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Michael C. Marchand. BSc (Hons)

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789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

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This thesis describes original work by Michael Charles Marchand, which was completed during the period of registration as a postgraduate/PhD student of the University of Leicester. No part of this work has been previously submitted for a higher degree at this or any other University.

Signed.....M. Marchand.....Date.31st March 2004.

Fetal Programming of Renal Morphology and Function.

Michael C. Marchand.

ABSTRACT.

Previous epidemiological evidence from a number of studies supports the hypothesis that the risk of essential hypertension, coronary heart disease and non-insulin dependent diabetes is, in part, programmed by intrauterine nutritional status. An increasing number of human studies indicate that the developing kidney is particularly vulnerable to the adverse effects of fetal growth retarding influences. In animals growth retarding diets or other insults, which have an impact on the development of cardiovascular functions, also appear to impact upon nephron number.

In this study, the feeding of a 9% casein diet to pregnant rats, a mild protein restriction, reduced nephron number in the offspring, which progressively declined with age compared to those exposed to an 18% control diet. At weaning low-protein exposed offspring had hypertension and evidence of renal insufficiency. On natural death, the kidneys from aged male rats exposed to both low-protein and control maternal diets had a higher incidence glomerulosclerosis and renal disruption than females.

Supplementing the maternal 9% casein diet with 3% glycine, 1.5% urea and 3% alanine in the rat normalised nephron number in the offspring. Only the addition of glycine in the maternal low-protein diet prevented the appearance of high blood pressure in the offspring.

In this study it has been demonstrated that in humans, those of a low birth weight or ponderal index, a marker of fetal undernutrition, had evidence of increased glomerular permeability, but not elevated blood pressure at age 10. This association was not evident at age 12 or in a separate cohort of young adults.

It is possible that hypertension and a reduced nephron reserve are not causally associated. The evidence from this thesis suggest that prenatal undernutrition may programme renal structure in later life, but that renal programming is not one of the primary mechanisms leading to hypertension.

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Onward.

Contents.

Chapter 1.

Introduction.

1.1. Cardiovascular disease (CVD) mortality.

In 2003, cardiovascular disease (CVD) accounted for over 245,000 deaths in the U.K. CVD remains the single largest cause of death and the single main cause of premature death in the U.K. and most westernised nations (British Heart Foundation, 2003). This equates to over 120,000 deaths from coronary heart disease (CHD) and over 66,000 from stroke (Chief Medical Officer, 2001; British Heart Foundation, 2003). In the U.K. CVD accounts for approximately 35 million lost working days, with a cost to industry of nearly £3 billion. The estimated cost to the National Health Service (NHS) and the personal social services is in the region of £3.8 billion (Department of Health, 1998). Only Ireland and Finland have a higher rate of CVD mortality (British Heart Foundation, 2003).

The causes of cardiovascular disease are multifactorial, and may in part be determined by genetics (See section 1.2). In the U.K there are clear regional variations with CVD mortality higher in the north compared to the south (Barker, 1998; Department of Health, 1998; British Heart Foundation, 2003). It is widely asserted that CVD is largely determined by avoidable adult lifestyle factors, such as smoking, high salt and saturated fat intake, obesity, stress and physical inactivity (Department of Health, 1998; British Heart Foundation, 2003) (See section 1.3). One of the major factors, which have been related to the risk of CHD and stroke, is raised blood pressure (hypertension)(Stamler & Stamler, 1993). Individuals with hypertension are three times more likely to develop or suffer a stroke, and twice as likely to die from these diseases than those with normal levels (Chief Medical Officer, 2001; Padwal *et al.,* 2001; Kannel, 1996; Klag *et al.,*

1996). It is estimated that lowering the mean systolic pressure in a population by 10 mmHg would correspond to a 30% reduction in total attributable mortality (Rose, 1985).

Maintenance of normal blood pressure is dependent on the balance of cardiac output and peripheral vascular resistance, which in turn have a number of regulatory mechanisms and their derangement may play a part in essential hypertension (Beevers *et al.*, 2001) (Figure 1.1).

Arbitrary measures are given to blood pressure to determine the clinical condition of an individual (Table 1.1). Blood pressure increases with age in westernised populations and is higher in particular ethnic populations (Lightstone 2003). Screening adults to detect hypertension early and initiate treatment before the onset of target organ damage appears highly cost and clinically effective (Littenberg, 1995).

Table 1.1. Classification of blood pressure for adults aged 18 years and older (not taking anti-hypertensive drugs or suffering from acute ill health).

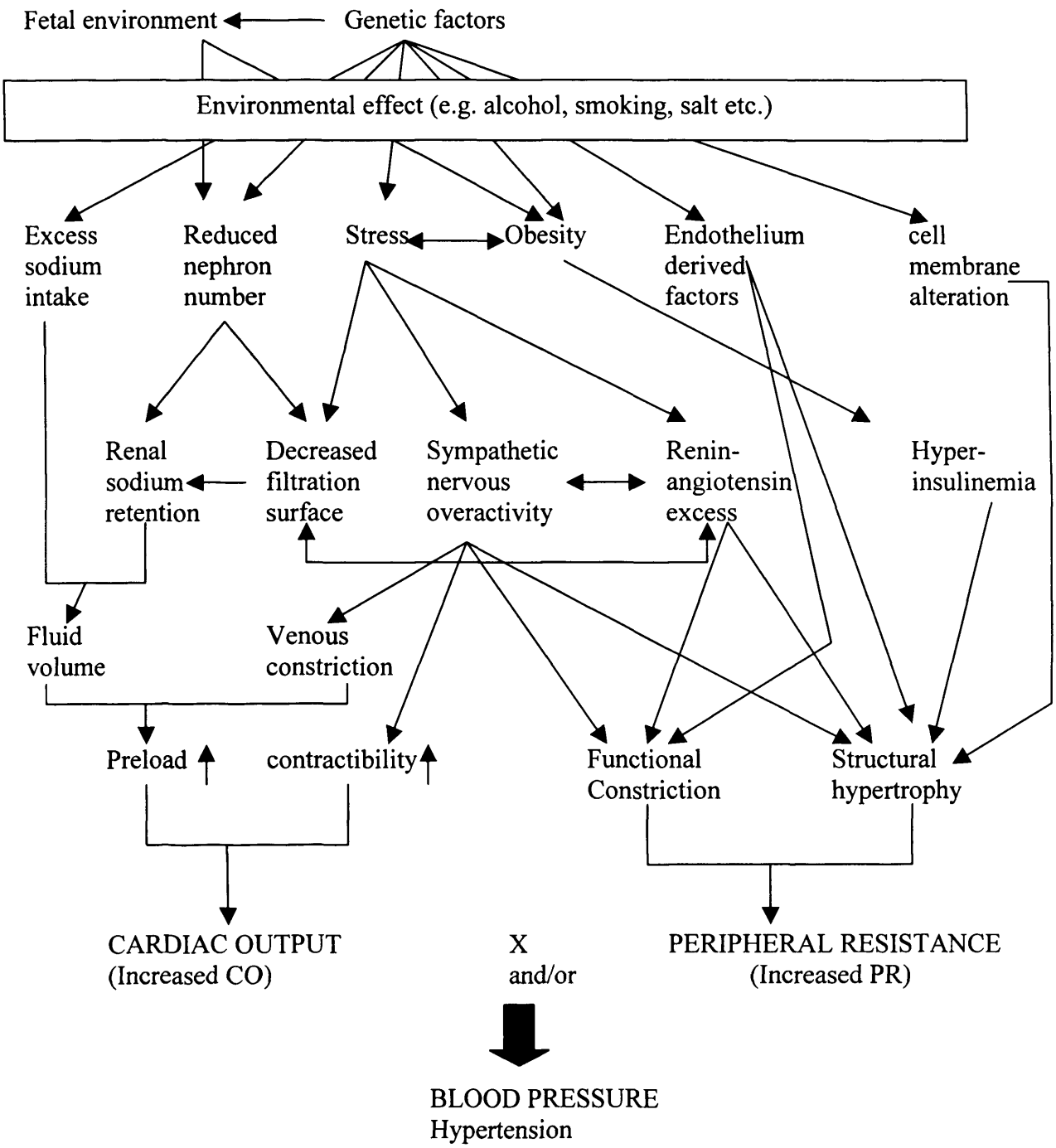
Category	**Systolic, mm Hg**	**Diastolic, mm Hg**
Normal	**<130**	**<85**
High Normal	**130-139**	**85-89**
Hypertensive	**>140**	**>90**

Adapted from: The Fifth Report of the Joint National Committee on Detection, Evaluation, and Treatment of High Blood Pressure (1993).

1.2. Hypertension and the kidney.

The Joint National Commission report (1993), defines human hypertension at a level of ≥ 140/90 mm Hg. This classification has been widely adopted and categorises hypertension into 4 groups of severity (Table 1.2) (Mulrow, 1999).

Figure 1.1. *Factors involved in the control of blood pressure and the pathophysiology of hypertension.*



Modified from Kaplan (1998). Environmental and genetic factors can contribute at any point in the diagram.

Table 1.2. Classification of hypertension for adults aged 18 years and older (not taking anti-hypertensive drugs or suffering from acute ill health).

Category of Hypertension	Systolic mm Hg	Diastolic mm Hg
Stage 1 (mild)	**140-149**	**90-99**
Stage 2 (moderate)	**160-179**	**100-109**
Stage 3 (severe)	**180-209**	**110-119**
Stage 4 (very severe)	**≥210**	**≥120**

Adapted from, The Fifth Report of the Joint National Committee on Detection, Evaluation, and Treatment of High Blood Pressure (1993).

The causes of hypertension in 95% of patients are unknown and in these cases the condition is termed 'essential-hypertension' (Brown, 1997; Lote, 2000). A small number may have an underlying renal or adrenal disease that explains the raised blood pressure and this is termed secondary hypertension (Beevers *et al.,* 2001; Lote, 2000; Wilkinson, 1994). Renal disease includes renal artery stenosis (renovascular hypertension), intrinsic renal disease (renal hypertension) and rarely, primary hyperaldosteronism (Lote, 2000).

The impact of the kidney on hypertension is well defined in both animal and human studies. In animals, experiments using the classical two-kidney, one clip Goldblatt hypertension model or renal artery stenosis clearly indicate that impairment of renal function results in elevated blood pressure (Dall'Aglio *et al.,* 1995; Pykonen *et al.,* 1986; Morton & Wallace, 1983). In humans, patients with high blood pressure and terminal nephrosclerosis become normotensive if bilaterally nephrectomised and transplanted with kidneys from a normotensive donor (Stuyver *et al.,* 1991).

The role of the kidney in the determination of the risk for hypertension can be inferred from genetically hypertensive rat strains. Renal transplants from the Milan hypertensive, the

spontaneously hypertensive and the Dahl salt-sensitive hypertensive rats into compatible strains of normotensive rats similarly provoked the recipients to develop hypertension (Churchill *et al.,* 1992; Dahl & Heine, 1975; Fox & Bianchi, 1976; Kawabe *et al.,* 1978; Kopf *et al.,* 1993; Rettig *et al.,* 1993). When a spontaneously hypertensive rat receives a renal transplant from a normotensive strain, blood pressure is reduced, highlighting the possible role of genetic factors in determining blood pressure.

Some single genes have been linked to the development of essential hypertension and involve elements of the renin-angiotensin-aldosterone system (Dluhy, 2002). However, Beevers *et al.,* (2001) and O'Shaughnessy, (2000), favour multiple gene involvement. The genetic causes e.g. Liddles syndrome, Gordons syndrome, apparent mineral corticoid excess, pseudohypoaldosteronism, of hypertension, impact on renal sodium handling, supporting the concept that hypertension is caused by impaired ability to excrete excess dietary sodium (Dluhy, 2002; Luft *et al.,* 2003; Tomson, 2000).

1.3. Environmental factors relating to hypertension.

Unmodifiable risk factors for cardiovascular disease include age, male sex, ethnicity, and family history (Padwal *et al.,* 2001). Dietary and behavioural factors are potentially the most modifiable determinants of hypertension. The Intersalt study measured 24-hour urine electrolytes and blood pressure in over 10,000 men and women aged 20-59 in 50 places worldwide. A positive correlation between sodium (Na^+) excretion and both systolic and diastolic blood pressure was observed. The correlation became stronger with age related increase in blood pressure and Na^+ excretion (Intersalt Cooperative Research Group, 1988; Elliot *et al.,* 1996). In the U.K. the average salt intake is about 9g a day, 3g more than the recommended amount. This

equates to 3.5g of sodium daily, 1.5g more than the recommended amount for good health (Food Standards Agency, 2003). This high ingestion of salt has been associated with the increased consumption of processed foods (Food Standards Agency, 2003). Folkow, (2003), points out that the salt content of processed foods should be declared and kept low because 'salt sensitivity' is not uncommon and is easily ingested by the individual, but impossible to eliminate.

Gordon & Kannel, (1983) and Thun *et al,* (1997), found that a high consumption of alcohol was related to elevated blood pressure and cardiovascular disease, although moderate consumption induced a lower blood pressure than in those who did not consume any. There is a strong correlation between raised serum cholesterol and CHD (Neaton & Wentworth, 1992). Replacing saturated fat with polyunsaturated fat was found to prevent coronary events and the adoption of a 'Mediterranean diet' and fatty fish improved survival. Such dietary interventions compared favorably with drug treatments for hypertension (Sacks & Katan, 2002).

Epidemiological evidence linking cigarette smoking with cardiovascular disease is over-whelming. Pittilo, (2000), has demonstrated that smoking is associated with blood vessel wall damage including endothelial injury. Waters *et al.,* (1996), found smoking actually accelerates coronary progression. Vascular damage and the progressive reduction in elasticity through smoking may be one contributory factor in essential hypertension.

Moderate dietary restriction in animal studies (See Section 1.6.3) have been shown to impair vascular reactivity in pregnant and non-pregnant rats and may influence maternal cardiovascular adaptation in pregnancy (Koumentaki *et al.,* 2001; Itoh *et al.,* 2002). Ozaki *et al.,* (2001) have demonstrated that offspring of rats exposed to these diets also have vascular dysfunction and hypertension. These moderate dietary restrictions feed to the pregnant rodent dam also produce offspring with a reduced kidney size, nephron compliment and hypertension (Langley-Evans *et al.,* 1999; Nwagwu *et al.,* 2000)(See section 1.7). It may be possible that the

observed hypertension in the offspring is a direct result of remodeled arteries and altered functional response as observed by Ozaki *et al.,* (2001). However, altered growth and reduced functional capacity of a kidney is also a mechanism associated with hypertension (Nwagwu *et al.,* (2000). It is possible that the hypertension observed is in part a consequence of both arterial damage and impaired kidney development and that both elements contribute mechanistically, resulting in a rapid decline toward CVD.

Obesity has, in the last two decades, reached epidemic prevalence and is an independent risk factor for hypertension (Coatmellec-Taglioni & Ribiere, 2003; Hall *et al.,* 2003). Excess weight gain may be responsible for 65-75% of the risk of essential hypertension, but the precise mechanisms of obesity related hypertension remain unclear (Coatmellec-Taglioni & Ribiere, 2003; Hall *et al.,* 2003). Excessive visceral adipose tissue may physically compress the kidneys promoting loss of nephron function, further increasing arterial pressure, possibly leading to severe renal disease (Hall *et al.,* 2003). Metabolic Syndrome (also referred to as Syndrome X) is a cluster of risk factors for CHD that have an underlying metabolic causation, with central obesity the prime candidate for these metabolic alterations. Increased abdominal adiposity contributes to dyslipidemia, hyperglycemia and hypertension and in a proportion of metabolic syndrome cases, there is also beta-cell dysfunction leading to the clinical manifestation of diabetes mellitus (Vega, 2004). The leading cause of end-stage renal disease (ERSD) is diabetic nephropathy (Pastan & Bailey, 1998). Adipose tissue can secrete pro-inflammatory factors, pro-insulin resistance factors and other cytokines and hormones that can contribute to hypertension and impaired fibrinolysis (Nestel, 2004; Vega, 2004).

1.4. The Developmental Origins of Health and Disease.

A number of epidemiological studies in the late 1980's carried out by Barker and colleagues led to the original 'fetal origins of adult disease' hypothesis, which proposed that CHD and allied disorders originate through adaptive responses to under nutrition in fetal life (Barker, 1998). These adaptations are proposed to permanently change the structure and function of organs in ways that lead to later disease (Barker, 1999). Developmental programming is the process whereby a particular stimulus or insult, in the fetus or neonate, acts during a sensitive period of development to establish a long term or permanent biological response (Lucas, 1991).

Barker *et al.,* (1990), reported that high blood pressure in a cohort of elderly men was associated with low weight at birth. Further studies showed that similar associations could be demonstrated in women and in younger individuals (Wincup *et al.,* 1989; Barker *et al.,* 1992). Studies in a wide range of populations throughout the world indicate that specific characteristics at birth are predictive of increased blood pressure and elevated risk of cardiovascular mortality in adult life (Barker, 1998; Curhan *et al.,* 1996; Forrester *et al.,* 1996; Gennser *et al.,* 1988; Godfrey *et al.,* 1994a; Law *et al.,* 1996; Rich-Edwards *et al.,* 1995; Stein *et al.,* 1996; Yiu *et al.,* 1994). Birth and placental weight, abdominal and head circumference and ponderal index are all measurements that can be related to blood pressure in later life. In essence, disproportionate retardation of intrauterine growth resulting in a baby that is, either small, thin or short, produces a greater risk of hypertension in adult life (Barker *et al.,* 1989; 1990; 1995; Barker, 1998; Fall *et al.,* 1995; Law *et al.,* 1996; Martyn *et al.,* 1995; 1996a; Osmond *et al.,* 1993; Vijayakumar *et al.,* 1995).

Godfrey *et al.,* (1994b), in a study of 10 year-old Jamaican children suggested that raised blood pressure was related to the lowest maternal haemoglobin concentrations (anaemia is an

index of general undernutrition), triceps skinfold in early pregnancy and the lowest gestational weight gains. Campbell *et al.,* (1996), reported that in a retrospective cohort of British men in their mid-forties, blood pressure was highest in those individuals whose mothers consumed a high carbohydrate, low protein diet in pregnancy. However, some caution is necessary in interpreting these epidemiological findings (section 1.4.1).

The risk of disease is compounded if the pregnancy is associated with disproportionately large placenta (Barker *et al.,* 1990). A large body of epidemiological evidence now suggests that in addition to raised blood pressure (Barker *et al.,* 1990; Law *et al.,* 1993), and coronary heart disease risk (Fall *et al.,* 1995), a non-genetic predisposition to non-insulin dependent diabetes (Barker *et al.,* 1993; Phillips *et al.,* 1994), asthma (Shaheen, 1997), and osteoporosis (Cooper *et al.,* 1997) may be acquired through exposure to growth retarding influences *in utero.*

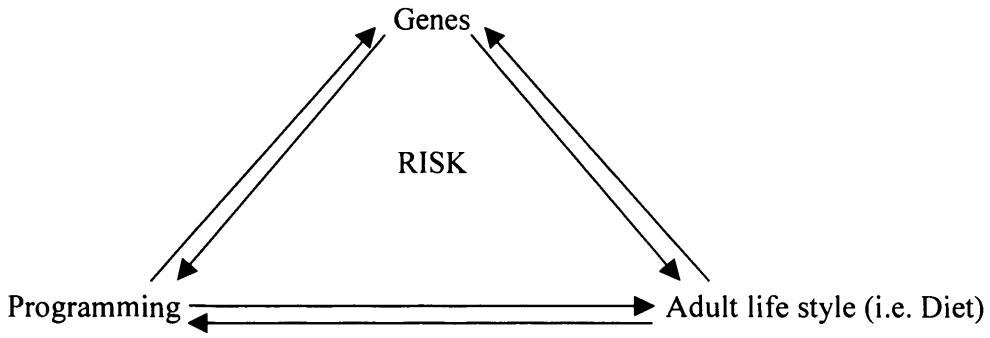
The primary determinant of fetal growth *in utero* is the genetic background of the individual. Studies in humans and animals suggest that the environment may limit this and in particular by the nutrients and oxygen the fetus receives from the mother (McCance & Widdowson, 1974; Gluckman *et al.,* 1990; Jackson, 2000). The birth size of babies born after ovum donation was essentially found controlled by the recipient mother's body and nutritional environment it afforded. The birth weight of the babies was strongly related to the weight of the recipient mother, with heavier mothers having larger babies, however birth weight was not related to the weight of the women who donated the eggs (Brooks *et al.,* 1995). This mirrors the classical experiments of Walton & Hammond, (1938) who demonstrated the influence of maternal restraint in crosses of shire horses and Shetland ponies.

Jackson, (2000a) indicates that each individual has a genetic endowment at conception that sets the limits on the capacity or metabolic function and the extent to which this capacity is achieved or indeed constrained is determined by the environmental experience. The

consequences of these experiences throughout life express themselves as achieved growth and body composition, hormonal status and metabolic function for one or other function. At any time in life the response to an environmental challenge is determined by an interaction amongst these factors and when the metabolic capacity to cope is exceeded, the limitation in function is exposed and expressed as diseases. Jackson, (2000a) points out that the effect of any adverse environmental exposure is likely to be more marked during early life on the development of the embryo and fetus, than in later life. This is likely to exert a fundamental effect on the development of metabolic capacity.

By factoring in developmental programming an interrelationship between, genes, adult lifestyle and programming emerges to produce a triad of interactions. The overall impact of each of these three contributions to total risk is difficult to estimate or assume any generalizations (Figure 1.2). A review by Han and Carter (2001), on the control of growth and development of the feto-placental unit acknowledge classical gene targeting within fetal developmental programming. They propose that enhanced DNA microarray technology will enhance the understanding of how fetal and placental growth is controlled. Simply expressed, those at the lowest risk may have genetic protection against disease, were exposed to ideal conditions during fetal development, and lead a healthy adult life style. Opposing this, individuals at greater risk will combine a genetic predisposition to disease with an adverse fetal environment coupled with a series of poor adult lifestyle factors (Langley-Evans, 2004). Maternal nutritional status is suggested to be the main developmental programming influence in the 'Barker hypothesis' and has been shown in both human and animal pregnancies to modify intrauterine growth and impact significantly on overall weight and proportions at birth (Godfrey & Barker, 1995).

Figure 1.2. *Developmental programming and the determination of disease risk.*



Adapted from Langley-Evans, (2004).

1.4.1. Criticisms of the Developmental Origins of Health and Disease Hypothesis.

The 'developmental origins of health and disease hypothesis', was so revolutionary from the classically held views of CVD, that it is only appropriate for the hypothesis to be rigorously examined and tested. Initial criticisms of the 'Barker hypothesis' suggested that people exposed to a poor prenatal environment are more likely to be exposed to a disadvantaged postnatal environment (Bartley *et al.,* 1994). The postnatal environment is known to have an effect upon the risk of disease. Furthermore, Barker and colleagues did not actually measure the baby's or mother's nutritional intake and simply assessed the baby's subsequent growth and birth weight as a measure of nutritional status (Paneth & Susser, 1995). Barker, (1994), has shown that the association between fetal growth and adult disease has been observed in all social economic groups and are independent of postnatal life style factors that may affect disease.

However, failure to account for, or incorrectly adjust for confounding factors, such as sex, height, parental socio-economic status, current socio-economic status, parental blood pressure, alcohol consumption, race and gestational age, has received particular criticism (Joseph &

Kramer, 1996; Kramer, 2000; Huxley *et al.,* 2002). For example, most studies using current weight as a confounding factor in regression coefficients were found by Huxley *et al.,* (2002), to exaggerate the strength of the inverse relationship between birth weight and blood pressure. The meta-analysis conducted by Huxley *et al.,* (2002) of 55 studies that reported regression coefficients of systolic blood pressure (with 48 further studies that reported only directional association) suggested that an over-estimation of the association between low birth weight and increased blood pressure had been made. Huxley *et al.,* (2002) weighted estimate of 0.6 mm Hg lower systolic blood pressure per 1 kg higher birth weight is much lower than that of Barker and colleagues' 2-4 mm Hg lower systolic blood pressure per 1 kg higher birth weight.

Kramer & Joseph, (1996), suggest that Barker and colleagues have a tendency to restrict their reference citations to studies that support their hypothesis yet at the time there was a sufficient number of published studies whose results did not support (Whincup *et al.,* 1992; Lucas & Morley, 1994; Matthes *et al.,* 1994) or even contradicted the fetal origins hypothesis (Seidman *et al.,* 1991). Initial concerns about the potential for selection bias (Kramer & Joseph, 1996) have been largely dispelled by large cohort studies, with small losses to follow up (Kramer, 2000). However, Huxley *et al.,* (2002) maintain that the hypothesis remains primarily supported through a publication bias whereby small studies with extreme results are more likely to be published. Huxley *et al.,* (2002) conclude that the influence of birth weight on later blood pressure, in humans, is unreliable.

The initial 'Barker hypothesis' enabled an emerging life science to examine just how poor nutrition may programme hypertension. As with all novel ideas the concept of programming is constantly evolving not only in the methodology and statistical analysis employed, but also in the understanding of the many factors that may influence developmental plasticity *in utero.* Programming factors should not necessarily be considered the principal mechanism behind the

early development of disease, but one of many working in concert (See Section 1.4). This supports the necessity to explore developmental programming using animal models. Animal studies may enable the causal nature of ' fetal programming' to be identified, whilst controlling for confounding factors.

1.5. Developmental programming.

Developmental programming in the context of perinatal determinants of human disease is a relatively new concept. Evidence of non-nutritional developmental programming has been available for some time, such as how the climate can affect the number of functional sweat glands in the human (Diamond, 1991), or how the incubation temperature of the American alligator egg can influence sex (Deeming & Ferguson, 1992). Barker (1998) describes the system during critical periods of development, or 'windows' as being plastic and sensitive to the environment, followed by a loss of plasticity with fixed functional capacity. 'Windows' are essentially periods of rapid cell division within a tissue. Mammalian cells undergo a number of cell divisions before further replication becomes impossible or apoptosis is initiated (Jennings *et al.,* 2000). An insult or stimulus at these critical periods may cause the developing tissue to be programmed and thus alter ultimate physiology and functional capacity. In humans the majority of organs are fully developed *in utero.* If critical periods of programming relate to periods of cell division, then in the human, most if not all the critical periods would occur during fetal life. In other species, cell division may occur before and after birth.

A recent paper by Bateson *et al.,* (2004) indicates that individuals developmentally adapted to one environment may, however, be at risk when exposed to another when they are older. The offspring's responsiveness to their mother's condition before birth may generally

prepare individuals so they are best suited to that environment. In conclusion, Bateson *et al.,* (2004) promote the concept that a fuller understanding of the patterns underlying human developmental plasticity in response to early nutrition and other environmental factors will have implications for the administration of public health.

In humans, organs required in a critical functional sense during fetal life, such as the heart, brain and liver, undergo differentiation and growth during the early phases of development. Other systems *in utero* such as the kidney and lung are involved in processes that are very different to those in postnatal life and undergo rapid growth and development in later gestation ready for birth. The exposure of the fetus to adverse conditions in early gestation may hence give rise to an individual prone to heart disease and in late gestation it may predispose the individual to renal disease. A recent study by Painter *et al.,* (2003) examined people exposed to the Dutch famine of 1944/1945 and found those exposed to famine in mid-late gestation had higher rates of microalbuminuria prevalence compared to those born outside the famine. Mid-late gestation is a period of rapid glomerular development and exposure to famine at this critical time may result in a reduced glomerular endowment, and increase the risk of developing microalbuminuria as an adult (Mackenzie & Brenner, 1995).

1.6. Nutrition and developmental programming.

1.6.1. Nutrition and fetal growth in humans.

The role of maternal nutrition as a developmental programming stimulus appears to be of great importance from a public health perspective, particularly as an optimal diet for pregnancy remains undefined. Comparison of normal birth weight ranges in various populations strongly

suggests that factors other than genetics may influence uterine growth. Mean birth weight is typically higher in westernised populations compared to underdeveloped countries and this implies that differences in birth weight are a product of nutritional differences (Barker, 1998). Mathews *et al.,* (1999), suggest that among reasonably well-nourished women of industrialised countries, maternal diet in pregnancy has at most a small impact on placental and birth weights and that malnutrition may have greater impact on fetal growth in developing countries. Doyle *et al.,* (1992) suggested that maternal nutrient intakes have a greater impact on birth outcomes from less affluent populations. However a recent study by Langley-Evans & Langley-Evans, (2003), observed that in well-nourished populations, maternal nutrient intakes are less important determinants of fetal growth than genetic factors and other maternal constraints such as smoking, physique and socio-economic status.

In humans, associations between maternal nutritional status and birth weight are difficult to demonstrate, but studies of periods of famine or extreme hardship during pregnancy do indicate that nutrition has some impact on birth weight. Wartime famines have been associated with lowered birth weights (Antonov, 1947; Smith, 1947) as have seasonal variations in food availability. Prentice *et al.,* (1981; 1987), has shown food availability in the Gambia varies greatly between the wet season (1250 kcal/day) and the dry season (1480 kcal/day). Babies born in the wet season are lighter by 160g than those born during the dry season.

Matthews *et al.,* (1999), reported that macro- and micronutrient intakes in early and late pregnancy did not impact upon birth weight. These findings were recently supported on a study of 300 women, where little significant impact of maternal diet upon either birth weight or proportions at birth was found (Langley-Evans & Langley-Evans, 2003). Godfrey *et al.,* (1996) assessed 538 women's nutritional status in early and late gestation. High carbohydrate consumption in early gestation resulted in babies of lower birth weight. Low intakes of meat and

dairy protein in late gestation were also associated with lower birth weight. This effect was very small, with a maximal change of 217g in birth weight, with the average infant birth weights in the UK being 3500g. The study was suggestive of an interaction between protein and carbohydrate within the maternal diet and its possible influence on fetal growth. Human studies do show some indices of nutritional status are associated with ponderal index, which in part reinforce some of Barker and colleague's assertions. Disproportion at birth may therefore be a more critical indicator of later disease risk than birth weight (Langley-Evans & Langley-Evans, 2003).

1.6.2. Nutrition and fetal growth in animal studies.

Fetal growth in sheep is significantly retarded by reductions in maternal protein and energy intakes (Mellor, 1983; Harding *et al.,* 1994). Placental growth appears to be stimulated by undernutrition and this is interpreted as an adaptation to optimise substrate supply to the fetus (Harding *et al.,* 1994). Lechner, (1984; 1987) and Lecluier, (1985), found periods of maternal starvation in guinea pigs resulted in severe retardation of fetal growth. Dwyer *et al.,* (1992), found a 40% reduction of *ad libitum* maternal food intake equally limited the growth of the guinea pig fetus and placenta. Severe limitation of maternal food intake in the rat impacts upon overall fetal and placental size. Ariyuki, (1987), observed a significantly reduced body weight by up to 55% in the full term rat fetus with maternal starvation for 3-7 days in late gestation. Similarly, Woodall *et al.,* (1996), found with a maternal diet limited to 30% ad libitum, significantly constrained fetal and placental growth and lowered weight at birth.

Studies in rats have generally focused upon maternal protein restriction. Low protein diets are made isoenergetic through the addition of carbohydrate and provide an interesting parallel to human studies that have identified interactions between maternal protein and carbohydrate intake

as determinants of placental and birth weight (Godfrey *et al.,* 1996). The National Research Council (1978), recommend the amount of protein for a non-pregnant adult rat is 9% and that during pregnancy this should be raised to 13%. The feeding of a 4-6% protein diet was consistently shown to reduce fetal and placental weights in late gestation and eventual birth weights in rats, relative to offspring exposed to a maternal diet of between 24-30% protein (Hastings-Roberts & Zeman, 1977; Morgan & Naismith, 1977; Sharder & Zeman, 1970; Zeman & Stanbrough, 1969). Langley-Evans *et al.,* (1996a), found that fetal and placental growth is accelerated between days 12-20 of gestation if dams are pre-habituated and exposed to a mild protein restriction of 9% casein compared to controls of 18% casein. This is an effect in part dependent on the plane of maternal nutrition prior to conception (Langley-Evans *et al.,* 1996c). Between days 20 and 22 (full-term) the growth of the low protein exposed fetus is attenuated. In 18% casein control (protein replete) animals, weight doubles over the last two days of gestation. At full-term 9% low protein exposed animals are typically of low-to-normal birth weight with disproportionate effects on the trunk and peripheral organ growth and a decrease in body elongation (Langley-Evans *et al.,* 1996 a,c). Langley-Evans (1999), notes that 'with a disproportionate retardation of growth of the peripheral organs and effectively a small short neonate at birth, associated with an enlarged placenta, the rat exposed to a low protein diet is a good correlate of the individuals in the human population identified as most at risk of developing cardiovascular disease'.

Langley-Evans & Nwagwu (1998) observed that low-protein maternal feeding from the day of conception resulted in a less pronounced period of accelerated growth in early to mid-gestation compared to those on a pre-habituation low protein diet. Fetuses exhibited no evidence of disproportionality, but were born with low birth weight and were smaller than controls at 20 days gestation. Kwong *et al.,* (2000), found that maternal exposure to a low-protein diet only for

the discrete period from conception to pre-implantation of embryos (day 0-4.25) was sufficient to promote the developmental origins of hypertension.

1.6.3. Nutritional programming of blood pressure.

Persson & Jansson (1992) performed one of the first animal experiments to test the 'Barker hypothesis'. Pregnant guinea-pigs had a single uterine horn ligated to specifically retard growth in one uterine horn. Arterial ligation produced retarded growth, in the region of 40-60% compared to their unaffected siblings from the contra-lateral, untreated horn. Pups from the ligated horn had higher blood pressures at three months compared to their siblings. The reduction of birth weight by 50-60% however, only accounted for approximately 10mmHg increase in blood pressure and thus was relatively modest.

Langley & Jackson (1994) produced a range of diets ranging from 18% protein, to be compared against 6%, a severe protein restriction in the pregnant rat, to 9%, a mild protein restriction and to 12%, the minimal requirement for the pregnant rat (Clarke *et al.,* 1977). All diets were equal in energy with the deficit in protein supplied with extra carbohydrate. A 9% casein low protein diet, providing the protein for a non-pregnant rat, in pregnancy was found to elevate blood pressure in offspring compared to those exposed in utero to an 18% casein control diet. The hypertensive state was evident from as early as weaning at 3-4 weeks of age (Langley-Evans *et al.,* 1994). Hypertensive offspring were also generated from mothers exposed to the minimal nutritional requirement of a rat pregnancy at 12% protein (Langley & Jackson, 1994). Elevations of systolic blood pressure in the order of 8-30mmHg compared to controls have been consistently reported (Langley-Evans *et al.,* 1994; 1996 a,b,c,e,f; 1999; Langley-Evans & Nwgwu, 1998; Sherman & Langley-Evans, 1998, Nwagwu *et al.,* 2000). The hypertensive effect

of the 9% casein maternal diet on offspring compared to 18% casein control has also been successfully reproduced in mice (Dunn *et al.,* 2001). Feeding 9% low protein diets over discrete periods of time, early, mid or late gestation (days 0-7, 8-14, 0-14, 15-22 or 8-22) had a similar effect on blood pressure as protein restriction from conception to birth (days 0-22) (Langley-Evans *et al.,* 1996c). The greatest effects were observed when low protein feeding was targeted at the later stages and throughout pregnancy, although relatively minor periods of mild maternal undernutrition, at any stage of pregnancy may exert a negative effect upon the cardiovascular system of the fetus and does not depend upon any critical or vulnerable 'window' (Langley-Evans *et al,* 1996c).

A similar finding by Woods (2000) was observed in rats exposed to a 5% MLP diet during the second half of gestation. The blood pressure of the offspring was significantly elevated compared to those exposed to a 5% MLP diet during the first half of gestation. Vehaskari *et al.,* (2001), found the feeding of a 6% MLP diet significantly elevated blood pressure through out the first 10 months of rat life compared to those exposed to a 20% maternal protein diet. The maternal feeding of a 54% high protein diet compared to a 20% protein diet did not elevate blood pressure in offspring at 4-weeks of age (Zimanyi *et al.,* (2002). The hypertension observed in the rats exposed to a maternal low protein diet *in utero* lasts throughout life and may be a contributor to a reduced life span (Aihie Sayer *et al.,* 2001). The feeding of a low protein diet does not modify the blood pressure in the non-pregnant or pregnant rat, only the offspring (Langley-Evans *et al.,* 1994). This indicates that low-protein feeding infers a developmental programming response on the offspring.

Animal studies consistently replicate these observations. However, studies that have failed to show an association between low-birth weight and subsequent hypertension (Matthes *et al.,* 1994; Lucas & Morley, 1994) have relatively small sample sizes (330-758 respectively).

However, by virtue of the fact no association was present contradicting the overwhelming global evidence supporting the 'Barker hypothesis' it may also be subject to publication bias.

1.6.4. Different dietary protocols and the developmental programming outcome.

Protein is unlikely to be the only nutrient of consequence in the programming of hypertension in the rat. In adjusting the protein content of the diet it is necessary to adjust the carbohydrate content to maintain equivalent energy content. For example the 9% casein low-protein diet used by Langley-Evans *et al.,* (1996c) contains 14% more carbohydrate when the protein content is restricted by 50%.

1.6.4.1. Fats.

The standard experimental diets of Langley-Evans and colleagues, use corn oil, rich in linoleic acid (LA), as the fat source and a 2:1 (10% w/w) mixture of starch and sucrose as the carbohydrate source (Langley-Evans *et al.,* 1996a). Langley-Evans, (1996) found that the feeding of mainly saturated fat (9% coconut oil+ 1% corn oil) during rat pregnancy increased blood pressure of the offspring independently of any protein effect. Langley-Evans & Jackson (1996) found normotensive rats, exposed to an 18% casein control diet *in utero* became hypertensive when fed a coconut oil diet. The increase in blood pressure was approximately 30 mmHg. No further increase in blood pressure was found in those rats exposed to a 9% diet *in utero* and already hypertensive.

Experimental diets rich in animal derived saturated fat (25% w/w) compared to control (5% w/w) produced hypertension in the female offspring only (Khan *et al.,* 2003). Maternal high-

saturated diets have also been found to produce abnormal vascular function, plasma lipid disturbance, and altered vascular fatty acid content (Gerber *et al.,* 1999; Ghosh *et al.,* 2001; Khan *et al.,* 2003). This is suggestive of a programming effect for fats.

When Lucas *et al.,* (1996) fed an 8% casein diet in which the fat source was soya oil, (LA rich) and the carbohydrate was provided as glucose: starch (17:2 w/w), to pregnant rats, no elevated blood pressure was recorded in the offspring. Linoleic and linolenic acids give rise to a different series of eicosanoids and may exert positive effects on maternal cardiovascular haemodynamics and subsequent nutritional supply to the fetus (Langley-Evans, 1999; 2000).

In the rat, dietary LA deprivation increases blood pressure (Rosenthal *et al.,* 1974; ten Hoor & van de Graaf, 1978; Schoene, *et al.,* 1980), whilst supplementation has a hypotensive effect (ten Hoor & van de Graaf, 1978; Schoene, *et al.,* 1980; MacDonald *et al.,* 1981). The hypotensive actions of LA appear to be mediated through prostacyclins and throboxanes, which have important vasoactive properties (as reviewed by Narumiya *et al,* 1999). Rats exposed to a commercially supplied diet containing 22% or 9% casein *in utero,* had no changes in blood pressure at 4-weeks, compared to those on the Langley & Jackson diet. One explanation forwarded is the commercial diet contains fat in the form of soya oil and whilst the composition of soya oil and corn oil do not differ greatly in their linoleic acid and oleic acid, which actually accounts for 80% of the fatty acids in these two diets, soya oil provides three fold more α-LA than corn oil (Langley-Evans (2000). The difference between the commercial diet and the diet used in this thesis in terms of programming blood pressure may be n-3 fatty acids. Soya oil in the commercial diet may give rise to prostanoids, which differ in their inflammatory and cardiovascular properties (Langley-Evans, 2000). Previous work using fish oil rich in n-3 fatty acids promote different blood pressure responses (in non-pregnant rats) compared to diets based on n-6 fatty acids in corn oil. Furthermore, the commercial diet provides half the fat of the diet

used in this thesis and the difference is sufficient to offset the effects of the n-3 fatty acid profiles. Evaluation of the long-term effects of maternal dietary fat upon blood pressure is of critical importance.

Fats have an important role in the vascular system. Phospholipids are a major component of cell membranes and cholesterol is a major component of all animal cells. Lipid prostaglandins contribute to the inflammatory response of injured cells, intensifying the effects of other potent vasodilators and promoting the emigration of phagocytes to the area (Tortora & Grabowski, 2003). However the role of fats can also be highly detrimental to vascular function and health in humans, such as low-density lipoproteins (LDL), which contain more cholesterol than its transporter protein when entering the blood vessels. High saturated fat diets, increased age or persistent hypertension and other detrimental environmental factors cause atheromas to develop within the blood vessel walls. These gradually migrate toward the endothelial lining and enlarge. Later, cholesterol and other lipids accumulate in the abnormal smooth muscle cells to produce plaques that bulge into the lumen as they develop. Nutrient exchange is compromised and the cells are replaced with scar tissue. Atherosclerosis is progressive, degenerative leading to eventual occlusion and possibly death (Sherwood, 1993; Sacks & Katan, 2002). Interestingly the rat is known to be resistant to the effects of atherosclerosis, even when subjected to high fat diets (Langley-Evans *et al.,* 1996f). The level and type of fat in the maternal rat diet employed in this thesis may commute a contributory programming effect in the offspring's resultant blood pressure, even with high levels of n-6 fatty acids present. More experimental evidence is required. Culling at 4-weeks in this thesis is early in the rat life and should avoid any potential disturbance in the offspring vasculature as having a detrimental effect on renal data examined.

Langley-Evans & Jackson (1996) also found that normotensive rats exposed to a maternal diet of 18% casein had significantly increased blood pressure when their drinking water

was replaced with 1.5% sodium chloride. Hypertensive rats exposed to a maternal 9% casein diet were not sensitive to salt loading, with no further increases in blood pressure noted. Spontaneously hypertensive rats are also able to resist the further hypertensive effects of salt loading by virtue of enhanced rates of sodium excretion (Ledingham *et al,* 1990). The interaction between dietary salt, blood pressure and nephron endowment in the rat offspring is not considered in this thesis, as the primary aim is to assess dietary developmental programming of renal morphology and subsequent function. At no time does the research outlined in this thesis attempt to unduly stress the reno-vascular system of the offspring. This does not exclude sodium loading as an experimental protocol in future studies using the maternal diets outlined in Chapter 2.

1.6.4.2. Methionine.

The Langley & Jackson, (1994) low protein diet provides 0.5g methionine/100g diet, compared to the commercial low protein diet (H9) of 0.08g/100g representing a six-fold difference. Rees (2002) has argued that the methionine content of the diet is excessive.

In studies of rats and mice fed a maternal low protein diet during pregnancy, maternal circulating threonine concentrations were reduced by 50-60% at day 19 of gestation possibly due to an increase of activity in the pathway which metabolises homocysteine produced by the trans-sulphuration of methionine (Rees *et al.,* 2000; Petrie *et al.,* 2002. An increase in threonine oxidation is characteristic of an excess of methionine in the diet as the requirement to oxidize excess homocysteine also increases the oxidation of threonine (Girard-Globa *et al.,* 1972, Rees *et al.,* 2000). The 9% casein, maternal low protein (MLP) diet provides 145% of the actual methionine requirement of a pregnant rat, but less than a third of the cysteine requirement. A

similar excess of methionine and low provision of cysteine occurs in the 18% casein control diet. The ratio of methionine to casein in the MLP diet is 5.55% compared to 2.77% in the corresponding control diet. However, when the protein: methionine ratio was balanced for control (18% casein diet with 0.25% methionine) and low protein (9% casein with 0.08% methionine), those exposed to maternal low protein still produced hypertension, suggesting methionine may not be a critical determinant of the programming effect (Pickard, 1999).

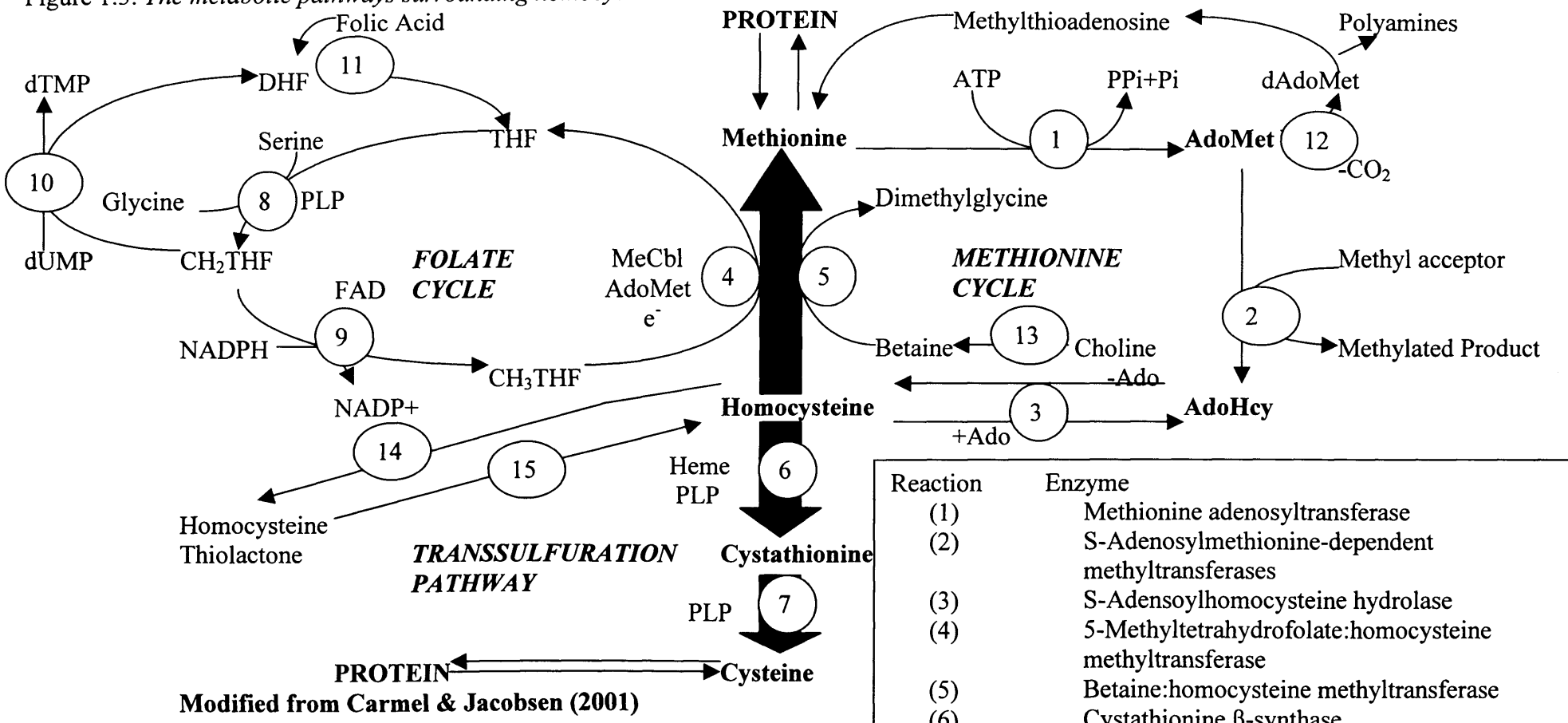
Rees, (2002, points out that animals fed casein based semi-synthetic diet must produce at least 60% of their cysteine requirement through this pathway. Casein does provide 9 essential amino acids (Lehninger, 1981) (Appendix 1) and the amino acid composition is considered reasonably adequate (Reeves, 1997), but may not be sufficient to meet rodent requirements for growth and pregnancy. The limitation of casein is its shortage of sulphur amino acids and in order to meet requirements the use of L-cystine is recommended over the use of DL-methionine in the AIN-93 rodent diet. This was formulated to improve the performance of animals that consume them and substituted the previous version AIN-76A (Reeves *et al.,* 1993). When casein is used at 200 g/kg and L-cystine at 3.0g/kg diet (AIN-93G formulated for growth. Reeves *et al.,* 1993) there are sufficient concentrations of each essential amino acid to provide the National Research Council (NRC)(1978) recommendations. The AIN-93M diet developed for maintenance of the adult rodent of 140g/kg of casein and 1.8g of L-cystine provides more than enough of the sulphur amino acids required (NRC, 1978). The estimated requirement for sulphur amino acids has been raised from 6.0g/kg to 9.8g/kg diet in the fourth revised edition of the NRC Nutrient Requirements of Laboratory Animals (1995). The AIN-93G diet, casein falls short even with additional L-cystine and remains below the recommended amount for growth (Reeves, 1997). Reeves (1997) points out that an additional 1.6g of sulphur amino acid will have to be added to L-cystine already present and L-methionine may be the amino acid of choice as high

concentrations of L-cystine compromise copper metabolism (Kato *et al.*, 1994).

There may be important metabolic consequences of methionine excess. Low protein feeding may limit the availability of many essential and conditionally essential amino acids and the capacity for the maternal liver to process excess methionine may be exceeded in early pregnancy. (See section 1.6.4.3). One pathway for homocysteine metabolism involves methionine synthase. Methylene tetrahydrofolate is utilized in the re-synthesis of methionine. If folic acid is limiting and substantial quantities of methylene tetrahydrofolate are used for the re-methylation of homocysteine the folic acid pool becomes depleted (James *et al.*, 1997). Rees, (2003) points out that an excess of methionine or homocysteine is similar to dietary folate deficiency.

Methionine can normally be cleared through conversion to homocysteine, in the presence of a methyl donor, and the homocysteine is then converted to cysteine in a number of reactions comprising the trans-sulphuration pathway (Figure 1.3). Low protein feeding may limit the capacity to generate methyl donor amino acids and homocysteine levels may rise. Homocysteine depends on cystathionine synthase and cystathionine γ-layse (SDH) for removal. Rees & Hay, (2001) found that cystathionine synthase remained constant during rat pregnancy whereas SDH activity changed. Dams exposed to a normal diet during pregnancy had a high SDH activity for the first 4 days, which had fallen to 25% of the starting level by day 11. The decline probably spares amino acids to support increasing demands for fetal growth. In low-protein exposed dams SDH activity was 25% less than at starting by day 4 of pregnancy demonstrating the need to conserve amino acids earlier (Rees & Hay, 2001). Circulating homocysteine levels are doubled in low-protein fed dams compared to those on control diets (Rees, 2003) The limiting effect of the low-protein diet on the dam affords no maternal nutrient buffer to the offspring and the potential consequence of a disturbed homocysteine metabolism may arise (Jackson, 1999; Rees, 2002).

Figure 1.3. *The metabolic pathways surrounding homocysteine.*



Reaction	Enzyme
(1)	Methionine adenosyltransferase
(2)	S-Adenosylmethionine-dependent methyltransferases
(3)	S-Adensoylhomocysteine hydrolase
(4)	5-Methyltetrahydrofolate:homocysteine methyltransferase
(5)	Betaine:homocysteine methyltransferase
(6)	Cystathionine β-synthase
(7)	Cystathionine γ-lyase
(8)	Serine hydroxymethyltransferase
(9)	5,10-Methylenetetrahydrofolate reductase
(10)	Thymidylate synthase
(11)	Tetrahydrofolate dehydrogenase
(12)	S-Adenosylmethionine decarboxylase
(13)	Choline dehydrogenase
(14)	Methionyl-tRNA synthetase
(15)	Paraoxonase

The recycling of homocysteine back to methionine may be limited in protein-restricted animals as this depends on the presence of a pool of methylated folates. Glycine provides the methyl groups required for the synthesis of the methylene tetrahydrofolate and determines the availability of methylated folates (Rees, 2002). Jackson, *et al.,* (1997b) found maternal glycine status in human population studies to be limiting. Placental *de novo* glycine supply to the fetus is dependent upon serine with folate as a cofactor (Bennett & Jackson, 1998). Furthermore, folate deficiency is known to limit the production and availability of glycine (Arnstein and Stankovic, 1956). The liver and kidney have an alternative re-methylation pathway to that of the trans-sulfuration pathway utilising betaine (trimethylglycine) as the methyl-group donor substrate (Wilcken & Wilcken, 2001) Zlotkin & Anderson, (1982) note that in early life excess methionine removal maybe compromised, as the enzymes of the trans-sulphuration pathway are largely not expressed in fetal tissue (See section 1.9.3. Homocysteine).

At present it is unclear whether increased levels of methionine in the maternal low protein diet play a pivotal role in the programming of blood pressure. A recent study by Langley-Evans *et al.,* (2003a) found no significant differences in systolic blood pressures when comparing 18% casein and 9% casein containing 0.5% methionine (Langley & Jackson, 1994) to a high methionine diet (18% casein, containing 1% methionine) and low methionine diet (9% casein containing 0.25% methionine).

1.6.4.3. Conditionally essential amino acids.

Traditionally amino acids have been classed as essential and non-essential in chemical and metabolic terms. Amino acids considered nutritionally essential indicate that each has a specific structural feature, the synthesis of which cannot be catalyzed by mammalian enzymes.

The reverse applies to non-essential amino acids although Reeds (2000), points out that a true nonessential amino acid can only be synthesised *de novo* non-amino acid source of nitrogen and an appropriate carbon source.

Amino acids termed 'conditionally essential' are used to imply that there are measurable limitations to the rate at which they can be synthesised. When this limit is attained, the amino acid in question becomes an essential component of the diet (Reeds, 2000). These limitations can result from a number of factors. Firstly, the synthesis of these amino acids requires the provision of another amino acid, either as the carbon donor or as a donor of an accessory group. Thus, the ability of the organism to synthesise a given conditionally essential acid is set by the availability of its amino acid precursor (Reeds, 2000). Jackson *et al.,* (1981) demonstrated this in the maintenance of the glycine supply of the milk fed mammal. The demand for this conditionally essential amino acid necessitated in an increase in the synthesis of serine, its precursor.

Secondly, some amino acids may be synthesised in only a limited number of tissues. For example, Wakabayashi *et al.,* (1994) and Wu *et al.,* (1997), point out that the synthesis of both arginine and proline are crucially dependent on intestinal metabolism and that dietary as opposed to systemic, amino acid precursors are obligatory (Beaumier *et al.,* 1995; Berthold *et al.,* 1995; Brunton, *et al.,* 1999; Murphy *et al.,* 1996 and Stoll *et al.,* 1999). Thus, alterations in either intestinal metabolism or the route of the nutrition can have a critical bearing on the ability of the organism to synthesise these amino acids (Reeds, 2000).

Thirdly, the majority of evidence suggests that in the presence of abundant quantities of the appropriate precursors, the quantities of conditionally essential amino acids that can be synthesised may be quite limiting (Castillo *et al.,* 1993; Fukagawa *et al.,* 1996). It can be argued there are circumstances under which the metabolic demands for amino acids rise to values that are beyond the biosynthetic capacity of the organism through either excessive stress (Jaksic *et al.,*

1991) or immaturity or lack of the necessary enzymic activity (Gaull *et al.,* 1972).

Reeds (2000) and more recently Fürst and Stehle (2004) conclude that essential, non-essential and those considered conditionally essential are all critically important to physiological function and health.

1.7. Renal developmental programming.

In man the number of nephrons varies considerably between 0.3 to 1.5 million (Mackenzie & Brenner, 1995; Lote, 2000). This is supported by Nyengaard & Bendtsen (1992) that put the average figure in man at 617,000. In the rat nephron number varies between 22,000-45,000 (Bertram *et al.,* 1992; Gilbert *et al.,* 1987; 1991; Merlet-Benichou, 1999).

The relationship of nephron number to blood pressure may be important. In genetically hypertensive rats, nephron number is typically 25% lower than in normotensive strains and in the PVG strain, which is resistant to hypertension, nephron number is greater than seen in other normotensive strains (MacKenzie & Brenner, 1995). It was initially proposed that structural alterations to the developing kidney might play a critical role in the prenatal programming of blood pressure (MacKenzie & Brenner, 1995). The hypothesis advanced was that maternal nutritional status might determine the nephron complement of the kidneys, and that total nephron supply is a factor determining the susceptibility of an individual to hypertension (MacKenzie *et al.,* 1996). Any insult interfering with nephrogenesis in early life may reduce the glomerular filtration rate (GFR) seen in the mature organ. Kidneys with a reduced nephron number increases pressure within individual glomeruli to maintain normal excretory function. Nephrons are subject to damage in response to high local pressures, leading to glomerulosclerotic lesions resulting in a vicious cycle of nephron loss and further increase in blood pressure (Brenner, 1985).

The kidney would appear to be a likely target for the programming effects of maternal and fetal undernutrition. As an organ that differentiates and grows rapidly at a relatively late stage in gestation, the kidney is extremely vulnerable to the adverse effects of growth retarding influences at this time. As any deficit at birth cannot be reversed (in humans), late gestational effects of undernutrition upon the kidney may play a pivotal role in the developmental programming of blood pressure.

1.7.1. Evidence for renal programming in humans.

The incidence of hypertension and stroke related mortality is significantly higher among African-Americans than in white Caucasians (Fogo, 2003). Autopsy studies suggest that the hypertension-prone black population characteristically have smaller kidneys, which is generally accepted as being indicative of lower nephron number (Pesce *et al.,* 1994). Australian aborigines are a population in which rates of renal failure are 21 times higher than the Australian Caucasian population. Singh *et al.,* (2001), demonstrated that in aborigines aged between 5-70 years, birth weight was significantly related to kidney volume. Ultrasound examination showed that individuals of lower birth weight tended to have thinner kidneys, but of normal length. These finding were supported at autopsy, with Australian aborigines kidneys being smaller than Australian Caucasians (Hoy *et al.,* 2003a). In contrast, Falkner *et al.,* (1998) found no association between birth weight and later blood pressure within African Americans.

Direct histological studies support the view that that fetal growth retardation exerts particular effects on the kidney that may predispose an individual to hypertension in later life. Hinchcliffe *et al.,* (1992) studied the kidneys of stillborn, growth retarded (IUGR) human infants compared to appropriate for gestational age (AGA) infants and found that the IUGR group had

35% fewer nephrons than AGA infants. Manalich *et al.,* (2000) studied 18 low birth weight infants compared to 17 normal birth weight infants and noted reduced nephron number and increased glomerular volume in the growth retarded group. Both renal variables were strongly correlated with birth weight and were independent of sex and race. These histological studies are supported by data collected through ultrasound examination of the fetal kidney. Konje *et al.,* (1996), reported that in late gestation, the kidneys of IUGR fetuses were particularly vulnerable to growth failure and had an elongated, thin morphology, which is consistent with the findings of Hoy *et al.,* (2003b) for both children and adults.

Studies evaluating renal function in relation to birth weight conducted on populations exposed to famine tend to disagree with the Barker hypothesis. Yudkin *et al.,* (1997) and Yudkin *et al.,* (2001) found no relationship between microalbuminuria and birth weight in individuals exposed to the Dutch famine of 1944-1945 during fetal development. Similarly, no influence of maternal malnutrition during the siege of Leningrad (1941-1944) on albumin excretion rate in the offspring was found, even though average birth weights fell by up to 18% (Antonov, 1947). A recent study by Painter *et al.,* (2003), noted an association between microalbuminuria in adult offspring in those exposed to the Dutch famine in mid-gestation, but this was not related to birth weight.

1.7.2. Evidence of renal programming in animal studies.

Early studies of the effects of fetal exposure to low protein diets (Hall & Zeman, 1968; Zeman, 1968; Merlet-Bénichou *et al.,* 1994) indicated that the developing rodent kidney is vulnerable to such insults, with lower numbers of nephrons and reduced functional capacity. These experiments tended to utilise dietary regimes that were extremely severe causing a

pathological response rather than a physiological one. Lucas *et al.,* (2001) demonstrated that rats exposed to a 50% intrauterine food restriction exhibited reduced nephron number, increased glomerular diameter, impaired renal function and earlier onset of glomerulosclerosis. The feeding of a 9% protein diet, which represents the equivalent of protein demand for a non-pregnant rat (Clark *et al.,* 1977), results in a nephron deficit at birth that extends into post-natal life (Langley-Evans *et al.,* 1999). Nephron number in rats exposed to a 9% casein diet was significantly reduced when compared to control animals, from 4 weeks of age. An evaluation of the impact of the timing of protein restriction upon the kidney indicated that mid- to late-gestation was a critical period for the programming of renal reserve (Langley-Evans *et al.,* 1999). Similarly, Vehaskari *et al.,* (2001) noted that feeding a 6% low protein diet over the final 9 days of gestation impaired renal development and promoted nephron deficit. In the rat, nephron number continues to increase until day 11 postnatally, but the effects of prenatal undernutrition persist and this postnatal development phase does not overcome the earlier influence of maternal diet.

The low-protein rat model is far from the extreme famine models discussed previously. Primarily, the manipulation of the nature and balance of nutrients help elucidate mechanisms by which intra-uterine programming of adult disease may occur. Subtle differences in the composition of the low-protein rat diet and its evaluation on the maternal substrate to adequately supply nutrients for fetal growth may be more representative of the levels of undernutrition experienced in human populations. Whilst Mathews *et al.,* (1999) suggest that women are reasonably well nourished in industrialised countries (See Section 1.6.1) the precise nature and composition of diet, enhanced by potent socio-economic factors, may cause marginal undernutrition, which may be sufficient to be considered limiting.

Rats exposed to low protein diets *in utero* are subject to progressive deterioration of renal function at a rate that exceeds that seen in aging control rats. Blood urea nitrogen concentrations,

urine output and albumin excretion were significantly higher in rats that were prenatally undernourished than in controls (Nwagwu *et al.,* 2000), despite the apparent maintenance of normal GFR (Langley-Evans *et al.,* 1998). This is consistent with the hypothesis advanced by Mackenzie & Brenner, (1995) as this suggests a vicious cycle of nephron loss and declining function.

Langley-Evans *et al.,* (2003b), demonstrated that the effects of prenatal nutrition upon the developing kidney can be replicated in larger species. In a study of sheep, the feeding of 50% of daily maintenance intake to twin bearing ewes during specific periods of pregnancy resulted in a reduction of birth weight in lambs when the nutritional insult was in late gestation (day 85-115). Nephron number was positively related to weight at birth and was significantly reduced in lambs subject to nutritional insults in earlier pregnancy (day 30-70). The ovine fetal kidney, as in rodents, is sensitive to maternal nutritional status, although the critical period tends to be in early gestation, compared to late gestation in rats.

The administration of the synthetic glucocorticoid, dexamethasone to pregnant rats not only induces hypertension in their offspring (Benediktsson *et al.,* 1993), but also decreases nephron number in the offsprings' kidneys and impairs adult renal function (Celsi *et al.,* 1997). A recent study by Wintour *et al.,* (2003) found early fetal exposure to dexamethasone resulted in hypertension and fewer nephrons within sheep. Impaired nephrogenesis resulted in mean glomerular volume being larger in those exposed to dexamethasone compared to controls.

Animal studies clearly demonstrate that prenatal undernutrition has long-term effects upon both blood pressure and renal function. Whilst programming influences in fetal life lead to later elevations of blood pressure and appear to generally reduce nephron number, hypertension and impaired renal development may merely coincide and not be necessarily related in a causal manner. Rats exposed to a maternal low-protein diet for the first seven days *in utero* went on to

develop raised blood pressure (Langley-Evans *et al.,* 1996c). Histological examination of these kidneys shows that a normal complement of nephrons is present (Langley-Evans *et al.,* 1999).

1.8. Mechanisms of developmental programming.

The mechanism through which undernutrition in mid-late pregnancy induces gross structural changes in the kidney is unknown. The observation of reduced functional units within the tissue is similar to effects of fetal undernutrition in other tissues. Low protein feeding during pregnancy and lactation produces a mature pancreas, with fewer islet cells and a reduced functional capacity (Snoeck *et al.,* 1990; Dahri *et al.,* 1991). Similar effects have been observed in the brain, with changes in volume and neuronal densities of specific centers within the hypothalamus (Plagemann *et al.,* 2000). The existence of similar general changes in a broad range of tissues, in response to a diverse range of nutritional insults is suggestive of a single common programming mechanism that may operate *in utero.*

1.8.1. Glucocorticoids.

Glucocorticoids are steroids, which can freely diffuse across the placenta. They are potent growth-retarding agents that also regulate the expression of many genes (Kitraki *et al.,* 1995; Mendelsohn *et al.,* 1982; Meyer *et al.,* 1979; Meyer, 1985; Schellenberg *et al.,* 1988). In order for the fetus to develop along its genetically determined pathway, the conversion of active glucocorticoids to their inactive forms is essential. The placental isoform of 11β-hydroxysteroid dehydrogenase (11β-HSD_2) plays a key role in protecting the fetus from the actions of maternal glucocorticoids *in utero* (Edwards *et al.,* 1996).

Langley-Evans *et al.,* (1996e) found the activity of 11β-HSD_2 in placentas of rats fed a low-protein diet to be reduced, relative to control activities at days 14 and 20. Langley-Evans *et al.,* 1996d), demonstrated an increase of glucocorticoid action in the tissues of fetal rats that continued postnatally. Rats exposed to a maternal low-protein diet *in utero*, exhibited abnormalities of the HPA axis and were hypersensitive to glucocorticoid hormone action through increased glucocorticoid receptors in vascular and brain tissue (Langley-Evans *et al,* 1996d). Similarly expression of 11β-HSD_2 mRNA was reduced in sheep placenta following undernutrition (Whorwood *et al,* 2001). Using the 11β-HSD_2 inhibitor carbenoxolone in rat pregnancy, Lindsay *et al.,* (1996) & Langley-Evans (1997c) were able to produce hypertensive offspring from a protein replete diet.

These observations have led to the hypothesis that fetal over-exposure to glucocorticoids of maternal origin may provide a mechanism for the programming of hypertension (Langley-Evans, 1997a; Bertam *et al.,* 2001). In order to test this, maternal glucocorticoid production in pregnancy was prevented by both surgical (Gardner *et al,* 1998) and pharmacological adrenalectomy using metyrapone (Langley-Evans *et al.,* 1996b; Langley-Evans, 1997b). The feeding of low protein diets to these rats lacking corticosterone failed to induce hypertension in the resulting offspring. Importantly, Langley-Evans (1997b) found that corticosterone replacement in these pregnant rats restored the hypertensive effect of the low-protein diet. This demonstrates that the production of corticosterone by the maternal adrenal is a prerequisite for the developmental programming of hypertension by the maternal diet.

The role of glucocorticoids in fetal developmental programming and health is comprehensively established (Reviewed Langley-Evans, 1999). Ongoing research in glucocorticoid exposure was being extensively tested in animal models from a number of research groups during the time of this investigation, with particular reference to rat nephrogensis

(McMullen & Langley-Evans, 2003). It was deemed unnecessary to test for glucocorticoids in the primary investigation into low-protein feeding and nephrogenesis within the rat (Chapter 3). In addition, assessing maternal glucocorticoid levels in pregnant dams on novel low-protein diets through tail arterial collection was considered to stressful and may possible contribute to either termination or unnecessary expose of the developing fetus to stress hormones conferring an altered developmental programming response. The potential increase of stress in the dam may yield non-conclusive result. This does not exclude it from future experiments using these diets (See section 2.2.1 and Chapter 4) where the focus is to establish the role of maternal glucocorticoids and placental 11β-HSD_2 on fetal development and organogenesis.

1.8.2. The renin-angiotensin system.

Several components of the renin-angiotensin system (RAS) operating at systemic and local levels are regulated through glucocorticoid action and may provide another mechanism by which long-term blood pressure is programmed. The production of angiotensinogen by the liver and the expression of angiotensin-converting enzyme (ACE) in the lung and other tissues (Mendelsohn, *et al.,* 1982) and the expression of angiotensin II (A-II) receptors (AT_1 and AT_2) are all increased by glucocorticoid action (Provencher, *et al.,* 1995). However, epidemiological evidence does not strongly implicate defects of RAS in the intrauterine programming of hypertension. Konje *et al.,* (1996), found plasma renin activity from umbilical cord blood was significantly higher in IUGR babies compared to AGA babies. In contradiction to this, Martyn *et al.,* (1996b) found that active and inactive renin concentrations to be greater in 50-53 year old men who were heavier at birth.

Langley & Jackson, (1994) and Langley-Evans & Jackson, (1995) observed adult rats

exposed to low-protein diets *in utero* consistently exhibit raised ACE activity in lung tissue and plasma. Hypertensive rats exposed to a maternal low-protein diet are hypersensitive to glucocorticoid action and maintenance of high blood pressure by angiotensin II may be through increased angiotensin II receptors (Langley-Evans *et al,* 1996d). Sahajpal & Ashton (2003) found renal angiotensin II AT_1 receptor protein expression in the kidney glomerulus was significantly greater in low protein exposed rats compared to controls.

Sherman and Langley-Evans (1998), observed that early administration of the ACE inhibitor captopril postnatally to low-protein exposed rats, maintained their normotensive state for up to 8 weeks after treatment cessation. Sherman & Langley-Evans, (2000), further studied the effects of the angiotensin II receptor (AT_1) antagonist (losartan) on offspring exposed to a low-protein maternal diet. Administration of losartan postnatally (between 2-4weeks) to low protein-exposed offspring prevented blood pressure elevation. The observed effect was maintained into adult life. This suggests that intrauterine glucocorticoid exposure may increase sensitivity of angiotensin II in early postnatal life, which in turn establishes lifelong raised blood pressure.

The local actions of RAS, which promote vasoconstriction and increase vascular resistance, are opposed by vasoactive prostaglandins, PGE_2 and PGI_2. Hypertensive rats exposed to a maternal low protein diet *in utero* exhibit alterations in renal prostaglandin metabolism and clearance. Urinary excretion of PGE_2 by low protein exposed rats was approximately double that of control rats. Renal 15-hydroxy prostaglandin dehydrogenase activity was also significantly reduced in these hypertensive animals (Sherman & Langley-Evans, 1998). This suggests a compensatory response of the kidney to oppose the vasoconstrictor activity of angiotensin II.

1.8.3. Homocysteine

Elevated levels of homocysteine have long been associated with coronary heart disease (McCulley, 1969; Mudd *et al.,* 1985). Evidence supports the concept that feeding a maternal low-protein diet during the pregnancy of rats and mice, disturbs methionine-homocysteine metabolism and that these disturbances may exert programming effects on the developing fetus (Rees, 2002) (As described in 1.6.4.2). Such programming may be associated with a rise in maternal homocysteine. Elevated levels of homocysteine in humans are associated with obstetric complications including pre-eclampsia, recurrent miscarriage and neural tube defects (Nelen *et al.,* 2000). Homocysteine could exert direct toxic effects upon the embryonic or fetal tissues.

1.8.3.1. Homocysteine and DNA methylation as a mechanism of programming.

With significant amounts of homocysteine being produced during the conversion of methionine to cysteine there is the risk of developing maternal hyperhomocysteinaemia (Figure 1.2). Homocysteine could exert direct toxic effects upon the embryonic or fetal tissues. Being a pro-oxidant molecule homocysteine, can induce the auto-oxidation of other amino-acids and small molecules (Blundell *et al.,* 1996). Oxidative damage to cells during development may impair the capacity for cell proliferation and activate apoptosis, which may have a permanent and profound effect upon the number and type of cells present within tissues (Sohar & Weindruch, 1996).

The fetus lacks cystathionine β-synthase the only means by which to eliminate homocysteine that may cross the placenta is through the synthesis of S-adenosyl homocysteine, which is then remethylated to S-adenosyl methionine (SAM) (VanAerts *et al.,* 1995). Increased

SAM levels have been shown to stimulate growth (VanAerts *et al.,* 1994) and it is suggested that this may account for the more rapid early growth exhibited by fetuses exposed to the maternal low-protein diet (Rees *et al.,* 2000).

Another potential mechanism for programming upon the developing fetal cells involves intermediates of the methionine-homocysteine cycle that determine the capacity of cells to methylate DNA (Bird, 2002; Friso & Choi, 2002; Rees, 2002). Methyl groups are needed to produce new DNA and promote cell division, so sufficient methyl donors are required to support the rapid growth of the embryo and conceptus. S-adenosyl-methionine (SAM) has the capacity to methylate the cytosine residues of DNA. Excess levels of this metabolite may lead to DNA hypermethylation and inappropriate silencing of genes during critical periods of development (Detich *et al,* 2003). Rees *et al.,* (2000) have shown that livers of fetal rats exposed to a maternal low-protein, diet have higher levels of methylated DNA compared to controls.

1.9. Aims.

The primary objective of this thesis is to assess the possible long-term functional effects of impaired renal development during fetal life. It is important to establish whether a reduced renal reserve promotes hypertension or the presence of elevated blood pressure promotes acceleration in abnormal renal function. Little is known about what constitutes an optimal maternal diet to sustain appropriate growth and development of the fetus. If nutrition is to be considered the primary determinant underlying developmental programming of nephrogenesis and latter blood pressure a clearer understanding of the programming mechanisms needs to be established. To fully justify animal experimentation it is important to evaluate any findings in humans in order for developmental plasticity and programming to benefit public health. The

essential aims are to:

1) Extend previous observations of developmental renal programming in an established rat model.
2) Address the limiting effect of the low-protein rodent diet on developmental renal programming.
3) Explore parallels between the programmed renal vascular disorders of the rat and early markers of disease in human cohorts.
4) Evaluate renal function in human volunteers using non-invasive methods.
5) Determine markers of renal function and their relationship if any to:
    i) Blood pressure as an indicator of cardiovascular disease.
    ii) Characteristics at birth indicative of restraints on growth *in utero.*

1.10. Hypotheses.

Chapter 3.

It is hypothesised that renal structure and function are determined by prenatal nutrition and that this is the key mechanism in the programming of hypertension.

Chapter 4.

It is hypothesised that the addition of glycine to the limiting maternal low protein diet will normalise blood pressure in maternal low-protein fed rats at 4 weeks, normalise nephrogenesis with no evidence of impaired renal function present.

Chapter 5.

It is hypothesised that in humans of a lower weight or size at birth will exhibit increased blood pressure and show evidence of increased glomerular permeability.

1.10.1. Testing the hypotheses.

Chapter 3.

The offspring of the 9% low-protein feed rat dam will have nephron number, and functional characteristics of the kidney assessed in relation to weight at birth and blood pressure at 4-weeks. Low-protein exposed pups may exhibit increased hypertension, urinary creatinine clearance (proxy for glomerular filtration rate), altered renal function and a reduced nephron compliment compared to 4-week old rats exposed to an 18% protein diet *in utero.* An association between a reduced nephron endowment and hypertension may be found in rats exposed to a maternal low-protein diet *in utero.*

Nephron endowment will be compared between 9% low-protein and 18% protein exposed rats at different time points through life. Nephron endowment in low-protein exposed rats will consistently remain lower throughout their lifespan compared to those exposed to an 18% protein diet *in utero.* Rats exposed to a 9% low-protein diet *in utero* may exhibit increased evidence of glomerular damage.

Chapter 4.

Using the established rat model the additional impact of supplementing differing forms of

nitrogen in the maternal 9% low-protein diet will be compared in 4-week old offspring. One of additional nitrogens added to the maternal low protein diet will be in the form of glycine a conditionally essential amino acid for pregnancy and a methyl donor. Blood pressure, markers of renal function and tissue composition, nephron compliment and glomerular volume will be assessed.

The additional nitrogen's provided to the maternal 9% low-protein diet might correct the previously observed hypertensive effect in 4-week old rats and normalise nephrogenesis. The type of nitrogen added to the diet may provide further understanding of the mechanisms through which developmental programming may occur through dietary mechanisms.

Chapter 5.

Markers of renal function and hypertension in relation to size and weight at birth will be assessed in Caucasian children aged 10 and 12 years from an existing cohort of study (The Princess Anne Hospital Growth Study) and young adults aged 18-22 years from a new cohort. The ages are determined to be before and post puberty to avoid the variance of accelerated growth and hormonal changes during this time. The age range of the young adults is below previous studies looking at individuals in middle age to avoid any impact environmental factors may have on the variables studied. Observations on renal function and hypertension observed in the child cohort will be compared to those in the adult cohort.

Chapter 2.

Methods.

2.1. Chemicals.

The suppliers of chemicals and reagents were Sigma-Aldrich Company Ltd, Poole, Dorset, UK, unless otherwise stated in the text.

2.2. Animals.

Animal facilities at the University of Nottingham and the University of Southampton were utilised for separate investigations. All animal procedures were conducted in accordance with the Home Office Animal Act (1986) under Home Office License (License holders: Dr. S.C. Langley-Evans, University of Nottingham and Prof. A.A. Jackson, University of Southampton). Experimental rats were of the Wistar strain, supplied by Harlan, UK (Belton, Leicestershire, UK). The animals were housed in wire mesh and perspex cages at a constant temperature of 21°C on a 12 hour light / dark cycle with free access to food and water at all times. Non-pregnant animals were fed *ad libitum* standard, non-purified, laboratory rat chow (CRME, Special Diet Services, Cambridge, UK) (Appendix 2).

2.2.1. Manipulation of diet in pregnancy.

Virgin female Wistar rats, weighing between 200-250g, were housed with a stud male in a wire bottom cage and inspected twice daily. Conception was detected by the appearance of a semen plug on paper placed beneath the cage. This was recorded as day zero of pregnancy, with

the female being immediately transferred to single housing and randomly assigned to one of five semi-synthetic experimental diets. Feeding of a semi-synthetic diet prior to conception has been shown not to alter metabolic or physiological responses in the resulting offspring and so habituation to the diets prior to pregnancy was not an element of this protocol (Langley-Evans *et al.,* 1996c). Maternal weight gain and food intakes were recorded daily. At delivery, all mothers returned to standard laboratory rat chow (CRME, Special Diet Services, Cambridge, UK). All litters were culled to a maximum of 8 pups to reduce variability in suckling. Offspring thus differed only in terms of prenatal dietary experience.

All experimental diets were made within in-house facilities as described by Langley & Jackson (1994) (Table 2.1). Casein, the sole protein source in the semi-synthetic diets, is low in cysteine and methionine and in order to avoid sulphur deficiency all diets were supplemented with 0.5g/100g D, L-methionine. The energy content of the low protein diet (MLP) was balanced with that of the control diet (CON) by increased provision of starch and sucrose. All ingredients were mixed dry, bound with water, formed into small cakes (approx. 40-60g) and oven dried at 60°C for 48 hours. Diets were stored at –20°C for up to three months prior to use.

The protocol for feeding pregnant females utilised an 18% casein control diet (CON) and a 9% casein low protein diet (MLP), as reported by Langley & Jackson (1994) (Chapter 3). In order to assess the effects of additional amino acids within the MLP diet protocol on offspring the following diets were also administered: 9% casein with 3% (w/w) glycine (MLPG) 9% casein with 3% (w/w); alanine (MLPA); 9% casein with a 1.5% (w/w)(due to its double carbon bond) of urea (MLPU). The additional amino acid groups were equal in nitrogen content, which was equivalent to a diet containing 12% (w/w) casein (Chapter 4) (Table 2.1). The inclusion of a maternal 12% casein diet was not appropriate as this has already been shown to induce programming effects on the offspring (Langley & Jackson, 1994). The aim was to address the

limiting maternal low-protein diet with additional nitrogen and assess developmental programming effects in the offspring.

Table 2.1. *Composition of diets.*

	Diet.				
	CON	MLP	MLPG	MLPU	MLPA
Casein (g/100g)	18.0	9.0	9.0	9.0	9.0
Starch (g/100g)	42.5	48.5	45.5	45.5	45.5
Sucrose (g/100g)	21.3	24.3	24.3	24.3	24.3
Cellulose (g/100g)	5.0	5.0	5.0	5.0	5.0
Corn oil (g/100g)	10.0	10.0	10.0	10.0	10.0
AIN76 Vitamins (g/100g)	0.5	0.5	0.5	0.5	0.5
AIN76 Minerals (g/100g)	2.0	2.0	2.0	2.0	2.0
Choline chloride (g/100g)	0.2	0.2	0.2	0.2	0.2
D, L-Methionine (g/100g)	0.5	0.5	0.5	0.5	0.5
Glycine (g/100g)			3.0		
Urea (g/100g)				1.5	
L-Alanine (g/100g)					3.0

AIN= American Institute of Nutrition.

CON= 18% casein control diet.

MLP= 9% casein low protein diet.

MLPG= 9% casein low protein diet + Glycine.

MLPU= 9% casein low protein diet + Urea.

MLPA=9% casein low protein diet + Alanine.

All offspring had their sex determined by genito-anal distance shortly after birth. Each litter was randomly reduced to a total of eight (4 males and 4 females) and the remainder were culled by decapitation. At 4 weeks all rats were coded, weaned and housed in single sex groups of between 4 and 8 animals.

2.2.2. Determining blood pressure and pulse in rats.

Systolic blood pressure and pulse rate were measured using the indirect tail-cuff method (IITC, model 229 BP monitor, Linton Instrumentation, Diss, Norfolk, UK), as reported by Langley & Jackson (1994). This method of blood pressure determination yields values comparable to those obtained by direct cannulation (Bunag, 1973). Prior to measurement all animals were acclimatised for two hours to a temperature of 27°C. This ensured that none of the animals became heat stressed, which occurs at 30°C in the rat, whilst permitting the tail artery pulse to be recorded. Conscious rats were placed into an appropriately sized perspex restraint tube and allowed to settle in a quiet environment. Langley-Evans *et al.,* (1996c) have previously shown that the rat can take up to 2 minutes to habituate to the restraint tube and that the blood pressure will remain stable if recordings are completed within 7 minutes. Exposure to the restraint tube occurred only once, as training to the procedure had previously been shown to have no effect on subsequent readings (Sherman & Langley- Evans, 1998). The tail cuff was rapidly inflated to 300 mmHg to record blood pressure. The tail artery pulse was recorded by a photoelectric cell on cuff deflation at 3mmHg/second. The average of five recordings was taken. Systolic blood pressure was determined using IITC software, which calculates systolic and mean pressures using a preset algorithm. This reduced the influence of observer subjectivity on the measurement.

Measurement of blood pressure in rats using indirect tail-cuff methodology has been criticised as the stress associated with the restraint used to make the measurement may lead to artefacts (van Vliet *et al.,* 2000). Jamieson *et al.,* (1997), also reported that tail-cuff and intra-arterial measurements, when measured simultaneously in conscious rats, showed a broad disagreement of up to 15 mmHg. It was noted that the lack of agreement might be due to deficiencies in either, or both approaches. Van Vliet *et al.,* (2000) suggest that any observed blood pressure effect noted with the tail-cuff systems should be confirmed using intra-arterial measurements as the recognised "gold standard". Gardner *et al.,* (1997), has shown that MLP-induced hypertension is noted in anaesthetised rats subject to carotid cannulation as with the indirect tail–cuff method. The consistency of reports from our laboratory with data from other models, which have utilised intra-arterial measurements of blood pressure (Vehaskari *et al.,* 2001; Woods *et al.,* 2001), further support these findings.

2.2.3. Urine collection in the rat.

4-week-old rats were placed into metabolic cages to collect a 24-hour urine sample. Urine was funnelled through a mesh filter to a small container at the bottom of the cage. After 24 hours the sample was weighed and stored at –80°C for a period of up to 6 months prior to biochemical analysis. In order to assess food particulate contamination of the urine, samples of the semi-synthetic diet were placed in a fresh, divided human urine sample for 24 hours. No difference in acidity between the human and 4 randomly selected rat urine samples was noted. The semi-synthetic diet did not appear to dissolve in the urine, even at 22% sucrose. This may be due to the slow baking procedure employed in the production of the diet and the length of time particles were in the urine. Independent T test showed no significant difference between albumin concentration in 4 contaminated and 4 normal human urine samples after 24 hours (P=0.783).

2.2.4. Culling and tissue harvesting.

All animals were culled by CO_2 asphyxiation followed by cervical dislocation. Blood was collected by cardiac puncture, transferred to heparin tubes, gently inverted and placed immediately on ice. The kidneys were rapidly dissected, weighed and snap-frozen in liquid nitrogen before being stored at –80°C until required for further analysis. Blood was centrifuged at 4°C for 10 minutes at 2500 rpm. Plasma was drawn off into aliquots before being stored at –80°C until required for further analysis. The left kidneys from rats held in metabolic cages for the 24-hour urine collection were fixed in buffered formalin (Sigma-Aldrich, UK) prior to histological analysis. All analyses took place within six months of sample collection.

2.3. Determination of nephron number.

Two different methods were employed to determine nephron number. The staining method (Section 2.3.1) was labour and resource intensive, allowing only limited numbers of kidneys to be processed at any one time. The maceration method (Section 2.3.2) was adopted for later studies as it combined greater reproducibility with greater throughput of kidneys within a day.

2.3.1. Histological staining method.

Fixed kidneys were used to determine nephron number using the method of Langley-Evans *et al.,* (1999). The tissues were removed from formalin and placed in a processing and embedding cassette with lid (Macrosette, Simport Ltd, Canada). The kidneys were placed in 70% ethanol solution overnight before being transferred to 90% ethanol for 1 hour. They were then

drained prior to a further hour with fresh 90% ethanol. Kidneys were then placed in ethanol for 2 hours, followed by a further 2 hours with fresh ethanol and then a further hour with fresh ethanol. Finally the kidneys were placed in chloroform to dehydrate and left in a fume cupboard overnight. The kidneys were then embedded in paraffin wax.

For embedding, kidneys were removed from chloroform and placed in fresh filtered molten wax (Tissue Prep 2, Fisher Scientific, UK) at 65°C for 1 hour. Kidneys were transferred to fresh molten wax for 1 hour and the procedure was then repeated for a further hour. Kidneys were then placed on their side (saggital plane), in metal trays resting on ice. The processing cassette was clipped on top of the metal tray before fresh molten wax was poured into the unit. The wax was allowed to set overnight in a fridge at 4 °C.

Serial 5μm saggital sections were cut through the hilar plane on a microtome (820 microtome, American Optical Corporation, USA). One section in 100 was retained for histological examination. To prevent folds when mounted the sample sections were floated in a water bath at a constant 40°C, and then collected on a non-coated microscopic slide (Gold Star, Chance Propper Ltd., UK). The slides were left to dry overnight before being stained with Harris haematoxylin and eosin as described by Zeman (1968)(Appendix 3), mounted in DPX fluid (Fisher Scientific, UK), and covered with a glass cover slip (Chance Propper, Ltd., UK). The slides were left in a fume cupboard overnight to set.

The number of sections cut from each kidney was carefully recorded. Using a magnification of 10x (Meiji microscope, Japan), all nephrons, identified by their glomeruli in each section, were counted. As reported by Langley-Evans *et al.,* (1999), 3-5 sections selected from the full range of sections for each kidney were counted and the average nephron number/section from these counts multiplied by the total number of sections cut. This gave an estimation of the total nephron number per kidney. Whilst this method of nephron estimation does not represent the Gold Standard stereological technique and may slightly overestimate

nephron number (Howard, 1997), Langley-Evans *et al.,* (1999) reported no significant difference in estimates made using the two techniques. Indeed the nephron numbers determined using this technique by Langley-Evans et al., (1999) tend to be lower than many reports of nephron number in rats (Zeman, 1968, Hellmann *et al.,* 1998; Merlet-Benichou *et al.,* 1994; 1999; Vehaskari *et al.,* 2001). Intra-assay variation was 1.1%, inter-assay variation was 1.17% based upon five repeat counts of the same sample.

2.3.2. Maceration method.

Nephron number was determined using the maceration method as described by Welham *et al.,* (2002). Whole kidneys were placed in 2ml 1M HCl and incubated in a water bath for 30 minutes at 37°C. The kidneys were then placed in 10ml sodium phosphate buffer solution pH 10, and homogenised using a hand held glass pestle and mortar. 20µl of vortexed homogenate was transferred in duplicate to a non-coated microscopic slide (Gold Star, Chance Propper Ltd., UK) and covered with a separate cover slip (Chance Propper, Ltd., UK). Using a magnification of 10x under phase contrast (Meiji microscope, Japan), glomeruli were counted and the mean number recorded. The mean was multiplied by 500 to give an estimation of total nephron number. Intra-assay variation was 2.1% based upon five repeat counts of the same sample.

2.3.3. Assessment of glomerular injury in aged kidneys.

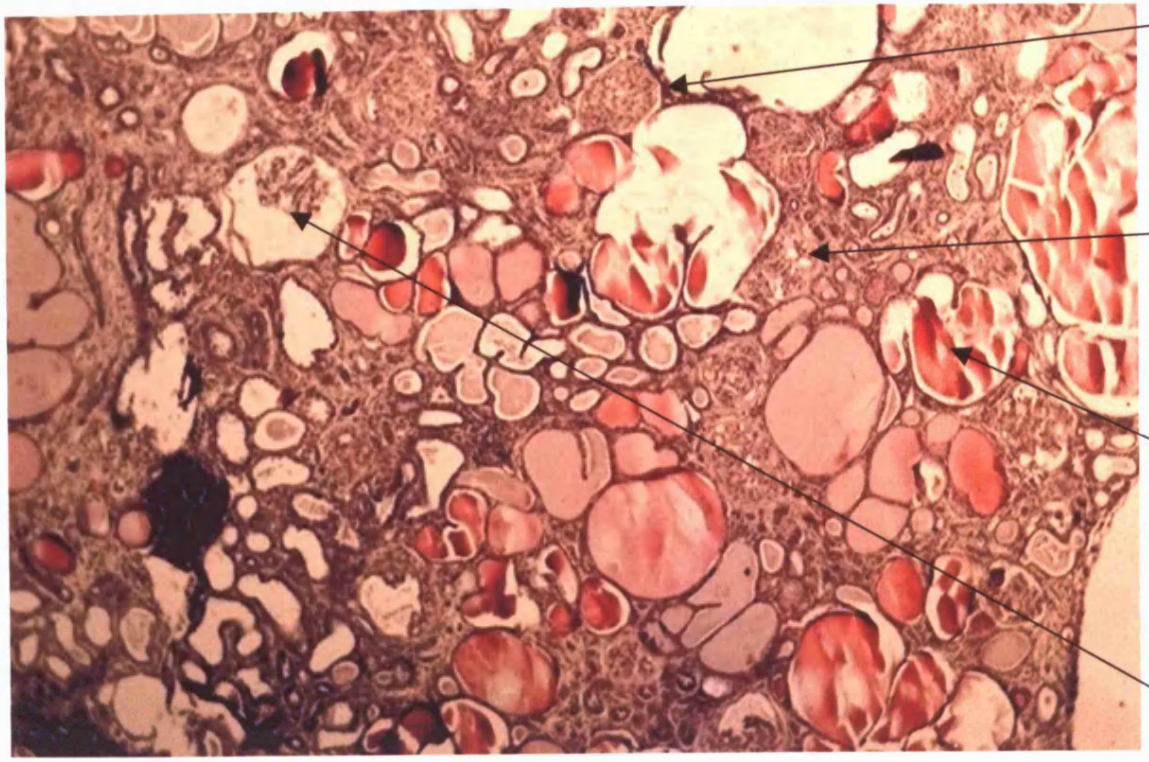
For the assessment of glomerular injury, kidneys were sectioned parallel to the corticomedullary axis prior to processing and staining as described in 2.3.2. Serial 10µm horizontal sections parallel to the corticomedullary axis were cut. A semi-quantitative scoring method was employed to determine glomerular condition under a light microscope. One hundred

random glomeruli per slide were characterised as histologically normal, or showing evidence of mild glomerulosclerosis; e.g. blebbing (Hostetter *et al.,* 1981), severe glomerulosclerosis; e.g. casts (Greenfeld *et al.,* 1997), or hypertrophied; e.g. 50% larger than average diameter (Brenner, 1985). Data was recorded as a mean percentage of glomeruli of each type within kidney sections (Photomicrograph 2.1).

2.3.4. Determination of glomerular volume in rat kidneys at 4 weeks.

Glomerular volume was estimated using the method of Elias *et al.,* (1962). One hundred glomeruli from one randomly selected 5μm section per kidney (intra-kidney glomerular volume variation 1.8%) section were measured in 2 planes using a lens graticule. The glomeruli were randomly selected and comprised both cortical and juxtamedullary; a difference in volume between the two was not established. From the two measurements, accurate to within 0.25μm, the geometric mean diameter (d) was calculated. Glomerular volume was determined for a 100 samples calculated using the equation $V=4/3 \times \prod \times (d/2)^3$ and the mean was determined for each animal.

Photomicrograph 2.1. Histological classification of damaged glomeruli in aged rat kidneys.



1. Normal glomerulus.
2. Mild glomerulosclerosis, evidence of blebbing.
3. Severe glomerulosclerosis with casts.
4. Hypertrophic, diameter 50% bigger than normal.

25x magnification.

2.4. Recruitment of human subjects.

All studies using human subjects were conducted under ethical approval from the University of Southampton Medical Ethics Committee and from Northampton Medical Research Ethics Committee (Appendix 6).

2.4.1. The Princess Anne Hospital Growth Study.

Sixty-four healthy children took part in the study. This consisted of 57 children (n=28 male and n=29 female) at age 10 and 59 children (n=27 male and n=32 female) at age 12. Five of the children examined at age 10 were non-contactable at age 12, so an additional seven were recruited (n=2 male and n=5 female). All of the children were recruited from the larger Princess Anne Hospital Growth Study (Mr. T. Wheeler, Reader in Obstetrics and Gynaecology, The Princess Anne Hospital, Coxford Road, Southampton, UK), which recruited 390 pregnant women and their babies. At birth the children were grouped in quintiles based on birth weight. The children used for this study were those contactable from the lower quintile, middle quintile and the upper quintile at age 10 and 12 years (Chapter 5). Maternal details and birth characteristics such as birth weight, length, head circumference, ponderal index and placental weights were available for each of the children, as were measurements of height and weight. Blood pressure readings were only available at age 10.

Parents of the children on the Princess Anne Hospital Growth Study were contacted to arrange urine collections at 10 years of age (Dr. F. Anthony, University of Southampton) and 12 years of age (Dr. R. Sherman, University of Southampton). Sterile containers were provided and sent by post. On the specified day, children collected a single morning sample in their own home. These samples were then refrigerated at the family home and collected on the same day. The

samples were acidified using 6M HCl as a preservative and then stored at –80°C. Acidification at a ratio of 1:1000 (v/v) did not appear to have an impact on the parameters that were measured. Three random urine samples were provided. Each sample was divided, with one half acidified. T-test analysis for urinary albumin indicated no significant difference between the samples, based on 6 repeat samples. Similarly, T-test analysis for urinary creatinine indicated no significant difference between the samples, based on 6 repeat samples. Storage of urine over a 6 month period with repeated thawing and freezing at monthly intervals did not significantly alter albumin and creatinine concentrations.

Urinary albumin concentrations were measured in each of the samples (as described in section 2.5.1) and expressed per mg creatinine (as described in section 2.5.2). Urinary specific gravity was also recorded (as described in section 2.5.5). Urinary sodium and potassium concentrations were measured in children aged 12 (as described in section 2.5.6) and expressed per mg creatinine (as described in section 2.5.2).

2.4.2. The Northampton young adult study.

A power calculation was used to determine the sample size necessary for this investigation (Rosner, 1995). This was based upon the mean excretion of albumin:creatinine 0.28±0.03mg/mg found in the population of children studied at age 10 (as described in 2.4.1). Seventeen adult subjects at 80% power was determined as the number necessary to detect at a 100% difference in excretion across the range of birth weights and 40 were examined. In the study of children the observed increase across the full range was over a 100%.

Forty healthy young Caucasian adults aged between 18 and 22 years (21 male and 19 female) took part in the study. Individuals of Afro-Carribbean origin were excluded, as they tend to have smaller kidneys, attain higher blood pressure in response to sodium loading and display a

reduced capacity to excrete sodium load (Luft *et al.,* 1979). Pesce *et al.,* (1994), found that individuals of African origin had larger glomerular diameters compared to Caucasians, which implies fewer nephrons. Individuals of Asian origin were also excluded, as they tend to be smaller as adults and weigh less at birth, even three generations after settling in the United Kingdom (Margetts *et al.,* 2002). Given the small sample size, reduction of possible confounders was an important issue.

All of the participants were recruited from University College Northampton, Park Campus, Boughton Green Road, Northampton, UK. On recruitment, questionnaires were sent to both the participants and their mothers (Appendix 4a & b). The questionnaires provided key information regarding the mother's pregnancy and the participants' current health and factors of lifestyle, such as smoking and alcohol consumption that may have an acute response on blood pressure elevation. Self-reporting by mothers has been found to be generally consistent with medical records (Curhan *et al.,* 1996). Exclusion criteria for the study included conditions, which may potentially alter fetal development, renal function or place the participant outside the normal distribution for size at birth. These included premature birth (i.e. gestation less than 37 weeks) (Bennett, 1999), pre-eclampsia (Dildy *et al.,* 1991) or non-insulin-dependant-diabetes-mellitus (Hollingsworth & Resnik, 1988; Roberton, 1992), during gestation. Participants were excluded if hypertensive, diabetic or having a known renal condition such as one kidney, or adrenalectomy. Participants on any long-term prescribed medication that might have interfered with the biochemical assays were also excluded.

Participants had their recorded birth weight in pounds and ounces converted to kilograms. Male and female birth weights were then divided at the 33^{rd} and 66^{th} Centile (as described by Power *et al.,* 2003). These formed the lower, middle and upper thirds of birth weight. Participants had their height (m) and clothed weight (Kg) (Seca alpha model 770, Germany) recorded. The participant's body mass index was calculated ($BMI=Weight/Height^2$), and body surface area (to

correct for creatinine clearance as described in section 2.5.3) determined using nomograms based upon age, height and weight (Dubois & Dubois, 1916).

A 10ml venous blood collection was made from the cephalic, median cubital or the basilic vein using the Vacutainer® method. Each Vacutainer® contained heparin. The blood sample was centrifuged (3,500 rpm) at 4°C for 5 minutes. Plasma was extracted and frozen at –80°C for up to three months prior to biochemical analysis. After the blood collection, participants were requested to lay down on a flat couch with limited stimuli for 30 minutes prior to blood pressure and pulse being measured.

2.4.3. Determining blood pressure and pulse in humans.

Systolic and diastolic blood pressure and pulse rate were measured using a digital sphygmomanometer with auto inflation cuff (Digital BP Meter UA-731, Japan). The maximal cuff pressure selected was standardised at 160mmHg. All blood pressure and pulse recordings were taken in triplicate, with intra-measurement variation for diastolic recordings at 2.3 %, systolic at 4.9% and pulse at 1.7%. The cuff was placed over the brachial artery of the left arm and secured prior to automated recording. All participants were requested not to smoke or drink caffeine-based products for at least 2 hours prior to testing. All recordings were performed with the participant having lain on a couch for 30 minutes in a warm room with limited stimuli (Perry & Potter 1997). Systolic and diastolic pressures were expressed in mmHg and pulse in beats per minute (bpm).

2.4.4. Urine collection in humans.

Within 24-48 hours of the blood sample participants performed a 24-hour urine collection. Human adults were given a 2-litre container acidified with 2ml 6M HCl to collect a 24-hour urine sample. Participants were advised to refrain from exercise (Tesch et al., 1989), maintain good hydration and try to have a meat free diet during the clearance (Gambino, 1995).

The first void of day one was discarded, but the time recorded. All the urine, including the final specimen voided at the end of the 24-hour period was collected and the finish time recorded. The volume of the specimen was recorded to enable creatinine clearance to be determined (as described in section 2.5.3). Urinary albumin concentrations were measured in each of the samples (as described in section 2.5.1) and expressed per mg creatinine (as described in section 2.5.2). Creatinine clearance was expressed in ml/minute (as described in section 2.5.3). Urinary specific gravity was also recorded (as described in section 2.5.5).

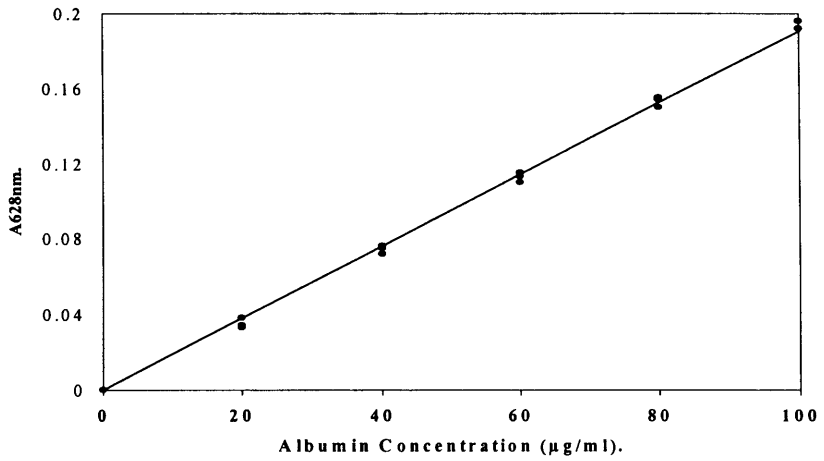
2.5. Determination of plasma and urinary chemistry.

2.5.1. Determination of albumin.

Plasma and urinary albumin were determined using the Bromcresol Green (BCG) method (Doumas & Biggs, 1972) (Sigma-Aldrich, UK). All reagents are described in Appendix 5. All standards and samples were tested in triplicate. Plasma and urine was centrifuged for 1 minute at 2500 rpm. 1 ml of BCG reagent was added to 50 µl of urine or plasma and vortex mixed. Absorbance at 628nm was read on a spectrophotometer (Shimadzu UV-160, Japan). The albumin concentrations of urine samples were determined from a standard curve (0-5mg/ml) prepared from bovine serum albumin (Figure 2.1).

Intra-assay variation was 1.38%, while inter-assay variation was 0.05%, based on six repeats of the same sample. In order to determine potential variation in albumin excretion throughout the day four samples were collected at random time points. No significant variation in albumin concentration was observed. No significant difference in urinary albumin concentration was observed between four samples repeatedly frozen and thawed up to a period of six months.

Figure 2.1. *Albumin standard curve.*



2.5.2. Determination of creatinine.

Plasma and urinary creatinine was determined using Sigma Diagnostics procedure number 555 based upon the alkaline picrate method (Bowers & Wong, 1980). All reagents are described in Appendix 5. All samples were assayed in triplicate. Urine was centrifuged for 1 minute at 2500 rpm. 1ml of colour reagent was added to 100 µl of blank (distilled water), creatinine standard (3mg/dl) or 1:20 diluted urine sample (distilled water). Plasma (100 µl) was assayed undiluted. The tubes were vortex mixed and allowed to stand in the dark for 8-12 minutes at room temperature. The samples were read on a spectrophotometer (Shimadzu UV-160, Japan), at 500 nm and the absorbance recorded. These values represented Standard A, and Sample A. 33µl acid reagent was added to all tubes, which were vortex mixed and allowed to

stand at room temperature for 5 minutes. Absorbance at 500 nm was recorded and these values represented Standard B, and Sample B.

$$\text{Creatinine mg/dL} = \frac{\text{Standard A-Standard B}}{\text{Sample A- Sample B}} \times 3$$

Intra-assay variation was 1.53%, and inter-assay variation was 1.90% based upon six repeats of the same sample of urine. Intra-assay variation was 0.2%, and inter-assay variation was 1.8% based upon five repeats of the same sample of plasma.

2.5.3. Determination of creatinine clearance.

Creatinine clearance was determined as a proxy of glomerular filtration rate from a timed 24-hour urine collection (section 2.5.2) and was expressed in ml/minute creatinine clearance = [(urine volume per minute x urine creatinine)/serum creatinine] x (1.73/surface area of body m^2) (Jacobs *et al.,* 1996).

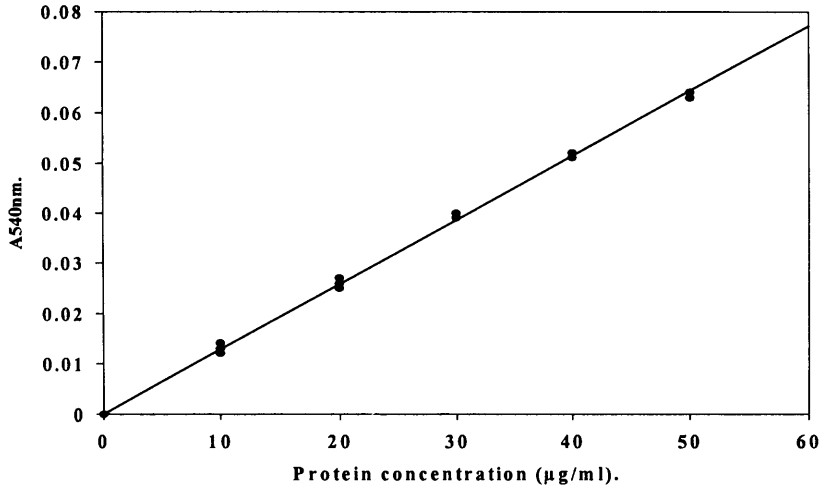
2.5.4. Determination of urinary and tissue protein content.

Urinary and tissue protein content was determined using the Bradford assay (Bradford, 1976), adapted for a microplate reader. All reagents are described in Appendix 5. All standards and samples were assayed in duplicate. Urine was centrifuged for 1 minute at 2500 rpm. Tissue was weighed and buffer was added to give 100 mg tissue/ml of buffer. The tissue and buffer solution was hand homogenised on ice before being centrifuged at 4000 rpm at –4°C for 20 minutes. Supernatant was removed and frozen at –80°C until biochemical analysis. Prior to

biochemical analysis the sample was thoroughly thawed and centrifuged at 2000 rpm for 3 minutes. Tissue homogenate was diluted to 1:10 with 0.1m NaOH. 200 µl of Bradford reagent was added to 10 µl of standard or sample. Absorbance was measured on a plate reader (Bio-Rad, UK), at 540 nm. Protein concentrations for urine and tissue were determined from a standard curve (0-0.5 mg/ml) prepared from bovine serum albumin. (Figure 2.2).

For urine intra-assay variation was 2.22%, while inter-assay variation was 2.93%, based on six repeats of the same sample of urine. For tissue intra-assay variation was 5.83%, while inter-assay variation was 4.77%, based on six repeats of the same sample of tissue.

Figure 2.2. *Protein standard curve.*



2.5.5. Determination of urine specific gravity.

Specific gravity of urine was measured as a marker of hydration status and the relative proportions of dissolved solid components to the total volume of the specimen. Combined with

other renal functional tests it may act as a marker for glomerular functional status (Jacobs *et al.*, 1996).

Urine was centrifuged for 1 minute at 2500 rpm. 20 µl of urine sample was analysed in duplicate. The refractive index ratio was determined using a refractometer (Adams, 1983). Intra-assay variation was 0.001% based on 10 measurements of the same sample.

2.5.6. *Determination of urinary sodium and potassium content.*

Urinary sodium and potassium was determined using flame emission photometry and used as a measure of Na^+ and K^+ excretion. Urine was centrifuged for 1 minute at 2500 rpm and diluted in de-ionised water for sodium, 1:1000ppm and potassium, 1:100ppm. The sodium and potassium concentrations of urine were determined from a standard curve (0-100ppm) (Appendix 5)(Figure 2.3, Figure 2.4). All samples were performed in duplicate and expressed in g/dl. Intra-assay variation for sodium was 0.3% and Intra-assay variation for potassium was 0.5% based on 5 repeats of the same sample.

Figure 2.3. *Sodium standard curve.*

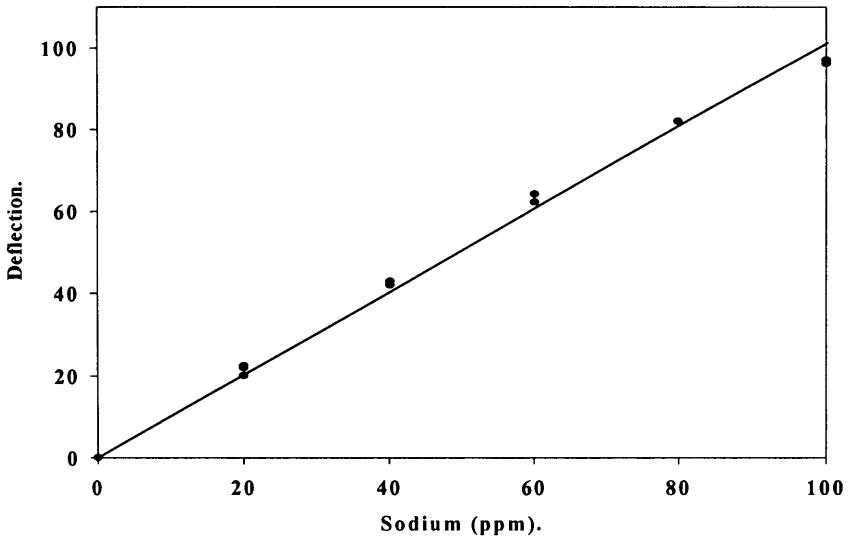
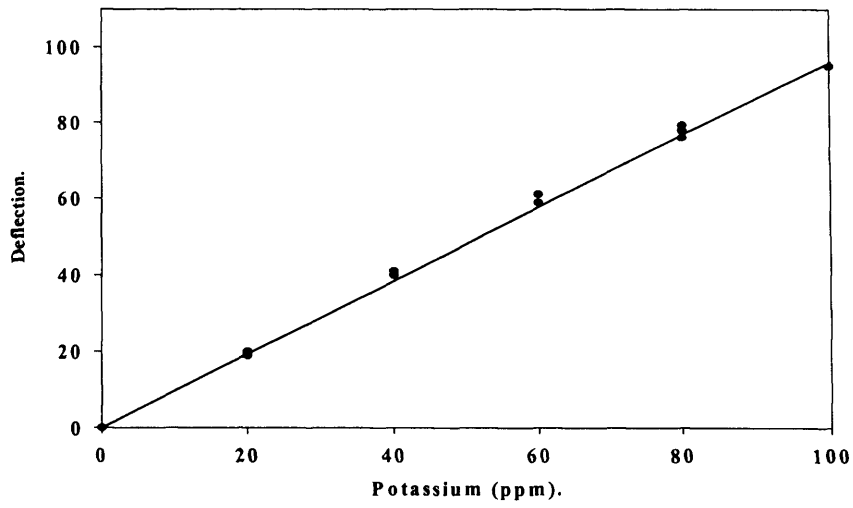


Figure 2.4. *Potassium standard curve.*



2.5.7. Determination of Blood Urea Nitrogen.

Blood urea nitrogen (BUN) was determined using Sigma Diagnostics procedure number 63-UV based on the method of Tiffany *et al.,* (1972). All reagents are described in Appendix 5). BUN estimation in this application is used as an indicator of renal functional status. BUN provides an index of the relative production of urea by the liver and its elimination by the kidneys (Owen *et al.,* 1993). All samples were assayed in duplicate. 900µl of reagent were added to 10µl of sample and 1ml of calibrator (40mg/dl urea nitrogen) was prepared at 37°C. The sample absorbance was recorded at 30 and 90 second intervals at 340nm and the values automatically calculated on a spectrophotometer (Shimadzu UV-160, Japan) using the following equation:

$$\text{Urea} = \frac{\Delta A/\text{min of unknown}}{\Delta A/\text{min of calibrator}} \times \text{calibrator value}$$

$$\Delta A/\text{min} = (A2 - A1)$$

Where A1= absorbance at first read time.

A2= absorbance at last read time.

Values are expressed in mg/dl. Intra-assay variation was 1.3% based on 6 repeats of the same sample.

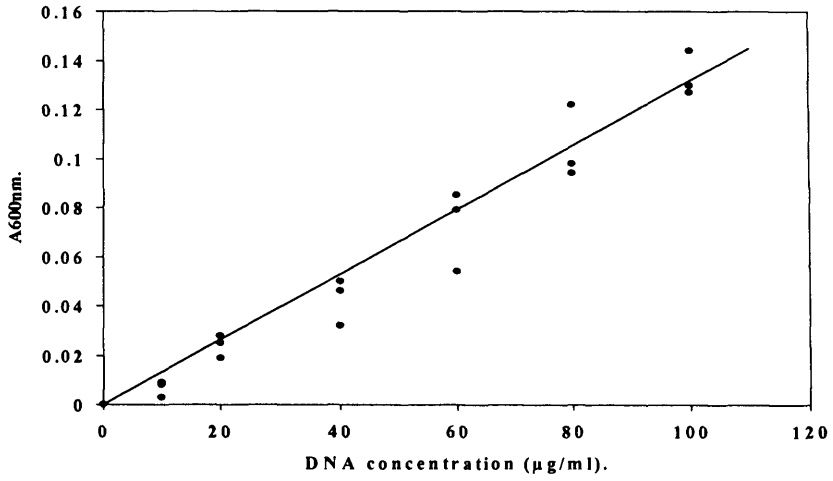
2.6. Renal Biochemistry.

2.6.1. Determination of DNA content.

DNA was assayed using a modification of the method of Burton (1956). Tissue samples were prepared as for the protein assay (section 2.9.1). All samples were assayed in duplicate and standards in triplicate. 250 µl of 2M NaOH was added to 250 µl of standard or tissue sample (100mg tissue/ml) in glass tubes and vortex mixed. After incubation for 1 hour at 37°C, 1 ml of 4M of perchloric acid (BDH, UK) was added and the tubes were vortex mixed. The solution was incubated for 20 minutes and then allowed to cool to room temperature. 0.6 ml of DPA reagent (Appendix 5) was added and all tubes were left in the dark overnight. 1.5 ml of pentyl acetate (Sigma-Aldrich, UK) was added and the tubes were thoroughly vortex mixed. Once settled, the organic blue phase was removed and its absorbance was read at 600 nm on a spectrophotometer (Cecil 1010, UK). DNA concentration in tissue was determined from a standard curve (0-100 µl) (Figure 2.5). The renal protein:DNA ratio was used to provide an indicator of cell size and number.

Intra-assay variation was 17.64%, based on eleven repeats of the same sample.

Figure 2.5. *DNA standard curve.*



2.6.2. Determination of angiotensin converting enzyme activity.

Angiotensin-converting enzyme (ACE) activity was determined in plasma and kidney using an adaptation of the direct spectrophotometric method of Ronca-Testoni (1983), based upon furylacrylphenylalanylglycylglycine (FAPGG) as the substrate. The ACE assay was performed using 96-well flat-bottomed microtitre plates. 25µl of plasma or 50µl of kidney homogenate was incubated with 200µl of 1 mM FAPGG dissolved buffer. Each sample assayed had a corresponding blank containing sample plus 200µl buffer (Appendix 5). The decrease in absorbance was followed at 340nm using a microtitre reader (Bio-Rad, UK), incubating the plate at 37°C. Plasma samples were incubated for 30 minutes, whilst kidney samples were incubated for an hour. ACE activity was determined from the equation:

$$\text{ACE activity units/l} = \frac{V \times 1000}{M \times S \times d} \times \text{change in A340/min}$$

Where, V= final reaction volume, S= sample volume, M= maximal change in A340 for complete hydrolysis of 1mM FAPGG, d= light path length. ACE activity of plasma was expressed as units per litre and in kidney homogenates as units per mg protein.

Intra-assay variation for kidney ACE was 3.22%, based upon seven repeats of the same sample.

Intra-assay variation for plasma ACE was 5.61% based upon seven repeats of the same sample.

2.6.3. Determination of kidney NAD-dependent prostaglandin dehydrogenase activity.

Prostaglandin activity in homogenised kidney (section 2.3.2) was determined using the method of Sherman *et al.,* (1999). The standard for the assay was 15 keto-PGE_2, concentration range of 0-20 nmole. 100µl of tissue sample with 900µl of reagent (Appendix 5) incubated at 37°C for 90 minutes. The reaction was stopped with 2ml 0.5 NaOH and the samples were read on a spectrophotometer (Shimadzu UV-160, Japan), at 500 nm and the absorbance recorded. All samples were assayed in duplicate with a blank for each sample of 100µl of tissue sample with 900µl of blank reagent (Appendix 5). Activity was expressed as nmoles 15 keto-PGE_2 formed/minute/mg protein. Intra assay variation was 6.37% based on six repeats of the same sample.

2.7. Statistical Methods.

All data is represented as mean ± SEM within the tables and figures. The statistical package SPSS (version 10) was used in all analyses. Statistical significance was set at $P<0.05$.

2.7.1. Data analyses of the rat.

Male and female rat data are shown separately, if there are litter effects, otherwise the data is combined. The data for chapter 3 and 4 was analysed using one-way and two-way analysis of variance (ANOVA) with an LSD test applied for post-hoc analysis where significant interactions were indicated by the ANOVA result. Data was analysed using individual pups and the average value for each litter as the dependent variable. If litter data was not available then the data was analysed using individual pups. Kidney data from neonatal, 2, 4 and 20-week studies were analysed using independent-samples T test. Aged rat kidneys were analysed using non-parametric independent-samples test with a Mann-Whitney U test applied due to the semi-quantitative method employed to determine glomerulosclerosis.

2.7.2. Data analyses of humans.

The children used for this study were grouped in to quintiles of birth weight and recruited from those contactable from the lower, middle and the upper quintiles at age 10 and 12 years (as described in section 2.4.1, & Chapter 5). The young adults were split into 3 groups at the 33rd and 66th centile based on birth weight (as described in section 2.4.2, & Chapter 5)(Power *et al.*, 2003). Markers of renal function, albumin creatinine and albumin:creatinine ratio were represented dichotomised at the 80th centile. This was derived from the observation that the significant excretion of urinary albumin at age 10 was in the top 20% of the population studied. Measurements for size and weight at birth were represented dichotomised at the 20th centile due to sample size. The majority of human subjects were above the low-birth weight point of <2500g (Department of Health, 2001). The data was analysed using one-way and two-way analysis of

variance (ANOVA) with an LSD test applied for post-hoc analysis where significant interactions were indicated by the ANOVA result, and Independent-samples T test.

Chapter 3.

Age-related renal function and morphology in the rat exposed to a maternal low protein diet *in utero.*

3.1. Introduction.

Maternal undernutrition has been identified as a contributory factor in determining intrauterine growth retardation (Godfrey, 1998). In humans, the kidney, an important regulator of blood pressure, appears extremely vulnerable to the adverse effects of growth retarding factors *in utero* (Hinchliffe *et al.,* 1992; Konje *et al.,* 1996; Sane *et al.,* 2001). Maintenance of renal haemodynamic function in a kidney with a reduced renal reserve is proposed to raise systemic blood pressure promoting an irreversible progression to renal failure (MacKenzie & Brenner, 1995). Animal studies strongly support these findings (Langley-Evans *et al.,* 1999; Nwagwu *et al.,* 2000).

A broad range of approaches has been taken for the study of fetal programming within animal models. These include nutritional manipulations in the rat, mouse, guinea pig and sheep pregnancy that have focused upon limiting specific nutrient intakes, or have applied a global nutrient restriction (reviewed Langley-Evans, 1999). Despite the diversity of the models that have been studied it is consistently noted that the manipulations of the nutrient supply (Langley-Evans, 1999; 2000) to the developing fetus results in elevated blood pressure in postnatal life, with an observed reduction in nephron number (Langley-Evans *et al.,* 1999; Vehaskari *et al.,* 2001) and glomerular filtration rate (Nwagwu *et al.,* 2000). The most extensively studied of these models has employed a mild restriction of maternal protein intake in pregnant rats (Langley & Jackson, 1994). This results in accelerated fetal growth in early gestation followed by late gestation growth

retardation, culminating in a low to normal weight at birth (Langley-Evans *et al.,* 1996a). The offspring of rats fed a low protein diet in pregnancy exhibit a number of endocrine, metabolic and physiological abnormalities by the age of weaning (Langley-Evans, 1999). The feeding of a 9% casein low protein diet in rat pregnancy induces hypertension in the offspring (Langley & Jackson, 1994; Langley-Evans *et al.,* 1994; 1996a,b,c). The hypertension observed in rats exposed to a low-protein diet *in utero* lasts throughout life (Langley-Evans & Jackson, 1995) The kidneys of offspring exposed to a mild maternal protein restriction in mid-late gestation exhibit impaired nephrogenesis and develop a pattern of function that suggests the existence of progressive glomerulosclerosis (Nwagwu *et al.,* 2000; Langley-Evans *et al.,* 1999).

At present, our understanding is that programming influences in fetal life lead to later elevations of blood pressure and appear to generally reduce nephron number. Hypertension and impaired renal reserve may merely coincide and may not necessarily be related in a causal manner (Langley-Evans *et al.,* 1999). Using the established low protein animal model of Langley & Jackson (1994), the aim of this study is to evaluate the impact of the low protein diet upon blood pressure, kidney morphology, organ development and markers of renal function over longer periods of the rat lifespan. It is hypothesised that renal structure and function are determined by prenatal nutrition and that this is a key mechanism in the programming of hypertension.

3.2. Protocol.

In order to investigate nephron compliment at differing times during the rat lifespan kidneys were derived from four separate investigations examining the effects of the low protein-feeding model (Langley & Jackson, 1994). For the purpose of this chapter the data is combined and the source of the tissue will be recognised in the appropriate table/figure. This enables

efficient and economic use of the laboratory animal. Importantly, tissues generated independently of the thesis author do not permit any bias in either the experimental protocol or the interpretations of the findings. By deriving tissue from other experimental studies it may be possible to demonstrate the consistency of low-protein feeding on developmental programming.

3.2.1. Source of rat kidneys.

3.2.1.1. Source 1.

Rat kidneys examined at two, four and twenty weeks, were supplied from a study that found evidence of a progressive deterioration in renal function and increased systolic blood pressure in rats exposed to a maternal low-protein diet *in utero* (Nwagwu *et al.,* 2000). In this study rats were examined at time points four and twenty weeks. Blood pressure was elevated at all three time points in the low-protein exposed animals relative to controls. Renal morphometry showed the 4 week old rats exposed to the low-protein diet, as having significantly smaller kidneys, being both shorter and wider than controls. Four week-old rats exposed to the low-protein diet had significantly reduced creatinine clearance compared to control. Both renal morphometry and creatinine clearance at the other time points were not influenced by prenatal diet. Urinary output, albumin excretion and blood urea nitrogen levels were all significantly greater in low-protein exposed animals compared to controls.

3.2.1.2. Source 2.

The aged rat kidneys where supplied from the study of Aihie Sayer *et al.,* (2000) which found that prenatal exposure to a maternal low-protein diet shortened life span. Organs were

harvested after animals were allowed to die without intervention if there was no evidence of pain distress or discomfort. Animals rapidly losing weight or distressed necessitated euthanasia. The average female rat life span at 92±4 weeks was significantly longer than the male at 74±2 weeks. Female rats exposed to low-protein diets *in utero* had their lifespan significantly reduced by 11%. A similar but non-significant trend was found in male offspring with control living an average 76±3 weeks and low-protein 73±3 weeks. Animals exposed to a low-protein diet *in utero* had significantly raised blood pressure at 4 weeks. Low-protein exposed animals tended to be lighter than controls.

3.2.1.3. Source 3.

The neonatal kidneys were supplied from a study by Langley-Evans *et al.,* (2002) assessing fetal exposure to a maternal low-protein diet and the immune system. The size of the thymus relative to body weight was significantly related to the number of circulating neutrophils. On delivery, each litter was culled to a maximum of eight pups. Those culled at birth provided kidneys for this study. The rats from this study were Sprague Dawley, all others were of the Wistar strain.

3.2.1.4. Source 4.

A preliminary animal study was performed to obtain maternal and parturition data, 4-week blood pressure, pulse and organ data, kidneys and 24 hour urine samples. The data is combined with other renal data form the other sources. It is designed to include the maternal component into to the statistical analysis to observe any effect that litter of origin may have on the on the data generated.

3.2.2. Animal husbandry.

Feeding and daily monitoring of pregnant dams was conducted by Dr. M. O. Nwagwu and blood pressure recording was conducted by Dr. S. C. Langley-Evans from the study of Nwagwu *et al.,* (2000). Feeding, daily monitoring of pregnant dams and blood pressure recording for the Aihie Sayer *et al,* (2001) study, were conducted by R. L. Dunn, Animal Technician, Southampton General Hospital, Institute of Human Nutrition, Tremona Road, Southampton, SO16 7PX. The author was responsible for the histology of kidneys from Nwagwu *et al.,* (2000) and Aihie Sayer *et al.,* (2001) (Source 1 & 2).

Feeding and daily monitoring of pregnant dams, blood pressure and pulse recording, urine collection and organ harvesting for this study and for the neonatal kidneys from Langley-Evans *et al.,* (2002), was conducted by Dr. Simon Langley-Evans, Reader in Human Nutrition, The University of Nottingham, Division of Nutritional Biochemistry, Sutton Bonington Campus, Loughborough, LE12 5RD. The author was responsible for the histology and biochemical analyses (Source 3 & 4).

3.3. Methods.

Animals for this preliminary study (Source 4) were of the Wistar strain (Harlan, UK Belton, Leicestershire, UK). Dams weighing between 211-243g were fed standard laboratory rat chow prior to conception (CRME, Special Diet Services, UK) (Appendix 2). Four rats were fed 18% casein control diet (CON) and four a 9% casein low protein diet (MLP) throughout pregnancy. Maternal weight gain and food intake were recorded daily. At parturition (n=5; n=3 CON; n=2 MLP), all rats transferred to standard laboratory rat chow (CRME, Special Diet Services, UK) throughout the suckling period. Total litter size was n=28 CON and n=18 MLP.

Two MLP fed dams failed to conceive and appeared to have reabsorbed their litters. 4 MLP exposed rats died postnatally, the cause of death was determined to be the mother. One CON fed dam had a vaginal plug but did not actually conceive. Offspring were weaned onto standard laboratory rat chow (CRME, Special Diet Services, UK) at 4 weeks of age. These animals differed only in terms of their prenatal dietary experience (Langley-Evans, 1999).

At 4 weeks of age offspring blood pressure and pulse was determined using the tail cuff method (section 2.2.2). At four weeks all offspring (n=18 males; n=11 females CON and n=9 males; n=5 females MLP), were placed in standard metabolic cages for a 24-hour urine collection (Section 2.2.3). Samples collected were used to determine urinary albumin, creatinine and creatinine clearance (sections 2.5.1, 2 & 3). Albumin values were corrected for creatinine content.

All offspring (n=29 CON, n=14 MLP) were weighed at four weeks before being sacrificed by CO_2 asphyxiation followed by cervical dislocation. Blood was collected by cardiac puncture to determine albumin, creatinine concentrations and blood urea nitrogen (BUN) (sections 2.5.1, 2 & 7). Liver, spleen, kidneys, heart and lungs were rapidly excised and weighed. Organ weight was adjusted against body weight (Section 2.2.4).

Left kidneys (n=29 CON, n=14 MLP) were used to determine nephron number using the maceration method (section 2.3.2). The maceration method was used to determine nephron number in neonatal, two, four and twenty week kidneys. The renal sectioning method was used to determine glomerular condition in aged rats (section 2.3.1).

3.3.1. Statistical analysis

All data is represented as mean ± SEM within the tables and figures. Data was analysed using individual pups as cases where no maternal data was available (Sources 1, 2. 3). Data was

analysed using the average value for each litter as the dependent variable where litter data was available (Source 4). Data for males and females are shown separately when litter effects are present. Differences among dietary groups based upon litter effects were sought using one-way ANOVA. Data was combined, as insufficient female numbers were present to perform post hoc analysis between litters of origin. Analysis of variance indicated that sex had a significant impact upon some of the variables studied and this is shown in the data. The data was analysed using two-way analysis of variance (ANOVA) with an LSD test applied for post-hoc analysis where significant interactions were indicated by the ANOVA result, unless otherwise stated. Kidney data from neonatal, two, four and twenty week studies were analysed using independent-samples T test. Aged rat kidneys were analysed using non-parametric independent-samples test with a Mann-Whitney U test applied. The statistical package SPSS (version 10) was used in all analyses. Statistical significance was set at $P<0.05$.

3.4 Results.

3.4.1. Maternal data.

Maternal food intake and weight gain was measured throughout pregnancy. Average weekly food consumption was less in low-protein dams but was not statistically significant compared to controls. Total mean weight gain in control dams was 40g greater than low-protein dams but was not statistically significant. Control dams gradually increased in weight over the course of pregnancy. Average weekly food consumption and weight gain remained consistent throughout pregnancy (Table 3.1).

Average litter size was the same for the different dietary groups (Table 3.2). Two-way ANOVA adjusted for litter of origin indicated that both diet ($F=37.189$, 1df, $P=0.001$) and litter

(F=38.773, 1df, P=0.001) had a significant effect on birth weight (Table 3.4). MLP birth weights were significantly lighter than those animals exposed to CON when adjusted for litter of origin (P<0.001) (Table 3.4). ANOVA indicates the interaction of birth weight between the litters of origin with no single litter having an overall effect on the data (Table 3.4).

Table 3.1. *Maternal weight gain and food consumption in pregnancy.* (Source 4).

Dietary Groups		CON	MLP	
At parturition		*n.* 3	*n.* 2	
Start weight.	(g)	230±9	240±2	P=0.444
Weight gain week 1.	(g)	51±4	33±6	P=0.145
Weight gain week 2.	(g)	48±4	42±7	P=0.548
Weight gain week 3.	(g)	77±8	60±3	P=0.160
Total weight gain.	(g)	175±14	135±2	P=0.164
Average food consumption.				
During week 1.	(g/d)	35±6	25±1	P=0.269
During week 2.	(g/d)	35±3	26±2	P=0.068
During week 3.	(g/d)	43±9	26±3	P=0.192

Data are shown as mean ± SEM for *n* observations.

3.4.2. Blood pressure and pulse rate. (Source 4).

The systolic blood pressures of male and female offspring are shown combined and separately and corrected for litter of origin (Table 3.3). The systolic blood pressures of combined offspring exposed to MLP, were significantly higher (22 mmHg) than offspring exposed to a

CON maternal diet (P<0.001). When blood pressure was corrected for body weight a similar pattern between dietary groups exists and is significant (P<0.001).

Table 3.2. *Litter size from dams exposed to varying maternal dietary composition.* (Source 4).

		Diet	
		Control	MLP
Total offspring	*n.*	29	17
Male/female	*n.*	18/11	9/8
Average litter size		9	9

Male MLP exposed rats have a significantly higher systolic blood pressure (32 mmHg) compared to CON (P<0.001). MLP male systolic pressure corrected for body weight was 32 mmHg higher than CON (P>0.001). In MLP females systolic pressure and systolic pressure corrected for body weight was higher than in CON, but fails to gain significance. ANOVA indicates the interaction of combined (male and female) systolic blood pressure between litters of origin. MLP litters had significantly elevated systolic blood pressure compared to CON litters (Table 3.4).

The pulse rate of offspring was measured at four weeks using the tail cuff method. Within the dietary groups the pulse rates of male and female offspring were similar (Table 3.3). ANOVA indicates the effects of pulse between litters of origin (F=3.943, 4df, P=0.009). One CON litter had a significantly lower combined (male and female) pulse rate compared to the other CON litters (P<0.01), but not the MLP litters (Table 3.4).

Table 3.3. *Blood pressure, pulse rate, birth and body weights of rats exposed to control or low protein diets in utero adjusted for litter of origin.* (Source 4).

Maternal Diet.		Birth Weight.		Body Weight. At 4 weeks.	Systolic Blood Pressure.	Systolic Blood Pressure/ Corrected For Body Weight.	Pulse Rate. (BPM)
	n.	(g)	*n.*	(g)	(mmHg)		
Litter mean							
CON	29	6.4±0.1	29	112±6	110±9	110±2	431±7
MLP	17	5.5±0.1***	14	117±3	132±4***	135±4***	419±11
CON MALE	18	6.4±0.1	18	117±3	109±3	109±3	439±8
MLP MALE	9	5.8±0.1***	9	124±5	141±4***	141±5***	422±12
CON FEMALE	11	6.4±0.1	11	108±3	112±4	111±4	423±15
MLP FEMALE	8	5.8±0.1***	5	109±4	124±6	126±6	415±20

Data are shown as mean ± SEM for *n* observations adjusted for litter of origin. *denotes significantly different to CON at P<0.05, *** denotes significantly different to CON at P<0.001. Two-way ANOVA indicated a significant effect of diet upon male and female rat birth weight, F=37.189, 1df, P=0.001 and blood pressure at 4 weeks, F=21.606, 1df, P=0.001. A significant effect of sex was indicated for body weight at 4 weeks, F=8.903, 1df, P=0.005. A significant effect of litter was indicated for birth weight, F=39.773, 1df, P=0.001 and body weight F=6.647, 1df, P=0.014 (See Table 3.4). Correction factors for body weight; Combined 115.5g Male 119.7g Female 108.6g. Correction factors for litter effect; Combined 5.0698; Male 5.0741, Female 5.0625. Systolic blood pressure corrected for body weight correlation coefficient; Combined 0.6562, 40df, P=0.001; Male 0.7770, 24df, P=0.001; Female 0.4660, 13df, P=0.080.

Table 3.4. *Association between litters for birth weight, body weight, blood pressure, pulse and combined organ weights adjusted for body weight (males and females) in the rat exposed to differing dietary composition in utero.* (Source 4).

Litter of Origin	CON	CON	CON	MLP	MLP
Number	1	2	3	1	2
Total Offspring M/F *n.*	5/1	9/2	4/7	5/3	5/5
Litter mean.					
Average birth weight.	6.93±0.11 [b,c,d,e]	6.05±0.7 [d,e]	5.98±0.6 [d,e]	6.50±0.09	5.15±1.2
At 4 weeks.					
Body weight (g)	122±3.0 [b,d,e]	104±3.0 [c,d]	116±4.0 [d]	138±6.0 [e]	111±3.0
Blood Pressure (mmHg)	110±5.0 [d,e]	116±3.0 [c,d,e]	103±4.0 [d,e]	141±6.0	131±5.0
Pulse (Bpm)	442±14	450±10	394±11 [a,b]	426±12	425±10
Heart (%)	0.57±0.07	0.58±0.01	0.55±0.02	0.53±0.03	0.54±0.01
Right Kidney (%)	0.51±0.01	0.55±0.02	0.55±0.02	0.56±0.01	0.54±0.01
Left Kidney (%)	0.50±0.02 [b,c,d]	0.57±0.01	0.58±0.02 [e]	0.55±0.01	0.52±0.01
Lung (%)	1.19±0.12	1.01±0.04	1.21±0.09	1.00±0.07	1.05±0.02
Liver (%)	4.83±0.07 [b]	5.90±0.24 [c,d,e]	5.12±0.17	4.99±0.07	4.90±0.08
Spleen (%)	0.53±0.02	0.50±0.03	0.47±0.02	0.50±0.03	0.52±0.03

Data are shown as mean ± SEM for observations. [a] significantly different to CON 1, [b] significantly different to CON 2, [c] significantly different to CON 3, [d] significantly different to MLP1, [e] significantly different to MLP2 ($P<0.05$). One-way ANOVA indicates a significant effect between litters for birth weight, $F=45.935$, 4df, $P=0.001$, body weight at 4 weeks, $F=12.793$, 4df, $P=0.001$, Blood pressure, $F=11.718$, 4df, $P=0.001$, Pulse, $F=3.943$, 4df, $P=0.009$, Left kidney, $F=5.287$, 4df, $P=0.002$ and Liver, $F=6.677$, 4df, $P=0.001$.

3.4.3 Body weight and Organ size. (Source 4).

Offspring had their body weights recorded at four weeks, prior to sacrifice and organ removal (Table 3.3). Body weight and litter of origin was added as a covariate. The values are adjusted to a combined body weight of 115.5g, males 119.7g and females 108.6g. Correction factors for litter effects are included at the bottom of the relevant tables.

The body weights in offspring exposed to the CON diet were lighter at 4 weeks than those exposed to an MLP diet but this was not significant. Two-way ANOVA adjusted for litter of origin indicated sex had a significant effect on body weight at four weeks, with males being significantly heavier than females ($P<0.01$) (Table 3.3). ANOVA indicates the interaction of body weight at 4 weeks between litters of origin (Table 3.4) No single litter was found to have an overall effect on the body weight data at 4 weeks. Being lighter at birth and heavier at four weeks coincides with an increased systolic blood pressure (SBP) in MLP exposed animals.

Male and female organ weights in proportion to body weight were similar in both CON and MLP animals. Two-way ANOVA corrected for body weight and litter of origin indicated that sex and maternal diet had no significant effect on organ weights, but as litter effects were present in the analysis of combined organ data, organ data is shown separately for males and females (Tables 3.5, 3.6). The small size of the female sample did not permit a post hoc analysis between the litters and the interaction between the litters of origin cannot be shown so the organ data is shown combined (Table 3.4). ANOVA indicates no individual litter had an overall effect on organ data (Table 3.4).

Table 3.5. *Organ weights of 4-week old male rats exposed to diets of differing composition in fetal life.* (Source 4).

		Maternal diet.	
		CON	MLP
Male	*n.*	18	9
Litter mean.			
Heart.	(%)	0.58±0.03	0.52±0.04
Right kidney.	(%)	0.54±0.01	0.58±0.02
Left kidney.	(%)	0.54±0.01	0.57±0.02
Lung.	(%)	1.04±0.05	1.10±0.08
Liver.	(%)	5.30±0.13	5.04±0.19
Spleen.	(%)	0.51±0.02	0.50±0.03

Organs weights are proportional to body weight. Data are shown as mean ± SEM for *n* observations and adjusted for body weight and litter of origin.

Litter had a significant effect on left kidney weight, F=4.634, 1df, P=0.042 and lung weight F=8.486, 1df, P=0.008.

Correction factor for body weight 119.7g and litter, 5.0741.

Table 3.6. *Organ weights of 4-week old female rats exposed to diets of differing composition in fetal life.* (Source 4).

		Maternal diet.	
		CON	MLP
Female	*n.*	11	5
Litter mean.			
Heart.	(%)	0.55±0.02	0.56±0.03
Right kidney.	(%)	0.54±0.01	0.54±0.02
Left kidney.	(%)	0.55±0.01	0.53±0.02
Lung.	(%)	1.22±0.07	0.96±0.10
Liver.	(%)	5.09±0.12	5.13±0.20
Spleen.	(%)	0.48±0.02	0.50±0.03

Organs weights are proportional to body weight. Data are shown as mean ± SEM for *n* observations and adjusted for body weight and litter of origin.

Two-way ANOVA adjusted for body weight and litter indicated body weight had a significant effect on liver F=9.850, 1df, P=0.009.

Correction factors for body weight 108.6g and litter 5.0625.

3.4. Nephron number.

3.4.4.1. Nephron number of 4-week old rats. (Source 4)

Left kidneys were used to determine nephron number (Figure 3.1). Within the dietary groups nephron number did not differ between male (18,582±957) and females (19,730±1.242) and so the data was combined for analysis (F=0.489, 1df, P=0.489). Two-way ANOVA adjusted

for body weight and litter indicated rats exposed to a MLP diet had significantly less nephrons than those exposed to CON (P<0.001) (Figure 3.1). ANOVA indicated a significant effect of nephron number between litters, with control litters having a significantly elevated nephron compliment compaired to those litters exposed to an MLP diet (P<0.01) (Figure 3.1.1). There was an association between blood pressure and nephron number at four weeks of age. Rats with a lower compliment of nephrons had a higher systolic blood pressure (Figure 3.2).

3.4.4.2. Neonatal nephron number. (Source 3).

Kidneys and birth weight data were supplied from randomly selected pups culled at birth. The organs were supplied fixed and whole from the study of Langley-Evans *et al.,* (2002). No maternal data available so the offspring are treated as individual cases. Nephron number was significantly less in the neonatal kidneys of pups exposed to a maternal low protein diet from conception to day 7 compared to CON (P<0.05) (Table 3.7).

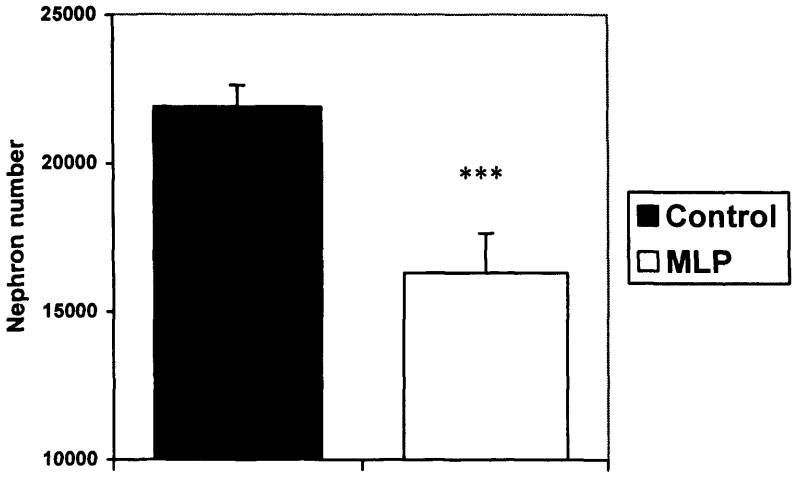
Table 3.7. *Nephron number, body and kidney weight in the rat neonate exposed to maternal diets of varying composition.* (Source 3).

Maternal Diet.		CON	MLP (day 0-7)	MLP (day 8-14).
n.		10	3	4
Nephrons/kidney.		2276±103	1617±262*	1963±273
Birth weight	(g)	5.80±0.1	5.63±0.3	5.50±0.35
Kidney weight(g)		0.028±0.001	0.025±0.002	0.024±0.002
Kidney/bodyweight	(%)	0.49±0.02	0.45±0.03	0.45±0.001

Data are shown as mean ± SEM for *n* observations.* denotes significantly different to CON, P<0.05. Two-way ANOVA indicated a significant effect of diet on nephron number in the neonate kidney; F=5.981, 2df, P=0.017. CON= 18% casein, control diet, MLP (day 0-7)= 9%

casein, low protein diet fed only between days 0-7 during pregnancy, MLP (day 8-14)= 9% casein, low protein diet fed only between days 8-14 during pregnancy.

Figure 3.1 *Nephron number in 4-week old rat offspring exposed to control or low protein diets in utero adjusted for body weight and litter of origin.* (Source 4).



Litter size. CON male n=18, CON female n=11, from n=3 litters. MLP male n=9, MLP female n=5 from n=2 litters. *** denotes significant against CON, P<0.001. Correction factors for body weight 115.5g and litter of origin 5.0698.

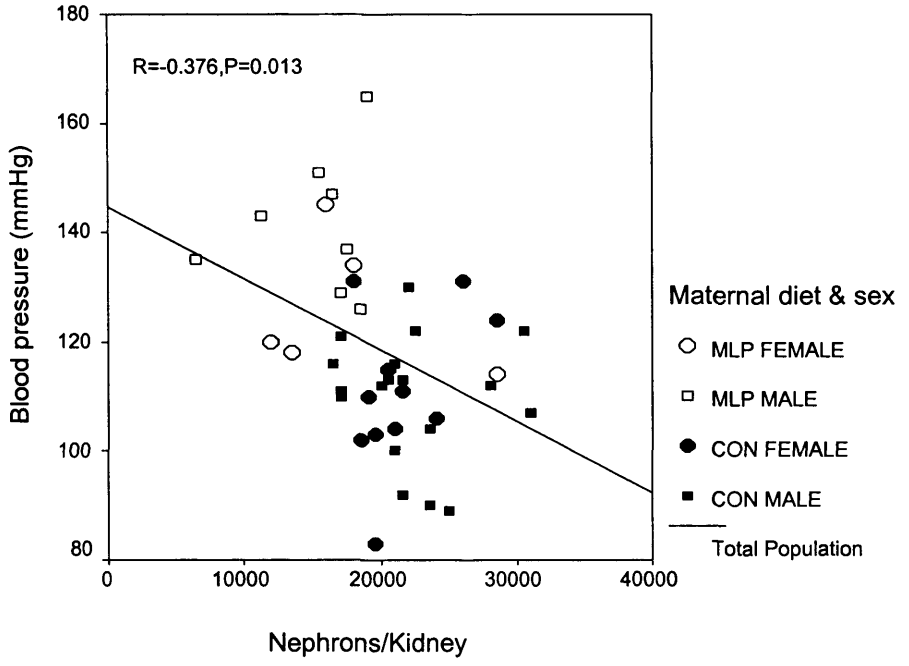
Two-way ANOVA adjusted for body weight and litter of origin indicated that diet had a significant effect on nephron number, F13.582, 1df, P=0.001. No effect of litter (F=0.354, 1df, P=0.556) or body weight (F=0.413, 1df, P=524) was present.

Figure 3.1.1. *Interaction between litter of origin and nephron number* (Source 4).

LITTER of Origin	CON 1	CON 2	CON 3	MLP 1	MLP 2
Litter Mean. Nephron Count.	23313± 1690 [a,a]	21168± 1135 [b,b]	21611± 1047 [b,b]	15833± 1931	16667± 1910

Data are shown as mean ± SEM. CON [a] is significant at P<0.01 [b] at P<0.05 compared to MLP. The first superscript represents the significant effect of that litter over MLP litter 4, and the second superscript over MLP5. One-way ANOVA indicated an interaction between litter of origin and nephron number (F=4.196, 4df, P=0.007).

Figure 3.2. *The association between nephron number and blood pressure in 4-week old rats.* (Source 4).



n. = 18 CON males (18% casein diet), *n.*=11 CON females (18% casein diet), *n.* = 9 MLP males (9% low protein diet), *n.*=5 MLP females (9% low protein diet).

Data shows that there is a significant relationship between systolic blood pressure and nephron number. Two-way ANOVA adjusted for body weight and litter of origin indicates diet has an effect on nephron number, F12.625, 1df, P=0.001 and blood pressure, F27.941, 1df, P=0.001.

3.4.4.3. Nephron number in rats at 2, 4 and 20-week time points (Source 1).

Kidneys were supplied fixed and whole from the study of Nwagwu *et al.,* (2000) (Litter of origin data not available). At time points two, four and twenty weeks, MLP exposed rats had significantly less nephrons compared to CON rats (P<0.001) (Table 3.8). Nephron number at four weeks of age was significantly greater than nephron number at two weeks (P<0.01). This data indicates that nephron number remains significantly different but also declines with age in both groups.

3.4.4.4. Glomerular condition in aged kidneys. (Source 2).

Kidneys were supplied fixed and whole from the study of Aihie Sayer *et al.,* (2001) (Litter of origin data not available). The male kidneys show extensive renal ablation, hypertrophy and an altered architecture indicative of chronic renal sufficiency and possibly end-stage renal disease compared to females. Males of both CON and MLP groups exhibited more evidence of glomerular injury than females of the same group (P<0.001). Males exposed to a low protein diet *in utero* appeared to be more susceptible to mild glomerulosclerosis than control (P<0.05) (Figure 3.3.1, Figure 3.3.2). Nutritional programming of renal morphology in fetal life appears to promote age related glomerulosclerosis in the male, but not the female rat (Photomicrograph 3.1). Other tubular structures within the kidney were not assessed.

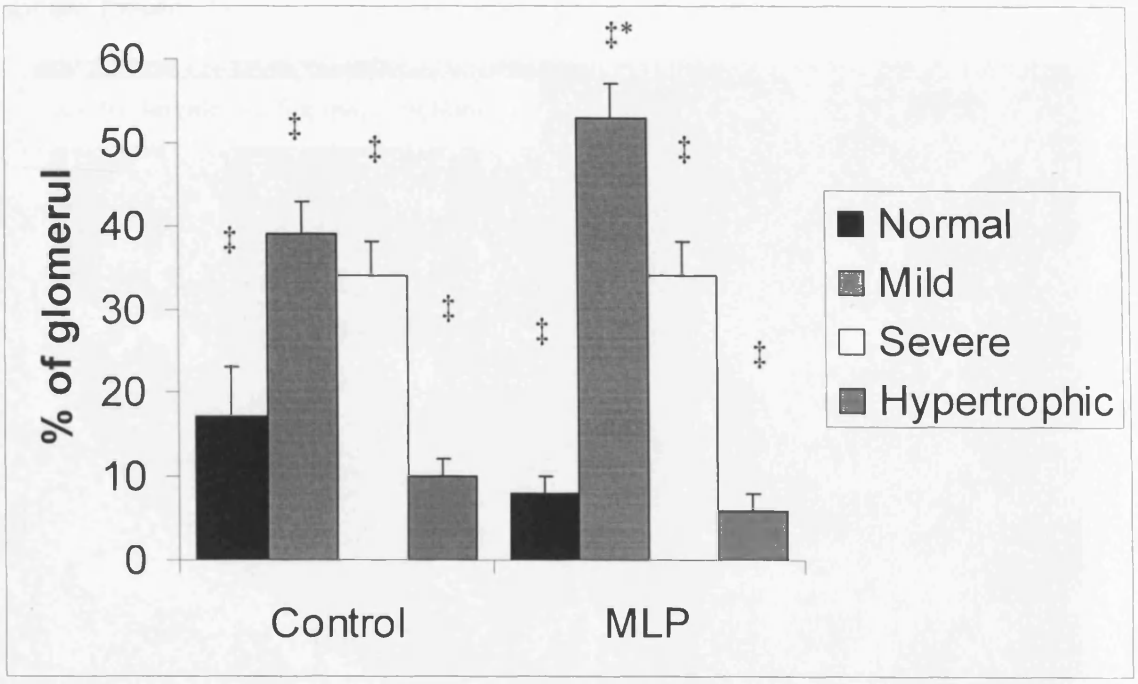
Table 3.8. *Nephron numbers in rats exposed to control or low protein diets in utero* (Source 1).

Nephron Number.

Maternal Diet.	*n.*	CON	*n.*	MLP
Age (Weeks)				
2	3	18833±1377	5	9950±837 ***
4	15	22594±3595†§	16	16375±2849***
20	3	16625±2875	7	10107±2402***

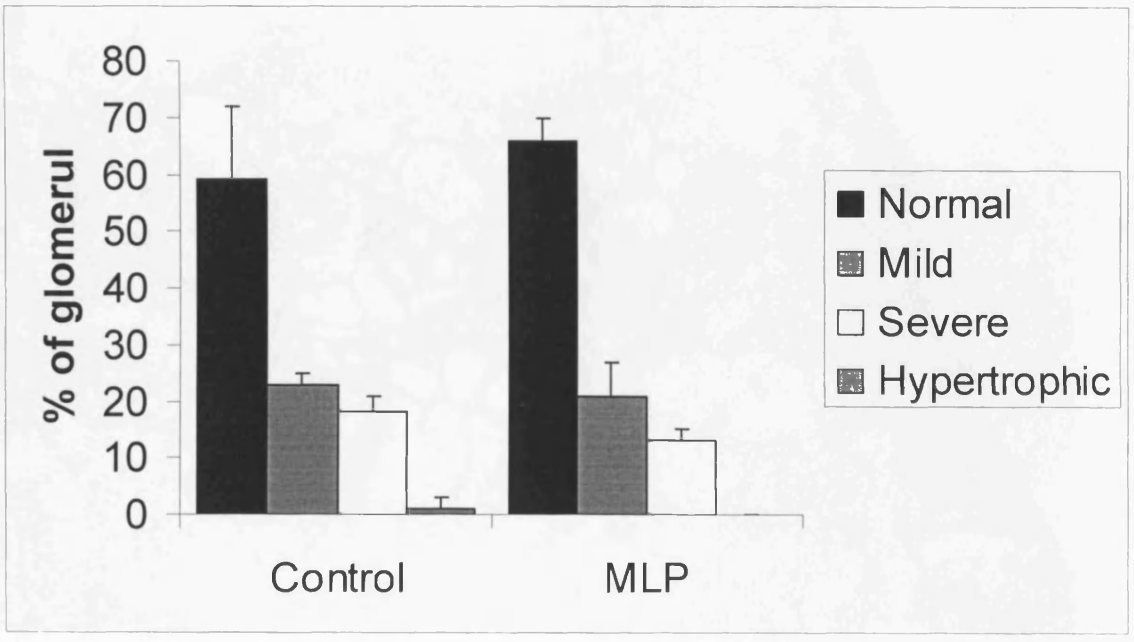
Data are shown as mean ± SEM for *n* observation. ***denotes significantly different to CON at P<0.001. †indicates significantly different to 2 weeks at P<0.01; § indicates significantly different to 20 weeks at P<0.001. Two-way ANOVA indicated a significant effect of diet upon combined rat nephron number; F51.338, 1df, P=0.001 and combined rat age upon nephron number F18.348, 2df, P=0.001 CON= 18% casein, control diet, MLP= 9% casein, low protein diet fed throughout pregnancy.

Figure 3.3.1. *Histological assessment of kidneys from aged male rats.* (Source 2).



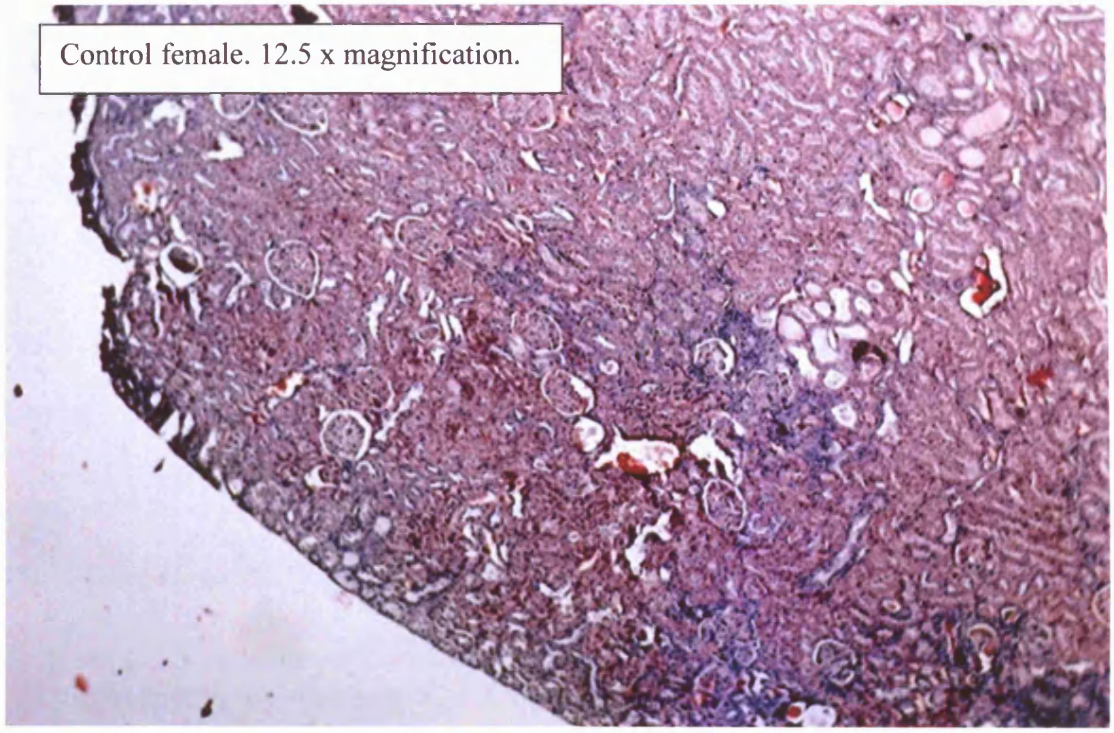
n.= 7 rats per group. * denotes P<0.05 between groups. ‡ denotes male vs. female from same dietary group (Figure 3.4.2.) P<0.001. (Analysed using non-parametric, Mann Whitney U).

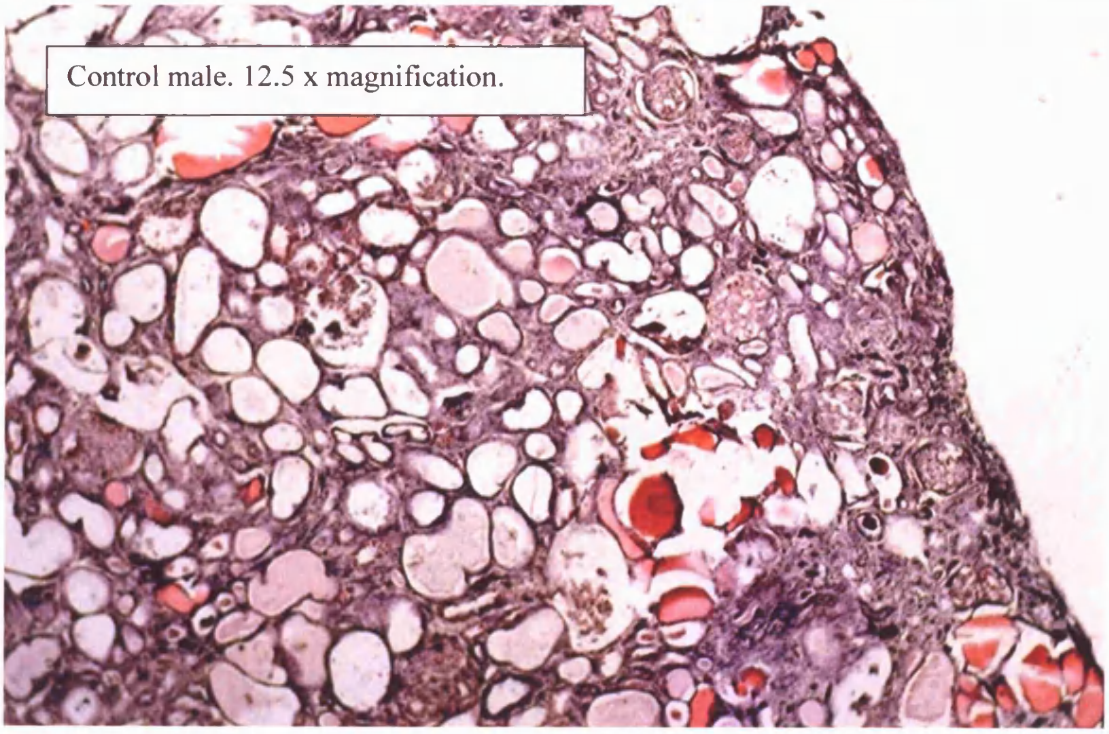
Figure 3.3.2. *Histological assessment of kidneys from aged female rats.* (Source 2).



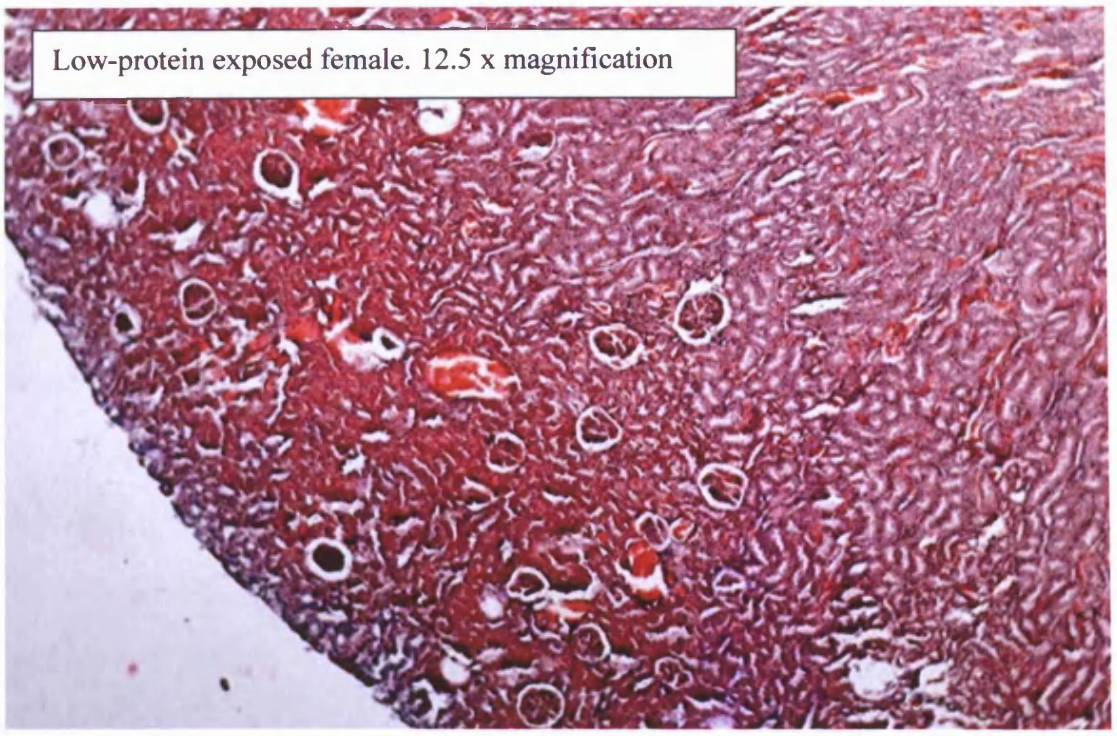
n.=4 rats per groups.

Photomicrograph 3.1. *Renal morphology of aged rats exposed to diets of differing composition in fetal life.* (Source 2).

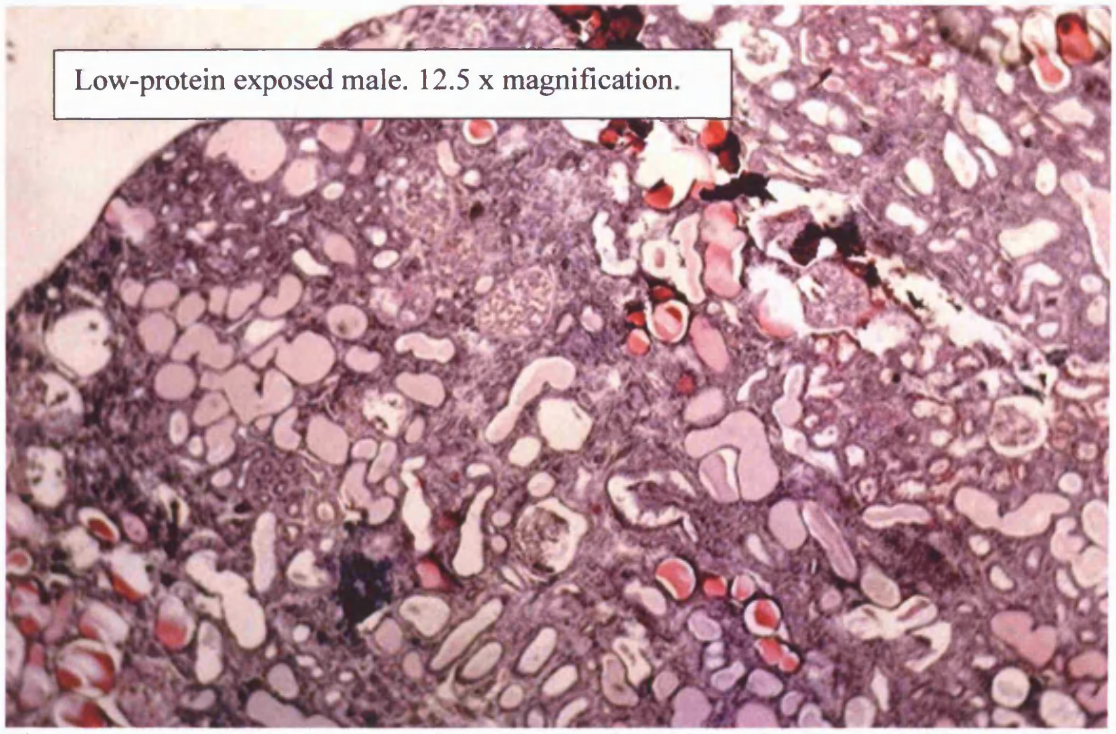




The structural architecture in the control male kidney is hypertrophic, exhibiting gross morphological disruption and glomerulosclerosis in comparison to the female.



Low-protein exposed female. 12.5 x magnification



Low-protein exposed male. 12.5 x magnification.

The structural architecture in the MLP male kidney is hypertrophic, exhibiting gross morphological disruption and glomerulosclerosis in comparison to the MLP female.

3.4.5. Renal function measures.

Renal function was assessed in terms of glomerular permeability (albumin excretion and creatinine clearance) and blood urea nitrogen (BUN) concentrations in offspring at four weeks. As litter of origin had a significant effect on the combined values the data is shown separately for males and females.

Assessment of urinary albumin excretion as a marker of glomerular permeability indicated no significant difference between CON and MLP. Plasma albumin was significantly reduced in MLP exposed male rats ($P<0.05$). Plasma creatinine was significantly higher in MLP male ($P<0.01$) and female rats compared to CON ($P<0.05$). Creatinine clearance is lower in MLP rats compared to CON, but was not significant. ANOVA indicates creatinine clearance in the combined data was elevated significantly in a single CON litter (Table 3.11). The single effect of this litter upon the data may be a reflection of the low numbers employed in this preliminary study. No other single litter had an effect on the variables measured.

A significant inverse trend between plasma creatinine concentrations and nephron number ($r=-0.330$, $P=0.041$) was present, suggesting that a reduced renal reserve promotes renal insufficiency and possibly disease (Figure 3.4).

Table 3.9. *Urinary and plasma biochemistry of 4-week old male rats exposed to diets of differing composition in fetal life.* (Source 4).

		Maternal diet.	
		CON	MLP
Litter size for males	*n.*	18	9
Litter mean			
Urinary albumin.	(mg/dl)	0.29± 0.01	0.26± 0.02
Urinary creatinine.	(mg/dl)	0.64± 6.1	0.72± 7.4
Urinary albumin/			
Creatinine ratio.	(mg/mg)	0.29± 0.01	0.27± 0.02
Plasma albumin.	(mg/ml)	7.37± 0.12	7.00± 0.20*
Plasma creatinine.	(mg/dl)	0.71± 0.07	1.09± 0.09**
Creatinine clearance.	(ml/min/kg)	0.58± 0.08	0.42± 0.11
Blood urea nitrogen/	(mg/l)	5.87± 0.54	4.92± 0.92
Urine output 24-hours	(ml)	8.50± 0.73	9.62± 1.08
Urine volume per minute/			
kg body weight	(µl/kg)	50.7± 0.54	49.2± 0.92

Data are shown as mean ± SEM for *n* observations. Adjusted for litter of origin. *denotes significantly different to CON at P<0.05, ** denotes significantly different to CON at P<0.01. Independent T test indicates a significant difference between dietary groups for plasma albumin (P<0.05) and creatinine (P<0.01). Two-way ANOVA adjusted for litter of origin indicated that diet was significantly different to plasma creatinine, F=7.899, 1df, P=0.010 in 4-week old male rats. Litter of origin had a significant effect on blood urea nitrogen, F=10.213, 1df, P=0.005 and urinary output over 24 hours, F=5.023, 1df, P=0.035. Litter correction factor 5.0370.

Table 3.10. *Urinary and plasma biochemistry of 4-week old female rats exposed to diets of differing composition in fetal life.* (Source 4).

		Maternal diet.	
		CON	MLP
Litter size for females	*n.*	11	5
Litter mean.			
Urinary albumin.	(mg/dl)	0.26± 0.02	0.24± 0.04
Urinary creatinine.	(mg/dl)	0.54± 8.9	0.60± 1.3
Urinary albumin/			
Creatinine ratio.	(mg/mg)	0.26± 0.02	0.24± 0.04
Plasma albumin.	(mg/ml)	7.45± 0.01	7.37± 0.15
Plasma creatinine.	(mg/dl)	0.81± 0.09	1.24± 0.15*
Creatinine clearance.	(ml/min/kg)	0.36± 0.05	0.37± 0.08
Blood urea nitrogen/	(mg/l)	5.11± 0.48	4.10± 0.76
Urine output 24-hours	(ml)	9.15± 1.16	9.96± 1.73
Urine volume per minute/			
kg body weight	(µl/kg)	58.2± 6.7	62.6± 10.2

Data are shown as mean ± SEM for *n* observations. Adjusted for litter of origin. * denotes significantly different to CON at $P<0.05$.

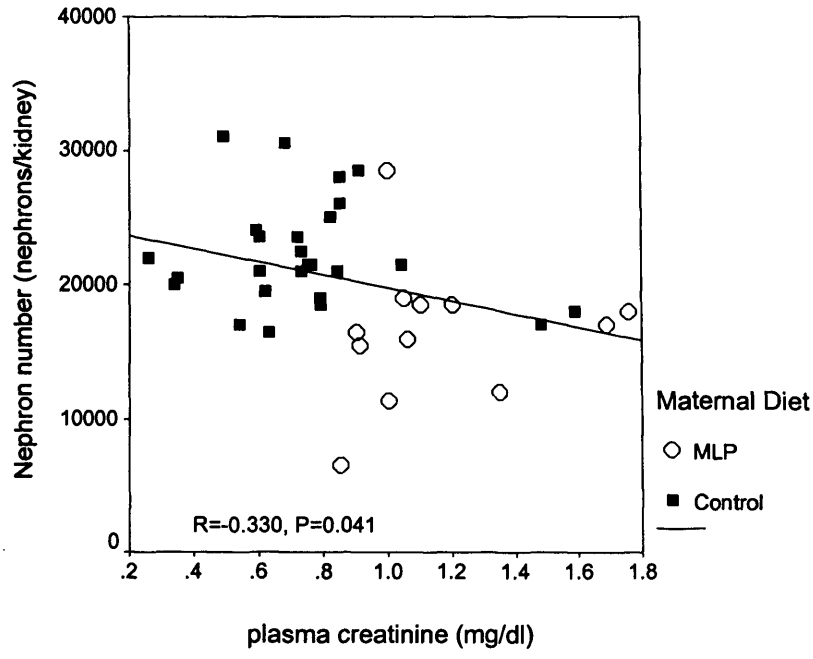
Independent T test indicates a significant difference between dietary groups for plasma albumin ($P<0.05$) Two-way ANOVA adjusted for litter of origin, indicated that diet was significantly different to plasma albumin $F=619$, 1df, $P=0.035$. Litter correction factor 5.000.

Table 3.11. *Association between urinary and plasma biochemistry and litter of origin in combined (males and females) 4 week old rats exposed to differing dietary composition in utero.* (Source 4).

Litter of Origin		CON 1	CON 2	CON 3	MLP 1	MLP 2
Litter mean.						
Urinary albumin.	mg/dl	0.26±0.03	0.28±0.02	0.30±0.02 [e]	0.23±0.01	0.27±0.02
Urinary creatinine.	mg/dl	0.54±12.6 [e]	0.66±5.70	0.59±5.73	0.54±8.60 [e]	0.80±6.01
Urinary albumin/ Creatinine ratio.	mg/mg	0.26±0.03	0.28±0.02	0.30±0.02 [d]	0.23±0.01	0.27±0.02
Plasma albumin.	mg/ml	7.30±0.13	7.45±0.09	7.58±0.11 [d,e]	6.99±0.03	7.09±0.15
Plasma creatinine.	mg/dl	0.90±0.15 [b]	0.56±0.09 [d,e]	0.72±0.05 [d,e]	1.06±0.06	1.34±0.22
Creatinine clearance.	ml/min/kg	0.42±0.08	0.75±0.19 [a,c,d,e]	0.41±0.03	0.40±0.05	0.41±0.08
Blood urea nitrogen.	mg/l	6.80±0.79 [c,e]	5.31±0.75	4.09±0.49 [d]	6.75±1.27	3.75±0.00
Urine output 24-hrs	ml	10.9±1.60 [c]	7.70±0.96 [d,]	7.32±0.59 [d,e]	12.1±1.18	8.50±1.25
Urine volume per minute/ Kg body weight.	µl/kg	63.0±10.2 [c]	52.0±5.76	45.56±3.70 [d]	64.4±5.62	53.4±7.32

Data are shown as mean ± SEM for observations. [a] significantly different to CON 1, [b] significantly different to CON 2, [c] significantly different to CON 3, [d] significantly different to MLP1, [e] significantly different to MLP2 ($P<0.05$). One-way ANOVA indicates a significant effect between litters for plasma creatinine, $F=4.448$, 4df, $P=0.005$, blood urea nitrogen, $F=3.320$, 4df, $P=0.022$, urinary output 24-hours, $F=3.920$, 4df, $P=0.009$.

Figure 3.4. *An association between plasma creatinine and nephron number in 4-week old rats exposed to diets of differing composition in fetal life.*



Total offspring at 4 weeks *n.* 29=CON, *n.* 14=MLP.

The data indicates that the lower the renal reserve the greater the increase in plasma creatinine levels.

3.5. Summary of results.

Overall weight gain and food consumption between MLP and CON dams during pregnancy was consistent. Systolic blood pressure was lower in offspring exposed to the maternal CON compared to MLP. Offspring exposed to an MLP diet had fewer nephrons at birth, two, four and twenty weeks compared to CON. Assessment of glomerular condition in aged kidneys highlighted males as being more susceptible to glomerulosclerosis than females.

3.6. Discussion.

The aim of this preliminary investigation (Source 4) was to examine the impact of fetal exposure to protein restriction on kidney morphology, function and hypertension in rats at 4 weeks. Nephron number was assessed at various time points throughout the rat life and the tissue was provided from other studies using the same low-protein model (Source 1,2,3). The relatively mild nutrient restriction provided to the dam raised blood pressure and reduced the nephron compliment in rat offspring at 4 weeks. This is consistent with previous findings (Langley-Evans *et al.,* 1999; Woodhall *et al.,* 1996; Vehaskari *et al.,* 2001). A strong inverse relationship between blood pressure and nephron number was observed in 4-week old rats. MLP exposed males had the highest recorded systolic blood pressure, even when corrected for body weight and litter effects. This is consistent with Ozaki *et al.,* (2001), who found dietary restriction in pregnant rats caused hypertension in both male and female offspring, but more pronounced in the male. However, renal function did not appear compromised in the 4-week old rat as previously reported (Nwagwu *et al.,* 2000). Nephron compliment in MLP exposed rats remains compromised throughout life, although males from both dietary groups exhibit a greater disturbance in renal architecture. To date no published literature or data has been found detailing this observation.

In previous studies (Merlet-Benichou *et al.,* 1994; Langley-Evans *et al.,* 1996a,b,c; 1999; Nwagwu *et al.,* 2000), the pup has been the statistical unit measured for the programming effects of a low-protein diet only the mother consumed. This statistical approach has recently been criticised (Walters & Edwards, 2003). The preliminary investigation into the effects of maternal low-protein feeding on hypertension and nephron number in 4-week old rats included the litter of origin into the statistical analysis. This only seems appropriate, as it is the maternal environment that is subjected to the dietary insult. Comparisons between litters indicate no single litter had an overall effect on either blood pressure or nephron number and clear differences between CON

and MLP litters was demonstrated. Nephron numbers at 4-weeks determined by the maceration method are broadly similar to those observed using the stereological method (Jones *et al.*, 2001).

The feeding of a mild protein restricted diet during the rat pregnancy is known to have no appreciable effect on maternal food intake (Langley-Evans *et al.*, 1996a) with the outcome of pregnancy tending not to be adversely affected by the fact that litter sizes do not differ significantly (Langley-Evans *et al.*, 1994; Kwong *et al.*, 2003). Even though the preliminary study used only a small numbers of dams to generate offspring, average litter size was equal between the dietary groups.

Birth weight was found to be different between the groups, with MLP exposed offspring being significantly lighter than CON. The male birth weight is heavier than females in both dietary groups. This is consistent with previous findings from other studies where offspring exposed to a low-protein diet *in utero* are of low-to-normal weight, but importantly have exhibited evidence of disproportion at birth (Langley-Evans *et al.*, 1996a). In this study MLP pups weighed less at birth than CON, by weaning their body weight is significantly heavier than CON and males are significantly heavier than females. This rapid acceleration of postnatal growth is consistent with the findings of Langley-Evans *et al.*, (1996c), Desai *et al.*, (1996) and Kwong *et al.*, (2000). This study may have benefited from measuring pups at birth in conjunction with weight to identify any possible disproportion between the dietary groups. The capacity to compensate for a low birth weight by subsequent accelerated weight gain may further influence the mechanisms of programming that may impinge on health in later life (Jones & Friedman, 1982; Anguita *et al.*, 1993; Petry *et al.*, 1997).

The birth weight of rats exposed to a maternal low protein diet between days 0-7 and 8-14 of gestation did not significantly differ from control animals as previously observed in Langley-Evans & Nwagwu, (1998). Low rat numbers for this section of the investigation possibly confounds any statistical difference.

Rats exposed to a low-protein diet *in utero* between day 0-7 of gestation had significantly reduced nephron endowment compared to CON. This is different from the findings of Langley-Evans *et al.,* (1999), who found exposure to a low-protein diet from day 0-7 of gestation produced a nephron reserve similar to controls. Rat nephrogenesis begins around day 14 of gestation. Comparing the data from this investigation with that of Langley-Evans *et al.,* (1999), the feeding of a low-protein diet between days 0-7 produced a nephron reserve similar to those in this investigation. However, this investigation produced a nephron compliment in offspring of 1963±273 when the maternal low-protein diet was feed between days 8-14, compared to the nephron reserve of 713±51 for the same period of maternal low-protein feeding as observed by Langley-Evans *et al.,* (1999). The low nephron reserve in offspring feed a maternal low-protein diet between days 8-14 as observed in Langley-Evans *et al.,* (1999), may be accounted for. Maternal low-protein diets have been associated with enhanced deletion of mesenchymal cells (embryonic precursor of the kidney) at the start of nephrogensis within the rat (Welham *et al.,* 2002). The low numbers of rats studied in this investigation may explain the high nephron numbers reported for maternal low-protein feeding between days 8-14. Langley-Evans *et al.,* (1999) had 17 offspring to determine nephron reserve compared to just 4 in this investigation and that this may confound the statistical analysis. Secondly, Langley-Evans *et al.,* (1999) used the histological staining method (section 2.3.1) compared to the maceration method employed in this investigation (section 2.3.2). Whilst both methods are comparable it would be of interest to replicate the Langley-Evans *et al.,* (1999), employing both the maceration and preferably the stereological technique to determine nephron number (Howard, 1997; Jones *et al.,* 2001).

There is some evidence to suggest that protein restriction induces changes in gene expression within the fetal kidney. Rees & Hay, (1998), noted that the growth retarding gene, *gas6* was up regulated in late gestation, a time coincident with the greatest impairment of nephrogenesis. Further determination of nephron compliment should also correspond with an

evaluation of specific gene profiles (Rees & Hay, 1998), and the level of apoptosis present in renal cells (Welham *et al.,* 2002). The findings in this study for blood pressure and nephron endowment do not differ from previous investigations using either a global or mild nutrient restriction or experiments which have examined exposure to maternal corticosteroids on developmental programming of hypertension and renal structure.

The weights of 4-week old kidneys from the preliminary investigation are similar in the MLP and CON exposed rats contradicting the findings of Nwagwu *et al.,* (2000) where the MLP kidney was found to be significantly lighter. This does not reflect previous held assumptions in both animal and human studies in that a smaller kidney has a reduced nephron complement. Relative size should not be used as a predictor of nephron number. Mild protein restriction during critical periods of kidney development may result in non-functional cells being laid down and maybe perturbed by accelerated postnatal growth. It is therefore important to assess functional capacity with measurements of organ size and morphology. Simple morphometric assessment of kidney size could not be determined due to the time the kidneys were in a preservative. Such measurements may have proved more conclusive of overall kidney size if combined with weight.

Significantly reduced nephron endowment in MLP exposed animals compared to CON at two, four, and twenty weeks supports the biochemical measures of blood composition and urinary indicators of renal function observed by their source Nwagwu *et al.,* (2000) (Source 1). The low number of litters and sample size of the preliminary investigation does not permit a detailed analysis of urinary and plasma biochemistry that could be considered sufficient to compare with data from previous studies. However, plasma creatinine is significantly higher in MLP exposed rats and also inversely related to nephron number. As plasma creatinine is freely filtered by the glomerulus, and not reabsorbed by the renal tubules it increases when renal function decreases (Lefebvre *et al.,* 2002). Gender associated differences in creatinine production are largely attributable to differences in muscle mass (Brenner & Rector, 2000). The presence of elevated

plasma creatinine may be explained by the larger number of males in this study coupled by their rapid postnatal growth. Raised plasma creatinine in MLP females suggest that low-protein exposure *in utero* may also confer altered postnatal growth and indicates that this may be through addition of increased muscle tissue (Gale *et al.,* 2001; Ozanne *et al.,* 2004; Singhal *et al.,* 2003). This data suggests that the kidneys of 4-week old rats exposed to MLP diet *in utero* may be exhibiting renal insufficiency by virtue of a reduced nephron reserve, but the lack of conclusive evidence from other markers of renal function mean no firm conclusions can be drawn. An assessment of the glomerular structure in MLP exposed animals may be necessary to ensure they are exhibiting normal development.

In the current study systolic blood pressure is inversely related to nephron number and confirms the observations of Manalich *et al.,* (2000), in human populations. A reduced nephron reserve requires an increase in systemic blood pressure in order to maintain the GFR and the excretory capacity of the kidney. Glomerular hyperthrophy is commonly seen as a marker of renal injury in a variety of experimental models (Samnegard *et al.,* 2001), and is believed to be a response, which maintains filtration surface area in the face of a reduction in the number of functional units. Glomerular capillary pressures would increase promoting glomerulosclerosis and further nephron loss in a sustained cycle of renal injury and increasing hypertension (MacKenzie & Brenner, 1995).

Histological examinations of aged kidneys (Source 2) show both MLP and CON males as having greater evidence of glomerulosclerosis and renal damage than females. The observed renal architecture in the aged rat is consistent with other studies (Brenner, 1985; Greenfeld *et al.,* 1997). Pronounced renal disease in the male supports the assertion of Aihie Sayer *et al.,* (2001), that increased blood pressure in the MLP rat may reduce lifespan. This does support the hypothesis forwarded by Mackenzie *et al.,* (1996) and whilst male kidneys are obviously exhibiting gross morphological changes possibly brought about by increasing blood pressure in

order to maintain renal function with a reduced renal reserve, this does not appear to be the case in females.

Urinary and plasma biochemical analysis by Aihie Sayer *et al.,* (2001) (source 2) would have enabled a detailed functional analysis to be conducted on these organs. Ozaki *et al.,* (2001), found increasing blood pressure in both males and females at 60, 100 and 200 days. Male blood pressure was consistently higher than females throughout these time points. In the rat, male gender is associated with more rapid progression of renal disease independent of blood pressure, dietary protein intake, or serum lipid levels (Kang *et al.,* 2004). It has been proposed by Kang *et al.,* (2002; 2004) that estrogen may protect female rats in progressive renal disease by stimulating vascular endothelial growth factor (VEGF) expression and maintaining a healthy intra-renal vasculature. The premature death of males (Aihie Sayer *et al.*, 2001) combined with the known pattern of renal deterioration and especially the increase in urinary output as observed in this study and that of Nwagwu *et al.,* (2000) might alternatively be explained by the development of diabetes. Several studies using a low-protein diet, of differing compositions, have demonstrated intrauterine programming of pancreatic structure (Snoeck *et al.,* 1990), islet function (Dahri *et al.,* 1991), and defects of glucose insulin metabolism (Ozanne *et al.,* 1996). Humans with diabetes usually have hypertension and progressive renal disease (UK Prospective Diabetes Study Group, 1999). Assessment of insulin sensitivity in the aged rat may have highlighted another mechanism by which nephron adaptation and subsequent deterioration occurred.

Mackenzie & Brenner, (1995) proposed that programming of renal structure might underlie associations between maternal undernutrition and later hypertension. Impairment of nephrogenesis due to fetal nutrient restraint was envisaged as raising local and systemic blood pressure as a means of maintaining essential renal haemodynamic functions. The present data, demonstrating long-term nephron deficit and its inverse relationship between hypertension are consistent with this hypothesis.

Further work in the low-protein rat model needs to evaluate the limiting nature of the diet and assess whether hypertension and a reduced renal reserve are causally associated.

Chapter 4.

Nutritional programming of systolic blood pressure and renal development in the rat.

4.1. Introduction.

Maternal undernutrition has been identified as a contributory factor in determining intrauterine growth retardation (Godfrey, 1998). In humans, the kidney, an important regulator of blood pressure, appears extremely vulnerable to the adverse effects of growth retarding factors *in utero* (Hinchliffe *et al.,* 1992; Konje *et al.,* 1996 and Sane *et al.,* 2001). Maintenance of renal haemodynamic function in a kidney with a reduced renal reserve is proposed to raise systemic blood pressure promoting an irreversible progression to renal failure (MacKenzie & Brenner, 1995) (Chapter 3).

Animal studies strongly support these findings (Langley-Evans *et al.,* 1999; Nwagwu *et al.,* 2000). The feeding of a 9% casein low protein diet in rat pregnancy accelerates fetal growth in early gestation, but impairs growth in late gestation (Langley-Evans *et al.,* 1996a). Offspring of a low-normal birth weight consistently exhibit hypertension (Langley & Jackson, 1994; Langley-Evans *et al.,* 1994; 1996a,b,c; & Langley-Evans, 1996). The kidneys of offspring exposed to a mild maternal protein restriction in mid-late gestation have impaired nephrogenesis and develop a pattern of functional measures that suggest the existence of progressive glomerulosclerosis (Nwagwu *et al.,* 2000; Langley-Evans *et al.,* 1998). It has now been demonstrated in the previous chapter that low nephron endowment at birth leads to a progressive deterioration of the kidney's essential structure and possibly function over time, which may lead to a shorter lifespan in males (Aihie Sayer *et al.,* 2001).

At present our understanding of what constitutes an optimal maternal diet for growth and development is limited. The feeding of low protein diets to pregnant rats has been argued to limit the availability of one or more essential amino acids (EAA)(Rees *et al.,* 1999). Evidence indicates that when the protein content of the diet is reduced progressively, the availability of total nitrogen, or the ability to maintain adequate formation of specific non-essential amino acids (NEAA), is likely to limit normal growth and function (Jackson, 1995; 1999). During fetal and neonatal life, the demands for glycine for growth are substantial. Glycine availability appears to be limited, indicating that the capacity for *de novo* formation is not adequate to meet demands (Jackson *et al.*, 1981). The use of 5-L-oxoproline as a marker for glycine status has indicated a limitation on its availability during pregnancy and in the newborn (Jackson *et al,* 1997a; Persaud *et al.,* 1997; Jackson *et al.,* 1997b). In humans, metabolic adaptations occur in response to the consumption of low protein diets. In order to form adequate amounts of NEAA an enhanced retention of urea-nitrogen occurs through the metabolic activity of colonic microflora. This increases the nitrogen available in the body for the formation of NEAA (Jackson, 1995; Meakins & Jackson 1996; Meakins *et al.,* 1998; Jackson, 2000). In rats, the practice of refection means that both EAA and NEAA formed by colonic microflora from salvaged urea-nitrogen are potentially available to enhance dietary quality for the animal (Torrallardona *et al.,* 1996a). When dietary protein intakes fall in adults, they become progressively less able to synthesise glycine. This limitation can be overcome in humans if additional nitrogen is provided in the diet by compounds as simple as urea (Meakins *et al.,* 1998).

In the rodent model an inadequate provision of EAA in the low protein diet may cause the programming of higher blood pressure in offspring (Rees *et al.,* 1999). However, the inability to form adequate amounts of NEAA, due to the lack of non-essential nitrogen in the diet, might limit maternal, placental and fetal function, rather than any specific EAA (Harding, 2001; Jackson 1999). The Langley & Jackson (1994) low protein diet provides 0.5g methionine/100g

diet, compared to the commercial low protein diet of 0.08g/100g, representing a sixfold difference. Rees (2002) has argued that the methionine content of the diet is excessive. There may be important metabolic consequences of methionine excess. Low protein feeding may limit the availability of many essential and conditionally essential amino acids and the capacity for the maternal liver to process excess methionine may be exceeded in early pregnancy and homocysteine levels may rise. The limiting effect of the low-protein diet on the dam affords no maternal nutrient buffer to the offspring and the potential consequence of a disturbed homocysteine metabolism may arise (Jackson, 1999; Rees; 2002). The recycling of homocysteine back to methionine may be limited in protein-restricted animals as it depends on the presence of a pool of methylated folates. Glycine provides the methyl groups required for the synthesis of the methylene tetrahydrofolate and determines the availability of methylated folates (Rees, 2002).

Using the established low protein animal model of Langley & Jackson (1994), the aim of this study is to evaluate the role of non-essential nitrogen in the form of glycine, urea and alanine in the low protein diet in programming blood pressure, kidney morphology, organ development and markers of renal function. It is hypothesised that the addition of glycine to the MLP diet will have a beneficial effect on blood pressure, nephrogenesis and renal function.

4.2. Protocol.

4.2.1. Animal husbandry.

Feeding and daily monitoring of pregnant dams in this study and blood pressure recording was conducted by R. L. Dunn, Animal Technician, Southampton General Hospital, Institute of Human Nutrition, Tremona Road, Southampton, SO16 7PX. The author was responsible for the manufacture of diets, collection of tissue samples, histology and biochemical analyses.

4.3. Methods.

Twenty-nine virgin female Wistar rats, weighing between 200-250g were mated and randomly allocated to one of five isocaloric experimental diets for the duration of pregnancy. Animals of the Wistar strain (Harlan, UK Belton, Leicestershire, UK) were fed standard laboratory rat chow prior to conception (CRME, Special Diet Services, UK) (Appendix 2). Seven rats were assigned to 18% casein control diet (CON), six to 9% casein low protein diet (MLP), five to 9% casein plus 3% glycine (MLPG), six to 9% casein plus 1.5% urea (MLPU) and five to 9% casein plus 3% alanine (MLPA) (Table 2.1). Glycine supplements were provided to determine the impact of this conditionally essential amino acid for pregnancy upon hypertension and renal abnormalities previously associated with feeding an MLP diet in rat pregnancy. The MLPU group was included to control for the effects of additional nitrogen provided with glycine supplementation. The MLPA group represented a control for the addition of supplemental non-essential amino acids to the MLP diet. Maternal weight gain and food intake were recorded daily. At parturition (n=25) (One 18%, one 9% and two 9% + urea failed to show), all rats transferred to standard laboratory rat chow (CRME, Special Diet Services, UK) throughout the suckling period and their offspring were weaned onto this diet at four weeks of age. These animals differed only in terms of their prenatal dietary experience (Langley-Evans, 1999). Recent data suggests that the protein content of milk produced within 12 hours of parturition is similar in control and MLP fed animals (Bellinger *et al.,* 2003). This suggests animals exposed to a MLP do not differ in their postnatal dietary experience and cross fostering may not be necessary.

At four weeks of age offspring blood pressure and pulse was determined using the tail cuff method as described in Chapter 2. At four weeks offspring (n=19 CON, n=17 MLP, n=15 MLPG, n=16 MLPU, n=16 MLPA), were placed in standard metabolic cages for a 24-hour urine collection (section 2.2.3). Where possible two males and two females from each litter were

placed in the metabolic cages as a representative sample. Urine samples collected were used to determine albumin, protein and creatinine (section 2.5.1 & 2). Albumin and protein values were corrected for creatinine content.

All offspring (n=43 CON, n=41 MLP, n=34 MLPG, n=32 MLPU, n=36 MLPA) were weighed at 4 weeks before being sacrificed by CO_2 asphyxiation followed by cervical dislocation. Blood was collected by cardiac puncture to determine angiotensin converting enzyme activity (ACE) and blood urea nitrogen concentrations (sections 2.5.7 & 2.6.2). Brain, liver, spleen, adrenals, kidneys, heart and lungs were rapidly excised and weighed (section 2.2.4). Organ weights are expressed as a percentage of body weight.

Left kidneys (n=10 CON, n=9 MLP, n=10 9% MLPG, n=9 MLPU, n=10 MLPA) were weighed at four weeks and used to determine nephron number using a histological staining method (section 2.3.1). Glomerular volumes were calculated for comparison between dietary groups (section 2.3.4). The right kidneys (n=10 CON, n=15 MLP, n=14 MLPG, n=16 MLPU, n=13 MLPA) were frozen under liquid nitrogen and stored at –80°C prior to the determination of the protein/DNA ratio as an indicator of cell size and number (section 2.6.1), ACE, activity (section 2.6.2) within the kidney tissue.

4.3.1. Statistical analysis.

All data is represented as mean ± SEM within the tables and figures. Data was analysed using the average value for each litter as the dependent variable. Data for male and female are shown separately when litter effects are present. Analysis of variance indicated that sex had a significant impact upon some of the variables studied and is shown in the data. The data was analysed using one-way and two-way analysis of variance (ANOVA) with an LSD test applied for post-hoc analysis where significant interactions were indicated by the ANOVA result, unless

otherwise stated. The statistical package SPSS (version 10) was used in all analyses. Statistical significance was set at $P<0.05$.

4.4 Results.

4.4.1. Maternal data.

Maternal food intake and weight gain was measured throughout pregnancy. Average daily food intake remained consistent between the groups (Table 4.1). Food consumption was significantly higher in the first week of pregnancy compared to the third week in the glycine ($P<0.01$) and alanine ($P<0.05$) fed dams. Food consumption in other dietary groups followed a similar trend but was not statistically significant. Maternal weight gain was significantly elevated between weeks 1-3 ($P<0.05$) in CON, between weeks 1-2, 2-3 and 1-3 in MLPG ($P<0.01$), between weeks 1-3 in MLPU ($P<0.05$), and weeks 1-2 in MLPA ($P<0.05$) fed dams (Table 4.1). Dam start weights, even though randomised before the feeding of experimental diets, indicates they were not well matched for body weight; MLPG ($P<0.05$), MLPU ($P<0.001$) and MLPA ($P<0.05$) are significantly heavier than CON and MLPU was significantly heavier than MLP ($P<0.05$) and MLPA ($P<0.05$). This may have an impact on the variables studied and the subsequent interpretation of the data generated. In future studies, once the animals are randomised a statistical analysis should be performed to ensure their start weights are not mismatched.

ANOVA indicated there was no significant difference in the average number of pups in the litters for the different dietary groups ($F=0.419$, 4df, $P=0.793$) (Table 4.2). Not all birth weight data was recorded within 12 hours of parturition. Potential fluctuation of neonatal weight after this time is a probability and insufficient to provide detailed statistical analysis. However,

the average of those birth weights recorded are in line with previous studies (Langley-Evans *et al.*, 1999).

Table 4.1. *Maternal weight gain and food consumption.*

Dietary groups	CON	MLP	MLPG	MLPU	MLPA
At parturition *n.*	6	5	5	4	5
Start weight. (g)	209±2	216±1	224±4*	234±6*§ *f*	222±3*
Weight gain week 1. (g)	29±4	23±5	14±3*	19±4	22±2
Weight gain week 2. (g)	40±6	31±3	35±2 [b]	30±2*	32±2 [a]
Weight gain week 3. (g)	49±7 [a]	47±12	59±2 [b,c]	55±5 [a]	NDA
Total weight gain. (g)	118±6	101±13	108±4	104±9	NDA
Food consumption.					
During week 1. (g)	21.5±2	24.0±5	26.0±1	21.5±2	26.0±1
During week 2. (g)	19.5±1	18.5±2	20.5±1	20.0±3	18.5±3
During week 3. (g)	18.5±2	21.5±1	18.5±1 [b]	18.0±2	17.0±1[a]

CON= 18% casein, MLP= 9% casein, MLPG= 9% casein + glycine, MLPU= 9% casein + urea, MLPA= 9% casein +alanine. NDA=No Data available.* indicates significantly different to CON, ‡ significantly different to MLPG, † significantly different to MLPU, § significantly different to MLP and *f* significantly different to MLPA ($P < 0.05$).[a] Indicates significantly different to week 1 ($P<0.05$) [b] Indicates significantly different to week 1 ($P<0.01$) [c] indicates significant weight gain between weeks 2 and 3 ($P<0.01$). One-way ANOVA indicated a significant effect between maternal starting body weights, $F=6.551$, 4df, $P=0.01$.

Table 4.2. *Litter size between dietary groups.*

Dietary groups	CON	MLP	MLPG	MLPU	MLPA
Total offspring *n.*	43	41	34	32	36
Male/female *n.*	20/23	21/20	17/17	17/15	20/16
Average litter size.	8	8	9	10	10

Data are shown as mean ± SEM for *n* observations.

4.4.2. Blood pressure and pulse rate.

Two-way ANOVA indicated that diet (F=12.353, 4df, P=0.001) and sex (F=3.998, 1df, P=0.047) had a significant effect on blood pressure within the dietary groups and is shown separately for litter mean, males and females (Tables 4.3). MLP Systolic blood pressure adjusted for litter of origin was significantly elevated compared to CON and MLPG animals (P<0.001). MLPU (P<0.001) and MLPA (P<0.01) animals had significantly higher blood pressure compared to CON and MLPG (P<0.001). Male offspring exposed to MLP diet, was significantly higher (15 mmHg) than offspring exposed to a CON maternal diet (P<0.01). Male rats exposed to MLPU diet had systolic blood pressures significantly higher than those exposed to CON (P<0.05). The systolic blood pressure in male offspring exposed to MLPG was significantly lower (16-23 mmHg) than those exposed to a maternal MLP, MLPU (P<0.001), and MLPA diet (P<0.01).

When corrected for body weight MLP exposed female blood pressure becomes significantly elevated compared to CON (P<0.05). Female rats exposed to MLPU (P<0.001) and MLPA (P<0.01) had systolic blood pressures significantly higher than those exposed to CON. Female MLPU exposed animals had a systolic blood pressure 10 mmHg higher than male MLPU animals. Similarly MLPA exposed animals had a systolic blood pressure 12 mmHg higher than MLPA males. The systolic blood pressure in female offspring exposed to MLPG was

significantly lower (18-27 mmHg) than those exposed to a maternal MLP (P<0.01), MLPU and MLPA diet (P<0.001) (Table 4.3). When blood pressure was corrected for body weight (F=4.901, 1df, P=0.028) and litter effects (correction factors in table 4.3), a stronger pattern emerges between dietary groups (F=11.146, 4df, P=0.001) and the sexes (F=6.624, 1df, P=0.01). Systolic blood pressure corrected for body weight and for litter of origin was significantly elevated in MLP animals compared to CON and MLPG (P<0.001). MLPU and MLPA animals had a systolic blood pressure corrected for body weight significantly higher than CON and MLPG (P<0.001). Glycine supplementation appeared to prevent the development of relative hypertension normally associated with MLP feeding. This association persists when blood pressure is corrected for body weight and litter effects.

Two-way ANOVA indicated that diet had a significant effect on pulse (F=8.059, 4df, P=0.001) The pulse was significantly lower (33-47 Bpm) in offspring exposed to a MLPA diet compared to those exposed to CON, MLP (P<0.001), MLPU and MLPG (P<0.05). When corrected for litter effects. The same trend is present in male rats exposed to varying maternal diets. In female rats the pulse rate is significantly lower (24-32 Bpm) in offspring exposed to MLPA diet compared to CON (P<0.01), MLP and MLPU (P<0.05).

4.4.3 Body weight and Organ size.

Body weights are shown controlled for litter of origin (Tables 4.3). Organ weights are shown proportional to body weight. Litter of origin and sex was found to have an effect on some of the organs weights so the data is shown separately for males and females (Table 4.4, 4.5). As body weight was added as a covariate, the values are adjusted to a body weight of 77.2g=males; 69.8g=females. Correction factors for litter effects are included at the bottom of relevant tables. Overall litter size had a significant effect on body weight (F=17.707, 1df, P=0.001).

Table 4.3. *Body weight, blood pressure and pulse rate at 4 weeks of age in male and female offspring of rats exposed to diets of varying amino acid content in fetal life.*

Maternal diet	CON	MLP	MLPG	MLPU	MLPA
Parturition *n.*	6	5	5	4	5
Male/female *n.*	20/23	21/20	17/17	17/15	20/16
Body weight (g)					
By litter mean	82±2	68±2*	68±2*	81±2§‡ *f*	70±2*§
Male	85±3	69±3*	72±3*	88±3§‡ *f*	76±3*
Female	79±2	62±2*	65±3*	75±2§‡ *f*	69±3*§
Systolic blood pressure (mmHg)					
By litter mean	108±3	121±3*‡	98±3*	124±3*‡	120±3*‡
Male	106±4	121±4*‡	98±4	119±4*‡	114±3‡
Female	110±4	120±4‡	102±4	129±4*‡	126±3*‡
SBP (mmHg) corrected for body weight.					
By litter mean	105±5	125±4*‡	98±5	115±5‡	114±4‡
Male	104±5	124±2*‡	101±4	115±5‡	115±4‡
Female	108±4	121±4*‡	103±4	128±4*‡	126±4*‡
Pulse					
By litter mean	400±7 *f*	397±6 *f*	388±8 *f*	386±7 *f*	353±7
Male	401±11 *f*	401±10 *f*	386±10 *f*	379±11 *f*	337±9
Female	400±8 *f*	394±8 *f*	380±8	392±9 *f*	368±8

Data are shown as mean ± SEM for *n* observations per group. * indicates significantly different to CON, ‡ significantly different to MLPG, † significantly different to MLPU, § significantly different to MLP and *f* significantly different to MLPA ($P < 0.05$) (continued over leaf).

Table 4.3. *continued,*

Two-way ANOVA indicated diet had a significant effect on male body weights, F=8.021, 4df, P=0.001, female body weights, F=11.498, 4df, P=0.001, male blood pressure, F=5.969, 4df, P=0,001, female blood pressure, F=7.508, 4df, P=0.001, male blood pressure corrected for body weight, F=4.863, 1df, P=0.030, female blood pressure corrected for body weight F=7.517, 4df, P=0.001, male pulse F=7.518, 4df, P=0.001. One-way ANOVA indicates the significant interaction between body weights, F=15.204, 4df, P=0.001, blood pressure, F=12.154, 4df, P=0.001, blood pressure corrected for body weight, F=5.167, 4df, P=0.001, pulse, F=9.468, 4df, P=0.001 and litter of origin.

Correction factors for body weight, total sample, 73.7g, male, 77.1g, female, 69.8g.

Correction factors for litter of origin, total sample, 16.09, male, 15.80, female, 16.36.

Systolic blood pressure corrected for body weight correlation coefficient; total sample -0.0827, 168df, P=0.284; Male –0.2092, 81df, P=0.058; Female 0.0422, 84df, P=0.700.

4.4.3 Body weight and Organ size (continued).

Body weight (litter mean) in offspring exposed to the CON diet was significantly heavier (12-14g) than those exposed to a MLP, MLPG, and MLPA diets (P<0.001). Only those exposed to MLPU had a similar weight to CON and significantly heavier to MLP, MLPG and MLPA (P<0.001) (Table 4.3). No single litter was found to have an overall effect on body weight data (F=11.498, 4df, P=0.001). Male offspring body weights followed a similar trend to the litter mean data. Those exposed to a CON maternal diet was significantly heavier (9-16g) than those exposed to MLP (P<0.001), MLPG (P<0.01) and MLPA (P<0.05). Male offspring exposed to a maternal MLPU diet had a significantly heavier body weight (12-19g) than MLP, MLPG (P<0.001), and MLPA (P<0.01) (Table 4.3). Female offspring body weights followed a similar

trend to the litter mean data. Those exposed to a CON maternal diet had a significantly heavier body weight (10-17g) than MLP, MLPG, and MLPA ($P<0.001$). Similarly, female offspring exposed to a MLPU maternal diet had a significantly heavier body weight (11-13.6g) than MLP ($P<0.001$), MLPG ($P<0.01$), and MLPA ($P<0.05$). Two-way ANOVA indicated that males were significantly heavier than females ($P<0.001$).

Differences in body weight did not mirror the systolic blood pressure (SBP) findings. Those animals exposed to a MLP diet were the smallest and had significantly higher SBP compared to CON. However, the MLPG exposed group, which are of a similar size to MLP, had the lowest SBP. MLPA animals were significantly bigger than MLP and those exposed to an MLPU diet that were of a similar size to CON yet both had a similar raised SBP to those exposed to a MLP.

Male heart, right kidney, lung and liver weights did not differ between dietary groups (Table 4.4). The male MLPU left kidney is significantly heavier than MLP ($P<0.05$). ANOVA indicated no single litter had an effect on left kidney weight ($F=0.973$, 4df, $P=0.427$). Male MLP ($P<0.05$) and MLPG ($P<0.01$) spleen is significantly heavier than MLPU. ANOVA indicated a significant interaction between diet and male spleen weights ($F=3.870$, 4df, $P<0.007$), with no single litter group having an overall effect. The male MLPG and MLPA brain is significantly lighter than MLP ($P<0.05$). ANOVA indicated a significantly strong interaction between MLP and other dietary group brain weights ($F=5.661$, 4df, $P<0.001$). This was not the only litter group having an effect. This may reflect the relative preservation of brain growth in MLP animals. The male MLPU and MLPA adrenal was significantly lighter than CON ($P<0.05$). ANOVA indicated no single litter had an effect on adrenal weight ($F=1.894$, 4df, $P=0.120$).

Table 4.4. *Organ weights for male rats exposed to varying amino acid composition in fetal life adjusted for litter of origin.*

Maternal diet.

Organ		CON	MLP	MLPG	MLPU	MLPA
Litter mean						
Heart.	(%)	0.61±0.03	0.62±0.02	0.58±0.03	0.62±0.03	0.60±0.02
Right kidney.	(%)	0.52±0.02	0.50±0.02	0.51±0.02	0.55±0.02	0.53±0.02
Left kidney.	(%)	0.50±0.02	0.48±0.02	0.49±0.02	0.53±0.02§	0.52±0.02
Lung.	(%)	0.83±0.04	0.88±0.03	0.81±0.04	0.81±0.04	0.78±0.04
Liver.	(%)	4.60±0.25	5.29±0.22	5.07±0.27	4.91±0.26	5.28±0.23
Spleen.	(%)	0.39±0.02	0.41±0.02 *f*[c,d]	0.42±0.02 *f*[d]	0.38±0.02	0.36±0.02
Brain.	(%)	2.06±0.04	2.07±0.03[a,b,c,d]	1.97±0.03§	2.06±0.03	1.98±0.03§[a]
Adrenals.	(%)	3.40±0.002	3.10±0.002	3.08±0.002	2.79±0.02*	2.86±0.002*

Data are shown as mean ± SEM for *n* observations per group. Organ weights adjusted for body weight and litter effects. * indicates significantly different to CON, § significantly different to MLP and *f* significantly different to MLPA ($P < 0.05$). [a] indicates significantly different to CON, [b] significantly different to MLPG, [c] significantly different to MLPU, [d] significantly different to MLPA ($P < 0.05$) for difference between litters using one-way ANOVA. Two-way ANOVA indicated diet had a significant effect on spleen, F=2.536, 4df, P=0.048. Body weight had a significant effect on heart, F=8.002, 1df, P=0.006, brain, F=378.566,1df, P=0.001 and adrenals, F=7.469, 1df, P=0.008. Litter had a significant effect on brain, F=6.972, 1df, P=0.010 and adrenals, F=6.385, 1df, P=0.014.

Correction factors for litter; heart, right/left kidney, lungs, liver, 15.80; spleen, brain, 14.39; adrenal 15.63.

Table 4.5 *Organ weights for female rats exposed to varying amino acid composition in fetal life adjusted for litter of origin.* Maternal diet.

Organ		CON	MLP	MLPG	MLPU	MLPA
Litter mean						
Heart.	(%)	0.60±0.03§	0.70±0.03[a,b,c,d]	0.54±0.03§	0.63±0.03	0.58±0.03§
Right kidney.	(%)	0.50±0.03§	0.61±0.03[a,b]	0.47±0.03§	0.55±0.03	0.53±0.03§
Left kidney.	(%)	0.48±0.03§	0.59±0.03[a,b]	0.45±0.03§	0.52±0.03	0.50±0.03§
Lung.	(%)	0.86±0.06§	1.04±0.05[a,b,c,d]	0.75±0.06§	0.86±0.05§	0.78±0.05§
Liver.	(%)	4.69±0.28§	5.86±0.25[a,b,c,d]	5.08±0.31§	4.90±0.27§	4.79±0.26§
Spleen.	(%)	0.35±0.02	0.40±0.02[a,b,c,d]	0.38±0.02[c,d]	0.35±0.01§	0.35±0.01§
Brain.	(%)	2.16±0.03[b,c,d]	2.22±0.03[a,b,c,d]	2.13±0.03§[c]	2.18±0.02	2.17±0.05
Adrenals.	(%)	2.65±0.012§	6.27±0.010	3.55±0.013	2.68±0.011§	3.32±0.011

Data are shown as mean ± SEM for *n* observations per group. Organ weights adjusted for body weight and litter effects. § significantly different to MLP ($P < 0.05$). [a] indicates significantly different to CON, [b] significantly different to MLPG, [c] significantly different to MLPU, [d] significantly different to MLPA ($P < 0.05$) for difference between litters using one-way ANOVA. Two-way ANOVA indicated diet had a significant effect on heart, F=4.286, 4df, P=0.004, right kidney, F=3.344, 4df, P=0.014, left kidney, F=3.328, 4df, P=0.015, lung, F=4.682, 4df, P=0.002, liver, F=3.123, 4df, P=0.020. Body weight had a significant effect on brain, F=279.073, 1df, P=0.001. Litter had a significant effect on right kidney, F=7.122, 1df, P=0.009, left kidney, F=5.604, 1df, P=0.021, Lung, F=7.264, 1df, P=0.009, spleen, F=4.298, 1df, P=0.042 and body weight, F=11.403, 1df, P=0.001.

Correction factors for litter; heart, right/left kidney, lungs, liver, adrenal 16.19; spleen, brain, 14.82.

4.4.3 Body weight and Organ size (continued).

Female MLP hearts was significantly heavier than CON (P<0.05), MLPG (P<0.001) and MLPA (P<0.01) Table 4.5). ANOVA indicated female offspring from MLP litters had a significant effect on heart weight over all other dietary groups (F=4.627, 4df, P=0.002).
The female right kidney from MLP rats was significantly heavier than CON (P<0.05), MLPG (P<0.001), and MLPA (P<0.05). ANOVA indicated female offspring from MLP litters had a significant effect on right kidney weight over CON and MLPG dietary groups (F=2.694, 4df, P=0.037). The female left kidney from MLP rats was significantly heavier than CON (P<0.05), MLPG (P<0.01), and MLPA (P<0.05). ANOVA indicated female offspring from MLP litters had a significant effect on left kidney weight over CON and MLPG dietary groups (F=2.940, 4df, P=0.025).

The female MLP lung was significantly heavier than all other dietary groups (CON P<0.05; MLPG P<0.001; MLPU P<0.05, MLPA; P<0.001). ANOVA indicated female offspring from MLP litters had a significant effect on lung weight over all other dietary groups (F=4.306, 4df, P=0.003). The female MLP lung was significantly heavier than all other dietary groups (CON P<0.01; MLPG P<0.05; MLPU P<0.05, MLPA; P<0.01). ANOVA indicated female offspring from MLP litters had a significant effect on liver weight over all other dietary groups (F=3.437, 4df, P=0.012). The female MLP spleen was significantly heavier than MLPU and MLPA (P<0.05). ANOVA indicated female offspring from MLP litters had a significant effect on spleen weight over all other dietary groups (F=7.364, 4df, P=0.001). Two way ANOVA indicated sex had a significant effect on spleen (F=9.522, 1df, P=0.002), with male spleens being heavier than females (P<0.001).

The female MLP brain was significantly heavier than MLPG (P<0.05). ANOVA indicated a significant interaction between dietary groups and female spleen weights (F=16.012,

4df, P<0.001), with no single litter group having an overall effect. Two-way ANOVA indicated sex had a significant effect on brain (F=5.503, 1df, P<0.020) with female brains being heavier than males (P>0.05). The female MLP adrenal was significantly heavier than CON and MLPU (P<0.05). ANOVA indicated female offspring from MLP litters had no significant effect on adrenal weight over all other dietary groups (F=2.014, 4df, P=0.100).

4.4.4. Nephron number and glomerular volume.

Left kidneys were used to determine nephron number (Table 4.6). Within dietary groups nephron number did not differ between males (11266±730) and females (10568±800) so the data was combined for analysis. Offspring exposed to a MLP diet had significantly fewer nephrons (29-37%) compared to CON, MLPG, and MLPU (P<0.05). ANOVA indicated offspring from MLP litters had a significant effect on nephron number over all other dietary groups (F=2.580, 4df, P=0.051). Supplementation of MLP with nitrogen from any of these sources appeared to correct the associated deficit of renal reserve and MLPG, MLPU and MLPA had similar nephron numbers to CON animals. Nephron numbers between dietary groups persisted after adjustment for litter of origin.

Left kidneys were also used to determine glomerular volume (Table 4.6). MLPA exposed animals had significantly reduced glomerular volume compared to CON (P<0.001), MLP (P<0.05), MLPG, and MLPU (P<0.01). ANOVA indicated offspring from MLPA litters had a significant effect on glomerular volume over all other dietary groups (F=4.507, 4df, P=0.01). There was no evidence of glomerular hypertrophy, glomerulosclerosis, or blebbing associated with fetal undernutrition at 4 weeks of age.

Table 4.6. *Nephron number and glomerular volume in rats exposed to diets of varying amino acid composition in fetal life.*

Maternal diet.	Nephron number (nephrons /kidney).	Nephron number (Litter Mean)	Number of glomerular measured. *n*	Glomerular volume (μm^3).
CON	12007±2160	11367±2611	684	198 ± 15
MLP	8078±3980*‡†	8078±3980†	1000	175 ± 12 *f*
MLPG	11414± 3917	10768±4088	988	191 ± 6 *f*
MLPU	12892±4055	12892±4055	886	191 ± 9 *f*
MLPA	10318±3065	10625±3835	905	144 ± 9

Data are shown as mean ± SEM for *n* observations per group. * indicates significantly different to CON, ‡ significantly different to MLPG, † significantly different to MLPU, § significantly different to MLP and *f* significantly different to MLPA ($P < 0.05$), for glomerular volume the same scripts apply ($P<0.001$). Correction factor for litter 15.95.

ANOVA indicated a significant effect of diet on nephron number, F=2.580, 4df, P=0.051 and glomerular volume, F=4.507, 4df, P=0.01.

To assess whether the maintenance of renal size by MLP exposed rats, despite a significantly lower nephron number, could be attributed to gross changes in cell size or number, right kidneys were used to determine tissue DNA, protein and DNA/protein ratio (Table 4.7). Two-way ANOVA indicated sex was not a significant factor and the data for male and female animals was combined. Taking tissue DNA content as a marker of cell number, combined data highlights kidneys from MLPG exposed animals as having significantly more cells (%) than MLP

and MLPU ($P<0.05$) and 57% more than CON. MLPA exposed animals had a similar renal DNA content to MLPG animals but this is not significant when compared to other dietary groups. When adjusted for litter of origin a similar trend persists but fails to be significant (Table 4.7.1) In the combined data, protein in the kidney tissue was significantly higher in MLPU offspring compared to those exposed to MLP and MLPG ($P<0.05$). Those exposed to CON and MLPA had similar renal protein content. A similar trend persists when adjusted for litter of origin, but only MLPU exposed animals had significantly more protein in kidney tissue than MLPG ($P<0.05$).

Taking tissue protein content in relation to DNA content as a crude marker of cell size, the combined data reveals animals exposed to MLPG have significantly more cells of a smaller size compared to CON, MLP and MLPU ($P<0.05$) (Table 4.7). The trend persists when adjusted for litter of origin, but is not significant between dietary groups (Table 4.7.1).

4.4.5. Renal function measures.

Renal function was assessed in terms of blood urea nitrogen concentrations (BUN) (Figure 4.1) and glomerular permeability (albumin/creatinine excretion) in offspring at 4 weeks (Figure 4.2) and shown corrected for litter of origin (Figure 4.1.1. & 4.2.1.).

Two-way ANOVA indicated litter of origin and sex had no significant effect on BUN and the data is combined. BUN was significantly elevated in MLP relative to control animals and MLPU ($P<0.05$). The three groups of MLP animals for which nitrogen supplements were provided had similar BUN to the CON group. MLPG had significantly higher BUN than MLPU ($P<0.05$). This significant trend persists when adjusted for litter effects, with MLPG BUN being significantly elevated compared to CON ($P<0.01$) (Figure 4.1.1). This suggests that that this marker of renal function was normal in all groups except MLP, the only group to show any

(Continued)

Table 4.7. *Kidney DNA and protein content and renal, plasma angiotensin converting enzyme activity.*

Maternal Diet	DNA (μg/g tissue)	DNA (μg/Kidney)	Protein (mg/g tissue)	Protein (mg/kidney)	DNA/ Protein Ratio (μg/mg)	Renal ACE (units/mg protein)	Plasma ACE (units/l)
CON	621 ± 144	260 ± 55	34.0 ± 3.1	14.6 ± 1.5	18.8 ± 4.8	1.88 ± 0.42	2.24 ± 0.59
MLP	546 ± 93	170 ± 31‡ *f*	36.0 ± 2.7	11.6 ± 1.5	15.9 ± 2.8	2.11 ± 0.40	2.50 ± 0.27
MLPG	1091 ± 210†§	384 ± 77	32.9 ± 4.4	11.7 ± 1.7	51.6 ± 20.9*§†	2.63 ± 0.51	4.43 ± 0.73*§
MLPU	537 ± 74	238 ± 38	38.1 ± 3.1	16.9 ± 1.6‡§	15.3 ± 2.1	2.89 ± 0.42	5.24 ± 0.66*§*f*
MLPA	933 ± 231	334 ± 76	34.6 ± 2.7	13.4 ± 1.2	28.1 ± 6.7	3.06 ± 0.46	3.33 ± 0.61

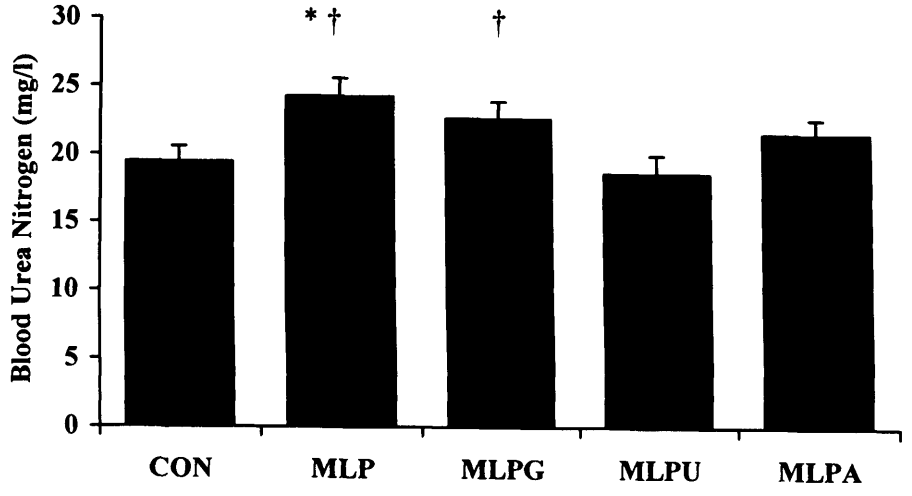
Data are shown as mean ± SEM for *n* observations per group. * indicates significantly different to CON, ‡ significantly different to MLPG, † significantly different to MLPU, § significantly different to MLP and *f* significantly different to MLPA ($P < 0.05$). ANOVA indicated a significant effect of diet on DNA (μg/g tissue) $F=2.642$, df 4, $P=0.042$; DNA (μg/Kidney) $F=2.228$, df 4, $P=0.076$; Protein (mg/kidney) $F=2.231$, df 4, $P=0.066$; DNA/protein ratio $F=2.228$, df 4, $P=0.076$; Plasma ACE (units/L) $F=4.291$, df 4, $P=0.004$.

Table 4.7.1. *Kidney DNA and protein content and renal, plasma angiotensin converting enzyme activity (adjusted for litter of origin).*

Maternal Diet	*n.*	DNA (μg/g tissue)	DNA (μg/Kidney)	Protein (mg/g tissue)	Protein (mg/kidney)	DNA/ Protein Ratio (μg/mg)	Renal ACE (units/mg protein)	Plasma ACE (units/l) Male/Female	
Litter mean								Male	Female
CON	10	623 ± 198	264 ± 50	34.0 ± 3.8	14.4 ± 1.8	19.3 ± 12.1	1.78 ± 1.14	3.08 ± 0.98	1.29 ± 0.67
MLP	15	515 ± 188	172 ± 69	37.7 ± 3.6	13.0 ± 1.7	12.9 ± 11.4	1.50 ± 1.40	1.98 ± 0.91	1.62 ± 0.81
MLPG	14	1070 ± 232	361 ± 86	31.7 ± 4.5	10.6 ± 2.1	58.5 ± 14.2*§†	4.90 ± 1.50	4.96 ± 0.92§	4.12 ± 3.53§*[a,b]
MLPU	16	537 ± 154	237 ± 57	37.9 ± 3.0	16.7 ± 1.4‡	15.7 ± 9.4	3.03 ± 0.90	5.14 ± 0.78§	5.86 ± 3.23§*[a,b]
MLPA	13	920 ± 180	323 ± 66	33.9 ± 3.5	12.6 ± 1.6	28.6 ± 11.0	3.19 ± 1.15	4.04 ± 0.82	3.29 ± 2.69§*[c]

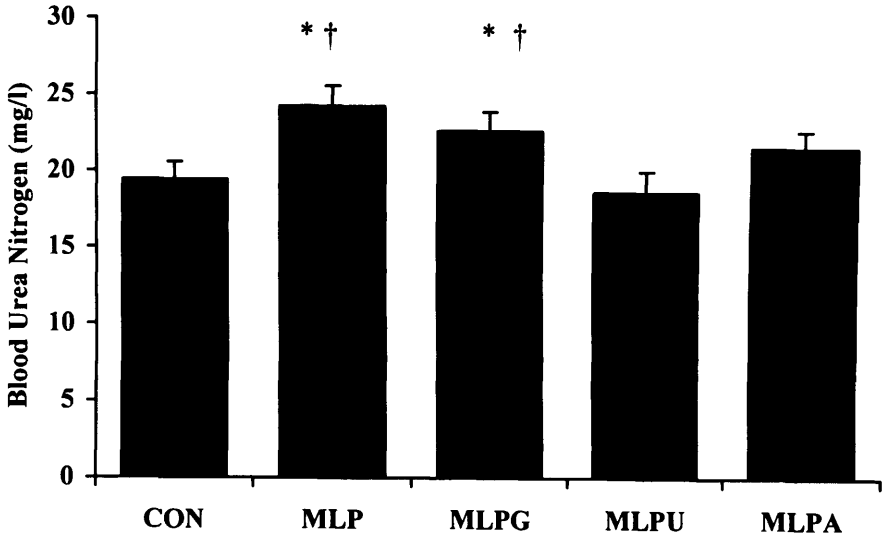
Data are shown as mean ± SEM for *n* observations per group adjusted for litter effects. * indicates significantly different to CON, ‡ significantly different to MLPG, † significantly different to MLPU, § significantly different to MLP and *f* significantly different to MLPA ($P < 0.05$). Two-way ANOVA indicated diet has a significant effect on plasma ACE ($F=7.416$, 4df, $P=0.001$) and origin of litter had a significant effect on plasma ACE ($F=10.996$, 4df, $P=0.002$). Litter correction factors; plasma ACE male 15.30, female, 14.34, DNA, protein 15.08, renal ACE 14.94. [a] indicates significantly different to CON, [b] significantly different to MLP, [c] significantly different to MLPU ($P<0.05$) interactions between litter means.

Figure 4.1. *Blood urea nitrogen at 4-weeks of age.*



* indicates significantly different to CON, † significantly different to MLPU (P <0.05). ANOVA indicated a significant effect of diet on BUN (F=3.111, df 4, P=0.020).

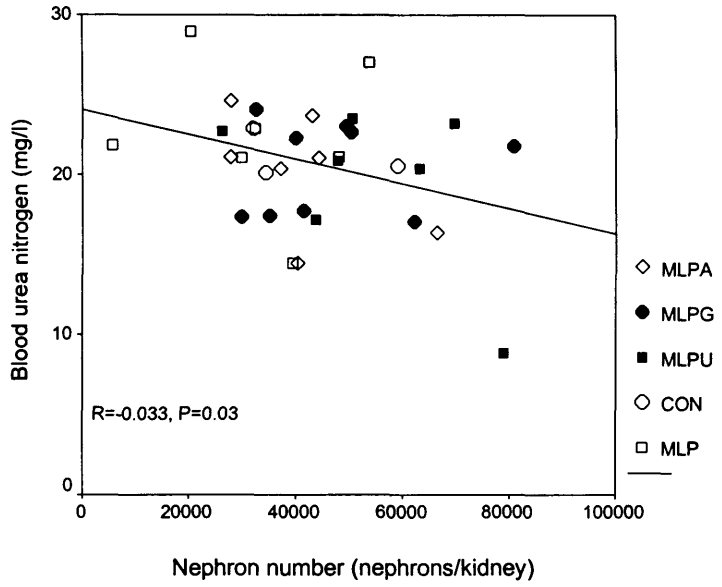
Figure 4.1.1. *Blood urea nitrogen at 4-weeks of age adjusted for litter of origin.*



* indicates significantly different to CON, † significantly different to MLPU (P <0.05). ANOVA indicated a significant effect of diet on BUN (F=3.111, df 4, P=0.020).

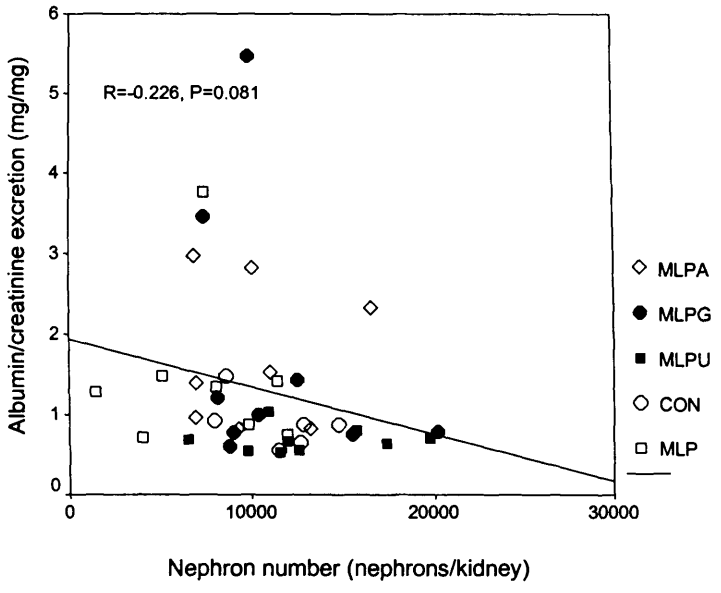
histological evidence of renal impairment. BUN was significantly correlated with nephron number (R=-0.033, P=0.03) (Figure 4.3).

Figure 4.2. *BUN excretion in relation to nephron reserve in rats exposed to varying maternal diets.*



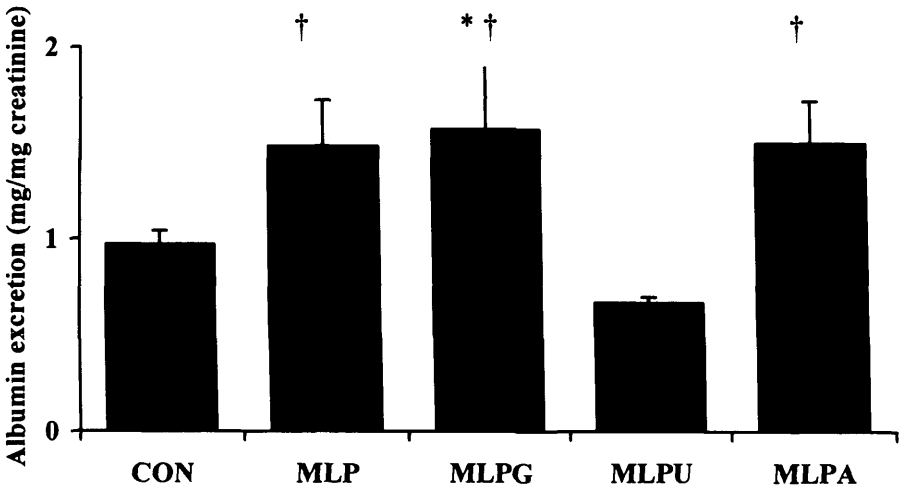
Assessment of albumin excretion as a marker of glomerular permeability provided a slightly different pattern (Figure 4.3). Two-way ANOVA indicated litter of origin and sex had no significant effect on albumin excretion (corrected for creatinine) and the data is combined for analysis. Offspring exposed to a MLPU diet excreted significantly less albumin compared to other dietary groups (P<0.05). When adjusted for litter of origin MLP animals excrete significantly more albumin than CON (P<0.05) (Figure 4.3.1). Whilst not significant, a trend between albumin excretion and nephron number (r=-0.226, P=0.081) was present (Figure 4.3).

Figure 4.3. *Albumin excretion in relation to nephron reserve in rats exposed to varying maternal diets in utero.*



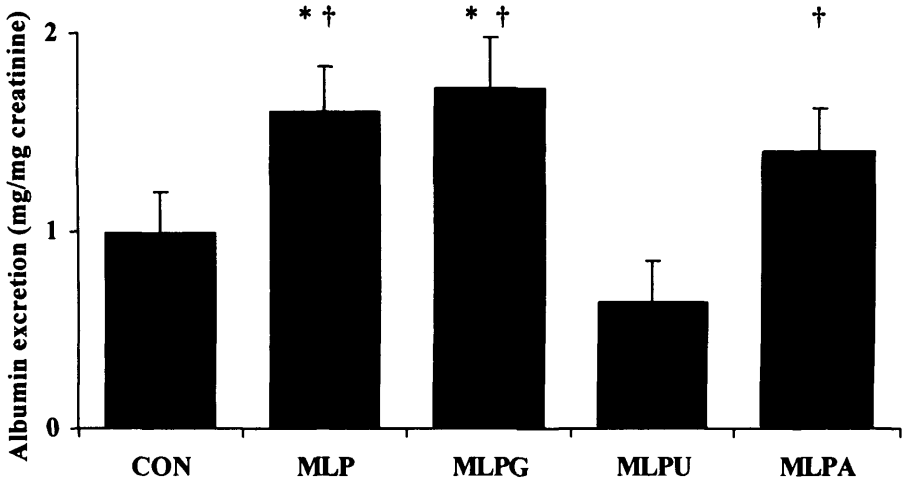
The activity of plasma ACE was not elevated relative to control animals in either MLP or MLPA groups. MLPU groups had significantly higher plasma ACE activity compared to CON ($P<0.05$), MLP ($P<0.001$) and MLPA groups ($P<0.05$). MLPG offspring had significantly higher plasma ACE activity than CON and MLP ($P<0.05$) (Table 4.7). This was not reflected in renal ACE activity, which was similar in all groups (Table 4.7). Two-way ANOVA indicated litter of origin had a significant effect plasma ACE $F=10.996$, 4df, $P=0.002$, and the data is shown separately for both males and females (Table 4.7.1). Male MPLG and MLPU plasma ACE was significantly elevated compared to CON ($P<0.05$). ANOVA indicated no significant difference between litters of origin and male plasma ACE ($F=1.107$, 4df, $P=0.367$). Female plasma ACE was significantly elevated in MLPG ($P<0.05$), MLPU ($P<0.001$), and MLPA ($P<0.05$) compared to CON. Similarly, female plasma ACE was significantly elevated in MLPG ($P<0.01$), MLPU ($P<0.001$), and MLPA ($P<0.05$) compared to MLP. ANOVA indicated a significant difference between litter of origin and plasma ACE in females ($F=4.294$, 4df, $P=0.008$), with no single dietary group having an overall effect (Table 4.7.1).

Figure 4.4. *Albumin excretion at 4 weeks.*



Data are shown as mean ± SEM for *n* observations. * indicates significantly different to CON, † significantly different to MLPU (P <0.05). ANOVA indicated a significant effect of diet on albumin excretion at 4 weeks (F=3.677, 4df, P=0.009).

Figure 4.4.1. *Albumin excretion at 4-weeks of age adjusted for litter of origin.*



Data are shown as mean ± SEM for *n* observations. * indicates significantly different to CON, † significantly different to MLPU (P <0.05). ANOVA indicated a significant effect of diet on albumin excretion at 4 weeks (F=4.149, 4df, P=0.005).

No significant difference in kidney prostaglandin activity was observed between dietary groups (Table 4.8).

Table 4.8. *Kidney prostaglandin PGE_2 activity in rats exposed to varying maternal dietary supplements.*

Maternal diet.	CON	MLP	MLPG	MLPU	MLPA
nmoles15 Keto-PGE_2 formed /minute/					
mg protein	0.34±0.06	0.40±0.08	0.39±0.10	0.32±0.08	0.42±0.10
Litter mean	0.37±0.11	0.32±0.10	0.49±0.12	0.32±0.08	0.45±0.09

Data are shown as mean ± SEM for *n* observations per group.

4.5. Summary of results.

Maternal weight gain was consistent in all dietary groups throughout pregnancy. Food intake was lower in the last week of pregnancy compared to the first. Systolic blood pressure was lower in offspring exposed to the maternal CON and MLPG diet compared to the other dietary groups. Only offspring exposed to an MLPA diet had a significantly lower pulse rate. Offspring exposed to an MLP diet had fewer nephrons than those exposed to other dietary groups, yet had similar cell number to other dietary groups except those exposed to an MLPG, which have high cell content. The effects of maternal protein restriction upon blood pressure and upon the kidney can be dissociated. Corrected urinary albumin excretion was less in those offspring exposed to CON and MLPU compared to the other groups. Maternal dietary exposure had an important effect upon organ weight, especially in MLP exposed females.

4.6. Discussion.

The feeding of a maternal low protein diet (MLP) has been shown in this study to exert programming effects upon blood pressure and the kidney, which is consistent with the results of Chapter 3 and other studies (Langley Evans *et al.,* 1996a,b,c; Langley-Evans *et al.,* 1999). In the present study a decrease in the protein content of the diet from 18% (CON) to 9% (MLP) during pregnancy resulted in the development of higher blood pressure by 4-weeks of age in the offspring. When non-essential nitrogen was added to the 9% casein diet consumed during pregnancy, there were marked effects on blood pressure, pulse, body weight and organ weight of the offspring, which varied depending on the form in which the nitrogen was provided.

The hypothesis under test in this study was that supplementation of MLP diet with glycine would reverse the effects of protein restriction upon renal and cardiovascular endpoints. Glycine may be viewed as a limiting amino acid in the MLP diet due to the increased demand, which occurs during pregnancy. The major demands for amino acids in pregnancy are to drive growth and tissue deposition. Amino acids are required in uneven amounts and glycine demands may be 2-10 times greater than those for other amino acids (Widdowson *et al.,* 1979). Glycine is required for a number of critical metabolic pathways, in which it is utilised as a fundamental building block (Jackson, 1991). Of special importance amongst these, are the demands for glycine in the synthesis of structural proteins such as collagen and elastin. Glycine is also used specifically to form compounds that carry out critical functional roles during growth and metabolism, such as DNA, RNA, nucleotides, haem, bile, creatine, glutathione (Jackson, 1991). Glycine has also been shown to be cytoprotective (Yatzidis *et al.,* 1996). Feeding the MLP diet may thus result in the down-regulation of glycine flux through particular pathways, which ultimately perturbs the normal development of organs and impacts upon long-term physiology and metabolism.

Fetuses exposed to MLP diet exhibit hypermethylation of DNA in the liver (Rees *et al.,* 2000). This may alter the expression of key genes involved in the development of hypertension, and may be a consequence of homocysteinaemia resulting from programming of the sulphur amino acid metabolism. Glycine may overcome these effects by permitting the inter-conversion of homocysteine and methionine. The MLP diet contains relatively more methionine to nitrogen, compared to the CON diet (Rees, *et al.,* 1999). Any excess of methionine over the immediate requirement would need to be degraded. An increased additional flow through the catabolic pathway would utilize glycine in the process. This would place a competitive demand on the availability of glycine for other pathways (Jackson, 1999; Harper *et al.,* 1970). Therefore the supplementation of MLP with this amino acid during pregnancy may alleviate some of the developmental programming effects of the diet.

The addition of nitrogen to the MLP diet as either urea (MLPU) or alanine (MLPA) had no effect upon systolic blood pressure, with similar levels recorded to that of MLP animals. When the extra nitrogen was provided to the mother in the form of glycine, the blood pressure of offspring was significantly lower, and similar to the level in offspring exposed to CON. Perturbation of folate metabolism, similar to glycine deficiency, through the administration of methotrexate during rat pregnancy leads to impaired elastin formation in the aorta, with permanent changes in the elastic properties of the great vessels, which would predispose to a higher blood pressure in the offspring (Berry, 1978; Berry & Looker, 1973). Similar functional changes have been noted in hypertensive individuals and in the offspring of rats exposed to low protein diet during pregnancy (Greenwald *et al.,* 2001; Martyn & Greenwald, 1997). In this study the addition of glycine to the maternal low-protein diet may have been sufficient to corrected any vascular deficiency, possibly correcting the elastin content of the arteries. This supports the concept that glycine is limiting in MLP feed rats and is conditionally essential for pregnancy. Analysis of the aortas from the varying dietary groups will need to be conducted to confirm this

supposition. Despite nephron number being similar in MLPG rats to CON, this normalisation of blood pressure was accompanied by evidence of glomerular permeability that appeared to be equivalent to that observed in the MLP group. The effects of maternal protein restriction upon blood pressure and upon the kidney can be therefore dissociated. To date no published literature has demonstrating this observation.

The MLPU and MLPA, which had similar blood pressures to MLP animals, contrastingly both had normal nephron number and renal function. This indicates that programming of blood pressure via other mechanisms; the up-regulation of the renin-angiotensin system, for example, may operate independently of the kidney (Sherman & Langley-Evans 2000). It has been shown previously that MLP feeding limits fetal growth in late gestation (Langley-Evans *et al.,* 1996a) and that the kidney is an organ particularly sensitive to this retardation of growth (Langley-Evans & Nwagwu, 1998, Langley-Evans *et al.,* 1999). As MLPU and MLPA animals maintained their raised blood pressure, it would be of interest to evaluate HPA axis status and renin-angiotensin system activity in these animals.

The present study found that MLP feeding had no effect on renal ACE activity, which is consistent with earlier reports (Nwagwu *et al.,* 2000). Subsequently, MLP feeding had no effect renal prostaglandin activity at four weeks, which is inconsistent to previous reports (Sherman & Langley-Evans, 1998; Langley-Evans, 1999) and may be compounded statistically by the sample size analysed. Raised blood pressures in MLPU and MLPA groups were not associated with elevated renal ACE activity, although increase activity may be present elsewhere and cannot be excluded (Langley-Evans & Jackson, 1995). Elevated plasma ACE activity was noted in MLPU, MLPA exposed animals, but as this was also noted in normotensive MLPG exposed rats, it may not explain the hypertension. A significant inverse association between BUN activity and nephron number is observed, supporting the assertion that it is a marker of functional impairment relating to a reduced renal reserve (Nwagwu *et al.,* 2000).

The correction of the nephron number induced by MLP feeding in all three nitrogen supplemented groups indicates that the reduction of renal reserve may be related to cell proliferation and differentiation of nephron precursors. Oxidative damage to cells, through excessive maternal homocysteine during development, may impair capacity for cell proliferation and active apoptosis (Sohar & Weindruch, 1996). Welham *et al.,* (2002), has demonstrated that protein restriction in rat pregnancy is associated with increased apoptosis of the mesenchymal cells at the start of rat metanephrogenesis. In addition, DNA hypermethylation may cause inappropriate silencing of genes during critical periods of development (Detich *et al.,* 2003). Recently, Rees, (2003), found specific increases in DNA methylation in fetuses exposed to a high methionine diet. In a recent study by Langley-Evans *et al.,* (2003a), the impact of a maternal low-protein diet upon the kidney was not related to the methionine content of the diet. Nephron number at birth and weaning was identical in 18% casein with 0.5% methionine and 18% casein with 1% methionine. Similarly, reducing the methionine content of the low-protein diet (0.25%) did not normalise nephron number. This suggests other programming mechanisms are at work and requires further investigation.

The increase in nephron number observed in hypertensive MLPU and MLPA groups relative to MLP alone may have been explained by the presence of non-functional nephrons. This does not appear to be the case, as renal function is maintained at a similar level to control animals and there is no evidence of glomerular hyperthrophy. Distribution of cortical and juxtamedullary nephrons was not determined. It is not clear whether with age these animals would develop glomerular injury and exhibit impairment of function. This would be secondary to their hypertension and not a causal factor in its development. Normalised nephron number observed in MLPU animals may be explained through increased salvage of urea-nitrogen by the metabolic activity of colonic microflora (Jackson, 1995; 2000b), In the rats the process of refection enables bacterially synthesized amino acids to contribute to the overall dietary quality of the host

(Torrallardona *et al.,* 1996a,b). Although urea is not a particularly efficient source of non-specific nitrogen, it can be used to meet requirements at marginal levels of dietary protein consumption (Jackson, 1995, 2000b). Certainly in this study, the addition of urea to the MLP diet appeared to protected organogenesis and relative size of the body in line with controls and is suggestive of a restored amino acid supply. It remains unclear at this stage how additional alanine corrected renal deficit and further analysis is required to determine precise metabolic pathways.

There was no obvious reason for the impairment of renal function in the normotensive MLPG group. The assessments of tissue DNA and protein were suggestive of subtle changes in renal structure, but these were not evident from the histological examination, or gross observation of organ size. An assessment of the ultrastructure of the glomeruli may be necessary to explain their increased permeability to albumin. MLP animals had a low nephron reserve and heavier kidneys compared to other dietary groups, yet had a similar DNA/protein ratio. This may indicate that that early structures that should differentiate to form nephrons failed to do so and have been replaced by other cell types and structures. The method employed to measure DNA (Burton, 1959; section 2.6.1) is widely used despite its high coefficient of variance. The data was randomly sampled between dietary groups to avoid any bias and to reduce the likelihood of an essay error. Another method is to count the number of nuclei of the histology slides used to determine nephron number, and then calculate cell number.

It is suggested that in animals with reduced renal reserve, systemic blood pressure rises secondary to local increases in single nephron pressure, an adaptation that preserves renal function (MacKenzie & Brenner, 1995). The low systemic blood pressures of MLPG animals do not seem consistent with such an adaptation. The additional measurement of glomerular volume provides an indicator of the size of the functional units within the kidney. Nephron number is inversely related to birth weight in humans (Manalich *et al.,* 2000). Glomerular hyperthrophy is commonly seen as a marker of renal injury in a variety of experimental models (Samnegard *et al.,*

2001) (Chapter 3) and is believed to be a response, which maintains filtration surface area in the face of a reduction in the number of functional units. At 4-weeks those exposed to MLPA diet have a significantly smaller glomerular volume compared to other dietary groups. Whilst no evidence of glomerulosclerosis or hypertrophy is evident, the smaller nephron may be more susceptible to increased local glomerular pressure to maintain filtration, possibly promoting a more pronounced deterioration of function and morphology over time (Chapter 3). MLPA rats had the lowest pulse rate in the offspring compared with any other dietary group. This cannot be explained by looking at the known physiological factors and requires further detailed investigation.

Maternal dietary exposure had an important effect upon postnatal growth of the offspring, with the MLP group having a reduced body weight at 4 weeks and consistent with other studies (Langley-Evans *et al.,* 1996a; Kwong *et al.,* 2000), but is in direct contrast to observed body weight in MLP exposed animals compared to controls at 4-weeks in the previous chapter. MLPG animals also had a significantly reduced body weight to control animals, despite the fact that the maternal environment was significantly heavier than control dams at the onset of pregnancy. Similarly, MLPA animals had body weights lower than CON and MLP. Only MLPU animals had a body weight the same as CON. Assessing body weight in conjunction with blood pressure, nephron endowment, and organogenesis it is possible to elucidate the considerable demands for glycine that occurs during MLP pregnancy. Additional maternal glycine appears only sufficient to meet maternal metabolic requirements and correct specific organ structures within the developing fetus. Subsequently there may be insufficient pool available to drive overall growth. As urea has no competitive stress from the excess of methionine in the maternal diet it is sufficient to drive overall growth and organ development within the fetus. This is further evidence that urea supplementation can restore amino acid supply in the maternal environment in

sufficient quantities. Males from this investigation were heavier than females, which is consistent with previous studies (Aihie Sayer *et al.,* 2001, Kwong *et al.,* 2000).

Organ weights are not symmetrical with body weight, even when showing organs in proportion to body weight and correcting for body weight in the analysis to compensate for any variations with in the dietary groups. Generally, organ weights tended to be heavier in MLP expose animals compared to other dietary groups despite their low body weight. This observation is more pronounced in the female MLP animals compared to male offspring when adjusted for litter of origin. Female offspring exposed to additional nitrogen seem to ameliorate the effects of low-protein feeding on organogenesis and this needs to be fully investigated. As seen in chapter 3, aged female kidney morphology appears generally preserved compared to males. It may be the general preservation of the female offspring's organ and vascular development that may ensure a suitable future environment in which to reproduce successfully and thus drive species survival.

Changes maybe specific for each dietary intervention and in relation to an identified function. Therefore, it would be of value to assess whether these enlarged organs associated with MLP feeding have a similar functional capacity and morphology as control and nitrogen supplemented low-protein exposed animals.

In the present experiment, the hypertensive effect of a low protein diet during pregnancy on blood pressure of the offspring was effectively reversed by the supplementation of glycine. The addition of nitrogen in the form of non-essential amino acid, urea or alanine did not impact upon blood pressure, demonstrating that the beneficial effect on blood pressure is specific. This is supported in a recent study by Brawley *et al.,* (2003a) that found supplementing the maternal low-protein diet with glycine, reversed vascular dysfunction, improved NO release and reduced circulating plasma homocysteine, possibly having a protective effect upon the maternal circulation, which may further protect the fetus from abnormal programming of the cardiovascular system. This suggests, that the availability of glycine during pregnancy exerts a

specific effect upon the capacity of the mother to provide a suitable nutritional environment for the development of the cardiovascular system of her offspring. This is consistent with the hypothesis advanced by Rees *et al.,* (2000). Evidence from human studies supports this view. The modest addition of dietary methionine to a low-protein diet in non-pregnant women appears to place a stress on the relative availability of glycine (Meakins *et al.,* 1998). Therefore, methionine or homocysteine may have a damaging effect upon the maternal, placental or fetal circulation, and glycine acts to correct this process.

A study by Dunn *et al.,* (2003), supplementing the low protein diet with five times more folate than is normally present, found that it reversed the effect of hypertension, possibly by facilitating an increase the endogenous formation of glycine. Folate supplementation in a low protein diet has also been found to restore vascular dysfunction in small mesenteric arteries of female adult rat offspring (Dance *et al,* 2003). Interestingly, in a recent study by Brawley *et al,* (2003b), folate supplementation in the low protein diet was found to restore uteroplacental blood flow in pregnant dams, possibly enhancing nutrient supply to the developing fetus.

The availability of different forms of nitrogen, and the ability of the mother, placenta and fetus to convert these to a pattern that meets the metabolic demands of the fetus, appear to play an important role in the ability of the fetus to achieve normal growth and development. It would be of interest to follow MLPG exposed animals to a greater age to observe the development of renal injury and later cardiovascular status. Crowe *et al.,* (1995) found iron restriction in rat pregnancy produced offspring which in early postnatal life had lower blood pressures than offspring of rats fed an iron replete diet, but developed hypertension in later life. It may be possible that glycine supplementation of MLP merely delays the onset of hypertension rather than preventing the normally observed blood pressure increases. Measurements made on maternal amino acids, NEAA status and that of the placental supply line may elucidate potential pathways or the mechanisms by which metabolic demand is sustained for the fetus and possibly how this is

anticipated. Certainly recording maternal, placental and fetal homocysteine and methionine levels within the varying dietary groups may have further supported the work of Rees *et al.,* (1999). Furthermore, evaluating levels of DNA methylation in the maternal and offspring tissue within the varying dietary groups may have supported the work of Rees *et al.,* (2000) and potentially indicate other mechanisms by which developmental programming may occur.

The role of glucocorticoids as a mechanism for programming was not a remit for this thesis (section 1.8.1). To the authors knowledge there is no studies identifying the effects of glucocorticoids within an MLP model with additional nitrogen. As no indication of how glucocorticoids and nitrogen may interact in relation to developmental programming it may prove interesting to explore in future work.

Chapter 5

Renal function and its relation to size at birth in children and young adults.

5.1. Introduction.

Barker, (1996), observed that individuals of a low weight at birth develop hypertension and coronary heart disease in later life. At present, our understanding is that programming influences in fetal life lead to later elevations of blood pressure and appear to generally reduce nephron number (Chapter 3 & 4). Observation of renal function made within the animal model is combined with invasive exploration that enables a detailed analysis of function and physiology to be conducted. This is not possible in living human subjects. However, applying the methodologies used in the animal model it may be possible to elucidate the physiological characteristics through non-invasive functional assessments made within the human. This is important, especially if developmental programming is going to be considered as a potential risk factor for non-communicable adult disease and effective interventions are to be employed in the future.

Hinchcliffe *et al.,* (1992), studied the kidneys of stillborn, growth retarded (IUGR) human infants compared to appropriate for gestational age (AGA) infants and found that the IUGR group had 35% less nephrons than the AGA infants. Manalich *et al.,* (2000) observed a reduced nephron number and increased glomerular volume in the kidneys of growth-retarded infants. These histological studies are supported by data collection through ultrasound examination of the fetal kidney. Konje *et al.,* (1996) reported in late gestation, the kidneys of IUGR were elongated with a thin morphology.

The hypothesis advanced by MacKenzie *et al.,* (1996), suggested that total nephron supply is a factor determining the susceptibility of an individual to hypertension. A reduced nephron number promotes an increased pressure within single nephrons to maintain GFR. If

sustained, focal glomerulosclerosis occurs, resulting in nephron loss. The animal enters a cycle of rising pressure to sustain haemodynamic function with progressive and irreversible renal injury (Brenner *et al.*,1988). Supporting evidence for this comes from a recent autopsy study by Keller *et al.*, (2003), demonstrating that patients with a reduced nephron number suffered primary hypertension.

The link between the hypotheses of Barker, (1996) and Mackenzie *et al.*, (1996) is obvious. The kidney is an organ, which goes through critical development in late gestation in both animals (Langley-Evans *et al.*, 1996a) and humans (Konje *et al.*, 1996). Growth retarding influences, such as poor nutrition, upon the fetus at this time, may compromise renal development. A reduced renal reserve at birth will not be reversed (MacKenzie *et al.*, 1996) so the individual begins life with a pre-programmed predisposition to progressive renal disease and essential hypertension.

Animal studies strongly support these findings. Despite the diversity of the models that have been studied, manipulations to nutrient supply of the developing fetus generally result in a smaller or thinner neonate (Langley-Evans *et al.*, 1996a; chapter 3) and elevated blood pressure postnatally (Langley-Evans *et al.*, 1999; Vehaskari *et al.*, 2001; Chapter 3 & 4). Renal examinations show the kidneys to be of an altered size and shape, exhibiting progressive functional deterioration (Nwagwu *et al.*, (2000) which contain fewer nephrons (Langley-Evans *et al.*, 1999; Chapter 3 & 4).

Hypertension and impaired renal reserve may merely coincide and may not necessarily be related in a causal manner as hypothesized by MacKenzie & Brenner, (1995). Drawing upon the findings from previous animal experiments the aim of this study is to evaluate the role of size and weight at birth, as a crude marker for fetal undernutrition (Barker, 1999) upon blood pressure, and markers of renal function within human children at ages 10 and 12 years and in young adults.

It is hypothesised that humans of a lower weight or size at birth will exhibit increased blood pressure and evidence of microalbuminuria, a marker of incipient renal disease.

5.2. Methods.

A total of 104 human subjects from two separate Caucasian populations were studied. These included 64 healthy children, and 40 healthy adults.

5.2.1. Children at ages 10 and 12.

At 10 years of age, 57 children (n=28 male: n=29 female) were recruited. At 12 years of age 59 children (n=27 male: n=32 female) were recruited. Five of the children recruited at age 10 were non-contactable at age 12, so an additional seven were recruited (n=2 male: n=5 female). All children were recruited from the Princess Anne Hospital Growth Study (as described in 2.4.1) (Robinson *et al.,* 1991). The combined group represents all the children recruited n=64 (Table 5.1). All children were born at full term (39.8±0.2 weeks). At birth the children were placed in quintiles based on birth weight. The children used for this study were those contactable from the lower, middle and the upper quintile at age 10 and 12 years. The birth weight ranges were 2430g-4920g at age 10 and 1900g-4920g at age 12. Characteristics of size at birth such as weight, length, head circumference, ponderal index and placental weights were available for each child as were measurements of height and weight at ages 10 and 12. Blood pressure measurements were only available at age 10. Body Mass Index (BMI) was calculated from height and weight. At age 10 BMI ranged from 13.49-23.83 m^2 and at age 12 BMI ranged from 14.16-30.03 m^2. Urine samples were provided at ages 10 and 12 (as described in section 2.4.3). Urinary albumin concentrations were measured in each sample (as described in section 2.5.1) and expressed per

mg creatinine (as described in section 2.5.2) to determine glomerular permeability. Total protein concentrations were measured in urine samples provided at age 12 (as described in section 2.5.4) and expressed per mg creatinine (as described in section 2.5.2) to detect proteinuria. Urinary sodium and potassium concentrations were measured in each sample provided at age 12 (as described in section 2.5.6). Due to the random urine sample provided, sodium and potassium concentrations were expressed per mg creatinine (as described in section 2.5.2). Urinary specific gravity was also recorded to determine hydration status and solute concentration (as described in section 2.5.5).

5.2.2. Statistical analysis

All data is represented as mean ± SEM within the tables and figures. Data for male and female are shown separately and combined. Measurements for size and weight at birth were also represented dichotomised using the 20^{th} centile for birth weight and ponderal index as a cut-off point. Markers of renal function were also represented dichotomised using the 80^{th} centile for albumin, creatinine and albumin/creatinine ratio as a cut-off point. Two-way analysis of variance indicated that sex had a significant impact upon some of the variables studied. The data was analysed using both one-way and two-way analysis of variance (ANOVA) with an LSD test applied for post-hoc analysis where significant interactions were indicated by the ANOVA result, and using independent-samples T test, with a confidence interval set at 95%. The statistical package SPSS (version 10) was used in all analyses. Statistical significance was set at $P<0.05$.

5.3 Results.

5.3.1. Gestation length, birth weight and measurements of postnatal size.

The combined data set represents all the children from the PAHGS sampled at birth (Table 5.1). The data showed that gestational length was associated with birth weight. Children from the lower quintile for birth weight had significantly shorter gestational time than those from the upper quintile ($P<0.05$). Two-way ANOVA indicated sex was not a significant influence upon weight at birth ($P=0.710$) (Table 5.1) The maternal placental weight was significantly associated with gestation length ($P<0.001$) and birth weight ($P<0.001$). Children from the lowest quintile for birth weight had a significantly lighter maternal placenta than those from the middle ($P<0.01$) and upper quintile ($P<0.001$). The placental weight of children from the upper quintile of birth weight was also significantly heavier than those from the middle quintile ($P<0.001$) (Table 5.1).

Length (crown/heel) of the child was significantly associated with birth weight ($P<0.001$). Children from the lowest quintile of birth weight were significantly shorter at birth than middle ($P<0.001$) and upper quintiles of birth weight ($P<0.001$). The lengths of children at birth from the upper quintile of birth weight were also significantly longer than those from the middle quintile ($P<0.05$). Head circumference of the child was significantly associated with birth weight ($P<0.001$). Children from the lowest quintile of birth weight had a significantly smaller head circumference than those from the middle ($P<0.001$) and upper quintile ($P<0.001$). Those from the upper quintile had a significantly larger head circumference than those from the middle quintile of birth weight ($P<0.001$). Two-way ANOVA indicated sex was not a significant determinant of either postnatal length ($P=0.584$) or head circumference ($P=0.280$) (Table 5.1).

Table 5.1. *Gestation length, placental size, birth weight and measures of postnatal size.*

Birth weight mean for quintile (g)	*n.*	Gestation length (weeks)	Placental weight (g)	Length (crown/ heel) (cm)	Head Circum. (cm)	Ponderal Index (m^2)
Combined	64	(combined data represents all the children sampled)				
2814±59	21	39.1±0.3 †	505±0.3*†	47.8±0.3 *†	34.2±0.3*†	25.8±0.3 *†
3432±24	22	39.9±0.3	611±0.2‡	50.3±0.3 ‡	35.3±0.1‡	27.8±0.5‡
4190±65	21	40.4±0.3	783±0.3	51.6±0.4	36.9±0.3	30.6±0.6
Age 10	57					
2863±47	16	39.5±0.3 †	513±0.3* †	48.0±0.2*†	34.4±0.2*†	25.9±0.4†
3424±24	21	40.1±0.2	604±0.2‡	50.3±0.3‡	35.3±0.1‡	27.0±0.5‡
4201±67	20	40.5±0.3	787±0.3	51.7±0.4	37.0±0.3	30.6±0.7
Age 12	59					
2817±72	16	38.9±0.5*, †	536±0.3*†	47.9±0.4*†	34.1±0.3*†	25.6±0.3 *†
3432±24	22	39.9±0.3	611±0.2‡	50.3±0.3‡	35.3±0.1‡	27.0±0.5‡
4190±65	21	40.4±0.3	783±27	51.6±0.4	36.9±0.3	30.7±0.6

Data are shown as mean ± SEM for *n* observations. * denotes significantly different between the lower and middle tertile, † between the lower and upper tertile, ‡ between the middle and upper tertile ($P<0.05$). * denotes a significantly difference between lower and middle tertile, † between the lower and upper tertile, ‡ middle and upper tertile ($P<0.01$).

Two-way ANOVA indicated that birth weight was significantly associated with length of gestation ($F=11.428$, 1df, $P=0.001$), placental size ($F=11.585$, 1df, $P=0.001$), crown/heel length ($F=21.938$, 1df, $P=0.001$), and head circumference ($F=47.688$, 1df, $P=0.001$).

5.3.2. Size at birth and BMI at ages 10 and 12 years.

Weight at birth was significantly related to BMI ($P<0.05$) and weight ($P<0.05$) at age 10, but not height. Length at birth was significantly associated with height at age 10 ($P<0.01$). Ponderal index was also significantly associated with height ($P<0.01$) at age 10, but not weight or BMI (Table 5.2). Similarly, weight at birth was significantly related to BMI ($P<0.05$) and weight ($P<0.05$) at age 12. Length at birth was significantly related to height ($P>0.001$) at age 12, as was ponderal index ($P<0.01$) (Table 5.3). Two-way ANOVA indicated sex was not a significant predictor of BMI at age 10 (male 17.6±0.4; female 18.0±0.6) and 12 (male 19.6±0.7; female 20.5±0.8). No significance between body weight and sex was observed at age 10 (male 33.4±1.0; female 34.0±1.3) and 12 (male 45.7±1.9; female 47.9±2.1) so the data for males and females was grouped and does not alter the overall data. This suggests that the growth trajectory post-partum remains consistent with relative size at birth. Head circumference at birth was not associated with any markers of size at ages 10 and 12.

5.3.3. Renal function in 10 year old children.

Urinary albumin excretion was significantly elevated in children at age 10 in relation to lower weight at birth ($P<0.05$). Sex was not a significant predictor of urinary albumin ($P=0.666$). Those from the lower quintile of birth weight excreted significantly more urinary albumin adjusted for creatinine than those from the middle quintile ($P<0.05$) and the upper quintile ($P<0.01$) (Table 5.4). Those from the lower quintile for length at birth excreted significantly more albumin adjusted for creatinine than those from the middle quintile ($P<0.05$). Those with a head circumference from the middle tertile excreted significantly more albumin than those from the upper tertile ($P<0.05$), but not the lower. The urine collected at ages 10 and 12 years was a single

sample. The urinary creatinine values as part of a renal function test acts as a crude marker for completeness of a 24-hour urine collection (Jacobs *et al,* 1999). Creatinine is freely diffused through the kidney and varies between age, sex and height (Lote, 2000; Walser, 1987). Glomerular disease, such as diabetic nephropathy, manifests itself with an excess of protein in the urine. Small loses are detectable as microalbuminuria, usually developing 10-15 years after hyperglycemia (Glassock & Brenner, 1994). The albumin: creatinine ratio is used to determine microalbuminuria, which is considered a surrogate marker for renal disease (Viberti, 1995).

A birth weight and ponderal index under the 20th centile was significantly associated with raised albumin (corrected for creatinine) excretion at age 10 ($P<0.01$) Whilst not significant individuals under the 20th. Centile for birth weight, birth length and head circumference also excreted more albumin (Table 5.5). However, children at age 10 identified as exhibiting microalbuminuria (as described in 2.7.2) had a significantly lower birth weight and ponderal index ($P<0.01$) than those considered be of normal size at birth (Table 5.6). Maternal placental weight whilst not significant was lighter in those children classed as having microalbuminuria. Gestation length had no effect on albumin excretion.

At age 10 years, 6.3% of children had low (<1.010) urinary specific gravity, 62.5% high (>1.020) urinary specific gravity with only 20.3% falling within the normal range as specified by Jacobs *et al.,* (1996). Specific gravity was significantly correlated with albumin excretion at age 10 ($R=-0.355$, $P=0.008$) and may be suggestive of proteinuria (Jacobs *et al.,* 1996). Specific gravity was not significantly associated with quintiles of birth weight ($P=0.702$) and no differences between the sexes were observed ($P=0.707$). No significant difference was observed between normal and high excretion (micoalbuminuria) for systolic ($P=0.228$) and diastolic ($P=0.179$) blood pressure and pulse ($P=0.284$)

Table 5.2. *Size and weight at birth in relation to size and weight at age 10.*

Tertile	*n.*	Height	Weight	BMI
Birth weight (g)				
<3319	16	137.7±1.9	33.1±1.9	17.3±0.6 †
3320-3785	21	135.6±1.3	31.9±1.2 ‡	17.3±0.5
>3786	20	138.8±1.1	36.1±1.1	18.7±0.5
Ponderal Index (m^2)				
<26	19	138.0±1.6	31.3±1.5 †	16.3±0.5* †
26-29	19	137.1±1.4	35.0±1.5	18.3±0.6
>29	19	136.9±1.2	35.2±1.2	18.8±0.6
Birth length (cm)				
<48	19	135.8±1.5 †	32.6±1.7	17.5±0.6
48-50	20	136.5±1.2	33.4±1.1	18.0±0.6
>50	18	139.9±1.3	35.2±1.5	17.9±0.6
Head Circum. (cm)				
<34	20	137.0±1.5	32.9±1.4	17.4±0.6
34-35	19	137.3±1.5	33.9±1.5	17.8±0.5
>35	18	137.6±1.3	34.4±1.4	18.2±0.7

Data are shown as mean ± SEM for *n* observations. * denotes significantly different between the lower and middle tertile, † between the lower and upper tertile, ‡ between the middle and upper tertile ($P<0.05$). * denotes a significantly difference between lower and middle tertile, † between the lower and upper tertile, ‡ middle and upper tertile ($P<0.01$).

Two-way ANOVA indicate birth weight was significantly related to BMI ($F=4.214$, 1df, $P=0.05$) and weight ($F=4.383$, 1df, $P=0.05$), postnatal length was significantly related to height ($F=10.816$, 1df, $P=0.002$) and ponderal index is significantly related to height ($F=6.622$, 1df, $P=0.013$) at age 10.

Table 5.3. *Size and weight at birth in relation to size and weight at age 12.*

Tertile	*n.*	Height	Weight	BMI
Birth weight (g)				
<3339	16	1.50±0.03	43.7±2.8	19.1±0.8
3340-3809	22	1.52±0.02	45.9±2.2	19.8±0.8
>3810	21	1.52±0.02	48.7±2.0	21.0±0.8
Ponderal Index (m^2)				
<26	19	1.53±0.02	43.8±2.2	18.6±0.7 †
26-29	20	1.51±0.02	46.5±2.5	20.2±0.8
>29	20	1.50±0.02	48.6±2.3	21.4±0.8
Birth length (cm)				
<49	19	1.48±0.02 †	43.5±2.7	19.5±0.8
49-51	22	1.52±0.02	46.5±1.9	20.2±0.8
>51	28	1.55±0.01	49.2±2.3	20.5±0.9
Head Circum. (cm)				
<35	21	1.52±0.02	46.3±2.5	19.9±0.8
35-36	19	1.51±0.02	45.8±2.2	19.8±0.7
>36	19	1.51±0.02	46.9±2.3	20.5±0.9

Data are shown as mean ± SEM for *n* observations. * denotes significantly different between the lower and middle tertile, † between the lower and upper tertile, ‡ between the middle and upper tertile ($P<0.05$). Two-way ANOVA indicate birth weight was significantly related to BMI ($F=5.901$, 1df, $P=0.043$), and weight ($F=4.323$, 1df, $P=0.043$), postnatal length was significantly related to height ($F=10.694$, 1df, $P=0.002$) and ponderal index is significantly related to height ($F=8.117$, 1df, $P=0.006$).

Table 5.4. *Measures of size and weight at birth and associations with markers of renal function at age 10.*

Tertile	*n.*	Albumin (mg/dl)	Creatinine (mg/dl)	Albumin/ Creatinine (mg/mg)
Birth weight (g)				
<3319	16	0.042±0.004***	121.4±16.7	0.44±0.08
3320-3785	21	0.029±0.006	127.8±13.4	0.22±0.05*
>3786	20	0.028±0.004	127.8±13.5	0.22±0.03*
Ponderal Index (m^2)				
<26	17	0.038±0.006	126.7±16.8	0.33±0.07
26-29	20	0.032±0.005	116.8±13.4	0.30±0.06
>29	20	0.028±0.004	141.8±12.8	0.21±0.04
Birth length (cm)				
<48	17	0.038±0.005	134.1±15.8	0.37±0.08
48-50	18	0.028±0.005	128.0±16.9	0.22±0.05*
>50	22	0.031±0.005	124.7±11.0	0.24±0.04
Head Circum. (cm)				
<34	16	0.028±0.006	111.1±13.7	0.24±0.06
34-35	19	0.038±0.005	132.4±16.9	0.37±0.07***
>35	22	0.029±0.005	137.8±11.7	0.21±0.03

Data are shown as mean ± SEM for *n* observations. * denotes significantly different from the lower tertile, ** denotes significantly different from the middle tertile, *** denotes significantly different from the upper tertile ($P<0.05$). Two-way ANOVA indicate urinary albumin excretion was significantly elevated at age 10 (adjusted for BMI at age 10) in relation to a lower weight at birth ($F=5.463$, 1df, $P=0.023$)

Table 5.5. *Albumin excretion at age 10 in dichotomised groups for size and weight at birth.*

Birth measurement.		*n.*	Albumin/creatinine (mg/mg)	*P.*
Birth weight.				
-low birthweight.	<2980 g	12	0.45±0.10	
-high birth weight.	>2981 g	45	0.24±0.03	P<0.01
Ponderal Index.				
-low ponderal index.	<25.13 Kg/m^2	12	0.42±0.09	
-high ponderal index	>25.14 Kg/m^2	45	0.23±0.03	P<0.001
Birth length.				
-short length.	<48.3 cm	12	0.39±0.10	
-long length.	>48.4 cm	45	0.25±0.03	P=0.077
Head circumference.				
-small circum.	<34.3 cm	9	0.35±0.09	
-large circum.	>34.4 cm	48	0.26±0.09	P=0.384

Data are shown as mean ± SEM for *n* observations. Low and high groups for birth weight, ponderal index and short, long, small and large groups for birth length and birth head circumference were defined on the basis of 20th centile cut-offs.

5.3.4. Measurement of size and weight at birth and blood pressure in children aged 10.

No association between systolic blood pressure, pulse and markers for size and weight at birth were observed in children aged 10 (Table 5.7). Only those individuals from the middle tertile of head circumference had a significantly increased diastolic pressure than those from the lower (P<0.01) and upper tertile (P<0.05). There is no association between weight at age 10 and

systolic (P=0.124) or diastolic blood pressure (P=0.222), and BMI at age 10 and systolic (P=0.70) and diastolic blood pressure (P=0.135).

Table 5.6. *Child microalbuminuria and its association with size and weight at birth.*

Indices of size at birth	*n.*	Normal excretion 45	High excretion (microalbuminuria) 12	*P.*
Birth weight.	(g)	3702±98	3173±13*	P=0.006
Ponderal Index.	(m^2)	28.9±0.6	26.4±0.5*	P=0.002
Birth length.	(cm)	50.3±0.4	49.3±0.5	P=0.134
Head circum.	(cm)	35.8±0.2	35.2±0.3	P=0.183
Placental weight.	(g)	660±28.0	595±43	P=0.244
Gestational length.	(days)	279.3±1.5	280.9±2.8	P=0.586

Data are shown as mean ± SEM for *n* observations. * denotes significantly different to Normal (P<0.01). Children were assigned to high excretion or normal excretion groups based on the 80^{th} centile as a cut-off point.

Table 5.7. *Blood pressure, pulse and size at birth.*

Tertile	*n.*	Systolic Pressure (mmHg)	Diastolic Pressure (mmHg)	Pulse (BPM)
Birth weight (g)				
<3319	16	97.2±1.9	54.6±1.5	80.8±2.3
3320-3785	21	95.1±1.5	53.1±1.5	81.4±2.4
>3786	20	98.0±1.6	54.5±1.0	80.1±2.5
Ponderal Index (m^2)				
<26	19	95.9±1.9	54.5±1.6	80.7±2.3
26-29	19	97.8±1.3	54.0±1.5	78.2±2.5
>29	19	96.3±1.7	53.6±1.1	81.7±2.3
Birth length (cm)				
<48	19	96.0±1.9	53.8±1.5	79.3±2.0
48-50	20	96.6±1.5	53.5±1.2	79.6±2.4
>50	18	97.5±1.6	54.9±1.5	81.8±2.8
Head Circum. (cm)				
<34	20	95.0±1.7	51.9±1.3	78.7±2.3
34-35	19	99.1±1.6	57.4±1.4‡*	82.3±2.3
>35	18	96.1±1.6	52.9±1.2	79.7±2.6

Data are shown as mean ± SEM for *n* observations. * denotes significantly different to upper tertile ($P<0.05$) ‡ denotes significantly different to the lower tertile ($P<0.01$)

5.3.5. Renal function in 12 year old children.

Two-way ANOVA indicated there were no significant associations between birth weight, ponderal index, birth length, head circumference and urinary albumin (adjusted for creatinine) excretion at age 12. There was no significant difference between the sexes, and the data is combined (Table 5.8). Unadjusted urinary albumin excretion was significantly higher in the lower quintile of birth weight compared to the upper ($P<0.05$). Individuals with a high albumin excretion (80^{th} centile for albumin adjusted for creatinine) showed no significant difference between measurements of birth weight and size compared to those exhibiting low excretion (Table 5.9). Whilst not significant, those with increased albumin excretion at age 12 did have a heavier birth and placental weight and a longer gestation length than those considered to have a lower excretion. Similarly, measures for size and weight at birth below the 20^{th} centile were not significantly related to albumin (corrected for creatinine) excretion at age 12 (Table 5.10). Urinary total protein was assessed to provide a broader spectrum for analysis with measures of birth size and weight. Individuals from the lower tertile for ponderal index had a significantly higher excretion of unadjusted total protein than those from the upper tertile ($P>0.05$) Individuals from the middle tertile of birth length had a significantly reduced total urinary protein (adjusted for creatinine) concentration compared to those from the upper tertile ($P<0.05$) (Table 5.8). No other associations between markers of size and birth weight were observed for total protein excretion. This observation suggests that the increased albumin excretion, in relation to birth weight and size that was clearly present at age 10 had disappeared by age 12.

At 12 years of age, 8.5% had low (<1.010) urinary specific gravity, 61% high (>1.020) urinary specific gravity with 30.5% falling within the normal range as specified by Jacobs *et al.,* (1996). Increased specific gravity may be suggestive of low hydration status as excess urinary protein was not present (Jacobs *et al.,* (1996).

Table 5.8. *Measures of size and weight at birth and its association with protein excretion at age 12.*

Tertile		Albumin	Creatinine	Albumin/ Creatinine	Total protein	Total prot./ creatinine
	n.	(mg/dl)	(mg/dl)	(mg/mg)	(mg/ml)	(mg/ml)
Birth weight (g)						
<3339	16	0.70±0.03	149.3±7.80	0.50±0.03	0.09±0.02	0.06±0.01
3340-3809	22	0.60±0.04	143.1±10.8	0.50±0.05	0.09±0.01	0.07±0.01
>3810	21	0.60±0.03*	144.6±11.0	0.50±0.03	0.07±0.01	0.05±0.01
Ponderal Index (m^2)						
<26	19	0.7±0.03	151.7±9.80	0.5±0.06	0.10±0.01	0.07±0.01
26-29	20	0.6±0.05	141.3±10.2	0.5±0.03	0.09±0.02	0.06±0.01
>29	20	0.6±0.02	143.2±11.0	0.5±0.03	0.07±0.01*	0.05±0.01
Birth length (cm)						
>43 <49	19	0.7±0.03	147.0±5.80	0.5±0.03	0.09±0.02	0.06±0.01
>49 <51	22	0.6±0.04	149.2±10.7	0.4±0.03	0.07±0.01	0.05±0.01
>51 <55	18	0.6±0.04	138.8±13.3	0.5±0.06	0.09±0.01	0.07±0.01‡
Head Circum. (cm)						
<35	21	0.6±0.03	141.2±6.90	0.5±0.03	0.08±0.01	0.06±0.01
35-36	19	0.7±0.04	162.3±8.90	0.4±0.02	0.10±0.02	0.06±0.01
>36	19	0.6±0.04	132.9±13.6	0.5±0.06	0.08±0.01	0.06±0.01

Data are shown as mean ± SEM for *n* observations. * denotes significantly different from lower tertile ($P<0.05$) ‡ denotes significantly different from the middle tertile ($P<0.05$).

Table 5.9. *Albumin excretion at age 12 in dichotomised groups for size and weight at birth.*

Birth measurement.		*n.*	Albumin/creatinine (mg/mg)	*P.*
Birth weight.				
-low birthweight.	<2980 g	13	0.45±0.4	
-high birth weight.	>2981 g	46	0.47±0.3	P=0.680
Ponderal Index.				
-low ponderal index.	<25.13 Kg/m^2	14	0.49±0.8	
-high ponderal index	>25.14 Kg/m^2	45	0.46±0.2	P=0.550
Birth length.				
-short length.	<48.3 cm	9	0.48±0.3	
-long length.	>48.4 cm	50	0.47±0.3	P=0.875
Head circumference.				
-small circum.	<34.3 cm	11	0.48±0.3	
-large circum.	>34.4 cm	48	0.46±0.3	P=0.785

Data are shown as mean ± SEM for *n* observations. Low and high groups for birth weight, ponderal index and short, long, small and large groups for birth length and birth head circumference were defined on the basis of 20^{th} centile cut-offs.

Table 5.10. *Albumin excretion and its association with size and weight at birth.*

Indices of size at birth		Normal excretion *n.* 45	High excretion (microalbuminuria) 12	*P.*
Birth weight.	(g)	3514±87	3608±18	P=0.621
Ponderal Index.	(Kg/m^2)	28.6±0.5	27.6±0.6	P=0.649
Birth length.	(cm)	50.0±0.3	50.6±0.5	P=0.327
Head circum.	(cm)	35.5±0.2	35.8±0.5	P=0.544
Placental weight.	(g)	648±20.2	677±58.5	P=0.565
Gestational length.	(days)	278±1.7	283±2.8	P=0.141

Data are shown as mean ± SEM for *n* observations. * denotes significantly different to Normal (P<0.01). Children were assigned to high excretion or normal excretion groups based on the 80th centile as a cut-off point.

Two-way ANOVA indicated that urinary sodium excretion was significantly higher in the lower quintile for birth weight compared to the middle (P<0.01) and upper quintile (P<0.01) (Table 5.11). Sodium excretion was significantly associated with BMI (P<0.05), but not sex (P=0.150). Similarly, potassium excretion was significantly higher in the lower quintile of birth weight compared to the middle (P<0.01) and upper quintile (P<0.01). Two-way ANOVA indicated a difference between the sexes for potassium excretion (P<0.01), but no association with BMI was present. When sodium and potassium concentrations were corrected for urinary creatinine all significant associations with size and weight at birth were lost. However, males from the lower quintile of birth weight excreted more sodium and potassium corrected for creatinine than both the middle and upper quintile, but not significantly. No trend was apparent for females (Table 5.11).

Table 5.11. *Urinary sodium and potassium excretion in males and females at 12 years of age.*

Sex	*n.*	Sodium (g/dl)	Sodium/ Creatinine (g/mg)	Potassium (g/dl)	Potassium/ Creatinine (g/mg)
Male					
<3185	6	10.4 ± 1.5	0.08 ± 0.02	38.5 ± 10.5	0.28 ± 0.08
3186-3539	13	7.4 ± 1.0*	0.05 ± 0.01	20.9 ± 3.4*	0.14 ± 0.02
>3540	8	7.0 ± 0.7*	0.05 ± 0.01	16.2 ± 1.9*	0.10 ± 0.01
Female					
<2985	10	8.9 ± 1.3	0.06 ± 0.01	20.4 ± 2.8	0.14 ± 0.02
2986-3825	7	6.0 ± 0.7*	0.06 ± 0.01	18.7 ± 4.0*	0.19 ± 0.05
>3826	13	6.7 ± 0.5*	0.06 ± 0.01	16.7 ± 1.6*	0.15 ± 0.03

Data are shown as mean ± SEM for *n* observations. * denotes significantly different to the lower quintile for birth weight in sodium and potassium, P<0.01. Two-way ANOVA indicated a significant effect of quintiles of birth weight on sodium excretion (F=6.206, 2df, P=0.004), potassium excretion (F=6.634, 2df, P=0.003) and sex was a significant influence upon potassium excretion (F=7.192, 1df, P=0.010).

5.3.6. Young adults.

The 40 healthy young adults (n=21 male: n=19 female) aged between 18 and 22 (19.6±0.2) years were recruited from University College Northampton (as described in section 2.4.2). They represent an early post-puberty cohort with minimal environmental influences. All were born at full term (39.7±0.2 weeks). The young adults were then allocated into three groups based on birth weight which ranged from 2260-4400g (as described in section 2.4.1, 2.7.2) Out of

the original 44 participants recruited, one parental questionnaire was not returned and one mother had a history of pre-eclampsia with her pregnancy. Both were excluded from the study. Two participants failed to attend their appointment on two separate occasions, giving a sample population of 40. One participant failed to supply a 24-hour urine sample and one a blood sample.

12.5% (n=5) of mothers reported having high blood pressure during pregnancy, but were included. 80% (n=32) of deliveries were normal, 7.5% (n=3) caesarean and 12.5% (n=5) forceps. For 52.5% (n=21) of the mothers this was their first child, 35% (n=14) their second and 10% (n=4) their third. For one mother it was her sixth. 39 mothers reported following no specific diet during pregnancy (i.e. lacto-vegetarian, vegan or fruitarian), one mother reported 'other', but failed to specify.

32.5% (n=13) of participants reported being smokers, with 95% (n=38) consuming alcohol. Smoking and alcohol consumption were not significantly associated with the height, weight and blood pressure measurements that were taken. BMI ranged from 18.98-31.46 m^2. Blood samples were taken to determine blood urea nitrogen concentration as an indicator of renal functional status (as described in section, 2.5.7.), serum albumin as a marker of renal disease (as described in section 25.1.) and serum creatinine to calculate creatinine clearance, with creatinine from the 24-hour urine sample (as described in section 2.5.2. & 2.5.3.) A 24-hour urine sample permitted albumin concentrations to be measured in each sample (as described in section 2.5.1) and expressed per mg creatinine (as described in section 2.5.2) to determine glomerular permeability. Urinary specific gravity was also recorded to evaluate hydration status and solute concentration (as described in section 2.5.5).

5.3.7. Gestation length, birth weight and adult size.

In the adult population those in the lower third for birth weight had significantly shorter gestation length than those from the middle third ($P<0.05$). Weight at birth was significantly related to gestation length ($P<0.05$) Male birth weight was significantly heavier than females ($P<0.01$) (Table 5.11). Weight at birth was also significantly related to BMI in the young adults. Those born in to the lower third of birth weight had a significantly lower BMI than those from the middle ($P<0.01$) and upper third ($P<0.05$). Male BMI was significantly greater than female BMI ($P<0.05$).

BMI indicated that 10% were under weight, 57.5% were of a healthy weight, 27.5% were pre-obese and 5% were obese (International Obesity Task Force, 1998). Individuals from the upper third of birth weight were significantly taller than those from the lower third ($P<0.001$). Males were significantly taller than females ($P<0.001$). Individuals from the lower tertile of birth weight remained significantly lighter in weight than those from the middle ($P<0.01$) and upper tertile ($P<0.01$). Males were significantly heavier than females ($P<0.001$) (Table 5.12). The preponderance of smaller females compared to larger males will skew the combined data and this should be considered only representative for the whole cohort. Male and female data is shown separately to reflect this.

5.3.8. Renal function in young adults.

Two-way ANOVA indicated that both birth weight and thirds of birth weight were not significantly related to urinary albumin excretion (Table 5.13). Females excreted significantly more urinary albumin than males ($P<0.05$). Males excreted significantly more creatinine than females ($P<0.05$). No significant associations between thirds of birth weight and creatinine

clearance were observed. No significant association between those classed as having a high albumin excretion (above the 80th centile) and birth weight was observed (P=0.657) (Table 5.14).

40% of the adults had a low (<1.010) urinary specific gravity, 17.5% high (>1.020) urinary specific gravity, whilst 40 % fell within the normal range as specified in Jacobs *et al.*, (1996). This may suggest, in the absence excess urinary protein, that the majority have a good hydration status (Jacobs *et al.*, (1996).

Table 5.12. *Weight at birth, gestation length and adult height, weight and BMI.*

Birth weight Tertile	*n.*	Gestation (weeks)	Height (m)	Weight (kg)	BMI (m^2)
Combined (males/females).					
<3160	14	39.2±0.3	164.5±2.4	61.1±2.3	22.5±0.6
3161-3463	13	40.1±0.3*	171.8±2.4*	77.0±3.6‡	26.0±0.1‡
>3464	13	39.8±0.2	178.4±3.3‡	79.2±4.3	24.8±0.9*
Male.					
<3246	3	38.7±0.9	172.7±3.8	72.4±7.4	24.2±1.7
3247-3633	8	39.8±0.4	175.6±2.3	84.7±3.1	27.5±0.9
>3634	10	39.9±0.2	181.9±3.5	82.8±4.7	24.9±1.0
Female.					
<2896	11	39.4±0.4	162.3±2.5	58.0±2.5	22.0±0.6
2897-3240	5	40.4±0.4	165.8±2.4	64.9±3.8	23.7±1.4
>3241	3	39.7±0.3	166.5±1.8	67.4±7.9	24.3±2.4

Data are shown as mean ± SEM for *n* observations. * denotes significantly different to the lower tertile for birth weight (P<0.05) ‡ denotes significantly different to the lower tertile for birth weight (P<0.01).

Two-way ANOVA indicated significant difference between the sexes for birth weight (F=8.555, 1df, P=0.006), height (F=14.899, 1df, P=0.001), weight (F=17.022, 1df, P=0.001) and BMI (F=4.776, 1df, P=0.036)

Table 5.13. *Urinary albumin and creatinine excretion in young male and female adults.*

Birth weight tertile.	*n.*	Albumin (g/dl).	Creatinine (mg/dl).	Albumin/ creatinine (mg/mg).	creatinine clearance/ adj. body surface area (ml/min).
Combined (males/females).					
<3160	14	0.09±0.008	107.23±17.9	0.10±0.01	0.10±0.01
3161-3463	13	0.09±0.007	122.09±20.1	0.09±0.01	0.12±0.01
>3464	13	0.09±0.006	110.08±16.4	0.10±0.01	0.11±0.01
Male.					
<3246	3	0.10±0.02	140.4±12.2	0.08±0.02	0.13±0.01
3247-3633	8	0.09±0.01	154.0±27.1	0.07±0.01	0.12±0.02
>3634	10	0.09±0.01	105.7±16.5	0.10±0.02	0.11±0.01
Female.					
<2896	11	0.09±0.01	98.2±22.0	0.11±0.01	0.09±0.01
2897-3240	5	0.08±0.01	71.0±6.3	0.12±0.01	0.12±0.02
>3241	3	0.09±0.01	123.1±51.0	0.09±0.03	0.12±0.02

Data are shown as mean ± SEM for *n* observations. Two-way ANOVA indicated a significant effect of sex on urinary albumin excretion, adjusted for creatinine (F=5.370, 1df, P=0.027), and urinary creatinine excretion (F=5.524, 1df, P=0.025). BMI had a significant effect on the amount of urinary creatinine excreted (F=5.268, 1df, P=0.028).

Table 5.14 *High albumin excretion in young male and females adults in relation to weight at birth.*

Birth weight (g)		High albumin excretion	Low albumin excretion	
Combined	*n.*	9	30	*P.*
Birth weight		3230±0.20	3313±0.08	P=0.657
Male	*n.*	2	18	
Birth weight		3940±0.45	3434±0.10	P=0.141
Female	*n*	7	12	
Birth weight		3027±0.17	3127±0.12	P=0.625

Data are shown as mean ± SEM for *n* observations.

Two-way ANOVA indicated thirds of birth weight had no significant effect on serum albumin (P=0.527) and serum creatinine (P=0.640), either adjusted or unadjusted for BMI. Males excreted significantly more serum albumin (P<0.001) and serum creatinine (P<0.001) than females when adjusted for BMI (Table 5.13). Independent samples T test indicated that comparing BUN activity between the lower and the upper third of birth weight there was a significant difference (P<0.048) (Table 5.15).

5.3.9. Blood pressure in young adults.

Blood pressure was not significantly related to birth weight, when adjusted for BMI (systolic P=0.250; diastolic, P=0.722). Two-way ANOVA indicated BMI was associated with systolic blood pressure (P<0.001), but not diastolic pressure (P=0.176). Those from the lower tertile for birth weight had a significantly elevated resting pulse compared to the middle tertile

(P=0.45) (Table 5.16). No significant difference was observed between normal and high excretion of albumin for systolic (P=0.528) and diastolic (P=0.429) blood pressure and pulse (P=0.065).

Table 5.15. *Serum albumin, creatinine and BUN in young adults.*

Birth weight Tertile	*n.*	Serum albumin (mg/dl)	serum creatinine (mg/dl)	BUN (mg/dl)
Combined (males/females).				
<3160	14	8.02±0.1	0.8±0.03	10.8±1.1
3161-3463	13	8.20±0.2	0.9±0.10	10.2±1.7
>3464	13	8.01±0.1	0.9±0.06	15.2±1.8*
Male.				
<3246	3	8.30±0.17	0.9±0.09	11.3±2.9
3247-3633	8	8.44±0.07	1.1±0.12	9.70±1.9
>3634	10	8.17±0.07	0.9±0.07	15.2±1.9
Female.				
<2896	11	7.95±0.11	0.8±0.03	10.7±1.2
2897-3240	5	7.85±0.32	0.7±0.10	10.9±3.2
>3241	3	7.80±0.44	0.7±0.08	15.0±5.7

Data are shown as mean ± SEM for *n* observations. * denotes significantly different to the lower tertile for birth weight (P<0.05).

Two-way ANOVA indicated that sex had a significant effect on serum albumin (F=12.266, 1df, P<0.001) and serum creatinine (F=12.261, 1df, P=0.001).

Table 5.16. *Blood pressure and pulse in young adults.*

Birth Weight Tertile	*n.*	Systolic pressure (mmHg)	Diastolic pressure (mmHg)	Pulse (BPM)
Combined (males/females).				
<3160	14	106.7±2.9	61.1±1.3	70.3±2.9
3161-3463	13	109.6±2.7	61.0±2.8	61.2±3.1*
>3464	13	112.2±3.3	57.5±2.0	63.6±2.3
Male.				
<3246	3	115.7±3.1	58.3±1.9	61.7±3.8
3247-3633	8	113.7±3.2	61.1±4.2	60.8±4.3
>3634	10	114.0±3.1	56.9±2.3	61.6±2.7
Female.				
<2896	11	104.3±3.3	61.9±1.5	72.6±3.3
2897-3240	5	103.0±3.2	60.8±3.1	62.0±5.1
>3241	3	106.3±10.8	59.7±4.1	70.3±2.2

Data are shown as mean ± SEM for *n* observations. * denotes significantly different to lower quintile for birth weight ($P<0.05$).

5.4. Summary of results.

Gestational length is associated with size at birth. Birth weight may predict later BMI. Albumin excretion was higher in those of a low-birth weight and ponderal index at age 10. This association is lost at age 12 and in the young adults. A lower birth weight was not associated with increased blood pressure.

5.5. Discussion.

The aim of this investigation was to examine the impact of size and weight at birth on renal function and hypertension at various time points within humans. Previous studies have demonstrated that at ages between 3-11 years birth weight was inversely related to both systolic and diastolic blood pressure (Whincup *et al.,* 1992; 1995; Law *et al.,* 1992; Hashimoto, *et al.,* 1996; Moore *et al.,* 1996; Taylor, 1997; Yiu *et al.,* 1999). A similar finding was observed in military conscripts aged 18 from Sweden that had an inverse relationship between birth weight and blood pressure (Nilsson *et al.,* 1997). The data in this study indicates that size and weight at birth and blood pressure are not consistent with these previous findings, or those of the low-protein animal model (as reviewed by Langley-Evans, 1999; Chapters 3 & 4).

The principle failure to replicate these previous observations is both cohorts are significantly smaller than those where an association was previously found. Furthermore, children from the Princess Anne Hospital Growth Study (PAHGS) where grouped into quintiles with only those from the lower, middle and upper tertiles examined may have strengthened the observations made. Whilst the PAHGS children were from the same area, the young adults studied were from all regions of the U.K. studying at University College Northampton. This may have increased the variability from a social-economic perspective and from potential regional dietary variations. A large cohort from the same area would have enabled for better control of these variables. The addition of maternal data and markers of relative size at birth (ponderal index) would have enabled more detailed analysis of the young adults studied.

Large population studies focus on single ethnic groups to reduce potential social, welfare, diet quality and quantity and physiological variables that may arise (First World Congress on Fetal Origins of Adult Disease, 2001). In this instance, developmental plasticity of the offspring may be specific to drive survival in that particular environment which enables sexual maturity to

be reached and subsequent reproduction (Prof. Waterlow: 17th. International Congress of Nutrition-criticism of third world feeding programmes). When such individuals migrate their underlying metabolic and physiological adaptations, may be tested beyond its predetermined functional capacity and will manifest itself as disease (Bateson *et al.,* 2004; Fogo, 2003).

However, it has been demonstrated that an inverse association between glomerular permeability, as assessed by albumin excretion with both birth weight and ponderal index in 10-year old children was present. To date no other published literature has been found and this is the first time glomerular permeability has been demonstrated in low, but normal birth weight humans that are non-diabetic. This observation was lost at age 12, possibly masked by the onset of a pubertal growth spurt, and in the young adults. One observation at age 12 in both males and females is that those from the lower quintile of birth weight excrete significantly more sodium and potassium in their urine than those from the middle and upper quintiles. The significance was lost when corrected for creatinine, but the trend was still present. As these children exhibited increased albumin excretion at age 10 a subtle disturbance in the in renal tubular function might be present (Guillery & Robillard, 1999), but as no plasma sodium or potassium concentrations were measured it is impossible to fully evaluate (Jacobs *et al.,* 1996). Furthermore, kidneys and tubular structures are known to scale to size in mammals (Prothero, 1996). This might suggest that the kidneys and the renal tubular structure are smaller in these lower birth weight children. Lampl *et al.,* (2002) using ultra-sound found AGA fetuses that were relatively thin at birth had relatively smaller kidneys for their size in late gestation. It was also noted that the relative size of the kidney increased with the increasing birth weight tertile.

However, it does bring into question whether blood pressure and renal development are causally associated. Systemic blood pressure is proposed to rise secondary to local increases in single nephron pressure, an adaptation that preserves renal function where nephron complement is low (MacKenzie & Brenner, 1995). Manalich *et al.,* (2000), found the volume of glomeruli in

low birth weight neonates at autopsy were larger when compared to those from a normal birth weight. This may further support the concept that those of a lower birth weight or ponderal index in this study possibly had fewer nephrons. Whilst glomeruli volume could not be determined in this investigation of living humans it may account for the increased glomerular permeability observed in the absence of raised blood pressure and therefore independent. Whilst nephron number cannot be obtained ultra-sound measurements of kidney size might have provided a useful comparison with known markers of renal function (Hoy *et al.,* 1998). Additional metabolic (Singer, 2001), and endocrine measures (Bertrum *et al.,* 2001; Sherman *et al.,* 1999) may have helped evaluate renal function further in the populations studied.

No association between lower birth weight and increased albumin excretion was observed in the young adults. Those above the 80^{th}.centile for albumin excretion were 83g lighter at birth, but this was not significant. Creatinine clearance was similar amongst the tertiles for birth weight and BUN was found to be elevated in the upper tertile compared to the lower tertile for birth weight. As BUN excretion is within the normal range (5-20 mg/dl from age 1-40), with no additional evidence of elevated blood pressure or renal disruption, kidney function appears normal in these individuals.

Assessing maternal birth weight, BMI and thinness in this study may have indicated possible maternal constraints, that placed upon the fetus may influence size at birth (Godfrey *et al.*, 1999). The current study indicates that gestational length is significantly associated with birth weight in both the populations studied. It is apparent from the data that a longer gestational period increases relative size at birth and is consistent with the findings of Laor *et al.,* (1997). In the child cohort maternal placental size tends to follow fetal size. It has been previously shown that an increase in placental size in relation to a relatively low weight at birth was associated with increasing blood pressure in adults (Barker *et al.,* 1990; Barker *et al.,* 1992, Law *et al.,* 1992; Moore *et al.,* 1996) and CHD (Martyn *et al.,* 1996a). This cohort does not show any evidence of

disproportion in placental size and postnatal measurements of head circumference and length are in relation to the quintile of birth weight.

An interesting observation from both cohorts is that birth weight is predictive of later weight and BMI and this may reflect a genetic predisposition for growth. Length and weight at birth were also associated with child height and weight. This supports the observations of Seidman *et al.,* (1997), who found birth weight was positively associated with BMI at age 17 and Sorensen *et al.,* (1997), in a large cohort aged 20. This suggests that the growth trajectory post-partum and post-puberty remains consistent with relative size at birth and may reflect a genetic predisposition. Simple anthropometric measures throughout early childhood may have indicated whether any 'catch up' growth in the lower birth weights was present. This has been associated with CHD in adults (Eriksson, *et al.,* 1999) and early increased central adiposity (Ong *et al.,* 2000). Mechanisms that signal and regulate early catch-up growth in the postnatal period may influence associations between small size at birth and risk factors for disease in adult hood. A recent paper by Power *et al.,* (2003) suggests that studies investigating postnatal growth effects on adult health should ideally examine the full growth trajectory.

As a measure of relative size BMI gives no indication of the distribution of body fat (McCarthy *et al.,* 2003). In children and adults centralised or upper body fat carries an increased risk for metabolic complications (Daniels *et al.,* 2000; Taylor *et al.,* 2000). Furthermore, Prentice, (1998) argues that the fixed classifications for BMI in children are inappropriate as there are profound changes from birth to adulthood and recommends separate charts for males and females (Cole *et al.,* 1995) as they are less affected by the onset of puberty. In the young adult cohort studied, over 30% had a BMI that is associated with being over weight (International Obesity Task Force, 1998). BMI in the young adults was strongly correlated with systolic blood pressure. Individuals from this group that were classed as overweight or obese had a significantly increased blood pressure than those classed as a healthy weight ($P<0.05$). Walker *et al.,* (1998) observed

parental blood pressure might be an important confounding factor in the relationship between low birth weight and subsequent hypertension, supporting the hereditary influence of genetics on developmental programming (Reviewed by Young, 2001). Furthermore this highlights the need to include parental data in the statistical analysis. Studies have shown a positive correlation between microalbuminuria and blood pressure in hypertensive adult humans (Pontrmoli *et al.*, 1996; Deckert *et al.*, 1993). In a study of adults increasing microalbuminuria excretion was significantly related to proportional increase in central adiposity (Liese *et al.*, 2001). The programming of obesity has been shown in low birth weight animals (Vickers *et al.*, 2000), and humans (Dunger, 2003), but as none of the cohort studied from the lower weight at birth, were obese as children or young adults this cannot be supported.

Obesity, accelerated catch up growth and environmental factors may interfere a strong influence upon studies based on free-living human populations in relation to uterine development. This is important when considering the data evaluated in this study. It does support a key concept in developmental programming forwarded by Bateson *et al.*, (2004). The multifactorial nature of blood pressure regulation and internal homeostasis may possibly correct the glomerular permeability observed in the child cohort at age 10 and this may last well into early adulthood. It may be that certain life style factors coupled with any genetic predisposition to elevated blood pressure or gradual increase in age may exacerbate this programmed feature and make the individual more prone to early renal dysfunction and subsequent hypertension. In this instance low birth weight and glomerular permeability could be considered causal in the development of CVD. It would be of immense value to assess the children of the PAHGS in early adulthood and middle age to assess whether those of a smaller weight and size at birth exhibited early microalbuminuria go on to develop hypertension.

Investigating maternal diet was not apart of this study. However, Godfrey & Barker, (1995), suggest that the major programming influence upon the fetus is the maternal nutritional

status. This assumption is based on relatively scant data of populations subjected to severe famine during wartime conditions (Smith, 1947) and retrospective studies, which in the broadest sense are unreliable as the precise amount and type of food consumed cannot be accurately measured (Campbell *et al.,* 1996; Godfrey *et al.,* 1994b). Studies of relatively well-nourished populations in the United Kingdom indicate that the normal variation in nutrient intakes during pregnancy has little effect upon infant weight and proportions (Mathews *et al.,* 1999; Langley-Evans & Langley-Evans, 2003) Between 1942 and 1944, nearly 400 pregnant women were recruited to the Oxford Nutrition Survey (Conducted by Hugh Sinclair), a dietary study to determine whether the wartime dietary rations were sufficient to prevent nutritional deficiencies. Detailed biochemical and clinical assessments were conducted on the women, with follow up until after delivery. A detailed analysis of this maternal and postnatal data combined with a detailed assessment of CHD risk factors in the offspring 50 years later found no evidence to support the hypothesis that birth weight or maternal nutrition in pregnancy are associated with CHD risk in adult life (Huxley & Neil, 2004).

Assessing adult disease in relation to birth weight through inadequate maternal diet is essentially flawed due to the exposure of potential environmental effects that a free living human may be exposed to over a lifetime and any genetic predisposition of the offspring to particular diseases. Using birth weight alone as a marker of undernutrition is simply not sufficient and a broader range of measures and observations need to be made. This supports the need for ongoing animal research that may elucidate potential mechanisms by which developmental programming of the fetus may occur. These can be directly applied to future research in humans. Such observation may help in the future to determine an optimal diet for pregnancy in humans. Longitudinal studies in humans need to apply the observations made in animal studies to reduce variables and place the necessary scientific measurements in to the study design that could fully evaluate the potential mechanisms by which, if any, developmental programming may occur in

the offspring. For example, if differing constituents of maternal diet are known to have a differing programming effect in rat offspring (Chapter 4), then in human studies the offspring to be studied should be grouped according to the main maternal dietary features and importantly the maternal environment should be included in the statistical analysis. The Princess Anne Hospital Growth studied was started at the time the initial Barker hypothesis was being developed. Now with the substantial amount of evidence emerging from both animal and human studies far greater controls and measures can be made that may interpret the function tested with greater clarity and a new cohort should be developed.

Chapter 6.

6.1. General discussion.

The aim of this thesis was to further investigate the effect prenatal influences may have on the kidney in both the rodent model and humans. The feeding of a maternal low-protein diet during pregnancy (Langley & Jackson, 1994) consistently produces offspring with hypertension and a reduced renal reserve (Chapter 3 & 4). In this thesis litter of origin has been included in the statistical analysis as the pregnant dam was the only recipient of the modified synthetic diet. A review of published literature indicates this to be the first time such a statistical approach has been used to assess renal morphology, function and blood pressure within the rat model. This method strengthens some of the observations made in the preceding chapters and confirms previous studies looking at nephron reserve and blood pressure that only accounted for the offspring in the analysis.

A significant inverse relationship between nephron number and blood pressure was shown at 4-weeks of age in the preliminary rat study (Chapter 3). Evidence of renal insufficiency in the MLP exposed rat pup was also present at 4-weeks of age. Nephron number in the rat (supplied from the study of Nwagwu *et al.,* (2000) was found to progressively decline with age with MLP exposed rats having significantly less nephrons than CON. This complimented previous renal functional assessments made prior to culling (Nwagwu *et al.,* 2000). Importantly, blood pressure progressively increased overtime, significantly in the MLP exposed rat (Nwagwu *et al.,* 2000). Combining the observations from this study and that of Nwagwu *et al.,* (2000) supports the hypothesis advanced by MacKenzie *et al.,* (1996) in that total nephron supply is a causal factor in determining the susceptibility to develop hypertension.

Histological examination of aged kidneys from the study of Aihie Sayer (2001) showed both MLP and control males as having greater evidence of glomerulosclerosis and renal damage

than females. MLP animals also exhibited progressively elevating blood pressure. This in part supports the hypothesis forwarded by MacKenzie *et al.,* (1996) (Chapter 3). The offspring in this investigation only differed in terms of their prenatal dietary exposure therefore the observation of a disturbed renal architecture in the male aged rat is novel. It may explain in the absence of genetic and environmental influences, why males as observed in human populations are more susceptible to the hypertension and CHD. Certainly in the study of Ozaki *et al.,* (2001), MLP males exhibited significantly elevated blood pressure and vascular disturbance than MLP females over their life course. Evaluating the renal morphology of these rats would be highly beneficial to contrast with the observations in this thesis.

This investigation only assessed the gross morphology of the kidney removed at the time of death. Urinary and plasma biochemical analysis and assessment of GFR throughout various time points of the rat lifespan may have supported any observed changes in the renal morphology. Removing kidneys at these time points may indicate the way in which kidneys progressively deteriorate, by examining other renal structures along with nephrons. Whilst the female aged kidney seems relatively preserved, functional assessments may indicate otherwise. Evaluating relative changes in other organ size, such as the heart, may help ascertain other physiological adaptations that are occurring in order to maintain renal function. Nwagwu *et al.,* (2000), conducted simple measurements of renal morphometry on 4-week old kidneys and found them to be shorter and broader compared to control animals, which supported the work of Konje *et al.,* (1996) in humans. This investigation only looked at the weights of the kidney in proportion to body size and it may have been more appropriate to measure the lateral and transverse length to evaluate the gross morphology of the organ.

Interestingly, female MLP exposed rats in chapter 4 had generally heavier organ weights compared to CON. In view of the preserved renal morphology observed in aged females it must be questioned to whether this organ enlargement is an adaptive mechanism to meet marginal diets

experienced *in utero* and that preservation of relative function is in order to sustain successful reproduction and species survival. It may provide clues to how developmental programming influenced by direct interaction of the mother within the environmental might play in evolutionary adaptation.

The feeding of low-protein diets to pregnant dams is limiting not only in the availability of one or more essential amino acids but also in the excess of methionine (Rees *et al.*, 2000). This may contribute in conjunction with the mild protein restriction to the developmental programming of nephron compliment and hypertension as observed in chapters 3 & 4. One of the aims of this thesis was to address the limiting effect of the low-protein rodent diet. Supplementing the maternal low-protein diet with glycine, urea and alanine, found that glycine was sufficient to prevent the appearance of high blood pressure in the offspring. This may be indicative of the methionine-homocysteine pathway playing a critical role in the cardiovascular programming effect of an MLP diet. Glycine levels in the MLP diet were sufficient to convert excessive maternal dietary methionine and correct fetal vasculature and nephrogenesis but not drive overall growth. In comparison urea, salvaged by refection, was sufficient to drive growth, organogenesis and nephrogensis in the offspring.

Examination of the kidneys of these offspring indicated that nephron number was normalised by the addition of the three nitrogen supplements (Chapter 4). Despite nephron number being similar in MLPG exposed rats to controls, this normalisation of blood pressure was accompanied by evidence of glomerular permeability and an assessment of ultrastructure of the glomeruli is necessary. Those exposed to MLPU and MLPA, which had similar blood pressures to MLP animals, contrastingly had both normal nephron number and renal function. This supports the concept that specific constituents of the maternal diet are the principle mediators promoting programming responses in the offspring. The availability of different forms of

nitrogen, and the ability of the mother, placenta and fetus to convert these to a pattern that meets the demands of the fetus need to be fully investigated.

The effects of maternal protein restriction upon blood pressure and upon the kidney can be dissociated. This is a novel implication is in direct contrast to the hypothesis forwarded by MacKenzie *et al.,* (1996), which may need to be reviewed. Female offspring exposed to additional nitrogen seem to ameliorate the effects of the low-protein diet on organogenesis. It would therefore be necessary to repeat this study over a longer period of time to assess renal function. As observed in Chapter 3, with the apparent preservation of renal structure in females, it is important to evaluate any differences between sexes that may occur through the additional supplementation of nitrogen. It would be important to evaluate over time whether MLPU or MLPA animals would develop glomerular injury and exhibit impairment of renal function. This would be secondary to their hypertension and not a causal factor in it development. In a long-term study it would be of benefit to follow the low-blood pressured MLPG pups to see if they remain resilient to elevated blood pressure as they age and to track their renal function.

This investigation has furthered the understanding of what constitutes an optimal maternal diet for fetal growth and organ development in the rat. This may have strong implications for human pregnancy and specifically investigations assessing the constituents of maternal diet on fetal growth and any subsequent follow up of the children with regard to non-communicable disease attributed to a lower than normal weight or size at birth.

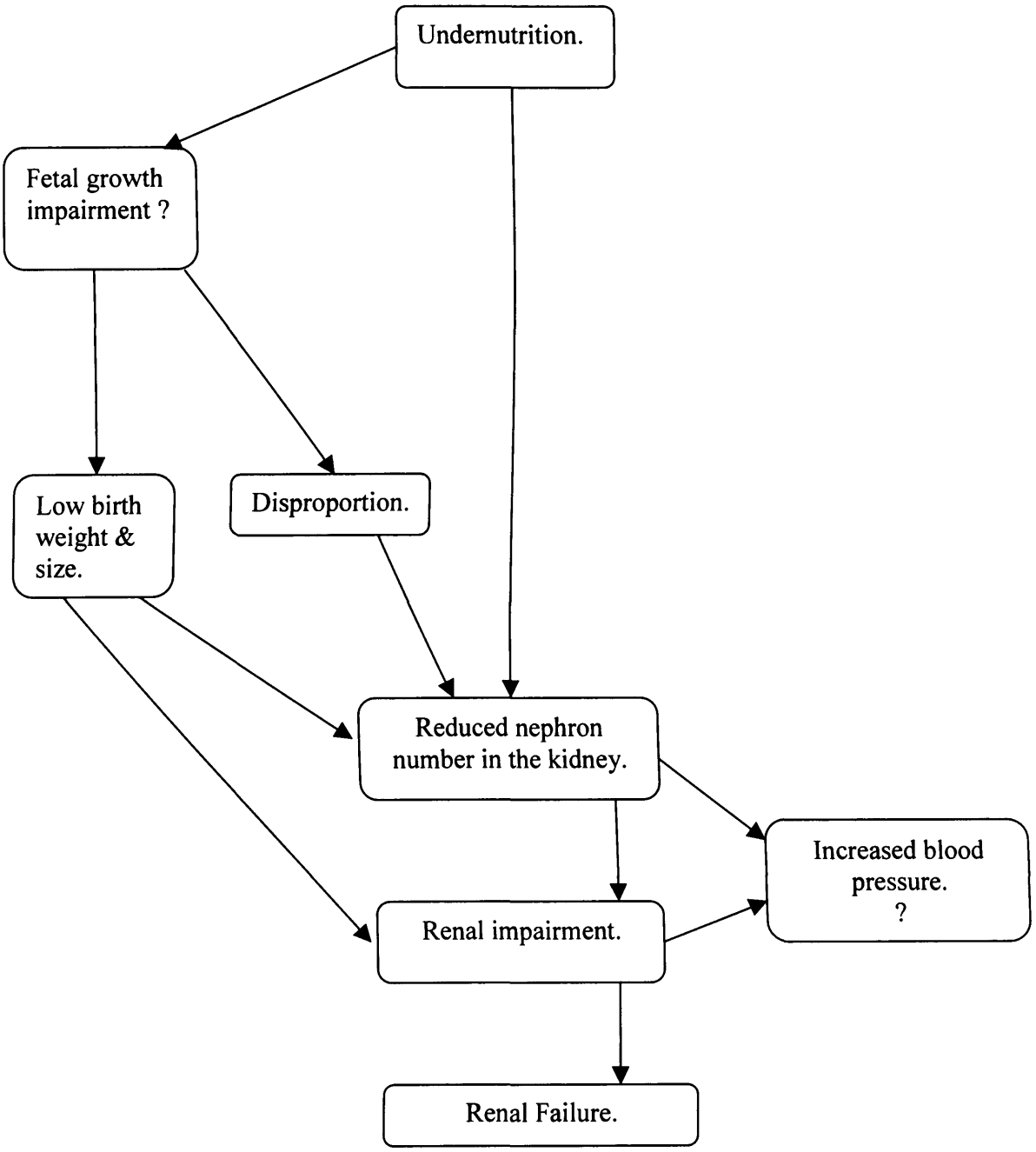
Programming of renal reserve may represent only one of a number of mechanisms, which may act in concert, or may prove, to be secondary to other fetal responses to undernutrition. In both rat studies (Chapters 3 & 4), the physiological, metabolic and endocrine functions of renal tissues should be examined in greater depth. For example, Bertram *et al.,* (2001), demonstrated an up-regulation of angiotensin II receptors within renal tissue following prenatal protein restriction. This would be expected to exert major effects upon the developing kidney structures

in fetal life (Goto *et al.,* 1994; Ray *et al.,* 1994; Alcorn *et al.,* 1996; Woods, 2000), and postnatally to increase blood pressure (Sherman, 1999). Assessment of DNA methylation in the maternal and offspring tissue may have indicated a potential mechanism by which renal reserve may have been programmed (Rees *et al.,* 2000).

With a number of research groups investigating the consequence of maternal diet on developmental plasticity and programming of non-communicable disease, additional tissue or samples generated from animal studies should be forwarded to particular specialist. This would enable greater use of all animal tissue and generate additional data. For example investigating the organs generated from the novel diets employed in chapter 4, may highlight other programming responses not considered in this thesis.

In the human study a lower birth weight and ponderal index was associated with increased glomerular permeability at age 10, which may have been indicative of a reduced renal reserve (Hincliffe *et al.,* 1992; Manalich *et al.,* 2000; Chapter 5). This association was lost at age 12 and not demonstrated in the young adult population. No association between low birth weight and raised blood pressure was observed. No association between markers of renal function and blood pressure was observed. This in part supports the findings in the Chapter 4. The glomerular permeability of the children at age 10 in the absence of elevated blood pressure reflects that of the MLPG exposed rat. The supposition is that the maternal diet may have been adequate in one aspect of nutrition, but not another. This is why future assessments of fetal growth and birth weight in humans should be viewed with a profile of the maternal nutrients consumed. This may help evaluate how certain aspects of the maternal diet may be considered limiting by comparing fetal development and subsequent size and weight at birth. One of the key problems associated with investigating free-living humans is the fact that less invasive techniques are employed and a greater degree of variability is introduced.

Figure 6.1. *Possible mechanisms involved in the programming of nephron reserve in the rat and human and its possible association with elevated blood pressure.*



In order for developmental programming to be considered in humans to the point where interventions during pregnancy result in healthier offspring and adults less prone to non-communicable disease (Bateson *et al.,* 2004) on going research is required. Sufficient knowledge generated from previously conducted animal and humans studies is available to design, develop and conduct a large scale, long-term follow up investigation in to the developmental origins of adult disease. This would involve a number of professions working collectively, but could bring about significant changes in the future of public health. If nutrition is such a powerful mediator of developmental programming, dietary education needs to be promoted.

In summary, the limiting low-protein diet is known to result in a low birth weight with rapid postnatal growth in the rat (Chapter 3) (Figure 6.1). Rats exposed to a maternal low diet consistently exhibit a reduced a renal reserve (Chapter 3, 4). Similarly in humans, low birth weight fetuses have been found to have at autopsy a reduced nephron compliment, with no urinary congenital malformations (Manalich *et al.,* 2000). It may be possible that the children of a low birth weight and smaller ponderal index investigated in this thesis have a reduced renal reserve. This may be accounted for as they exhibit evidence of glomerular permeability at age 10 (Chapter 5). In the MLP rat a reduced renal reserve is associated with evidence of renal impairment as early as weaning. Nephron number in the rat declines with age possibly resulting in end stage renal failure in the male. Similar, observations are made in human populations show that aging is associated with a progressive deterioration of renal function and structure (Anderson *et al.*, 1986), and that independent risk factors such as hypertension can play a contributory role in the progression of renal damage (Remuzzi & Bertani, 1990; 1998). Arterial stiffness is another age related phenomenon associated with increased major cardiovascular events (Heijden-Spek *et al.,* 2000). In the human study there was no evidence of elevated blood pressure with increased glomerular permeability at age 10. Similarly in the rat, modification of the MLP diet with additional nitrogen indicated that the programming of renal reserve, function and blood pressure

are determined by independent mechanisms and not causally related. Furthermore, any observed renal disturbance in the children of a low size and weight at birth may not be associated with elevation in blood pressure by the relative compliance of the arteries (Giovanni de Simone *et al.*, 1997).

The association of a reduced renal reserve and primary hypertension with increased left ventricular mass as observed in middle aged Caucasians at autopsy (Keller *et al.*, 2003), does not account for any environmental factors that may cause the elevation of blood pressure. Smoking, lack of exercise and obesity may elevate blood pressure independently of the kidney, but place additional stress upon this organ that may lead to impaired function gradually resulting glomerulosclerosis. This effect may possibly be enhanced if the nephron complement is lower. With a rapid decline in function, blood pressure would then increase in a continuing cycle leading to progressive nephron loss (MacKenzie & Brenner, 1995) (Figure 6.1).

An adequate maternal supply of nutrients for growth and development of the fetus *in utero* is of prime importance. Marginal differences in nutrition give rise to a number of potential insults that can alter the structure and function of a number of important organs within the developing fetus. The potential number of organs and regulatory mechanisms that are disturbed in the conceptus may work in unison to promote a rapid decline in their general health as adults, when subjected to either genetics or environmental changes Determining an optimal maternal diet for pregnancy is far from concluded, but has major importance worldwide. This thesis has demonstrated the effects a maternal low-protein diet can have on nephron development and associated blood pressure and how with the additional supplementation of nitrogen some of the associated programming effects may be abolished. In humans, exposure to an adequate supply of essential nutrients *in utero,* may generate individuals less prone to renal dysfunction, diabetes, hypertension and coronary heart disease. This in turn may reduce the amount of medical intervention and long-term health care required and may economic benefits.

6.2. Conclusion.

The work of McMullen & Langley-Evans, (2003) using a reduced methionine content of the maternal low-protein diet still produced elevated blood pressure and a lowered nephron compliment in the offspring. It may be important to conduct future research in the animal models using their dietary protocol (Chapter 4-discussion). Whilst the Langley & Jackson (1994) maternal diet has been considered a relatively mild restriction in terms of its protein content, the work of Rees, (2002; 2003) and this thesis demonstrate this is not the case. It needs to be questioned whether the rodent low-protein diet is really representative of human diets? The low-protein diet is so limiting that it can only be considered experimental. Whilst it does elicit developmental plasticity in the fetus and provide an insight in to programming mechanisms the recommendation of this thesis is to review the current rodent low protein diet. The experimental diet should reflect the marginal differences experienced within human populations.

The Langley & Jackson (1994) diet utilised in this thesis uses the AIN76 mineral and vitamin mix, which has been superseded by the AIN93 (Reeves, 1997). It would be of value to substitute the AIN76 for the AIN93 mineral and vitamin mix in the maternal 18% and 9% casein model to evaluate nephron compliment and blood pressure in the offspring.

6.3. Future work.

The work in this thesis has predominantly examined nephron number, volume and markers of renal function that may be indicative of increased glomerular permeability. The feeding of a maternal low-protein diet consistently produces offspring with a lower nephron reserve, but to date no published data is available to indicate whether the nephrons present are structurally normal in comparison to controls. The addition of glycine to the maternal low-protein

diet restored nephron number in the offspring at a similar level to CON, but functional characteristics indicated glomerular permeability equivalent to MLP. The addition of urea and alanine to the MLP diet corrected nephron reserve and renal function in the offspring. The type of nitrogen added my have specific characteristics of growth and development during nephrogenesis as glomerular volume in MLPA animals were smaller than all other dietary groups.

It is clear from the work in this thesis experimental diets have differing effects on nephrogenesis and renal function that go beyond simply determining nephron number. It is now important to examine the ultra structure of glomeruli in experimental animals using an electron microscope. Initially it would be used to observe any glomerular structural differences that may be present, if any, between dietary groups. To take kidney programming further the development and functional capacity of other renal structures, such as the Loop of Henle, needs to be assessed. This is important as the MLP diet has been shown to effect the general cardiovascular properties of the offspring, but to date no published literature is available that has looked at reno-vascular structures in developmental programming. In order to develop accurate or even novel methodologies, kidneys from larger species, such as sheep, should be utilised and then scaled down for the rodent.

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Appendix 1.

Composition of casein (Based on Lehninger, 1981)

199 residues total.

Alanine	9
Valine	11*
Leucine	17*
Isokacine	11*
Proline	17*
Methionine	5*
Phenyalanine	8*
Tryptophan	2*
Lysine	14*
Arginine	6
Histidine	5*
Glycine	9
Serine	6
Cysteine	0
Threonine	5*
Tyrosine	10
Asparagine	8
Glutamine	14
Asparagine	7
Glutamicacid	25

* Denotes essential amino acid.

Appendix 2.

The composition of standard laboratory CRME rat chow (CRME, Special Diet Services, Cambridge, UK).

Nutrient.	**Concentration.**
Protein	174 g/kg
Fat	31 g/kg
Fibre	123 g/kg
Pectin	13 g/kg
Hemicellulose	68 g/kg
Cellulose	33 g/kg
Lignin	10 g/kg
Starches	448 g/kg
Sugars	47 g/kg
Calcium	8 g/kg
Total Phosphorus	6.4 g/kg
Phytate Phosphorus	2.1 g/kg
Available Phosphorus	4.3 g/kg
Sodium	3 g/kg
Chlorine	3.2 g/kg
Magnesium	2.2 g/kg
Potassium	6.4 g/kg
Iron	110 mg/kg

Appendix 2. (continued).

Nutrient.	**Concentration.**
Copper	16 mg/kg
Manganese	70 mg/kg
Zinc	65 mg/kg
Cobalt	280.9 µg/kg
Iodine	603 µg/kg
Selenium	217 µg/kg
Fluoride	9 mg/kg
Retinol	5104.1 µg/kg
Vitamin A	16843.4 µg/kg
Cholecalciferol	80.7 µg/kg
Vitamin D_3	3228.6 µg/kg
α-tocopherol	93.8 mg/kg
Vitamin E	103.2 mg/kg
Vitamin B_1	16.8 mg/kg
Vitamin B_2	13.6 mg/kg
Vitamin B_6	18.5 mg/kg
Vitamin B_{12}	83.5 µg/kg
Vitamin C	8 mg/kg
Vitamin K_3	184.9 mg/kg
Folic Acid	4.4 mg/kg
Nicotinic Acid	78.7 mg/kg

Appendix 2. (continued).

Nutrient.	**Concentration.**
Pantothenic Acid	26.1 mg/kg
Choline	951 mg/kg
Inositol	2355.4 mg/kg
Biotin	369.1 μg/kg
β-Carotene	0.9 mg/kg

Appendix 3.

Haematoxylin and eosin staining procedure.

Stage	Solution	Time
1	Histo-clear	10 mins.
2	Histo-clear	10 mins.
3	Absolute Alcohol	2 mins.
4	Absolute Alcohol	2 mins.
5	70% Alcohol	2 mins.
6	70% Alcohol	2 mins.
7	Tap Water	1 min.
8	Harris Haematoxylin	10 mins.
9	Tap Water	2 mins.
10	Acid Alcohol	4 secs.
11	Tap Water	Max. 5 mins.- "TO BLUE"
12	Eosin	5 mins.
13	Tap water	Rinse.
14	70% Alcohol	2 mins.
15	70% Alcohol	2 mins.
16	Absolute Alcohol	1 min.
17	Absolute Alcohol	1 min.
18	Histo-clear	4mins.
19	Histo-clear	4mins.

Acid Alcohol was prepared as described in Appendix 4.

Histo-clear (BDH, UK),replaces Xylene.

Appendix 4a.

Relationship between adult renal function and prenatal growth.
Mothers Questionnaire. STRICTLY CONFIDENTIAL.
SUBJECT NUMBER.....

1. What was your childs birth weight? lbs_____ oz_____OR
   Kilos_____ g_____

2. Was your child born earlier than the full 40 weeks?
   YES ☐
   NO ☐
   IF YES, HOW MANY WEEKS
   GESTATION_____

3. Was this your first child? (if not please indicate their position with relation to other siblings and include birth dates).
   1st. ☐
   2nd. ☐
   3rd. ☐
   MORE ☐ HOW MANY?_____

4. Whilst pregnant with this child did you experience any of the following?
   Diabetes ☐
   Pre-eclampsia ☐
   High blood pressure ☐

5. What kind of delivery did you have?
   Normal ☐
   Caesarean section ☐
   Forceps ☐
   Ventouse ☐

6. Prior to and during pregnancy did you follow any specific diet?
   Lacto-vegetarian ☐
   Vegan ☐
   Fruitarian ☐
   Other ☐ state_____

7. Did the child participating in this study require any special treatment/medication as soon as they were born?
   NO ☐
   YES ☐ Please give details_____

Thankyou for taking the time to fill out this questionnaire.

Appendix 4b.

Relationship between adult renal function and prenatal growth.

Volunteers Questionnaire. (STRICTLY CONFIDENTIAL)

SUBJECT NUMBER.....

1. What is your date of birth? 2. What is your gender? Male ☐ Female ☐

3. Do you have any of the following medical conditions,

High blood pressure (hypertension)?	YES ☐ NO ☐
Diabetes?	YES ☐ NO ☐
A Kidney condition ?	YES ☐ NO ☐
Coronary heart disease?	YES ☐ NO ☐

IF YES PLEASE GIVE MORE DETAILS.

_____

_____

_____

4. Have you ever had surgery on your kidneys?

YES ☐

NO ☐

IF YES PLEASE GIVE MORE DETAILS.

_____

_____

_____

_____

5. Does any member of your family suffer from the following listed conditions. If the answer is YES please indicate relationship to you (e.g. Grandfather on mothers side, uncle-fathers brother).

High blood pressure (hypertension)?	YES ☐ NO ☐	_____
Diabetes?	YES ☐ NO ☐	_____
A Kidney condition ?	YES ☐ NO ☐	_____
Coronary heart disease?	YES ☐ NO ☐	_____

6. How many units of alcohol do you drink on average per week? (1 unit of alcohol equals ½ pint of ordinary strength lager/beer/cider, a 25ml pub measure of spirits or a small glass of wine)

NONE	☐
BETWEEN 0 & 10	☐
BETWEEN 10 & 15	☐
BETWEEN 15 & 20	☐
MORE THAN 20	☐

7. Do you smoke? Yes ☐ How many per day do you smoke?_____

No ☐

Appendix 5.

Acid Alcohol.

Acid alcohol was prepared for histological staining (haematoxylin and eosin stain), using Carltons Histological Technique, 1967.

1ml concentrated HCl in a 70% alcohol solution.

Albumin assay.

Bovine serum albumin (BSA) for urinary and plasma determination;

Standard: 1mg/ml BSA made up in 0.1M NaOH.

Reagent: Bromcresol Green, 0.30 mmol/L, in buffer and non-reactive stabilizer pH. 4.2 (Sigma Diagnostics).

Angiotensin converting enzyme.

For plasma and tissue determination;

Reagent: 10mg n-(3-[2-furyl] acryloyl)-phe-gly-gly) made up in 25ml 0.05M Tris-HCl, 0.3 M NaCl, pH 7.4 buffer.

Harjanne, A (1984) Clin. Chem. **30**: 901

Bradford assay.

For urinary and tissue determination;

Reagent: 200 mg Coomassie blue dissolved in 100 ml 95% ethanol. 200 ml phosphoric acid (grade ACS reagent >85%) was added before being made up to 2.5 L in distilled water. Solution was stored for 1 week prior to use and remained stable for up to 2 months in a brown bottle.

Blood Urea Nitrogen (BUN).

For plasma determination;

Reagent: α-Ketoglutarate 7.5 mmol/L; nicotinamide adenine dinucleotide >0.20 mmol/L urease (Jack Bean) >8700 U/L; glutamate dehydrogenase (microbial) >580 U/L) Reagent also contains buffer (pH 8.35 at 25°C) and sodium azide, 0.05%, as presevative. (Sigma diagnostics)

Creatinine assay.

For urinary and plasma determination;

Standard: creatinine, 3.0 mg/dL, in hydrochloric acid, 0.02 N.

Reagent: creatinine colour reagent (picric acid, 0.6%, sodium borate and surfactant) was mixed with sodium hydroxide solution 1.0 N at a ratio of 5:1.

Acid reagent: (Sigma Diagnostics).

DNA reagents.

For tissue determination;

DNA Standard: salmon sperm nuclei (type II-S): 400 µg/ml solution.

DPA reagent: 40 g/L diphenylamine 0.1 ml/L paraldehyde were made up to 1 L with glacial acetic acid.

Homogenisation of kidney tissue.

Homogenisation buffer: 30 mM KCl, 10 mM of potassium phosphate pH of 7.4.

Sodium.

For urinary determination;

Standard: 2.45g of sodium chloride (AR grade) added to 1 liter of deionised water (1:1000ppm).

Potassium.

For urinary determination;

Standard: 0.93g of potassium chloride (AR grade) added to 1 liter of deionised water (1:100ppm).

NAD-dependent prostaglandin dehydrogenase.

For tissue determination;

Standard: 1mg 15 ketoPGE$_2$ dissolved in 2mls ethanol and diluted with Tris-HCl pH 10 at a ratio of 1:71.5.

Reagent: 1mg of Prostaglandin E$_2$ dissolved in 2mls of ethanol and diluted with Tris-NAD (1.0mM NAD in Tris HCl pH 10) at a ratio of 1:250. Reagent 1.0mM NAD, 5.7μm Prostaglandin E$_2$, 1M Tris-HCl p.H.10.0.

Blank reagent: 1.0mM NAD, Tris-HCl pH 10.

NORTHAMPTON MEDICAL RESEARCH/ ETHICS COMMITTEE
Chairman: Dr Robin Sheppard
Secretary: Mrs Michelle Spinks

Northamptonshire NHS
Health Authority

Highfield
Cliftonville Road
Northampton
NN1 5DN

Tel: 01604 615000
Fax: 01604 615010

Our Ref: FE/MS/01/98

30 January 2002

Appendix 6

Mr Michael Marchand BSc(Hons)
University College Northampton
Centre for Healthcare Education
Park Campus
Boughton Green Road
NORTHAMPTON NN2 7AL

Dear Mr Marchand

01/98 Relationship between adult renal function and prenatal growth

Thank you for your recent letter, in response to the concerns raised by the Committee at their meeting on 10 January in relation to the above study.

Following consultation with members of the Committee, I am pleased to confirm that your response is in accordance with the Committee's requirements, and that Formal Ethical Approval has therefore been granted for the study to proceed.

I confirm that the Northampton Medical Research/Ethics Committee operates according to Good Clinical Research Practice (GCP) principles, and enclose a copy of the Committee's Constitutions and Standing Orders.

You will find details enclosed regarding a Regional funded project to record and analyse projects that have been submitted to this Ethics Committee. The letter enclosed explains the project in more detail. Please take time to read it, before completing the survey. Your participation is useful and necessary to the completion of a mapping exercise of research (any research) that is proposed, planned or taking part in Northamptonshire. Your record of using resources would be helpful in shaping future funding of research and development in the county.

To complete our records regarding the project, please complete and return the form accompanying this letter.

Please let me know if the study has to be terminated or any ethical considerations arise which need to be discussed further by the Committee.

Yours sincerely

Michelle Spinks
Secretary, Northampton Medical Research/Ethics Committee

Publications from this thesis.

M.C. Marchand, S.C. Langley-Evans, T. Wheeler, F. Anthony, R.C. Sherman, M.O. Nwagwu, A. Dewar (2001) Albumin excretion in relation to characteristics at birth as a marker of reno-vascular programming. *Proceedings of the Nutrition Society,* **60**: 50A.

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