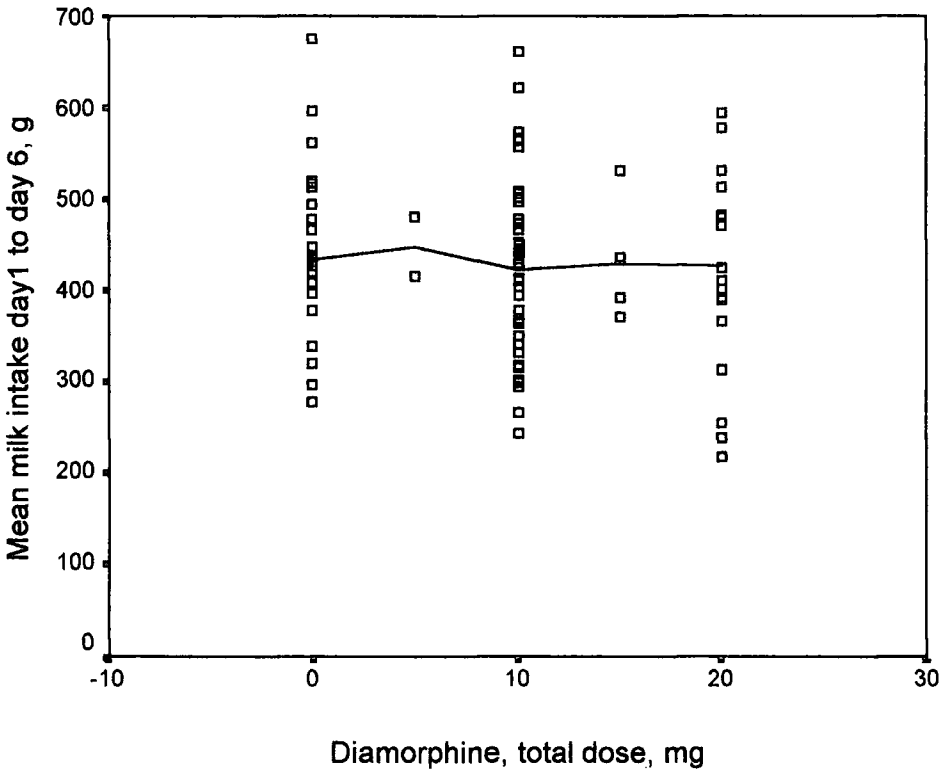
In this study, seventy-six of the mothers had intra-muscular injections of diamorphine during the course of their labour. There is limited research on morphine use in labour and its possible effect upon the feeding behaviour of the neonate. However, several studies have examined another opioid, pethidine, which has been shown to make the infant drowsy (Emde, Swedberg, & Suzuki, 1975), which affects breast seeking and breast feeding behaviours (Ransjo-Arvidson et al., 2001). Diamorphine can be given in 5 or 10 mg doses at three hourly intervals. The total dose administered to each woman was calculated and used in the analysis. A scatter plot was drawn and a lowess line fitted to the data to see if the administration of intra-muscular diamorphine to the mother during labour was related to the milk intake of the infant on the first day of life (Figure 3.13). Inspection shows no relationship between the two.

Figure 3.13 Relationship between milk intake on day one and the total dose of diamorphine administered to the mother.



Other opioids (pethidine) have been found to make the infant drowsy over the first few days of life (Belsey et al., 1981; Nissen et al., 1995). In case the possible carry over effects of the analgesia did not become apparent until after the first 24 hours, a scatter plot was drawn to examine the relationship between the total dose of diamorphine and the mean milk intake over the first six days of life (Figure 3.14). There was no obvious relationship between mean milk intake from day 1 to day 6 and diamorphine dose.

Figure 3.14 Relationship between mean milk intake, day 1 to 6 and the total dose of diamorphine administered to the mother



Half the mothers had an epidural anesthetic during their labour. Rosenblatt et al. (1981) found a decreased motor tone in the first 24 hours following birth in infants born to mothers receiving epidural anaesthesia in labour. A decreased motor tone could affect feeding. Initial assessment in the form of a scatter plot (Figure 3.15) suggests that there is no difference in the milk consumed over the first day of life in those infants of mothers that had an epidural compared to the infants of mothers that did not have an epidural. Similarly examination of the scatter plot of the mean milk intake from day one to day six in those infants of mothers that had an epidural compared to the infants of mothers that did not have an epidural showed no obvious difference (Figure 3.16)

Figure 3.15 Scatter plot comparing milk intake on day 1 in the infants of mothers that had an epidural anaesthetic and those that did not, with means

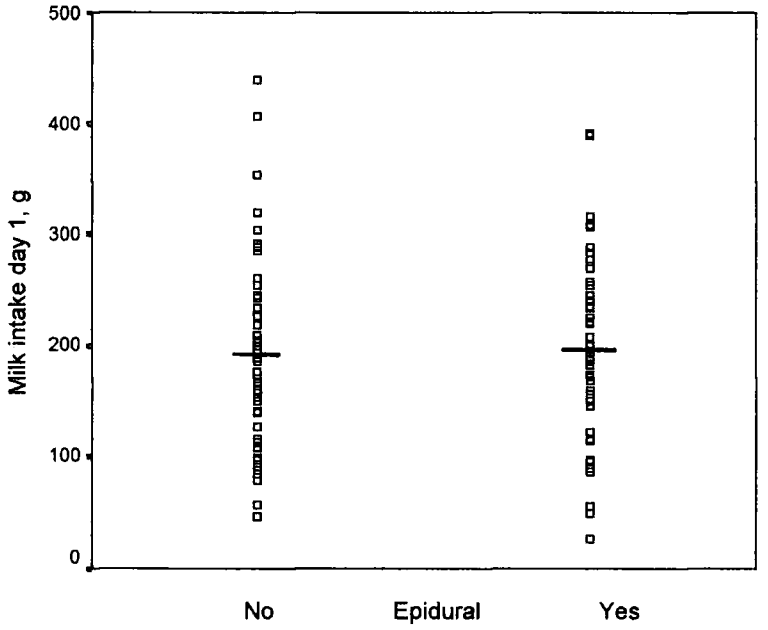
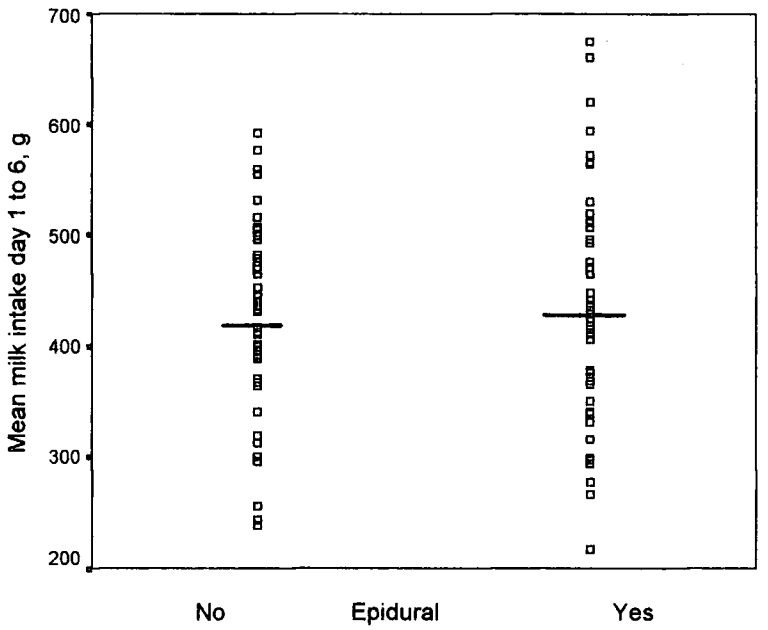


Figure 3.16 Scatter plot comparing mean milk intake from day 1 to day 6 in the infants of mothers that had an epidural anaesthetic and those that did not, with means

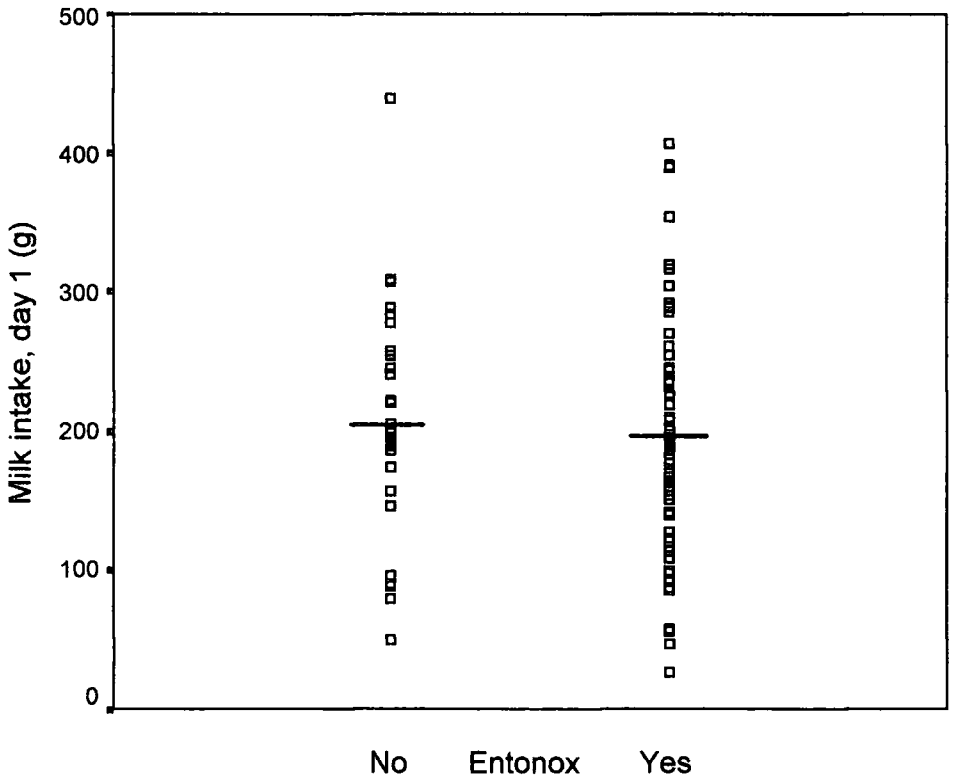


An independent sample t test confirmed that there was no significant difference in the milk intake on day one in infants of those mothers having an epidural (mean 198.7, SD

79.0) and those infants of mothers that that did not have an epidural anaesthetic anaesthetic (mean 194.7, SD 87.4); t = 0.239 with 98 df, p = 0.81. Likewise there was no difference in the mean milk intake from day one to day six in the infants of those mothers receiving an epidural anaesthetic (mean 431.5, SD 102.8) and those infants of mothers that did not have an epidural anaesthetic (mean 424.9, SD 84.9); t = 0.347 with 98 df, p = 0.73. Neither difference was statistically significant.

Sixty nine percent of the mothers used inhalational Entonox during their labour. As the effects of Entonox wear off very quickly (Stenger, Blechner, & Prystowsky, 1969), the milk intake was only examined for the first day of life. Figure 3.17 compares the milk intake on day one, in those infants of mothers that had inhalational Entonox and those that did not. Examination of the scatter plot shows no obvious difference in milk intake between the two groups. An independent t test confirmed that there was no significant difference in the milk intake on day one in infants of mothers that had Entonox (mean 191.9, SD 84.4) and those infants of mothers that did not (mean 207.3, SD 79.9); t = .855 with 98 df, p = .443.

Figure 3.17 Scatter plot comparing milk intake on day 1 in the infants of mothers that had inhalational Entonox and those that did not



A very small number of mothers had other forms of anaesthetic (4 women had a spinal anaesthetic and 2 had a general anaesthetic (GA)). Two scatter plots were drawn of milk intake on day 1 and mean milk intake day 1 to day 6 (Figures 3.18 & 3.19). The milk intake of the infants whose mother had a spinal anaesthetic are shown by squares and the milk intake of the infants whose mother had a general anaesthetic were shown by triangles. Both indicated that the milk intake of infants born to mother's who received either a spinal or general anesthetic to be comparable to the milk intake of other infants. Formal tests of statistical significance are unlikely to be informative with such small numbers.

Figure 3.18 Scatter plot comparing milk intake on day 1 in the infants of mothers that had spinal anaesthetic (squares), general anaesthetic (triangles) and remaining mothers

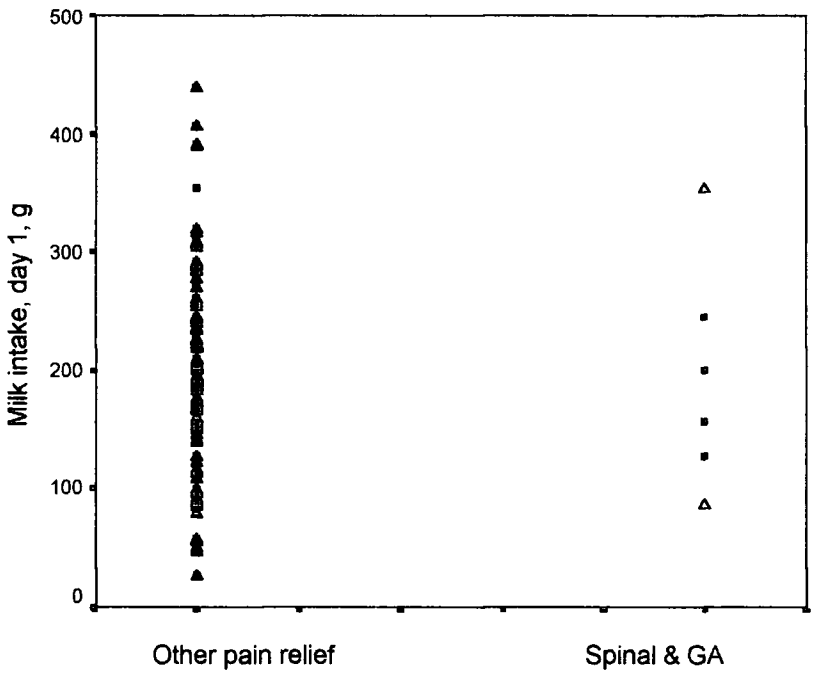
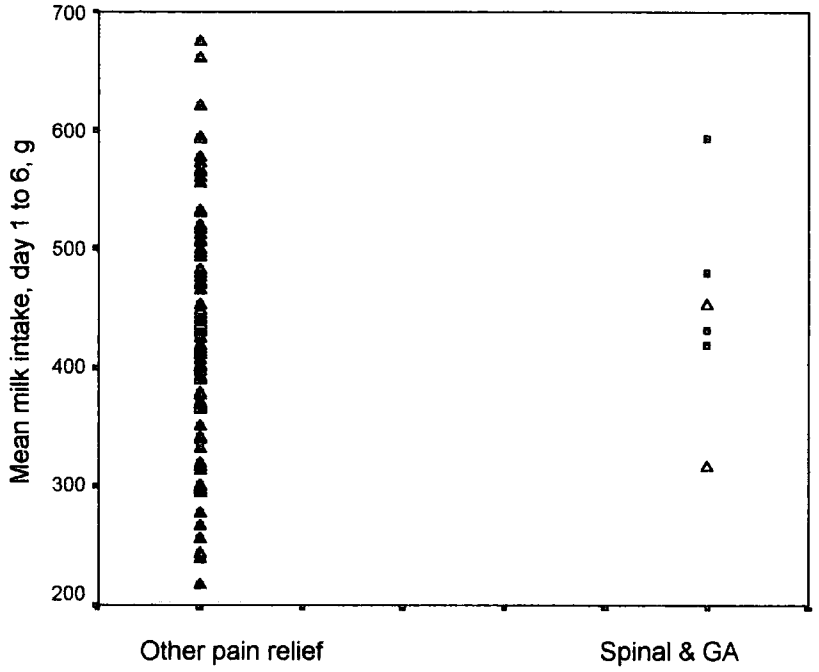


Figure 3.19 Scatter plot comparing mean milk intake day 1 to 6 in the infants of mothers that had spinal anaesthetic (squares), general anaesthetic (triangles) and remaining mothers



Many of the mothers had more than one form of pain relief in labour, so further analysis using multiple regression was carried out using all the forms of analgesia and anaesthesia administered in labour as predictor variables. The dependent variables used were milk intake on day 1 and mean milk intake from day 1 to day 6. These analyses will be presented later in the chapter. The length of labour was also included as a control variable. If labour becomes prolonged, the mother is more likely to have more analgesia or anaesthesia, which exposes the infant to more and may affect feeding in the early neonatal period. Birthweight was used as a control variable because of its previously demonstrated relationship with milk intake. Model 1 controlled for birthweight and the length of labour in minutes, which were entered as control variables. Model 2, the forms of analgesia and anaesthesia were entered into the analysis. The other five predictor variables were epidural anesthetic, IM diamorphine, inhalational Entonox, general anaesthetic and spinal anaesthetic (all of these were coded 0 = no, 1 = yes); the dependent variable was milk intake on day 1. The F change statistic for the comparison of the models tests whether there is a statistically significant effect of these five variables considered together. Results are shown in Table 3.14. Birth-weight and length of labour were not statistically significant in model 1. In model 2, the addition of the five other analgesia and anaesthesia predictor variables

caused R square to increase by 5.6% with a F change ratio of 1.1 with 5, 87 df, p = .383, which is clearly not significant. This means that none of the predictor variables had any significant effect upon the milk intake of the infant over the first day of life and for the purpose of this study can be excluded as possible confounding variables.

Table 3.14 Regression of milk intake (g) day 1 on diamorphine (mg), epidural, Entonox, general anaesthetic, spinal anaesthetic and length of labour, controlling for birthweight

	B	SE B	t	p
Model 1				
Constant	102.6	65.5	1.6	.120
Birthweight (kg)	33.1	18.9	1.8	.083
Length of labour (mins)	-0.04	.035	-1.1	.260
Model 2				
Constant	116.7	69.3	1.7	.096
Birthweight (kg)	31.3	19.1	1.6	.106
Length of labour	-0.05	.041	-1.3	.183
IM Diamorphine (mg)	16.3	19.7	.798	.450
Epidural	14.9	20.5	.758	.428
Inhalational Entonox	-24.6	19.2	-1.3	.203
General Anaesthetic	-117.3	80.3	-1.5	.148
Spinal Anaesthetic	-19.9	47.2	-.42	.674
Analysis of Variance				
Model 1				
	df	Sum of squares	Mean square	F	p
Regression	2	23095.4	11547.7	1.9	.158
Residual	92	564188.8	6132.5		
Total	94	587284.3			
Model 2					
Regression	7	55755.6	7965.1	1.3	.258
Residual	87	531528.6	6109.5		
Total	94	587284.3			
		Model 1	Model 2		
R		.198	.308		
R square		.039	.095		
Adjusted R square		.018	.022		
Standard error		78.3	78.2		
R square change		.039	.056		
F change		1.9	1.1		
p =		.158	.383		

Dependent variable: milk intake day 1, g

In case the effects of analgesia and anaesthesia upon milk intake did not become apparent until later, the same regression model was used, but the dependent variable was changed to the infants mean milk intake day 1 to day 6. Results are shown in Table 3.15. Birth-weight and length of labour in model 1 gave an R square value of .131 indicating that these variables accounted for 13.1% of the variation seen in mean milk intake from day 1 to day 6. Birth-weight was statistically significant ($t = 3.7$, $p<0.001$), but length of labour was not. In the second model, the addition of the five other analgesia and anaesthesia predictor variables caused R square to increase by 4.9% with a F change ratio of 1.0 with 7, 87 df, $p = .401$, which is clearly not significant. This means that none of the predictor variables had any significant effect upon the mean milk intake of the infant over the first six days of life and for the purpose of this study can be excluded as possible confounding variables.

Table 3.15 Regression of mean milk intake (g) day 1 to 6 on length of labour (mins), diamorphine (mg), epidural, Entonox, general anaesthetic and spinal anaesthetic controlling for birthweight

	B	SE B	t	p
Model 1				
Constant	168.3	74.8	2.2	.027
Birthweight (kg)	80.5	21.6	3.7	.000
Length of labour (mins)	-0.04	.040	-.917	.361
Model 2				
Constant	215.1	79.3	2.7	.008
Birthweight (kg)	73.8	21.9	3.4	.001
Length of labour	-0.04	.047	-.827	.411
IM Diamorphine (mg)	1.2	22.6	.051	.960
Epidural	.245	23.4	.011	.991
Inhalational Entonox	-34.7	21.9	-1.6	.118
General Anaesthetic	-95.7	91.9	-1.0	.301
Spinal Anaesthetic	50.1	53.9	.928	.356

Analysis of Variance

Model 1

	df	Sum of squares	Mean square	F	p
Regression	2	111454.2	55727.1	6.9	.002
Residual	92	737638.7	8017.8		
Total	94	849092.9			
Model 2					
Regression	7	152963.9	21851.9	2.7	.013
Residual	87	696129.0	8001.5		
Total	94	849092.9			

	Model 1	Model 2
R	.362	.424
R square	.131	.180
Adjusted R square	.112	.114
Standard error	89.5	89.4
R square change	.131	.049
F change	6.9	1.0
p =	.002	.401

Outcome variable: mean milk intake day 1 to day 6

3.4 Summary of main results for chapter 3

This chapter has presented results on the milk intakes of the infants, and considered some of the confounding variables that were taken into account in the study. The principal results were as follows:

1) Average milk intake increased daily from day 1 to day 7.

2) There was no sex difference in milk intake.

3) Daily milk intake was significantly related to the number of feeds per day.

4) Milk intake on each succeeding day was significantly related to the previous day's milk intake, and the number of feeds on each succeeding day was significantly related to the number of feeds consumed on the previous day.

5) Birthweight was significantly associated with the amount of milk consumed over the first six days of life.

6) There was no relationship between length of labour and the amount of milk consumed on the first day of life, or on the amount of milk consumed over the first six days of life.

7) There was no relationship between the infants Apgar score at five minutes and milk intake on the first day of life.

8) There was no relationship between maternal analgesia or maternal anaesthesia and milk intake on the first day of life or on the amount of milk consumed over the first six days of life.

Chapter 4 Results

Infant size at birth, growth up to twelve weeks of age and milk intake

4.1 Growth

4.1.1 Reliability of measurements

Two of the principal hypotheses of this study concerned the growth of infants. This chapter summarises data on their growth, and considers its relationship with milk intake. As previously described in the methodology the author attended several growth clinics and was trained by an auxologist in the anthropometric measurement of infants (Chapter 2, section 2.3.1). The measurements of weight and head circumference have previously been found to be the most reliable, and the measurement of length the least (Johnson, Engstrom, & Gelhar, 1997).

A small study was conducted to ascertain the reliability of length measurement. Eleven infants aged between 4 weeks and one year of age were measured three times on the same occasion and the mean and standard deviation of their measured length calculated for each infant. These results are shown in Table 4.1.

Table 4.1 Infant length measured three times, mean and SD (cm)

Infant	Length 1	Length 2	Length 3	Mean (cm)	SD (cm)
1	77.1	77.0	76.9	77.00	0.10
2	63.3	63.3	63.4	63.33	0.06
3	54.0	54.2	53.6	53.93	0.31
4	51.5	51.4	51.3	51.40	0.10
5	66.4	66.5	66.2	66.37	0.15
6	54.4	54.5	54.4	54.43	0.06
7	52.8	52.7	52.8	52.77	0.06
8	63.2	63.1	63.0	63.10	0.10
9	77.0	77.0	76.8	76.93	0.12
10	74.8	74.6	74.6	74.67	0.12
11	60.0	60.0	60.2	60.07	0.12

The pooled (averaged) within-subject SD is 0.12. So its variance is that squared, which is 0.0144. Two measurements were made, one at birth and one at seven days, so the error variance associated with the difference is 0.0144 + 0.0144 = 0.0288 cm^2 (Mosteller, Rourke, & Thomas, 1961). The SD of the difference is the square root of that, 0.16cm. The average gain in length over the first seven days (given later in Table 4.3) was 51.2 – 49.8 = 1.4 cm. So the measurement error is about 10% (11.4%) of the difference in length over the first seven days. Over the period from birth to twelve weeks the average change was 10.5cm (Table 4.3), so the error is about 1% (1.5%) of the difference.

4.1.2 Calibration of scales

Another important factor in a study of this type is the accuracy of the equipment being used. As described in the methodology (chapter 2.3), all of the scales used in the study were calibrated using M 3 calibration weights to ensure that they were weighing accurately. This procedure was performed before the start of the study and three times during the course of the study. The scales were checked with calibration weights of 1000g, 2000g, 3000g, 4000g and 5000g. The observed weights for each scale were subtracted from the expected weights. Because these differences (with one exception, scale 2 in Oct. 00) were all in the same direction, a simple average of the differences (mean difference) is an acceptable summary measure of errors. Table 4.2 gives the difference and mean difference for each scale.

To give an example for the first scale (Homescale, Rachel) in Table 4.2, the observed weights minus calibration weights, both in g are 1000 – 1000 = 0g; 2002 – 2000 = 2g; 3000 – 3000 = 0g; 4002 – 4000 = 2g; 5002 – 5000 = 2 g. The mean difference (error) is 1.2g.

Table 4.2 Calibration of scales on four separate occasions, difference and mean difference for each scale

	Home scale	Scales on delivery suite										
Oct.00	Rachel	no 1	no 2	no 3	no 4	no 5	no 6	no 7	no 8	no 9	no 10	no 11	no 12
	0	0	-5	0	0	-5	0	5	5	0	0	10	-160
	2	-10	5	0	0	-10	0	10	0	-10	0	15	-400
	0	-10	10	0	0	-5	0	10	10	-10	5	20	-360
	2	-10	5	0	0	-10	0	10	10	-10	5	30	-430
	2	-15	5	0	0	-15	0	10	5	-15	0	40	-285
Mean difference	**1.2**	**-9.0**	**4.0**	**0.0**	**0.0**	**-9.0**	**0.0**	**9.0**	**6.0**	**-9.0**	**2.0**	**23.0**	**-327.0**

	Home scale	Scales on delivery suite									
Jan.01	Rachel	no 1	no 2	no 3	no 4	no 5	no 6	no 7	no 8	no 9	no 10
	0.0	0.0	0.0	0.0	0.0	0.0	-10.0	0.0	0.0	0.0	0.0
	0.0	-5.0	5.0	0.0	5.0	0.0	-10.0	0.0	0.0	-5.0	0.0
	2.0	-5.0	10.0	0.0	10.0	-5.0	-5.0	0.0	-5.0	-10.0	0.0
	0.0	-5.0	10.0	0.0	10.0	0.0	-10.0	0.0	-5.0	-10.0	0.0
	0.0	-5.0	10.0	0.0	15.0	0.0	-5.0	0.0	-5.0	-10.0	0.0
Mean difference	**0.4**	**-4.0**	**7.0**	**0.0**	**8.0**	**-1.0**	**-8.0**	**0.0**	**-3.0**	**-7.0**	**0.0**

	Home scale	Scales on delivery suite									
Apr.01	Rachel	no 1	no 2	no 3	no 4	no 5	no 6	no 7	no 8	no 9	no 10
	0.0	0.0	-5.0	0.0	0.0	5.0	0.0	0.0	0.0	0.0	0.0
	0.0	0.0	-5.0	5.0	0.0	0.0	0.0	-5.0	10.0	0.0	0.0
	2.0	0.0	-5.0	0.0	0.0	5.0	0.0	-5.0	0.0	0.0	0.0
	0.0	0.0	-5.0	5.0	0.0	5.0	0.0	-10.0	10.0	0.0	0.0
	0.0	0.0	-5.0	10.0	0.0	5.0	0.0	-10.0	10.0	0.0	0.0
Mean difference	**0.4**	**0.0**	**-5.0**	**4.0**	**0.0**	**4.0**	**0.0**	**-6.0**	**6.0**	**0.0**	**0.0**

	Home scale	Scales on delivery suite									
Jul.01	Rachel	no 1	no 2	no 3	no 4	no 5	no 6	no 7	no 8	no 9	no 10
	0.0	0.0	0.0	-10.0	10.0	0.0	0.0	-5.0	0.0	10.0	0.0
	0.0	-5.0	0.0	-10.0	0.0	0.0	0.0	-5.0	5.0	20.0	0.0
	0.0	-5.0	0.0	-10.0	0.0	0.0	0.0	-5.0	0.0	30.0	0.0
	0.0	-10.0	0.0	-10.0	10.0	0.0	0.0	-5.0	5.0	30.0	0.0
	0.0	0.0	0.0	-10.0	10.0	0.0	0.0	-5.0	10.0	30.0	0.0
Mean difference	**0.0**	**-4.0**	**0.0**	**-10.0**	**6.0**	**0.0**	**0.0**	**-5.0**	**4.0**	**24.0**	**0.0**

During the calibration before the start of the study, one scale (Oct.00, no 12) was found to be weighing light by a large mean difference of –327g. This was brought to the attention of the Delivery Suite Manager and the scale was immediately removed from clinical use and sent for repair. This scale did not return during the course of the study. This finding shows how important calibration of scales is, both in research and clinical practice. Although each scale was labeled and numbered at the first calibration, unfortunately by the second calibration these labels had been washed off. So I was unable to determine which scale was which on each calibration or which scale was used when weighing the infant. However this was of no consequence as the other scales were in any case moved from delivery room to delivery room, as there were only ten scales for 16 delivery rooms. So it was not possible to know which scale had been used for a particular infant. By the second calibration another scale had been removed from delivery suite as it was unable to hold its charge when the electricity supply was removed.

The mean difference of the scales that were used in the study (excluding the scale withdrawn from the delivery suite) varied from –10g to 24g. Forty-three of the 45 scales had a mean difference ranging from – 10g to +9g, which is a 19g possible error rate. The other two scales had a mean difference of 23 and 24g. The weight of the lightest infant at birth in the study for this thesis was 2510g; she gained 40g between birth and seven days and 2416g between birth and 12 weeks. Therefore 47.5% of weight gain between birth and seven days and 0.8% of weight gain between birth and 12 weeks of age could be attributed to error alone based on a 19g error. The change in weight of only nine infants fell within + or – 19g at seven days of age and could therefore be explicable on scale error alone (on pessimistic assumptions). The change in weight from birth to seven days in the infants in this study was from –262g to +374g. By 12 weeks the least weight gain for the infants in the study was 984g and the maximum weight gain was 4106g, an error rate of 19g would account for 1.9% of weight gain in the slowest growing infant and 0.5% of weight gain in the fastest growing infant.

4.1.3 Measurements at birth, seven days and twelve weeks

Measurements of weight, length and head circumference were taken at birth, seven days of age and twelve weeks of age. Growth is one of the principal outcome variables in this study and was used in future analyses. Two procedures were used to analyse growth in this study. Firstly weight in kg on a continuous scale was used, adjusted for birthweight in a regression analysis as a measure of weight gain. Secondly weight in z scores on a dichotomous scale was used, adjusted for birthweight. This identified slow growing infants and is also adjusted for sex as the z scores are sex specific.

All birth measurements were taken within two hours of birth. All seven-day measurements were taken between 168 and 172 hours. The twelve-week measurements were subject to more variation due to matters beyond the author's control. For example, one family moved to Germany and did not return until the infant was 16 weeks of age and two families moved house and took some time to track down. Five families did not respond to the telephone or letters and follow up took place after several attempts at contact. Sixty one percent of the study sample was measured at exactly 12 weeks of age, 16% were measured at 12 weeks and 1 day, 9% were measured at 12 weeks and two days and 6% were measured at 12 weeks and 3 days. This meant that 92% were measured within three days of attaining 12 weeks. The remaining 8% were measured between 12 weeks and four days and 16 weeks of age. Because some of the 12 week weights were delayed, a new variable was created called 'twelve week age'. This was the exact age of the infant at the 12 week measurement and was used in future analyses to take this into account.

Summary statistics for weight, length and head circumference at birth, at seven days and at twelve weeks are shown in Table 4.3.

Table 4.3 Summary statistics of measurements at birth, seven days and twelve weeks: raw data

		Weight (kg)	Length (cm)	Head Circumference (cm)
Birth	Mean	3.458	49.8	35.3
	SD	0.433	1.8	1.2
	Minimum	2.510	45.0	32.6
	Maximum	4.615	54.0	39.0
7 days	Mean	3.483	51.2	35.7
	SD	0.427	1.8	1.0
	Minimum	2.468	45.5	33.4
	Maximum	4.614	55.0	38.0
12 weeks	Mean	5.958	60.3	40.6
	SD	0.743	2.2	1.1
	Minimum	4.074	53.5	37.4
	Maximum	8.092	66.0	43.9

4.1.4 Comparison of sample data to z scores of the British Growth Standards

To ensure that the study sample was representative of the general population, weights, lengths and head circumferences were converted to z scores. This was achieved by using a computer package available from the Child Growth Foundation based on statistics collected for the 1990 British Growth Standards (Cole, 1995; Freeman, Cole, Jones, White, & Preece, 1995; Preece, Freeman, & Cole, 1996). These statistics take into account both sex and age. Descriptive statistics are in Table 4.4.

Table 4.4 Summary statistics of measurements at birth, seven days and twelve weeks: z scores

N=100		Weight (z)	Length (z)	Head Circumference (z)
Birth	Mean	−.055	−.366	.37
	Median	−.204	−.521	.370
	SD	.908	.893	.916
7 days	Mean	−.436	−.108	.23
	Median	−.574	−.020	.165
	SD	.871	.915	.851
12 weeks	Mean	.070	.256	.010
	Median	− .011	.301	.048
	SD	.998	.942	.931

For the study sample to be equivalent to the British standards, the mean measurement should be zero. To check whether the z scores calculated for the study data were significantly different from the median of 0, a one sample t-test was used. The measurements that were significantly different from the population standards at birth were length ($t = -4.1$, with 99 df, $p < 0.001$) and head circumference ($t = 4.3$, with 99 df, $p < 0.001$). At seven days of age weight ($t = -5.0$, with 99 df, $p < 0.001$) and head circumference ($t = 2.7$, with 99 df, $p < 0.01$), and at 12 weeks of age length ($t = 2.7$, with 99 df, $p < 0.01$) were all significantly different.

The mean birth weight of the study sample was .05 of a standard deviation lower than that expected from the growth standard; this difference was not significant. The mean weight of the study sample at seven days was .44 of a standard deviation lower than the population standards and at 12 weeks it was .06 of a standard deviation greater than the population standards. This large difference at 7 days is significant, but the other difference is again slight. There are several potential reasons for the slight discrepancies observed. The difference noted at birth may be because the recruited study sample were formula fed infants only. These infants are more likely to be born to mothers from a lower social class and to mothers that smoke. The difference in weight observed at seven days may be due to the way the growth standard was constructed. The British Standards were based on the weights of infants at birth and at six weeks of age. The data in the interim period are derived by linear interpolation. Therefore, it

does not take into account the fact that most infants lose weight in the early neonatal period.

The mean z score for length at birth was significantly lower than the growth standards by .37 of a standard deviation, but nearer to the growth standards at seven days and significantly greater than the growth standard at 12 weeks. The discrepancy noted in the length measurements at birth may be because length is the least accurate measurement or may be due to the study sample being from a poorer social background with a high percentage of smoking mothers. The z scores for the head circumference measurements were .37 of a standard deviation higher than the population standards at birth and .23 of a standard deviation higher at seven days, but only slightly higher than the population standards at 12 weeks (.010). This is quite difficult to explain as anything other than a small sample effect.

One of the main aims of this study was to examine slow growth in relation to cord blood leptin and ghrelin. Slow growth can be detected by looking at the change in weight in z scores, as previously described by Ong et al. (1999). After converting the weights to z scores, a decline in the z scores of –0.67 was used to identify slow growth in their study; this is equivalent to crossing down one inter-centile space on the Child Growth Foundation 1996/1 growth chart (Cole, 1995; Preece et al., 1996). As this study was inter alia a replication of theirs, the same criterion for slow growth was used in the same analyses. The change in the weight z score was calculated at 12 weeks of age. Seventeen infants had crossed down one inter-centile space and were demonstrating slow growth over the period birth to 12 weeks.

Figure 4.1a shows two female infants that showed normal growth and Figure 4.1b shows two female infants that showed slow growth. Figure 4.2a shows two male infants that showed normal growth and Figure 4.2b shows two male infants that showed slow growth.

Figure 4.1a Two female infants showing normal growth between birth and twelve weeks of age using the criterion of Ong et al (1999)

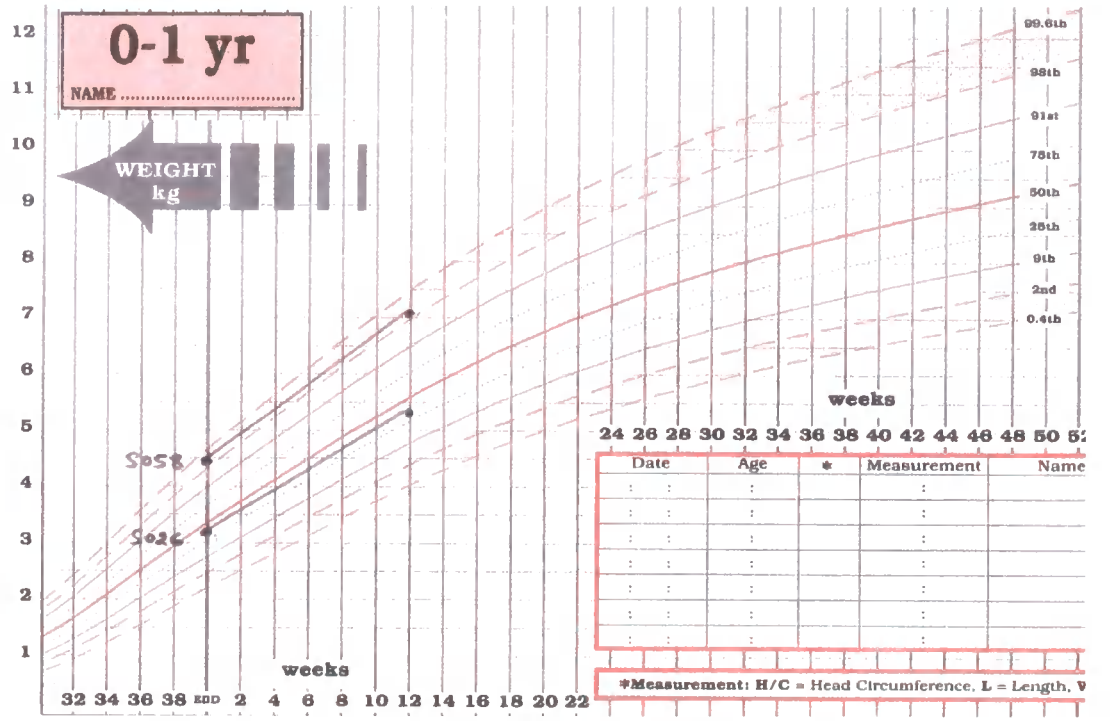


Figure 4.1b Two female infants showing slow growth between birth and twelve weeks of age

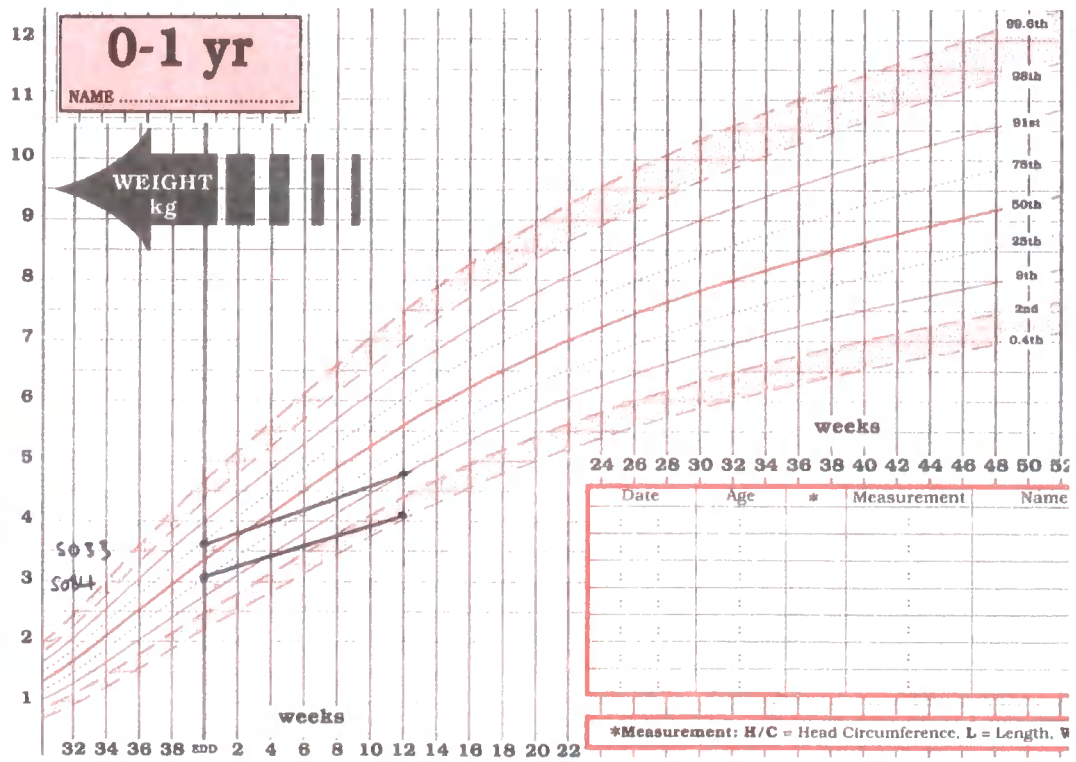


Figure 4.2a **Two male infants showing normal growth between birth and twelve weeks of age**

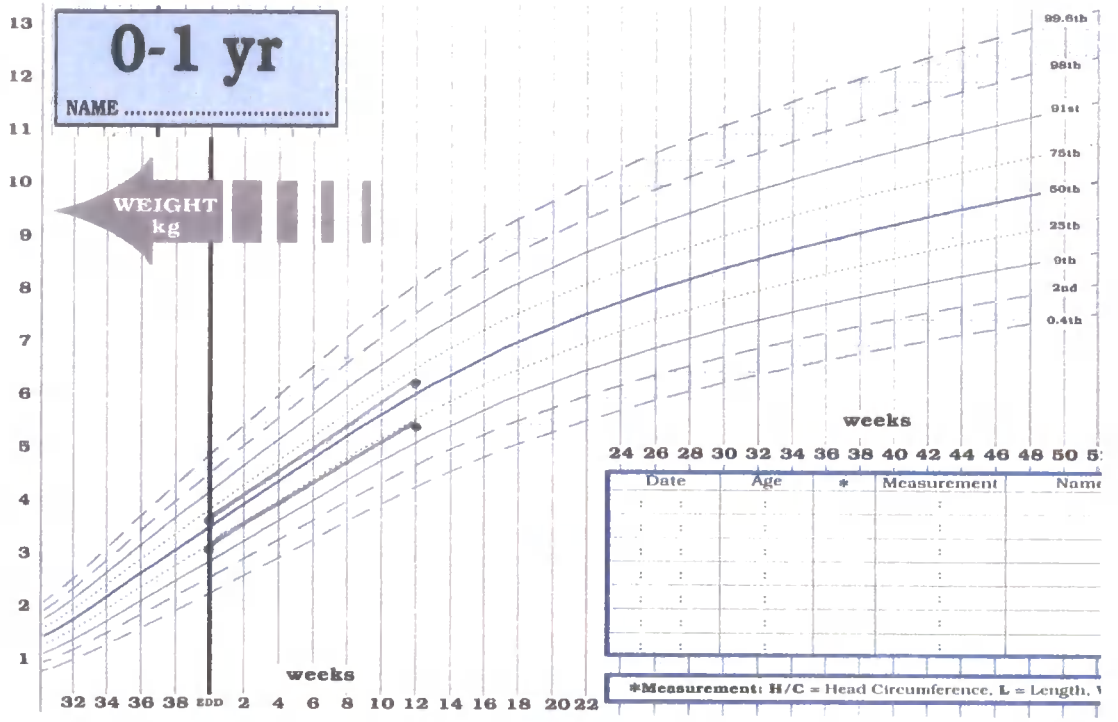
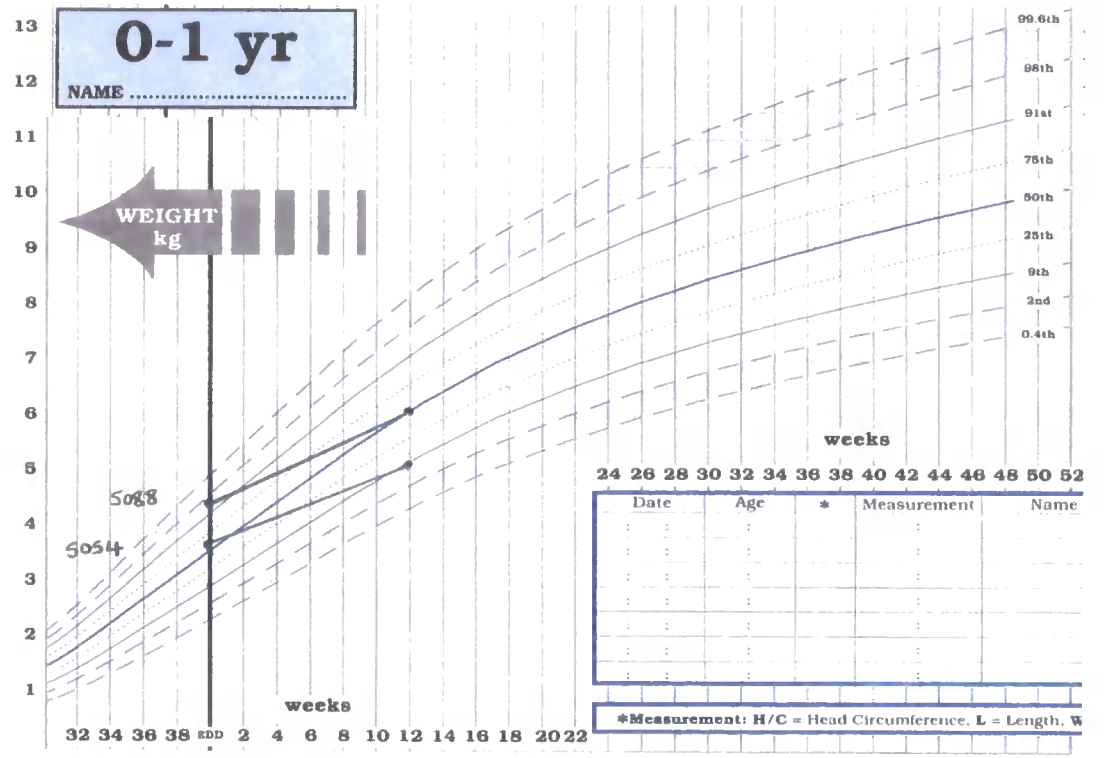


Figure 4.2b **Two male infants showing slow growth between birth and twelve weeks of age**



4.1.5 Determinants of size at birth and of infant growth

The main hypotheses of this thesis concern the relationships between cord blood leptin, ghrelin, milk intake and weight gain up to twelve weeks of age. Birthweight is a determinant of milk intake as shown in Chapter 3, section 3.2.4, and of growth in infancy up to twelve months of age (Cohen, Brown, Canahuati, Rivera, & Dewey, 1995; Kramer et al., 1985). Therefore factors that could potentially influence birthweight need to be examined and controlled for in subsequent analyses relating these hormones in cord blood to infant growth. Two important factors that have previously been found to influence the size of the infant at birth are parental size and maternal smoking.

Parental height has previously been related to the infant's weight at birth. However, because the size of the maternal effect is greater than the paternal effect (Kramer, 1987), more studies have shown a maternal influence (Elwood & Sweetnam, 1987; Hindmarsh, Geary, Rodeck, Kingdom, & Cole, 2002; Ounsted, Scott, & Ounsted, 1986; Strauss & Dietz, 1998) than a paternal influence (Klebanoff, Mednick, Schulsinger, Secher, & Shiono, 1998). In this study parental height was measured for 100 mothers and 58 fathers; the heights for the remaining 42 fathers were estimated by their female partners as described in the methodology (chapter 2.3.2). Summary statistics are presented in Table 4.5.

Table 4.5 Summary statistics for parental size (cm)

	Mean	SD	Median	Minimum	Maximum
Maternal height	161.9	6.3	161.5	149.5	179.4
Paternal height	176.9	7.8	177.8	159.0	193.0

Smoking in pregnancy also influences the weight of the infant at birth. Infants born to mothers who smoke are lighter than infants born to mothers who do not (Conter, Cortinovis, Rogari, & Riva, 1995; Elwood & Sweetnam, 1987; Godfrey, Barker, Robinson, & Osmond, 1997; Hindmarsh et al., 2002) and in one study they were found to be shorter at birth (Conter et al., 1995). Smoking in pregnancy was documented

from the case notes and each mother was asked verbally following delivery of the baby. As previously documented, 45% of the mothers smoked and 55% did not.

The contribution of parental height and smoking in pregnancy to the weight of the infant at birth was assessed by multiple regression analyses. In the first, maternal and paternal height and maternal smoking were entered as predictor variables in the regression with infant birthweight as the outcome variable. Table 4.6 shows the results of this analysis.

Table 4.6 Regression of infant birthweight on parental height and maternal smoking in pregnancy

		B	SE B	t	p
Constant		1.24	1.5	.84	.404
Maternal height (cm)		.017	.007	2.6	.010
Paternal height (cm)		−.0026	.005	−.49	.622
Mother smokes		−.266	.083	−3.2	.002
Analysis of Variance					
	df	Sum of squares	Mean square	F	p
Regression	3	2.7	.89	5.4	.002
Residual	96	15.9	.17		
Total	99	18.6			
R		.380			
R square		.145			
Adjusted R square		.118			
Standard error		.41			

Outcome variable: infant birthweight (kg)

From the data for this study, maternal height was a significant predictor of infant birthweight, but paternal height was not. Maternal smoking also significantly influenced the infant's weight at birth, showing that infants born to mothers that smoked in pregnancy were lighter.

This analysis was repeated but using infant length at birth as the outcome variable. Figure 4.7 shows the results of this analysis. Neither maternal nor paternal height significantly influenced the infant's length at birth. The effect of maternal smoking in pregnancy on birth length fell just short of significance (.06). These analyses show the detrimental effect of maternal smoking in pregnancy on birthweight (and probably on birth length).

Table 4.7 Regression of infant birth length (cm) on parental height (cm) and maternal smoking in pregnancy

		B	SE B	t	p
Constant		39.7	6.3	6.3	.000
Maternal height (cm)		.043	.03	1.6	.125
Paternal height (cm)		.019	.02	.85	.399
Mother smokes		−.660	.351	−1.9	.063
Analysis of Variance					
	df	Sum of squares	Mean square	F	p
Regression	3	19.3	6.4	2.2	.097
Residual	96	284.8	2.9		
Total	99	304.1			
R		.252			
R square		.063			
Adjusted R square		.034			
Standard error		1.7			

Outcome variable: infant length at birth (cm)

One of the outcome variables for the study for this thesis was infant growth, and as some studies have found that parental height was related to infant growth, it was important to assess this relationship and ascertain if parental height needed to be controlled for in future analyses. In this study, the contribution of parental height to infant weight at 12 weeks of age was assessed by multiple regression analyses. Birth length, birthweight, sex, 'twelve week age' maternal and paternal height were entered as predictor variables in the regression and the dependent variable was twelve week weight. The results are shown in Table 4.8.

Table 4.8 Regression of infant weight at 12 weeks on parental height, controlling for birthweight, birth length, sex and exact age at 12 weeks

	B	SE B	t	p
Constant	.89	2.6	.35	.730
Birth length (cm)	-.006	.04	-.14	.886
Birthweight (kg)	.97	.17	5.5	.000
Sex	.46	.11	4.1	.000
Twelve week age	.17	.08	1.9	.049
Maternal height (cm)	0.03	.009	.39	.699
Paternal height (cm)	0.06	.007	.94	.352

Analysis of Variance					
	df	Sum of squares	Mean square	F	p
Regression	6	27.0	4.5	15.2	.000
Residual	93	27.6	.29		
Total	99	54.6			

R	.704
R square	.495
Adjusted R square	.462
Standard error	.54454

Outcome variable: infant weight at twelve weeks, (kg)

This confirmed that infant sex and birthweight were significant predictors of weight gain to 12 weeks of age, but parental height had no influence on infant weight gain from birth to twelve weeks of age. There is, of course, an effect of maternal height operating through birthweight, as shown in Table 4.6. This analysis shows that there is no additional effect after birthweight is taken into account. Therefore parental height would not need to be controlled for in future analyses pertaining to infant weight gain as long as birthweight is taken into account.

Parental height has been related to the linear growth infants to 12 weeks of age (Hallman, Backstrom, Kantero, & Tiisala, 1971), male infants to one and two years (Smith et al., 1976), and female infants to two years (Smith et al., 1976). Therefore the same analysis was carried out as above, but using 12 week length as the outcome variable. The results are shown in Table 4.9.

Table 4.9 Regression of infant length at 12 weeks on parental height, controlling for birthweight, birth length, sex and exact age at 12 weeks

	B	SE B	t	p
Constant	16.9	6.1	2.8	.007
Birth length (cm)	.44	.10	4.3	.000
Birthweight (kg)	1.4	.42	3.4	.001
Sex	1.8	.27	6.8	.000
Twelve week age	.56	.20	2.9	.005
Maternal height (cm)	0.04	.02	1.9	.060
Paternal height (cm)	0.05	.02	3.0	.004

Analysis of Variance	df	Sum of squares	Mean square	F	p
Regression	6	338.0	56.3	33.5	.000
Residual	93	156.6	1.7		
Total	99	494.6			

R	.827
R square	.683
Adjusted R square	.663
Standard error	1.3

Outcome variable: infant length at 12 weeks (cm)

Birthweight, birth length, sex, twelve week age and paternal height were significant predictors of the length of the infant at twelve weeks. R square was .683 indicating that these variables account for 68.3% of the variance seen in infant length at 12 weeks of age. Maternal height was just short of significance (p=0.060). At 12 weeks the paternal influence on infant length appears to be greater than maternal influence, perhaps because the maternal influence is already reflected in the infants birthweight.

4.2 Relationship between milk intake and growth

4.2.1 Milk intake and growth up to seven days of age

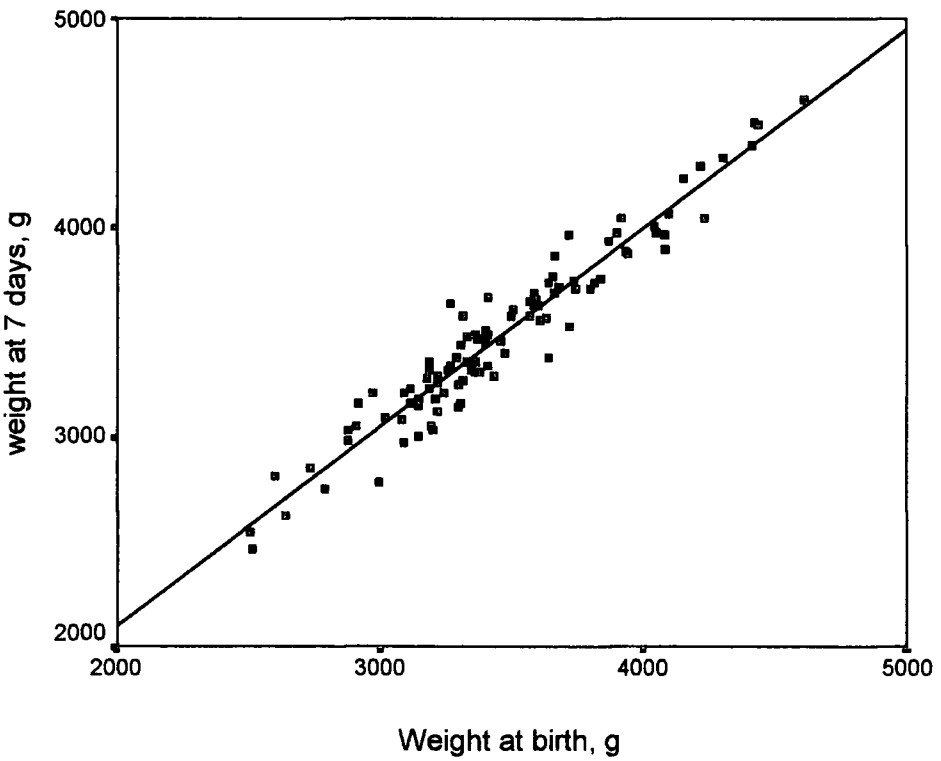
Many infants lose weight in the early neonatal period. Until recently the cause of the weight loss was deemed to be physiological and attributable to changes in the extracellular volume of water in the tissues. However with the advent of bio-electrical resistance studies, the weight loss was related to a decrease in total body water and body solids (Rodriguez et al., 2000). Breastfeeding infants have been shown to have a greater weight loss than formula fed infants (Avoa & Fischer, 1990; Dollberg, Lahav, & Mimouni, 2001; Maisels, Gifford, Antle, & Leib, 1988; Yamauchi & Yamanouchi,

1990), suggesting that type of feeding practice is a significant factor. The effect of type of feeding on weight loss was shown to be greatly reduced when breastfed infants were fed frequently and to demand (Jolly, Humphrey, Irons, Campbell-Forrester, & Weiss, 2000), with only 2 out of 21 breastfed infants losing weight by 3 days of age. This suggests that weight gain or loss might be related to milk intake. No studies have measured the degree of weight change in relation to milk intake. Therefore, weight change between birth and seven days of age was examined in relation to milk intake. Milk intake is related to birthweight, as was demonstrated in chapter 3, section 3.2.3, so this was used as a control variable.

Thirty eight percent of the infants in this study had lost weight by the seventh day, ranging from 20 to 260g. The weight of 5% of the infants remained within 10g of their birthweight and 57% of the infants had gained weight of between 10 to 370g by seven days.

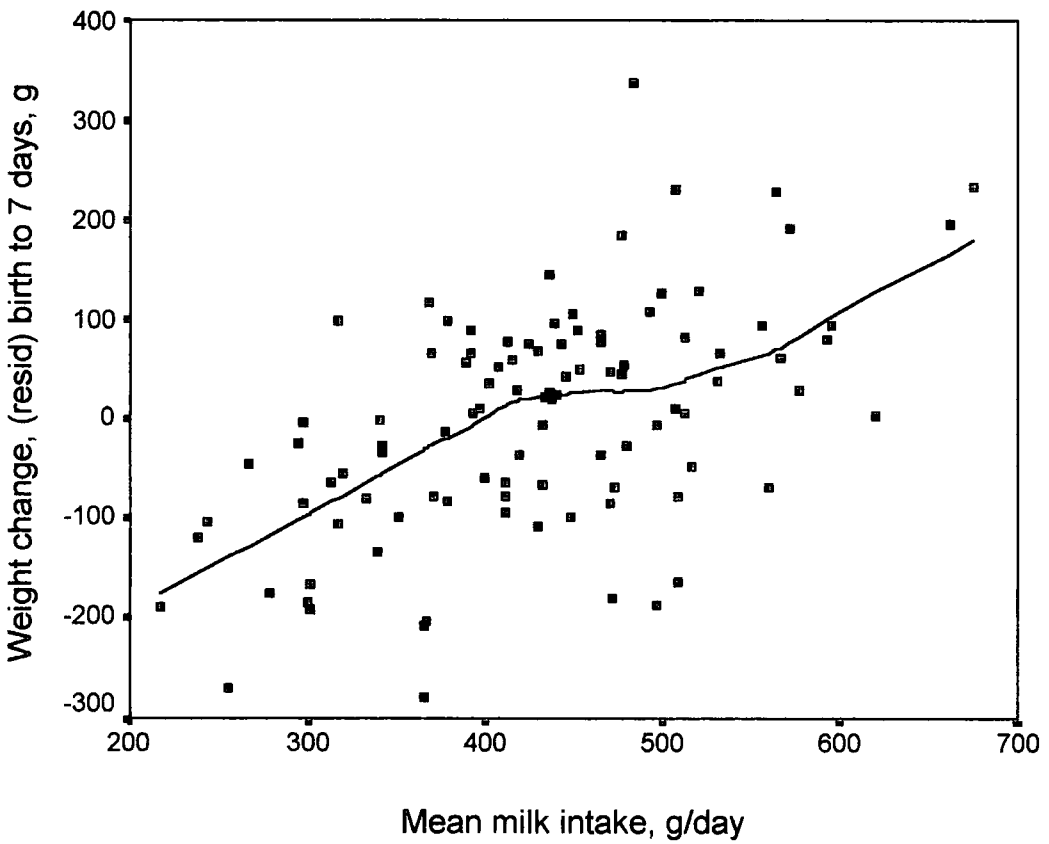
Figure 4.3 shows a scatter plot of weight at seven days against weight at birth. Inevitably there was a strong relationship between weight at birth and weight at seven days (Pearson's r = .96, $p<0.001$).

Figure 4.3 Relationship between weight at birth and weight at seven days



One way of assessing weight gain or loss at seven days is to calculate the difference in weight and relate this to milk intake. To allow for regression to the mean, this was achieved by calculating the difference between the value specified by the regression line and the achieved seven day weight on the y axis (Figure 4.3). The residuals are the difference between the specified values and the actual value. Figure 4.4 shows a scatter plot of the change in weight measured by the residuals and the mean milk intake for the infant, with a fitted lowess line. The lowess line is a non-parametric regression line (local averages) that makes no assumptions about the form of the relationship.

Figure 4.4 Scatter plot showing relationship between weight change (g) from birth to 7 days (measured by residuals) and mean milk intake (g), with lowess line



This graph shows clearly that the change in weight from birth to 7 days was related to the amount of milk the infant consumed. The infants with the lowest milk intake showed a weight loss, whereas the infants with the highest milk intake showed a weight gain.

The significance of this relationship was examined using multiple regression. Mean milk intake over the first six days of life was used as a predictor variable, controlling for birth-weight. The dependent variable was weight at seven days (Table 4.10). Birth-weight and mean milk intake over the first six days of life were each highly significant, with an R square of .95, accounting for 95% of the variance.

Table 4.10 Regression of seven day weight on mean milk intake controlling for birthweight

	B	SE B	t	p
Constant	.07	.08	.85	.399
Birthweight (kg)	.89	.02	38.6	.000
Mean milk intake (kg)	.77	.11	7.2	.000

Analysis of Variance					
	df	Sum of squares	Mean square	F	p
Regression	2	17.2	8.6	982.6	.000
Residual	97	.85	.008		
Total	99	18.1			

R	.97
R square	.95
Adjusted R square	.95
Standard error	.09

Outcome variable: seven day weight, kg

4.2.2 Milk consumption and growth up to twelve weeks of age

Since milk intake over the first week of life was a significant predictor of weight gain to seven days (seven day weight adjusted for birthweight), and energy intake at three months of age has previously been found to be a significant predictor of weight gain to 12 months of age (Stunkard, Berkowitz, Stallings, & Schoeller, 1999), it is possible that milk intake in the early neonatal period could predict weight gain up to 12 weeks of age. This was assessed by analyses using the six day milk intake as a predictor variable, controlling for birthweight and exact age at 12 weeks (Table 4.11). The six day milk intake was used because there was missing milk data on day 7 (as previously described in chapter 3, section 3.2.1).

Table 4.11 Regression of twelve week weight on milk intake day 6 controlling for birthweight and exact age at 12 weeks

		B	SE B	t	p
Constant		2.3	.47	4.9	.000
Birthweight (kg)		.96	.15	6.4	.000
Twelage		.16	.09	1.8	.071
Milk intake day 6 (kg)		.52	.47	1.1	.276
Analysis of Variance					
	df	Sum of squares	Mean square	F	p
Regression	3	22.1	7.4	21.8	.000
Residual	96	32.5	.34		
Total	99	54.6			
R		.64			
R square		.41			
Adjusted R square		.39			
Standard error		.58172			

Outcome variable:12 week weight, kg

The infants'milk intake on day six did not predict the infants' weight gain to 12 weeks of age. The only significant factor in this model was birthweight, which has previously been demonstrated.

4.3 Summary of main results for chapter 4

In this chapter, data on the weight gain and other growth measures of the infant were examined, and related to milk intake and other independent variables. The principal findings were:

1) Maternal height and smoking in pregnancy were significantly related to the infant's weight at birth.

2) Paternal height was significantly related to infant length at 12 weeks.

3) The mean z scores for weight and length of the infants in the study were .05 and .37 of a standard deviation lower than the British Growth Standards at birth, but .06 and .26 of a standard deviation higher by 12 weeks of age.

4) The mean z score for head circumference was .37 at birth and .09 at 12 weeks, higher than the standard at birth, but similar at 12 weeks.

5) 38% of infants had lost weight by day 7, 5% remained the same and 57% of the infants had gained weight.

6) Birthweight and milk intake over the first six days of life were significantly associated with weight gain to 7 days, accounting between them for 95% of the variance in weight gain.

Chapter 5 Results

Relationship between cord blood hormones, milk intake, feeding frequency, size at birth and growth up to twelve weeks of age

5.1 Cord blood leptin

5.1.1 Cord blood leptin, milk intake and feeding frequency

One of the main hypotheses of the study reported in this thesis is that infants with higher cord leptin levels would have a lower milk intake than infants with lower cord leptin levels. This hypothesis was based on the findings that infants with leptin deficiency (Montague et al., 1997, 1998; Strobel, Issad, Camoin, Ozata, & Strosberg, 1998) or leptin resistance (Clément et al., 1998) are hyperphagic and gain weight rapidly. These findings come, however from infants with major gene defects, and a key question is whether leptin levels in the normal range are related to milk intake and feeding frequency. No previous studies have directly looked at the relationship between cord blood leptin and feeding in the early neonatal period.

Leptin was measured in the cord blood of 49 females and 50 males. One female sample was accidentally lost during the immunoassay. Results are shown in Figure 5.1. Mean cord blood leptin was 10.1 ng/ml with a standard deviation of 6.7. The minimum and maximum values were 1.6 to 36.7 ng/ml. Because the distribution was skewed the raw values were transformed to natural logarithms (to base e) to produce a more normal distribution for use in further analyses. This is shown in Figure 5.2.

The relationship between cord blood leptin, feeding frequency and milk intake was initially assessed using several scatterplots, each fitted with lowess lines. The feeding variables have been previously defined in chapter 3, section 3.2.1. The six feeding variables used were 'time to first feed', 'amount of first feed', 'number of feeds day 1', 'the amount of milk consumed day 1', 'mean number of feeds day 1 to 6' and 'mean milk intake day 1 to 6'. These are shown in Figures 5.3a to 5.3f. Inspection of each scatterplot suggests that there was no relationship between cord blood leptin and the feeding variables measured. The data were summarized by the correlation of cord blood leptin (Pearson's r) with each feeding variable. Results are shown in Table 5.1.

Figure 5.1 Histogram showing distribution of cord blood leptin levels

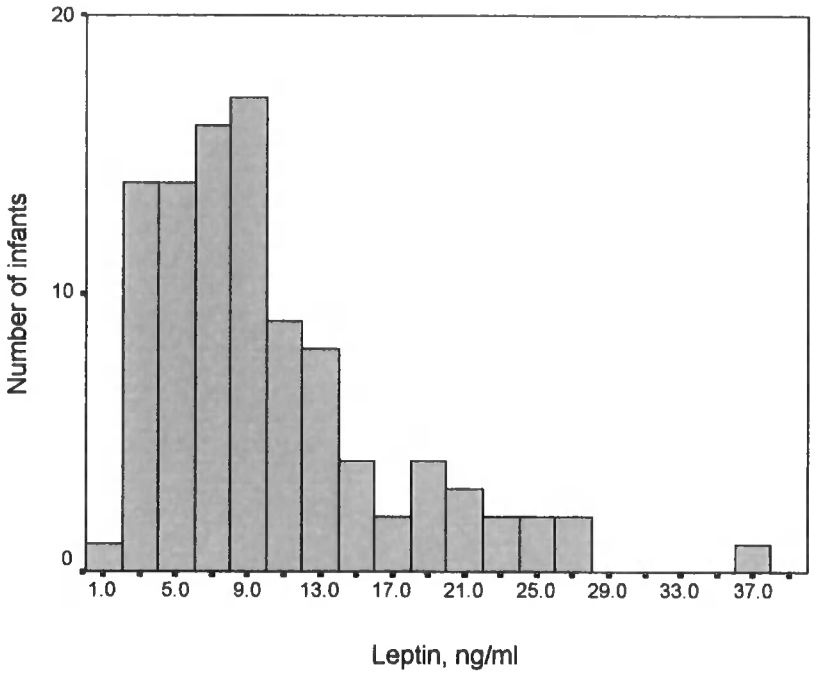
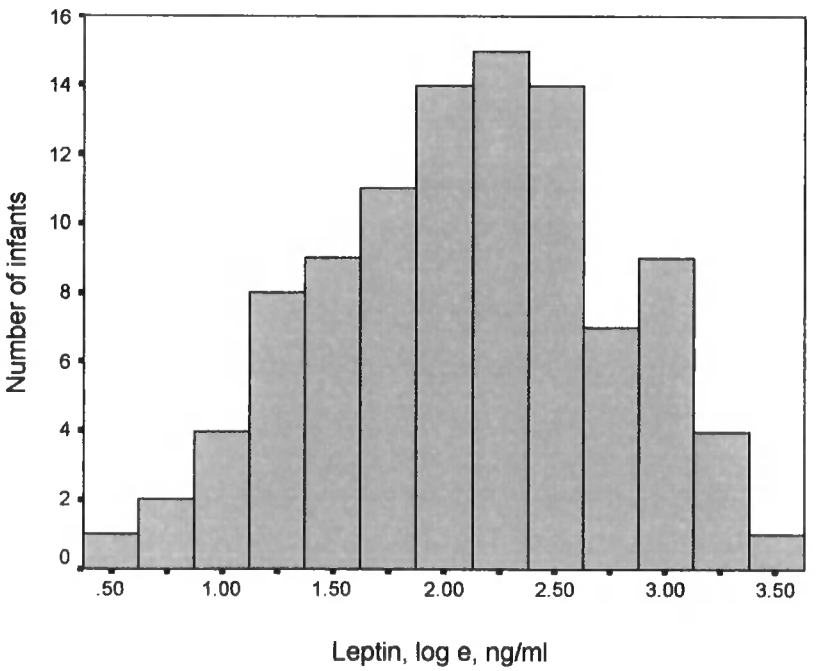


Figure 5.2 Histogram showing distribution of cord blood leptin levels following logarithmic transformation



Figures 5.3a-5.3f Scatterplots showing the relationship between cord blood leptin levels and the six feeding variables

Figure 5.3a Time to first feed (minutes)

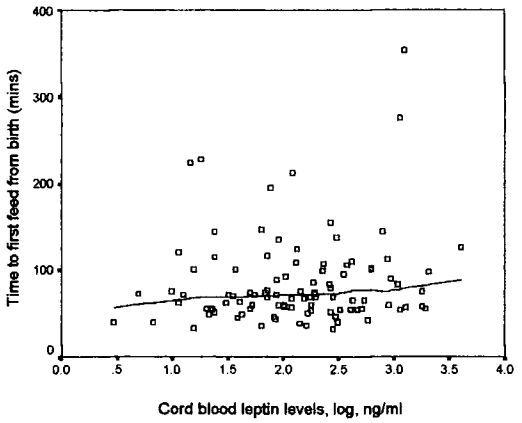


Figure 5.3b Amount of first feed (g)

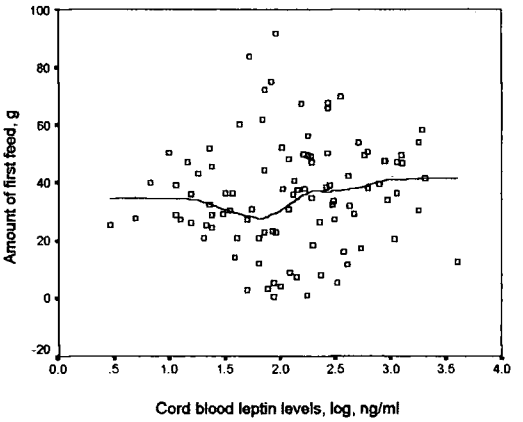


Figure 5.3c Number of feeds day 1

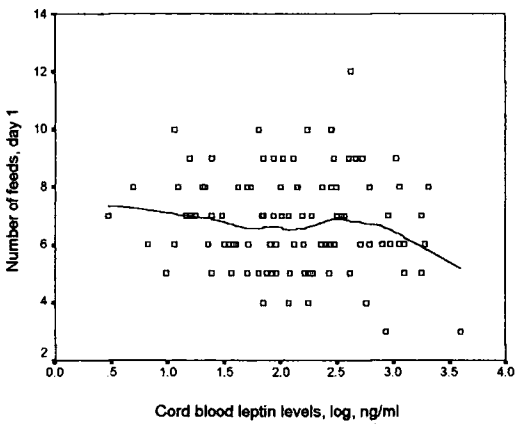


Figure 5.3d Milk intake day 1 (g)

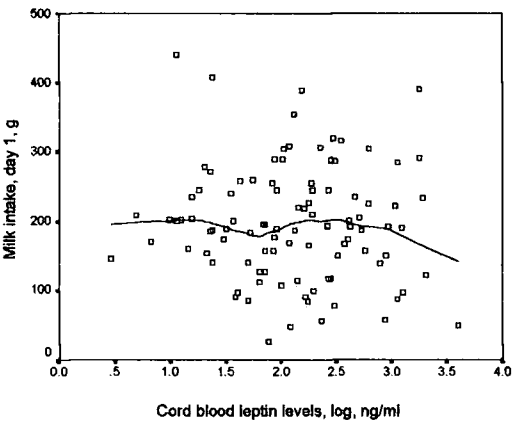


Figure 5.3e Mean number of feeds (day 1 to 6)

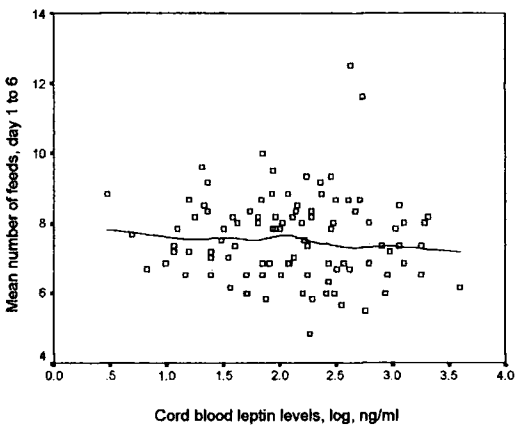


Figure 5.3f Mean milk intake (day 1 to 6, g)

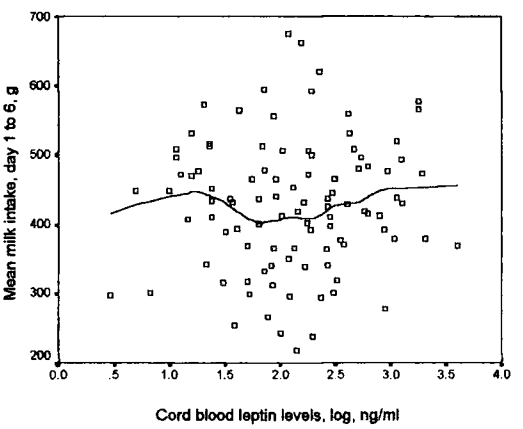


Table 5.1 Correlation of cord blood leptin (log) and feeding variables

N = 99	r	p
Time to first feed	.13	.216
Amount of first feed	.10	.323
Number of feeds day 1	−.11	.293
Milk intake day 1	−.06	.556
Mean number of feeds (day 1 – 6)	−.05	.657
Mean milk intake (day 1 – 6)	.06	.548

Table 5.1 confirms that there was no significant correlation between cord blood leptin and any of the feeding variables measured.

As milk intake has previously been shown in this thesis to be significantly related to birthweight (Chapter 3, section 3.2.3), it is possible that this relationship might be masking the relationship between milk intake and leptin. It was therefore important to conduct analyses that could control for birthweight. Two further regression analyses were carried out to examine the possibility of a relationship between cord blood leptin and milk intake. Milk intake on day 1 was used in the analyses in case leptin had a short duration effect. Mean milk intake on days 1 to 6 was used in case leptin had a longer term effect upon appetite regulation.

The first analysis was the regression of milk intake for day 1 on birth weight and cord blood leptin (Table 5.2a). There was no significant relationship between the infants milk intake on day 1 and birthweight ($t = 1.3$, $p = 0.189$) or cord blood leptin levels ($t = -1.3$, $p = 0.184$). The second analysis used the regression of the mean milk intake from day one to day six on birth weight and cord blood leptin (Table 5.2b). This showed a significant relationship between mean milk intake and birthweight ($t = 3.6$, $p = 0.001$), but no relationship with leptin ($t = -1.1$, $p = 0.274$). Therefore, in this study cord blood leptin was not significantly related to these feeding variables even after taking birthweight into account.

Table 5.2a Regression of milk intake (day 1) on cord blood leptin, controlling for birth weight

		B	SE B	t	p
Constant		105.4	66.8	1.6	.118
Birthweight (kg)		38.4	21.5	1.8	.077
Leptin (log e)		−19.1	14.3	−1.3	.184
Analysis of Variance					
	df	Sum of squares	Mean square	F	p
Regression	2	23961.3	11980.6	1.8	.179
Residual	96	647219.6	6741.9		
Total	98	671180.8			
R		.189			
R square		.036			
Adjusted R square		−.016			
Standard error		82.1			

Outcome variable: milk intake (g) day 1

Table 5.2b Regression of mean milk intake (day 1 to 6) on cord blood leptin, controlling for birth weight

		B	SE B	t	p
Constant		165.6	72.4	2.3	.024
Birthweight (kg)		86.4	23.3	3.7	.000
Leptin (log e)		−16.9	15.4	−1.1	.274
Analysis of Variance					
	df	Sum of squares	Mean square	F	p
Regression	2	112360.5	56180.2	7.1	.001
Residual	96	759880.8	7915.4		
Total	98	872241.2			
R		.359	.359		
R square		.129	.129		
Adjusted R square		.111	.111		
Standard error		88.9	88.9		

Outcome variable: mean milk intake (g) day 1 to 6

5.1.2 Cord blood leptin, size at birth and growth up to twelve weeks of age

Ong et al. (1999) showed that cord blood leptin levels were significantly related to infant weight gain up to four months of age. As there was no effect of cord blood leptin on milk intake in the above analyses, it was important to see whether this result of Ong et al could be replicated. Based on their data, one of the main hypotheses of this thesis

was that infants with higher cord blood leptin levels would have slower weight gain over the first three months of life.

Cord blood leptin has previously been related to birthweight, birth length and placental weight (Chapter 1, section 1.2.6), so this needs to be taken into account. The data were initially examined using simple correlation (Pearson's r) (Table 5.3) to examine the relationship between cord blood leptin and size at birth, 7 days and 12 weeks of age. Infant length and head circumference were included in the analyses to try and disentangle adiposity from linear growth, both of which are reflected in birthweight. At birth, cord blood leptin levels were significantly related to placental weight, weight and length. The relationship between cord blood leptin and weight was still evident at seven days, but the relationship with length weakened and became non-significant. There was no significant relationship between cord blood leptin and size at 12 weeks of age.

Table 5.3 Correlation of cord blood leptin (log e) and size at birth, seven days and twelve weeks

	N = 99	r	p
Birth	Placental weight (g)	.32	.001
	Weight (kg)	.45	.000
	Length (cm)	.21	.040
	Head circumference (cm)	.07	.482
7 days	Weight (kg)	.46	.000
	Length (cm)	.19	.056
	Head circumference (cm)	.15	.144
12 weeks	Weight (kg)	.16	.259
	Length (cm)	.05	.633
	Head circumference (cm)	−.06	.534

The relationship between cord blood leptin, placental weight, birthweight and length at birth were examined further by multiple regression analyses. Sex of the infant was

introduced as a predictor variable in this analysis as there is some previous evidence for sex difference in cord leptin levels. Figure 5.4 shows that the female infants had higher cord blood leptin levels than the male infants. An independent t test confirmed that there was a significant difference between female cord blood leptin levels (log e) (mean 2.3, SD .59) and male cord blood leptin levels (mean 1.9, SD .64); $t = 3.7$ with 97 df, $p < .001$. Therefore the predictor variables used were placental weight, birthweight, birth length and sex of the infants. The outcome variable was cord blood leptin levels. Table 5.4 shows this analysis. There was a significant effect of birthweight ($t = 4.4$, $p < .001$) and sex ($t = -5.0$, $p < .001$) on the cord blood leptin levels of the infant, but no effect of placental weight or birth length.

Figure 5.4 Box plot showing difference between cord blood leptin levels in male and female infants

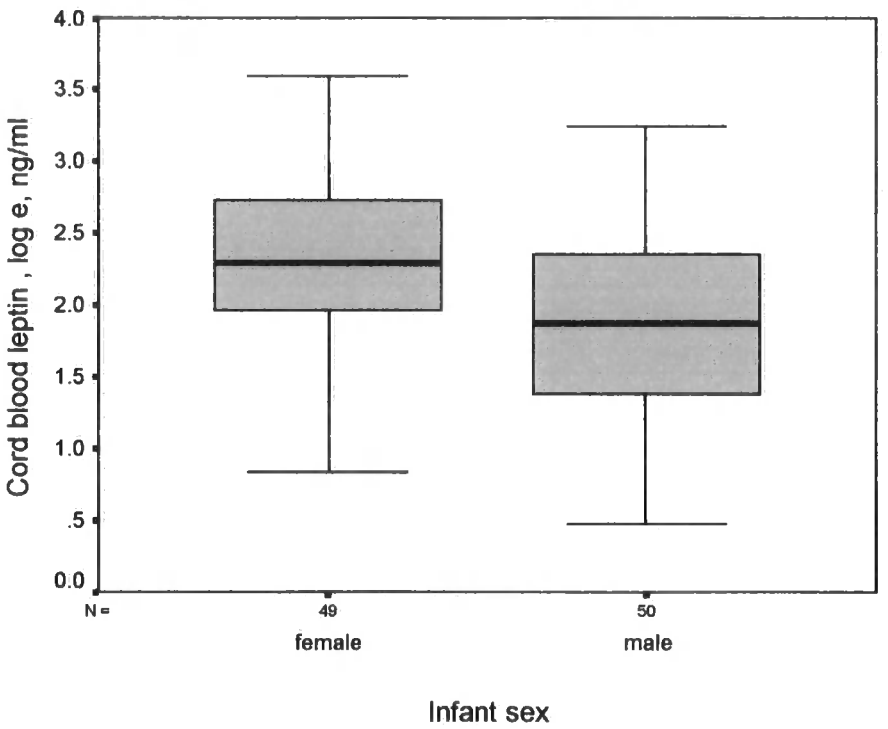


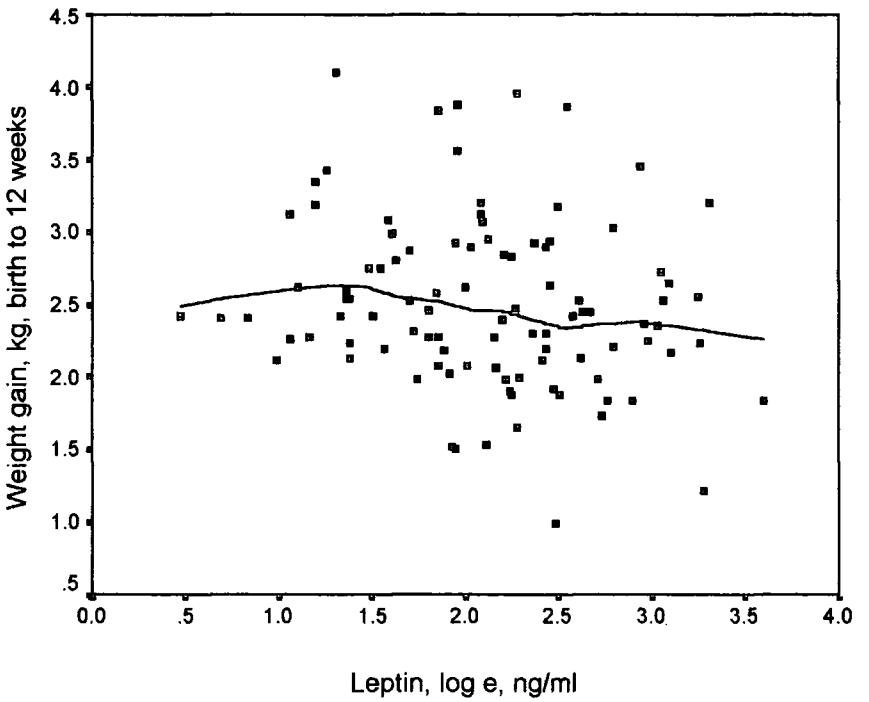
Table 5.4 Regression of cord blood leptin levels on placental weight, birthweight, birth length and sex of the infant

		B	SE B	t	p
Constant		1.5	1.7	.88	.381
Placental weight (g)		.0002	.000	.376	.708
Birthweight (kg)		.84	.19	4.4	.000
Birth length		-.004	.04	-1.1	.294
Sex		-.53	.107	-5.0	.000
Analysis of Variance					
	df	Sum of squares	Mean square	F	p
Regression	4	16.1	4.0	14.7	.000
Residual	94	25.6	.273		
Total	98	41.7			
R		.621			
R square		.385			
Adjusted R square		.359			
Standard error		.5221			

Outcome variable: cord blood leptin levels, ng/ml

As it has previously been shown that birthweight and sex are significant predictors of cord blood leptin levels (Table 5.4), and also of weight gain up to 12 weeks of age (Chapter 4, section 4.1.5). It was therefore important to control for these variables when testing the hypothesis outlined at the beginning of the chapter. To allow replication of the results of Ong et al (1999), the following analyses used the same procedure that they followed, which used weight gain as the outcome variable instead of weight at 12 weeks. Essentially, the analysis is the same, if we have two weights, say birthweight and 12 week weight, predicting 12 week weight from birthweight is the same as predicting change in weight to 12 weeks from birthweight (Plewis, 1985). A new variable was produced called 'weight gain', which was calculated by subtracting birth weight from the weight measured at twelve weeks. A scatter plot of the relationship between weight gain and cord blood leptin levels is presented in Figure 5.5. A lowess line was fitted to the data. For this relationship, $r = -.19$, $p = .066$, just short of significance.

Figure 5.5 A scatterplot with lowess line fitted, showing relationship between cord blood leptin and weight gain from birth to twelve weeks



Initial inspection of Figure 5.5 does not suggest a strong relationship between cord blood leptin levels and infant weight gain from birth to twelve weeks of age. One concern is that some of the infants were measured later than twelve weeks of age and would have had a greater weight gain in the extended period. To control for this the variable 'twelve week age' was used ('the exact age at the twelve week measurement' as described in Chapter 4, section 4.1.3), and the data were examined using multiple regression analysis (Table 5.5). This analysis examined the relationship between weight gain from birth to twelve weeks and four predictor variables, birth weight, sex, twelve week age and cord blood leptin levels. Birthweight was used as a control variable as it is itself related to weight gain (Fergusson, Horwood, & Shannon, 1980). There was a significant relationship between weight gain and sex ($t = 3.3$, $p = 0.001$) and twelve-week age ($t = 2.2$, $p = 0.031$), but no significant relationship between weight gain and birthweight ($t = -0.31$, $p = 0.758$) or cord blood leptin ($t = -0.71$, $p = 0.479$).

Table 5.5 Regression of weight gain on cord blood leptin, controlling for birthweight, sex and exact age at the twelve week measurement

	B	SE B	t	p
Constant	2.4	.44	5.3	.000
Twelve week age	.18	.08	2.2	.031
Sex	.41	.13	3.3	.001
Birthweight (kg)	.018	.15	.12	.905
Leptin (log e)	−.075	.11	−.71	.479

Analysis of Variance					
	df	Sum of squares	Mean square	F	p
Regression	4	6.3	1.6	5.3	.001
Residual	94	27.7	.294		
Total	98	34.0			

R	.430
R square	.185
Adjusted R square	.150
Standard error	.5425

Outcome variable: weight gain (kg), birth to twelve weeks

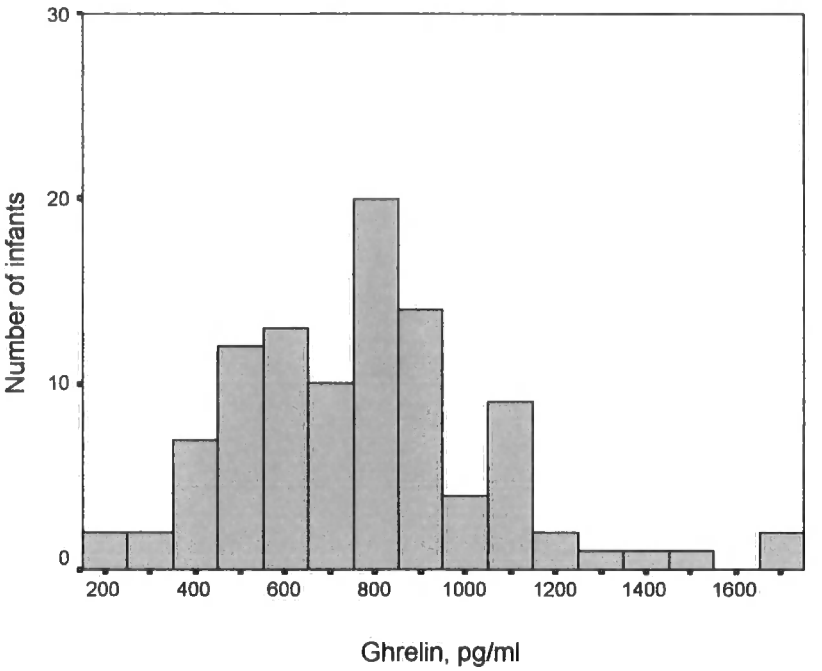
5.2 Cord blood ghrelin

5.2.1 Cord blood ghrelin, milk intake and feeding frequency

Ghrelin is a newly identified peptide that has been implicated in the control of appetite in adult humans. In adults higher levels of ghrelin induced hunger and food seeking behaviour (Wren et al., 2001) and ghrelin levels rose before feeding commenced (Shiiya et al., 2002). Ghrelin has recently been detected in the cord blood of the human infant but as yet its role in the infant is unclear (Castellino et al., 2002; Chanoine, K, & Wong, 2002; Coutant et al., 2002). No previous studies have looked at the relationship between cord blood ghrelin and feeding behaviour. It is possible based on the above evidence that infants with higher cord ghrelin levels will initiate feeding sooner and have a higher milk intake than infants with lower ghrelin levels. This was one of the main hypotheses of this study.

Mean cord blood ghrelin was 760.9 pg/ml with a standard deviation of 282.8; the minimum and maximum values were 210.0 to 1670.0 pg/ml. A histogram of the data shows a reasonably normal distribution (Figure 5.6).

Figure 5.6 Histogram showing distribution of cord blood ghrelin levels



The relationship between cord blood ghrelin, feeding behaviour and milk intake was initially assessed using scatterplots, each fitted with lowess lines. The variables classified as feeding behaviour have been previously defined in chapter 3, section 3.2.1. As in the analyses using leptin, the six feeding variables used were 'time to first feed', 'amount of first feed', 'number of feeds day 1', 'the amount of milk consumed day 1', 'mean number of feeds day 1 to 6' and 'mean milk intake day 1 to 6'. These are shown in Figures 5.7a to 5.7f. Inspection of each scatterplot suggests that there was no relationship between cord blood ghrelin and the feeding variables measured. The data were summarized by correlation of cord blood ghrelin with each feeding variable. Results are shown in Table 5.6.

Figures 5.7a-5.7f Scatterplots showing the relationship between cord blood ghrelin levels and the six feeding variables

Figure 5.7a Time to first feed (minutes)

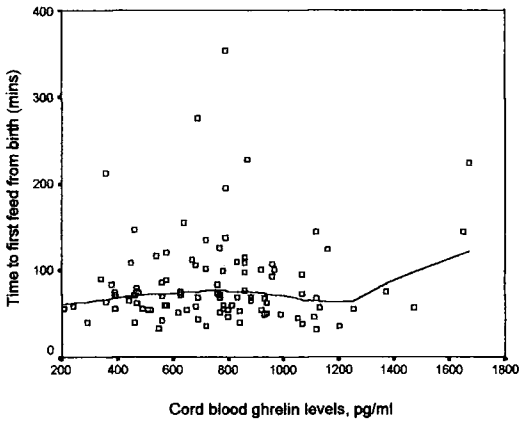


Figure 5.7b Amount of first feed (g)

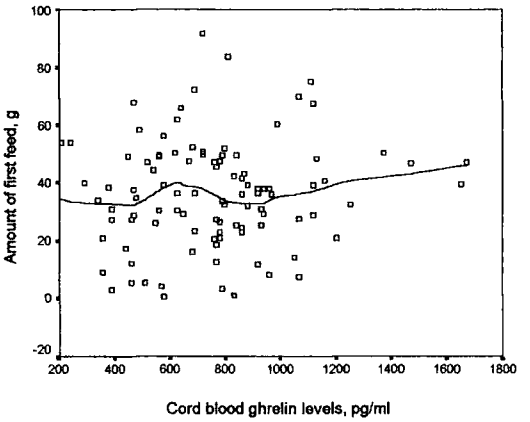


Figure 5.7c Number of feeds day 1

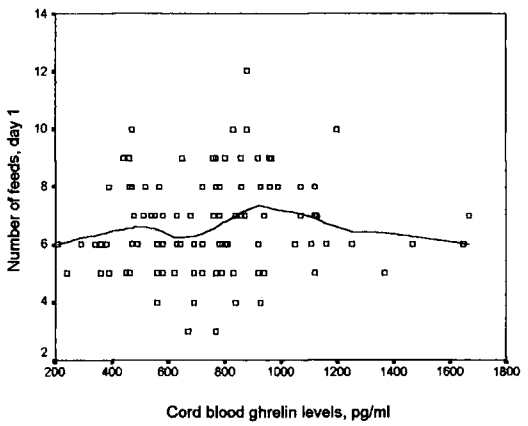


Figure 5.7d Milk intake day 1 (g)

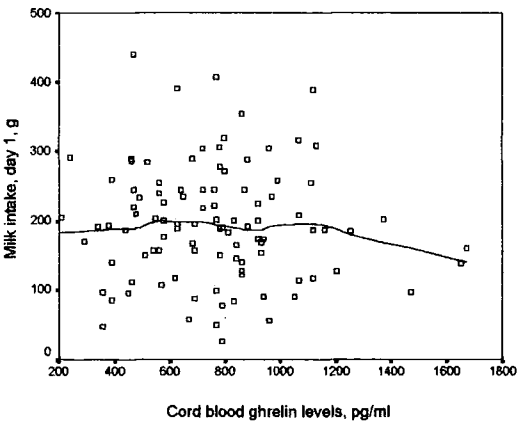


Figure 5.7e Mean number of feeds (day 1 to 6)

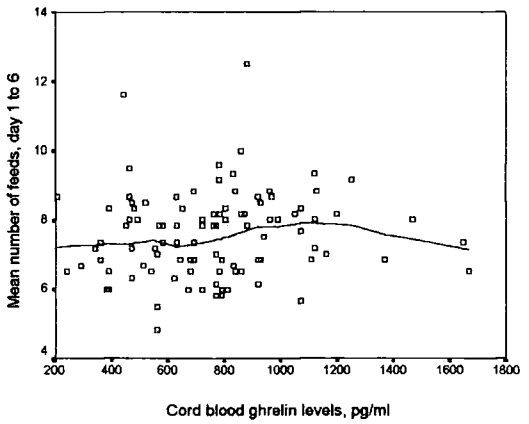


Figure 5.7f Mean milk intake (day 1 to 6, g)

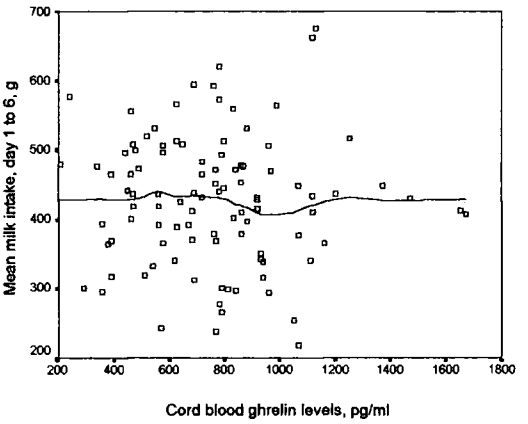


Table 5.6 Correlation of cord blood ghrelin pg/ml and feeding variables

N = 100	r	p
Time to first feed	.10	.298
Amount of first feed	.10	.319
Number of feeds day 1	.09	.361
Milk intake day 1	−.06	.541
Mean number of feeds (day 1 – 6)	.09	.387
Mean milk intake (day 1 – 6)	−.02	.867

Table 5.6 shows there were no significant relationships between cord blood ghrelin levels and any of the feeding variables measured.

The hypothesis was tested further by examining the regression of time to first feed on birth weight and cord blood ghrelin (Table 5.7). The relationship between time to the first feed and birthweight just failed to reach significance ($t = 1.9$, $p = 0.06$). There was no relationship between time to first feed and cord blood ghrelin ($t = 1.3$, $p = 0.199$).

Table 5.7 Regression of time to first feed on cord blood ghrelin pg/ml, controlling for birth weight

	B	SE B	t	p
Constant	−10.8	45.4	−.237	.813
Birthweight (kg)	22.7	11.9	1.9	.060
Ghrelin pg/ml	.024	.018	1.3	.199

Analysis of Variance					
	df	Sum of squares	Mean square	F	p
Regression	2	12299.8	6149.9	2.4	.099
Residual	97	251317.7	2590.9		
Total	99	263617.6			

R	.22
R square	.05
Adjusted R square	.027
Standard error	50.9

Outcome variable: time to first feed (minutes)

Adult humans administered exogenous doses of ghrelin showed an increased food intake (Wren et al., 2001). Two summary statistics of infant milk intake were used to examine the relationship between ghrelin levels and milk intake. These were total milk intake on day 1 and the mean milk intake for day 1 to day 6.

The regression of milk intake day 1 on birthweight and cord blood ghrelin is shown in Table 5.8a. There was no relationship between milk intake on day 1 and birthweight ($t = 1.1$, $p = 0.270$) or cord blood ghrelin ($t = -.47$, $p = 0.641$). A second analysis examined the relationship between mean milk intake day 1 to day 6 and the same two predictor variables (table 5.8b). The relationship with birthweight was statistically significant ($t = 3.6$, $p<.001$). The relationship with cord blood ghrelin was not statistically significant ($t = .282$, $p = 0.778$).

Table 5.8a Regression of milk intake on day 1 on cord blood ghrelin pg/ml, controlling for birth weight

	B	SE B	t	p
Constant	132.6	74.2	1.8	.077
Birthweight (kg)	21.6	19.5	1.1	.270
Ghrelin pg/ml	−.013	.030	−.47	.641

Analysis of Variance					
	df	Sum of squares	Mean square	F	p
Regression	2	11125.1	5562.6	.80	.450
Residual	97	670264.9	6909.9		
Total	99	681390.1			

R	.128
R square	.016
Adjusted R square	−.004
Standard error	83.1

Outcome variable: milk intake, g, day 1

Table 5.8b Regression of mean milk intake (day 1 to day 6) on cord blood ghrelin, controlling for birth weight

	B	SE B	t	p
Constant	161.5	79.4	2.0	.045
Birthweight (kg)	75.1	20.8	3.6	.000
Ghrelin (pg/ml)	9.00E-02	.032	.282	.778

Analysis of Variance					
	df	Sum of squares	Mean square	F	p
Regression	2	103259.9	51629.9	6.5	.002
Residual	97	769180.7	7929.6		
Total	99	872440.6			

R	.344
R square	.118
Adjusted R square	.100
Standard error	89.0

Outcome variable mean milk intake, g, (day 1to day 6)

5.2.2 Cord blood ghrelin, size at birth and growth up to 12 weeks of age

Ghrelin affects both appetite and growth. Hyperphagia and weight gain was shown in rats following a 12 day of continuous intracerebroventricular infusion of ghrelin (Nakazato et al., 2001). In human adults, intravenous injection of ghrelin resulted in a surge of growth hormone (Arvat et al., 2001; Broglio et al., 2001; Takaya et al., 2000). Both these findings suggest a role for ghrelin in growth, perhaps acting via its effect on appetite (Nakazato et al., 2001). In infants, cord blood ghrelin levels were positively related to cord levels of growth hormone (Castellino et al., 2002) suggesting a role for ghrelin in prenatal growth, although two studies showed that ghrelin levels were not related to birthweight (Castellino et al., 2002; Coutant et al., 2002) or length at birth (Castellino et al., 2002). There are as yet no studies that have examined the relationship between cord blood ghrelin levels and growth. One of the main hypotheses for this study was that infants with higher ghrelin levels would gain more weight over the first three months of life than infants with lower ghrelin levels. As birthweight is a significant predictor of weight gain, it was important to examine whether cord ghrelin levels were related to size of the infant at birth in the study data. Placental weight was also included as it is related to prenatal growth. The data were initially examined by simple correlation with Pearson's r (Table 5.9). There was no relationship between cord blood ghrelin and size at birth, size at seven days or size at 12 weeks.

Table 5.9 Correlation of cord blood ghrelin pg/ml and size at birth, seven day and twelve week measurements

	N = 100	r	p
Birth	Placental weight (g)	−.09	.339
	Weight (kg)	−.13	.207
	Length (cm)	−.04	.710
	Head circumference (cm)	.07	.495
7 days	Weight (kg)	−.12	.238
	Length (cm)	.04	.656
	Head circumference (cm)	.03	.797
12 weeks	Weight (kg)	−.03	.799
	Length (cm)	−.02	.881
	Head circumference (cm)	.05	.591

The data were further examined by multiple regression analysis using the variable 'twelve week age' to take into account the exact age of the infant (Table 5.10). The analysis examined the relationship between weight gain from birth to 12 weeks and four predictor variables, birth weight, sex, twelve-week age and cord blood ghrelin levels. Sex and twelve-week age have previously been identified as significant predictors of weight gain and were confirmed as such again in this regression. There was a significant relationship between weight gain and sex ($t = 4.2$, $p < 0.001$) and weight gain and twelve-week age ($t = 2.2$, $p = 0.033$), but no significant relationship between weight gain and birthweight ($t = -0.23$, $p = 0.817$) or weight gain and cord blood ghrelin ($t = 0.93$, $p = 0.356$).

Table 5.10 Regression of weight gain on cord blood ghrelin pg/ml, controlling for birthweight, sex and exact age at twelve weeks

	B	SE B	t	p
Constant	2.3	.49	4.5	.000
Twelve week age	.17	.08	2.2	.033
Sex	.46	.11	4.2	.000
Birthweight (kg)	-.029	.13	-.23	.817
Ghrelin (pg/ml)	–.00017	.00	.928	.356

Analysis of Variance					
	df	Sum of squares	Mean square	F	p
Regression	4	6.5	1.6	5.6	.000
Residual	95	27.6	.291		
Total	99	34.1			

R	.438
R square	.192
Adjusted R square	.157
Standard error	.5391

Outcome variable: weight gain (kg), birth to 12 weeks

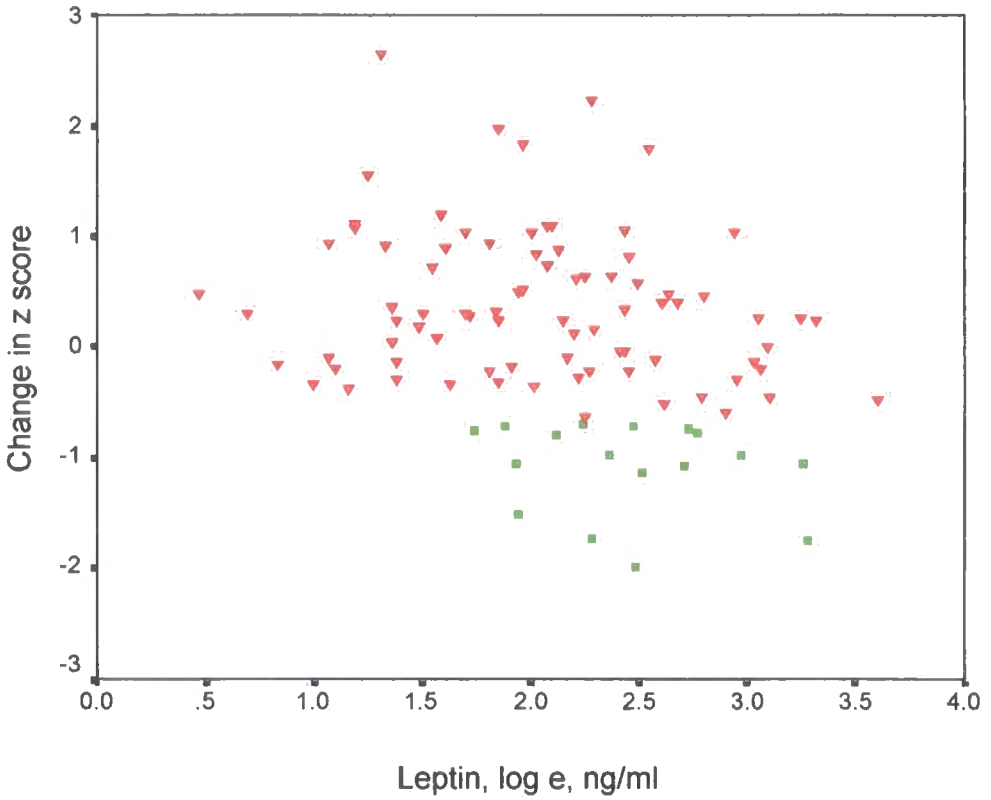
5.2.3 Cord blood leptin, ghrelin and slow growth

Since the previous analysis did not replicate Ong et al's (1999) results, it was important to examine this discrepancy using the same methods of analyses that they used. Ong et al. used an alternative method of looking at the weight gain data. This method was previously described in chapter 4.1.4. It identifies those infants that have shown the slowest growth defined by change in z scores. Ong et al. (1999) divided their sample into groups after converting their weights to z scores. They used a decline in the z scores of –0.67 to identify slow growth; this is equivalent to crossing down one inter-centile space on the Child Growth Foundation 1996/1 growth chart (Cole, 1995; Preece, Freeman, & Cole, 1996). They showed that the infants showing the slowest growth between birth and 4 months of age had higher cord blood leptin levels (Ong et al., 1999).

In this study, weight were converted to z scores using UK 1990 growth standards (Cole, 1995; Preece et al., 1996). The change in the weight z score was calculated using the 12 week measurement and took into account sex and exact age. This identified 17 infants who had crossed down one inter-centile space. One of the main hypotheses of

this study was that infants with higher cord leptin levels would gain weight more slowly. The data were firstly examined with a scatter plot. Figure 5.8 shows the change in z scores in relation to cord blood leptin levels.

Figure 5.8 Cord blood leptin levels and change in weight z scores from birth to 12 weeks. Infants shown by green squares crossed down one inter-centile space on the growth chart; infants shown by red triangles are the remainder of the group



The graph suggests that the infants that crossed down one inter-centile space (green squares) had higher cord leptin levels. Perhaps a more appropriate summary would be that none of the infants that showed slow growth had low leptin levels.

A second hypothesis of this study was that infants with higher cord ghrelin levels would gain more weight over the first twelve weeks of life. Figure 5.9 shows the relationship between ghrelin and weight change. The ghrelin levels for the infants showing the faster growth from birth to 12 weeks (crossing up one centile) were no different from the rest of the group. However, the infants demonstrating the slower

growth (green squares) had lower ghrelin levels. (Or, as in the case of leptin: none of the infants that showed slow growth had high ghrelin levels).

These relationships were summarized using a point-biserial correlation (Table 5.11).

Figure 5.9 Cord blood ghrelin levels and change in weight z scores from birth to 12 weeks. Infants shown by green squares crossed down one inter-centile space on the growth chart; infants shown by red triangles are the remainder of the group

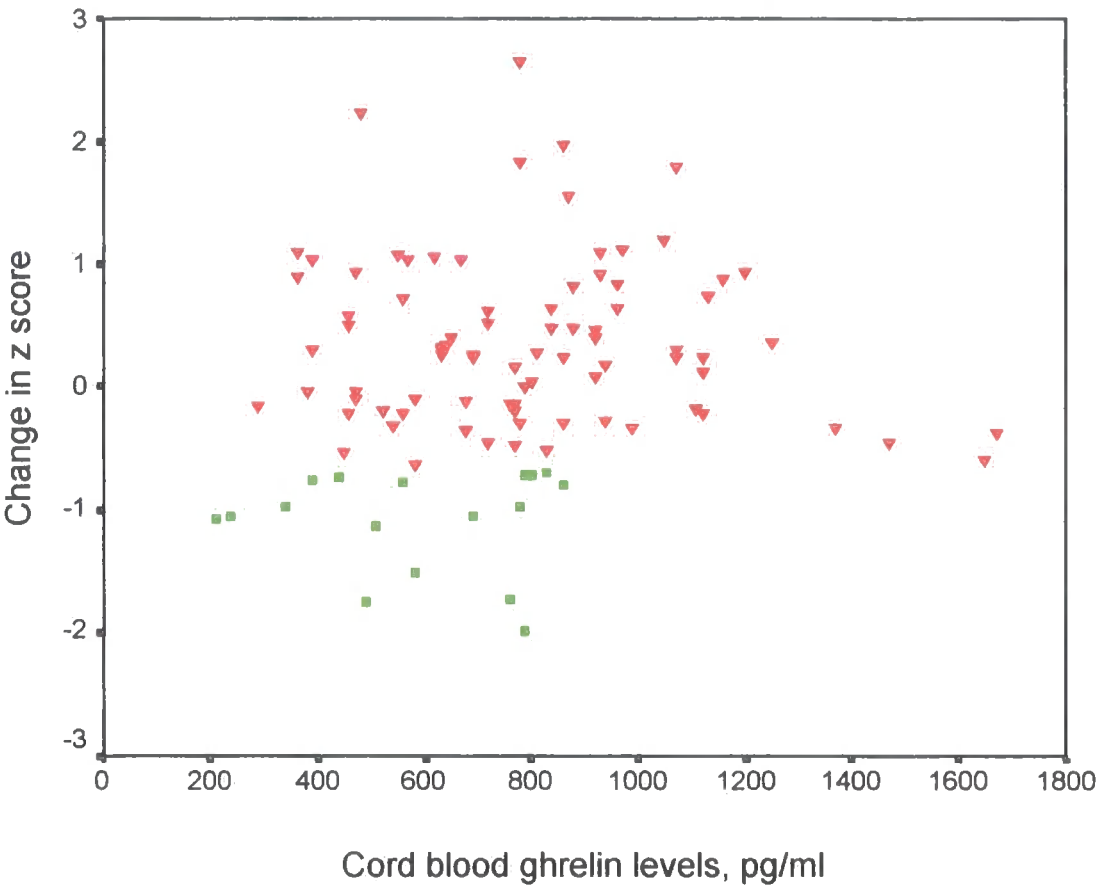


Table 5.11 Correlation of log leptin, ghrelin and change in weight (binary variable 1=slowest growing 17 infants, 0=83 others)

	Slow growth	Leptin (log e, ng/ml)	Ghrelin pg/ml
Birthweight z score	.25*	.51**	−.11
Slow growth		.24*	−.27**
Leptin (log)			−.14

* correlation is significant at the 0.05 level (2-tailed)

** correlation is significant at the 0.01 level (2-tailed)

There was a positive correlation between birthweight and slow growth, showing that infants of a higher birthweight were more likely to show slow weight gain over the first three months of life. Leptin was significantly correlated with birthweight, but ghrelin was not. Leptin and ghrelin levels were not significantly correlated with one another. Leptin levels were positively correlated with slow weight gain and ghrelin levels were negatively correlated with slow weight gain, showing that infants with higher leptin and lower ghrelin levels were more likely to have slower weight gain over the first three months of life.

Further examination of the data used logistic regression. The change in weight data was coded with an indicator variable. Those infants that crossed down a centile were assigned 1 (n = 17) and the remaining infants assigned 0 (n = 83). The first analysis examined the relationship between leptin and slow weight gain. Birthweight and ghrelin were used as control variables and leptin was entered last into the model as the predictor variable. The log-likelihood and chi-square statistics are presented in Table 5.12. Entered last into the logistic regression together with birthweight and ghrelin as control variables, the effect of leptin was not statistically significant (Table 5.12). The second analysis examined the relationship between ghrelin and slow weight gain, with birthweight and leptin entered as control variables and ghrelin entered last into the model as the predictor variable (Tables 5.12). Adjusted for birthweight and leptin, the relationship between ghrelin and slow weight gain was highly significant. Table 5.13 shows the parameter estimates. The value of exp β was .997, (95% CI 0.994 to 0.997)

indicating for each unit increase in cord ghrelin in pg/ml, the odds of the infant having a slower weight gain decreased by this factor.

Table 5.12 Log-likelihoods and chi-square statistics for regression of cord leptin and ghrelin on weight gain, whilst controlling for birthweight

Model summary	– 2 log (likelihood)	Chi-square	df	p
Analysis 1				
Block 1: Birthweight as z score	84.304			
Block 2: Ghrelin	76.401	7.9	1	<.01
Block 3: Log leptin	74.962	1.44	1	n.sig.
Analysis 2				
Block 1: Birthweight as z score	84.304			
Block 2: Log leptin	82.494	1.81	1	n.sig.
Block 3: Ghrelin	74.962	7.532	1	<.01

Table 5.13 Parameter estimates for Block 3 (same for both analyses)

	β	SE	df	p	Exp (β)	95.0% confidence intervals for Exp (β)	
Birthweight (z score)	.533	.383	1	.16	1.704	.80	3.61
Log leptin	.648	.548	1	.24	1.911	.24	1.91
Ghrelin	–.003	.001	1	.01	.997	.994	.999
Constant	–.809	1.519	1	.59	.445		

5.3 Cord blood insulin, milk intake, feeding frequency and size at birth

Milk intake and feeding behaviour were principle outcome variables in this study and as insulin has been shown to be closely related to feeding, it was therefore important to include it as a control variable, as well as interesting to examine its role in milk intake in its own right. In normal physiology, insulin levels rise following feeding and are necessary for glucose utilization, glycogen storage, lipolysis and liver and protein anabolism (Woods, Decke, & Vasselli, 1974). In newborn infants, plasma insulin levels rise following each feeding episode (Ogilvy-Stuart et al., 1998).

Cord blood insulin levels in this study are shown in Figure 5.10. Mean cord blood insulin was 4.5 mU/l with a standard deviation of 3.0. The minimum and maximum values were 0.9 to 15.8 mU/l. As the distribution was skewed, they were transformed to natural logarithms (to base e) to produce a more normal distribution for use in further analyses, as shown in Figure 5.11.

Figure 5.10 Histogram showing distribution of cord blood insulin levels

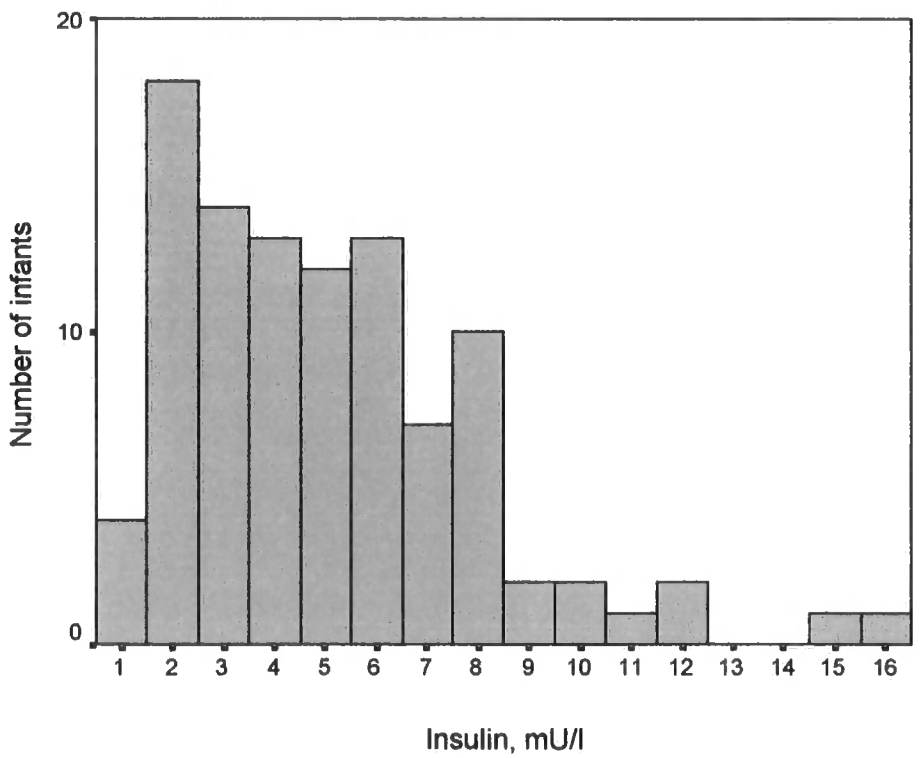
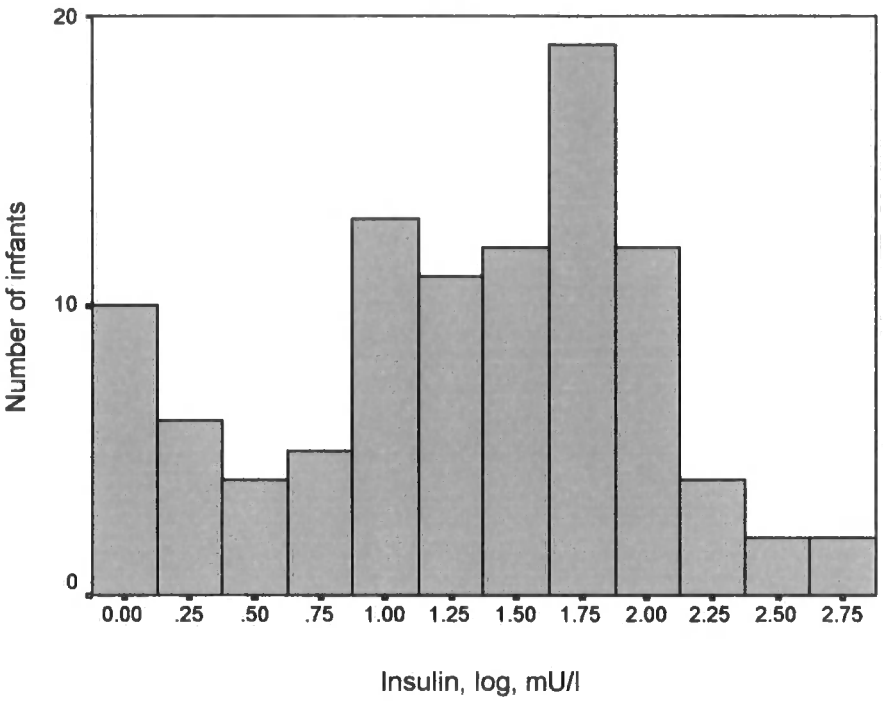


Figure 5.11 Histogram showing distribution of cord blood insulin levels following logarithmic transformation



The relationship between insulin levels and feeding were first examined using simple correlation (Table 5.14) as in previous analyses. Cord blood insulin levels were correlated with six feeding variables, time to first feed, amount of first feed, number of feeds on day 1, milk intake day 1, mean number of feeds and mean milk intake from day 1 to day 6. Cord blood insulin levels were significantly correlated with the mean number of feeds from day 1 to day 6; the correlation was negative so infants with lower cord blood insulin levels had a higher number of feeds over the six day period. This suggests that low insulin levels may be a stimulus to feed. This inverse relationship would be in keeping with the hyperphagic response observed in insulin deficient individuals (Schwartz, Woods, Porte, Seeley, & Baskin, 2000; Woods, Schwartz, Baskin, & Seeley, 2000).

Table 5.14 Correlation of cord blood insulin (log) and feeding variables (Pearson's r)

N = 100	r	p
Time to first feed	.01	.925
Amount of first feed	.01	.915
Number of feeds day 1	−.13	.197
Milk intake day 1	.07	.487
Mean number of feeds (day 1 – 6)	−.21	.038
Mean milk intake (day 1 – 6)	.11	.255

As shown by the study for this thesis birthweight is a significant predictor of mean milk intake and weight gain over the first week of life. Therefore the observed effect of cord blood insulin on feed frequency may be influenced by birthweight. Cord blood insulin levels have previously been related to birthweight (Ong et al., 2000), so it was important to ascertain if the same relationship existed in the data from the study for this thesis. The data was initially examined using simple correlation (with Pearson's r) (Table 5.15). Cord blood insulin levels were significantly correlated with the birthweight of the infant.

Table 5.15 Correlation of cord blood insulin and size at birth

	N = 100	r	p
Birth	Weight (kg)	.33	.001
	Length (cm)	.08	.425
	Head circumference (cm)	.09	.395

As cord insulin levels were significantly related to birthweight, it was important to incorporate birthweight into the analysis when examining the relationship of cord blood insulin and mean number of feeds day 1 to 6. This relationship was examined using regression analyses. The analysis examined the relationship between the mean number of feeds the infant consumed from day 1 to day 6 and two predictor variables, birthweight and cord blood insulin (Table 5.16). The relationship with cord blood insulin levels remained statistically significant after adjustment for birthweight (t =

−2.03, $p < 0.05$). Infants with lower cord blood insulin levels did have a significantly higher number of feeds from day 1 to day 6.

Table 5.16 Regression of mean number of feeds day 1 to day 6 on cord blood insulin (log), controlling for birth weight

	B	SE B	t	p
Constant	7.9	.989	7.98	.000
Birthweight (kg)	4.683E-02	.299	.16	.876
Insulin (log)	−.37	.181	−2.03	.045

Analysis of Variance					
	df	Sum of squares	Mean square	F	p
Regression	2	6.5	3.25	2.2	.116
Residual	97	143.2	1.48		
Total	99	149.7			

R	208
R square	.043
Adjusted R square	.024
Standard error	1.215

Outcome variable: mean number of feeds day 1 to 6

5.4 Summary of main results for chapter 5

In this chapter, the key hypotheses set out in the introduction were examined. The principal results were as follows:

1) Cord blood leptin levels were not related to any of the feeding variables measured in this study.

2) Cord blood leptin levels were significantly related to placental weight, birthweight and birthlength. There was a significant sex difference in leptin levels; female infants had higher leptin levels than male infants.

3) Cord blood leptin levels were not related to weight, length and head circumference at 12 weeks.

4) Cord blood leptin levels were not related to weight gain from birth to 12 weeks of age (controlling for birthweight).

5) Cord ghrelin levels were not related to any of the feeding variables measured in this study.

6) Cord blood ghrelin levels were not related to any of the growth measurements taken at birth, seven days or twelve weeks of life.

7) Lower cord blood ghrelin levels were significantly associated with slower growth from birth to three months of age.

8) Cord blood insulin levels were significantly related to the mean number of feeds consumed over the first six days of life; infants with lower insulin levels consumed more feeds.

9) Cord blood insulin levels were significantly related to the weight of the infant at birth.

Chapter 6

Discussion

6.1 Overview of chapter

Review of the literature in chapter one illustrated the recent progress that has been made in the understanding of appetite and body weight regulation. The advance of techniques in molecular biology, the knockout gene models in rodents and the discovery of rare human genetic disorders have identified several peptide hormones responsible for appetite regulation. However, there is a large gap in research concerning how these newly identified hormones function in normal human appetite. The study reported in this thesis investigated the role of leptin and ghrelin (measured in cord blood), in milk intake and feeding behaviour over the first week of life in normal infants, and the way in which they relate to growth over the first twelve weeks of life. This chapter will discuss the results presented in Chapters Three, Four and Five in relation to the aims and hypotheses outlined in Chapter One.

The four hypotheses of this study were:

I. Infants with higher cord leptin levels will have a lower milk intake than infants with lower cord leptin levels.

II. Infants with higher cord leptin levels will gain less weight over the first twelve weeks of life than infants with lower cord leptin levels.

III. Infants with higher cord ghrelin levels will initiate feeding sooner and have a higher milk intake than infants with lower ghrelin levels.

IV. Infants with higher cord ghrelin levels will gain more weight over the first twelve weeks of life than infants with lower cord ghrelin levels.

The procedures used to achieve these aims were:

a) Accurate documentation of feeding behaviour by measuring the amount of milk consumed at each feed and the number of feeds over the first week of life.

b) Measurement of weight, length and head circumference at birth, seven days of age and twelve weeks of age.

c) Measurement of leptin and ghrelin in cord blood using radioimmunoassay.

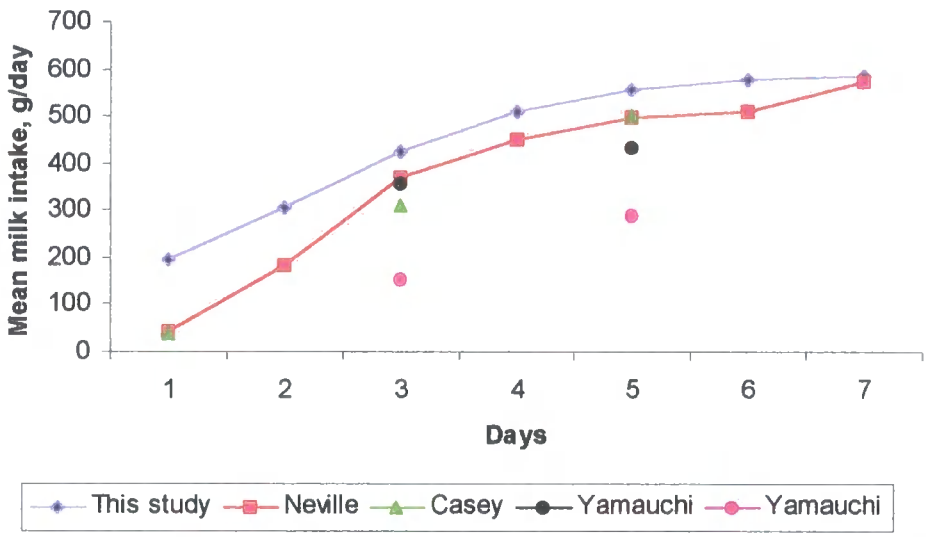
d) Measurement and control for relevant covariates.

6.2 Milk intake and feed frequency

The study reported in this thesis analysed feeding behaviour and milk intake in 100 formula-fed, term, healthy infants over the first week of life. Previous feeding studies have been based on smaller sample numbers (Casey, Neifert, Seacat, & Neville, 1986), a shorter study period (first two days of life, Dollberg et al. 2001) and mostly on exclusively breast-fed infants. The data from this thesis demonstrates that milk intake significantly increases over each twenty-four hour period over the first week of life in formula-fed infants. The pattern displayed is similar to that observed in breast and formula-fed infants over the first two days of life (Dollberg, Lahav, & Mimouni, 2001), breast-fed infants on days 3 and 5 (Yamauchi & Yamanouchi, 1990), breast-fed infants over the first five days of life (Casey et al., 1986) and breast-fed infants for the first seven days of life (Neville et al., 1988). However, the reported volumes of milk intake vary widely between these studies. The discrepancy in volumes between these studies could be accounted for by different methods of data collection, making direct comparison quite difficult. For example, Dollberg et al (2001) measured the infant's milk intake by 'test-weighing' the breast-feeding infants (adjustments were made for weight loss), and by estimation of the remaining milk left in the bottle following a feed in formula-fed infants. Yamauchi & Yamanouchi (1990) measured the infant's milk intake by 'test-weighing' of breast-feeding infants on days 3 and 5, dividing their sample by feed frequency. The two groups were infants that breast-fed between 0 and 6 times and infants that fed between 7 and 11 times. Casey et al (1986) measured milk intake on days 1, 3 and 5 in eleven infants, but data were available for only three infants on day one. Their study used 'test-weighing' of the infant to measure milk intake and the sample were only breast-fed infants, with no adjustments made to compensate for insensible water loss. Neville et al (1988) used 'test-weighing' of the infant and adjustment was made for insensible water loss but for comparison with other studies they also refer to figures before this adjustment was made.

This data in this thesis were based on direct weighing of pre-prepared bottles of formula milk before and after feeding, the sample number was larger and all the infants were bottle-fed. The formula-fed infants in the present study had higher milk intakes than the breast-fed infants from the previous three studies, as shown in Figure 6.1.

Figure 6.1 Comparison of mean milk intake (g/day) from formula fed infants from the study in this thesis (blue diamonds) with breast-fed infants from studies by Neville (red squares), Casey (green triangles) and Yamauchi (black and pink dots)



Data from the study by Yamauchi & Yamanouchi is illustrated twice because they divide their infants' milk intake into two groups by the number of feeds the infant consumed in the 24-hour period. The infants coded in black consumed 7-11 feeds and the infants coded in pink consumed 0-6 feeds in the 24-hour period. Milk intake on days 1 and 2 were noticeably lower in breast fed infants than the formula fed infants in this study. By days 3 and 5 the difference is much less, apart from the infants that consumed 0-6 feeds in the study by Yamauchi & Yamanouchi who had considerably lower breast milk intakes than the other breastfed infants on these days. This shows that the more frequent the infant fed, the more milk the infant consumed.

Direct daily comparison can only be made with one study (Neville et al. 1988) in which the authors recorded milk intake for seven days in breast-feeding infants. Lower levels

of milk intake were especially apparent in the breast-fed infants on days 1 and 2. On day 3 the breastfeeding infants had noticeably increased their milk intake to a much higher level. Between days 4 to 7, the milk intakes recorded for the breast-fed infants were more in line with the milk intakes recorded for the formula fed infants in the study reported in this thesis.

Only one study measured milk intake in formula fed infants (Dollberg et al. 2001), they compared the milk intake of 15 breastfed infants with 28 formula fed infants. Comparison could only be made on day 1 because of the method they used to report milk intake. They reported their data in cc/kg/day, which requires a daily infant weight as well as a daily volume of milk. Their breastfed infants consumed mean (SD) 9.6 (10.3) cc/kg/day, their formula fed infants consumed 18.5 (9.6) cc/kg/day and the infants in the study reported in this thesis consumed 57.4 (24.7) g/kg/day. To be able to compare the two studies, a conversion factor of weight of formula milk to volume was calculated (Appendix O). Using the conversion factor, the reported milk intake of 18.5 cc would be 19.02 grams, this difference is very small, so the two volumes can be compared. There was quite a large difference in milk intakes between the two studies.

Why might the recorded levels of milk intake between breast and formula-fed infants differ? Firstly comparison of milk intakes from infants 'test-weighed' and from direct measurement of the formula in bottles, leads to an underestimation of milk intake from 'test-weighing' (Borschel, Kirksey, & Hannemann, 1986). Borschel et al. showed that the milk intake measurements from test-weighing (not corrected for insensible water loss) were 87 to 93% of those estimated by direct measurement.

Secondly the lower levels of milk intake recorded for the first two days in breast-fed infants could reflect the normal physiology of lactogenesis. During the initial phase of lactation, colostrum is secreted. This is produced in small volumes and is of a rich consistency (Beischer & Mackay, 1986). On the third or fourth day 'the milk comes in' and the secretion of the breast changes from colostrum to milk that is produced in larger volumes. The amount of milk taken is lower in the early neonatal period because there is less milk available. As breast-feeding is a normal physiological process and formula feeding is an artificial substitute, relative to breastfed infants, formula-fed infants are overfed in the early neonatal period because they have a relatively unlimited supply of milk. Thirdly, as some studies only had small sample numbers, this would

give rise to large sampling errors. This was especially apparent in the study by Casey et al, (1986) who had only three subjects for day 1 of their measured intakes. Fourthly, the milk intakes of the infants in the study by Dollberg et al. (2001) may have been reduced by the study design. Although they stated that the infants were fed *ad libitum*, they were in fact kept to a strict 4 hourly schedule. This feeding regime and the fact that mothers and infants were kept apart could interfere with the onset of lactation in the breastfeeding mother. The breast-feeding studies and the study for this thesis measured showed similar volumes of milk taken once lactation was established in breast-feeding infants as previously shown in Figure 6.1.

The number of feeds consumed in the first twenty-four hours following birth varied between 3 to 12 in individual infants, with a mean of 6.7 (SD, 1.7). This was higher than the mean number of feeds in breast-fed infants (Yamauchi & Yamanouchi, 1990), which had a mean of 4.3 (SD, 2.5, range from 0 to 11). In the second twenty-four hours following birth, the means were similar. The mean (SD) for formula fed infants in this study was 7.3 (1.8) and for the breastfeeding infants of Yamauchi & Yamanouchi (1990) it was 7.4 (3.9). The difference observed in the number of feeds between the two studies in the first twenty-four hours might be because the mean for the latter study was reduced by some infants that did not feed at all in that time period. The range they reported was from zero feeds to eleven feeds.

The data presented in this thesis showed that milk intake in formula-fed infants was directly related to the number of feeds consumed; the more feeds consumed the higher the overall milk intake. This is in accord with the findings of Yamauchi & Yamanouchi (1990). They showed that breastfeeding frequency in the first twenty-four hours following birth was significantly related to milk intake measured on days 3 and 5; infants that breast-fed between 7 to 11 feeds in the first twenty-four hours had a much higher milk intake than infants fed between 0 to 6 feeds. They also showed a significant correlation between feeding frequency during the first 24 hours following birth and the subsequent 24 period. The data presented in this thesis showed that the milk intake for each succeeding day was related to the previous days milk intake and again that the number of feeds each day were related to the previous days number of feeds. These findings suggest that individual infants are moderately consistent in their feeding behaviour. It may also suggest that mothers are consistent in how they feed their infants, indicating an element of maternal control (Wright, 1981). Several factors

support a degree of infant control. The infants in the study reported in this thesis were fed to demand using 100 ml bottles of ready-made formula milk. No specific instructions were given to the mothers about how much to feed their infants. General instructions are found on tins of formula powdered milk to prepare 90 mls of feed for an infant weighing 3.5 kg between birth and two weeks of age. Midwives are advised to instruct the mother to feed the infant as much and as often as the infant wants. Over the first week of life in the study reported in this thesis (the time period of the measured formula), 100 mls of milk was available for the infant at each feed, the infant seldom took the whole amount. A few infants towards the end of the first week of life consumed the whole bottle and the mother opened another bottle of formula milk. The amount of milk infants consumed varied from feed to feed, and feed size gradually increased per day over the first week of life, suggesting that the infants were controlling their intake.

This data presented in this thesis also showed that birthweight was a significant predictor of the infants mean milk intake over the first week of life. This is in accordance with other studies that have shown relationships between body weight and energy intake (Cohen, Brown, Canahuati, Rivera, & Dewey, 1995; Davies, Wells, & Lucas, 1994; De Swiet, Fayers, & Cooper, 1977; Dewey, Heinig, Nommsen, & Lonnerdal, 1991; Fomon, Owen, & Thomas, 1964). Fomon et al (1964), showed that body weight significantly influenced milk intake in infants aged between 8 and 30 days. Milk intake was greatest when body weight varied between 3 and 5 kg, which is the period of most rapid growth. The finding that birthweight was clearly related to milk intake even over the first week of life was surprising considering this is a complex period when many adaptations to extra-uterine life are required.

Many studies report that infants initially lose weight in the early neonatal period (Avoa & Fischer, 1990; Dollberg et al., 2001; Maisels, Gifford, Antle, & Leib, 1988; Yamauchi & Yamanouchi, 1990). Breast fed infants appear to lose more weight by three days of age than formula fed infants. In one study breastfed infants lost approximately 7% of their initial birthweight compared to 4% in formula-fed infants (Maisels et al., 1988). The higher weight loss in breast fed infants has been attributed to the low volume of colostrum that is produced in early lacatation before the full milk supply is established. However, low milk availability cannot account for the weight loss in formula fed infants. Some studies attribute the weight loss to changes in the

volume and distribution of water in the body as well as to a low energy intake (Bauer, Cowett, Howard, vanEpp, & Oh, 1993; Heimler et al., 1993). Measurements of total body water by deuterium oxide dilution, extracellular volume by bromide solution and intracellular volume by the difference between total body water and extracellular volume in preterm infants showed that there were significant changes in body water distribution. Infants that lost more than 10% of their initial birthweight, lost extracellular volume, with no change in intracellular volume, and had a lower energy intake (Heimler et al., 1993). Infants that lost less than 5% of their birthweight showed a significant loss of extracellular fluid, a significant increase in intracellular volume and had a higher energy intake. This study demonstrated that weight loss was as a result of depletion of the extracellular volume in infants with a lower energy intake. Weight loss and loss of total body water content in the early neonatal period has also been associated with a greater diuresis (Bauer et al., 1993). Using bioelectrical impedance, term breastfed infants were shown to have a 5.7% weight loss, which was attributed to a decreased total body water content and a decrease in body solids (Rodriguez et al., 2000). Maximum weight loss in the study for this thesis by the seventh day was 7 %. Thirty eight percent of the infants had lost weight by the seventh day ranging from 20 to 260g. Six percent of the infants were within 10g of their birthweight and 56% of the infants had gained more than 10g from their birthweight. The most important finding was that the infants' change in weight was significantly related to their milk intake. So generally, the infants that had lost weight by the seventh day had consumed less milk. The reverse was true for weight gain; the infants that gained weight mostly had a relatively higher mean milk intake, although some infants had reasonably high milk intakes and lost weight. Why infants of the same birthweight show different milk intakes and diverse changes in weight remains unanswered. It may reflect infants' having different levels of appetite or different basal metabolic rates, affecting energy intake or energy expenditure, or maternal factors such as feeding practices, which could affect the change in weight. Also a proportion of the change in weight may also reflect changes in fluid distribution within intracellular and extracellular compartments and total body water content.

Analgesia and anaesthesia in labour has been postulated as a confounding variable in early feeding behavior. Newborns not exposed to any form of maternal analgesia or anaesthesia in labour show spontaneous rooting and sucking movements and commence suckling within one hour of age (Widstrom, Ransjo-Arvidson, Christensson,

& Mathiesen, 1987). However infants born to mothers who received mepivicaine via a pudendal block, intramuscular pethidine or an epidural showed less breast-feeding behaviour; they were less likely to show hand mouth movements or licking movements or to commence suckling (Ransjo-Arvidson et al., 2001). Some studies found that the opioid pethidine administered to the mother in labour made the infant drowsy over the first few days of life (Emde, Swedberg, & Suzuki, 1975), with poor muscle tone and slower reflexes (Hodgkinson & Husain, 1982). It is likely that these changes could affect milk intake by interfering with the mechanical action of feeding or appetite. Similar studies examining the effects of intramuscular diamorphine (the opioid used in the hospital in which this study was carried out in Newcastle) have not been conducted. None of the above studies measured the milk intake of the infant following the administration of analgesia and anaesthesia; they all concentrated on neonatal behaviours examined by reflexes, muscle tone and alertness. In the study reported in this thesis there was no relationship between milk intake over the first 24 hours, or mean milk intake over the first six days of life, and the analgesia and anaesthesia administered to the mothers in labour. The study reported in this thesis did not include neonatal behavioural testing, so whether the infants demonstrated poor muscle tone, drowsiness or attenuated feeding behaviours is unknown, but there was no effect of analgesia and anaesthesia on milk intake in the infants. This was reassuring and allowed exclusion of maternal analgesia and anaesthesia as a confounding variable. The literature review for this study identified a large gap in research of the effects of use of diamorphine in labour and its effects upon milk intake in breast and formula-fed infants and their subsequent neonatal behaviour. This is quite serious as 76 of the mothers in this study had diamorphine in labour, and suggests an area that needs examining.

Apgar scores reflect the infant's physical condition at birth (Apgar, 1953). Scores greater than 7 at five minutes are indicative of an excellent neonatal condition. The selection criterion for this study excluded any infants that had an Apgar score of less than 7 at five minutes of age. Consequently, the infants recruited into the study were term, healthy and had Apgar scores greater than 7 at five minutes of age, with a birthweight greater than 2500g and from mothers with no known medical complications. Only one infant was excluded from the study in the neonatal period due to a deterioration of physical condition. Several hours after birth this infant had difficulty maintaining its body temperature. This maladaptation to extra uterine life

could not have been anticipated from an Apgar score as it does not measure infant temperature. The data reported in this thesis showed no relationship between the infants Apgar score at five minutes and the infants milk intake over the first 24 hours of life. There was also no relationship between the length of labour and milk intake over the first twenty-four hours of life, or mean milk intake over the first six days of life. This was an encouraging finding, as the length of labour could stress and tire the fetus. The evidence from this thesis suggests it does not, at any rate to an extent that affects milk intake. There have been no previously published studies of this relationship.

The method used for measurement of milk intake for the study reported in this thesis had several advantages over methods in previous published studies. The milk was supplied in ready prepared bottles, which would reduce any error in reconstitution of feeds. The bottles were weighed before and after feeding, which was important as there was some variation in the weight of the bottles before feeding (up to 0.8g in SMA bottles and 5.7g in Cow & Gate bottles). This could have significantly influenced results when feeding volumes were low in the first few days of life. Using a feed chart concurrently with the labelled bottles was successful in that it brought attention to accidentally split milk, using bottles in the wrong sequence and lost bottles. It also allowed comparison of the maternal estimation of the amount of milk consumed by the infant with the actual weighed amount of milk consumed each feed. Correlation of estimated versus weighed milk intakes were calculated for a random ten percent sample of the infants. The results showed a correlation of between .90 and .99, ($p<0.001$). This indicated that mothers were highly accurate in their estimation of their infants' milk intake.

The main problem observed with the collection of milk intake data for this study was that 11 mothers ran out of milk on the seventh day, even though the instruction sheet for the mothers reminded them that it was important to contact the researcher if the milk supply became low. Following the first few mothers that ran out of milk, the study design was changed to supply the mothers with 56 bottles of milk instead of 48. Unfortunately this was still not enough. Future studies should consider an intermediate visit to the mother between birth and seven days. At this visit feeding frequency could be checked and milk supplies topped up if they are becoming low.

Two previous infant feeding studies have shown quite large drop out rates from recruitment to the end of the study period (Cohen, Brown, Canahuati, Rivera, & Dewey, 1994; De Carvalho, Robertson, Friedman, & Klaus, 1983). Cohen et al. recruited 453 women at the birth of their infant to exclusively breast feed for 16 weeks postpartum and then be randomized into different feeding groups for a further 10 weeks. At 16 weeks, due to the study eligibility criteria (which had to be followed from birth to 16 weeks) only 152 women were still eligible to participate. So they had lost 66% of their study sample by 16 weeks. Between 16 and 26 weeks a further 11 women dropped out (7%). So overall they lost 73 % of their sample. De Carvalho et al. recruited 47 mothers at the birth of their infant to take part in their study; by 15 days postpartum 23 mothers were eliminated for differing reasons, so they lost 48.9% of their study sample. By day 35 postpartum, another 8 mothers were eliminated, so they lost a total of 66% of their participants. All of these infants were lost during the course of the study. The drop out rate in the study reported in this thesis was not as high as the rates just described. In this study I approached 121 mothers on delivery suite to participate, 16 did not commence the study (5 because the infants did not fit the study criteria once delivered, 6 due to problems in obtaining a cord blood sample which was vital to the study, 4 as the mothers did not deliver their infants until the early hours of the morning, and 1 as the mother decided to withdraw before commencement of the study). One hundred and five mothers were entered into the study. During the first week, 5 infants were excluded: 1 became sick, 2 had their brand of formula milk changed and 2 withdrew for no apparent reason. So the drop out rate for this study was 4.8% during the first week and no further losses occurred over the remaining study period.

6.3 Infant feeding and growth

During the course of the study reported in this thesis, the size of infants at birth, at seven days and at twelve weeks of age was measured. Growth was a principal outcome variable for this study, so several determinants of size at birth and growth were considered.

The results reported in this thesis showed that smoking in pregnancy was a significant predictor of the infants weight at birth. There was also an indication that smoking had an effect upon infant length at birth, though this relationship fell just short of

significance as shown in Chapter 4.1.5. These findings are in accordance with other studies that have shown that infants born to mothers that smoke were lighter than infants born to non smoking mothers (Conter, Cortinovis, Rogari, & Riva, 1995; Elwood, Sweetnam, Gray, Davies, & Wood, 1987; Godfrey, Barker, Robinson, & Osmond, 1997; Hindmarsh, Geary, Rodeck, Kingdom, & Cole, 2002; Kramer, 1987). Some studies also showed that infants born to mothers that smoke were shorter at birth (Elwood et al., 1987; Godfrey et al., 1997; Hindmarsh et al., 2002). Why this study showed a significant effect of smoking upon birthweight, but less of an effect upon birth length, is likely to be because there is a graded effect of smoking upon growth (Elwood et al., 1987). In infants of mothers that smoked heavily (>25 cigarettes/day), there was a 9% deficit in birthweight, a 2% deficit in birth length and a 1.5% deficit in head circumference compared to infants of non-smokers (Elwood et al., 1987). Because the effect on length is smaller, a larger sample would be required for statistical significance. How smoking in pregnancy affects birth weight and birth length is still under debate. Several potential hypotheses have been put forward relating to the chemical composition of cigarette smoke. The first hypothesis is that nicotine causes constriction of the blood vessels in the uterus which affects uterine and placental blood flow (Economides & Braithwaite, 1994; Lambers & Clark, 1996). Nicotine increases maternal blood pressure and heart rate by constriction of the blood vessels, which in turn decreases uterine blood flow. This could affect the transfer of essential nutrients from the mother to the infant. Nicotine readily crosses the placenta into fetal compartments and concentrations are 15% higher than maternal concentrations (Lambers & Clark, 1996). The second hypothesis is that nicotine may suppress maternal appetite and maternal nutritional intake. Rats chronically exposed to nicotine by implantation of nicotine pellets showed a decrease in food intake compared to controls (Levin, Morgan, Galvez, & Ellison, 1987). Removal of the nicotine pellets resulted in a rebound hyperphagia and rapid weight gain. The effect upon appetite may be via meal size. Miyata et al. (2001) showed that rats exposed to infusion of nicotine had a lower food intake; meal number was unaffected, but the size of meals was reduced (Miyata, Meguid, Varma, Fetissov, & Kim, 2001). When the infusion of nicotine was stopped, the rats became hyperphagic and regained weight. The effect of nicotine withdrawal might explain the rebound hyperphagia and weight gain apparent in people who quit smoking (Young-Hwan, Talmage, & Role, 2002). In a large survey of 12,103 men and women in the USA, cigarette smokers were lighter and leaner than non smokers controlling for age and sex (Albanes, Jones, Micozzi, & Mattson, 1987).

This suggests that a component of smoke interferes with energy intake or energy metabolism, but how components of smoke directly interact with appetite control is unknown. If maternal smoking is affecting maternal appetite and maternal weight gain, this could have detrimental effects upon the fetus. During pregnancy, infants of mothers with an imbalanced diet, or malnutrition tend to show slower fetal growth (Godfrey et al., 1997; Hales & Barker, 2001) and intra uterine growth retardation (Strauss & Dietz, 1998). Elwood et al. (1987) documented other distinct differences in the diet of smoking mothers compared to non-smoking mothers. Smoking mothers ate less healthy foods such as meat, fruit and vegetables and were more likely to replace food with highly sugared tea. Although this finding may be due to a social class element, it still could adversely affect the infant's birthweight.

A third hypothesis concerning the relationship between smoking in pregnancy and birthweight involves carbon monoxide. Carbon monoxide is a chemical component of smoke. It forms carboxyhaemoglobin, which crosses the placenta and inhibits the release of oxygen into fetal tissues causing hypoxia (Lambers & Clark, 1996). Fetuses of smoking mothers subjected to hypoxia in utero, exhibit abnormal body growth patterns by 32 weeks of gestation (Lampl, Kuzawa, & Jeanty, 2003).

This data presented in this thesis (Table 4.6) showed that maternal height was significantly related to infant birth weight, but paternal height was not. Kramer (1987) in his meta-analysis examining the effect of parental height on infant birth weight showed a significant positive effect of both maternal and paternal height on infant birth weight. However, the effect of paternal height was considerably smaller. This might explain why the data reported in this thesis showed a maternal effect but not a paternal effect; because the magnitude of the effect was much smaller, a larger sample number would be required to show it. The study by Klebanoff et al. (1998) had height and weight measurements for 1063 fathers. Their larger sample number may account for their significant finding. In Kramer's (1987) meta analysis, out of the six studies identified three studies showed statistical significance. The smaller paternal effect is in accordance with the 'maternal-fetal conflict hypothesis'. This hypothesis proposed a conflict in *utero* between maternal genes, fetal genes resulting from the mother and fetal genes from the father (Barker, 1994; Moore & Haig, 1991). Natural selection in fetal genes would maximize fetal growth by increasing the transfer of nutrients to the infant. Fetal genes inherited from the father would make excessive demands on the

mother, depleting her energy stores to her detriment. Natural selection in maternal genes protects the mothers' survival and protects her ability to produce more offspring. Therefore maternal genes usually override the paternal fetal genes and hence constrain fetal growth.

This data presented in this thesis showed no sex difference in birth weight. This finding is contrary to the findings of the meta-analysis by Kramer (1987) who showed that on average male infants were 126.4g heavier than female infants at birth in developed countries and 93.1g heavier in developing countries. The most likely reason why the sex difference was not observed in the study for this thesis is that the sample number was insufficient to show an effect of this magnitude. However the study reported in this thesis did find a sex difference in infant growth. Male infants were heavier, longer and had a larger head circumference than the female infants by twelve weeks of age, as previously shown (Cohen et al., 1995; Kramer et al., 1985; Stunkard, Berkowitz, Stallings, & Schoeller, 1999). So sex of the infant was adjusted for in other analyses that examined effects of a predictor variable on weight gain. In the study reported in this thesis, paternal height was also a significant predictor of infant length at twelve weeks of age, but maternal height was not ($t = 1.9$, $p = 0.06$, just short of significance). Both paternal and maternal height has previously been related to infant length at 12 weeks (Hallman, Backstrom, Kantero, & Tiisala, 1971); the most likely reason for the apparent inconsistency is sample number.

The weight, length and head circumference measurements were converted to z scores to compare the study sample with the general population standard. The mean weight (-.055) and mean length (-.37) scores at birth were lower than the median of the British population standards at birth, but greater at 12 weeks of age ($z = .070$ & .26 for weight and height respectively). Length at birth and 12 weeks were the only measurements that were significantly different from the general population. There are two possible reasons for the difference in z scores. The infants from the study sample were formula-fed infants only. Formula-fed infants are more likely to be born to mothers that smoke (Shepherd, Power, & Carter, 2000). Infants born to mothers that smoke are shorter (Elwood et al., 1987; Godfrey et al., 1997; Hindmarsh et al., 2002) and lighter (Conter et al., 1995; Elwood et al., 1987; Godfrey et al., 1997; Hindmarsh et al., 2002). In the study for this thesis, 45% of the mothers smoked and these infants were lighter at birth and tended to be shorter. The second reason may be the reliability of measurements.

The difference in the mean weight measurements between this study and the British standards was small. The fact that the difference was small might reflect the great reliability of birthweight measurements (Johnson, Engstrom, & Gelhar, 1997). However the discrepancy in the mean length measurements at birth was quite large. The measurement of length has previously been shown to be the least reliable anthropometric measurement in infancy, especially when taken immediately after birth (Johnson et al., 1997).

'Slow growth' was defined in this study using a previously published criterion (Ong et al., 1999). Ong et al. used a decline in z scores of –0.67 to identify slow growth; this is equivalent to crossing down one inter-centile space on the Child Growth Foundation 1996/1 growth chart. Seventeen of the infants in this study demonstrated slow growth by this criterion. Slow weight gain is not usually pathological. Some studies report the heavier the infant at birth the slower the weight gain, and the later the age at which birthweight doubling occurs (Jung & Czajka-Narins, 1985; Neumann & Alpaugh, 1976), as infants heavier at birth tend to show 'regression to the mean' (Cole, 1995). However in the four example infants presented on growth charts in Chapter 4, (the two slowest growing males and two slowest growing females) only the male infant SO88 did so, starting between the 91st and 98th centile at birth falling to the 50th centile at 12 weeks. The other male infant S054 started just above the 50th centile at birth falling to just below the 9th centile. And in the two female infants, infant S033 started just below the 75th centile at birth falling to the 9th centile at 12 weeks and infant S084 started just above the 25th centile at birth falling to just above the 0.4th centile. These are quite significant centile crossings during the period of rapid weight change in infancy (Fomon et al., 1964). Formula fed infants on average gain 25 to 30 g a day during this time period (Kleinman & Committee on Nutrition, 1998). Further examination of the 17 slow growing infants identified that nine infants showed the described pattern of regression to the mean (7 males and 2 females). Eight infants showed crossing down centiles that did not meet this criterion (3 males and 5 females). The cause of the slow weight gain was not addressed by this study, but previous studies have suggested causes relating to parenting styles (Black, Hutcheson, Dubowitz, & Berenson-Howard, 1994) and difficulty with feeding (Altemeier, O'Connor, Sherrod, & Vietze, 1985; Dowdney, Skuse, Heptinstall, Puckering, & Zur-Szpiro, 1987; Drewett, Corbett, & Wright, 1999; Pollitt & Eichler, 1976).

6.4 Cord blood leptin, milk intake and growth up to twelve weeks of age

The first hypothesis tested in this thesis was that infants with higher cord blood leptin would have a lower milk intake compared with infants with lower cord blood leptin levels. The hypothesis was based on evidence from infants with congenital leptin deficiency (Montague et al., 1997, 1998; Strobel, Issad, Camoin, Ozata, & Strosberg, 1998). These infants had barely detectable leptin levels, were hyperphagic and gained weight rapidly in early infancy. This study, however, found no relationship between cord blood leptin levels and milk intake (or feed frequency) over the first week of life. There was no association between cord leptin and time to initiating feeding or between cord leptin and amount of first feed, number of feeds or milk intake for the first day of life. Nor was there any relationship between cord leptin and the mean number of feeds or milk intake for the first six days of life.

There are several reasons why cord blood leptin levels may not have been related to milk intake in this study.

Cord blood leptin comprises placental and fetal leptin. The placenta is a major source of leptin and appears to contribute to most of the circulating leptin within the fetal-placental unit (Henson et al., 1999; Hoggard, Haggarty, Thomas, & Lea, 2001; Masuzaki et al., 1997). This is supported by the observation that there is a large difference between the levels of leptin in cord blood and leptin levels measured in neonatal plasma. Serum leptin levels in the infant are approximately 90% lower in the neonatal period than in cord blood (Hytinantti, Koistinen, Koivisto, Karonen, & Andersson, 1999; Kirel et al., 2000; Marchini, Fried, Ostlund, & Hagenas, 1998; Matsuda et al., 1999; Schubring et al., 1998). A key issue, therefore, is whether the infants appetite would respond in the same way to placental leptin as to endogenous leptin produced by their own adipocytes.

Placental leptin and fetal leptin may be regulated differently. Although the structure of placental leptin is identical to adipose tissue leptin in its size, charge and immunoreactivity, and has a similar gene promoter controlling its mRNA synthesis, it has an additional 'placental-specific' upstream enhancer to uniquely influence placental leptin mRNA production (Bi, Gavrilova, Gong, Mason, & Reitman, 1997). This DNA sequence positively influences the expression of the gene in a tissue specific fashion.

This suggests that expression of the leptin gene is different in the placenta than in adipose tissue and therefore may respond to different regulators (Hoggard et al., 2001). Several observations suggest that placental leptin may not act in the same way as adipose leptin in non-pregnant physiology. However only maternal-placental observations are available to reason this point, but are relevant to this argument because the placenta is part of the maternal placental fetal unit and if placental leptin is regulated differently in maternal physiology then it may be regulated differently in placental fetal physiology. Firstly, maternal leptin levels were shown to rise by approximately 61% in the first 12 to 14 weeks of gestation of pregnancy when there was an average 3% decrease in body fat (Highman, Friedman, Huston, Wong, & Catalano, 1998). In the last gestation of pregnancy between 34 and 36 weeks gestation the leptin levels rose by 66% from pre-gravid levels and body fat had increased on average by 9%. These observations suggest that in the first trimester of pregnancy the rise in leptin levels was not related to body fat and in the last trimester the rise in leptin levels were disproportionate to the average increase in fat mass. The observation of a rise in leptin and a decrease in body fat in the first trimester of pregnancy contrasts with how leptin is regulated in normal non pregnant human physiology, where leptin is secreted primarily by the adipocyte (O'Rahilly, 1999) and correlates with percentage body fat (Considine et al., 1996). Although there is a degree of variability in leptin levels of adults of the same size in the general population (Wiegle et al., 1997), leptin levels invariably correlate with changes in body fat for a given individual. For example, if adults are fasted they show a loss of weight and a decline in plasma leptin levels (Wiegle et al., 1997), alternatively if adults are overfed for a period of time, they show an increase in body fat with a concomitant rise in leptin levels (Levine, Eberhardt, & Jensen, 1999).

A final consideration concerns the way in which leptin was measured. In the study reported in this thesis, cord blood leptin was measured by radioimmunoassay as 'total leptin', this is a combination of free leptin and leptin that is bound to a circulating fragment of its membrane receptor. The physiological actions of free and bound leptin are not yet fully understood. The proportion of bound leptin is lower in cord blood than at any other time period in human life, (Quinton et al., 1999). In normal physiology the ratio of bound to free leptin is determined by body fat, consequently lean people have lower total leptin levels with a higher proportion of bound leptin than obese people who have higher total leptin levels and greater levels of free leptin. When these individuals

were starved for 24 hours, both the lean and obese subjects showed a decrease in total leptin levels, with no significant change in bound leptin levels, but a significant decrease in free leptin levels (Sinha et al., 1996). When the subjects were refed the total leptin levels increased, with no alteration in the bound leptin levels, but a significant increase in free leptin levels. This study suggests that bound and free leptin behave differently in the experimental situations of fasting and refeeding. Free leptin is believed to more active, it is measurable in cerebrospinal fluid at levels much lower than in plasma or serum, and readily crosses the blood brain barrier and is therefore available to bind to the leptin receptors in the hypothalamus (Landt, Parvin, & Wong, 2000). The critical point appears to be what proportion of free leptin crosses from the blood to the cerebrospinal fluid, and is transported to the hypothalamus to inhibit feeding. This is not ascertainable in a non invasive study.

It has recently been argued that leptin should be viewed as a 'starvation hormone' rather than a 'satiety hormone' in that its role is to signal energy deficit rather than energy surplus (Prentice, Moore, Collinson, & O'Connell, 2002). Avoidance of starvation carries a greater survival need than the avoidance of obesity. This is especially apparent when leptin levels are low; the complete absence of leptin induces hyperphagia and weight gain in infants with negligible leptin gene expression (Montague et al 1997, Strobel et al 1998). However, in adult human obesity, high levels of leptin correlate with a high fat mass, and high circulating levels of leptin but do not appear to suppress appetite (Considine et al., 1996).

The second hypothesis of this thesis was that infants with higher leptin levels would gain less weight over the first three months of life than infants with lower leptin levels. Essentially this calls for a replication of the result of Ong et al, (1999), who showed an inverse relationship between leptin levels and growth up to four months of age and found this relationship was still apparent at two years of age. The study reported in this thesis did not find a relationship between cord blood leptin levels and weight gain up to three months of age. A direct comparison of these studies is summarized in Table 6.1. Both studies have similar design. The method in the study reported in this thesis has the advantage that the same researcher performed all the measurements, so was only subject to intra-examiner differences as opposed to the study by Ong et al. (1999), which was subject to both intra and inter examiner differences, which may have influenced reliability. The measurement of weight using electronic scales, however, is

subject to less error than most other anthropometric measurements in infancy (Johnson et al., 1997). The ranges for cord blood insulin were similar in both studies and the sample numbers were 136 and 100 respectively so variation in this should not have influenced statistical power very much. The only obvious differences between the two studies were in the reported levels of cord blood leptin. In the study by Ong et al. the leptin levels were lower than the leptin levels of the infants in the study reported in this thesis. Also it was impossible to ascertain the ratio of male to female infants at 4 months in the Ong et al. study and whether they controlled for sex. They commenced their study with 197 infants at birth (119 males and 78 females), falling to 136 infants at four months, so the numbers of male to female infants may be unbalanced. Boys gain weight faster than girls in early infancy and boys have lower leptin levels than girls. The infants from the study reported in this thesis were equally distributed into 50 male and 50 female infants and no effect of cord leptin on weight gain was found. The most likely explanation of the difference between the studies may be the variables controlled for. In one part of the paper of Ong et al, the authors say that weight gain was analysed allowing for exact age and birthweight, not controlling for sex. In the table presented, however, the authors say the regression analysis was adjusted for sex and age at visit, apparently not controlling for birthweight. In the present study, when the relationship between weight gain to 3 months and cord blood leptin levels were correlated without controlling for birthweight and sex, the result was close to significance ($r = -19$, $p = 0.066$). However birthweight and sex are significant predictors of weight gain and therefore need to be taken into account in weight gain analyses. Consequently, regression of weight gain on cord blood leptin levels, after controlling for birthweight and sex showed that cord leptin was not a significant predictor of weight gain but sex was. If Ong et al. (1999) have not controlled for birthweight or sex in their regression analysis as implied in their table, this could, perhaps account for the difference observed between the two studies.

Table 6.1 Comparison of Ong et al's study and study for this thesis

	Ong et al 1999	Present study
Measurements at birth	Taken within one day of birth Length (n=176) Head circumference (n=180) Birthweight from records (n=197) Measurements taken by trained members of research team	Taken within two hours of birth N=100 All measurements taken by same trained researcher
Measurement of growth	At 4 months Research clinic/members of research team N=136	At 3 months in infants home Same researcher N=100
Leptin ng/ml (median + range)	♀ 7.9 (2.6 – 42.1) ♂ 5.0 (1.1-19.0)	♀ 9.9 (2.3 – 37.0) ♂ 6.5 (1.6-26.0)
Insulin mu/l (median + range)	♀ 3.1 (1.2 – 17.2) ♂ 2.7 (0.8-11.9)	♀ 4.4 (1.0 – 16.0) ♂ 3.4 (0.9-12.0)

6.5 Cord blood ghrelin, milk intake and growth up to twelve weeks of age

The third hypothesis of this thesis was that infants with higher cord levels of ghrelin would initiate feeding sooner and have a higher milk intake than infants with lower ghrelin levels. However, the study reported in this thesis showed no relationship between cord blood ghrelin levels and the time to the infant's first feed, or to the infants milk intake on day 1, or the infants mean milk intake day 1 to 6. Also no relationship was found between cord ghrelin levels and the other feeding variables measured for this study (amount of first feed, number of feeds day 1, and mean number of feeds day 1 to 6). This finding was surprising as higher ghrelin levels have been shown to influence subjective ratings of hunger on visual analog scales in human adults (Arvat et al., 2001; Wren et al., 2001), increase energy intake in normal healthy humans (Wren et al., 2001)

and increase food seeking behaviour in rodents (Wren et al., 2000). Additionally children and young adults with Prader-Willi Syndrome have extremely high levels of fasting ghrelin levels and severe hyperphagia (Delparigi et al., 2002; Haqq et al., 2003). How can this be explained?

Firstly, because there was no relationship between ghrelin and time to first feed, this might suggest the measurement of ghrelin in cord blood may reflect a satiated state. The placenta provides continuous nutrition for the fetus up until the cord is cut at the time of delivery. If the cord blood level of ghrelin in all newborn infants reflects a satiated state it might not influence subsequent milk intake in the infant. The mean time to first feed was 86 minutes ranging from 32 to 353 minutes, showing infants became hungry at different times following delivery. Thirty eight percent of the infants fed within one hour following delivery, a further 48% fed between 1 and 2 hours following delivery and the remaining 12% feeding between 3 and 5 ¾ hours. Therefore there was time for ghrelin levels to rise before feeding was initiated.

Secondly, the study for this thesis related a normal physiological measurement to milk intake, whereas the other studies described were interventional and administered a dose of ghrelin and then measured appetite and food seeking behaviours. Many of the animal studies administered high doses of ghrelin by intraperitoneal, intracerebroventricular or intravenous routes (Wren et al, 2000, Nakazato et al, 2001,). The human studies administered ghrelin intravenously (Arvat et al., 2001; Broglio et al., 2001; Takaya et al., 2000; Wren et al., 2001). These studies would have produced pharmacological levels of ghrelin much higher than those observed in normal physiology and may have intensified the effect upon appetite. It is possible that the artificial higher levels of ghrelin were acting as a supra physiological stimulus to feed and that the changes in normal physiology would be more subtle and difficult to detect.

Thirdly, the increased appetite exhibited by the subjects with PWS are concomitant with fasting serum ghrelin levels three to four fold the normal fasting ghrelin levels observed in normal subjects (Delparigi et al., 2002; Haqq et al., 2003). These are pathological and may have an even more intensified effect upon appetite.

The final hypothesis for this thesis was that infants with higher cord levels of ghrelin would gain more weight over the first twelve weeks of life than infants with lower cord

ghrelin levels. This study found no relationship between cord blood ghrelin and weight gain when weight gain was analyzed as a continuous variable in linear regression analysis, but did find a relationship between ghrelin levels and slow growth when infants were classified into z scores and weight gain was determined by change in z scores (as defined by Ong et al, 1999). Infants that demonstrated the slowest growth (crossing down one centile on the growth chart) had significantly lower cord ghrelin levels.

High ghrelin levels sustained by continuous infusion have been associated with an increase in weight in mice (Tschop, Smiley, & Heiman, 2000). Likewise individuals with Prader-Willi syndrome have considerably elevated fasting ghrelin levels, become grossly obese in childhood and often die from complications of morbid uncontrollable obesity (Delparigi et al., 2002; Haqq et al., 2003). In addition, a recent study (Iniguez et al., 2002) showed that infants born small for gestational age (SGA) who grew poorly from birth to one year of age (crossing down a centile) had a larger decline in ghrelin levels from fasting following an intravenous glucose load. The SGA infants that showed the greatest increase in growth from birth to one year of age (crossing up a centile) showed less of a decline in their ghrelin levels from fasting following a glucose load. These findings support a role for ghrelin in the regulation of weight. Higher levels of ghrelin were related to faster weight gain and lower ghrelin levels were related to slower weight gain. The most striking finding in the data presented in this thesis was that none of the infants that showed slow weight gain had high ghrelin levels.

The mechanism of how ghrelin influences weight gain is unknown and the effect on weight gain in this thesis was not associated with an effect on milk intake. It is known that intravenous injection of ghrelin promotes a surge in growth hormone (Arvat et al., 2001; Broglio et al., 2001; Takaya et al., 2000), which is responsible for skeletal growth, suggesting there is an indirect link between ghrelin, growth hormone and weight gain.

6.6 Relationship between insulin, birthweight and feeding

The principal hypotheses on which this thesis was based concerned the role of leptin and ghrelin in early infant feeding, but as insulin has also been implicated as a hormone involved in hunger it could not be ignored. Intravenous and subcutaneous administration of insulin to rodents induced glucoprivic feeding (Orosco, Rouch, & Nicolaidis, 1994), and in humans hyperinsulinaemia has been associated with increased ratings of hunger on visual analog scales and an increased food intake (Rodin, Wack, Ferrannini, & De Fronzo, 1985). However insulin deficient individuals who have low or absent insulin levels are hyperglycaemic and hyperphagic (Schwartz, Woods, Porte, Seeley, & Baskin, 2000; Woods, Schwartz, Baskin, & Seeley, 2000). In view of insulin's potential role in feeding, insulin was also measured for this study. Cord blood insulin levels were positively related to birthweight and placental weight. This is in accord with some other studies (Christou et al., 2001; Ong et al., 2000), but not others (Hawdon, Aynsley-Green, Alberti, & Ward Platt, 1992; Ogilvy-Stuart et al., 1998). Why there is a discrepancy between these studies cannot be explained. However, there is increasing evidence to support a role for insulin as a growth factor in utero. Both fetal insulin and fetal adipose tissue rise during the last trimester of pregnancy (Nakae, Kido, & Accili, 2001), and the birth weight of the infant has been directly related to the amount of functioning fetal pancreatic tissue (Fowden, 1989). Both these findings and the positive relationship between cord insulin, birthweight and placental weight suggest that insulin influences growth in utero.

The second finding reported in Chapter 5, section 5.3 was an inverse relationship between cord blood insulin levels and mean number of feeds over the first week of life. Infants with lower cord insulin levels took more feeds per day than infants with higher cord insulin levels, thus suggesting that lower insulin levels are a stimulus to feed. It is well established that insulin deficient individuals are hyperphagic and the absence of insulin stimulates feeding (Schwartz et al., 2000; Woods et al., 2000).

This data presented in this thesis also demonstrated an inverse relationship between cord blood insulin and ghrelin levels (-.32, $p<0.05$). Infants with lower insulin levels had higher ghrelin levels. The importance of this finding is not clear; however in humans, insulin secretion is suppressed for a significant period following intravenous

injection of ghrelin (Wren et al., 2001), and in adults administered an insulin infusion whilst maintaining euglycaemia, there is a sharp drop in ghrelin (Saad et al, 2002). These findings make it difficult to ascertain whether insulin normally influences the actions of ghrelin or vice versa or whether the relationship is reciprocal, but definitely confirms an inverse relationship between them. Ghrelin gene expression has recently been identified in the insulin producing β cells of the pancreas (Volante et al., 2002), which might provide the link between the two. However, more research is required to understand the significance of this finding, especially in relationship to feeding behaviour and weight gain. The evidence suggests that both high levels of ghrelin (Wren et al., 2001) and low levels of insulin (Schwartz et al., 2000; Woods et al., 2000) stimulate hunger. The data presented in this thesis showed that infants with lower insulin levels consumed more feeds and had higher ghrelin levels, which might be reflect a greater appetite. In fact milk intake in this study was significantly related to the number of feeds, yet insulin levels were not related to milk intake. The only possible explanation is that a feeding episode is stimulated by insulin, but other factors control how much milk is taken. The data presented in this thesis showed that lower ghrelin levels were related to slower weight gain; this might be a consequence of the inverse relationship between ghrelin and insulin. The infants with the lower ghrelin levels had higher insulin levels and took fewer feeds, perhaps reflecting a lower appetite. Although care must be taken in interpretation of correlations, one could speculate that there is an indirect link between these three findings. There may be an interaction between insulin and ghrelin that stimulates feeding, which in turn influences weight gain. Future studies could examine this hypothesis further.

6.7 Future research

This study reported in this thesis showed that feeding can be accurately and successfully measured in term formula-fed infants. This finding supports the earlier work of Fomon et al. (1964), although the procedures he used have mainly been used to study milk intake in preterm infants not normal feeding. The advantage of measuring milk intake in the infant (until they start solids) is that milk is their only source of nutrition. Measurement is precise, the composition of the milk is known and there is no interference with natural feeding. In older children and adults, food intake is complicated by consumption of different energy foods, and emotional and learned eating behaviours which are much more difficult to measure. Using the methods

described in this thesis, further studies could be designed to look at energy intake in relation to specific appetite stimulating or appetite inhibiting hormones.

One of the main problems in measuring hormones in infant blood is that the procedure is invasive and unlikely to obtain parental or ethical approval. However, since completion of this study, two reliable non-invasive methods of leptin measurement have been developed and found to be valid. These are salivary (Groschl et al., 2001) and urinary measurements (Zaman et al., 2003); both significantly correlated with serum leptin levels. Perhaps in time non-invasive methods for ghrelin measurement will become available. A study could be designed to examine the role of these hormones in relation to feeding and growth. It seems apparent that the cord blood measurement of these hormones is complicated by placental production of leptin and differences between free and bound fractions, so leptin and ghrelin need to be measured either in urine or saliva. Measurement of these hormones and milk intake could be carried out after the first week of life in the neonatal period, as this would allow time for the placental hormones in the infant to be degraded and for the infants milk intake to settle into a more stable pattern. As these hormones have a relatively short half life, serial measurement of these hormones before and after a feeding episode over a twenty-four hour period would be needed to identify a direct effect of their influence upon feeding.

As this study looked at a normal population and identified a relationship between ghrelin and slow weight gain, it could be important to examine ghrelin levels in infants from nonstandard groups such as: premature infants, intra-uterine growth retarded infants and infants who fail to thrive who all display different growth velocities. The most direct access with minimal invasiveness would be to premature infants, as they are routinely cannulated so blood could be analysed for ghrelin levels before and after a feeding episode, and both energy intake and weight gain is accurately measured. These infants are also followed up after discharge from hospital on a regular basis. So quite accurate information could be obtained on their growth velocity in relation to their feeding behaviour. Growth tends to falter in infants with non-organic failure to thrive within the first few months of life; problems with feeding have been suggested as one of its possible causes. A prospective study could be designed to look at milk intake, feed frequency, weight gain and cord blood ghrelin levels in a large sample of term infants from a normal pregnancy similar to the study conducted for this thesis.

However, an additional assessment of feeding behavior by video recording of a feeding episode and a 24 hour record of milk intake could be integrated into the study design at different time points during early infancy. Weight could be measured at each visit. From this feeding problems and slow growth could be detected and related back to their initial cord blood ghrelin levels. This might show that cord ghrelin levels could be used as a predictor of non organic failure to thrive.

Literature review for this thesis identified two areas that were lacking research. The first was the use of the opioid diamorphine as an analgesic in labour and its effect upon the infant. A study design could incorporate both behavioural and milk intake measures over the first few days following delivery. Several assessors, unaware of what analgesia and anaesthesia had been given, could perform the behavioural assessment via video recordings. Milk intake could be measured as it was for this study. Women could not be randomized into a trial, as pain relief in labour would have to be maternal choice. Control would be needed for other anaesthetics used, as the study reported in this thesis mothers tended to have more than one form of pain relief. They tended to start using Entonox (nitrous oxide and oxygen), then proceed to diamorphine and, then half went onto epidural anaesthetic. The second area seriously lacking in research was the effect of length of labour on infant feeding behaviour, which could easily be investigated in a research study. Length of labour is routinely measured for all labouring mothers using a partogram on delivery suite.

6.8 Summary and conclusions

This study measured three hormones in cord blood (leptin, ghrelin and insulin) and examined their relationship with accurately assessed infant milk intake over the first week of life and weight gain over the first twelve weeks. The novel findings of this study were:

I. Weight gain or loss in the early neonatal period is a direct and significant consequence of milk intake consumed over that period.
II. Infants with lower cord blood insulin levels took more feeds over the first six days of life.
III. There was no relationship between cord blood leptin and milk intake over the first week of life.

IV. There was no relationship between cord blood ghrelin levels and milk intake over the first week of life.

V. There was no relationship between cord blood insulin levels and milk intake over the first week of life.

VI. Infants with low cord blood ghrelin levels gained significantly less weight from birth to twelve weeks of age.

VII. There was no relationship between cord blood leptin levels and weight gain from birth to twelve weeks of age.

Advances in molecular biology and genetic genotyping have identified that leptin and ghrelin are involved in complex signaling pathways within the central nervous system that converge upon the hypothalamus, where several peptides and neurotransmitters influence food intake and behaviour. The study reported in this thesis attempted to ascertain the role of cord blood leptin, ghrelin and insulin in infant feeding and growth. The study was successful in recruiting women into a complex study starting in labour, and in the measurement of milk intake and growth in formula-fed infants. It provides a comprehensive record of milk intake and number of feeds in a large sample in the first week of life. Anthropometric measurements of weight, length and head circumference were made at birth, seven days and twelve weeks of age by the same researcher, so provide dependable data for growth in formula-fed infants over that time period. The study clearly showed that weight gain and weight loss in the early neonatal period is related to milk intake. This finding challenges the accepted concept that the weight loss is entirely due to physiological water loss. The hormones leptin and ghrelin were measured in cord blood. These measurements were more difficult to interpret than first thought, because during the course of the study, advances in research meant that leptin in cord blood was identified to be of placental as well as fetal origin, and both bound and free. This might be why no relationship was found between total leptin and milk intake. Cord blood insulin was the only hormone that correlated with feeding behaviour; infants with lower insulin levels consumed more feeds over the first week. Ghrelin was the only hormone that correlated with growth; infants with lower ghrelin levels showed slower weight gain from birth to twelve weeks of age.

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Appendix A

Information handout for midwife

'Cord Blood Leptin Levels and Feeding in the First Seven Days of Life'

Hello, my name is Rachel James and I have a background in nursing and midwifery. I am hoping to undertake a study at the RVI looking at 'Cord blood leptin levels and feeding in the first seven days of life'

Leptin

Recent research in both rodent and human models has implied that leptin is an important regulator of energy balance and appetite control (*Zhang et al 1994, Farooqui et al 1999*). Leptin is the product of the ob gene and is secreted by the adipocyte reflecting the amount of fat tissue *(O'Rahilly 1999)*. After entering the CNS leptin binds to specific receptors in the hypothalamus activating a complex pathway that controls energy homeostasis. Children with **congenital leptin deficiency** have a mutation of the *Ob* gene, which results in absent, or low leptin levels, they exhibit marked hyperphagia and morbid obesity (Farooqui et al 1999). Treatment of one of these children with recombinant leptin diminishes hyperphagia and promotes weight loss.

Research into the role of leptin in appetite is still in its early stages. In pregnancy, we have evidence that leptin is produced in trophoblasts and amnion cells (*Masuzaki et al 1997)*. Serum leptin levels rise in the mother through pregnancy (*Masuzaki et al 1997)* and are positively correlated with maternal weight, BMI and fat mass (Butte et al 1997). Though as yet we are still unsure of its physiological role in pregnancy.

In the fetus, leptin is present in both the arterial and venous cord blood. *(Sivan et al 1997)*. These levels are independent of maternal leptin levels *(Schubring et al 1997)*. Cord blood leptin levels range from 2.0 to 84.5 ng/ml between 36 & 42 weeks gestation *(Matsuda et al 1997)*. Cord blood leptin levels positively correlate with birth-weight and placental weight *(Schubring et al 1997)*. In infants higher cord blood leptin levels are associated with lower weight gain over the period from birth to 4 months *(Ong et al 1999)*, thus suggesting that leptin controls appetite in some way. Long-lean infants tend to have lower leptin levels and gain more weight *(Fergusson 1980, Ong et al 1999)*.

Why leptin and cord blood

Infant feeding is initiated soon after delivery with infant milk intake and subsequent weight gain varying greatly between infants in the first week of life. The relationship between health and body weight is an area currently receiving considerable attention. It is thought that events occurring within the fetal environment are a precursor to disease in adulthood. Barker (1992) has found that certain factors such as IUGR and placental size are associated with cardiovascular disease in adult life.

This forms the basic hypothesis for my research, I wish to measure milk intake in infants for the first week of life and relate it to their cord blood leptin levels and subsequent growth to observe whether the leptin is having any effect upon appetite and feeding.

Aim: To explore the relationship between cord blood leptin levels and appetite in the newborn.

Hypothesis: Leptin levels at birth regulate milk intake by controlling feeding behaviour in early life.

Why I need to use bottle-feeding infants?
This is not a study encouraging bottle-feeding. It is very difficult to measure accurate milk intake in breast-feeding infants. This entails both the mother and baby being weighed pre and post feed. This would be very disruptive for new breast-feeding mums, especially during the night-time feeds. Appetite would be difficult to determine as milk production depends upon lactogenesis.

Study
- Only those mothers that have already firmly decided to bottle-feed will be approached.
- Recruitment before delivery either on the antenatal ward or in the early stages of labour at the discretion of the attending midwife.
- 100-120 singleton pregnancies, term infants 37-42 weeks gestation.

On delivery suite
- I need a sample of cord blood to measure cord blood leptin levels, IGF1 & for precious peptide analysis.
- Record placental weight & birth weight
- Length & head circumference of baby
- Record of APGARS, length of first and second stages of labour, analgesia and anaesthesia used in labour.

On the postnatal ward
An accurate record of the infant's milk intake for first 7 days of life.
Mums will be asked to keep a feed chart, recording the volume of milk left in the bottle and any vomiting. They will be asked to keep all bottles that they have used whilst in hospital, a suitable container will be provided to store them. I will collect them daily.

On discharge, I will provide milk of the same formula that they have chosen, sufficient to complete 7 days of feeding.

I will visit the mums on day 7 at their home to collect the remaining milk bottles and repeat the growth measurements of weight, length and head circumference.

I will also revisit the mothers and infants at their home at 12 weeks to repeat the growth measurements of weight, length and head circumference.

What I hope to find is that cord blood leptin controls appetite in the newborn, theoretically infants with low leptin levels should feed a lot where as infants with high leptin levels should feed less. Which would then support the fact that infants with higher leptin levels gain less weight.

Why is this important? This will give us further insight into the regulation of feeding. This is an initial study and depending on the outcome, future research could look at cord blood leptin levels and infant growth in breast-feeding infants.

Thank you for reading this handout, please feel free to approach me whilst I am in and around the maternity unit if you have any questions or comments.

Appendix B

Calibration of scales

Calibration of scales

	Home scales	Scales on delivery suite										
Date		23.10.00	23.10.00	23.10.00	23.10.00	23.10.00	23.10.00	23.10.00	23.10.00	23.10.00	23.10.00	23.10.00
	Rachel	no 1	no2	no3	no4	no5	no6	no7	no8	no9	no10	no11	no12
Model	Seca 727	Seca 727	Seca 727	Seca 727	Seca 727	Seca 727	Seca 727	Seca 727	Seca 727	Seca 727	Seca 727	Seca 727	Seca 727
	1.000	1.000	0.995	1.000	1.000	0.995	1.000	1.005	1.005	1.000	1.000	1.010	0.840
	2.002	1.990	2.005	2.000	2.000	1.990	2.000	2.010	2.000	1.990	2.000	2.015	1.600
	3.000	2.990	3.010	3.000	3.000	2.995	3.000	3.010	3.010	2.990	3.005	3.020	2.640
	4.002	3.990	4.005	4.000	4.000	3.990	4.000	4.010	4.010	3.990	4.005	4.030	3.570
	5.002	4.985	5.005	5.000	5.000	4.985	5.000	5.010	5.005	4.985	5.000	5.040	4.715

	Home scales	Scales on delivery suite									
Date	15 01 02	17 01 01	17 01 01	17 01 01	17 01 01	17 01 01	17 01 01	17 01 01	17 01 01	17 01 01	17 01 01
	Rachel	no 1	no2	no3	no4	no5	no6	no7	no8	no9	no10
Model	Seca 727	Seca 727	Seca 727	Seca 727	Seca 727	Seca 727	Seca 727	Seca 727	Seca 727	Seca 727	Seca 727
	1.000	1.000	1.000	1.000	1.000	1.000	0.990	1.000	1.000	1.000	1.000
	2.000	1.995	2.005	2.000	2.005	2.000	1.990	2.000	2.000	1.995	2.000
	3.002	2.995	3.010	3.000	3.010	2.995	2.995	3.000	2.995	2.990	3.000
	4.000	3.995	4.010	4.000	4.010	4.000	3.990	4.000	3.995	3.990	4.000
	5.000	4.995	5.010	5.000	5.015	5.000	4.995	5.000	4.995	4.990	5.000

Calibration of scales

	Home scales	Scales on delivery suite								
Date	03 04 01	04 04 01	04 04 01	04 04 01	04 04 01	04 04 01	04 04 01	04 04 01	04 04 01	04 04 01
	Rachel	no 1	no2	no3	no4	no5	no6	no7	no8	no9	no10
Model	Seca 727	Seca 727	Seca 727	Seca 727	Seca 727	Seca 727	Seca 727	Seca 727	Seca 727	Seca 727	Seca 727
1.000	1.000	0.995	1.000	1.000	1.005	1.000	1.000	1.000	1.000	1.000
2.000	2.000	1.995	2.005	2.000	2.000	2.000	1.995	2.010	2.000	2.000
3.002	3.000	2.995	3.000	3.000	3.005	3.000	2.995	3.000	3.000	3.000
4.000	4.000	3.995	4.005	4.000	4.005	4.000	3.990	4.010	4.000	4.000
5.000	5.000	4.995	5.010	5.000	5.005	5.000	4.990	5.010	5.000	5.000

	Home scales	Scales on delivery suite								
Date	11 07 01	11 07 01	11 07 01	11 07 01	11 07 01	11 07 01	11 07 01	11 07 01	11 07 01	11 07 01	11 07 01
	Rachel	no 1	no2	no3	no4	no5	no6	no7	no8	no9	no10
Model	Seca 727	Seca 727	Seca 727	Seca 727	Seca 727	Seca 727	Seca 727	Seca 727	Seca 727	Seca 727	Seca 727
1.000	1.000	1.000	0.990	1.010	1.000	1.000	0.995	1.000	1.010	1.000
2.000	1.995	2.000	1.990	2.000	2.000	2.000	1.995	2.005	2.020	2.000
3.000	2.995	3.000	2.990	3.000	3.000	3.000	2.995	3.000	3.030	3.000
4.000	3.990	4.000	3.990	4.010	4.000	4.000	3.995	4.005	4.030	4.000
5.000	5.000	5.000	4.990	5.010	5.000	5.000	4.995	5.010	5.030	5.000

Appendix C

Information leaflet for mothers

Appendix C: Information leaflet for mothers

For mother's intending to bottle-feed

I would like to invite mothers who are proposing to bottle feed their babies to take part in a study looking at cord blood leptin and ghrelin levels and feeding in the first seven days of life.

My name is Rachel James. I am a midwife and as part of my PhD project would like to look at why some babies get hungry and some don't. At the moment we believe this may partly be due to a chemical that is in the babies' blood that comes from the placenta and is in the babies' fat tissue. This chemical may then affect how hungry your baby gets. One way that we can look at this is by measuring the level of the chemical in the cord blood from the placenta and recording what your baby eats for the first seven days of life. Bottle-feeding provides a good opportunity to do this, as we can keep an accurate record of how much milk your baby takes.

The study is in three parts. The first is on labour ward where I or the midwife would take blood from the cord of the placenta after delivery (this does not involve you or your baby). Then I would need to measure your baby's length, weight and head circumference in the first hours of life.

I would ask you to record each feed your baby has from delivery until your baby is seven days old and to keep all the bottles from feeding, with whatever milk is left in them. As in hospital the milk of your choice in ready made sterilised bottles will be given to you to use together with teats, record sheets for recording the number of feeds. A container will be provided to store the bottles after feeding.

The second part of the study involves me visiting your home at a time convenient to you after your baby is 7 days old to pick up the record sheets and bottles and to weigh the baby. The third and last part of the study is me visiting you at home when your baby is 3 months old to weigh and measure your babies length.

The information from this study will give us a better understanding of the mechanisms involved in feeding and help us understand why some babies don't feed so well in the early days of their life.

If you might be willing to take part I would be very pleased to see you and discuss it further. Please tell your midwife who will contact me.

Appendix D

Patient consent form

Patient Informed Consent Form

Cord blood leptin levels and feeding in the first seven days of life.

I declare that I am willing to participate in the above study, which has been explained to me by the researcher Rachel James.

I am aware that I can withdraw my consent to participate in the study at any time without needing to justify my decision.

Mother's Signature: _____

Date:_____

Mother's Name:_____

Researcher's Signature:_____

Date:_____

Researcher's Name:_____

Appendix E

GP letter

R.J.A.James, RGN, RM, ADM,
BA (Hons), MA.
Department Of Psychology
Science Laboratories
South Road
Durham
DH1 3LE
Tel: 0191 374 2619

Dr.T.D. Cheetham, BSc, MD
MRCP, MRCP CH.
Consultant Paediatric Endocrinologist
Department of Paediatrics
Royal Victoria Infirmary
Queen Victoria Road
Newcastle upon Tyne
NE1 4LP
Tel: 0191 2824417

Date:

Re:

Dear Doctor

I am writing to let you know that your patient has agreed to be recruited into our study on feeding in the new-born.

The objective of our study is to examine the relationship between arterial cord blood leptin and the infant's milk intake over the first seven days of life. We hope that this will give us a better understanding of the nutritional status of the new-born and the mechanisms of feeding and some insight as to why some babies don't feed as well during the early days of their life.

The study involves taking a sample of arterial cord blood from the placenta after delivery, measuring infant length, weight and head circumference at the time of birth and then asking the mother to record all feeds for the following 168 hours. I will visit the mother at home after 168 hours to collect the records and to reweigh the baby and again at three months to record the baby's weight and length. Free Infant feed of the mother's choice will be provided over the first seven days.

The study is non-invasive and will pose no risk to either the mother or her baby.

Yours sincerely

Rachel James

Appendix F

Letter to GP/Midwife/Health Visitor

R.J.A.James, RGN, RM, ADM,
BA (Hons), MA.
Department Of Psychology
Science Laboratories
South Road
Durham
DH1 3LE
Tel: 0191 374 2619

Dr.T.D. Cheetham, BSc, MD
MRCP, MRCP CH.
Consultant Paediatric Endocrinologist
Department of Paediatrics
Royal Victoria Infirmary
Queen Victoria Road
Newcastle upon Tyne
NE1 4LP
Tel: 0191 2824417

Date:

Re:

Dear Doctor/Midwife/Health Visitor,

I am writing to let you know that your patient has agreed to be recruited into our study on feeding in the new-born.

The objective of our study is to examine the relationship between arterial cord blood leptin and the infant's milk intake over the first seven days of life. We hope that this will give us a better understanding of the nutritional status of the new-born and the mechanisms of feeding and some insight as to why some babies don't feed as well during the early days of their life.

The study involves taking a sample of arterial cord blood from the placenta after delivery, measuring infant length, weight and head circumference at the time of birth and then asking the mother to record all feeds for the following 168 hours. I will visit the mother at home after 168 hours to collect the records and to reweigh the baby and again at three months to record the baby's weight and length. Free Infant feed of the mother's choice will be provided over the first seven days.

The study is non-invasive and will pose no risk to either the mother or her baby.

Yours sincerely

Rachel James

Appendix G

Data Collection Sheet 1

Data Collection Sheet 1

Subject NO.	Subject ID

Mother	
Surname	
First name	
Known as	
Hospital No.	
D.O.B 00/00/00	
Address	
Tel no.	
GP's Name	
Address	

Appendix H

Data Collection Sheet 2

Data Collection Sheet 2

Subject No.	Subject ID

Gestation	Weeks Gestation	
Estimated by 19 wk scan		

Labour					
First painful contractions Time (24 hour clock)					
Vaginal Examinations in Labour	Time				
	Dilatation				
Time in 24 hr clock	Time				
Dilatation in cms	Dilatation				
Duration of 1st stage of labour (hours)					
Duration of 2nd stage of labour (hours)					

Analgesia					
Diamorphine	Time				
	Dose				
	Time				
	Dose				
Entonox					
Epidural					
Other					

Appendix I

Data Collection Sheet 3

Data Collection Sheet 3

Subject No.	Subject ID

Delivery		
Type of Delivery		
D.O.B		
Time of Birth		
Sex		
Name if known		
Apgars Time	Time Score	Time Score
Birthweight (g)		
HC (cm)		
Length (cm)		
Placental weight (g)		
Parity		
Smoker		
Diabetes		

Appendix J

Data Collection Sheet 4

Data Collection Sheet 4

Milk Type

Subject No.
Subject ID

Milk Intake

Weight in grams	1	2	3	4	5	6	7	8	9	10
Bottle before use										
Bottle after use										
Difference in grams										

Weight in grams	11	12	13	14	15	16	17	18	19	20
Bottle before use										
Bottle after use										
Difference in grams										

Weight in grams	21	22	23	24	25	26	27	28	29	30
Bottle before use										
Bottle after use										
Difference in grams										

Weight in grams	31	32	33	34	35	36	37	38	39	40
Bottle before use										
Bottle after use										
Difference in grams										

Weight in grams	41	42	43	44	45	46	47	48	49	50
Bottle before use										
Bottle after use										
Difference in grams										

Weight in grams	51	52	53	54	55	56	57	58	59	60
Bottle before use										
Bottle after use										
Difference in grams										

Appendix K

Instructions for mothers

Instructions for mothers

Thank you for agreeing to take part in my study.

In Hospital

I will sort out the milk for you on the delivery suite and transfer with you to the postnatal ward.

Feed your baby with the ready-made bottles, starting from the bottle labeled number 1.

Write on the feed chart

- Date
- Time
- Number of feed
- How much your baby takes (read the scale on the bottle)
- Please write down if the baby is sick or if you spill any milk or if any bottles accidentally get discarded.

Please keep the bottle with whatever milk is left in it and place it in the container provided.

At Home

Please follow the same routine at home.

I will arrange a time and date suitable to you to come and collect the bottles at the end of seven days of feeding.

Please follow the instructions on the bottle with regard to storage and don't use the bottle after it has been opened for one hour.

Please contact me Rachel James if you have any problems with regard to this study

0191 682 2826

Please contact me if you think you are going to run out of milk

Appendix L

Feed Chart

Appendix L: Feed Chart

Baby's Name Subject NO. Subject ID

Date	Time	Bottle Number	Amount Taken (mls)	Comments

Appendix M

Data Collection Sheet 5

Data Collection Sheet 5

Subject No.	
Subject ID.	

Baby's Name	

Weight and Length

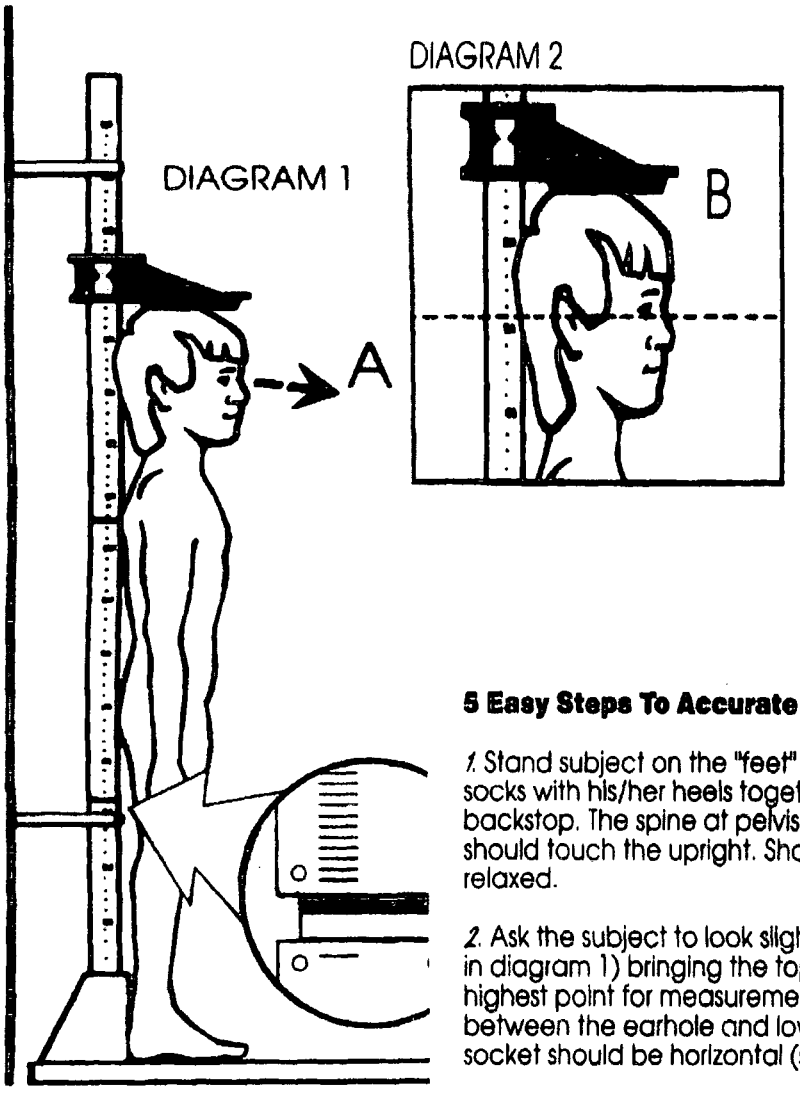
7 Day date	
7 Day Time	
HC (cm)	
7 Day Weight (g)	
7 Day Length (cm)	

12 wk date	
12 wk time	
HC (cm)	
12 wk Weight (g)	
12 wk Length (cm)	

Appendix N

Instructions for the Leicester Height Measure

The Leicester Height Measure



5 Easy Steps To Accurate Measurement

1. Stand subject on the "feet" without shoes or thick socks with his/her heels together firmly against backstop. The spine at pelvis and shoulder level should touch the upright. Shoulders should be relaxed.

2. Ask the subject to look slightly downwards (see 'A' in diagram 1) bringing the top of the head to the highest point for measurement. An imaginary line between the earhole and lower border of the eye socket should be horizontal (see 'B' in diagram 2).

3. Lower the measuring arm gently onto the subject's head and ask him/her to "breathe in and stand tall".

4. Read off measurement - arrowed on the upright - to the last completed millimetre. Do not round up! Consider repeating this step twice more to check the accuracy of your measurement.

5. Record, date and initial your measurement in the boxes provided on the subject's record sheet, PCHR or centile chart and plot the measurement on the chart.

Appendix 0

Weight to volume conversion for formula feed

A gram is a unit of mass in the metric system equivalent to a cubic centimetre and one millilitre of water at 4°c. Analysis of water with a balance accurate to ± 0.03g confirmed that 25mls of water weighed 25g. Analysis of infant formula, ten samples weighed in duplicate showed that 25 mls of feed weighed a mean of 25.7g (SD 0.02).

Weight of 25 mls of formula feed	
Weight 1	Weight 2
25.67	25.66
25.66	25.66
25.65	25.61
25.67	25.66
25.64	25.64
25.65	25.64
25.64	25.66
25.63	25.64
25.68	25.66
25.62	25.65