UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE

Infection, Inflammation and Immunity Division

The Effect of Maternal Nutrition on the Developmental Origins of Respiratory Disease.

by

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ABSTRACT

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THE EFFECT OF MATERNAL NUTRITION ON THE DEVELOPMENTAL ORIGINS OF RESPIRATORY DISEASE.

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Environmental challenges during early life have been shown to result in greater risk of chronic diseases such as diabetes and coronary disease in later life. Factors such as unbalanced nutrition before birth result in metabolic and structural adaptations that lead to persistent modifications to offspring phenotype. There is evidence that respiratory disease is influenced by developmental environment. Reduced fetal growth is associated with impaired lung development, increasing risk of developing asthma and Chronic Obstructive Pulmonary Disease (COPD) in later life. To investigate the mechanisms underlying these effects, it is necessary to utilise animal models that emulate the phenotypes observed in human studies.

The aim of this thesis was to investigate whether exposure to a maternal low protein diet *in utero* affects offspring lung morphology, bronchial hyperresponsiveness (BHR) and global methylation and/or gene expression in Wistar rats. Pregnant Wistar rats were allocated to either control (C, 18% casein) or protein restricted (PR, 9% casein) diet. Lung tissue was harvested (225 days) from male offspring (F1, (28 days, C=15; PR=10, 120 days, C=11; PR=7, 225 days: C=6; PR=6) F2, (28 days, C=24; PR=17) and F3 (21 days, C=16; PR=5)). Lungs were removed and the left lung was sectioned and haematoxylin and eosin stained, imaged at 10x magnification and using stereological methods, point counted to give volume fractionation of selected areas. Other morphological measurements were made to estimate surface area (10x magnification), and alveolar wall thickness (63x magnification). Primary bronchi were dissected out, mounted in a myograph and bronchoconstriction in response to a range of bronchoconstrictors and bronchodilators was assessed. RNA and DNA were extracted using TRIzol® and global methylation assessed using Methylamp™ Global DNA Methylation Quantification Ultra Kit.

There was no significant difference in either lung volume or lung structure between the F1 or F2 offspring of control of PR exposed mothers except for a significant increase ($p=0.046$) in the amount of smooth muscle around blood vessels in the protein restricted group in the F1 225 day old group. However, in assessment of constrictor responses of isolated bronchi, a significant difference in response between groups was found with both carbachol, an acetylcholine mimetic, and U44619, A thromboxane mimetic, with the F1 offspring from protein restricted mothers exhibiting significantly increased BHR at 75 days of age ($p=<0.001$) compared to controls. No significant difference in global DNA methylation in lung tissue was found between F1 of F2 offspring of mothers exposed to the control of PR diet during pregnancy. Gene expression levels of selected candidate genes in lung tissue were then assessed using qPCR and again no differences were found between groups.

In this study there is no evidence to suggest that *in utero* exposure to a maternal low protein diet has affected offspring lung physiology, structure, methylation patterns or gene expression. However, there could be differences in the amount of smooth muscle found around the vessels in response to this nutritional challenge as protein restricted animals appear to have more smooth muscle compared to controls. BHR was observed in the 75 day old rats indicating a possible shift towards a priming of asthma phenotype which could be induced with post natal allergen challenges.

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Declaration of Authorship

I, Shelley Ann Davis, declare that the thesis entitled 'The Effect of Maternal Nutrition on the Developmental Origins of Respiratory Disease' and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- This work was done wholly or mainly while in candidature for a research degree at this university;
- Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly attributed;
- Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- None of this work has been published before submission.

Signed: ……..

Date: ……

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Abbreviations

Abbreviation	Full word
A2M	Alpha-2-macroglobulin
ADAM33	A Disintegrin metalloprotease 33
ADRB2	Adrenergic, beta-2 receptor
Ang II	Angiotensin II
ANOVA	Analysis of Variance
Atp1α1 and Atp1α2	ATPase, Na^+/K^+ transporting, alpha 1 polypeptide
AUC	Area under the curve
B2M	Beta-2-microglobulin
BAC	Beta Actin
Bcl2	B-cell CLL/lymphoma 2
BHR	Bronchial Hyperresponsiveness
BMI	Body mass index
BPA	Bisphenol A
BRL	BRL37344
C	Control
CCh	Carbachol
cDNA	Copy DNA
CHRNA 3/5	α-nicotinic acetylcholine receptor
CFTR	Cystic fibrosis transmembrane conductance regulator
COPD	Chronic Obstructive Pulmonary Disease
CRC	Cumulative response curves
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1
DENND1B	DENN/MADD domain containing 1B
DEPC water	Diethylpyrocarbonate water
DOB	Dobutamine
DOHAD	Developmental Origins of Health and Disease
EDTA	Ethylenediaminetetraacetic acid
EPHX1	Microsomal, epoxide hydrolase 1
FAM13A	Family with sequence similarity 13, A

Abbreviation	Full word
FEV_1	Forced Expiratory Volume in one second
FEV_1/FVC	Forced Expiratory Volume in one second/ Forced Vital Capacity
FVC	Forced Vital Capacity
GMA	Glycidyl methacrylate
GPR126	G protein-coupled receptor 126
GR	Glucocorticoid receptor
GST	Glutathione S-transferase
GSTCD	Glutathione S-transferase, C-terminal domain
GSTM1	Glutathione S-transferase mu 1
H&E	Haematoxylin and eosin
HAT	Histone acetylases
HDAC	Histone deacetylase
HHIP	Hedgehog interacting protein
HMD	High Methyl Diet
HMOX	Heme oxygenase
HSD11B1 and HSD11B2	Beta-Hydroxysteroid dehydrogenase 1 and 2
HTR4	5-hydroxtryptamine receptor 4
ICAM-1	Intercellular adhesion molecule-1
IgE	Immunoglobulin E
IGF1 and IGF2	Insulin-like growth factor 1 and 2
IL1RN	Interleukin 1 receptor agonist
IL-13	Interleukin 13
ISO	Isoprenaline
IUGR	Inter Uterine Growth Restriction
IV	Intra Venous
KPSS	Potassium Physiological Salt Solution
LMD	Low Methyl Diet
LTBP4	Latent transforming growth factor beta binding protein 4

Abbreviation	Full word
Map2k5	Mitogen-activated protein kinase kinase 5
Mapk14	Mitogen-activated protein kinase 14
MMP	Matrix metalloproteinase
MR	Mineralocorticoid receptor
mRNA	Messenger RNA
ORMDL3	Orosomucoid 1-like 3
PAP	Papaverine
PBS	Phosphate Buffered Saline
PCDH	Protocadherin
PDE4D	Phosphodiesterase 4D, cAMP-specific
PEF	Peak Expiratory Flow
PPT2	Palmitoyl-protein thioesterase 2
PR	Protein Restricted
PSS	Physiological Salt Solution
Rpl10a	Ribosomal protein L 10a
SAL	Salbutamol
SERPINE2	Serpin peptidase inhibitor, E2
SFTPB	Surfactant protein B
SOD3	Superoxide dismutase 3
SWS	Southampton Women's Survey
TBE	Tris/Borate/EDTA Buffer
TGFB1	Transforming growth factor β1
THSD4	Thrombospondin, type 1, domain containing 4
TNF	Tumor necrosis factor
Tp53	Tumor protein P53
TRPV4	Transient receptor potential cation channel, 4
UBC	Ubiquitin C
UPC	Uncoupling protein
VCAM-1	Vascular cell adhesion molecule-1
VDBP	Vitamin D binding protein
VLA-4	Very Late Antigen-4

1. Introduction

1.1 Developmental Origins of Health and Disease, DOHAD

The DOHAD hypothesis proposes the idea that environmental fetal exposure can alter the epigenome of that individual, resulting in an altered phenotype. That this alteration is set to be advantageous for the environment the individual is exposed to during its life-time.

1.1.1 Evidence for DOHAD

Most of the evidence for the DOHAD hypothesis in humans comes from two different natural exposures. The two seminal studies that provide data to support the DOHAD hypothesis are the Hertfordshire cohort born between 1911 and 1930, and the Dutch Hunger Winter cohort who were born to mothers pregnant during the winter of 1944-1945.

For the Hertfordshire cohort the first chief health visitor and lady inspector of midwives for the county of Hertfordshire, Ethel Margaret Burnside (Syddall 2005), instigated the collection of in-depth data about all the children born in Hertfordshire while she was practicing there. This data included the birth weight, body length and head circumference of each new born. This data was then stored and forgotten until it was rediscovered years later. Records of birth weight, body length and head circumference provided surrogate information on early life nutrition. All of the individuals who could be traced and who were still living in Hertfordshire were recontacted and correlations between growth restriction during pregnancy and development of chronic diseases in later life were established (Syddall 2005). Positive correlations were found between a low birth weight, surrogate measure for reduced fetal nutrition, risk of developing type II diabetes, increased blood pressure (Hales 1991), and risk of developing metabolic syndrome (McKeigue 1998).

The Hertfordshire cohort has been used to link the development of chronic diseases to early life exposures, however this study has had to use surrogate measures, birth weight in relation to crown/rump length, to estimate if a nutrition restriction has occurred. The actual diet that the mother was exposed to is not available.

Another example of clear correlation between early life environmental exposures and risk of chronic disease in later life is an imposed nutritional restriction within a Dutch population who could not get food due to restrictions on movements during the Second World War. German occupation of the Dutch area that was affected lead to the formation of an allied response known as ‘Operation Market Garden’. Taking place over nine consecutive days (17-25 September 1944) airborne forces- Market, and ground forces- Garden, stormed the area to try and remove the German forces. After the failure of this attempt, mainly blamed on

the weather, the amount of food available was severely decreased. This was mainly due to the strike of the Dutch railway workers in an attempt to aid the Allied attack, but once the attack had failed the lack of transportation of food stuffs during this period caused massive shortages and many Dutch people starved to death during the following winter.

This cohort is especially relevant as the individuals were known to have experienced famine during pregnancy and anthropomorphic surrogate measures for nutritional intake are not used. The effects seen therefore are those induced by direct nutritional reduction only. Studies of this cohort have found that those individuals that had been exposed to famine during gestation had decreased glucose tolerance and increased insulin levels indicative of type II diabetes, which was independent of their current BMI, at both 50 and 58 years of age (Ravelli 1999; Painter 2006). Exposure to famine has also been linked with the development of obesity with women whose mothers were exposed to the Dutch Famine in early gestation showing significantly higher BMI by 7.4% (95% CI: 0.7%, 4.5%) than that of non-exposed women. Interestingly this association was not found in those individuals whose mothers were exposed during either mid or late gestation or in men (Ravelli 1999). This implied that there are developmental windows that affect the offspring differently if the exposure is experienced at different times within development. Although effects have been seen in some papers, others have reported no transgenerational effects and some have been sex linked with the trait only being passed down from mother to daughter and not being seen in the sons (Painter 2006).

Both of these cohorts provide evidence that development of many chronic diseases have a basis in early life. These findings have also been replicated in other cohorts throughout the world. This type of evidence has been found for ischemic heart disease in British, American, Indian, Swedish, Finish, Dutch, Icelandic and Danish populations (Huxley 2007), and for type II diabetes in Japanese, Indian, Icelandic, Swedish, Finnish, American, Dutch, Canadian, British and Chinese populations (Whincup 2008). Thus, the evidence to support the effect of early life in later development of chronic diseases in humans is substantial.

1.1.2 The DOHAD Hypothesis

Epidemiological evidence resulted in the proposal of the developmental origins of health and disease (DOHAD), or thrifty hypothesis (Hales 2001), that suggests that as an organism develops, its capacity for developmental plasticity decreases, but the effect of the environment increases, as seen in figure 1. This is a mechanism that allows the organism to adapt to the environment it should find itself being born into. This is an advantageous adaptation for the individual allowing it to utilise its environment effectively and reach reproductive age in peak condition. This can mean that the individual appears to have a normal phenotype but has actually adapted itself, and is at greater risk of developing diseases later in life.

This adaptive response allows the alterations to the phenotype of the organism within a single generation that then can then be altered back if the environment reverts back for its offspring. These alterations are suspected to be epigenetic in nature as there is no alteration within the DNA sequence and natural selection as a result of random mutagenesis occurs over much longer time frames. Some of the most obvious visual examples of an adaptive response to environment are known within mammals that have a short life-span, such as the meadow vole, meaning that they have two or more litters of young during a single year. The environmental exposures here are thought to be day length and temperature. This then alters the epigenetic profile changing the phenotype of the young to produce either the summer or winter coat (Gluckman 2005).

These adaptations are meant to be advantageous to the organism for example if there is a reduction in the availability of nutrition a small size coupled with a slower metabolism can help survival, however if there is an abundance of nutrition a larger size with increased metabolism would have advantages for reproduction (Bateson 2004).

Problems occur when the environmental exposure during development does not match that in which the organism encounters in its adult life. This mismatch in environments is thought to be the basis of increased risk of disease within these individuals (Godfrey 2007).

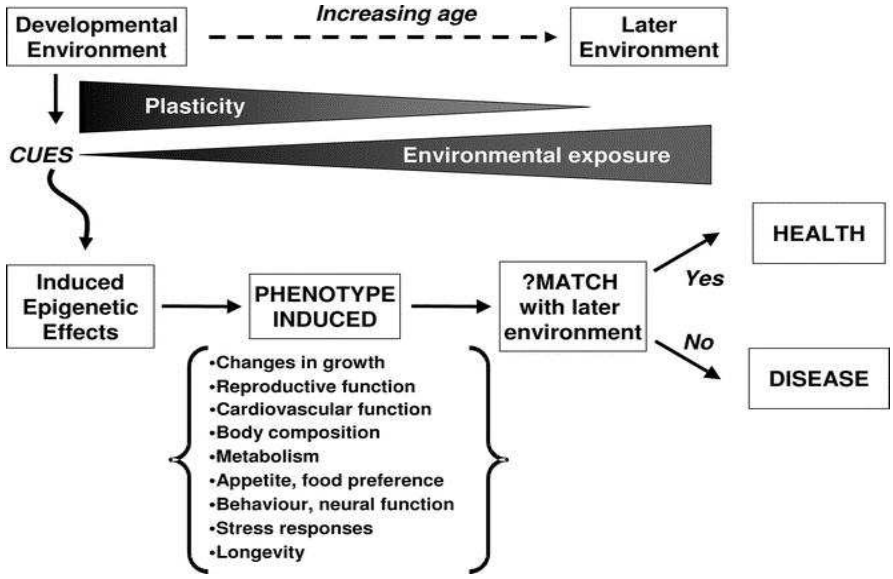

Figure 1: Diagrammatic representation of the effect of the environment during development. As the age of the individual increases its developmental plasticity decreases, this is a natural developmental stage which prevents differentiated parts of the body altering themselves. While this happens, the effect of environmental exposure increases, this allows for the growing fetus to alter some of its phenotypes to become better adapted to the environment it will experience when outside the womb. This adaptive programming can cause an increase in the individuals' disease risk if the environment it is adapted for is not the actual environment that it experiences during its lifetime. Reproduced from (Godfrey 2007).

Diseases known to be associated with increased risk from altered maternal environment such as under nutrition include decreased glucose tolerance, type II diabetes, coronary artery disease (Painter 2006) and obesity (Ravelli 1999). As an organism is developing its epigenetic profile has to adapt as each level of development is reached and then passed. It is at this time that the organism is most susceptible to any interference that may affect it at this level. This does not mean that this is the only time during the organism's life span that the epigenome will be altered but it is the most susceptible time for changes to occur.

1.1.3 Animal Models of DOHAD

Given that in humans studies, to establish the effects of environmental exposure during mothers or grandmothers pregnancy and to investigate the mechanisms behind these, would take many years, a number of animal models for developmental programming effects have been developed (Nathanielsz 2006). In general these models alter the extent or content of nutrition that is allowed to reach the fetus across the placenta.

Animal models allow for the direct effect of nutrition to be studied, as with the Dutch Hunger Winter cohort. The model of reduction of a single food group within the food source is more representative of a typical situation found within normal human situations. The reduction of total nutrition, such as the Dutch Hunger Winter Famine, is more severe and requires unusual circumstances for exposure within a human population.

Implementing a nutritional challenge can be done in several ways. One of these is placental ligation, where the blood flow is restricted from the placenta leaving less to reach the fetus, thus reducing the total nutrition it receives. Another way is to alter the amount, or balance, of nutrients the mother is allowed during pregnancy. This could be altering the total calorific intake to the dam, total under-nutrition, or it could be that the normal animal food is replaced with another type that contains less of a certain food group, such as protein, and is then made up with another group to keep the overall diet isocalorific.

As detailed later in this chapter, the timing of the challenge has an effect on the phenotype of the offspring. This is due developmental windows. Exposure to a dietary challenge in the early stages of gestation can effect the organs of the fetus while an exposure seen in later gestation will not effect the organs as they are already formed but could effect the birth weight as the later period is involved with increasing the size of the fetus and not the development of the fetus. This is important to note with the Hertfordshire cohort as it used birth weight as a surrogate measure for fetal nutrition. Due to these effects, with nutritional challenges it is important to note the time and type of challenge that is given.

1.1.4 Offspring Effects

1.1.4.1 Maternally Inherited Offspring Effects

Many studies have looked at the effect of the maternal line, usually through nutrition, as in the above examples. As there are no other human examples than those previously discussed, the following studies look at data from animal models. Most of these studies have utilised a rat model. Severe dietary challenges have been used to look at different aspects of nutrition alteration in the development and function of the organs and tissues found to be associated with disease. For example, an increase in vasoconstriction, but no change in vasodilation, was found in male rats whose dams were given a severe restriction of total nutrition in-take from conception until term, and then a high fat diet, with 45% of their energy in-take derived from fat, for the offspring until culled (Torrens 2008). These rats had endothelial dysfunction, a symptom of cardiovascular disease and metabolic syndrome. This study involved four different groups, those on control diet throughout, birth dams and offspring; those dams restricted nutrition during pregnancy but offspring on control; those dams on control diet and the offspring on a high fat diet from birth until culling, and those dams on restricted nutrition during pregnancy and then their offspring on the high fat diet from birth until culling. Interestingly it was the two groups where the dams experienced nutrition restriction during their pregnancy that showed endothelial dysfunction showing that the level of maternal nutrition is as important as the high fat diet in these vascular alterations.

The reduction of methyl groups within the pregnant dams diet, essential for epigenetic changes such as DNA methylation at CpG sites to be made, resulted in male offspring with an almost 40% higher concentration of insulin within their system when compared with controls, indicating a shift towards type II diabetes (Maloney 2011). This diet was given to dams from three weeks prior to conception and for the first five days of gestation. When plasma homocysteine levels, a source of methyl groups that is provided endogenously, were checked, those rat dams that had been fed a methyl-deficient diet had increased levels. This could be due to the dam trying to make up for the loss of methyl groups within the diet by supplementing with her own during this period (Maloney 2011) .

The utilisation of models involving the reduction of one source of essential food is fairly common place within the literature, with one of the most common being a reduction in protein. This also acts as a methyl-donor source. The effects of different levels of reduction within this food group have been studied in several different organs. Gressens et al. (1997) used a 75% reduction of protein, which was supplemented within the diet to produce an isocalorific result between the control and treatment group rats, from confirmation of pregnancy until 2 weeks of gestation to look at the development of the brain in the resulting offspring. They found that there was no change in the overall rat brain weight; however the development of the brain was altered, smaller cortical thickness, in the animals on the low protein diet. This difference only

occurred at 2 weeks postpartum and all the animals' brains were similar at the next observed time point indicating some sort of catch up growth within the treatment animals (Gressens 1997).

A smaller reduction of protein, around 50% but keeping an isocalorific total diet, in mice was given to three different groups (Chen 2009). Reduced protein was given from conception until the end of lactation for one group, from conception until birth for another, and the final group was on the control diet throughout. The first group describes a diet where the offspring experience low protein diet throughout its early life, mothers pregnancy and then after until weaning, the second experiences a low protein diet and then a normal diet allowing a period of catch-up growth which was not present in the first group. This had an effect on the life-span of the offspring with the first group have a shorter life than those in the second group. When compared to control, the first group had improved insulin sensitivity, showed lower organ weights, e.g. heart and liver, and had smaller body weights up to three weeks of age.

The same level of dietary restriction, 50% maternal protein, has been used in several rat studies, although the exposure has been made over different time periods. When this diet was given for two weeks before conception until term, the offspring from the protein restricted group had increased blood pressure and levels of glucocorticoid enzymes within their brain and liver, and had decreased binding capacity of type II glucocorticoid receptors (Langley-Evans 1995). It was postulated within this paper that the reduction in the binding capacity of these receptors could be the reason behind the increase in blood pressure within these individuals. Other studies have started dams on a 50% protein restricted diet from conception until term. One study found a decrease in the level of methylation of both the PPARα and GR promoters of the male offspring from the protein restricted dam group. This was found to persist through to the F2 generation as well (Burdge 2007). The same diet run over the same time frame showed hypertension within the male offspring but not in the female offspring (Woods 2003).

Interestingly several of these studies looked at the effect of maternal nutrition on both female and male offspring, however most effects are seen within the male offspring. This could suggest a sex linked effect.

1.1.4.2 Paternally Inherited Offspring Effects

Fewer studies have been undertaken examining the effect of paternal programming. In humans the male line seems to affect the offspring differently in some organs depending on the sex of the offspring. A study by Pembrey et al. looked at the effect of smoking on the BMI of the male's offspring. It shows that if the male started smoking early in life, before the age of 11 years, their sons, but not their daughters had an increased BMI at the age of 9 (Pembrey 2006). More recently, Arshad et al. (2011) studying a longitudinal

birth cohort from the Isle of Wight found that for childhood asthma, maternal asthma was significant in girls, but not in boys, while paternal asthma was associated with asthma in boys, but not in girls (Arshad 2011).

Animal models have also been utilised to examine at the effect of altered diet on male line inheritance. In the rat, effects of a high fat diet have been studied. Males were exposed to the high-fat diet from 4 weeks of age until 13 weeks, when they were mated to the females who remained on a control diet. This dietary exposure altered the phenotype of the fathers making them fat, with an increase in their glucose tolerance and insulin resistance indicating type II diabetes development. The daughters of these males were shown to have increased glucose tolerance but a decrease in their insulin production, due to dysfunction of the beta cells. This indicates a shift in their metabolism towards both type I, reduced insulin production, and type II, glucose tolerance, diabetes (Ng 2010).

A mouse model was also used to look at the male effect on metabolic systems. The diet that is used in this study is of more interest as it involves a reduction of approximately 50% in protein content. Male mice were randomly allocated to each diet which was introduced after weaning, around 21 days, and continued until 9-12 weeks of age when males were mated with females who had remained on the control diet. Both male and female offspring had altered gene expression of genes in the cholesterol and lipid metabolism pathways in liver tissue. However the number of animals in each group was small and the effect may not have been due to the reduction in protein but by an increase in sucrose that was also present in the diet. The methylation levels within the parental (F0) sperm were examined and found to be different dependent on the litter from which they came, but were not different dependent on the dietary exposure (Carone 2010).

1.1.4.3 Transgenerational Effects

The effect of epigenetics has been observed over more than just a single generation. For example, in humans, transgenerational effects have been observed where the initial environmental exposure occurred in F0 generation and was still present in F2. In this study F0 exposures such as poor nutrition or smoking during the slow growth period of the F0 generation, resulted in effects on life expectancy and growth through the male line and female line in the F2 generation, although there had been no further exposure (Pembrey 2006; Kaati 2007).

Studies of human cohorts have also provided evidence of transgenerational effects of environmental exposure on chronic disease risk. Kaati and colleagues studied a Swedish cohort of descendants of a population from northern Sweden where food availability within the population could be established from harvest records. In this cohort, sex dependent effects of poor food availability were evident, with their grandmothers' nutrition affecting their granddaughters' length of life and the grandfathers' affects their grandsons'. In general terms if there was a poor period of food availability before puberty then the F2,

granddaughters and sons, had an increased risk of death at a younger age, i.e. an increased mortality risk ratio (Kaati 2007). This study shows that there are risk factors from nutrition that can be effective through both the female and male lines and that this can be seen transgenerationally.

From studies it appears that the female line can affect the offspring for several generations, while the male line only appears to effect the next generation. The reason for this could be due to the development of the sperm. As mentioned above the female ova are laid down during their mother's conception and their gestation, leaving markers from this period. The male sperm is developed as the male hits puberty. This means that the environment that the male experiences during gestation is not present and so will have little or no effect when compared with that of the female line.

1.1.5 Mechanisms of DOHAD

As previously mentioned, organisms are predicted to be able to change their phenotype between one generation and the next. This led to the proposal that these changes were due to epigenetic alterations. These epigenetic changes are not permanent and do not alter the DNA sequence. They are usually caused by additions of chemical groups to key residues within regulatory sections of a gene, or of a histone protein causing it to coil or relax allowing transcription factors to copy the genes around that histone.

Changes within a gene promoter or enhancer region will usually result in a reduction of expression of the gene, however this is not always the case and some epigenetic markers increase the expression of the gene they are attached to. These changes occur naturally during development allowing for different genes to be switched on and off as required. They are essential for the organism's development.

The term epigenetics refers to changes in phenotype or an organism at either the molecular (e.g. gene expression), cellular or physiological level (e.g. appearance) that are heritable mitotically and/or mieotically. These changes are caused by chemical group additions to the DNA, or changes in the way DNA is packed within the nucleus (chromatin structure), rather than changes in the actual DNA sequence. The term was created (Waddington 1956) to describe how a developing embryo's genetic information would interact with the environment to determine development (Dolinoy 2006). This mechanism could account for part of the missing heritability within the genome. Epigenetic effects are reversible and so can be switched on and off depending on the needs of the cell at that point in time. Epigenetic mechanisms involve post-translational modification of histones, e.g. acetylation and methylation, methylation of DNA bases and the production of non-coding microRNAs.

The addition of methylation or acetylation residues to the histone subunits alters their configuration and secondary structure allowing change between an open chromatin structure, euchromatin, and a closed chromatin structure, heterochromatin. This alteration in structure changes accessibility of the DNA to

transcription factors that regulate mRNA transcription, and hence gene expression. Methylation of DNA bases tends to occur at CpG sites in the genome. The combination of the cytosine residue (C) followed by the phosphate link (p) then a guanine residue (G) is needed for the binding of a methyl group to the cytosine residue. Methylation of the DNA can prevent or alter the number of transcription factors binding to the DNA. There are areas of the genome that contain many CpG sites following each other. These areas are known as CpG islands and are usually associated with the promoters of genes. The addition of a methyl group to the DNA does not automatically mean that those genes associated with that promoter region have their expression reduced or stopped; it can also produce an increase in the expression of that gene.

1.1.5.1 Histones

To help protect and regulate DNA within the nucleus the DNA strand of bases are kept in tight structural hold. The first level of organisation of this structure is a double helix which holds the two stands of a single chromosome together. This double helix coil is then wrapped around structures known as histones. Histones are a complex of eight subunits of protein that form a ring shape (Strahl 2000). When the DNA is unwound from these proteins transcription can occur as the required apparatus can only get to the DNA once it has been unwound. This enable the cell to keep the DNA organised in as compact a structure as possible. Histone subunits undergo a number of post-translational modifications including acetylation and methylation at specific residues in tails that protrude from the nucleosome core. Collectively, these combinations of post-translational modification are referred to as the 'histone code', and through the recognition of this code by a range of chromatin remodelling proteins, regulate chromatin confirmation, and hence gene expression.

1.1.5.1.1 Histone Acetylation

Histone acetylation is the addition of an acetyl group to the chromatin that holds the DNA coiled around the histones. This is mediated by enzymes known as histone acetyltransferase (HAT) **(Ito K 2005)**. Histone acetylation generally favours an open chromatin configuration allowing gene transcription.

1.1.5.1.2 Histone Methylation

This is similar to acetylation except that a methyl group is added to one of the chromatin proteins. The enzyme used to do this is known as Histone methyl transferase (Strahl 2000). There are several enzymes that can reverse this methylation step (Swiqut 2007).

1.1.5.1.3 Histone Deacetylase, HDACs

Histone deacetylase's (HDAC's) are enzymes which remove acetyl residues from the histones. The addition of acetyl residues to specific residues on the N-terminal tails of histones generally favours a closed

chromatin configuration preventing gene transcription. Given this, HDAC enzymes are critical to control gene expression within the cells. There are several classes of HDACs, class I includes HDACs 1, 2, 3 and 8, class II includes HDACs 4, 5, 6, 7, 9 and 10, class III includes SIRT 1, 2, 3, 4, 5, 6 and 7 and class IV includes HDAC 11 (De Ruijter 2003; Senqupta 2004; Dokmonovic 2007).

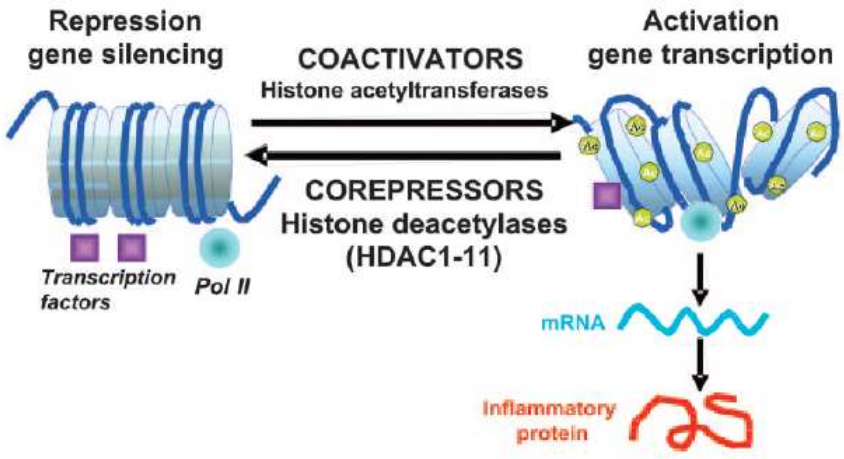

Figure 2: Action of HDAC and HAT enzymes on the configuration of chromatin. Reproduced from(Barnes 2006).

1.1.5.2 Micro-RNAs

Non-coding microRNAs are short lengths of messenger RNA, around 20 base pairs in length, which do not code for any protein. These short non-coding RNAs bind to specific combinations of nucleotides often found in the 3' UTRs of messenger RNAs to prevent or reduce translation of the RNA into the nascent protein (Tate 1993; Peterson 2004; Makeyev 2008).

1.1.5.3 DNA Methylation

DNA methylation is the addition of methyl groups to cytosine bases in the DNA. This is done by an enzyme known as DNA methyltransferase (DNMT) of which there are three subtypes. These are DNMT1, DNMT2 (now known as TRDWT1) and DNMT3, with family members DNMT3A, DNMT3B and DNMT3L. (Langer 2004) DNMT1, 3A and 3B methylate DNA in mammalian cells to establish regulation of gene expression, DNMT3L is thought to methylate with the result of effecting maternal imprinting. DNMT2 (or TRDWT1) does not methylate DNA (Goll 2006).

Methyltransferases are used normally in the cell to regulate transcription. The addition of these groups prevents transcription from occurring and reduces the expression of the gene into which they have attached. Addition of methyl groups usually occurs in CpG islands in the promoters of genes. CpG islands are areas of DNA rich in both cytosine and guanine bases, and make up more than 50% of the genome. These areas are usually between 300-3,000 bases long. This interference may not totally prevent transcription of the gene

but can lower the total level of the resultant protein within the affected tissues. Other enzymes, such as DNA demethylase (Bhattacharya 1999), in the cell can be used to reverse this effect by removing the methyl group. It is the effect of the disease which alters the natural balance of these two sets of antagonistic enzymes and results in the phenotype displayed by the disease. The methylation status of the genome can be affected by diet, environment, naturally occurring mutations and pre-existing inherited methylation patterns (Jirtle 2007).

Methylation patterns can also be inherited from either parent as well as being affected by environmental cues. Another example of methylation is imprinting. This form of methylation is non-Mendelian but is parent of origin dependent and does involve inherited patterns. It involves the alteration of gene expression to ensure correct levels are found in the offspring, such as on the X-chromosome (Jirtle 2007).

1.2 Asthma

Asthma is a respiratory disease that causes reversible airway inflammation when in the presence of a certain stimuli. It is a complex disease that involves many different genes and their interaction with the environment. There are several forms of asthma: childhood asthma that begins in childhood and the patient grows out of it over time, persistent asthma where the disease is diagnosed during childhood and persisted during that individual's life, and adult onset asthma (McLean 2011).

1.2.1 Clinical Symptoms

An individual may exhibit no symptoms when there is no stimulus. However once the individual has been exposed to that stimulus, whatever it may be, symptoms become apparent. The clinical symptoms include wheezing, coughing, shortness of breath and tightness in the chest. Not all of these symptoms may occur at the same time, and some may never occur.

Wheezing can be heard using a stethoscope with each breath. It can be difficult to tell if the wheezing is due to asthma, or resulting from a respiratory infection. Persistent wheezing is usually not due to an infection. Coughing is common as the airways of asthma sufferers tend to produce more mucus than normal airways. Shortness of breath and tightness in the chest could also be from respiratory infection but are common in asthma as the lungs become inflamed.

Asthma can be triggered by many different types of stimuli and it is these many, and varied, causes that make treating asthma difficult. Some causes of asthma are listed in table 1.

Table 1: Causes of asthma exacerbations.

Cause	**Aspects of cause that are triggers.**
Allergens	Most asthma exacerbations occur in response to allergic reactions to common allergens. These include: Furry and feathered animals that release dander, small skin particles, that can result in irritation to the airways. Salvia and urine can also cause problems Mould and Fungi: spores released from these organisms. Pollen: especially from plants such as trees and grass House-dust Mite: specifically their droppings which can build-up in carpets, bedding, soft furnishings and soft toys Food: allergy can develop for many foods including cow's milk, eggs, fish, nuts and yeast, as well as some colourings and preservatives.
Air Pollutants	This is mainly from car exhaust fumes
Cold and Viral infections	This is one of the most common cause of asthma exacerbations
Emotions	Any emotion that increases the breathing rate, such as stress or extreme laughter, can increase the risk of inhaling a stimulus
Exercise	Exercise also increases the breathing rate
Hormones	Usually only around the time of puberty and in females

Medicines	These are mostly anti-inflammatory drugs, such as ibuprofen
Smoking	Many of the toxins in cigarette smoke can cause irritation to the lungs and result in asthma exacerbations
Weather	Exposure to a cold environment can cause irritation to the airways

1.2.2 Diagnosis

The presence of asthma symptoms does not mean that the individual has asthma and so a physician's diagnosis is important. While there is no definitive biochemical or physiological test that can diagnose asthma, physicians will diagnose based on reported symptoms, spirometery, evidence of bronchial hyperresponsiveness and response to treatment. It is difficult to diagnose asthma in very young children as there is no clearly defined way to differentiate between wheeze, caused by infection, and wheeze caused by asthma.

1.2.2.1 Spirometry

Spirometry is used to measure lung function. To measure lung function, an individual takes a large breath in and expels forcefully through a tube connected to a spirometer. The first second gives the forced expiratory volume in one second (FEV_1). This can give an indication of an obstructive pattern within the airways as the peak will be lower and the amount of breath left for the long exhale will be larger if airways obstruction is present. The long continuation of the breath gives an estimate for FVC (Forced Vital Capacity). This is the total amount of air able to be forcefully released from the lungs. If the individual has a poor volume for the FEV_1 but is able to continue the long breath afterwards this is an indication of a restricted pattern, most commonly associated with asthma. A ratio for the two measurements is usually also taken FEV_1/FVC (Forced Expiratory Volume in one second/Forced Vital Capacity) although this is mostly used in the diagnosis of COPD (Chronic Obstructive Pulmonary Disease) (Pellegrino 2005).

The results have to be normalised for patient age, sex, height and weight as these all contribute to the FEV_1. It is also important to identify the ethnic ancestry of the patient as the expected values are different depending on descent. For example total lung capacity, vital capacity and residual volume are around 12% lower in a black population than in a Caucasian one (Lanese 1978).

Spirometric results give an indication of the type of lung function problem as well as the severity of the condition. Figure 3 shows the spirometric results from different types of lung function complication, with the accompanying lung function measures in the table 2.

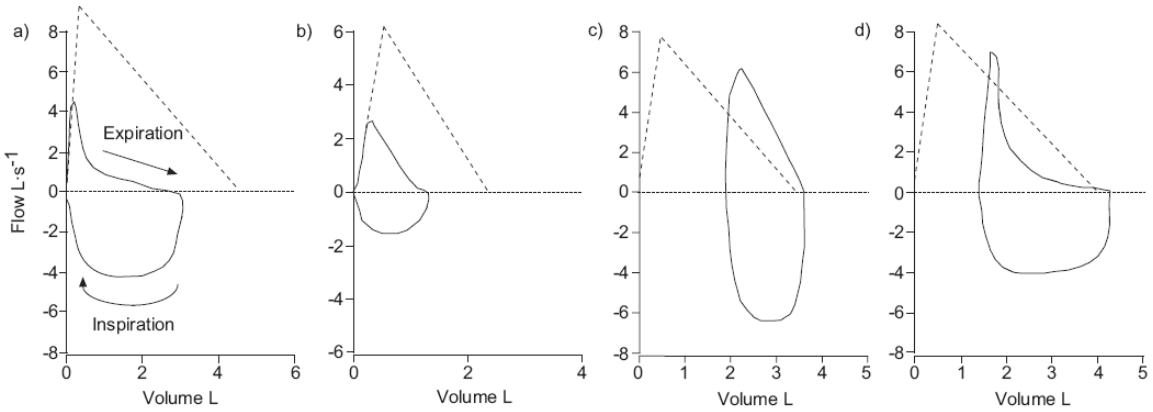

Figure 3: Spirometry result curves; for A) Obstructive, B) Normal, C) Restrictive and D) Mixed lung function complications. Reproduced from (Pellegrino 2005)

Table 2: Spirometry result values; for A) Obstructive, B) Normal, C) Restrictive and D) Mixed lung function complications. Reproduced from (Pellegrino 2005)

Lung Function complication	**FEV_1 %** (Forced Expiratory Volume in one second)	**FEV_1/FVC %** (Forced Expiratory Volume in one second/ Forced Vital Capacity)	**PEF %** (Peak Expiratory Flow)	**TLC %** (Total Lung Capacity)
A Obstructive	38	46	48	101
B Normal	57	73	43	96
C Restrictive	66	80	79	62
D Mixed	64	64	82	72

The obstructive lung function results show a decrease in the FEV1 and FEV1/FVC ratio values, of 38% and 46% respectively. This is due to the reduction in flexibility of the airways reducing the volume of air that can escape in the first second of the test and increasing the time it takes to evacuate the total volume of air. An example of an obstructive lung disease is Chronic Obstructive Pulmonary Disease (see section 1.3).

The normal lung function results show that usually half the predicted value of air is released in the first second, the FEV1/FVC ratio is above 70% with around 43% of the peak expiratory flow and almost all of the total lung capacity percentage.

The restrictive results show a high percentage for the FEV1 and ratio values but a lower total lung capacity value. This is due to the small airways being involved. The first expiratory blast is not affected but the long breath is usually not able to be completed. An example of a restrictive lung disease is asthma

The mixed results show a low FEV1/FVC value and a reduced TLC value.

Spirometry is a well known and often used method to help diagnose both different forms of lung disease as well as the severity of that disease, especially in the case of obstructive disorders. If an individual has normal parameter for their spirometry results but has had symptoms for a long period of time a methacholine challenge may be used to help with diagnosis.

1.2.2.2 Methacholine Challenge

One of the most commonly used diagnostic tests or asthma is a methacholine challenge, also known as a provocation test. This uses inhaled methacholine, an acetylcholine mimetic, as an agent to constrict the bronchi (Beckett 1992). This drug is diluted to form a concentration gradient going from no drug up to a possible value of 32mg/ml (Crapo 2000). Once the individuals FEV_1 (Forced Expiratory Volume in one second) has fallen by 20% of their normal flow the test is stopped and bronchodilators are administered until the FEV_1 value has normalised. It is the concentration of methacholine that is required to reach this 20% reduction that gives an indication of asthma for that individual as most asthmatics tend to show enhance constriction in response to methacholine challenge, this is termed bronchial hyperresponsiveness (BHR) (Birnbaum 2007). Bronchial hyper-reactivity testing can also be carried out using alternative constrictor agents such as histamine and adenosine.

1.2.3 Treatment

There are three main groups of drugs that are used for asthma treatment, all of which are aimed at relieving the narrowing of the airways by inflammatory infiltrates, as seen in figure 4. These are I) inhaled glucocorticoids, II) β-agonists and III) cysteinyl-leukotriene inhibitors (Drazen 2000).

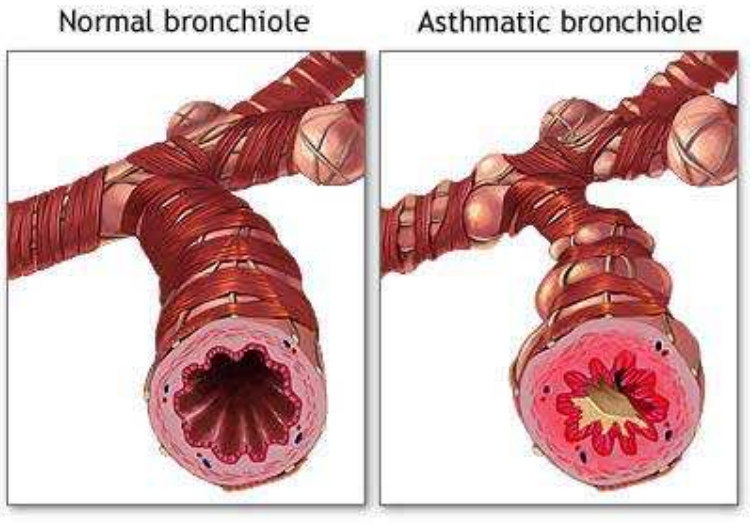

Figure 4: Normal and asthmatic bronchi, showing the narrowing of the lumen of the airway caused by inflammatory infiltrates, remodelling and increased mucus production. Reproduced from (AllreferHealth 2009)

Inhaled glucocorticoids have multiple mechanisms of action and are the mainstay of current asthma therapies. They are naturally occurring molecules that have two main functions with in the body. The first is a reduction in pro-inflammatory mediators and the second is an increase in the production of anti-inflammatory mediators. The glucocorticoid proteins bind to their receptors which dimerise and translocate to the nucleus, where they bind to specific response elements in gene promoters, enhancing transcription of genes encoding anti-inflammatory proteins and inhibiting transcription of pro-inflammatory mediators

Glucocorticoids have a very narrow therapeutic index as they are toxic to the body. This means that there are several side-effects that occur with their use. These include immunosuppression, insulin resistance, osteoporosis, glaucoma and cataracts. Hence most glucocorticoids are delivered topically to the airways through the use of inhaler devices. However there are some asthmatics that do not respond to glucocorticoids. These individuals are known as glucocorticoid resistant and require other types of treatment for their asthma medication. (Kamada 1996)

B2-agonists are effective bronchodilators that bind to β2 adrenergic receptors that are expressed in the airways and cause the smooth muscle to relax by increasing the intracellular level of cAMP. There are two main types of β2-agonists, long-acting and short-acting. Long-acting β2 agonists usually last up to 12 hours after they have been given and are used as a preventative medication. They are prescribed for the long term control of symptoms within persistent asthma, nocturnal asthma and to help prevent exercise-induced asthma. Short-acting β2-agonists have a much shorter half-life and only work for up to an hour after administration. They work in the same way as the long-acting form and are used as rescue medication for sudden bronchoconstriction.

Cysteinyl-leukotriene inhibitors are used in asthma treatment as they prevent the action of leuoktrienes that are one of the main pro-inflammatory mediators. These lipids are released from leukocytes at sites of inflammation. They stimulate the production of molecules that cause micro-vascular leakage, allowing eosinophils into the airways and increasing the inflammatory response as well as being potent bronchoconstrictors (Ducharme 2004) .

1.2.4 Prevalence

The prevalence of a disease gives an idea of the number of patients within a given population that have that disease at a given time. Respiratory diseases are one of the largest disease groups in the UK. In 2004 around 1,942 out of every 10,000 males and 2,516/10,000 females had a respiratory disease. It is the second most common cause of emergency admissions in the UK. In the UK the prevalence of asthma in 2004 for males was 409/10,000 people and 484/10,000 for females. There were around 1.381 deaths caused by asthma in 2004 (British Thoracic Society 2007).

Around one third of the UK population have experienced wheeze in the last year. Asthma diagnosis is higher in children (21%) than in adults (15%). Recent wheeze and diagnosed asthma have increased since 1960 and have given the UK the highest asthma prevalence in the world. Hospital admissions for asthma made up 12% of the total admissions in 2004/2005, and around 10% of doctor consultation visits (British Thoracic Society 2007).

1.2.5 Pathogenesis

Asthma may be classified as atopic (extrinsic) or non-atopic (intrinsic), based on whether symptoms are precipitated by allergens (atopic) or not (non-atopic). Atopic inflammation results from specific IgE mediated immune responses to allergens. Atopy can also cause other allergic conditions such as eczema, hay fever and allergic conjunctivitis. While the majority of asthmatics are atopic, some individuals exhibit non-atopic asthma. This results in the same clinical symptoms as atopic asthma but is often induced by factors such as anxiety, stress, exercise, cold air, dry air, hyperventilation, smoke, viruses or other irritants such as occupational exposures.

There are two stages of inflammation in asthma, the early-phase and the late-phase. The early-phase reaction involves cells, such as mast cells, that have IgE antibodies bound to the high affinity IgE Receptor (FcεR1) on their surface. Upon an encounter with an allergen and subsequent cross-linking of IgE receptors, pro-inflammatory markers are released, such as histamine, eicosanoids and reactive oxygen species. These molecules induce the contraction of the smooth muscle around the airways, an increase in mucus secretion and induce vasodilation. (Bousquet 2000)

The late-phase reaction occurs between 6 and 9 hours after the initial encounter. This involves the recruitment to the airways, and activation of, eosinophils, CD4+ T cells, basophils, neutrophils and macrophages. The recruitment of these cells from the blood is facilitated by increased expression of adhesion molecules due to the action of inflammatory cytokines. The up-regulation of adhesion molecules (CD11a, CD11b, CD18, VLA-4, ICAM-1 and VCAM-1) is all critical for the induction of an inflammatory response. (Elias 1999)

Asthma reactions are dependent on $CD4^+$ T lymphocytes that have been skewed to a Th2 phenotype. It is the Th2 cytokines, IL-4, IL-5, IL-9 and IL-13; that drive asthma pathogenesis. IL-4 skews the T cells towards a Th2 phenotype and results in an increase in serum IgE through inducing isotype switching in B-cells, IL-5, granulocyte-macrophage colony stimulating factor and IL-9 are involved in the production of eosinophils. IL-13, like IL-4, also promotes switching of B-cells to an IgE producing phenotype and is responsible for the change in the airways towards hyperresponsiveness and mucus metaplasia all of which results in airway remodelling (Wills-Karp 2004).

1.2.6 Remodelling

The severity and chronicity of inflammatory episodes lead to remodelling of the airways. This is because the airways become damaged and inflammatory cells infiltrate to repair the damage that has occurred. In the case of asthma these instances of damage are often close together and the first set of inflammatory mediators has not been dispersed before the next set arrives. This build-up of inflammatory mediators causes the remodelling seen in the asthmatic airway. The protective effect works through two ways, one is to regenerate the tissue that has been damaged and the other is to replace that that has been too damaged to repair, this leads to the formation of scar tissue. This remodelling causes thickening around the airways altering their structure (Vignola 2000).

Remodelling within the airway involves an increase in the number of goblet cells, which increases mucus production, causing the coughing symptom of the diseases as the individual tries to clear it. The number of epithelial cells also increases and this is not site specific for injury, but throughout the luminal surface area. This forms a thicker layer of epithelial cells making the lumen smaller (Törmänen 2005). This reduces the airflow and increases the airway surface tension making it more likely to collapse (Bousquet 2000).

The amount of smooth muscle around the bronchi is also seen to increase in airways of asthmatic patients and results from both hyper- and hypoplasia of the smooth muscle cells. This feature of airway remodelling is not usually seen in either chronic bronchitis or chronic obstructive pulmonary disease. This increase in muscle mass around the airways could be due to inflammatory mediators driving cell proliferation, or purely by the increase in the work undertaken by these muscle cells as they constrict the bronchi. The increase in smooth muscle cells during bronchoconstriction results in increased airway resistance. Computer models have predicted that within asthmatic airways these muscle cells only have to shorten by 40% to completely close the airway lumen (Bousquet 2000). Within normal lungs airway resistance reaches a plateau but continues to increase in asthmatic lungs as the airways close. This links in with spirometery FEV_1 readings where the value for this levels off in normal lungs but continues to decrease in asthmatic lungs (Vignola 2003).

The extra-cellular matrix is the area between the cells that compose the airway. It consists of fibrous proteins, such as collagen and elastin, structural and adhesive proteins, such as fibronectin and laminin, as well as a hydrated polysaccharide gel (Bousquet 2000). It is the composition of this that greatly alters airway structure and strength.

Thickening of the basement membrane is also seen in asthma remodelling. This membrane is made up to two layers, the basal lamina and the lamina reticularis. It is the lamina reticularis that is thickened within asthma (Bousquet 2000). This can be seen even in the early stages of asthma. This thickening occurs

due to the deposition of immunoglobulins and collagen. Both collagen-I and collagen-III, increases within the extra-cellular matrix around the bronchi.

Remodelling involves the small airways first and progresses to affect the larger airways and has been linked with changes in vasculature.

Within an asthmatic lung the vessels are larger which increases the thickness of the airway wall. This is due to high levels of inflammatory mediators, such as prostaglandins and adenosine, as well as cytokines and growth factors. The presence of these molecules drives expression of the vessel growth gene VEGF. The inner diameter of vessels increases as well coinciding with an increase in proliferating endothelial cells.

1.2.7 Genetic Basis of Asthma

Familial and twin studies suggest that there are strong underlying genetic factors which cause those individuals with asthma to be susceptible (Hemminki 2007). It has been suggested that approximately 60% of asthma risk is genetically based and the other 40% due to environmental exposures (Duffy 1990). Asthma has been found to run in families, with an increased risk of the offspring developing asthma if it is present within the family already (Burke 2003). However the overall effect of non-asthmatic older siblings is protective for younger children as they have a higher rate of exposure to external allergens (Leadbitter 1999). It has also been noted that the risk of developing asthma is increased for males when compared with females (Kay 2001). This may suggest that one or more asthma susceptibility genes could be sex linked.

Many studies have been undertaken that have looked into the genes associated with asthma. There are two main genetic study types: 1) hypothesis based candidate gene studies, and 2) hypothesis independent studies, such as genomewide linkage studies in families and more recently Genome Wide Association Studies (GWAS).

Candidate gene studies select a gene whose protein is known to play a role in one of the processes involved within the disease. Genetic variation, usually Single Nucleotide polymorphisms (SNPs), within the gene encoding the protein, which may affect gene expression or protein function, is then assessed for association with the disease or related phenotypes. There have been many genes shown to be associated with asthma utilising this approach including genes involved in regulation of atopic immune responses, oxidant stress and tissue repair mechanisms. (Holloway 2010)

While candidate gene studies increase understanding about individual susceptibility to disease, in general they do not provide more information on disease pathogenesis as by definition, the protein encoded was already known or suspected to be involved in the disease to be selected as a candidate for genetic analysis. In contrast, hypothesis independent genomewide approaches have the potential to identify novel genes that encode unknown proteins that play a role in disease pathogenesis. For example, in asthma,

gemonewide linkage analysis in family based samples has identified several novel genes e.g. ADAM33 and PCDH1 as having a role in asthma pathogenesis.

The gene encoding a disintegrin metalloproteinase 33 (ADAM33) was the first gene to be identified for asthma using positional cloning (van Eerdewegh 2002) and has been associated with the development of smooth muscle around the airways. It has also been implicated in remodelling and associated with asthma (Ober 2006; Vercelli 2008). Links have also been made with development of COPD in Asian populations (Sadeghnejad 2009; Xiao 2010), and protocadherin-1 (PCDH) (Koppelmann 2009) genes as being associated with asthma.

PCDH is a membrane bound protein that is found at all cell-cell boundaries. It is involved in neural cell adhesion and so may play an important role in the development of the neuronal system. The protein itself includes an extra-cellular region that contains seven cadherin-like domains, a transmembrane region and a C terminal cytoplasmic region. Any cells that express this protein are prone to aggregation activity. This protein has been linked with bronchial hyperresponsiveness in Asthmatics from several Caucasian populations (Koppelmann 2009).

GWAS studies utilise hundreds of thousands of SNPs across the genome to test for association with the phenotype in question. Recent GWAS studies in asthma have identified a number of novel genes of unknown function as being associated with asthma susceptibility. For example, the first novel asthma susceptibility locus to be identified by GWAS contains the orosomucoid 1-like 3 (ORMDL3) and Gasdermin-like (GSDML) genes on chromosome 17q12-21 (Moffatt 2007). In this study, 317,000 SNPs in 994 subjects with childhood on-set asthma and 1243 non-asthmatic controls were genotyped. Subsequent studies in ethnically diverse populations have replicated the association between variation in the chromosome 17q21 region (mainly rs7216389) and childhood asthma (Holloway 2010).

1.3 Chronic Obstructive Pulmonary Disease, (COPD)

Chronic Obstructive Pulmonary Disease (COPD) is an irreversible inflammatory disease of the lungs that is typically late onset with most COPD occurring after 40 years of age. COPD is becoming a leading cause of morbidity and mortality in the UK. At present it is the 5th biggest killer in both the UK and world wide (National Statistics 2006). However it is estimated that it will become the 3rd most common cause of death in the UK in 2020 (Murray 1997). COPD is diagnosed as a restrictive deficit in lung function that is not reversible. The GOLD (the Global initiative for chronic Obstructive Lung Disease.) defines COPD as 'a disease state characterised by airflow limitation that is not fully reversible. The airflow limitation is usually both progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gasses' (Pauwels 2001). There are a number of pathophysiological processes that can lead to a diagnosis of COPD including chronic bronchitis, remodelling of small airways and fibrosis, and emphysema.

1.3.1 Clinical Symptoms

The very early stages of COPD are usually undetectable by the individual. Mild (GOLD stage I) COPD has a diagnostic criteria in terms of reduced lung function, but generally patients experience no symptoms. Typically, the first symptoms exhibited by patients with moderate COPD (GOLD stage II) include shortness of breath when completing every day tasks. This is usually attributed to age and smoking, as smoking is the strongest risk factor for development of COPD. Patients with moderate COPD have reduced lung function and the symptoms become much more noticeable. There may also be instances of coughing, congestion and wheeze. The ability to complete everyday tasks is drastically effected and exacerbations begin to occur. The final stage of COPD (Severe, GOLD stage III/IV) often requires supplementary oxygen therapy, and any exacerbations that occur can be fatal.

The reason behind the breathlessness is the inability of the lungs to efficiently remove the air that is already present before the next breath is taken. As seen in figure 5, a normal lung will expand during a period of exercise with the amount of gas exchanged increased. A COPD lung increases the amount of air coming in but is unable to increase the volume going back out. This means that the overall gas exchange is nowhere near the volume of that seen in the normal lung. A limit to the amount of gas volume alters the partial pressure of each gas and reduces the efficiency of gas exchange from the lung to the blood reducing the amount of oxygen available to the body. This leaves the individual breathless.

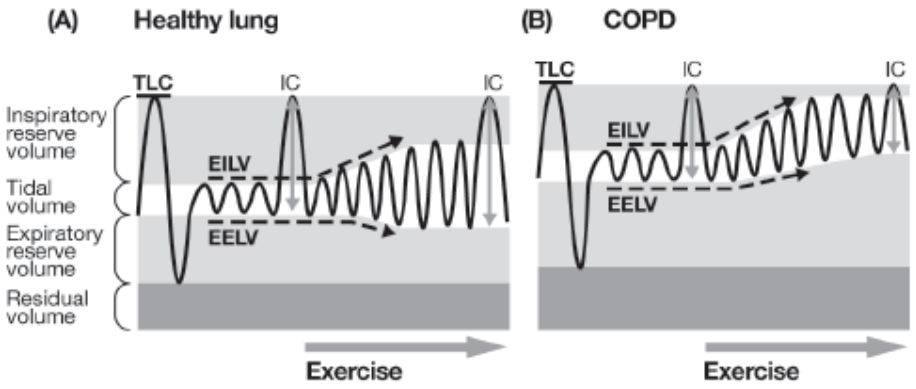

Figure 5: Spirometery readings with exercise. As exercise continues the normal lung increases its gas exchange volume by having more gas taken in as well as by removing more gas in the exhale. The COPD lung is able to increase the volume of gas in but not the volume that is released. This reduces the efficiency of the gas exchange and leaves the individual breathless. Reproduced from(Jones 2011).

1.3.2 Diagnosis

COPD is difficult to diagnose as its symptoms are also common signs of ageing, however the number of people being diagnosed with COPD in the UK is on the increase, in part, due to the improved methods of diagnosis (Tinkleman 2007). The most common way of diagnosing COPD is by spirometery, with FEV_1/FVC to be below that of 70% or 0.7. The values for this ratio are measured by spirometery readings as described in section 1.2.2.1. This disease shows an obstructive pattern, as seen in figure 6. The individual is unable to expel the normal volume of air in the first second of the challenge, making the initial peak less, and the volume being expelled is higher for longer over the rest of the test due to this.

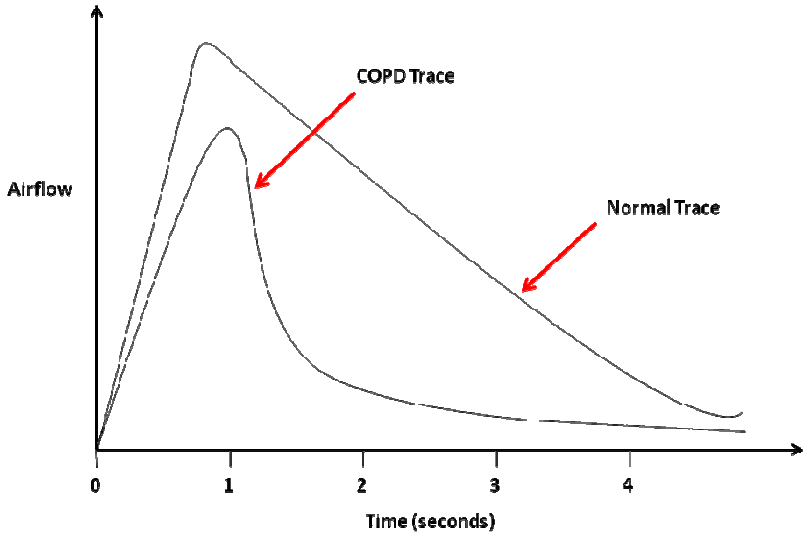

Figure 6: Stylised spirometery trace comparing a normal lung with a COPD lung.

Once this ratio is known the individual is given a severity rating as dictated by the global initiative for chronic obstructive lung disease (GOLD) standard. A ratio of less than 0.7 where the FEV_1 is more than or equal to 80% of that predicted is considered as mild (class I), if the FEV_1 is between 50 and 79% of that predicted it is considered moderate (class II), between 30 and 49% severe (class III) and if the FEV_1 is less than 29%, or less than 50 % but with chronic respiratory failure, the individual is considered to be very severe (class IV). Figure 7 shows a normal healthy lung on the left and a smoking, COPD patient's, lung on the right. The COPD lung is black due to the deposits left behind from smoking.

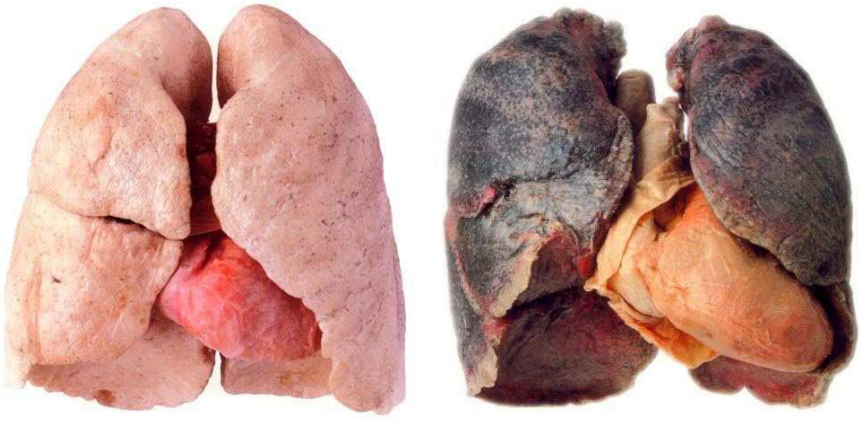

Figure 7: Normal healthy lung compared with COPD, smoking lung. The normal healthy lung on the left with a larger overall size and pink healthy tissue, the COPD lung on the right is smaller and the black discolouration is caused through smoking, the biggest risk factor for COPD. Reproduced from(mylot 2005).

Under-diagnosis of COPD is thought to be very common. It is estimated that around only 25% of COPD cases are diagnosed (Manfreda 1989; Lundback 1991; Viegi 1991). This is due to a lack of physicians' awareness of the symptoms and factors involved in COPD, as well as a possible perception that the condition was self-inflicted. The use of spirometers as a diagnostic tool is low and the education available on COPD is minimal. The patients themselves are also a factor as they believe that the symptoms are just a result of their age and life style and do not push for an actual diagnosis (Jones 2011). A study by Tinkleman et al. (2007) indicated that introducing screening of smokers over the age of 40 the number of cases of diagnosed COPD could increase by 10-20% (Tinkleman 2007).

1.3.3 Treatment

There is no treatment that has been shown to stop the progression of decline in lung function as this is a natural process. This means that only the symptoms of the disease can be treated. It is important to note that although corticosteroids can be used to treat asthmatic inflammation they do not work for inflammation in COPD, this aspect of the disease will be discussed further in section 1.3.9. However the

biggest risk factor in COPD is smoking and this increases the rate of lung function decline. The best way to reduce the rate of reduction is to stop smoking. A study by Kohansal *et al.* showed that in healthy individuals that had never smoked lung growth peaked earlier in women than in men and those women also had a slower rate of decline. Smoking was found to increase the rate of decline and the susceptibility of individuals to smoking varied (Kohansal 2009). The only help that can be given to individuals suffering from this disease is to treat symptoms.

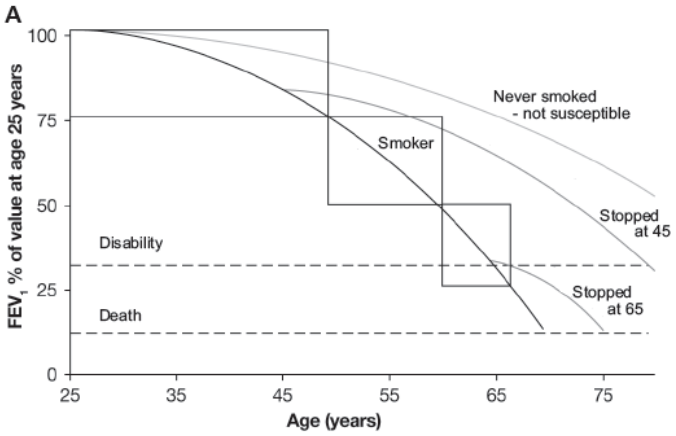

Figure 8: Rate of lung function decline is reduced by stopping smoking. The graph above goes from peak lung function, at 25 years, to 75 years, and shows the effect smoking, and the cessation of smoking has on the decline of an individuals lung function. Reproduced from(Jones 2011).

Treatment of the symptoms depends on the severity of the disease classified by the GOLD standard. If classified as mild (class I) the only treatment is to stop known risk factors, such as smoking, and having an annual flu vaccination to help prevent exacerbations. Moderate COPD (class II) sufferers are advised to take the same precautions as class I with the added treatment of bronchodilators as needed. These bronchodilators work in two different ways, long-acting β2 adrenergic receptor agonists, (e.g. salmeterol and formoterol) help to relieve airway narrowing of the airways through their β2 stimulation, relaxing the smooth muscle surrounding the airways. Long-acting muscarinic antagonists (e.g. tiotropum) work on the cholinergic tone of the airways reducing the contraction of airway smooth muscle aiding reduction in hyperinflation.

Long-acting β2 agonists are known to improve symptoms and reduce the need for rescue medication (Ramirez-Venegas 1997; Mahler 1999; Campbell 2005; Derom 2007). It is also known that formoterol has a faster onset of action then salmeterol (Cote 2009), however if the patient is taking a standard daily dose this has no effect. As the disease progresses the prescribed dose of twice daily is given to act as a base line treatment to prevent exacerbations.

There is only one long-acting muscarinic antagonist commonly used in COPD treatment, tiotropium. This drug is the one most commonly used as a maintenance drug as it is only used once a day and has a 24 hour effect resulting in bronchodilation (Briggs 2005; van Noord 2005; Wedzicha 2008; Brusasco 2011). The use of both a long-acting β2 agonist and a long-acting muscarinic antagonist has been found to be more effective than either type on in own (van Noord 2005; Cazzola 2009; Singh 2011).

If classified as severe (class III), inhaled corticosteroids are added to the treatment plan in response to exacerbations. The final level of severity, very severe (class IV), includes all the treatments for the lesser degrees of severity as well as long term oxygen for respiratory failure and the consideration of surgical intervention.

Treatment with oxygen therapy increases the partial pressure of oxygen within the lungs, meaning that more is passed from them into the blood system and then on to the rest of the body. By this stage of the disease the individual needs this increase in oxygen as they would suffocate otherwise. This is known as chronic respiratory failure.

Surgical intervention involves the removal of sections of the lung where the elastase has been destroyed. The alveoli in these areas are larger and have no elastic recoil and so air is not pushed back out of the lungs. This means that the efficiency of gas exchange is reduced and less oxygen enters the blood stream. By removing the affected tissue, the over all efficiency within the lung increases allowing better gas exchange within the lung which provides more oxygen for the body.

1.3.4 Incidence

In the UK the incidence of COPD was estimated to be between 25,000-30,000/ million in 2007 (Blanc 2007). This figure has increased recently due to better methods of detection. Once the initial diagnosis has been made the severity of the condition is assessed and given a score using a predetermined scale. One of the biggest concerns with COPD is the level of under diagnosis. The reasons behind this have been detailed in section 1.3.2.

Interestingly a study by Marco, 2007, found that the incidence of COPD in a population of young adults, 20-44 years old, was 2.8 cases/1,000/year (de Marco 2007). This figure was derived from data whose source was the European Community Respiratory Health Survey II.

1.3.5 Prevalence

A global estimate of COPD prevalence has been given as 9-10% in individuals over the age of 40 years (Halbert 2006). In the UK the prevalence of COPD was thought to be around 1.5 million but a study on a sample population resulted in a much higher figure with 13.% of individuals over 35 having COPD (Shahab 2006). This equates to 3.4-3.8 million people nationally. A study from China gave a prevalence of 8.2% within

its population of over 40 years (Zhong 2007), and a Danish population study found their prevalence of COPD to be 12% between the ages of 45 and 84 years (Hansen 2008). Within the USA morbidity from COPD is estimated at 4% making it the 4th leading cause of death (Hurd 2000). The prevalence of COPD is different in different countries as shown in figure 9. The increase in the number of individuals with this disease is partly due to better health care increasing the mean age of the population as well as a better understanding of the disease as it becomes more common.

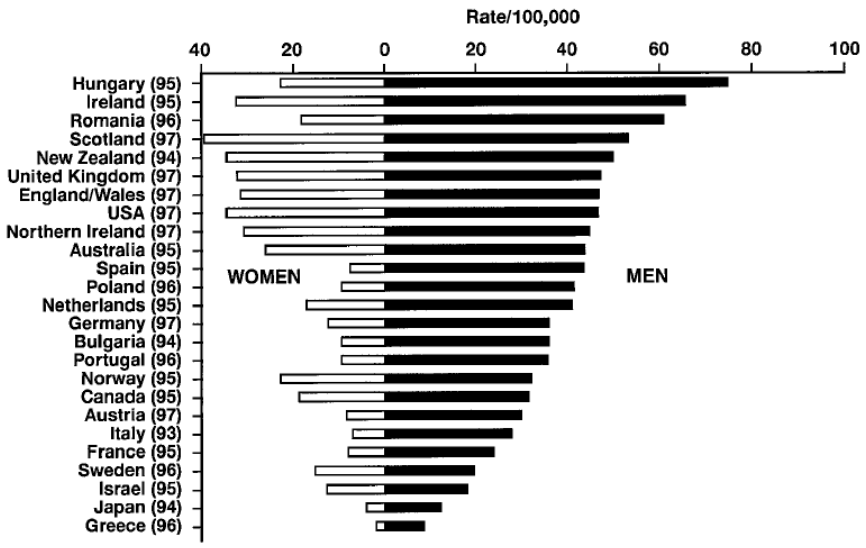

Figure 9: Differences in deaths caused by COPD, from several different countries for individuals between 35-70 years of age. This figure was based on data from the World Health statistics (WHO). Reproduced from(Hurd 2000).

1.3.6 Pathogenesis

The biggest cause of COPD is the oxidative stress that the lungs endure from the free radicals that are present in tobacco smoke and other exposures such as particulate air pollution. These lead to the release of cytokines that cause inflammation which leads to remodelling. Changes in the structure of the lung in COPD occur in two main areas. Firstly, in the small airways, inflammation drives goblet cell metaplasia, smooth muscle hyperplasia, and subepithelial fibrosis, resulting in narrowing of the airways, limiting airflow and is termed chronic bronchitis (Saetta 1994). Breathing out becomes harder for the individual as the pressure within the chest causes the airways to collapse instead of expand. This leads to hyperinflation of the lungs.

Airway fibrosis is the thickening of the wall of the airway (figure 10). The reduction in contractibility within the airway, caused by fibrosis, impedes the body's natural reaction to harmful stimuli and exacerbating factors.

In the alveoli, the inflammatory response causes destruction of the lung, a process known as emphysema. Emphysema is a pathological diagnosis defined by permanent enlargement of airspaces distal to the terminal bronchioles. This causes a significant decline in the alveolar surface area available for gas exchange. In addition, loss of alveoli leads to airflow limitation by both a decrease in elastic recoil, and airway narrowing as a result of the loss of the alveolar supporting structure. This reduces the ability of the lungs to push out air on the exhale, as there is no elastic recoil when lungs relax, resulting in a decrease in vital capacity as well as a decrease in FEV_1. Emphysema is due to alterations in the deposition of the matrix around the alveolar tissue. Less elastin is present reducing the lungs elastic recoil effect, and this destruction of the alveoli is due to the action of proteases, such as neutrophil elastase. Damage to the small airways structure and those that support the alveoli means that airways are prone to collapse as the patient breathes out and limits their ability to push air out due to reduction in elastic recoil of the alveoli. This is not reversible and the only option for severe emphysema is surgery (Geddes 2000).

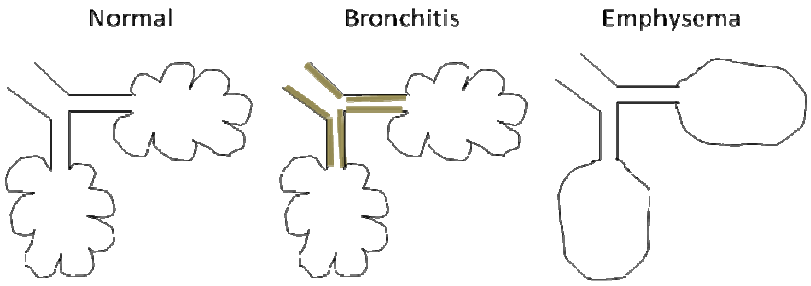

Figure 10: Normal airways compared with those with bronchitis and emphysema. The normal bronchi exhibit wide airways with large alveolar having a maximum surface area, bronchitis shows the thickening around the airways, fibrosis, and emphysema shows the decrease in surface area of the alveolar due to the breakdown in structure.

The rate of decline in lung function is normally at a steady rate until an exacerbation occurs. An exacerbation results from an inflammatory reaction to exogenous stimuli, that is often, in contrast to asthma, non-responsive to steroid treatment. The difference in steroid responsiveness between COPD and asthma exacerbations may be due to epigenetic influences that may be causal or develop due to COPD (Barnes 2006). These exacerbations cause a drop in lung function that is never regained and a steady rate of decline continues from this point after the exacerbation (Eaton 2009).

1.3.7 Genetic Basis of COPD

As previously mentioned, the highest risk factor for COPD in the developed world is smoking. However not all smokers develop COPD. This suggests that there is another underlying factor which causes those individuals with COPD to become susceptible to the disease. There is increasingly strong evidence that one such underlying risk factor for COPD is genetically based (Kueppers 1977; Silverman 1998; Gottlieb 1999).

Many studies have been undertaken that have looked into the genes associated with COPD. As with asthma these studies are of two main types, candidate gene and hypothesis independent approaches.

COPD candidate gene studies have identified several genes that are associated with COPD. For example, association was found with susceptibility to COPD and genetic variants in the gene encoding matrix metalloproteinase-12, this protein had originally been chosen as a candidate gene as they are known to degrade extra cellular matrix. It has been linked with airway inflammation and remodelling, as seen in COPD disease progression (Molet 2005; Xie 2005).

Using genomewide linkage studies in families has been more difficult for COPD due to the late stage of onset of the disease making recruitment of multigenerational families difficult. However, using early onset COPD (0 years of age), the Boston early onset COPD study identified the gene encoding serine protease inhibitor E2 (SERPINE2) (DeMeo 2006). This gene had been previously linked with respiratory disease as it, in balance with its antagonistic partners- serine proteases, regulates the lung extra cellular matrix. It has been proposed that an imbalance between these two sets of proteins can alter the matrix and result in degradation giving an emphysemic phenotype (Zhu 2007; Kim 2009; Kim 2011).

GWAS studies have more recently identified several genes as being associated with COPD. For example, using a GWAS approach Pillai et al. (2009) found genes such as the α-nicotine CHRNA3/5 receptor and hedgehog-interacting protein (HHIP) to be associated with FEV_1.

The identification of CHRNA3/5 as a COPD susceptibility gene highlights an important issue when studying the genetics of COPD. Most individuals are smokers, and so the genes identified could be associated with smoking and not COPD directly. HHIP is involved in development of the lung during embryogenesis and would not necessarily have been thought to be linked with the lung later in life, or disease state (Pillai 2009).

1.4 DOHAD and the Respiratory System.

1.4.1 DOHAD and Lung Function

Respiratory diseases are classically defined by pulmonary lung function, and lung function is in turn dependent on *in utero* environment. From birth, lung function increases until around 22 years of age, where it peaks and then steadily declines, as seen in figure 11. Thus lung function in adulthood is in part determined by lung development, as even with a normal rate of decline, lung function at a particular age is determined by peak lung function, which in turn is determined by lung development (Postma 2011) .

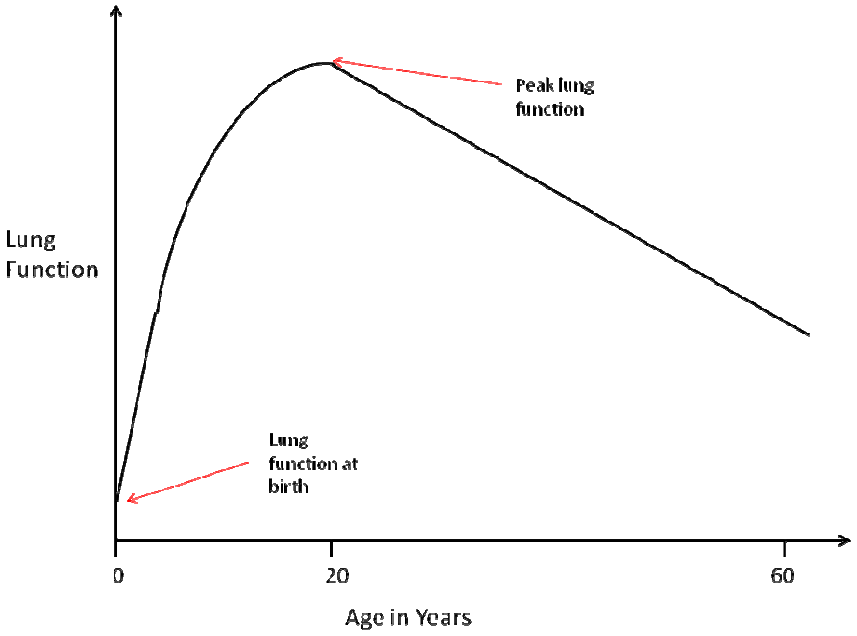

Figure 11: Interpretation of the Fletcher-Peto diagram.

While there are no studies that have looked at the effect of maternal nutrition on lung function across the life course in humans, studies have correlated early life exposure measurements with lung function measures cross-sectionally at a number of ages. By putting the data from all of these studies together the effect of lung function can be seen from birth up to the age of 65 years. Figure 12 shows how each of these studies fits into the overall age range for lung function.

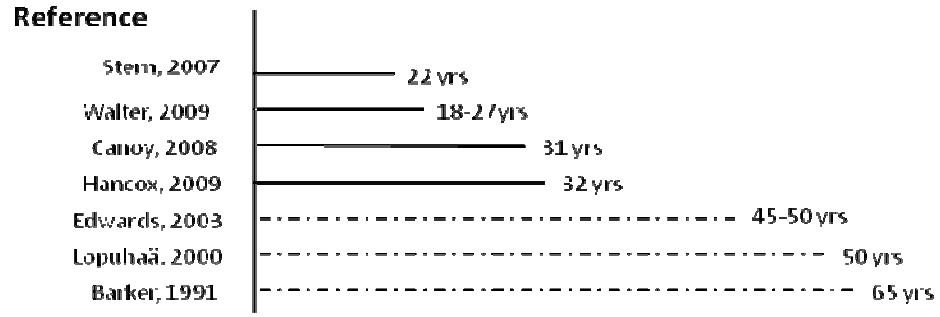

Figure 12: Overview of studies looking at the lung function during different time periods. The dashed lines represent a study that has data from birth and then has no further data until the age given.

Figure 12 summarises a number of studies that have information from birth and lung function at one or more points later in life. A number of studies have correlated birth weight (as a surrogate marker of maternal nutrition) with respiratory phenotypes in later life. An increase in the risk of becoming hospitalised due to any respiratory illness was also found to be linked with a low birth weight (Barker 1991; Lopuhäa 2000; Edwards 2003; Canoy 2007; Hancox 2009; Walter 2009). Each of these studies have looked at the effect of a low birth weight on lung function and at different ages during an individuals life, but there are no repeated measures of lung function throughout the life course. Therefore it is not possible to precisely identify how poor nutrition (as indicated by birth weight) leads to poor lung function in adulthood as it could be poor *in utero* growth of reduced childhood lung growth leading to a lower peak lung function, or alternatively altering responses to postnatal exposures (e.g. smoking) giving rise to a faster rate of decline in lung function from the peak.

The key study highlighting the importance of *in utero* lung development of lung function across the life course is the study of Stern et al. (2007) that looked at the lung function of individuals at birth through to age 22 years. In this study, subjects who had poor lung function (infant Vmax(FRC) in the lowest quartile) also had lower values for FEV_1/FVC (-5.2%, p<0.0001), FEF25-72 (-663 mL/s, p<0.0001), and FEV_1 (-233mL, p=0.001) up to age 22, after adjustment for height, weight, age, and sex compared to those in the upper three quartiles combined. The magnitude and significance of this effect did not change after additional adjustment for wheeze, smoking, atopy, or parental asthma. This highlights that poor airway function at birth was predictive of poor lung function in adulthood (Stern 2007).

1.4.2 Animal Models and DOHAD in the Respiratory System

As discussed in section 1.1.3, in order to provide causality for particular exposures or to investigate mechanisms behind the effects of exposures in developmental programming, it is necessary to utilise animal models. One of the closest models to a human lung is that of sheep in terms of timing of lung development during gestation. In sheep placental restriction, by umbilicoplacental embolisation, carried out from day 120

of gestation until term (147 days), leads to low birth weight compared to controls (Maritz 2004). Later tests, at the age of 8 weeks and 2 years postnatal, found that the treated sheep achieved the same body weight as the controls but that the structure of the lungs was altered. Restricted sheep had 28% less alveoli than controls and the lungs were 10% smaller in size. The alveolar walls were also found to be thicker. The result of these two structural changes meant that both the surface area of the lungs and lung elastic recoil were reduced.

Similar findings have been reported in rats. Two studies in rats, using differently timed 50% dietary restrictions with the dams, produced alterations in the lungs of the offspring. The first study started the dietary restriction at day 10 of gestation until term. These pups weighed less than the controls at 21 days of age but more at 9 months indicating some catch up growth. The lung weights were not different but the restricted group had a smaller number of alveoli and increased alveolar wall thickness (Karadag 2009). The second study involved a shorter exposure with dams given the restricted diet in the last trimester of their pregnancy. The offspring were found to have reduced volume fraction for air spaces, as well as a reduced surface area to volume ratio for the restricted group. The lung surfactant lipids were also reduced (Chen 2004). These studies show that an alteration in the diet during gestation can alter the structure of the lungs as well as changing the environment within the lungs, such as the surfactants present.

1.4.3 Development Origins in Asthma

There is a range of evidence that supports the hypothesis that asthma susceptibility is, to a large extent, determined by developmental influences. These include epidemiological studies in humans as well as evidence from animal models

1.4.3.1 Epidemiological Evidence for Developmental Origins of Asthma

As for the study of the role of developmental programming in many chronic diseases, most studies providing evidence for developmental programming in asthma rely on anthropomorphic measures, such as birth weight; head circumference; body length or ponderal index as surrogate markers of maternal nutritional status.

The matching up of these measures gives an indication of nutritional availability. If the birth weight is small but the other measures are larger this would indicate that the offspring should be larger and that body weight has been sacrificed to preserve brain/head development. It is important to have this set of measurements to be able to distinguish between small individuals and those experiencing *in utero* growth restriction.

Kurukulaaratchy et al. (2005) showed in a prospective birth cohort from the Isle of Wight that risk of early transient wheeze was increased by both maternal environmental tobacco smoke exposure in pregnancy (OR=1.58; 95% CI: 1.02-2.45), and low birth weight (3.65; 1.27-10.52) (Kurukulaaratchy 2003). However, this was not associated with persistent wheeze (asthma) at the age of 10. Leadbitter et al. (1999) studying a longitudinal cohort from New Zealand, found that after adjustment for other factors, recent asthma symptoms were positively associated with birth length (p=0.04) but not with head circumference. The adjusted odds ratio for asthma in the previous two years in infants with a birth length of 56cm or more was 6.4 (95% CI 2.0-19.8).Infants with a low birth weight of less than 3.0 kg had an odds ratio for reported asthma of 0.2 (95% CI 0.0-0.6). Associations between anthropomorphic measures and total IgE levels (as a marker of atopy) were also seen in this cohort. While the authors of this study acknowledge that precision of the findings is limited by the small numbers in the extreme categories of each birth parameter, the results are consistent with intrauterine programming of the developing respiratory and immune systems (Leadbitter 1999). In support of the observations of Leadbitter et al. (1999), Shaheen et al. (1999) studying the 1970 British Cohort Study, showed that the prevalence of asthma at 26 years fell with increasing birth weight (Shaheen 1999). More recently, Bierg et al. (2011) have shown that in a Dutch birth cohort, while low birth weight alone was not associated with increased risk of diagnosis of asthma at age 10, there was a strong interaction of low birth weight and prenatal-smoking on the risk of physician-diagnosed asthma. They suggest that this observation indicates that airway inflammation from prenatal smoke exposure induces obstructive symptoms more easily in the underdeveloped airways of low birth weight children (Bjerg 2011).

One marker of intrauterine growth restriction is a rapid weight gain after birth. Several studies have now shown that rapid weight gain after birth is associated with poor lung function and increased risk of asthma. Lucas et al. (2004) showed in infants from the Southampton women's Survey (SWS) cohort that lung function in normal-term infants aged 5-14 weeks were not related to birth weight or infant feeding but fell by 3.2% per standard deviation increase in infant weight gain, suggesting that lower rates of fetal growth and higher rates of early infancy weight gain are associated with impaired lung development (Lucas 2004). Subsequently, they also measured birth weight and length and conditional fetal head and abdominal circumference growth velocities calculated from antenatal ultrasound measurements, in a larger sample from the SWS cohort (n=1548). At 3 years of age, a rapid growth trajectory during 11-19 weeks gestation followed by late gestation growth faltering was associated with atopy, suggesting that influences affecting fetal growth may also alter immune development. A lower early fetal growth trajectory was also associated with non-atopic wheeze, possibly reflecting an association with smaller airways (Pike 2010). More recently reduced fetal growth in this cohort has also been associated with increased BHR at age 6 (Pike 2011).

In addition to anthropomorphic studies, most epidemiological studies have shown that the *in utero* period is critical for determining subsequent risk of asthma. Maternal exposure to a range of exposures including tobacco smoke (Haley 2011), paracetamol (Garcia-Marcos 2011), and house hold chemical use (Choi 2010) have all been associated with increased asthma in the child. In relation to tobacco smoke exposure, this provides some evidence that these exposures may even act to increase risk of asthma transgenerationally. In a study by Li *et al.* (2005) the risk of developing asthma, both start of symptoms and persistent, has been linked with maternal smoking during pregnancy, with an odds ratio of 1.5. However this risk is reduced to nothing if the mother stopped smoking before she became pregnant. Interestingly however, grand maternal smoking during the mother's fetal period was associated with increased asthma risk in her grandchildren (Li 2005).

1.4.3.2 Animal Model Evidence for Developmental Origins of Asthma

Several studies have now examined the effect of maternal environmental exposure during pregnancy on the induction of allergic lung inflammation in animals postnatally. Mice born to mothers exposed to cigarette smoke from three weeks prior to conception until birth show alterations in their lung following postnatal challenge with either house dust mite or inhaled PBS with remodelling of the airways with increased smooth muscle, collagen and increased goblet cells with the house dust mite exposure (Blacquiere 2009). However, this requires postnatal challenge as no differences were observed at baseline in lung structure. This exposure of *in utero* cigarette smoke also induced increased bronchial hyperresponsiveness to methacholine and increased numbers of neutrophils and mast cells in the lung (Blacquiere 2009).

In a seminal study, Hollingsworth *et al.* (2008) exposed pregnant mice to a diet rich in methyl donors, such as Genistein (Hollingsworth 2008). An increase in methyl donors in the diet of mice dams (High Methyl Diet , HMD) enhanced the development and severity of allergic airway disease (increased airway hyperreactivity, lung lavage eosinophilia and IL-13, higher concentrations of serum IgE, OVA-specific IgE, and OVA-specific IgG1 and histologic evidence of allergic airways disease) in the F1 progeny following sensitisation and challenge with the allergen ovalbumin in comparison to offspring or mother exposed to a low methyl donor diet (LMD). Furthermore, the effect of *in* utero dietary modification on the allergic airway phenotype was paternally transmitted to the F2 generation with more eosinophilic airway inflammation and higher concentrations of total serum IgE in F2 offspring of male F1 mice exposed *in utero* to a HMD. In this study, the authors also used a genomic approach to assess DNA methylation in lung tissue from F1 mice with severe allergic airway disease (lavage eosinophils <75%) exposed *in utero* to a HMD and F1 mice with minimal allergic airway disease (lavage eosinophils >25%) exposed *in utero* to a LMD. This showed an increase in the level of methylation, particularly in the promoter of the RunX3 gene which translated through

to a decreased expression of the protein in lymphocytes and correlated with functional differences observed in lymphocyte function. This could be reversed by exposure of splenocytes *in vitro* to a demethylating agent (5-Azacytidine) which increased mRNA expression and protein level of RunX3 were both increased (Hollingsworth 2008).

1.4.4 Developmental Origins of COPD

As for asthma, there is a range of evidence that supports the hypothesis that COPD susceptibility is, to a large extent, determined by developmental influences. These include epidemiological studies in humans as well as evidence from animal models

1.4.4.1 Epidemiological Evidence for Developmental Origins of COPD

Epidemiological studies have shown that susceptibility to COPD is associated with markers of fetal growth such as anthropometric measurements at birth (Barker 1991). As described above, fetal growth and duration of gestation influence lung development and the study by Stern et al. (2007) highlights that poor airway function in the newborn period, which is dependent on fetal lung development, is a significant predictor of poor adult lung function, and potentially COPD (Stern 2007). Equally, poor adult lung function and increased risk of death from COPD are also associated with lower birth weight, again suggesting an effect of maternal undernutrition modulating *in utero* development (Barker 1991; Lopuhäa 2000; Edwards 2003; Canoy 2007).

In the study of the Hertfordshire cohort, Barker et al. (1991) showed that small birth weight (≤5.5 lb) carried an increased risk of developing COPD at age 60 (standard mortality ratio (SMR) of 131), where as a normal range birth weight (6.5-7.5 lb) did not carry this risk (SMR of 80). There are also implications for respiratory health, with those of a low birth weight (≤6.5 lb) having an increased risk (OR = 1.83) of suffering from bronchitis or pneumonia. There was also a correlation found between (FEV_1) and birth weight but not (FVC). This links in with the development of the lungs as FEV_1 components are present in the lung *in utero,* while FVC components develop after birth. In keeping with this rationale, FVC values were decreased if the individual suffered from respiratory infection during their childhood (Barker 1991). This is characteristic of an obstructive disease.

A study by Lopuhäa and colleagues found that of those mothers who experienced famine while pregnant, the highest correlation between exposure and development of COPD was during the early (1.5 (0.9 to 2.6)) and mid trimesters (1.7 (1.1 to 2.6)). If the woman was exposed during the last trimester of her pregnancy there was no correlation with the development of COPD (0.9 (0.5 to 1.5). Exposure to famine in the early or mid trimester resulted in and increased risk of developing all types of respiratory complications,

such as infections in childhood. The level of IgE with these individuals was not altered and so a push towards atopic affects is not seen in this study (Lopuhäa 2000).

1.4.4.2 Animal Model Evidence for Developmental Origins of COPD

There are no studies within the literature, at the time of print, which has investigated the developmental origins of COPD within an animal model.

1.5 Animal Models

Animal models are used as a surrogate for humans. This is due to ethical reasons as well as practical reasons. One of the biggest advantages of using an animal model is the complete control over the exposure, diet and/or challenge that the animal experiences. It is also possible to get the results faster as the life span of the model animal is usually much shorter than that of humans. As this study is interested in the effect on the lung development from maternal environmental exposure up to late adulthood, to encompass possible effects on risk of COPD, a human study looking at a single generation would be an investment of over 60 years. With an animal model this time-span can be vastly reduced depending on the species used.

1.5.1 How Appropriate are Animal Models

It is important to compare the animal model being used back to the human model trying to be emulated. This is essential because if the animal model being used has no relevance to that seen in humans then there is no reason to be using that model. There are several animal models which have been used to help investigate the possible effect a certain treatment or environment may have upon the human system. These models vary depending on the area of interest of the study, which animal is closest to displaying the processes being looked at, or the effects, as seen in humans.

If the study is looking at an effect relevant to all mammals then mice, rats, dogs or sheep may be used. For example mice can be used to model the effect of gene expression when in the presence of interfering RNAs. Sheep have been used as a model of human development to examine the effect of single and repeated doses of corticosteroids on lung function, arterial pressure and growth (Moss 2002). In another study (Bederson 1986) rats were used to refine the best way to examine the effect of middle cerebral artery occlusion as seen in human stroke victims. Rats are a common choice for animal experiments as they have a short life span, so breeding new generations takes less time, and they tend to have larger litters giving a good number of animals to increase the power of the study. There are a few disadvantages with using rats, the main one being that the rats are not fully developed at birth, unlike humans. A commonly used animal model that is fully developed at birth are sheep, not only are these animals fully developed at birth but they have large fetuses which allow investigation at earlier time points.

The model used in each investigation has to relate to humans as well as being as economical as possible. The reason for economic choices in models may have to be considered in terms of number as well as overall cost. Will the study have more impact with a larger number of smaller animals, for example rats, than with a smaller number of larger animals, such as sheep?

As the level of human comparison increases within the animal model it become harder to obtain the ethical permission to carry out any investigations. The animals also increase in size and the complexity of care and treatment levels increase, increasing the cost. Therefore the animal that was chosen to model our maternal nutrient challenge was the Wistar rat. These rats have been used for many years as a model of human relevance.

1.5.2 Comparison Between Wistar Rat and Human Lung development.

Human lung development begins in the womb when the fetus makes breathing movements. The first three stages of lung development occur before birth while the last and longest stage occurs after birth, as seen in figure 13. The first stage is known as the Pseudoglandular stage. It occurs between the fifth and eighteenth week after fertilisation. During this phase the bronchiole tree is laid down and the premature airways become lined with cubic epithelium. The second stage is known as the Canalicular stage. It occurs between the sixteenth and twenty-seventh week of development. During this stage the epithelial cells differentiate and the blood-air barrier is formed. The last stage before birth is known as the Saccular stage. It occurs from the twenty-fourth week until birth. During this stage the airspaces expand and lung surfactant is starting to be produced. Once born the lung starts to be used and the next stage of development occurs. This stage is known as alveolaristion. It occurs from birth up until the child is approximately five years old. This stage mainly consists of an increase in the foundation structure of the lung (Burri 1984).

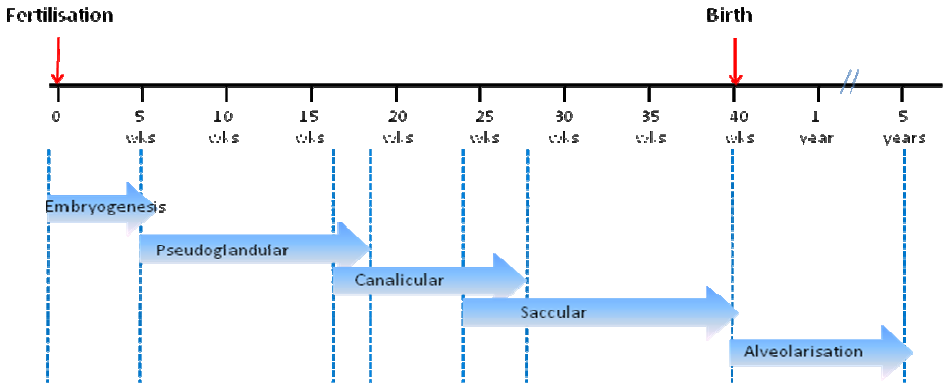

Figure 13: Diagrammatic representation of the stages of human lung development. Lung development starts five weeks after fertilisation and continues until the child is around five years old. Over this period of time there are four defined stages. Each stage has to occur in order but the next stage can begin before the previous one has ended.

Rat lung development follows a similar pattern as that of the human, as seen in figure 14. It starts at a similar stage with the lung having no alveoli before birth. There is not much change in the lung structure for the first four days after birth. During days four to seven proliferation occurs in the thick walled secondary alveolar septa. The surface area of the lung then continues to increase until about day thirteen. During this period, around day ten, respiratory bronchioles are formed. From day thirteen until the lung finishes development, about day 28, the secondary septa elongate creating an increase in lung volume which coincides with a decrease in volume density (Powell 1980).

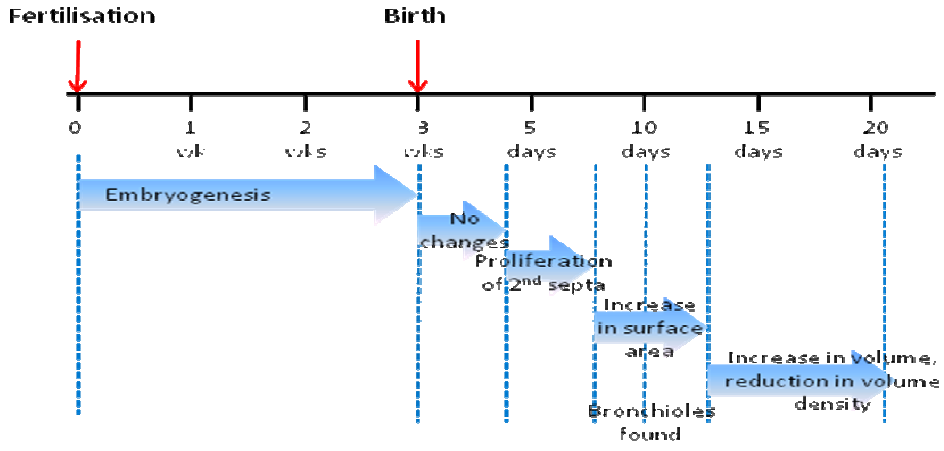

Figure 14: Diagrammatic representation of the stages of rat lung development. Lung development starts three weeks after fertilisation and continues until the rat is around 20 days post partum. Over this period of time there are four defined stages. Each stage has to occur in order but the next stage can begin before the previous one has ended.

The average life span of a human is 80 years (Sowell 2003) with lung development taking 5.6 percent of that time. The average life span for the Wistar rat is about three years (Goodrick 1982) with lung development taking 13.5 percent of that time. As can be seen the development of the rat lung takes up twice as long as the human lung development as a percentage of total life span. However when looking at

the stages of development, it is seen that some of the stages that occur before birth in the human, occur after birth in the rat. This raises significant considerations for utilisation of the rat as a model for human lung development. Firstly prenatal environmental exposures in the rat may not show effects on later stages of lung development that occur postnatally in the rat but prenatally in the humans. Secondly, changes in early postnatal environment may have greater effects on adult lung function in rats than in humans. Despite these limitations the stages of development that the lungs of both organisms mature through are similar. Use of the Wistar rat as a model for human development is well established and has been used to model development programming of the lung previously (Chen 2004).

1.5.3 Models of Intrauterine Growth Restriction Models, IUGR

As implied, intrauterine growth restriction models reduce, or restrict the growth of the fetus while *in utero*. There are several ways to induce this challenge to the offspring. The most common forms include placental ligation, total nutrient reduction, or a reduction in a specific food group within the diet, and standardisation of litter size. Some of these methods are used in conjunction with each other or as ways of standardising the protocol to refine challenge effects.

Placental ligation involves removal of the nutrients via ligation of the placenta. This method does not involve any alterations to the mother's diet and results in total restriction of nutrients flowing to the offspring. Disadvantages of this method include increased stress levels to the mother, as she is often being handled, and being more labour intensive as the ligation requires an operation to be preformed on the pregnant rats. Wigglesworth (1974) used placental ligation by attaching a silk ligature to the lower end of the uterine horn restricting the growth of the fetuses that are closest to this horn. This was undertaken on day 17 of the pregnancy and the rats were culled 4-5 days afterwards. By doing this in this way the control and IUGR animals were both exposed to the same external factors reducing the error between the two groups. This model results in up to 60% growth retardation of the IUGR fetuses, if they survived, at the extreme end of the scale, but were normally found to have between 15% and 30% reduction in growth when compared with the control animals (Wigglesworth 1974).

A total reduction in the food intake by the mother also produces restricted growth in the offspring. This is undertaken by limiting the amount of food that the mother has access to. It is less labour intensive than the placental ligation technique but can only be used as a global reduction in the nutrients taken in by the mother. Ozaki et al. used total nutrient reduction of 30% on the rats from day 0, initially found to be pregnant, to day 18. This study used 10 control rats and 11 restricted rats. It found that restricted rats had significantly increased blood pressure ($p=<0.01$) at age 60 days. The rats also showed adverse effects in their cardiovascular function (Ozaki 2001).

To try and refine the effect of a single compound in the development of an organism, the reduction of a single food group linked with that compound may be undertaken. This method involves replacement of the usual rat food chow with one that has an altered concentration of the tested food group. This method is less labour intensive than the placental ligation but costs are increased due to the altered diet that the rats eat. Kalenga et al. used the reduction of casein (12% and 8%) in the maternal diet during pregnancy. This study used 11 control rats and 23 IUGR rats. It found that this model resulted in the restricted rats having a reduced lung volume, although the lung parenchyma was mature, and the growth of the rats was retarded (Kalenga 1995).

All epidemiological evidence within humans has been based on growth restriction and within animals, evidence comes from IUGR. This can lead to some confusion and can result in interchangeable terminology.

There is a lot of variability within the models described, but most are looking at unbalanced nutrition. This means that other studies, such an increased fat model, have been put into the same category, however these are not IUGR but over nutrition.

1.5.4 Langley-Evans Model.

An early study from 1983 initially proposed the dietary challenge of a low protein maternal diet. This study used four different percentages within the diet of protein (6, 8, 12 and 18%) (Resnick 1983). This study found that animals from a low protein maternal diet showed a brain-sparing phenotype that was not reversed when the pups were cross fostered to foster mothers who had been given an increased protein diet, 25%, indicating that the alterations had occurred *in utero*.

The Langley-Evans model, proposed almost ten years later, is used as the basis for this study. The first description of these studies was published in 1994 (Langley 1994). In a subsequent study protein restricted rats showed a larger fetal and placental size at 20 weeks gestation, but there was no final effect on birth weight. No difference in total protein within the brain was seen at either 20 days gestation or at birth, however levels of glucocorticoid enzymes were altered with a lower concentration in the low protein rats for the three areas of the brain studied. Interestingly the receptors for each of these enzymes did not have an altered concentration. Although the measurements were done by proxy, using alterations in the concentration of resulting compounds (Langley-Evans 1995). Although the study used different time points for culling, 20 days into gestation; at birth; weaning and 7 weeks after birth, the dietary components used in the Langley-Evans study are the same that were used in the diet for this investigation. Exact details of the dietary components are listed in table 3.

Dietary Components	Control Diet (%)	Protein Restricted Diet (%)
Casein	18	9
Cornstarch	42.5	48.5
Sucrose	21.3	24.3
Corn Oil	10	10
Cellulose Fibre	5	5
AIN-76 mineral mix	2	2
AIN-76 vitamin mix	0.5	0.5
DL-Methionine	0.5	0.5
Choline Chloride	0.2	0.2

Table 3: Component break down of both Control and Protein restricted diets, for the duration of pregnancy of the Wistar rats. Reproduced from (Langley-Evans 1995).

One of the important things to note with this study is that the deficit in protein is counterbalanced by an increase in the carbohydrate content. Therefore any effects seen could be due to restricted protein or increased carbohydrate.

1.5.5 Previous Studies Using Langley-Evans Model

Studies that have utilised the Langley-Evans dietary model have seen a decrease in the life span of those rats whose mothers were exposed to a low protein diet during their pregnancy. Interestingly this if the offspring were born small and did not experience a period of catch-up growth their life span was increased, however if the period of catch-up growth was present then their life span was reduced (Langley-Evans 2006). Those offspring exposed to a low protein maternal diet during pregnancy also showed a preference for fatty foods during their life span(Bellinger 2004; Engeham 2010).

Studies of individual tissues within these rats observed alterations in both liver and renal function. Hepatic gene promoters exhibited altered methylation giving rise to differing levels of gene expression, which was found to be transgenerational, in F2 and F3 generations (Burdge 2007; Hoile 2011). These alterations could be counteracted by the supplementation of folic acid within the diet (Lillycrop 2010). A decrease in nephron number as well as an increase in blood pressure was found transgenerationally, F1 and F2, in male offspring (Harrison 2009).

Blood pressure has been extensively studied using this model. An increase in male offspring blood pressure has been consistently seen with this diet (Swali 2010), and this has also been found to be transgenerational, F2 (Torrens 2008). These alterations in blood pressure can be counter-acted by dietary supplementation with folate (Torrens 2006). A possible mechanism for this alteration in blood pressure was proposed by an increase in the expression level of prostaglandin E(2)coupled with impaired degradation (Sherman 1999). Differences in bone growth were also observed with exposure to maternal protein restriction (Mehta 2002).

As the thrifty hypothesis has proposed differences to be an epigenetic effect, (Hales 2001) as supported by altered hepatic gene promoter methylation, the levels of homocysteine were considered, but there was no alteration in these levels found. (Langley-Evans 2006) It was also suggested that any methylation changes would be gene specific and not genome-wide (Engeham 2010).

1.5.6 Summary of animal model considerations for DOHAD Models

To summarise this section Nathanielsz highlighted ten main principles within developmental programming that need to be considered (Nathanielsz 2006). These are presented below together with evidence that chronic human disease, in particular respiratory disease, is subject to the effects of developmental programming.

1. **Critical periods of vulnerability**: This has been exampled in the fact that exposure to famine within the Dutch Hunger Winter cohort has been linked with mortality from COPD if the exposure occurred during the early or mid-trimester of pregnancy but not late (Lopuhäa 2000).
2. **Permanent effects**: an example of this is given by increased glucose tolerance and insulin production in individuals that experienced nutritional restrictions during gestation but have these diabetes symptoms at the age of 50 (Painter 2006).
3. **Activity dependant**: this aspect is looking at the use of organs by the fetus before it is born, such as fetal breathing movements. Studies in sheep have shown that if these movements are depressed for any reason then the lung does not mature as expected (Harding 1993).
4. **Structural changes in important organs**: a protein alteration within the diet caused different parts of rat brain to develop differently, altering the size and the ability of some receptors to bind their substrates (Langley-Evans 1995).
5. **Key role for placenta**: low placental weight increases risk of developing chronic heart failure later in life (Barker 2010).
6. **Fetal compensation carries a price**: rat dams fed a low protein diet during their pregnancy produced offspring that had lower birth weights than the control. However if the offspring were then fed a low protein diet until they were weaned their life-span was longer than those offspring given a normal diet for this period (Chen 2009).
7. **Attempts after birth to reverse the consequences of programming may have their own unwanted consequences**: this is a very similar point to the previous one, where any attempt at altering the consequences of programming can lead to deleterious outcomes in the long run.
8. **Fetal cellular mechanisms often differ from adult ones**: This is of particular note in studies looking at the lung as the environment in which the fetal lung grows is very different to that which it

experiences once born. This is due to the concentration changes of oxygen presenting each environment.

9. **Effects of programming may pass across generations**: an increased risk of developing asthma was found to be linked to grand maternal smoking, during the gestation of the mother, for the grand children (Li 2005).

10. **Different effects in males and females**: after a low protein diet was given during pregnancy to rat dams, hypertrophy was seen in male but not female offspring (Woods 2003).

Each of these points can alter the effect on the offspring and the following generations. One of the easiest factors that can be altered to produce a programming effect is nutrition. Several of the examples given previously have shown the effect of altered nutrition on the development of the lungs in both humans and animal models. These effects have been linked with development of respiratory disease throughout the individual's life.

2. Materials

2.1 Physiology and Morphometry

All reagents were stored at room temperature, unless otherwise stated. All chemicals were molecular Biology or Biotechnology grade.

Table 4 Reagents

Reagent	Cat no:	Supplier
Absolute Ethanol	51976	Sigma
Calcium Chloride	262244w	BDH
Choral Hydrate	C/4280/53	Fisher Scientific
Eosin Yellow	HD1320	R A Lamb
Formalin	00600E	Surgipath
Haematoxylin	411161000	Acrōs Organics
Paraffin Wax	08605E	Surgipath
Pertex	178290	Histolab
Potassium Alum	A/1840/60	Fisher Scientific
Sodium Dodecyl Sulphate	S/P530NC/48	Fisher Biochemicals
Sodium Hydroxide	S-8045	Sigma
Sodium Iodate	S8379	Sigma
10 x TBE Buffer	EC-860	National Diagnostics
Xylene	X/0250/17	Fisher Scientific

Table 5 Plastic Ware

Plastic ware	Cat no:	Supplier
Cover Slips (22 x 40mm)	MNJ-350-0605	Menzel-Gläser
Universal Tubes	CW3900	Alpha Laboratories
Petri Dishes	633102	Greiner Bio-one
Plastic Cassette	M490-10	Simpat
Slides	Colour frosted Ends	Knittel-Glöeser

Table 6 Apparatus

Apparatus	Serial no:	Location	Supplier
Camera	Coolpix 4500	Lab 19, Level B, Link Block	Nickon
Hypercentre Tissue Processor	unknown	Lab 21, level B, Link Block	R A Lamb
Microscope	196862	Lab 19, Level B, Link Block	Leica
Microtome	unknown	Lab 23, Level B, Link Block	Leitz
Drying Oven	unknown	Lab 20, level B, Link Block	Leec

Table 7 Software

Program	Version	Supplier	Web-link
SPSS	14.0	SPSS Inc	www.spss.com

Table 8 Miscellaneous

Description	Supplier
Scalpel & Blades	-
Tube Clamp	-
Tubing	-
Sterile Tweezers	-

2.2 Bronchial Hyperresponsiveness

All reagents were stored at room temperature, unless otherwise stated. All chemicals were molecular Biology or Biotechnology grade.

Table 9 Reagents

Reagent	Cat no:	Supplier
Calcium Chloride	C7902	Sigma
Potassium Chloride	P4504	Sigma
Magnesium Sulphate	M1880	Sigma
D-Glucose	G5767	Sigma
EDTA	E1644	Sigma
Potassium Phosphate	P5379	Sigma
Sodium Bicarbonate	S8875	Sigma
Carbachol (stock $1x10^{-2}$M, kept at -20°C)	C4384	Sigma
U46619 (stock $1x10^{-4}$M, kept at -20°C)	T0516	Sigma
Histamine (stock $1x10^{-2}$M, kept at -20°C)	H7125	Sigma
Angiotensin (stock $1x10^{-4}$M, kept at -20°C)	A9525	Sigma
Isoprenaline (stock $1x10^{-2}$M, kept at -20°C)	I5627	Sigma
Papaverine (stock $1x10^{-2}$M, kept at -20°C)	P3510	Sigma
Salbutamol (stock $1x10^{-2}$M, kept at -20°C)	S8260	Sigma
BRL 37344 (stock $1x10^{-4}$M, kept at -20°C)	B169	Sigma
Prazosin (stock $1x10^{-2}$M, kept at -20°C)	P6656	Sigma

Table 10 Plastic ware

Plastic ware	Cat no:	Supplier
1.5ml Tubes	616201	Greiner Bio-one
10ul Pipette Tips	771288	Greiner Bio-one
100, 200, 1000ul Pipette Tips	772288/739288/740288	Greiner Bio-one
1ml Syringe	300013	BD Plastipak
20ml Syringe	300613	BD Plastipak
Petri Dishes	633102	Greiner Bio-one

Table 11 Apparatus

Apparatus	Serial no:	Location	Supplier
Microscope	-	Vascular Lab, Level C, IDS Building	Zeiss
Lights	-	Vascular Lab, Level C, IDS Building	Zeiss
Myograph	-	Vascular Lab, Level C, IDS Building	Danish Myo Techniologies

Table 12 Software

Program	Version	Supplier	Web-link
Chart	5.0	AD Instruments	www.adinstruments.com
SPSS	14.0	SPSS Inc	www.spss.com

Table 13 Miscellaneous

Description	Supplier
Scalpel & Blades	-
Metal Spatulae	-
Funnel	-
Pins	-
Philip Head Screw Driver (size 2.0)	-
Dissection Scissors	-
Small Sealable Plastic bag	-
Wire (size 40µm)	-
Forceps	-
Writing Ink	-
Sterile Tweezers	-

PSS and KPSS solutions 1 Litre

Reagent	PSS	PSS Concentrations (mM)	KPSS	KPSS Concentrations (mM)
NaCl	6.954g	12	-	-
KCl	0.350g	0.47	9.222g	12.37
$MgSO_4$	0.288g	0.2	0.288g	0.2
$NaHCO_3$	2.100g	0.25	2.100g	0.25
KH_2PO_4	0.161g	0.134	0.161g	0.134
D-Glucose	0.991	0.55	0.991g	0.55
EDTA	0.008	0.00273	0.008g	0.00273
$CaCl_2$	2.5ml	2.5	2.5ml	2.5

2.3 Methylation and Gene Expression

All reagents were stored at room temperature, unless otherwise stated. All chemicals were molecular Biology or Biotechnology grade.

Table 14 Reagents

Reagent	Cat no:	Supplier
Absolute Ethanol	51976	Sigma
DEPC Treated Water	N/A	N/A
2 x qPCR Mastermix (PD)	Mastermix-R	Primerdesign ltd
PCR Grade Water	04 909 631 001	Roche diagnostics, UK
Iso-propanol	I-0398	Sigma
Guanidine Hydrochloride	G-3272	Sigma
Sodium Citrate	S-4641	Sigma
Chloroform	BP1145-1	Fisher BioChemicals
TRIzol Reagent	15596-026	Invitrogen
Liquid Nitrogen	N/A	N/A

Table 15 Plastic ware

Plastic ware	Cat no:	Supplier
ABgene 96 well V-Bottomed storage plates	AB-1058	ABgene, UK
ABgene Thermo-Fast 384 Well Plates	TF-0384	ABgene, UK
Universal Tubes	CW3900	Alpha Laboratories
Matrix D Lysing Tubes	6913-100	Q-Bio Gene
1.5ml Tubes	616201	Greiner Bio-one
0.5ml Tubes	LT2172	Alpha Laboratories
0.2ml Tubes	LW2130	Alpha Laboratories
0.2ml Tube Strips	LW2500	Alpha Laboratories
10ul Pipette Tips	771288	Greiner Bio-one
100, 200, 1000ul Pipette Tips	772288/739288/740288	Greiner Bio-one
Petri Dishes	633102	Greiner Bio-one
2ml Screw Cap Tubes	CP5320WL	Alpha Laboratories
25ml Glass Pastets	760180	Greiner Bio-One
Pipette Filler	712912	Alpha Laboratories

Table 16 Commercial Laboratory Kits

Kit	Cat no:	Supplier
ImProm II™ - Reverse Transcription Kit	A3800	Promega, Southampton, UK
SYBRgreen™ Core qPCR Mastermix Kit	RT-SN10-05	Eurogentec, SA
DNAfree Kit	AM1906	Ambion, USA
Methylamp™ Global DNA Methylation Quantificantion Ultra Kit	P-1014	Epigenetek

Table 17 Apparatus

Apparatus	Serial no:	Location	Supplier
ABgene Plate Heat-sealer	951/2563	Asthma Genetics Lab	ABgene, UK
AB54-S Balance	1120133255	Asthma Genetics Lab	Mettler-Toledo, USA
BioPulverizer	59014H (cat no)	Asthma Genetics Lab	BioSpec Inc, US
Lightcycler480™ Real-time PCR Machine	1230	Asthma Genetics Lab	Roche diagnostics, UK.
Nano-drop Absorbance Spectrophotometer	unknown	Lab 18, Human Genetics	Nano-drop technologies, USA
MJ Research DNA Engine Tetrad Thermal cycler	TD002380	Asthma Genetics Lab	BioRad, USA
Labofuge 400 Plate Centrifuge	40340180	Asthma Genetics Lab	Heraeus, Germany
Ribolyser	unknown	Brook Lab	Thermo Fisher Scientific Inc
UV PCR Cabinet	MC 0239	Asthma Genetics Lab	Lab-Caire, UK
M-240R Bench-top Centrifuge	0000138-02-00	Asthma Genetics Lab	BOECO, Germany
Microplate Reader	L02186	Lab LF58, F level, South Academic Block	Molecular Devices
Incubator	unknown	Asthma Genetics Lab	New Brunswick Scientific

Table 18 Software

Program	Version	Supplier	Web-link
SPSS	14.0	SPSS Inc	www.spss.com
Roche Lightcycler480 Relative Expression Module	1.2	Roche Diagnosics, UK	www.roche-applied-science.com
Roche Lightcycler Basic Software	1.2	Roche Diagnosics, UK	www.roche-applied-science.com
SOFTmax PRO	3.0	Molecular devices	www.moleculardevices.com
BLAST Search		NCBI	www.ncbi.nim.gov/blast/Blast.cgi
Primer Express	2.0	Applied biosystems	
Universal Probe Assay Design	-	Roche Diagnosics, UK	www.roche-applied-science.com/sis/rtpcr/upl/index.jsp

Table 19 Miscellaneous

Description	Supplier
Foil	N/A
ABgene Clear Seal Diamond Plate Seals	ABgene, UK
ABgene Clear Seal Strong	ABgene, UK
Scalpel & Blades	-
Liquid Nitrogen Dewar	-
Metal Spatulae	-
Sterile Tweezers	-
RNAzap	Ambion, USA
RNAzap Wipes	Ambion, USA

3. Methods

3.1 Southampton Maternal Protein Restriction Model (PR)

To investigate the effects of maternal under nutrition on lung development, the Southampton model of maternal protein restriction (PR) was used. This diet employs a 50% reduction in the casein content (PR, 9% casein) in the isocaloric diet of pregnant dams from the day of confirmation of pregnancy until delivery, with control dams (C) receiving an 18% casein diet. It is important to note that the dietary challenge in these rats is during the entire pregnancy which means that there is no specific developmental window being targeted.

This diet is based on the Langley-Evans model of protein restriction and a detailed description was given in section 1.5.4. On confirmation of pregnancy, female Wistar rats were allocated to either the control or protein restricted diet. At delivery dams and pups were returned to standard chow and litters standardised to 8. The Home Office project license number for this investigation is 70/6457.

Lungs were harvested from male F1 offspring at the end of initial lung development (28 days), young adult age (35 days), adult age (75 days), adult age (120 days) and mature adult age (225 days). As can be seen in figure 14, there were 25 animals available in the F1 28 day old group, with 15 from the control diet and 10 from the protein restricted diet. These animals were studied for physiological and morphometric measures, as well as gene expression. There were 12 animals from the F1 35 day old age group, 6 from control and 6 from the protein restricted diet. 12 animals were also studied from the F1 75 day old age groups with 6 from the control and 6 from the protein restricted group. Both of these two groups were used for the BHR experiments only. There were 18 animals from the F1 120 day old age group, with 11 from the control diet and 7 from the protein restricted diet. These animals were studied for gene expression data only. There were 12 animals from the F1 225 day old age group, with 6 from the control diet and 6 from the protein restricted diet. These animals were collected for physiological and morphometric measures, as well as gene expression.

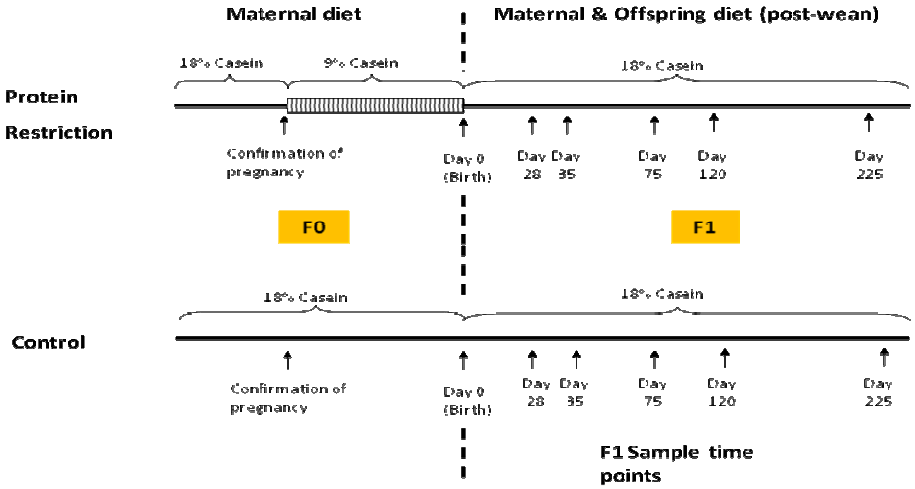

Figure 15: Diagrammatic representation of the two rat diets and the F1 culling time points for this investigation. Rats were randomly allocated control or protein restricted diet once pregnancy had been confirmed. They remained on this diet until they gave birth when they were all given the control diet. Offspring from these rats were culled at days 28, 35, 75, 120 and 225.

A further generation (F2) was then bred from the females F1 offspring bred to control males, and lungs were harvested from male F2 offspring at the end of initial lung development (28 days), figure 16. There were 41 animals available in the F2 28 day old group, with 24 from the control diet and 17 from the protein restricted diet. These animals were studied for physiological and morphometric measures, as well as gene expression.

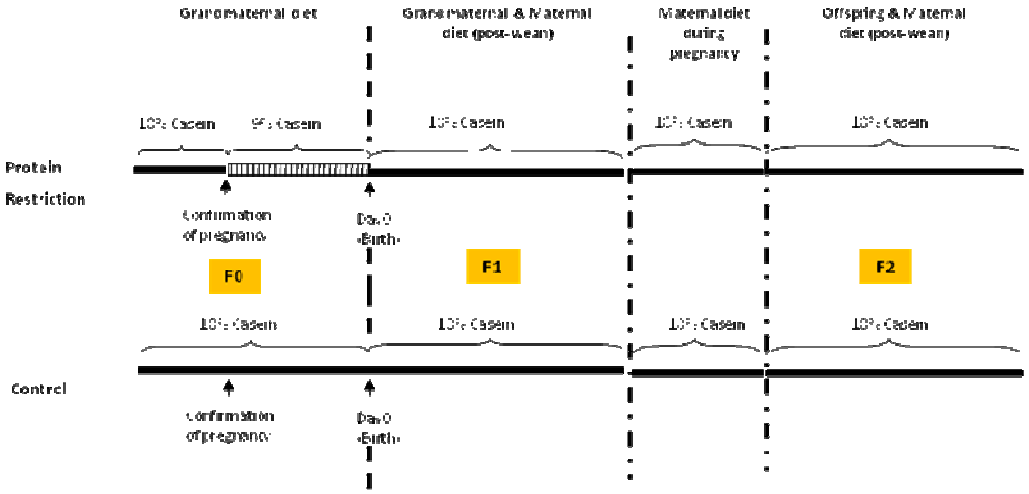

Figure 16: Diagrammatic representation of the two rat diets and the F2 generation for this investigation. Rats were randomly allocated control or protein restricted diet once pregnancy had been confirmed. They remained on this diet until they gave birth when they were all given the control diet. Offspring from these mice were then mated and the resultant generation culled at day 28, with no further dietary challenge. This created the F2 generation.

The females from this group (F2) were then bred with control males to produce the F3 generation from which the lungs were harvested from the males at 21 days, figure 17. There were 21 animals available in the F3 21 day old group, with 16 from the control diet and 5 from the protein restricted diet. These animals were studied for physiological and morphometric measures, as well as gene expression.

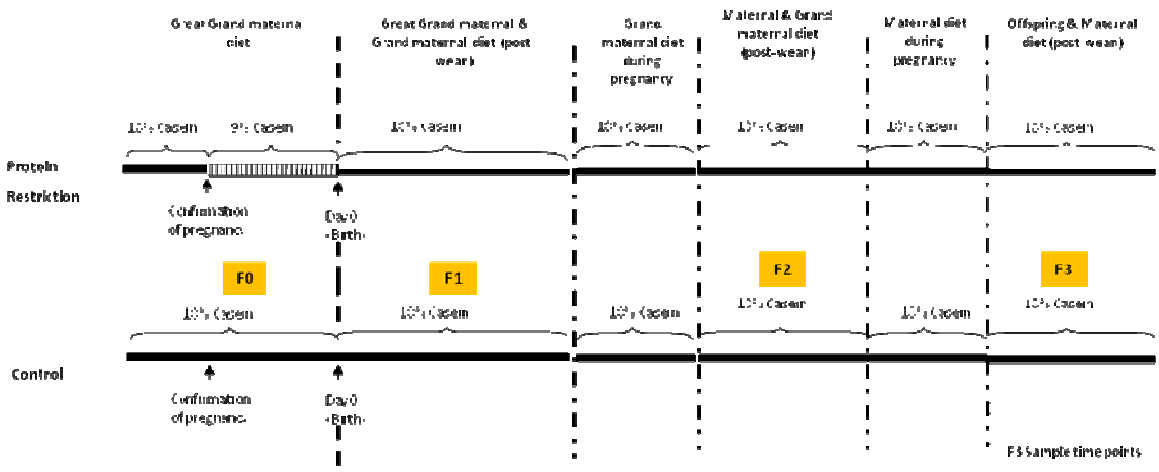

Figure 17: Diagrammatic representation of the two rat diets and the F3 generation for this investigation. Rats were randomly allocated control or protein restricted diet once pregnancy had been confirmed. They remained on this diet until they gave birth when they were all given the control diet. Offspring from these mice were then mated to produce the F2 generation. The F2 offspring were then mated and the resultant generation culled at day 21, with no further dietary challenge. This created the F3 generation.

The rats were bred until the third generation as these individuals will have had no exposure to the dietary challenge. The F1 are obviously present inside the womb of the mother when the challenge is being administered. The eggs that will form the F2 generation are also present at this time and so will be exposed. The F3 however are not present in any form at the time of the challenge. This means that any difference that is found in this generation will have to be due to inherited epigenetic effects, as opposed to direct effects of the exposure.

3.2 Physiology and Morphometry

3.2.1 Lung Volumes.

To assess lung volumes in F1 animals the left lung was removed and weighed to give the wet mass. A needle was then inserted into the primary bronchi of the lung and the valve opened to allow the formalin to fill the lung. Formalin was used as the fixing agent as its use in morphometric analysis of the lung is well established (Hayatdavoudi 1980; Tome 1990; Lang 1994). Following inflation the lung was again weighed and the volume of the fluid introduced into the lung was used to estimate the volume of the lung. The formalin was always introduced from the same height/pressure which was calculated as previously described (Weibel 1966) and shown below:

Lung fixing perfusion pressure:

Perfusion pressure = 25 cm of H_2O (1.36 cm of H_2O is equivalent to 1 mmHg)

= 18.4 mmHg (1 mmHg is equivalent to 133 Pa)

= 2447.2 Pa

Hydrostatic pressure [Pa] $P = \rho g h$ where g = gravity at 9.81 m/s^2

h = height [m]

ρ = practical gravity of fluid (m ÷ v) $[kg/m^3]$

ρ for 10% formalin = 1.03 g / ml

= 1030 kg/m^3 (water is 1000 kg/m^3)

Therefore:

$2447.2 = 1030 \times 9.81 \times h$

$h = 2447.2 \div 10104.3$

$h = 0.242$ m

= 24.2 cm

3.2.2 Tissue Preparation and Embedding

To ensure consistency, left lungs were removed and embedded for all animals. Paraffin wax was chosen for embedding as the gaps in the matrix are larger than those found in other embedding substances, such as GMA. This would allow for the potential utilisation of other stains e.g. trichrome staining, whose large dye molecules would be not able to access the tissue otherwise, as well as any immuno-staining that may be required during further investigation.

The left lung was kept in formalin, 10 times more formalin as tissue, volume per volume, for 48 hours, this was considered sufficient fixing time, as although the lung is a large piece of tissue, it had already been infused with formalin during estimation of lung volume (see section 3.1.4.1.1). The formalin was then removed and replaced with 70% ethanol solution for storage until embedding. Tissue was placed inside a plastic labelled cassette and then put into the reaction chamber of the Hypercentre tissue processor (R A Lamb). The tissue was bathed in 50% alcohol, 70% alcohol and 90% alcohol for 10 minutes each, followed by absolute alcohol for 50 minutes and then a mix of equal parts absolute alcohol and chloroform for a further 10 minutes. It was then placed into chloroform for 20 minutes before being put into the melted wax chamber at 60^{o}C for 20 minutes. It was then left in the chamber overnight, at 37^{o}C under a gentle vacuum which helps the solution to penetrate the tissue and remove small air bubbles.

Following the process above, the samples were placed in the wax bath. The correct base mould was chosen depending on the size of the tissue and a thin layer of wax was poured into it. It was kept on the warm spot on the machine (Hypercentre Tissue Processor, R A Lamb) until the sample had been removed from the cassette and orientated into the correct position in the mould with heated forceps. The mould was then put on the cold spot and the tissue sample held until the wax had set enough to hold the tissue. Following this, the mould was filled until three quarters full and then the labelled plastic top of the cassette was added to the top of the mould and both were completely filled with wax. The mould was left to set completely and then the wax embedded sample removed from the mould and stored until cut.

3.2.3 Cutting and Haematoxylin and Eosin (H & E) Staining

The paraffin block with the embedded lung was cut on a microtome (Leitz) in 5µm thick sections. To ensure that the beginning of the sectioning was random, which is essential for unbiased analysis, the first section taken was the first full section of the block that included tissue (Hyde 2007). Twenty sections were cut and mounted onto slides. Twenty sections were kept at this point as the whole lung had to be cut at one time and spare slides may be needed for further investigation. This made up layer A. One hundred 5µm sections were then cut and discarded. A new layer (layer B) of twenty sections was then cut. This was repeated until the whole lung had been cut through.

One slide from each layer was then stained using H & E (Brumley 1967; Holt 1988; Chen 2004).

Haematoxylin solution preparation:

Haematoxylin	1g
Distilled water (ROW)	1000mls
Potassium Alum	50g
Citric Acid	1g
Chloral Hydrate	50g
Sodium Iodate	0.2g

Eosin Yellow solution preparation:

Eosin Yellow	5g
Calcium Chloride	1g
Distilled water (ROW)	500mls

Sections were dewaxed in xylene for two minutes and then hydrated through graded alcohols for five minutes. Sections were stained in Mayers haematoxylin for ten minutes and then left in gently running cold tap water for five minutes to 'Blue'. Five minutes in 1% eosin solution followed which was then washed in gently running cold tap water for a further five minutes. Sections were dehydrated through graded alcohols and then cleared in Xylene before a cover slip was applied and the slide left to set before imaging.

3.2.4 Stereology

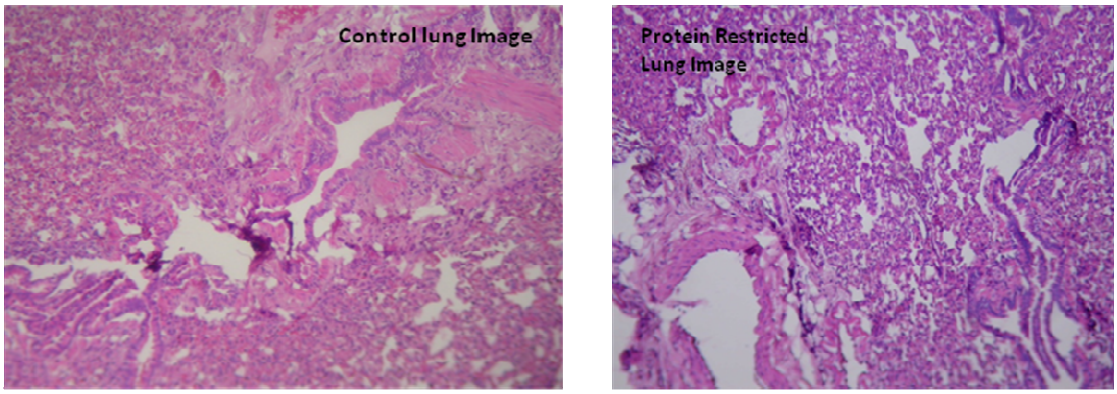

Figure 18: Overview of H and E images from the offspring rat lungs culled at 225 days old. These mature lungs show an offspring lung from the control *in utero* diet on the left and from the protein restricted diet on the right. These images were taken at x10 magnification.

To estimate the surface area of the lungs a volume density design was used based on Cavalieri's principle. The sections used were transverse uniform random. This means that one orientation will be chosen according to the design. For the measurements to be correct two other choices must be isotrophic (random). The first of these is achieved by starting the cutting of the sections at a random point (first full section of paraffin that contains tissue), the second is the placing of the grid onto the sample. The grid has to have one plane matching the vertical plane but it can then be randomly place onto the image in the other two directions, forward or back and left or right. All equations, method designs and grids have been taken from, or are based on, those referred to in 'Unbiased Stereology' (Howard 2005).

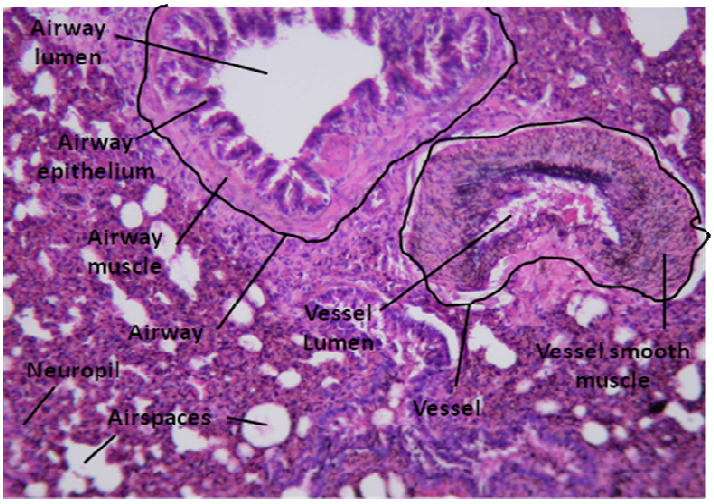

Figure 19: Overview of the lung structures showing an airway with its different components. In the middle, at the top of the image, a vessel on the right and air spaces in the bottom left of the image. Any tissue which is not of interest is labelled as Neuropil. This image was taken at x10 magnification.

3.2.4.1 Volume Fractionation

To estimate the volume of smooth muscle around the airway, point counting was used on an image taken at x10 magnification. This method gives a ratio of total volume to the volume of interest, in this case smooth muscle. The total number of points, from the randomly placed grid, which lie in the area of interest, e.g smooth muscle, and the number of points in the reference area, e.g. smooth muscle and the lumen and edge of the airway are counted. Because the final value is a ratio of the object of interest it does not matter it the airway lies at right angles to the section. The equation used for this estimation is:

$$\text{Volume density of area of interest} = \frac{\text{total number of points in the area of interest}}{\text{Total number of points in the area of reference}}$$

The grid used to determine if there is an alteration in the composition of the lungs has to be the correct grid for the size of the tissue, the thickness and number of sections looked at and the size of the

component which is being measured. Selection of the correct grid was undertaken using the 'nugget effect' equation. This equation gives a percentage error for the variance of an area. This can be altered by increasing the number of sections looked at or the number of sections cut from the original tissue. The nugget effect, which gives the error involved depending on how close the points are in the grid, can be altered by choosing a grid with either more or less points. One lung was chosen randomly and used to determine if this was an acceptable method to produce the required results. The first grid that was used tested was not correct as it had more than a 10% error in both the variance of area and the nugget effect. The second grid that was considered was used (see figure 20), as it produced an 8% error for the variance of area and a 0.5% error for the nugget effect. These are both below the 10% error that is considered acceptable with this method (Howard 2005). The rest of the lungs were then assessed following the same methods.

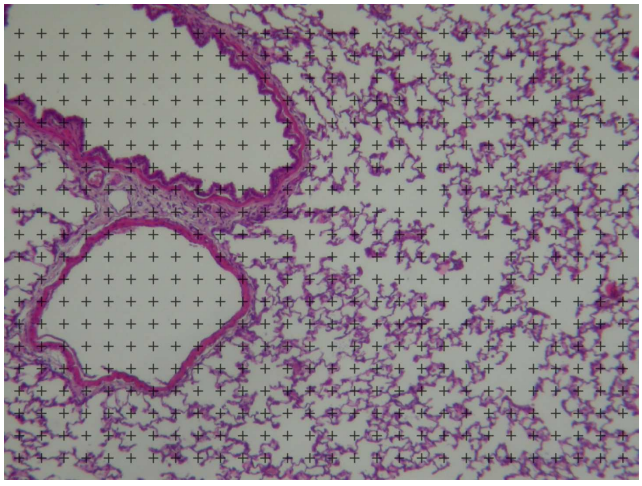

Figure 20: An image analysed from the 225 day old rat offspring with the point counting grid overlaid on top as seen during analysis. As the cross has a thickness the same point on the cross is used as a reference point for the cross. In the analysis the top right point of the cross line overlay was used. Depending on where this cross lands on the image the point is counted as in that tissue of interest. This image was taken at x10 magnification.

3.2.4.2 Surface Area

To estimate the surface area of the alveoli in the lungs the Cavalieri's principle was used at the same magnification but this time the points of intersection along an isotrophic line are used as the estimator, figure 21. The surface area is given by the number of points of intersections along this given line. This will give a surface area A^2 in a 3D unit of volume. The equation used for this estimation is:

$$\text{Surface area} = \frac{2 \times \text{total number of intersections with interface of interest}}{(\text{cycloid length}/\text{magnification})}$$

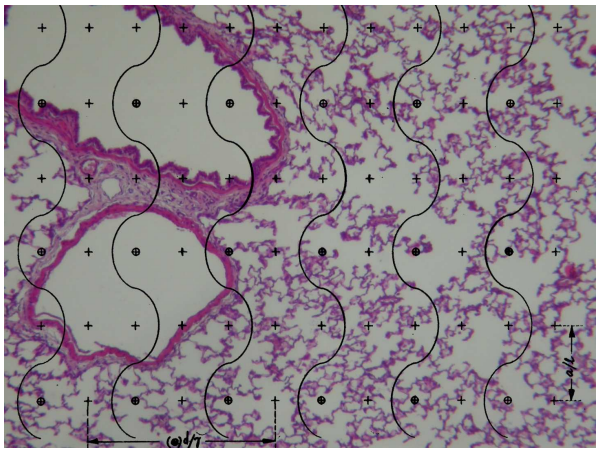

Figure 21: An image analysed from the 225 day old rat offspring with the cycloid grid for estimation of surface area overlaid on top. One side of the line is used to record the intercepts as the line has a thickness. The left hand-side of the line was used in this analysis. This image was taken at x10 magnification.

3.2.4.3 Alveolar Wall Thickness

To estimate the average thickness of the alveolar walls a grid, with lines at 45° angles and points of intercept at 90° to those lines, figure 22, was placed randomly over the lung image taken at a magnification of x63. Where the intercept of the line landed on an alveolar wall the thickness of that wall was measured and a geometric mean was used to compare between the groups.

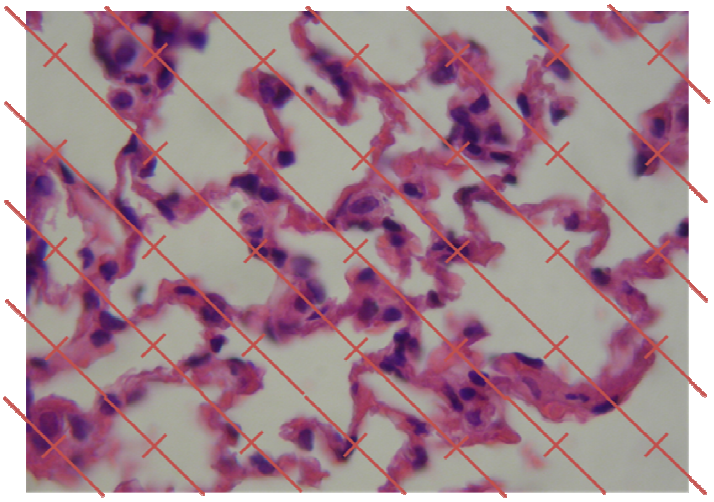

Figure 22: image of the alveolar walls from a 225 day old rat lung with the J-image grid for estimation of alveolar wall thickness overlaid on top. Where the smaller intersecting lines land on an alveolar wall, the thickness of that wall is measured along the same line as the small intersecting line. An average of these measurements is used to compare between groups. This image was taken at x63 magnification.

3.3 Bronchial hyperresponsiveness

3.3.1 Dissection of Bronchi from Rat Lungs.

Initially the lung is removed from the thoracic cavity into a resin dish, allowing for the tissue to be pinned keeping it steady. The tray is then filled with PSS buffer and the lungs cleaned by removing as much of the fat and blood clots from the outside of the lungs as possible, being careful not to damage the lungs while doing this. A dilution, 1:10, of water soluble black writing ink in PSS is used to fill a 1ml syringe and a G21 needle attached to the end. The end of the needle is carefully placed into the opening of the bronchi and the bronchi are then filled with the ink solution. This colours the bronchi making them distinguishable from pulmonary vessels.

The first order bronchi are followed into the lung and tissue is removed by lifting it away from the bronchial tube and cutting away from it with the lung kept steady by pinning the very edge of the lung to hold it in place. The bronchi branches very quickly. In this study on F1 generation, bronchi from 35 and 75 day old rats were used.

Once the bronchi have been removed from the lungs they are then cleaned as well as possible. This is essential to make it easier to mount the bronchi on to the wires of the myograph. The sections of tissue are cut into 2mm length pieces, as this is the length between the jaws within the myograph wells.

3.3.2 Mounting of Bronchi onto the Myograph.

Figure 23: Myograph set-up. The dissection is done using the microscope on the left. The myograph is in the middle with the four organ baths on the top and the computer linked to the myograph to monitor the tension on the wires is to the right.

The metal myograph wire was cut into 2cm long sections. It is important that the wire is not too long as there needs to be a space between the wire and the myograph well to be able to mount the bronchi. It is also important that the wire is not too long so that it will fit onto the screws within the wells. To make sure that there is enough wire, two more pieces than required were cut as the wire can bend and as it is so fine

may also break when tightening the screws. When the wire is cut there is a small kink where it was held taut when being cut. The wire is placed on the side with the kink at the bottom end nearest to the researcher to ensure correct orientation as it is difficult to push the kinked end through the tissue.

One piece of wire is then secured horizontally between the jaws within the myograph well, making sure that the kinked end of the wire is at the end with the screw that the tissue will be attached to first. The jaws can then be tightened just enough to hold the wire in place. One end of this wire, the end with the kink, can then be wrapped around the screw and the screw tightened but not too much as the wire may break. The well is then filled with 5ml of PSS buffer.

When this has been done the tissue can be pushed onto the wire. The jaws are opened and the tissue pushed into the space between them. Once this has been done the end of the wire can be secured onto the second screw, the same side but below the first attached screw and the wire tightened.

Once complete the second wire is then pushed through the bronchi tube, making sure the kinked end does not pass through the tissue. When the second wire has an equal distance showing on either side of the tissue, the jaws are gently closed to hold the wire in place as the wire is wrapped around the screws both top and bottom on the side. The wires are held parallel to each other and taught. As seen in figure 24.

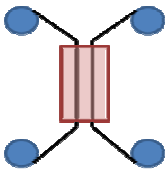

Figure 24: Bronchi tissue (pink rectangle) as positioned within the myograph bath. The two wires are secured onto the tension plates and passed through the bronchi lumen without being crossed/twisted together.

The wells are then put back into the myograph, over slips are placed over the wells and machine is turned on. A mix of 95% CO_2 and 5% O_2 is pumped into the buffer within the wells and the solution is heated to around 37^{o}C. The resting tension on the bronchi is around 1g, which translates into 4.9mN. The tension on the tissue is brought up to this level and the well is left for 1 hour to get up to temperature.

3.3.3 Testing of Tissue Reaction to Regents.

Additional PSS buffer and PSS with 2.5mM concentration of $CaCl_2$ are warmed up to 37°C and aerated with a mix of 95% CO_2 and 5% O_2, as above. At this time the myograph is turned on and the time marked to ensure that the tissue has achieved a stable tension. Once the tissue is stable the jaws are opened and the buffer is removed from the wells with a vacuum and 5ml of new KPSS is added to the wells.

Following this, a bronchoconstrictor agent is added at the desired initial concentration and the time of addition is recorded on the graph. Three minutes gap is left between additional doses of drug. If a different drug is to be tested, the solution is removed from the wells using the vacuum and the well is washed out three times with 5ml of PSS and the tissue is left to achieve a stable resting tension.

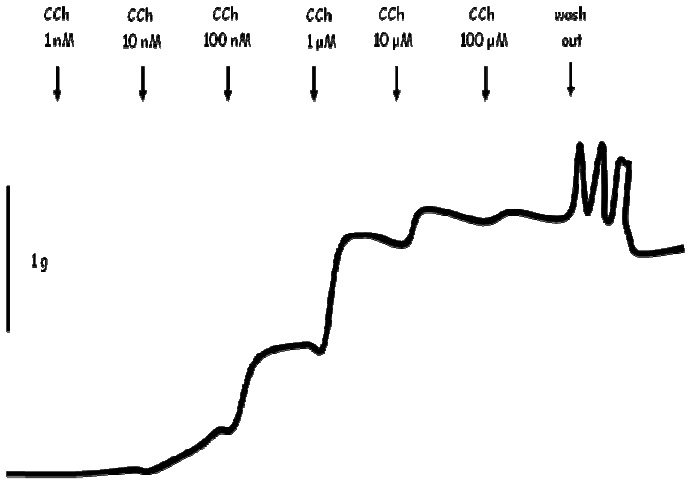

Figure 25: An example of a myograph readout. Increasing concentrations of the drug are added and left for 2 minutes. This example is showing the effects of a bronchoconstrictor, carbachol, therefore the tension of the bronchi increases with each addition. The 1g scale at side is shown for reference only.

The same process is used for the relaxation drugs but prior to the first concentration being added the bronchi are individually constricted to 80% of the maximum response to carbachol. This is done individually for each bronchi as an 80% constriction will differ between different bronchi due to length and tone of the bronchi segment. Serial dilutions are made from stock solutions for each drug of 0.01M. Dilutions contained 450µl of each concentration of $1x10^{-3}$, $1x10^{-4}$, $1x10^{-5}$ and $1x10^{-6}$M. These concentrations were added to the myograph wells as detailed in table 20.

Table 20: Dilution series from stock concentration $1x10^{-2}$M

Dilution	Volume added per well (µl)	Concentration in well
$1x10^{-6}$	5	1nM
$1x10^{-6}$	10	3nM
$1x10^{-6}$	35	10nM
$1x10^{-5}$	10	30nM
$1x10^{-5}$	35	100nM
$1x10^{-4}$	10	300nM
$1x10^{-4}$	35	1µM
$1x10^{-3}$	10	3µM
$1x10^{-3}$	35	10µM
$1x10^{-2}$	10	30µM
$1x10^{-2}$	35	100µM

Serial dilutions are made from stock solutions for each drug of 0.0001M. Dilutions contained 450µl of each concentration of $1x10^{-5}$, $1x10^{-6}$, $1x10^{-7}$ and $1x10^{-8}$M. These concentrations were added to the myograph wells as detailed in table 21.

Table 21: Dilution series from stock concentration $1x10^{-4}$M

Dilution	Volume added per well (µl)	Concentration in well
$1x10^{-8}$	5	10pM
$1x10^{-8}$	10	30pM
$1x10^{-8}$	35	100pM
$1x10^{-7}$	10	300pM
$1x10^{-7}$	35	1nM
$1x10^{-6}$	10	3nM
$1x10^{-6}$	35	10nM
$1x10^{-5}$	10	30nM
$1x10^{-5}$	35	100nM

Once the experiment has been completed, the well is emptied by vacuum and the wires and tissue removed. The wells are washed with 5ml acetic acid for 2 minutes to remove any calcium deposits left by the buffer. The well is then finally washed three times with 5ml of water.

3.4 Methylation and Gene expression

3.4.1 DNA, RNA and Protein Extraction

Firstly a tissue sample of approximately 100mg in weight was snap frozen in liquid nitrogen and homogenised using a bio-pulveriser steel hammer. Homogenised tissue was washed into Lysing Matrix-D tubes with 1.0ml trizol. Tubes were ribolysed for 20 seconds on speed setting 4.0 in the ribolyser-instrument (Thermo Fisher Scientific). This procedure was repeated at least twice or until the tissue had been fully homogenised. The homogenate was transferred to a clean 1.5ml micro-centrifuge tube. 0.2ml of chloroform was added per 1ml of trizol, shaken vigorously by hand for 15 seconds and then centrifuged at 12,000 x g (11,100 RPM) for 15 minutes at 4°C. RNA was isolated from the top aqueous layer while the bottom layer was kept on ice for DNA and protein isolation the following day. RNA was precipitated by mixing in 0.5ml of iso-propanol (ice-cold) per 1ml of TRIzol reagent. RNA was washed twice using 75% ethanol in Diethylpyrocarbonate (DEPC) water and the pellet was re-suspended in 20µl DNA-free master mix then incubated for 1 hour at 37°C. The master mix was from Ambion and consisted of 2µl 10x DNase I buffer, 1µl of rDNase I and 17µl of RNase-free water per tube/sample. 2µl DNase inactivation reagent was added, mixed and incubated for 2 minutes. Samples were centrifuged at 10,000 x g for 1.5 minutes at 4°C. The mix was transferred into a new 0.2ml tube and 30µl RNase-free water was added before the sample was split between two screw cap tubes with 25µl in each. Tubes were stored at -80°C.

After all of the remaining top aqueous layer was removed from the tubes, 0.3ml of 100% ethanol per ml of trizol was added to the DNA/protein phase left in the 1.5 ml tubes from the previous day. This was mixed gently via inversion and left to incubate for 3 minutes at room temperature. The top layer of phenol-ethanol supernatant was removed to a new 2ml screw cap tube and left on ice to be used later for protein isolation. The DNA pellet was washed twice with 1ml 0.1M sodium citrate in 10% Ethanol per 1ml trizol. Each wash step included a 30 minute incubation at room temperature with periodic mixing followed by centrifugation at 2,000 x g for 5 minutes at 4°C. The pellet was re-suspended in 75% ethanol and incubated at room temperature for a further 15 minutes. It was then centrifuged as before at 2,000 x g for 5 minutes at 4°C. The 75% ethanol was carefully removed and the pellet allowed to air dry. The pellet was then dissolved in 300µl TE and left overnight. The following day the tubes were centrifuged at ≥12,000 x g for 10 minutes before the solution was removed to a screw cap tube to be stored at -80°C.

The phenol-ethanol supernatant was aliquoted into equal volumes into new 2ml screw top tubes. Tubes were then treated in parallel for the following procedures. The proteins were precipitated with 750µl of iso-propanol per 0.5 ml trizol. Samples were incubated at room temperature for 10 minutes before being centrifuged at 12,000 x g for 10 minutes at 4°C. The supernatant was removed and pellet washed three

times with 1ml 0.3M Guanidine hydrochloride in 95% ethanol. Each wash step was incubated for 20 minutes at room temperature and then centrifuged at 7,500 x g for 5 minutes at 4°C. Once this was completed the tube was snap frozen in nitrogen and stored at -80°C. A flow diagram of this procedure can be seen in figure 26.

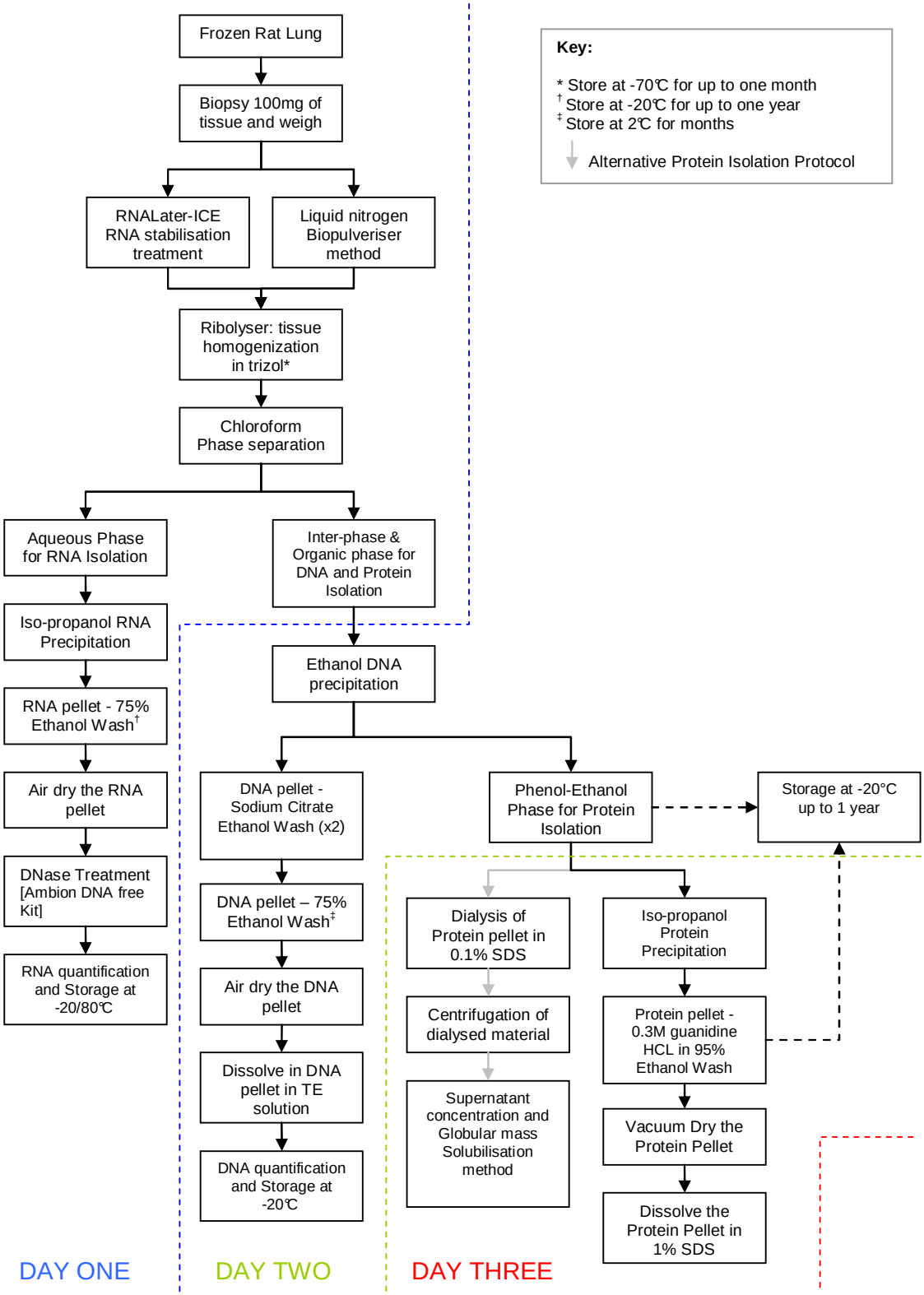
Frozen Rat Lung
Biopsy 100mg of tissue and weigh
RNALater-ICE RNA stabilisation treatment
Liquid nitrogen Biopulveriser method
Ribolyser: tissue homogenization in trizol*
Chloroform Phase separation
Aqueous Phase for RNA Isolation
Inter-phase & Organic phase for DNA and Protein Isolation
Iso-propanol RNA Precipitation
RNA pellet - 75% Ethanol Wash†
Air dry the RNA pellet
DNase Treatment [Ambion DNA free Kit]
RNA quantification and Storage at -20/80℃
Ethanol DNA precipitation
DNA pellet - Sodium Citrate Ethanol Wash (x2)
DNA pellet – 75% Ethanol Wash‡
Air dry the DNA pellet
Dissolve in DNA pellet in TE solution
DNA quantification and Storage at -20℃
Phenol-Ethanol Phase for Protein Isolation
Storage at -20°C up to 1 year
Dialysis of Protein pellet in 0.1% SDS
Centrifugation of dialysed material
Supernatant concentration and Globular mass Solubilisation method
Iso-propanol Protein Precipitation
Protein pellet - 0.3M guanidine HCL in 95% Ethanol Wash
Vacuum Dry the Protein Pellet
Dissolve the Protein Pellet in 1% SDS
DAY ONE
DAY TWO
DAY THREE
Key:
* Store at -70℃ for up to one month
† Store at -20℃ for up to one year
‡ Store at 2℃ for months
Alternative Protein Isolation Protocol

Figure 26: Flow chart describing DNA and RNA extraction by the Trizol method. The procedure for extraction through trizol takes three days. The tissue disruption and RNA isolation occurs on the first day, DNA isolation occurs on the third day and the last day is for protein isolation.

The DNA concentration for each sample was quantified using a Nanodrop 1000. Samples were then normalised to as close to 100ng/µl as possible in 10µl volumes with RNA free water.

3.4.2 Global Methylation Assay

The level of global methylation was measured using the Epigenetek, Methylamp™ Global DNA Methylation Quantification Ultra Kit. (Cat. No. P-1014) A diagrammatic representation of the assay methodology is shown in figure 27.

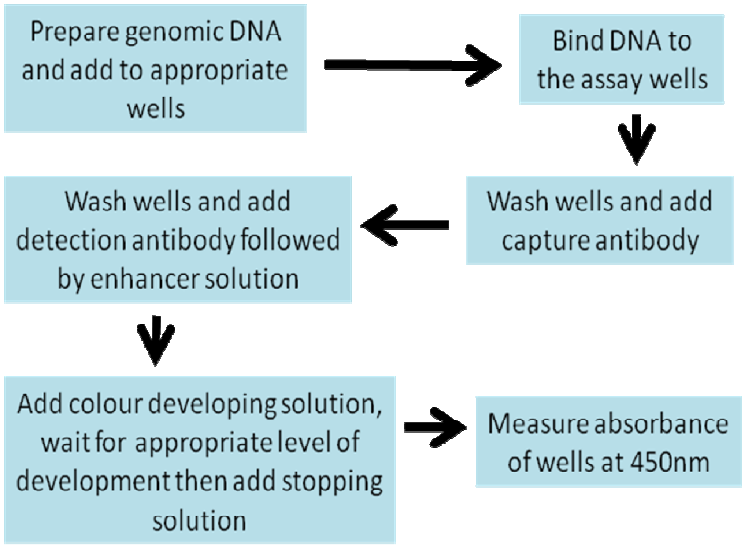

Figure 27: Diagrammatic representation of the Methylamp™ Global DNA Methylation Quantification Ultra Kit method.

DNA samples were normalised to 100ng/µl. 28µl of the DNA binding solution was added to every well that was being used. 2µl of each sample was then added to these wells and the wells were always in duplicate. Ten wells were left with no sample to form the standard dilution curves and the negative control. The positive control was diluted 1:20 with the DNA binding solution. This diluted solution was used to form the standard curve by diluting with the DNA binding solution again, the top concentration being 100% methylated DNA, then 75%, 50%, 25% and 0%. The 0% in the standard curve was just the binding solution. 2µl of each of these dilutions was added to the appropriate wells. 2µl of the negative control was added to the appropriate well.

The plate was shaken gently to make sure that the entire surface of the bottom of the well was covered. This is essential as the final measure is based on light absorbance that needs to be evenly distributed within the well to give a correct measurement. The plate was incubated at 37^{o}C with no humidity for 40 minutes to bind the DNA to the bottom of the well to stop it from being washed out during later stages. The plate was then incubated for a further 40 minutes at a higher temperature of 60^{o}C. This was to remove any excess solution from the wells. If there was any non-evaporated solution left it was removed using a pipette.

150µl of block solution was the added and incubated at 37°C for 30 minutes. Following this, the wells were washed three times with 150µl of the diluted wash buffer. The pH of this solution was between 7.2 and 7.5. The 150µl was aspirated within the wells 10 times for each wash.

Once the last wash had been removed the capture antibody (1000µg/ml) was diluted 1:1000 with the diluted wash buffer. 50µl of this diluted solution was added to the wells and incubated at room temperature for 60 minutes. Once complete, wells were washed four times by aspirating 150µl of the washing buffer.

The detection antibody (400µg/ml) was diluted 1:5000 with the diluted wash buffer and 50µl were added to each well. The plate was then incubated at room temperature for 30 minutes. The wells were then washed five times, by aspirating, with 150µl of the diluted washing buffer.

The enhancer solution was then diluted 1:5000 with the diluted wash buffer and 50µl added to each well. The plate was then incubated at room temperature for 30 minutes. The wells were then washed five times, by aspirating, with 150µl of the diluted washing buffer.

100µl of developer solution was added to each well and incubated at room temperature in a dark room for 1-5 minutes depending on the time required for the colour to change from a clear solution to a medium blue colour. Once this had happened 50µl of the stop solution was added. This stopped the developer solution from becoming too dark and turned the colour from blue to yellow. The plate was then wrapped in foil to prevent light from degrading the colour.

The plate was then taken to a microplate reader (Molecular Devices), unwrapped and the absorbance at 450nm wavelength was measured within 2-15 minutes of the addition of stop solution. There were two methods of determining the level of methylated DNA within the samples. The first method gave an estimate of the percentage of methylated DNA.

$$\text{Methylation \%} = \frac{(\text{Sample OD} - \text{Negative OD})/\ \text{rat GC content (42)}}{(\text{Positive control OD} - \text{Negative OD}) \times 10}$$

A more accurate measure for both % and ng of methylated DNA within the samples.

$$\text{Methylated DNA (ng)} = \frac{\text{Sample OD} - \text{Negative control}}{\text{Slope}} \times 100$$

$$\text{Methylation \%} = \frac{\text{Methylated DNA amount/ rat GC content (42)}}{\text{Sample DNA amount added}} \times 100$$

The second method was used to estimate the percentage of DNA that was methylated in the samples.

This method was used with pooled samples for each time point and treatment as well as individual samples for the F1 225 day old samples.

3.4.3 cDNA Generation from Extracted Rat Lung RNA.

The RNA concentration for each sample was quantified using a Nanodrop 1000. Samples were then normalised to as close to 500ng/µl as possible in 10µl volumes with RNA free water. Normalised sample RNAs were reverse transcribed using the Improm^{TM}II Kit from Promega, UK. All of these reactions contained negative water, no template controls, reverse transcription negative, lacking in the Improm II reverse transcriptase, and positive, Kanamycin RNA, controls. Samples and primers were mixed, as detailed in table 22. These 5µl volumes were incubated for 5 minutes at 70°C to denature double stranded RNA.

Master mix:	**Positive control** (µl)	**Negative control** (µl)	**Experimental sample (x1)** (µl)	**Experimental sample (x25)** (µl)
Positive control	2	0	0	0
Normalised RNA	0	0	2	2 per reaction
Oligo primer	1	0	0	0
Random primer	0	1	1	25
Nuclease free water	2	4	2	50
Final volume	5	5	5	125

Table 22: Sample and primer set-up for initiation of reverse transcription reaction of RNA samples.

As soon as this step had been completed the samples were put on ice ready for the next stage. Each sample then had the remaining solutions added, as in table 22. The total volume of the reaction was now 20µl. These volumes were then put into the thermo cycler on the following program:

- 25°C for 5 minutes
- 42°C for 1 hour
- 70°C for 15 minutes
- Cooled to 20°C to be removed.

cDNA was stored at -80°C until needed for qPCR.

Master mix:	**Positive control** (µl)	**Negative control** (µl)	**Experimental sample (x1)** (µl)	**Experimental sample (x25)** (µl)
Nuclease free water	4.2	8.8	7.3	182.5
5x ImProm II buffer	4	4	4	100
$MgCl_2$	4.8	1.2	1.2	30
dNTP	1	1	1	25
Ribonuclease inhibitor	0	0	0.5	12.5
ImProm II RTase	1	0	1	25
Final volume	15	15	15	375

Table 23: Second reaction set-up for the completion of the reverse transcription reaction.

3.4.4 Optimisation of Primer Sets.

Each set of primers were designed using PrimerExpress (version 2.0; Applied Biosystems) software and where appropriate the online Universal Probe Library assay design centre (www.roche-applied-science.com/sis/rtpcr/upl/index.jsp). Each design was aimed to cover a splice site which was common to all of the known transcripts of that gene were possible. Non-specific binding sites were avoided by running the primer designs through a BLAST search (www.ncbi.nim.gov/blast/Blast.cgi) to ensure the primer was specific to the genes being targeted.

Some primer sets had been previously optimised and validated within the laboratory (MJ Rose-Zerilli), new primer assays were validated as described in section 3.5.5.5. Primer sets for each gene are given in appendix 2.

3.4.5 qPCR Validation of Primers

Validation of primer sets was performed using pooled rat lung cDNA. When optimising these reactions the ideal combination should produce the lowest value of crossing threshold (Ct), or crossing point (Cp), was chosen. Primer concentrations were set-up using three different final concentrations each in combination as detailed in table 24.

		Reverse Primer		
		300nM	600nM	900nM
Forward Primer	300nM	Reaction 1	Reaction 2	Reaction 3
	600nM	Reaction 4	Reaction 5	Reaction 6
	900nM	Reaction 7	Reaction 8	Reaction 9

Table 24: Primer optimisation reaction set-ups for the differing primer concentrations.

The following annealing temperatures were tested 59°C, 60°C, 61°C and 62°C, as were the following magnesium chloride concentrations: 3, 2.5, 2, 1.5, and 1mM. Optimum primer concentrations were set at 900nM for the forward and reverse primers for the Taqman assays and at 1µM for the forward and reverse primers for the SYBR green assays. The optimum magnesium chloride concentration was set at 2mM for both assays, and the optimum annealing temperature was set at 60°C.

3.4.6 Quantification of Gene Expression by qPCR.

Alterations in the expression of different genes determine tissue type during development and can produce an altered phenotype in the organism. One way to identify and quantify gene expression differences is through quantitative polymerase chain reaction (qPCR).

There are two types of assays for the genes that are used in this investigation, SYBRgreen assays and Taqman (hydrolysis probe) assays. The SYBR green assay uses the total fluorescence within the wells when the two DNA strands have been parted before the annealing process to measure the amount of DNA within those wells. The Taqman assay uses the fluorescence emitted by the probe as it cleaved by the 5'-3' nuclease activity of the DNA polymerase as it displaces the probe from its binding site within the PCR amplicon to measure the amount of DNA within the well.

SYBRgreen Master Mix:

Reagents	**X1 reaction (µl)**	**Concentration**
10x Buffer	2	N/A
$MgCl_2$ (50mM)	0.8	2mM
dNTP mix (5mM)	0.8	0.2mM
Forward Primer (10µM)	2	1µM
Reverse Primer (10µM)	2	1µM
SYBRgreen Dye	0.6	N/A
Gold Star Taq	0.1	N/A
dH_20	12.7	N/A
cDNA	1	N/A
Volume	**20**	**N/A**

SYBRgreen amplification program:

- Taq activation:
    - 95°C for 10 minutes
- Amplification: (x55 cycles)
    - 95°C for 15 seconds
    - 60°C for 1 minute
    - 72°C for 1 second with a single data acquisition
- Melting:
    - 95°C for 15 seconds
    - 60°C for 15 seconds
    - 65°C for 1 second
    - 95°C increasing at 0.11°C/second with continuous data acquisitions
- Cooling:
    - 50°C for 1 minute

Taqman 2x qPCR Mater Mix:

Reagents	**X1 reaction (µl)**	**Concentration**
2x qPCR mix	5	N/A
Forward Primer (10µM)	0.9	0.9µM
Reverse Primer (10µM)	0.9	0.9µM
Probe (5µM)	0.4	0.2µM
dH_20	1.8	N/A
cDNA	1	N/A
Volume	**10**	**N/A**

Taqman amplification program:

- Pre-incubation:
    - 95°C for 10 minutes
- Amplification: (x45 cycles)
    - 95°C for 15 seconds
    - 60°C for 1 minute with a single data acquisition
- Cooling:
    - 40°C for 30 seconds

3.4.7 Estimation of Relative Gene Expression of Samples.

The optimum conditions for the primers were used with each gene on a pooled selection of samples. Samples were pooled according to age and treatment and tested in duplicate for each gene. This was done to find out if the relative level of gene expression was different in any gene between the treatment groups and minimise the waste in reagents. Each gene also contained a replicate of a calibrator sample that lies on the standard curves to ensure that the levels of expression seen can be correlated back to the original standard curves. If a significant difference was found between the groups then the individual samples were tested to ensure it is a real difference and not an abnormal sample skewing the outcome.

The relative level of gene expression is compared with housekeeping genes. These are genes whose expression should not change between the samples as they are present at almost equal levels throughout the tissue being looked at. More than one house keeping gene is used, BAC, B_2M and UBC are used in this investigation. The average expression of these genes is used to quantify the target genes expression. In this study the delta ct value was used to compare the expression levels.

3.5 Statistics

The same sets of statistics were used to determine the significance of the difference, if any, between the treatment groups. The tests used were a Students T test and an analysis of variance (ANOVA) test. The Students T test was chosen as there were two un-paired test variables, control or PR group, or testing of the same treatment group but between two time points, control group at F1 28 days against control group at F1 225 days. The outcomes for each testing group were continuous and the raw data showed a parametric test would be appropriate. A two tailed test was used as the direction of effect, of the dietary challenge was unknown.

The ANOVA test was chosen to for similar reasons to the T test, the data being normally distributed etc, but this test enabled the comparison of the data a crossed different time points while still comparing between the treatment groups.

Determination of normal distribution of raw data was determined using a Shapiro-Wilk test.

4. Results

4.1: Physiology and Morphometry

4.1.1 The DOHAD Hypothesis and Respiratory Disease

As previously described (see 1.1) maternal dietary challenges during pregnancy can alter offspring disease risk. This is due to the ability of the fetus to predict the best adaptation for its life once it is born, also known as the predictive adaptive response. In humans, evidence exists that suggests, changes in maternal nutrition during pregnancy results in altered lung development that may lead to increased risk of respiratory disease in the offspring (see 1.4). For example both asthma and COPD have been linked epidemiologically with markers of fetal growth such as anthropometric measurements at birth (Barker 1991; Leadbitter 1999). Fetal growth and duration of gestation influence lung development; recent studies in the Southampton Women's Survey reveal that lower rates of fetal growth are associated with impaired lung development in children (Lucas 2004). Airway function in the newborn period, which is dependant on fetal lung development, is also a significant predictor of asthma (Dezateux 1997; Palmer 2001; Håland 2006), adult lung function (Stern 2007), and potentially, COPD (Håland 2006). Shaheen et al. studying the 1970 British Cohort Study showed that the prevalence of asthma at 26 years increased with decreasing birth weight (Shaheen 1999). Equally, poor adult lung function and increased risk of death from COPD are also associated with lower birth weight, again suggesting an effect of *in utero* development (Barker 1991; Lopuhäa 2000; Edwards 2003; Canoy 2007). For example, studies of the effects of under nutrition in pregnancy resulting from the effects of the Dutch famine of 1944-1945, showed that exposure to famine during gestation resulted in increased incidence of COPD at age 50 (Lopuhäa 2000).

Studies of developmental programming in humans have limitations such as availability of tissue and the inherent timescale of cohort studies that need to span many decades to examine correlation between *in utero* exposures and the risk of late onset diseases such as COPD. As a consequence, a number of animal models have been utilised to explore the effects of developmental programming on a range of physiological parameters (Nathanielsz 2006).

The Southampton PR model, described in section 2.1, uses a decrease of 50% in the protein content of the rat dams' food from confirmation of pregnancy until birth of offspring. The decrease in protein is supplemented by an increase in carbohydrates to give an isocalorific diet. This model has been shown to induce a number of phenotypes that recapitulate effects of the DOHAD response in humans such as raised offspring blood pressure (Torrens 2008) and decreased nephron number within the kidneys (Harrison 2009).

4.1.2 Morphometry

Stereology is a method of making morphological measurements that can be applied to a 3D object to estimate a complete value for that 3D object. This method is used, as when inflated, the lung comprises approximately 85-90% airspaces and therefore the internal structure is vitally important (Weibel 2007).

There are four main considerations with this method. One is the thickness of the sections. To be used on a microscope the sections have to be thin which does not provide support for the tissue. This makes the tissue easily breakable and difficult to get accurate readings from if it is too thin. Secondly the sample size is small especially when compared with the entire lung organ. While for the rat this difference is not as accentuated as for analysis of human lung, it is still a consideration. Thirdly is consideration of specimen preparation. In the case of lungs, for accurate analysis it is important to ensure an equal, consistent degree of inflation of the lung samples when fixing. Furthermore, consideration needs to be made of the possible effects of unequal shrinkage and compression during sample processing (Weibel 2007).

The most commonly used stain for overall structure in morphometric investigations in the literature is haematoxylin and eosin (H&E) staining. Other stains that have been cited as used for this type of study are Congo Red, used to look at the large airways in rats (Vanacker 2001), Mason's trichrome, used to look at smooth muscle and basement membrane area of rats (Moir 2003), and alpha desmin/alpha vimentin, used to look at bronchiole profiles and areas of tissues in the airways of rats (Bonner 2000). H&E was used in this investigation as it is easier to define the areas of interest in comparison to other stains.

4.1.3 Aims

Given that: both 1) changes in body weight and, 2) changes in body composition, as seen with a brain-sparing effect, can be altered with maternal diet; in addition 3) increased risk of respiratory disease has been linked with both of these, we hypothesised that:

i. Maternal protein restriction will significantly alter body weight, lung weight and/or lung volume.

ii. Maternal protein restriction will significantly alter the fine structure of the lungs.

iii. These changes in lung structure will also be evident in the F2 and F3 generations.

To test these hypotheses, we aimed to extract the lungs from rats harvested at 28, 120 and 225 days of F1 animals and assess body weight, lung weight and lung volume. To establish if there were changes in the fine structure of the lungs we used stereology to measure volume fractionation, surface area and alveolar wall thickness, we also aimed to harvest the lungs from 28 day F2 and 21 day F3 generations and assess these measures in those as well.

4.1.4 Materials and Method

For materials see section 2.1

Animals were culled at F1 28 days, F1 225 days, F2 28 days and F3 21 days for this part of the investigation. See section 3.1 for details on the animal model. Comparisons between body weights, total lung weights and lung volumes were preformed using the method detailed in section 3.2.1. Lung tissue from the F1 28 day, F1 225 day and F2 28 day, rats was embedded, see section 3.2.2, in paraffin wax, cut and stained with H & E, see section 3.2.3, and stereological methods such as volume fractionation, see section 3.2.4.1, surface area, see section 3.2.4.2, and average alveolar wall thickness, see section 3.2.4.3, were used to compare the structure of the two treatment groups for the selected time points.

As described previously (see section 3.5) a Students-T test was used to determine the significance of any differences seen between the groups.

4.1.5 Results

In all graphs data is shown in box plot format with the middle line indicating the median value, the two edges of the box indicating the upper and lower quartile values and the tails coming from the box indicating the 95% values around the mean. Beyond these the circles indicate individual values that are more than two standard deviations from the median, and asterisks indicate individual values that are more then three standard deviations from the median.

4.1.5.1: Physical Data and Lung Volumes

4.1.5.1.1: Male 28 Day F1, 28 Day F2 and 21 Day F3 Rats.

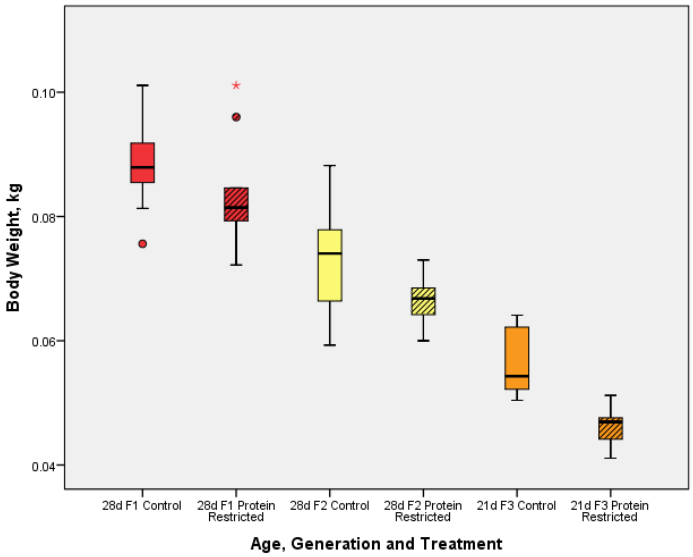

Figure 28: Comparison of the body weights (kg); of male rats 21-28 days old over three consecutive generations. (F1 28 days, C=15, PR=10: F2 28 days, C=24, PR=17: F3 21 days, C= 16, PR=5)

As can be seen in figure 28, the body weights of the animals increase with age, as expected. The F1 and F2 groups are older, 28 days, than the F3, 21 days. The growth difference between the two age groups almost doubles over the week between 21 days and 28 days. With all generations there is a trend towards a lower body weight in the PR group when compared to C, with the F3 generation having a significantly lower body weight in the protein restricted group than the controls. The difference in body weight was significant for the F2 ($p=0.001$) and F3 ($p=0.008$) generation with the use of the ANOVA test. This trend does not extend into the F1 and F2 older groups, implying that the rate of growth of the PR group is faster than that of the C group, i.e. the PR animals exhibit catch-up growth.

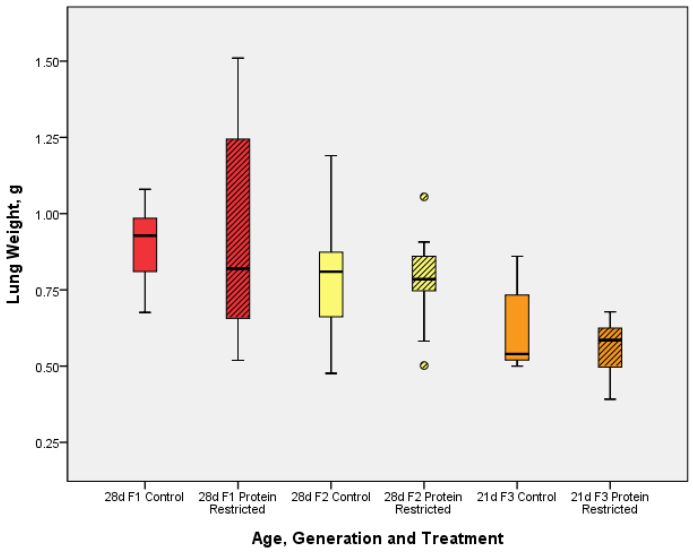

Figure 29: Comparison of total lung weights; from male rats 21-28 days old over three consecutive generations. (F1 28 days, C=15, PR=10: F2 28 days, C=24, PR=17: F3 21 days, C= 16, PR=5)

Total lung weights exhibit a similar pattern as that observed for whole body weight (figure 29). The difference in lung weight between either generations- or treatment groups- is not as extreme as that in body weight, with an increase of about 50% compared to 100% for bodyweight. There is no significant difference found between treatment groups for the t-test, however the F2 generation showed a p value of 0.044 when the ANOVA test was applied. There is a trend towards smaller lung weights in the protein restricted groups when compared with controls, although this is not as marked as that seen in body weight.

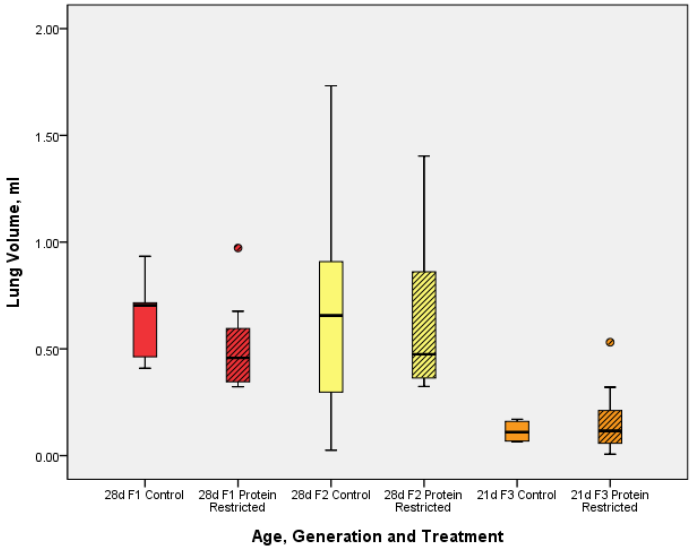

Figure 30: Comparison of lung volume; from those male rats ranging from 21-28 days old over three consecutive generations. (F1 28 days, C=15, PR=10: F2 28 days, C=24, PR=17: F3 21 days, C= 16, PR=5)

Lung volume also follows the age related pattern observed for body and lung weight, (figure 30) however the difference between the F3 groups and the other groups is much more extreme with the F1 and F2 volumes being approximately 4.5 times larger than those of the F3 rats reflecting the rapid postnatal growth in lung volume. Although there is no significant difference ($p=>0.05$, t-test and ANOVA) between the treatment groups they all follow the same trend with the protein restricted group exhibiting a lower lung volume than the control group in all generations.

4.1.5.1.2: Male and Female F1 225 Day Old Rats.

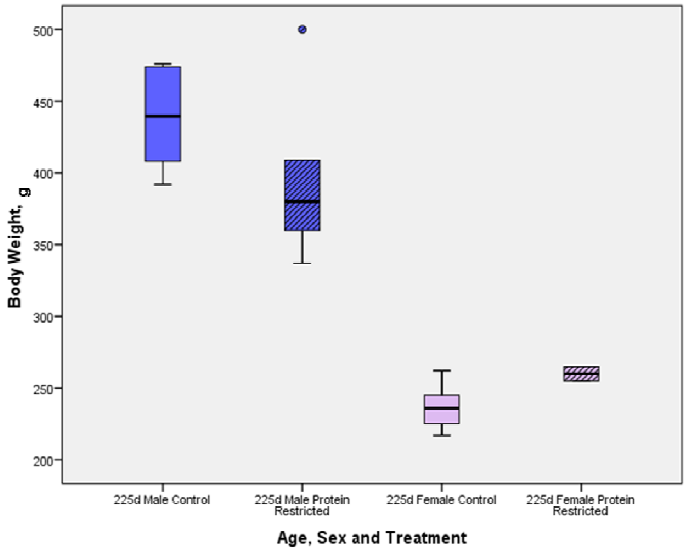

Figure 31: Comparison of body weight; of male and female rats aged 225 days old across treatment groups. (F1 225 days male, C=6, PR=6: F1 225 days female, C=6, PR=2)

As seen in figure 31, body weights of male and female rats at the age of 225 days is significantly different (p=0.003, t test) with the males being larger than the females. The body weights for male or females are not significantly different (p=>0.05, t test and ANOVA) between treatment groups. However there is a trend towards the control group weighing more than the protein restricted group in the male data, whereas this trend is reversed in the female data.

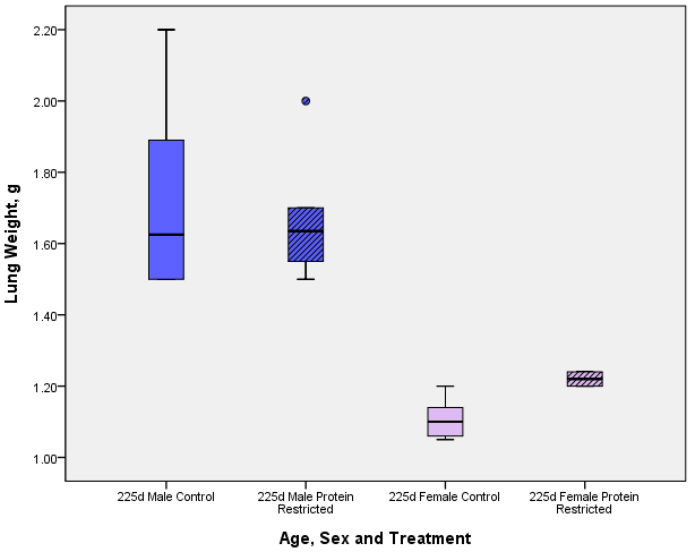

Figure 32: Comparison of total lung weight; from male and female rats aged 225 days old across treatment groups. (F1 225 days male, C=6, PR=6: F1 225 days female, C=6, PR=2)

There is almost no difference (p=>0.05, t test and ANOVA) between the total lung weights of the male 225 day old treatment groups, figure 32. There is a no significant difference (p=>0.05, t test and ANOVA) between the treatment groups in the female data with the controls being smaller than the protein restricted group. The females have a significantly lower total lung weight than the males (p=0.004, t test and ANOVA) which is as expected as the females are smaller in size than the males.

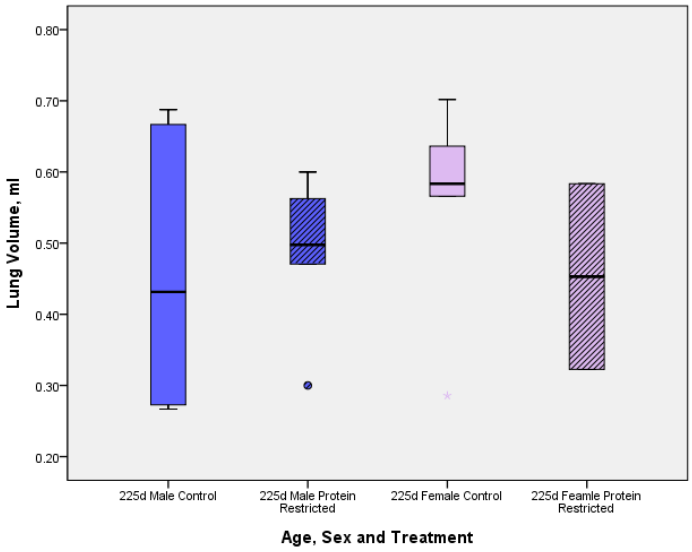

Figure 33: Comparison of lung volumes; from male and female rats aged 225 days old across treatment groups. (F1225 days male, C=6, PR=6: F1 225 days female, C=6, PR=2)

The lung volume data, figure 33, is more evenly spread with no significant difference (p=>0.05, t test and ANOVA) seen between treatment groups or sex. However the control group have a trend towards a smaller volume than the protein restricted group in the males, with this trend reversed in the females. Overall the lung volume of the females is lower than that of males, again as expected given the relative size of females compared to males.

4.1.5.2 Morphometry Results

Previous work on 120 day old male rats has established that maternal protein restriction results in clear changes in physiological parameters such as blood pressure (Brawley 2003) and altered gene expression in the lung (MJ Rose-Zerilli, personal communication and (Rose-Zerilli 2010)). Using the F1 28 day and the F1 225 day old offspring of the control and protein restricted mothers, the morphometric analysis of the lungs was undertaken to determine if there were differences between the two treatment groups. A third group was also analysed, F2 28 day old offspring, to assess the possibility of effects of maternal protein restriction manifesting in the F2 (grandchild) generation.

4.1.5.2.1: Volume Fractionation

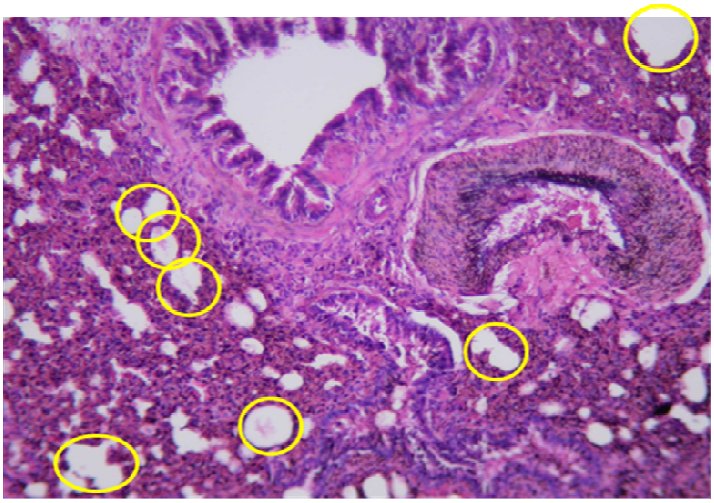

Figure 34: Rat lung H&E stain. Airspaces are highlighted by yellow rings. Magnification x10.

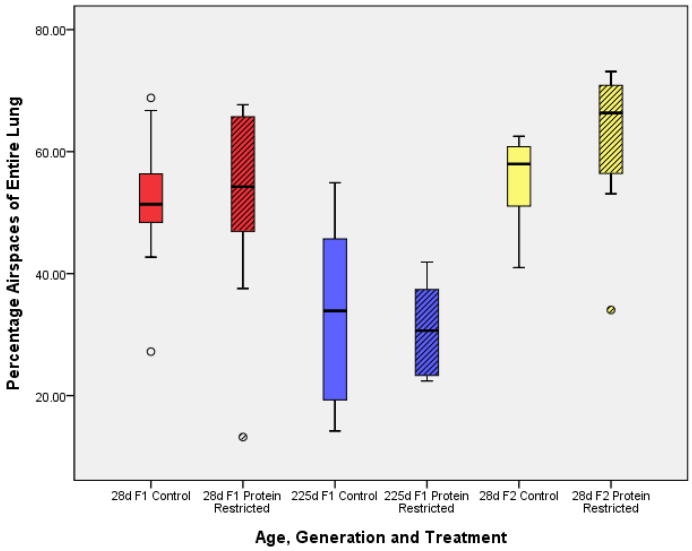

Figure 35: Box plot showing the total % airspaces of the entire lung; from PR and C groups for all age groups and generations of rats tested. There is a trend for increased airspaces in the C lungs but the range of data is larger in the PR group. The p value for the difference between the two groups is 0.146. (F1 28 days, C=13, PR=13; F1 225 days, C=6, PR=6; F2 28 days, C=15, PR=15)

No significant differences were found between the two treatment groups in the F1 28 day, F1 225 day or F2 28 day old offspring for percentage air spaces within the lung.

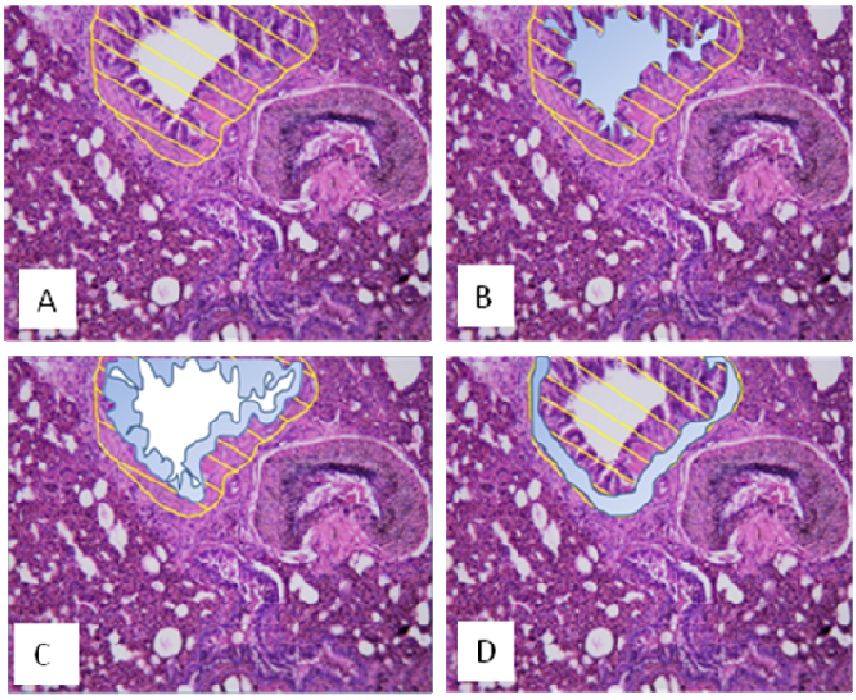

Figure 36: Rat lung H&E stained, A) shows the entire airway, B) shows the lumen of the airway in blue, C) shows the epithelium around the airway in blue and D) shows the smooth muscle around the airway in blue. Each of these airway components was compared between the treatment groups for all generations and age groups. Magnification x10.

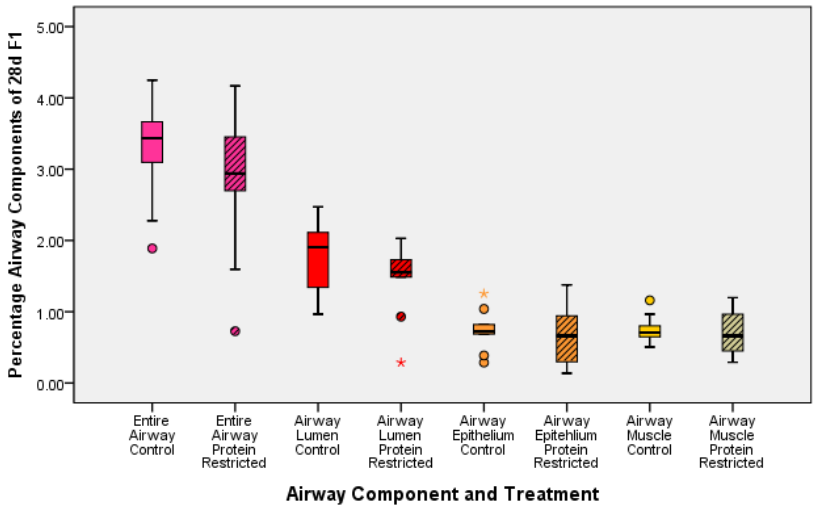

Figure 37: Box plot showing the total % airways, and their components, from both PR and C groups for the F1 28 day old rats. (F1 28 days, C=13, PR=13)

There was no significant difference seen between the treatment groups for any component of the airways in the F1 28 day old rats.

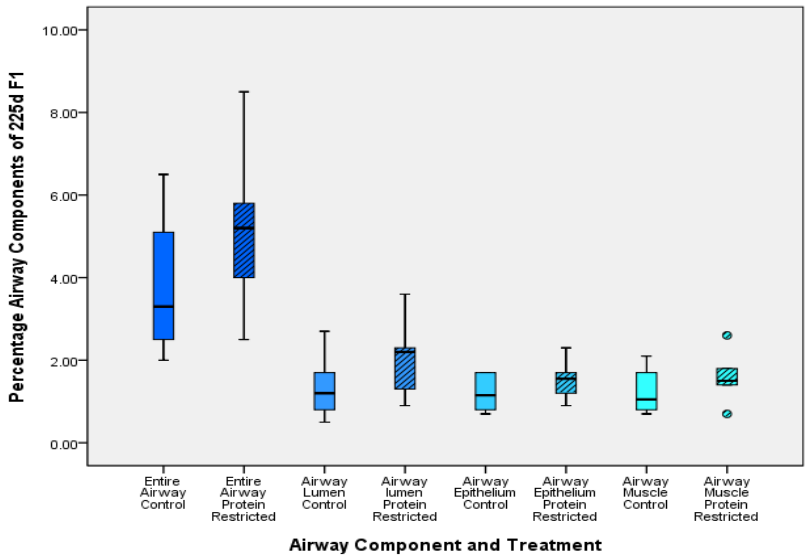

Figure 38: **Box plot showing the total % airways of the entire lung,** for the different airway components analysed, from both PR and C groups for the 225 day old rats. (F1 225 days, C=6, PR=6)

There was no significant difference seen between the treatment groups for any component of the airways in the F1 225 day old rats.

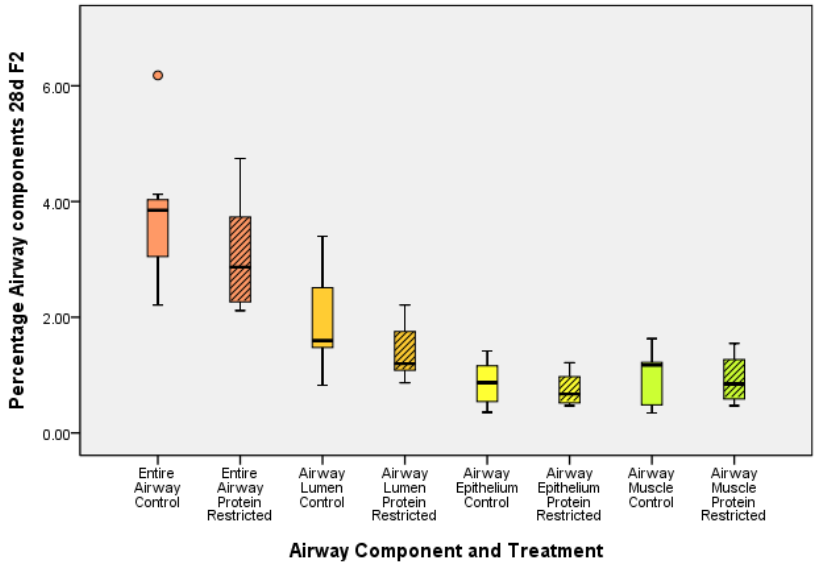

Figure 39: Box plot showing the total percentage of airways of the entire lung, for the different airway components analysed, from both PR and C groups for the 225 day old rats. (F2 28 days, C=15, PR=15)

There was no significant difference seen between the treatment groups for any component of the airways in the F2 28 day old rats.

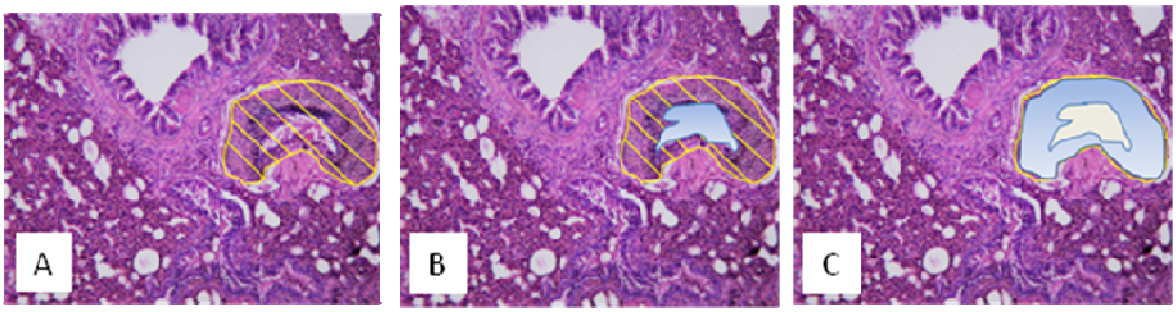

Figure 40: Rat lung H&E stained. A) The yellow ring with internal yellow lines shows the entire blood vessel, B) shows the vessel lumen in blue and D) shows the vessel muscle in blue. Magnification x10.

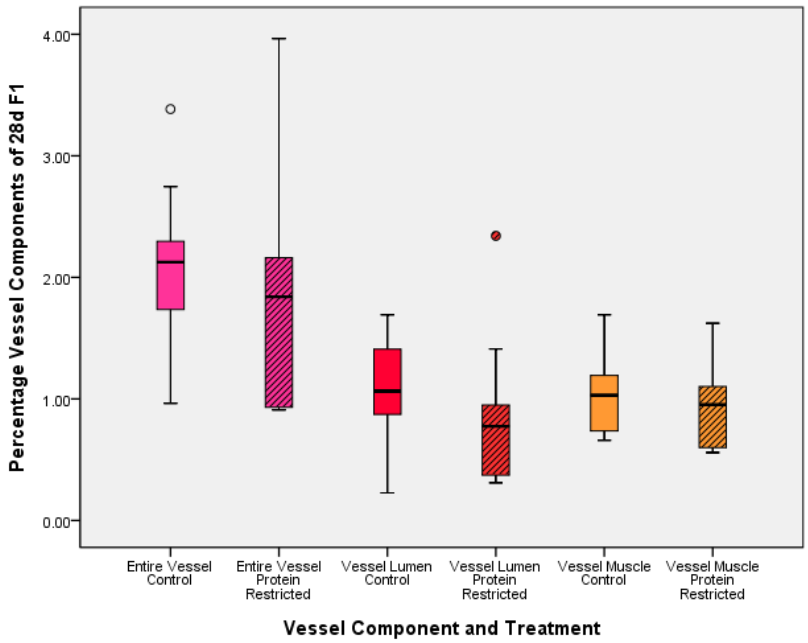

Figure 41: Box plot showing the percentage of vessel of the entire lung, from both PR and C groups for the F1 28 day old rats. (F1 28 days, C=13, PR=13)

There was no significant difference seen between the treatment groups for any component of the vessels in the F1 28 day old rats.

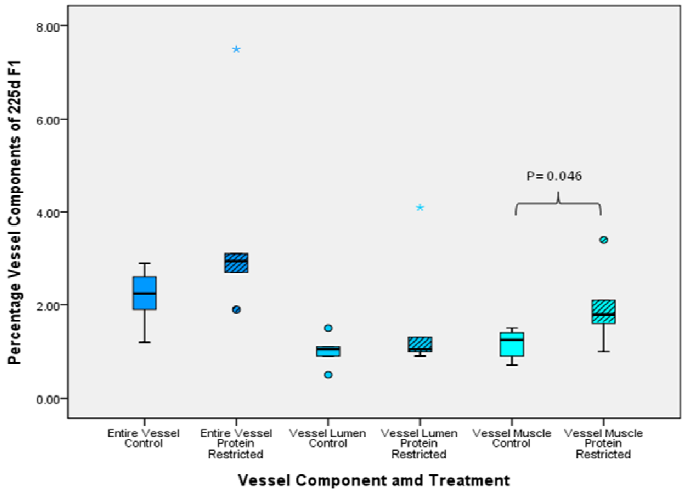

Figure 42: **Box plot showing the percentage of vessel of the entire lung,** from both PR and C groups for the F2 28 day old rats. (F2 28 days, C=15, PR=15) from both PR and C groups for the F1 225 day old rats. (F1 225 days, C=6, PR=6)

There was no significant difference between the treatment groups for the entire vessel or vessel lumen for the F1 225 day old rats, but there is a significant difference between the amounts of muscle around the vessels in these rats. The muscle around the PR rats is increased when compared with the C rats (p=0.046, t-test; p=0.024, ANOVA).

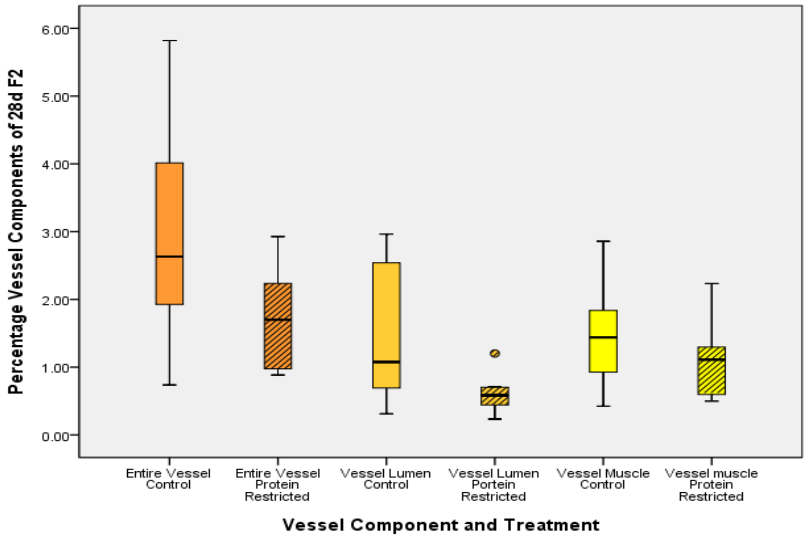

Figure 43: Box plot showing the percentage of vessel of the entire lung, for the different vessel components analysed, from both PR and C groups for the 225 day old rats. (F2 28 days, C=15, PR=15)

There was no significant difference between the treatment groups for the vessel lumen for the F2 28 day old rats, but there is a significant difference between the entire vessel and the amount of muscle around the vessels in these rats when described using an ANOVA test. The entire vessels and the amount muscle around the vessels in PR rats is increased when compared with the C rats (vessel, p=0.038; vessel muscle, p=0.005; ANOVA). This significance was not found when using a t-test.

4.1.5.2.2 Surface Area.

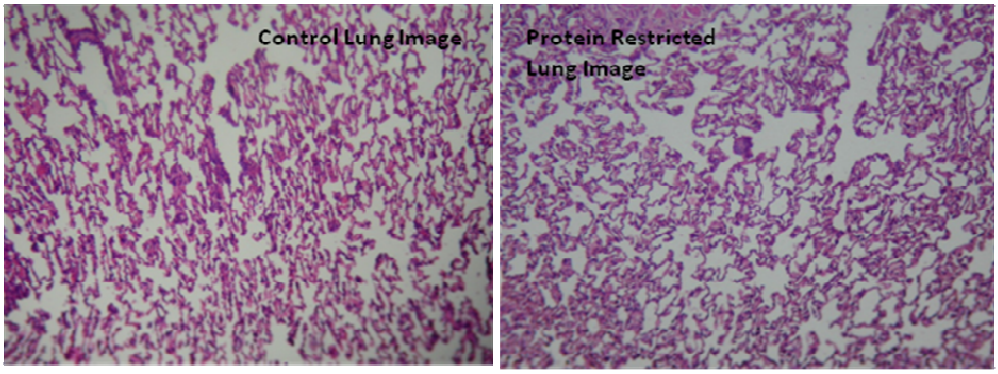

Figure 44: Overview of H and E images from the offspring rat lungs culled at 225 days old. These mature lungs show an offspring lung from the control *in utero* diet on the left and from the protein restricted diet on the right. Magnification x10.

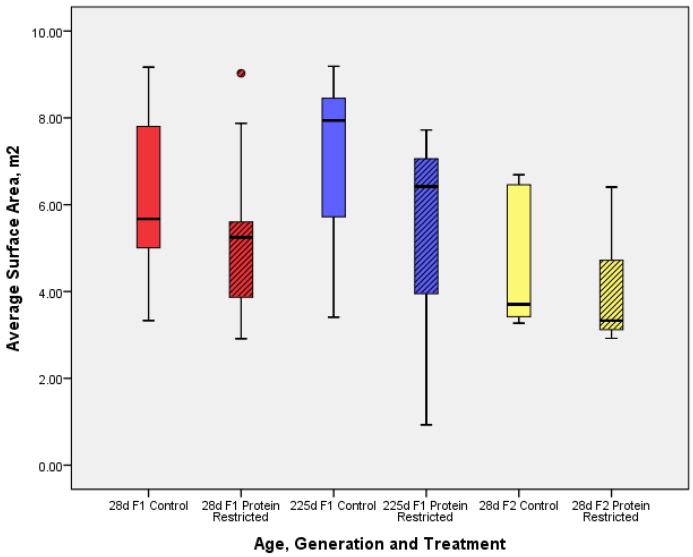

Figure 45: Box plot of average internal surface area, for all the age groups and generations of rats tested. (F1 28 days, C=13, PR=13; F1 225 days, C=6, PR=6; F2 28 days, C=15, PR=15)

There was no significant difference found between the two treatment groups when looking at the lung surface area for any of the F1 28 day old offspring, the F1 225 day old offspring or the F2 28 day old offspring. There is a trend towards a reduction in surface area in the PR lungs for both age groups. This difference is more pronounced in the adult, F1 225 day, age group.

4.1.5.2.3 Alveolar Wall Thickness.

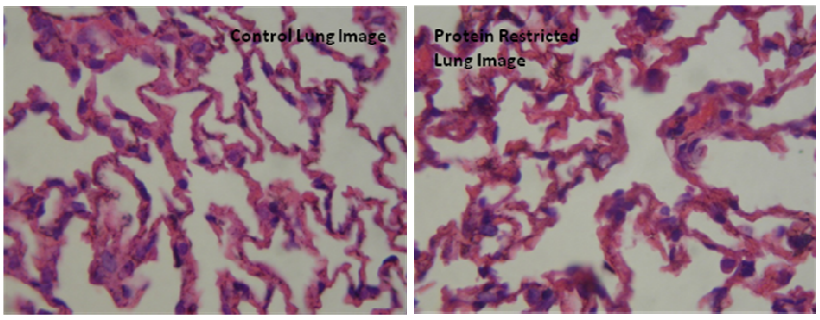

Figure 46: Overview of H and E images from the offspring rat lungs culled at 225 days old. These mature lungs show an offspring lung from the control *in utero* diet on the left and from the protein restricted diet on the right, of the alveolar walls. Magnification x63.

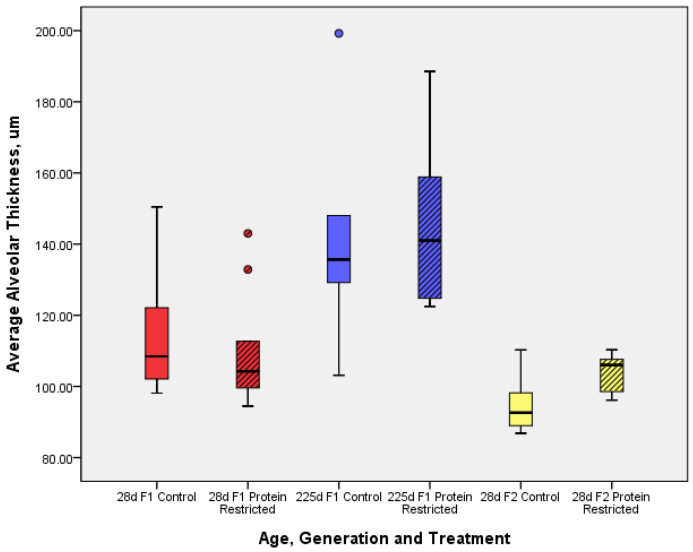

Figure 47: Box plot of average alveolar wall thickness, for all the age groups and generations of rats tested. (F1 28 days, C=13, PR=13; F1 225 days, C=6, PR=6; F2 28 days, C=15, PR=15)

There was no significant difference found between the two treatment groups when looking at the average alveolar wall thickness for the F1 28 day old, the F1 225 day old offspring or the f2 28 day old offspring. There is a trend towards a reduction in average alveolar wall thickness in the protein restricted group lungs for the 28 day F1 age group, which was then reversed in the adult 225 day F1 age group.

4.1.6 Discussion

4.1.6.1 Physiology Discussion

4.1.6.1.1 Body Weights

As expected, body weights increased with the age of the rats. There was no significant difference between body weights between the two treatment groups in any of the F1, F2, or F3 generations. However, there was a trend towards a lower body weight in the protein restricted groups in all generations. This was inline with previous observation of the effect of protein restriction (Torrens 2002; Rodford 2008), and although the difference is not significant between groups, this is likely to reflect the severity of dietary restriction in this model when compared with others in the literature where a significant difference in the body weights of the two groups has been observed (Chen 2004).

Body weight measurements from the 225 day old F1 rats showed that there was a significant difference between the male and female measurements. In the interests of efficiency only the male offspring were used for further analysis. The reasons for using the male animals instead of the females were, 1) Physiological measurements in females are known to alter with respect to their ovulation, therefore culling would have to be timed to match these cycles to reduce measurement variability if females were utilised; 2) some studies have shown that protein restriction induces changes in physiological phenotypes in males that are not reproduced in the female offspring, and as this is a preliminary study it is preferable to use the group that has previously exhibited physiological changes in response to PR (Woods 2003); and 3) the female offspring can be utilised to breed subsequent generations (F2, F3 etc) to study the effect of epigenetic inheritance.

Previously, there have been two significant studies that have utilised this precise model of Wistar rats with a 50% reduction in protein of maternal diet. A study of F1 offspring found a significant difference ($p=<0.01$, two-way ANOVA) in the birth weights of offspring, with the PR group (n=9) having a lower birth weight than controls (n=12) (Torrens 2002). A second study however, did not observe a significant difference in birth weights between groups (C=13, PR=15) (Torrens 2008). In this study the numbers for the F1 offspring at 28 days (C=5, PR=7) and 225 days (C= 6, PR=6) was lower than these studies, which may account for the lack of significant difference in birth weights between groups.

4.1.6.1.2 Lung Measurements

Total lung weight was found to be lower in younger rats, as expected, however the difference in the lung weights as the offspring age does not follow the same trajectory as observed for body weights. A larger difference in lung weight was observed between the 21 and 28 day old rats than between the 28 and 225 day old rats. This implies that the although the lung is still growing for part of the time between 28 days and 225 days, this growth is less than that seen between 21 and 28 days. This is in line with previous

observations suggesting the lung develops to maturity before the body does. (Powell 1980) This earlier maturation of the lung in the rat model is also seen in the human. The rat lung grows for 13.5% of a rat's total life span (Powell 1980; Goodrick 1982), whereas the human lung grows for 5.6% of a human's total life span (Burri 1984; Sowell 2003).

No significant difference in lung volumes between the two treatment groups at any age was seen. However there was a trend towards reduced volume in the protein restricted group compared to controls. Lung volumes followed a similar pattern to that of total lung weight, although there is a more marked difference between the younger age groups with the volume of the F3, 21 day old rat lungs, being half that of the 28 day old groups.

4.1.6.1.3 Summary

Several problems were identified in the method utilised to estimate lung volume. One of these was back flow of formalin around the needle inserted into the bronchi due to poor fit of the needle with the bronchi of these lungs, this was reduced by tying the needle into the bronchi but some fluid still escaped in some cases. A loss of formalin through small nicks in the lungs caused during dissection was also observed in some instances, and these lungs were removed from analysis. Partial inflation of the lung was also seen. It is assumed that because a non-flexible needle was used to introduce the formalin in to the lungs any deviation from the main path of the bronchi may have caused the needle to enter into a single branch of the lung. This resulted in only a single lobe inflating and not the entire lung. Over inflation of the lung was also observed. To try and remove these problems an altered method was developed.

By inserting the cannular needle into the trachea through the wall of this organ, with only the needle causing the breach, the hole was the correct size and no formalin was lost from the lung. As the needle was cannular, the steel needle could be removed and only the flexible sheath was left in the lung. This meant that the fluid was able to fill the lung completely with no partial inflation of a single lobe. The lungs were also inflated *in situ,* reducing the chance of cuts in the lung during dissection, and by leaving the rib cage and diaphragm as intact as possible, preventing over inflation.

4.1.6.2 Morphometry Discussion

A summary of the results found for each age group allows for a better understanding of the interpretation of the results. The F1 28 day old rats and the F2 28 day old rats were found to have no significantly different alterations in their lung structure between control and PR groups.

4.1.6.2.1 Volume Fractionation

The percentage of the lung that was found to be airspaces was slightly reduced in the PR rats. There was also a small difference in the percentage of the lung that constituted the total airways, with the PR rats

having a slightly higher value that that of the C group. The percentage of total airways was created using a measure of the lumen, epithelium and the muscle. It is interesting to note that the within this data, only the airway lumen was increased in the PR group when compared to the C group. As the other differences showed opposite direction of effect it implies that although the total airways were found to be slightly increased in the PR rats this is mainly due to the lumen of the airway. The percentage of the lung that comprised blood vessels was found to be increased in the PR rats and when broken down this appeared to be due to the increased amount of muscle that was found around the vessels.

No significant difference was found between treatment groups for the components of the lung structure for the 225 day old rats. The percentage of the lung that was found to be airspaces was slightly reduced in the PR rats. There was also a small difference in the percentage of the lung that constituted the total airways, with the PR rats have a slightly higher value that that of the control group. The percentage of total airways was created using a measure of the lumen, epithelium and the muscle. It is interesting to note that the within this data set only the airway lumen was increased in the PR group when compared to the control group. As the other differences were against the total airway result it implies that although the percentage of the lung that comprised total airways was found to be slightly increased in the PR rats this is mainly due to the lumen of the airway. The percentage of the lung that comprised blood vessels was found to be increased in the PR rats and when broken down this appeared to be due to the increased amount of muscle that was found around the vessels.

The amount of smooth muscle found around the vessels of the F1 225 day PR group of rats was significantly increased ($p=0.046$). This remodelling of the muscle around the vessels could potentially explain the previous observation of increased blood pressure in the PR F1 treatment group previously observed with this model (Torrens 2002; Torrens 2008). This increase in blood pressure was also observed in the F2 generation, however there was no alteration in the internal diameter of the vessels. Vessels from the PR group were found to have enhanced vasoconstriction. The finding that there is more muscle around the vessels in the PR lung means that they are able to constrict more than those in the control group and exert more pressure on the blood inside those vessels. However this does not provide an exact mechanism for the hypertension seen as the blood branches off into smaller vessels. If these vessels were seen to have more muscle around them then this would indeed explain the results as the smaller the vessels the higher the pressure the blood is already under. If the muscle surrounding the smaller arterioles in the lung was found to be also increased, then the effect of that muscle on the blood pressure would be magnified.

4.1.6.2.2 Surface Area

The surface area was decreased in the PR when compared to the control group for all time points and generations. When comparing between the age groups the surface area of the lung increased in the oldest 225 day rats. This implied that the lungs are still maturing in the 28 day old rats and a difference may be seen at an older age in the F2 generation.

4.1.6.2.3 Alveolar Wall Thickness

The control alveolar walls were thicker than those seen in the PR for the F1 28 day old rats, although not significantly. This trend is still apparent in the 225 day old rats but the difference between the two treatment groups is less. This trend is reversed in the F2 28 day rats with the PR group having thicker alveolar walls when compared with the control group.

4.1.6.2.4 Summary

All the trends in differences between percentages of total lung for the various airway components found in the 28 day old rats were the same direction in both the F1 and F2 generations for both treatment groups. However, these trends appeared to reverse in the older 225 day old treatment groups. This could indicate that a period of catch-up growth occurs in the PR group, resulting in a reversal of the trends in airway structure. Previous studies using more severe dietary changes have seen significant changes in airway structure in younger animals that resemble those observed between the 28 day old control and PR groups in this study(Chen 2004; Maritz 2004).

The results of the morphometric analysis of the F2 generation lungs showed changes in structure between control and PR similar to those observed in the F1 generation, however although still not significant, these effects appear to be more pronounced. A study by Maloney et al. (2011) examined the effect of a maternal diet low in methyl-donors on the development of diabetes through both decreased insulin production and insulin resistance in rats (Maloney 2011). In this model, the dietary challenge was started three weeks before the females were mated up until confirmation of pregnancy, plugging, at around 5 days post mating. In this study a low methyl-donor maternal diet for this time resulted in offspring that were more resistant to insulin. However this phenotype was only observed in male offspring and was more pronounced at the older time point of 51 weeks than at 26 weeks of age. This has similarities with humans, where development of type II diabetes, featuring insulin resistance, tends to occur later in life.

The timing of the dietary challenge here is of interest as the effect on offspring insulin resistance occurred despite the maternal dietary challenge being restricted to approximately the first 5 days of development. In contrast, the dietary challenge in the current study is only initiated on confirmation of pregnancy. However, while for the F1 generation, this means that the developing embryo is not exposed

during this critical early window, for the F2 generation, the eggs that will go on to form the F2 generation are being made during this time in the female F1 embryos. During the act of fertilisation gametes are stripped of all their methylation patterns and then these are reinstated early in blastocyst development (Cirio 2008). This may account for the apparently stronger effect of dietary challenges that occur in this early developmental window. It may also explain why there is an apparently stronger effect on lung morphometry in the F2 rats of maternal PR, in comparison to the effects seen in the F1 offspring.

4.1.6.2.4.1 Sample Size

A posthoc power calculation for a 5% difference between the two treatment groups, for airway muscle, indicated that a sample size of 60 would have been required to achieve statistical significance with an α=0.05 and 80% power. This means that the size of groups being using would have to be increased ten times and there were not enough animals in the study to accomplish this.

4.1.6.2.4.2 Vessel Smooth Muscle

The observed increase in smooth muscle, surrounding blood vessels in the lung, suggests a potential mechanism to explain the observation of hypertension in F1 and F2 offspring in the maternal PR model. Future studies could extent the analysis to smaller resistance arterioles and to blood vessels in other organs. This effect is not seen in the younger 28 day old rats. As such this effect could be due to natural aging, as with humans it is unusual to see problems such as hypertension in the young. If this is the case then the build-up of muscle around the vessels must be driven by an underlying reason such as an alteration in gene expression. This could be due to a failure to stop a gene from being expressed at the correct time, or it could be due to an increase in the expression of that gene at some point, possibly due to some altered epigenetic effect. To see if this is the case gene expression for this set of lungs will be the next step in this investigation.

4.1.6.2.5 Future Directions

If resources, time and money, had been inexhaustible, an investigation into the morphometry of the F3 lungs would have been of interest. In addition, the input of a challenge, either an allergen challenge or a straight forward forced constriction of the bronchi, with methacholine for example, would likely have resulted in a more pronounced alteration within the lungs.

4.2 Bronchial Hyperresponsiveness (BHR)

4.2.1 Introduction

A characteristic of asthmatic airways is reduced lung function. This is due to both the number and frequency of bronchoconstriction events that occur with asthma exacerbations, and the development of a degree of fixed airway obstruction as a result of airway remodelling. See section 1.2.7.

BHR is exaggerated bronchial constriction in response to nonspecific provocation, whether that is inhalation of various bronchoconstrictors, or due to environmental stimuli, e.g. exercise, dry or cold air, hypertonic or hypotonic aerosols (see section 1.2.2.2). Such hyperresponsiveness is characteristic of asthma, and is commonly used for clinical diagnosis, as asthmatics exhibit exaggerated bronchoconstrictor responses, to a methacholine or histamine, challenge. An increased airway responsiveness to stimuli results in an increase in the number of these types of events, which in turn, may lead to increased airway remodelling and disease progression.

Direct evidence for BHR being linked with developmental programming has not been reported. However, there is evidence for susceptibility to asthma *per se* being influenced by developmental programming. For example maternal exposure to a range of environmental exposures results in increased risk of developing asthma in childhood (Bertelsen 2010; Kozyrskyj 2011). Furthermore a number of studies have linked anthropomorphic measurements at birth (e.g. birth weight, head circumference and body length) to altered susceptibility to asthma in childhood (Barker 1991; Lopuhäa 2000; Edwards 2003; Canoy 2007; Hancox 2009; Walter 2009). One of the first studies to link low birth weight with BHR was undertaken in 1988. This study was interested in the link between asthma and premature birth, because of this the babies in the study were low birth weight due to premature delivery not low birth weight for normal gestational age, however the prevalence of BHR to histamine was significantly increased in the low birth weight group of children compared to the reference group (Chan 1988).

Exploration of the effect of any developmental challenge on rat airways has not been reported in the literature. There is a large body of evidence that has noted a hypertension phenotype, with altered vasoconstriction within offspring rats whose mothers were exposed to the low protein diet during their pregnancy (Rodford 2008; Torrens 2008). This effect has also been found to be transgenerational (Torrens 2008). This chapter will examine the effect of bronchoconstrictors: carbachol (an acetylcholine mimetic); U46619 (a thromboxane mimetic); angiotensin II and histamine, and bronchodilators: isoprenaline; salbutamol; BRL37344 and papaverine, on the rat bronchi to try and validate this model, and assess differences in responses for the protein restricted versus control treatment groups.

4.2.2 Aims

Given that 1) BHR is a key diagnostic measure for asthma and, 2) changes in asthma risk have been seen in response to maternal protein restriction and 3) this alteration in asthma risk is linked with BHR, we hypothesised that:

iv. Base-line readings from the rat bronchi will be similar to those found in humans.

v. Increased BHR will be present in the offspring from protein restricted mothers compared to controls.

vi. These changes in BHR will result in significantly different reaction of the bronchi to bronchodilators.

To test these hypotheses, we aimed to extract primary bronchi from lung samples harvested at 35 and 75 days of F1 animals and assess the reaction of these bronchi to a panel of both bronchoconstrictors and bronchodilators in order to establish if potential changes in BHR have been induced by maternal PR and if so does this alter the reaction of the bronchi to bronchodilators.

4.2.3 Materials and Methods

For materials see section 2.1.

4.2.3.1 Bronchial Responsiveness Testing

Animals were culled at F1 35 days, and F1 75 days for this part of the investigation. See section 3.1 for details on the animal model.

Following post mortem lungs were excised and placed in cold PSS (4°C). To better visualise the bronchi, the airways were flushed with PSS containing a few drops of ink. Segments of bronchi were dissected out, cleaned of surrounding tissue, cut into 2mm segments and mounted on the wire myograph. Segments of bronchi were maintained in PSS heated to 37°C and continually gassed with 95% O_2 and 5% CO_2. More detail for these methods is given in sections 3.3.1 and 3.3.2.

The optimal resting tension for rat bronchi was calculated by measuring the KPSS contraction at increasing resting tensions. The optimal resting tension for rat bronchi was found to be 1.5g of tension. Subsequently mounted bronchi were placed under 1.5g of tension and cumulative concentration response curves (CRCs) to the various agonists were constructed.

Bronchoconstriction was assessed by the construction of CRCs to the acetylcholine mimetic, carbachol (CCh, 1nM-100µM), the thromboxane mimetic, U46619 (1pM-1µM), angiotensin II (Ang II, 1pM-1µM) and histamine (1nM-100µM). Bronchi were then preconstricted with a sub-maximal dose of CCh equivalent to pEC_{80} (the concentration which produced 80% of the maximal CCh response) and relaxation was assessed to the non-selective β-adrenoceptor agonist isoprenaline, (ISO, 0.1nM - 100µM), the selective β_2-adrenoceptor agonist salbutamol (SAL, 0.1nM - 100µM), the selective β_3-adrenoceptor agonist BRL37344 (BRL, 0.1nM - 100µM), and the PDE inhibitor papaverine (PAP, 0.1nM - 100µM).

4.2.3.2 Statistics

As described previously (see section 3.5) a Students-T test was used to determine the significance of any differences in area under the curve (AUC) seen between the groups.

4.2.4 Results

Animals were used from the F1 generation at 35 and 75 days of age for this investigation. These animals were produced as detailed in section 3.1.

4.2.4.1 Resting Tension

Stepwise increases in resting tension produced greater constriction to KPSS up to a resting tension of 1.5g. At resting tensions greater than this, KPSS-induced constriction was reduced (see figure 48). All data is displayed with errors bars showing one standard deviation from the mean.

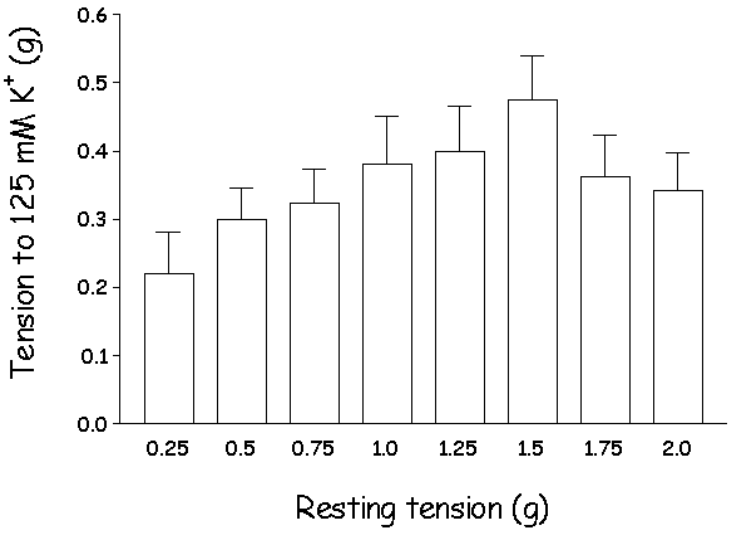

Figure 48: Response of 125mM KPSS to isolated bronchi at various resting tensions. (n=10)

4.2.4.2 Base-line Effects of Bronchoconstrictors on Rat Bronchi.

Initially the effects of four bronchoconstrictor agents were tested on bronchi obtained from the lungs of rats whose body weights were between 200 and 250g. The classical stimulator of bronchoconstriction in humans, histamine, failed to produce any response in a rat bronchi (see figure 49). Similarly the vasoactive peptide angiotensin II produced only a very modest constriction of isolated rat bronchi (see figure 50).

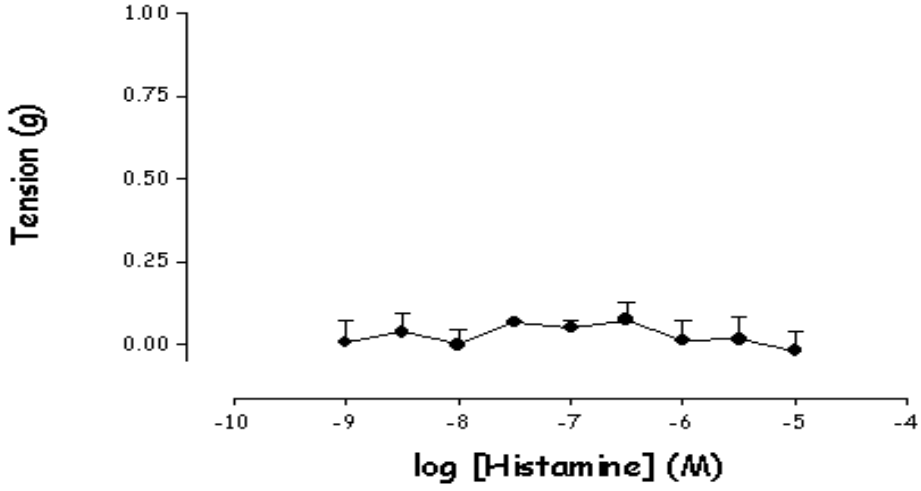

Figure 49: Cumulative addition of histamine to isolated rat bronchi (•, n=8)

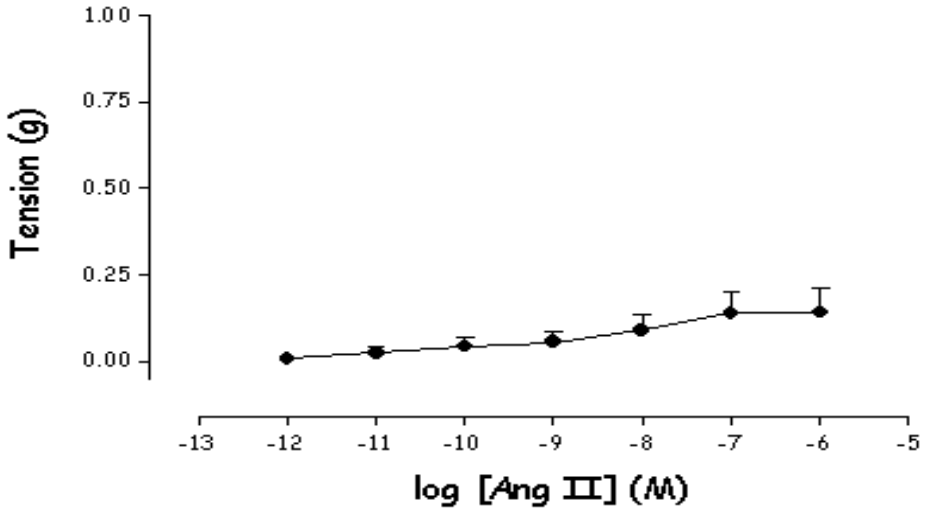

Figure 50: Cumulative additions of angiotensin II to isolated rat bronchi (•, n=8)

4.2.4.3 Base-line Effects of Dilators on Rat Bronchi

The non-selective adrenoceptor agonist isoprenaline produced a modest bronchodilation of isolated rat bronchi that was attenuated at higher concentrations (figure 51). To counter any α-adrenoceptor mediated constriction of the bronchi, responses where repeated in the presence of the α_1-adrenoceptor agonist, Prazosin (10μM) which abolished the biphasic response (figure 52).

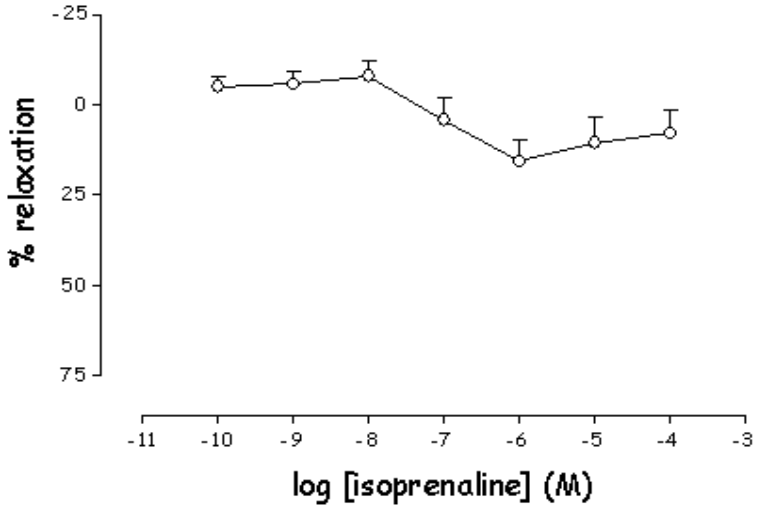

Figure 51: Cumulative addition of adrenoceptor agonist isoprenaline to isolated rat bronchi (o, n=8)

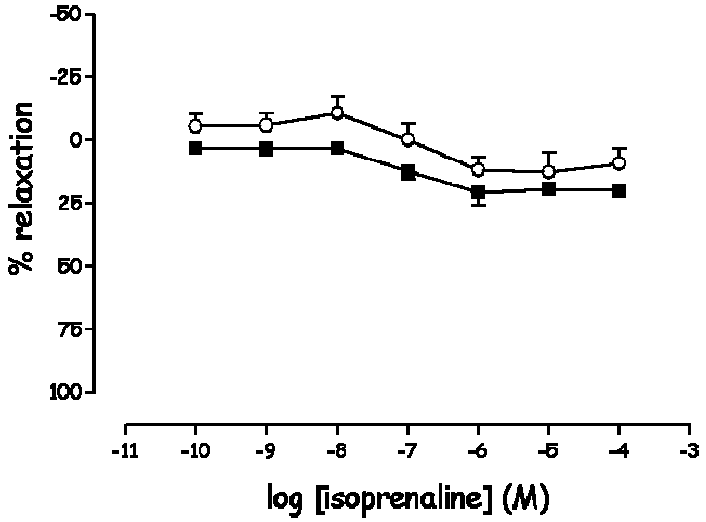

Figure 52: Cumulative addition of the non-selective adrenoceptor agonist isoprenaline to isolated rat bronchi in the absence (o, n=8) or presence of the α_1-adrenoceptor antagonist, prazosin (10μM; ■, n=3)

Similarly to isoprenaline, the selective β_2-adrenoceptor agonist salbutamol and the selective β_3-adrenoceptor agonist BRL37344 produced a modest dilation of isolated bronchi (figure 53 and 54). The cAMP activator papaverine had little response until the very top dose where it produced a complete abolition of the carbachol-induced tone (figure 55).

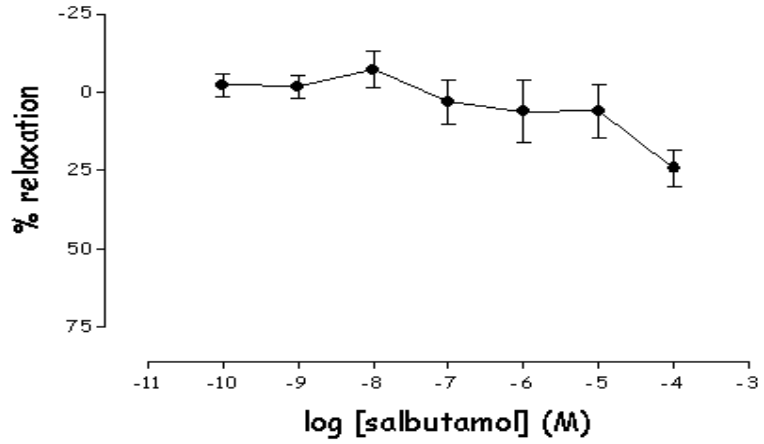

Figure 53: Cumulative addition of the selective β_2-adrenoceptor agonist salbutamol to isolated rat bronchi (•, n=8)

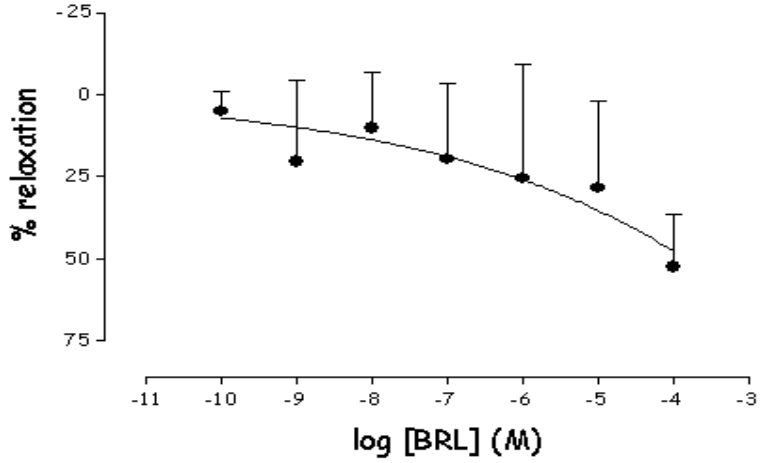

Figure 54: Cumulative addition of the selective β_3-adrenoceptor agonist BRL37344 to isolated rat bronchi (•, n=8)

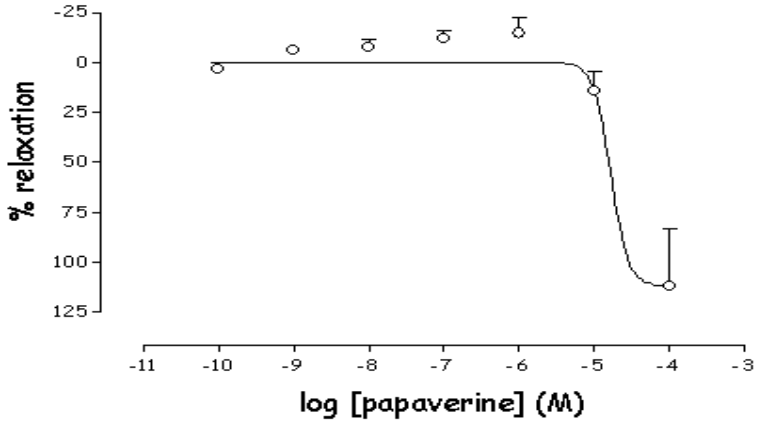

Figure 55: Cumulative addition of the cAMP activator papaverine to isolated rat bronchi (o, n=8)

4.2.4.4 Comparison of Protein Restricted and Control

4.2.4.4.1 Bronchoconstrictor Responses at Age 35 Days

In isolated bronchi from day 35 old rats, CCh produced a dose dependent constriction that was similar between PR and C groups (figure 56)

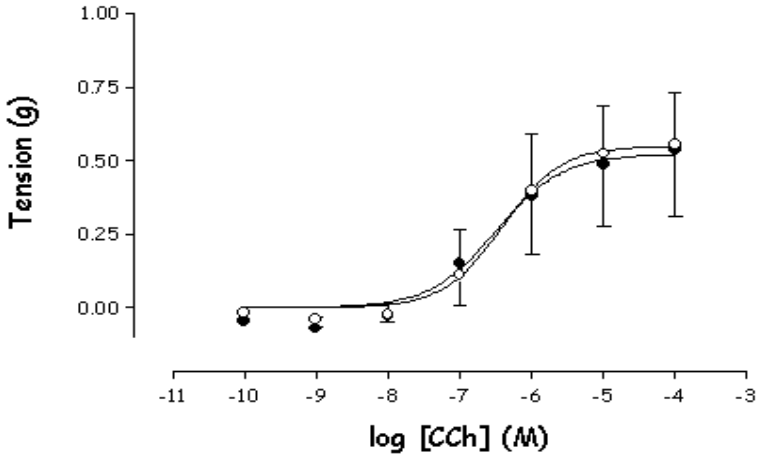

Figure 56: Cumulative addition of CCh to isolated bronchi from 35 day old male offspring from C (o, n=6) or PR (•, n=5) dams.

Similar to CCh, the thromboxane mimetic U46619 produced a concentration dependent constriction in isolated rat bronchi. There was a no difference in the constriction between the PR group and the control (figure 57; p=>0.05).

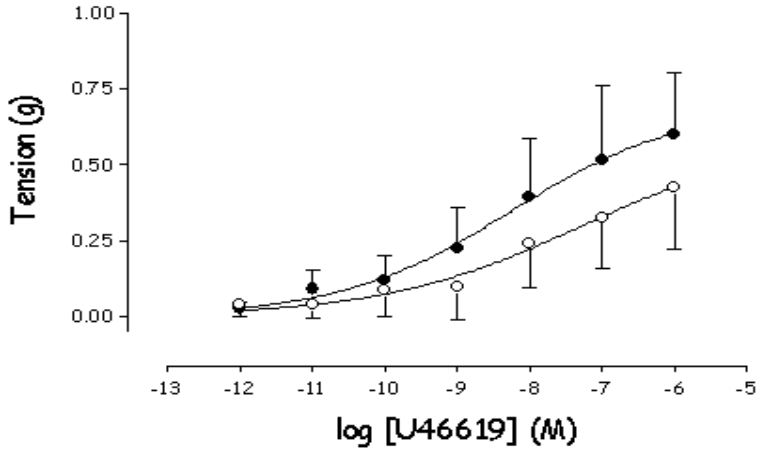

Figure 57: Cumulative addition of thromboxane mimetic U46619 to isolated bronchi from 35 day old male offspring from C (o, n=6) or PR (•, n=5) dams.

4.2.4.4.2 Bronchodilator Responses at Age 35 Days

The 35 day old bronchi relaxed to the isoprenaline in a concentration-dependent manner that was similar in both groups (figure 58). As seen previously, papaverine did fully abolish CCh-induced tone but only at the highest concentrations. There was no real difference seen between the two treatment groups (p=<0.05) (figure 59).

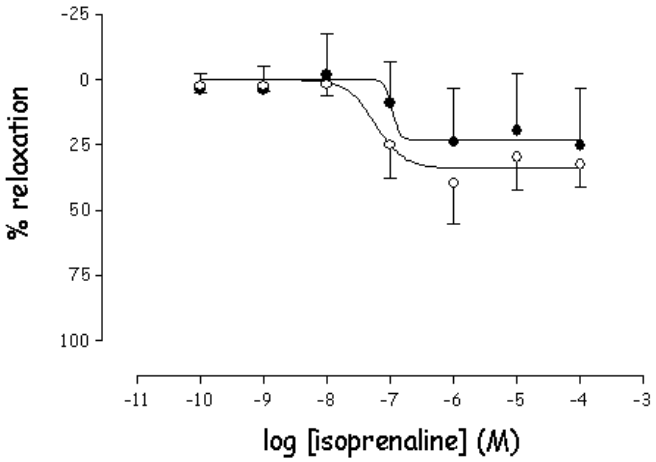

Figure 58: Cumulative addition of isoprenaline to 80% CCh constricted isolated bronchi from 35 day old male offspring from C (o, n=6) or PR (•, n=5) dams.

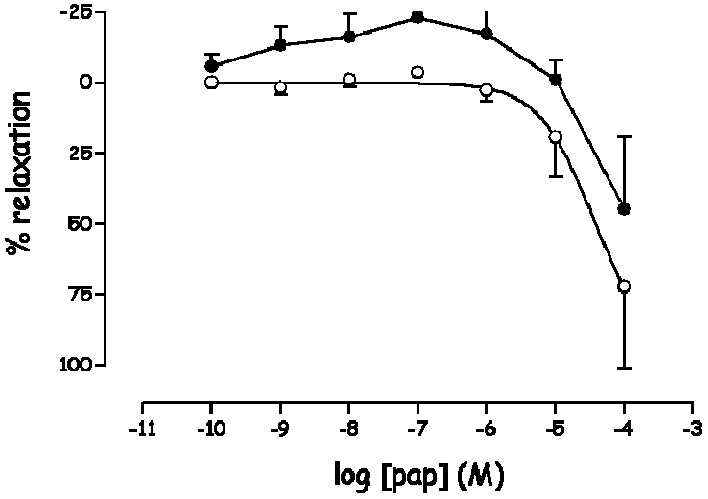

Figure 59: Cumulative addition of papaverine to 80% CCh constricted isolated bronchi from 35 day old male offspring from C (o, n=6) or PR (•, n=5) dams.

4.2.4.4.3 Bronchoconstrictor Responses at Age 75 Days

In isolated bronchi from day 75 old rats, CCh produced a dose dependent constriction that was significantly increased in PR compared to C groups (Figure 60; p=<0.0001).

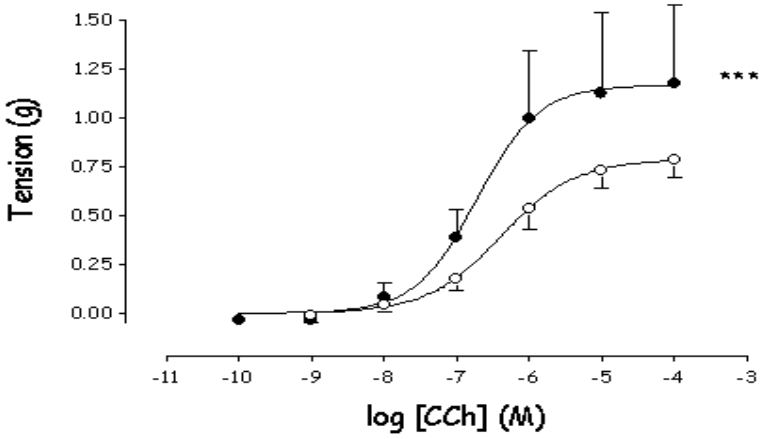

Figure 60: Cumulative addition of CCh to isolated bronchi from 75 day old male offspring from C (o, n=6) or PR (•, n=4) dams. *** indicates p<0.001 % max response C vs PR.

Similar to CCh, the thromboxane mimetic U46619 produced a concentration dependent constriction in isolated rat bronchi. There was a significant increase in the constriction in the PR group compared to C. (figure 61; p<0.001).

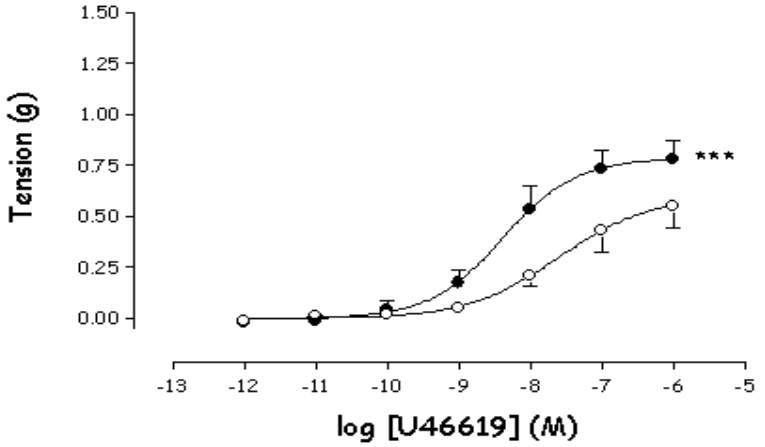

Figure 61: Cumulative addition of U46619 to isolated bronchi from 75 day old male offspring from C (o, n=6) or PR (•, n=4) dams. *** indicates p<0.001 % max response C vs PR.

4.2.4.4.4 Bronchodilator Responses at Age 75 Days

The 75 day old bronchi relaxed to the isoprenaline in a concentration-dependent manner that was similar in both groups (figure 62). As seen previously papaverine did fully abolish CCh-induced tone but only at the highest concentrations (figure 63). There was no difference seen between the two treatment groups (p=>0.05).

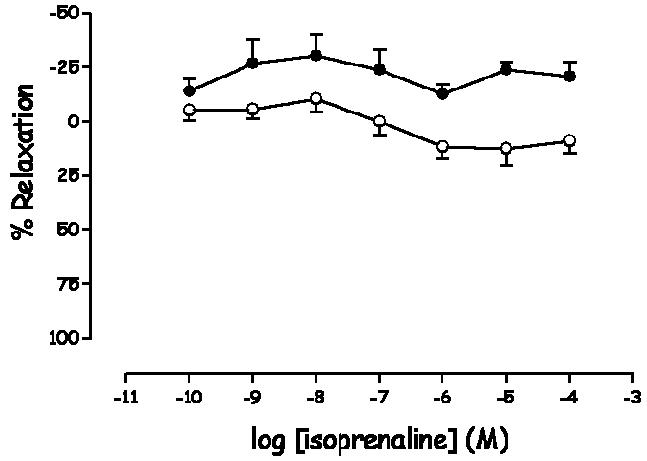

Figure 62: Cumulative addition of isoprenaline to 80% CCh constricted isolated bronchi from 75 day old male offspring from C (o, n=6) or PR (•, n=4) dams.

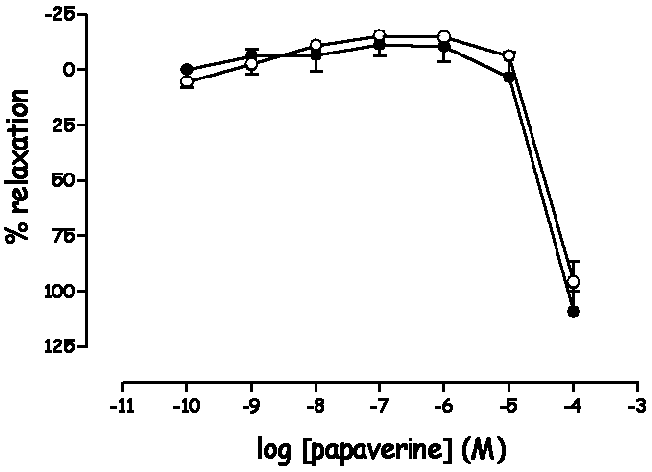

Figure 63: Cumulative addition of papaverine to 80% CCh constricted isolated bronchi from 75 day old male offspring from C (o, n=6) or PR (•, n=4) dams.

4.2.5 Discussion

4.2.5.1 Base-Line Bronchoconstrictors

As described in section 1.2.2.2, a number of bronchoconstrictors are used to assess the degree of bronchial hyperresponsiveness of airways in humans. Methacholine, an acetylcholine mimetic, is used most commonly for bronchial hyperresponsiveness testing in humans. Another acetylcholine mimetic, is carbachol, that also induces bronchial smooth muscle contraction (Forster 1993). Within the rat bronchi, the addition of carbachol to the wells caused a concentration-dependent constriction. Due to the constant, and concentration-dependant nature, of the constriction to this drug, it was chosen to achieve 80% maximum constriction of bronchi before assessment of bronchodilator responses.

Histamine is an alternative bronchoconstrictor often utilised in BHR testing in humans. Histamine induces bronchoconstriction by activating specific H_1 histamine receptors within the human lung (Bryce 2006). It causes an inflammatory response and also constriction of bronchial smooth muscle (Melgarejo 2010). However it has been established that within the rat lung the presence of histamine is immediately reduced/eliminated by histaminase (Rose 1940; Zeller 1942; Cotzias 1952), with the rate of histamine degradation dependent on the amount of lung tissue present (Zeller 1942). Thus the lack of response observed in the rat bronchi to histamine was expected.

Angiotensin II is found in all tissues of the human body but is present in the highest concentration in the capillaries of the human lung, and is a known vasoconstrictor (Nishioka 2011). In this investigation, angiotensin had no effect on bronchi, in line with previous observations by others (Sakai 2010). For example, a two week course of angiotensin treatment, 200ng/min/kg via subcutaneous infusion in the rat, results in remodelling of the arteries but has no effect on the airway (Ramsay 2000). Angiotensin receptors have been found to be expressed within the rat lung through RT-PCR methods. This may suggest that although the receptors are present, they may not be functional (Sakai 2010).

The effects of thromboxane can be mimicked by U46619, a stable synthetic analogue of prostaglandin PGH, by activation of phospholipase C. A previous study has shown that U46619 caused a bronchoconstriction effect 178x more potent than methacholine in humans (Jones 1992).

4.2.5.2 Bronchodilators

In humans constriction of the bronchi is treated with broncho-dilators such as selective β_2-adrenergic receptor agonists, e.g. salbutamol. Several different broncho-dilators were tested for their effectiveness in rat lungs. Almost all of these reagents affect α and β-adrenergic receptors that are found within the lungs of both humans and rats. None of the dilatory agents used in the rat lung worked with any great efficacy.

Salbutamol is a mainstay of asthma therapy. It is a selective β2-adrenergic receptor agonist (see section1.2.3) that is used to relieve broncho-spasm. When this drug was used to study vasodilation in the vasculature of the rat the only effect seen was in the hind-quarters (Gardiner 1991). The use of salbutamol (either aerosolised or by intravenous injection) to induce bronchodilation as assessed by lung function in a live rat, has shown that salbutamol is effective in improving airway function after bronchoconstriction induced by methacholine (Petak 1999). In the current study, treatment with salbutamol in the dose range 1nM-100μM did result in a slight relaxation of tension, but only by ~20%, conflicting with these previous observations. The difference between the studies is that in the study by Petak *et al.* the salbutamol was administered both orally and via IV injection and the lungs were still *in situ* within the rat. This could imply that relaxation of the bronchi in response to salbutamol requires a factor to be present that is in the lung tissue surrounding the bronchi but not in the bronchi themselves. Alternatively, this could also reflect differences in bronchodilator responsiveness between large and small airways, as the use of dissected bronchi to assess airway smooth muscle responses is restricted to large 1st generation bronchi.

Isoprenaline has been used as an asthma therapy in the past but is no longer used clinically as it is a non-selective β-adrenergic receptor agonist, activating both β_1- and β_2-adrenergic receptors. While this will induce bronchoconstriction, in the heart this will induce increased rate and force of cardiac smooth muscle contractions via its action on β_1-adrenergic receptors (de Monte 1993). Isoprenaline was expected to induce bronchodilation as it has previously been shown to induce bronchodilation in the rat (Mahajan 2010), and it did relax the bronchi slightly at a lower dose than salbutamol. However, as higher concentrations were added to the bath the bronchi constricted again to give no overall relaxation. It was thought that the isoprenaline may have been acting on the β-receptors to relax the bronchi, but then began to activate the α-receptors which caused the bronchi to re-constrict. To test this an α-receptor blocker was added to the bronchi bath before the isoprenaline dilution. The α-blocker used was prazosin, which was added to the wells at a final concentration of 10mM, before the Isoprenaline dilution series was started. This caused the bronchi to relax and the relaxation continued, supporting the initial suggestion that activation of α-receptors due to reduced selectivity at higher concentrations of isoprenaline underlies the constriction observed.

Papaverine is an opium alkaloid antispasmodic drug, that is a known vasodilator and is used as such to treat a number of conditions such as migraine (Victor 2003), erectile dysfunction (Yildiz 2011), and during

cardiac surgery to aid with heart catheterisation (Osman 2008). It has also been linked with the reduction of ACE inhibitor (enalapril) induced cough in guinea pigs (Ebrahimi 2006). Papaverine was expected to produce a positive control for the relaxation of the bronchi as it increases intercellular cAMP, through inhibition of phosphodiesterases (Rang 2007) leading to protein kinase A activation and decreased free [Ca^{2+}]. However, although papaverine did relax the bronchi completely, this was not in a dose dependent manner, and it was only with the addition of the top concentration (100μM) that relaxation was observed. The reason for relaxation at only the highest concentration of papaverine added could be due to the amount of free calcium within the bath. The PSS that is in the well surrounding the bronchial tissue has a calcium concentration of 2.5mM. That concentration may need the highest dose of papaverine to be effective.

BRL37344 is an agonist of an additional β-adrenergic receptor subtype present in the body, β_3-adrenergic receptors (Hodis 2011). The β_3-adrenergic receptor has been found within the rat lung and linked with the clearance of alveolar fluid (Li 2010). β_3-adenergic receptors have also been previously shown to mediate bronchodilation in canine airways (Tamaoki 1993). The bronchi did relax in a dose dependent manner although not completely. This is of interest as this indicated that β_3 receptors may be a potential therapeutic target for possible use in humans. Activation of β_3 receptors to produce a dilation of the bronchi would circumvent the problems with the use of β_2 receptors, such as salbutamol, as it would not affect the other organs within the body. However a previous study of isolated human bronchi showed that while BRL37344 did appear to exhibit weak bronchodilator effects, this was mediated by β_2-adrenergic agonist action (Martin 1994), suggesting that β_3-adernergic receptors do not mediate bronchial smooth muscle relaxation in humans.

4.2.5.3 Effects of Maternal Protein Restriction on Bronchoconstrictor and Bronchodilator Responses of Isolated Rat Bronchi

Both carbachol and thromboxane constricted the rat bronchi in a dose dependent manner. The younger time point (35 days) did not show any difference in their response between the two treatment groups. However in the older, 75 day, in PR rats a bronchial hyper-responsive phenotype was observed. A recent study has suggested that the asthmatic remodelling seen in the bronchi could be caused by mechanical stress responses in the airways induced by repeated constrictions, as this phenotype was seen in non-allergic inflammation in asthmatic individuals that underwent repeated methacholine challenge (Grainge 2011). In this study, individuals were exposed to three challenges 48 hours apart of either allergen challenge (bronchoconstriction with eosinophillic inflammation) or methacholine challenge (bronchoconstriction without inflammation). Remodelling of the airways was seen in both sets of challenged airways and not in control groups exposed to methacholine together with a bronchodilator or saline alone. This would suggest that if individuals exhibited increased bronchial hyperresponsiveness as a result of

developmental programming, this could lead to airway remodelling and predispose to development of asthma in childhood (Grainge 2011).

This is the first report of bronchial hyperresponsiveness following maternal protein restriction in the absence of any post-natal stimuli. The mechanism behind the contraction of smooth muscle, such as that surrounding the airways, is the phosphorylation of the myosin light chain which activates actin-activated myosin ATPase which is required for muscle contraction. The kinase responsible for this is the myosin light chain kinase, a calcium-calmodulin-activated kinase, and it is essential as it cannot be replaced by another kinase (Hong 2011). Due to its method of activation the calcium-calmodulin-binding site is the most important regulator for smooth muscle contraction. It is possible that maternal exposure to a low protein diet has altered the expression level of the myosin light chain kinase which has resulted in the BHR phenotype, or it may be that there are other structural changes that are making the calcium-calmodulin-binding site more accessible.

4.2.5.4 Future Work

Having seen a bronchial hyper-responsive phenotype in the 70 day old F1 rats it would be of interest to see if the same phenotype can be observed in F2 and F3 generations, as this would suggest that the effect may be transmitted transgenerationally, and in the case of an F3 effect, imply that this is epigenetic in nature (see section 1.1.5).

The ability to observe the effect of these drugs *in vivo* would give a better understanding of the reaction within the bronchi as they would be within their natural environment. The dissection of the bronchi from the surrounding lung tissue alters its biology as other mediators are not present and the structural support is also absent. To do this *in vivo* a lung function measure would have to be taken. There are a number of ways to assess lung function in and bronchial hyperresponsiveness in the rat. For example a common asthma inducing challenge for small mammals, such as rodents, includes inhalation, under anaesthesia, of with isoflurane with a low percentage of Ovalbumin, e.g. 2% (Nabe 2005).

In humans, asthma results from the interaction between inherited susceptibility and postnatal environmental exposure. To determine whether the observed effect of maternal protein restriction on bronchial hyperresponsiveness had relevance to human asthma, the observation of the effects of maternal protein restriction on lung function, and inflammatory, responses in an allergen challenge model such as that utilised by Raemdonck et al. would be of interest (Raemdonck 2011).

4.3 Methylation and Gene Expression

4.3.1 Methylation Introduction

As described in section 1.1.5, epigenetic mechanisms can alter the expression of genes reversibly. One such mechanism is the addition of methyl groups to the cytosine residue of a cytosine-guanine pair of bases. In a number of animal models, changes in offspring DNA methylation in a range of tissues have been observed in response to alterations in maternal diet. In the Agouti mouse (a(vy/a)), expression of the yellow coat colour is dependent on changes in DNA methylation of six CpG sites in a retrotransposon upstream of the transcription start site of the Agouti gene. The offspring of pregnant Agouti mice fed a diet supplemented with the phytoestrogen genistein shifted coat colour from yellow towards a pseudoagouti brown coat as a result of changes in methylation (Dolinoy 2006). The extent of this DNA methylation was similar in endodermal, mesodermal and ectodermal tissues, suggesting the exposure acted during early embryonic development. Furthermore, this genistein-induced hypermethylation persisted into adulthood, altering other common features of the Agouti phenotype, such as obesity. Supplementation of maternal diet with methyl donors has also been shown to alter methylation of a number of genes in inflammatory cells and lead to increased airway inflammation in a mouse model of asthma (Hollingsworth 2008). In the lung, exposure of pregnant females to a restricted diet (65% of ad libitum) has been shown to result in impaired endothelium-dependent pulmonary artery vasodilatation, and exaggerated hypoxia-induced pulmonary hypertension and subsequent right ventricular hypertrophy all associated with increased global lung DNA methylation (Rexhaj 2011).

In the rat, changes in maternal diet have also been shown to alter DNA methylation both globally, and in a gene specific manner. For example, increased folic acid coupled with reduced vitamin B_{12} in the maternal diet has been shown to reduce global DNA methylation in placental tissue (Kulkarni 2011). Maternal protein restriction has also been shown to result in altered renal structure accompanied by changes in methylation of the promoter for the gene encoding 11β-hydroxysteroid dehydrogenase (Mesquita 2010).

4.3.2 Gene Expression

As described above, there is evidence that maternal dietary exposures during pregnancy, such as protein restriction, can lead to altered DNA methylation and consequently changes in gene expression. A number of candidate genes were selected to assess changes in gene expression in response to maternal PR. These can be classified into four main groups: 1) histone deacetylases; 2) lung development; 3) asthma susceptibility genes and 4) COPD susceptibility genes.

4.3.2.1 Histone Deacetylase

Histone deacetylases (HDAC) such as the class II HDAC family members HDAC2, HDAC3 and HDAC8 remove acetyl groups ($O=C-CH_3$) from ε-N-acetyl lysine amino acids on histones which the DNA is coiled around. Acetylation of lysine residues increases the positive charge of histone tails, encouraging high-affinity binding between histones and the DNA phosphate backbone. This increased DNA binding results in a more condensed DNA structure, preventing access of transcription factors to DNA binding sites and hence reducing transcription.

HDAC2 is expressed as a single isoform from a gene encoded on chromosome 20q21 in the rat and is associated with the core histones H2a, H2B, H3 and H4 (Hollenbach 2002). In the lung HDAC2 has been shown to be reduced in COPD patients (Ito 2005) and in cystic fibrosis patients the transmembrane conductance regulator protein excreting cells are also effected by HDAC2 (Bartling 2009).

HDAC3 is highly involved in the regulation of cellular mitosis. It has been linked with microtubule attachment, centromeric deacetylation and sister chromatid cohesion (Li 2006; Eot-Houllier 2008; Ishii 2008). Similar to HDAC2, HDAC3 mRNA levels have been shown to be decreased in lung biopsies from COPD patients (Ito 2005) .

HDAC8 is known to be phosphorylated by the cyclic AMP-dependent protein kinase A and that the increase in phosphorylation inhibits HDAC8 resulting in the acetylation of the histones H3 and H4 (Lee 2004). HDAC8 has been used as a marker of smooth muscle differentiation and its distribution within the cell, following the stress fibre distribution in alpha-smooth muscle actin, implies some association with smooth muscle function, possibly through regulation of the contractile capacity of the smooth muscle cells independent of its function in regulation gene expression in the nucleus (Waltregny 2005). As with HDAC2 and HDAC3, HDAC8 has also been found to be decreased in COPD patients (Ito 2005).

Analysis of the HDAC8 gene in the rat is problematic as there is no annotated gene sequence, but using homology there is a sequence similar to that in humans.

4.3.2.2 Developmental Genes

The insulin-like growth factors 1 (IGF-1) and 2 (IGF-2) are key regulators of fetal growth and development. IGF1 is formed as a precursor that is bound by a receptor and then secreted. There are several known transcripts of this protein. In the rat, serum levels of IGF1 in the serum fluctuate, with a decrease leading up to birth, a dramatic increase in levels at day 8 which is then reversed back to previous level, day1, by post-partum day 15. IGF1 levels then continue to steadily decrease as the rat ages (Werner 1989). IGF-2 expression is controlled through maternal imprinting, with the paternally inherited allele being expressed (Reik 2000; Sasaki 2000; Arney 2003). Overall IGF-2 levels are reduced in the adult, when compared to fetal,

rat tissues (Lund 1986). A reduction in maternal food intake by 50% has been shown to lead to intrauterine growth restriction (IUGR) and an increase of both IGF-1 and IGF-2 mRNA levels in the lung (Chen 2007).

Tp53 is a well known essential protein that responds to diverse cellular stress resulting in cell cycle arrest, apoptosis and senescence. It is also involved in DNA repair and reacts to changes in metabolism. The importance of Tp53 in the cell cycle means that expression levels within cells must be carefully regulated (Lehnert 2001). This protein can be DNA bound and contains transcription activation, DNA-binding and oligomerisation domains. There are multiple variants due to alternative promoters and multiple splicing variants. These variants encode two distinct isoforms which can regulate activity. Tp53 expression levels have been linked with COPD, with increased pro-apoptotic Tp53 protein expression in lung tissue specimens from COPD patients in comparison to non-COPD smoking subjects (O'Brien 2007; Siganaki 2010) .

Uncoupling protein (UCP) is a mitochondrial uncoupling protein that separates the oxidative phosphate from ATP and dissipates the energy as heat. It is also known as the mitochondrial proton leak. UCP helps to facilitate the transfer of anions from the inner to the outer membrane. This reduces the membrane potential in mammals (Divakaruni 2011).

In sheep, gene expression levels of UCP in the lung peak at postnatal day 1 of age and then decline with age. The effect of total maternal nutritional reduction induced IUGR is to give rise to increased levels of UCP gene expression both prenatally and postnatally and the increased expression persisted until the last postnatal sampling point of 6 months (Gnanalingham 2005).

The role of this protein in the rat is similar as that seen in humans (Cassard-Doulcier 1998). The expression levels of UCP within the rat lung, increase at the point of birth and continue to increase up to three fold from day 1 until adulthood (Xiao 2004).

4.3.2.3 Asthma Genes

As described in section 1.2.8, A Disintergrin Metalloprotease 33 (ADAM33), is a membrane anchored protein and is structurally related to snake venom disintergrins. It has been implicated in cell-cell and cell-matrix interactions, including fertilisation, muscle development and neurogenesis. This type one transmembrane protein has been identified as an asthma susceptibility gene, with polymorphisms in the gene being associated with increased susceptibility to asthma and specifically, bronchial hyperresponsiveness (van Eerdewegh 2002; Schedel 2006), also to lung function decline in the general population (van Deimen 2011) and COPD (Sadeghnejad 2009; Xiao 2010). Tissue specific regulation of ADAM33 expression has been shown to be regulated by methylation of a CpG island within the promoter (Yang 2008).

Protocadherin 1 (PCDH1) is a membrane bound protein that is a member of the δ-protocadherin subgroup of non-clustered protocadherins and is found at all cell-cell boundaries. It is involved in neural cell adhesion and so may play an important role in the development of the neuronal system. The protein itself includes an extra-cellular region that contains seven cadherin-like domains, a transmembrane region and a C terminal cytoplasmic region. Any cells that express this protein are prone to aggregation activity. Genetic studies have shown association between variants in the PCDH1 gene and susceptibility to asthma and BHR in several Caucasian populations (Koppelmann 2009). Furthermore, these studies showed a potential interaction between these PCDH1 variants and maternal smoking during pregnancy, suggesting a role for this protein in early lung development (Koppelmann 2009). In the mouse, PCDH1 is expressed both in the developing and adult lung epithelium and the tight spatial and temporal regulation of PCDH1 expression suggests that it plays multiple roles not only during development, but also in mature tissues and organs (Redies 2008).

The glucocorticoid receptors (GR) allow glucocorticoid to bind to them in the cytoplasm and when bound they are translocated into the nucleus, once there they can bind to glucocorticoid response elements. These elements increase the transcription of certain genes. Most of these genes encode anti-inflammatory proteins, as seen in asthmatic inflammation. The GR response elements also have a secondary function which blocks the transcription of inflammatory encoding genes (Barnes 1998). The Southampton model of protein restriction found that the expression of the GR gene was increased within the liver of the PR offspring when compared to controls. This was found to be linked with a reduction of methylation in the GR promoter, these alterations were prevented by the addition of increased folic acid within the diet (Lillycrop 2005), this alterations was also found to be transmitted to the F2 generation without any further dietary challenge (Burdge 2007). A later study linked the decrease in methylation with a decrease in methyltransferase-1 expression (Lillycrop 2007).

When the animal model in this investigation was used to study hypertension, GR levels were found to be elevated in response to maternal PR (Bertram 2001). The importance of GR to lung development is highlighted by GR deficient mice that are embryonic lethal with under developed lungs partially filled with fluid (Cole 1995). This is unsurprising as cortisol is hugely important in the maturation of cells during development. It is given to premature babies to start the development of surfactant and single doses during specific windows have some particular long term consequences (Halliday 2010).

4.3.2.4 COPD Genes

Beta-Hydroxysteroid Dehydrogenase 1 (11β-HSD1), is a microsomal enzyme that catalyses the conversion of the stress hormone cortisol into its inactive metabolite cortisone. This protein is also able to catalyse this reaction in reverse, to produce cortisol from cortisone. This is essential as too much cortisol

results in central obesity. Variation within this gene has been linked with obesity and insulin resistance. This protein also has an equally high affinity for aldosterone (Tannin 1991). The level of cortisol within the lung is implicated in the maturation of the lung tissue (Suzuki 1998).

The protein has a similar effect within the rat and it has also been linked with blood pressure regulation (Lloyd-MacGilp 1999). As for GR and UCP2, IUGR in sheep has been shown to result in persistent increased 11β-HSD1 mRNA expression in the rat lung (Gnanalingham 2005). The rat homologue is around 77% identical to that found in humans (Tannin 1991). Beta-Hydroxysteroid dehydrogenase 2 (11β-HSD2), has a similar effect as 11β-HSD1, although it cannot reverse the reaction, going from cortisone back to cortisol. It is expressed predominantly in epithelial tissues, especially in the kidney and lung and is thought to prevently illicit activation of mineral corticoid receptors (MR). The 11β-HSD2 enzyme has been found to co-localise in rat lung tissue with both MR and GR receptors (Hirasawa 1997; Suzuki 1998). Within cells that have no MR involvement, 11β-HSD2 protects the cell from the growth inhibiting effects of cortisol. (Suzuki 1998) This is especially true during embryonic development.

The role of this protein in the rat is slightly different as it catalyses the transformation of corticosterone into 11-dehydrocorticosterone. This enzyme has been found in the terminal alveolar sacs of the rat lung at 21 days of age (Suzuki 1998). Bertram used the same rat model of maternal protein restriction and found that levels of 11β-HSD2 were reduced in protein restricted animals which inversely correlated with the levels of GR also present in relation to hypertension. These readings were taken over a large time span running from 2-20 weeks (Bertram 2001). Work looking at the early life origins of adult disease found that placental levels of 11β-HSD2 were linked with birth weight. It was also found that by inhibiting 11β-HSD2 the birth weight of the rat is also reduced and the adults developed hypertension and hyperglycaemia (Seckl 1997). The impact of this alteration in levels implies a link between early life origins and development of disease in both rat and human models.

The mineralocorticoid receptor (MR) protein, also known as NR3C2 (nuclear receptor subfamily 3, group C member 2), mediates the actions of aldosterone, including the increased reabsorption of sodium and consequently blood volume and pressure. It is a ligand dependent transcription factor which, upon ligand binding, dimerises and translocates to the nucleus where it binds to MR response elements and transcriptionally activates target genes (Strachan 2011). The ligands for MR include mineralcorticoids, such as aldosterone and deoxycorticoids, as well as glucocorticoids, like cortisol. Normally, the MR is “protected” from glucocorticoids by co-localisation of 11β-HSD2 that converts cortisol to inactive cortisone. In the lung, 11β-HSD2 co-localises with MR in airway epithelium, suggesting that 11β-HSD2 may play an important role in pulmonary mineralocorticoid activity, such as sodium and fluid transport (Suzuki 1998).

4.3.3 Aims

Given that 1) DNA methylation can be altered globally by changes in maternal diet, 2) changes in DNA methylation have been seen in response to maternal protein restriction and 3) this alteration in methylation can affect the phenotype of the individual, we hypothesised that:

vii. Maternal protein restriction will significantly alter global DNA methylation in lung tissue of the F1 offspring.

viii. These changes in global DNA methylation will also be evident in the F2 and F3 generations.

ix. These changes in DNA methylation will result in significantly different gene expression providing a mechanistic basis for the observation that maternal PR leads to altered lung physiology.

To test these hypotheses, we aimed to extract DNA and RNA from lung samples harvested at 28, 120 and 225 days of F1 offspring and assess the global DNA methylation and expression levels of candidate genes. To establish if potential changes in global methylation and/or gene expression induced by maternal PR were transgenerational in nature, we also aimed to harvest lung tissue from 28 day F2 and F3 generations and assess global DNA methylation and gene expression.

4.3.4 Materials and Methods

For materials see section 2.3

Rat dams were allocated either C or PR diet from confirmation of pregnancy until term, and resulting male offspring were culled at F1 28 days, F1 120 days, F1 225 days, F2 28 days and F3 21 days for this part of the investigation. See section 3.1 for details on the animal model. Extraction of the DNA, RNA and Protein has been detailed in section 3.4.1. The global methylation assay was in kit form and is detailed in section 3.4.2. Once the RNA had been extracted it was used to generate cDNA ready for use with the primer sets (See section 3.4.3.). Each set of primers used was optimised and validated using pooled rat cDNA from all lung samples (See section 3.4.4 and 3.4.5). Gene expression was compared between the two treatment groups for all time points (See sections 3.4.6 and 3.4.7.). As described previously (see section 3.5) a Students-T test was used to determine the significance of any differences seen between the groups, with $p<0.05$ considered significant.

4.3.5 Results

4.3.5.1 Pooled Global Methylation Results

Methylation of lung DNA was assessed in pooled samples for each treatment group from F1 28 day old, F1 120 day old, F1 225 day old, F2 28 day old and F3 21 day old rats.

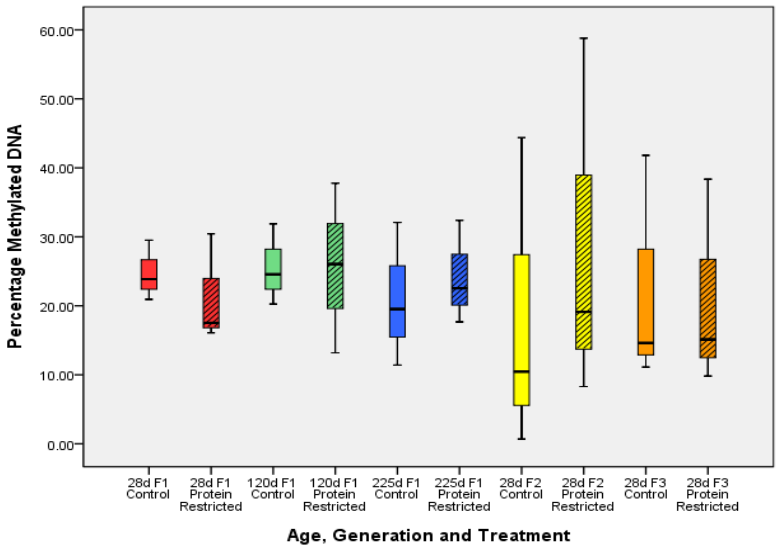

Figure 64: Pooled data for global methylation percentage at each time point for the different treatment groups. (F1, 28 days, n=25; F1, 120 days, n=18; F1, 225 days, n=12; F2, 28 days, n=41; F3, 21 days, n=21)

No significant differences were observed between treatment groups at the same time point or between time points for the same treatment group.

4.3.5.2 F1 225 Day Old Global Methylation Results

Methylation of lung DNA was assessed in individual lung samples from the F1 225 day old rat group.

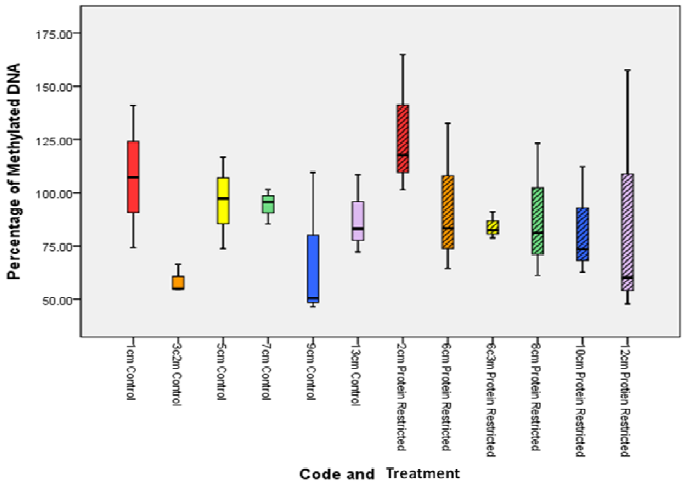

Figure 65: Individual sample data for the 225 day old rats. X axis gives individual animal ID and indicate allocation to control and protein restricted groups. (C, n=6; PR, n=6)

The F1 225 day old samples were chosen as the ones that were looked at individually as they showed the only significant difference in their lung structure. Each sample was measured in triplicate. The mean value for the methylation of the control group is 85.54, and the mean for the protein restricted group is higher, at 94.26 but not significantly different. There is a large variation in methylation levels between individuals within a treatment group and also between the triplicate samples from a single rat.

4.3.5.3 Sample Number and Type for Gene Expression

Table 25 below indicates the number of samples that were pooled into their separate generation, age group and treatment. The pooled samples were used for acquisition of gene expression data. To generate pools, 2µl of each pre-prepared cDNA samples were collected in fresh tubes dependent on age and treatment group allocation.

Table 25: Table shows the number of samples that was used in each pooled sample for gene expression.

Generation	Age (days)	Treatment	Number of samples
F1	28	Control	15
		Protein Restricted	10
F1	120	Control	11
		Protein Restricted	7
F1	225	Control	6
		Protein Restricted	6
F2	28	Control	24
		Protein Restricted	17
F3	21	Control	16
		Protein Restricted	5

4.3.5.4 Reference Genes.

When trying to quantify the relative expression of a gene it is important to make sure that the reference genes to which their expression is being compared are correct. A study by Cai et al. (2007) looked at the reference genes with a stable expression, within the different rat tissues, for use within low density arrays. The study looked at the best genes to use for several different tissues, including the lung. From this study the top five genes were, in order of preference, Rpl10a, Mapk14, FLJ20445, Taf91 and Map2k5 (Cai 2007).

The primer sets for these genes were obtained, however the genes proved difficult to optimise and were eventually replaced with other primer sets for other more recognised reference genes, β-actin, β-2-microglobulin and ubiquitin C. These were optimised and used to produce standard curves for the entire pooled sample of rat lung cDNA.

4.3.5.5 Standard Curves.

A standard curve was created for all the gene sets. The dilution series was created 1 in 4 with 5 dilutions giving the following concentrations.

Dilution	4^0	4^{-1}	4^{-2}	4^{-3}	4^{-4}	4^{-5}
Concentration	1	0.25	0.0625	0.0156	0.0039	0.00098

Standard curves were formed for the following genes:

- B_2M
- BAC
- UBC
- ATP11α1
- HDAC2
- HDAC3
- HSD11β1
- HSD11β2
- IGF1
- IGF2
- MR
- Tp53

A successful standard curve had an error of less than 0.2 and an efficiency of between 1.5 and 2.5. The duplicated samples should appear close together on the graph, with the samples points sometimes touch or over-laying another. The error value gives an idea of how close the replicated samples are. The efficiency value is ideally 2. This is because every time there is an amplification cycle the strands of cDNA should be copied by primers annealing on each strand, doubling the total amount of DNA in the reaction.

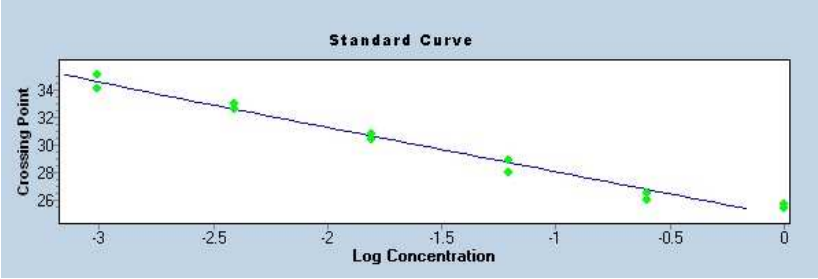

Figure 66: Example of a standard curve found for UBC on 04-05-2010. The error for this curve was 0.0482 and the efficiency was 2.039.

There was no amplification of the no template control samples, indicating that there was no contamination in the samples from other sources.

4.3.5.6 Comparison of Gene Expression.

Pooled samples for each treatment at each time point were then tested for their relative gene expression for each of the genes that had satisfactory standard curve data.

Each gene was compared to the expression of the reference genes for the same time point and expressed as a ratio of that reference gene's expression. Using the expression level data obtained from the reference genes a minimum detectable difference for the genes was calculated as shown in the example for BAC and UBC below:

$$= \frac{(\text{concentration of reference gene 1/concentration of reference gene 2})_{\text{control group}}}{(\text{Concentration of reference gene 1/concentration of reference gene 2})_{\text{treatment group}}}$$

= (23.13377/27.62174)
(22.95467/27.99596)

= 0.837520373/0.820000457

= 1.021365739

This indicates that any difference being tested that is above 1.022 is not due to experimental variability and could be real difference.

4.3.5.7 Gene Expression Levels Compared Between Treatment Groups.

4.3.5.7.1 Histone Deacetylases

HDAC2 expression within the lung was assessed in pooled samples for each treatment group from F1 28 day old, F1 120 day old, F1 225 day old, F2 28 day old and F3 21 day old rats.

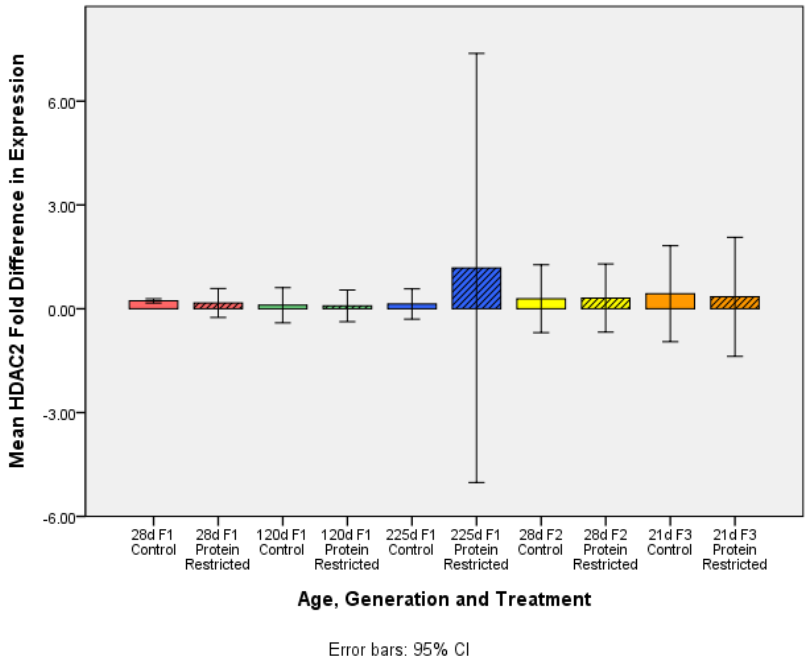

Figure 67: Gene expression data for HDAC2, showing the difference in expression between treatment groups at each time point using pooled samples. There were no significant differences (p=>0.05) between the expression levels of each treatment group or between each time point. (F1, 28 days, C, n=15, PR, n=18; F1, 120 days, C, n=11, PR, n=7; F1, 225 days, C, n=6, PR, n=6; F2, 28 days, C, n=24, PR, n=17; F3, 21 days, C, n=16, PR, n=5)

HDAC3 expression within the lung was assessed in pooled samples for each treatment group from F1 28 day old, F1 120 day old, F1 225 day old, F2 28 day old and F3 21 day old rats.

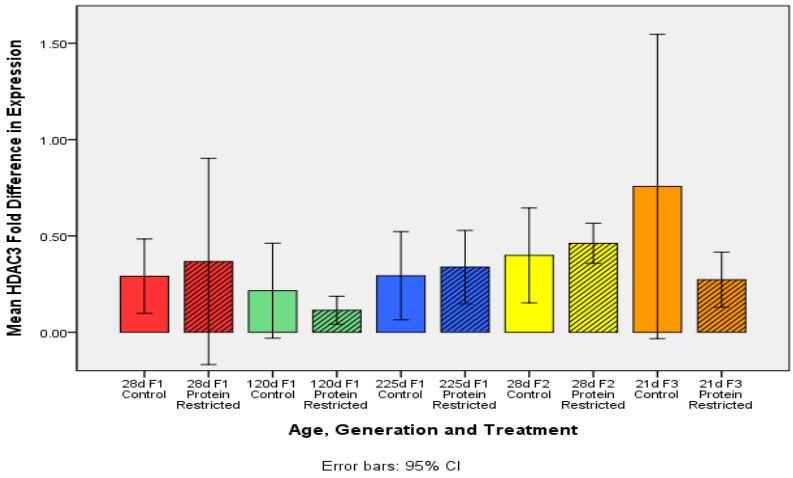

Figure 68: Gene expression data for HDAC3, showing the difference in expression between treatment groups at each time point using pooled samples. There were no significant differences (p=>0.05) between the expression levels of each treatment group or between each time point. (F1, 28 days, C, n=15, PR, n=18; F1, 120 days, C, n=11, PR, n=7; F1, 225 days, C, n=6, PR, n=6; F2, 28 days, C, n=24, PR, n=17; F3, 21 days, C, n=16, PR, n=5)

4.3.5.7.2 Developmental Genes

IGF1 expression within the lung was assessed in pooled samples for each treatment group from F1 28 day old, F1 120 day old, F1 225 day old, F2 28 day old and F3 21 day old rats.

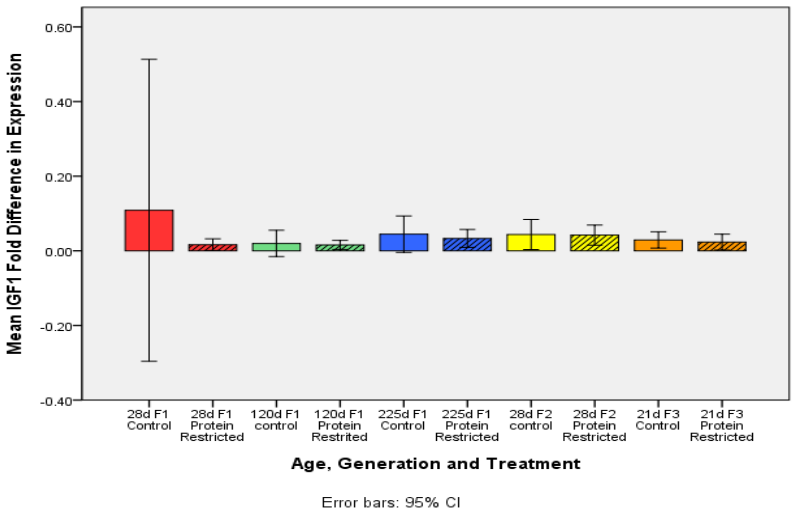

Figure 69: Gene expression data for IGF1, showing the difference in expression between treatment groups at each time point using pooled samples. There were no significant differences (p=>0.05) between the expression levels of each treatment group or between each time point. (F1, 28 days, C, n=15, PR, n=18; F1, 120 days, C, n=11, PR, n=7; F1, 225 days, C, n=6, PR, n=6; F2, 28 days, C, n=24, PR, n=17; F3, 21 days, C, n=16, PR, n=5)

IGF2 expression within the lung was assessed in pooled samples for each treatment group from F1 28 day old, F1 120 day old, F1 225 day old, F2 28 day old and F3 21 day old rats.

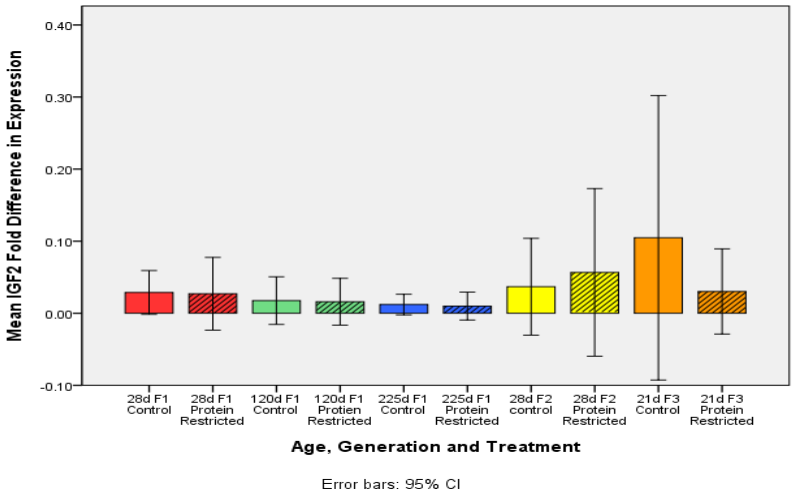

Figure 70: Gene expression data for IGF2, showing the difference in expression between treatment groups at each time point using pooled samples. There were no significant differences (p=>0.05) between the expression levels of each treatment group or between each time point. (F1, 28 days, C, n=15, PR, n=18; F1, 120 days, C, n=11, PR, n=7; F1, 225 days, C, n=6, PR, n=6; F2, 28 days, C, n=24, PR, n=17; F3, 21 days, C, n=16, PR, n=5)

Tp53 expression within the lung was assessed in pooled samples for each treatment group from F1 28 day old, F1 120 day old, F1 225 day old, F2 28 day old and F3 21 day old rats.

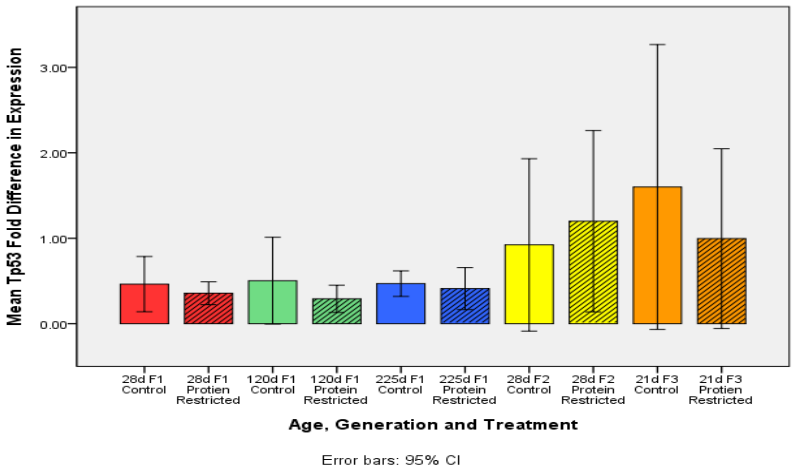

Figure 71: Gene expression data for Tp53, showing the difference in expression between treatment groups at each time point using pooled samples. There were no significant differences (p=>0.05) between the expression levels of each treatment group or between each time point. (F1, 28 days, C, n=15, PR, n=18; F1, 120 days, C, n=11, PR, n=7; F1, 225 days, C, n=6, PR, n=6; F2, 28 days, C, n=24, PR, n=17; F3, 21 days, C, n=16, PR, n=5)

4.3.5.7.3 COPD Genes

HSD11B1 expression within the lung was assessed in pooled samples for each treatment group from F1 28 day old, F1 120 day old, F1 225 day old, F2 28 day old and F3 21 day old rats.

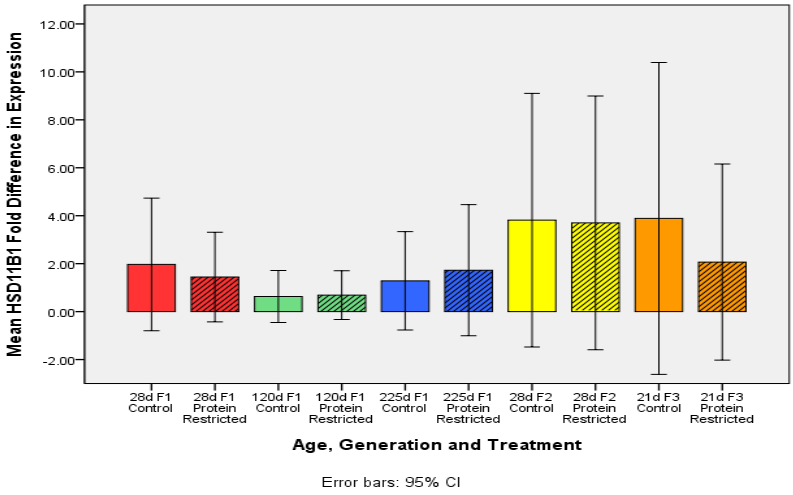

Figure 72: Gene expression data for HSD11B1, showing the difference in expression between treatment groups at each time point using pooled samples. There were no significant differences (p=>0.05) between the expression levels of each treatment group or between each time point. (F1, 28 days, C, n=15, PR, n=18; F1, 120 days, C, n=11, PR, n=7; F1, 225 days, C, n=6, PR, n=6; F2, 28 days, C, n=24, PR, n=17; F3, 21 days, C, n=16, PR, n=5)

MR expression within the lung was assessed in pooled samples for each treatment group from F1 28 day old, F1 120 day old, F1 225 day old, F2 28 day old and F3 21 day old rats.

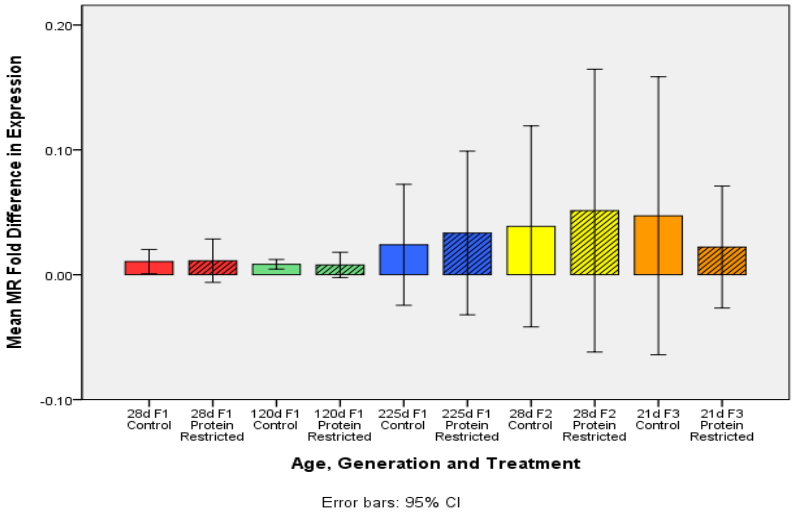

Figure 73: Gene expression data for MR showing the difference in expression between treatment groups at each time point using pooled samples. There were no significant differences (p=>0.05) between the expression levels of each treatment group or between each time point. (F1, 28 days, C, n=15, PR, n=18; F1, 120 days, C, n=11, PR, n=7; F1, 225 days, C, n=6, PR, n=6; F2, 28 days, C, n=24, PR, n=17; F3, 21 days, C, n=16, PR, n=5)

Table 26: Exact p values for the difference between each treatment group for each time point.

Sample	HDAC 2	HDAC 3	HSD 1	IGF 1	IGF 2	Mr	Tp53
F1 28d	0.306	0.611	0.539	0.431	0.899	0.891	0.291
F2 28d	0.879	0.393	0.951	0.906	0.567	0.718	0.461
F3 21d	0.669	0.115	0.376	0.480	0.241	0.446	0.270
F1 120d	0.766	0.214	0.873	0.681	0.892	0.850	0.208
F1 225d	0.278	0.551	0.609	0.434	0.722	0.647	0.439

4.3.6 Discussion

4.3.6.1 Methylation

No difference in the global methylation levels between the two treatment groups was shown and the level of methylation did not change with the age of the rat either. This does not mean that there is no difference in the methylation levels for certain genes that are involved in the process of development or disease within the lungs. It may be that there are a set of genes that have been differentially-methylated in the PR group when compared with the controls, but this is off-set by an increase in methylation in another set resulting in no overall change in global methylation.

There is also the possibility that there is an individual sample within the pooled rat samples that has an extremely altered level of methylation that has altered the position of the mean readings giving a false negative result, masking a real difference that could be observed if that individual was removed. To see if this was the case individual rat samples were assayed for their methylation levels within the F1 225 day old age group.

The F1 225 day old group was chosen as it showed the only significantly different result in the structural analysis. Again no difference was seen between the two treatment groups and there was no obvious outlier from a single individual that could have skewed the pooled results. It could be that the levels of methylation are altered only at the gene level and off-set by an opposite effect on another gene. To see if this was the case individual genes were selected to see if their expression levels had been altered.

Other studies have observed changes in methylation but these have either been with the addition of substances to induce a phenotype in an F0 animal, or the dietary challenges were more severe, such as high and low methyl-donor diets specifically tailored for the experiment. As such, results between this study and those are not directly comparable. It could be that the PR dietary challenge is not severe enough to produce an alteration in the global methylation profile.

There is also the possibility that DNA methylation changes are not involved but another form of epigenetic regulation such as histone modification is. For example, exposure to a maternal low protein diet has been shown to result in reduced p 16 mRNA expression in the mammary glands of F1 offspring which was due to histone modifications and not DNA methylation changes (Zheng 2011).

Any changes in global methylation are more likely to be due to alterations in Long Interspersed Nuclear Elements, LINE (Ohka 2011). These are repetitive elements in the DNA that can be methylated but do not have a specific function of related protein that is derived from them. Because of this it may be better to look at specific genes that are known to be involved in the process of interest. Therefore the purpose of

the gene expression analysis was to identify genes that were differentially expressed so gene specific methylation could be assessed.

4.3.6.2 Gene Expression

Some genes and the top five reference genes did not produce a valid standard curve. This may be because the cDNA was not of a high enough quality, although the RNA was put through a DNAse treatment twice to ensure that there was little or no DNA contamination within the samples. The RNA absorbance curves and ratios also suggested that these samples were not contaminated, but this could still be the cause of the lack of standard curve.

It could be that the primers for the sections of cDNA were not completely binding, and so a disruption of the copying was observed. The RT-PCR kit was used as per the kit instructions and so this stage of the procedure should have worked correctly. It may also have been that the cDNA samples had been freeze thawed too often and the lengths of cDNA had been broken down into smaller pieces that were not of a long enough length to give an accurate estimation of gene expression.

4.3.6.2.1 HDACs

There was no difference in the expression of the HDACs between the two treatment groups. This could mean that the process of histone acetylation is not involved in the mechanism behind the disease processes within the lung. However it may be that we are looking at the wrong gene. It may be that another gene that is involved in the regulation of HDACs has an altered expression. As the expression of these proteins has been shown to be altered in human disease (COPD) it may just be that this mechanism is not as important in baseline lung physiology in the rat.

4.3.6.2.2 Developmental Genes

Genes that are involved in the developmental process were of interest as if a gene that is meant to be switched off is still being expressed, or has an increased expression, this could lead to the alteration of the structure of the lungs or a more severe, or repressed, reaction to certain stimuli. No genes within this class were found to be differentially expressed between the two groups.

The lack of a difference in these could indicate that there is not a difference seen, or it could be due to the same factors as those discussed in relation to the HDACs above; wrong genes being looked at, other unlinked genes are regulating the process, or they are not the important genes within the mechanism being looked at. Alteration in the expression levels of developmental genes usually involve a difference in the structure of the tissue, in this case the lung, that is involved, even with complex disorders (Courchesne 2001). As there was no structural differences found in the morphometry section it was unlikely that these

genes were the ones whose expression was altered. The only difference that was noted was a difference in the expression profile of IGF2 between time points. As the rats age increased the expression level of this gene decreased, however due to the variance of the results this finding was not statistically significant.

4.3.6.2.3 Asthma Genes

There were no differentially expressed genes identified in this class of genes for this investigation. This could be due to a lack of challenge that is used in humans to activate these genes before initiation of the phenotype. It may be that the focus is on the wrong gene, with another being the cause of the disease outcome.

4.3.6.2.4 COPD Genes

As with the asthma genes there was no difference in expression within this group of genes either. It could be that the alteration of these genes is done through exposure to tobacco smoke, as it is the biggest risk factor for developing COPD. It may be that if these animals had been exposed to tobacco smoke alterations in gene expression would have been seen.

Association studies that have linked some of these genes with respiratory disease could have been picking up the association of another gene in which polymorphism predisposed to these diseases that was in linkage disequilibrium with the genes mentioned. This means that a gene very close in physical location was actually linked with the disease but that gene was not the one highlighted as it had less reason to be linked. It could also be that the genes being looked at are not affected but another that is in that genes functional pathway has been altered making it the regulatory step for the process.

In contrast with results seen previously from the gene expression of these rats the Hsd11β2, IGF1, IGF2, Pcdh1 and Tp53 genes were found to have significantly increased expression in the protein restricted rats, and the ADAM33, Atp1α1, Atp1α2 and GR genes were found to have a decreased expression in the protein restricted rats (Rose-Zerilli 2010). These results were only found between the 120 day old treatment groups as these were the only ones investigated. However, these results could not then be replicated in later samples. This could have been due fragile cDNA from these samples breaking down into small fragments that could then not be used as a template.

The difference between these results and those found in this investigation could be due to the age of the samples. The same lung tissue was used to extract the RNA and so it had been thawed again between the two extraction time points. It could also be that the primers used were older for this investigation.

4.3.6.3 Future Directions

Future work from this investigation could be to look at unique cell types using laser capture microdissection. This would give the gene expression profile from these individual cells. It could be that an increase in one gene in one type of cell is counteracted by a decrease in another cell type. By individually selecting a single cell type the unique gene expression profile will be revealed.

As the gene expression looked at within this investigation was targeted to look at specific genes the use of global gene expression profiles using microarrays could point us towards other genes that are either up or down steam of the ones looked at, that are the regulating step in the pathway. As asthma and COPD are complex diseases there are many more than one specific gene regulating their phenotype and so a global gene expression array gives a wider view point. For example one study used this approach to look at the gene expression profile of different parts of the rat brain to identify new pathways of degradation. This was done by extracting and purifying the RNA, from the isolated cell types of interest, and hybridising it to a rat genome 230 2.0 array (Affymetrix). The raw data was analysed and any differences in gene expression levels across the entire genome were discovered (Hedlund 2010).

An allergen exposure, asthma challenge, or a tobacco smoke exposure, COPD challenge, may have activated alterations in gene expression that are not seen before the animals have been challenged. There are models involving postnatal challenge that recapitulate features of asthma and COPD in the rat. For example repeated exposures to inhalation of 10g/L of either saline (control) or ovalbumin (challenge) for a period of 30 minutes will cause remodelling in rat airways (Birrell 2005).

5. General Discussion

5.1 Summary of Findings

5.1.1 Physiology and Morphometry

In response to a 50% reduction in protein content (in an otherwise isocalorific diet) of maternal diet from confirmation of pregnancy until birth, comprehensive morphometric analysis of the lungs found no significant differences between the control and PR groups in F1 28 day old, F1 225 day old or F2 28 day old male offspring in any morphometric measurements of lung structure. This contrasts with previous observations of the effect of maternal calorific restriction within the rat. Chen et al. (2004) found that body weight, lung volume and alveolar surface area were reduced in response to maternal nutritional restriction. However, there are significant differences between the study of Chen et al. and the current study in both the type and timing of the nutritional challenge. In the study of Chen et al., mothers experienced a 50% reduction in total food given, but only during the last trimester of gestation, from day 15-21 of gestation. This could account for the difference seen between studies in the effect of maternal nutritional restriction on lung development. As discussed in section 4.1 the Southampton protein restriction model reduces the protein content of the diet but the diet is isocalorific, through increasing the carbohydrate content, and no effects are seen on offspring bodyweight.

Equally, studies of the effects of reduced nutritional intake on offspring lung development in sheep have shown alterations within the lung. Reduced nutritional intake induced by umbilico-placental embolisation during late gestation, 120 days to term (147 days) has been shown to result in increased alveolar wall thickness, small number of alveoli and a greater volume density within the lungs of offspring. These alterations are more significant in animals at 8 weeks postpartum than those of a younger age (Maritz 2001). A later study undertaken using the same exposure found that the alterations seen at 8 weeks postpartum persisted at age 2 years, indicating that the effects of *in utero* diet can lead to changes in lung structure that persist into adult life (Maritz 2004).

5.1.1.1 Sample Size

One of the biggest limiting factors of this study was the number of samples that were included. In a *post-hoc* power calculation an estimated 60 animals would be required to accurately observe a significant difference between the two groups with respect to the smallest of the airways components, the airway muscle, with a power of 80% and α=0.05. The most likely reason for this large number of required animals is the amount of variation that is seen between rats.

Within this study the actual numbers of animals used was much lower, with sample groups selected based on previous observable differences in blood pressure between groups (Torrens 2008). However, it is interesting to note (while bearing in mind the differences between the studies noted above), that morphometric differences were seen in the study by Chen et al. (2004) with group sizes of 6-8. Undertaking a retrospective power calculation using the data from Chen et al. to observe the size difference noted with 80% power and α=0.05 would require a minimum group size of 8, if the PR model induced the same degree of effect as the 50% total nutrient restriction exposure. This implies that the difference between the two diets results in a difference in the size of effect seen.

5.1.1.2 Timing of Exposure

Changes in timing of diet given are also of importance as developmental windows alter during the progression of pregnancy. As evidenced by the study of the Dutch Hunger Winter cohort, exposure to famine during early and mid gestation had an effect on respiratory disease outcome in the offspring, but not late gestational exposure. If dietary restriction was started before conception, a reduction in protein could have a greater effect on the methylation profile of the offspring. This is due to the fact that the methylation patterns on the maternal and paternal gametes are stripped and then replaced as soon as fertilisation occurs. With a reduction in available protein providing fewer methyl groups, less methylation of the offspring genome could be a resulting effect.

Starting restriction at an earlier time point may also have an effect on the methylation patterns laid down in the gametes of the parents and hence alter the methylation pattern in the offspring. A study that reduced the amount of methyl donors given to rat dams, from 3 weeks prior to conception until 5 days after the birth of the offspring, observed a type II diabetes phenotype in the male, but not female, adult offspring (Maloney 2011).

In the rat a larger majority of the lung is developed after birth in comparison to humans (see section 1.5.2), and so increasing the time the diet is given, such as continuing on until weaning, could have a greater effect on the lung, as with the current dietary challenge plan the mothers are back onto a normal diet once the offspring have been born.

5.1.1.3 Type of Nutrient Restriction Exposure

Other alterations that could be included in this model are changes in the composition of diet given. As discussed above, other more severe diets, such as 50% total reduction in nutrition during the mothers' pregnancy, have been shown to alter the structure of the lungs (Chen 2004; Chen 2007). A more severe challenge is less likely to occur within normal conditions in the general population, but could give an indication of the types of effects that could be seen, and the mechanisms involved, in prenatal programming

of lung development. These could be then followed up in a more targeted approach in a model more reminiscent of normal exposure variation in the general population such as maternal PR. The other possibility is that although the airways appear normal they are more prone to damage when challenged with a *post-natal* stimulus and so they are 'primed' for respiratory disease.

5.1.1.4 Parent of Origin Effects

In humans transgenerational effects have been shown to be inherited in a sex linked manner, grandfather to grandson, and grandmother to granddaughter (Kaati 2007). This has also been observed in animal models where paternal (father's) diet exposure has been shown to result in epigenetic programming and phenotype changes. The use of a high fat diet given to rat sires, from weaning until sexual maturity, resulted in β-cell dysfunction in the daughters, which was found to be linked with the differential expression of several genes involved in the related pathways (Ng 2010). Another study, in mice, observed a paternal low protein diet, from weaning until sexual maturity, caused an increase in the expression of genes involved in lipid and cholesterol biosynthesis within the livers of the offspring; this was linked with an alteration in the methylation of the cytosines in this tissue (Carone 2010). Therefore it may be possible that paternal exposure may result in bigger phenotypic/methylation/gene expression changes than maternal exposure in male offspring as assessed in this study. Alternatively, larger differences in female offspring phenotype may be induced in response to maternal PR than those observed in male offspring in this study.

5.1.2 Bronchial Hyperresponsiveness

As previously mentioned (section 1.2.2), BHR is one of the defining characteristics of human asthma. This study observed increased BHR in the 75 day old F1 rats in response to maternal PR. However, a significant difference was not seen in the younger 35 day old rats. This effect, of alterations in lung phenotype being larger in older offspring, has also been seen in sheep (Maritz 2004). A possible explanation for this could be that the offspring sacrifice another system in order to achieve maturity in peak condition and this then declines as they age, and they can no longer stave off the effects of the *in utero* diet causing BHR. It could also be that the exposure of the rats to normal air currents and/or mechanical effects of stretch in postnatal life makes the BHR phenotype apparent, and that this takes time for the effects to develop.

The increase in BHR observed could be linked with the increase in smooth muscle that has been seen in the morphometric analysis of the F1 225 day old rats. In this group the PR rats had significantly increased smooth muscle ($p=0.046$) around the vessels within the lung. Although there was no difference seen in the airways components, the sample size could have been too low to identify this morphometrically, but was enough to result in the BHR phenotype seen in the offspring. Alternatively, as these rats have not been exposed to any stimuli, the bronchi have become hypersensitive to CCh due to epigenetic alterations and

that this would lead to increased bouts of constriction in a normal environment due to exposure to inhaled allergens.

However, while significant alteration in the reaction of the protein restricted group compared with the control groups to bronchoconstriction agents was observed, this does not imply that these phenotypic changes result from epigenetic differences induced by maternal PR, as hypothesised at the start of the investigation. Differences in the BHR phenotype may result from a direct effect of the *in utero* environment on lung development. To confirm if the BHR effect is indeed a result of epigenetic programming, a number of additional investigations would need to be made. Firstly, assessment of CpG specific DNA methylation differences in bronchial smooth muscle between groups. Secondly, if an observable difference in smooth muscle cell function *in vitro* between groups could be observed, then methylation marks could be removed by culturing cells in 5-deaza-Cytidine to assess whether this alters cellular phenotype. Thirdly, if the BHR phenotype could be shown to be transgenerational, with observable effects in the F2 and F3 generations, this would also imply that the phenotype was the result of epigenetic programming, specifically DNA methylation which is the only epigenetic mark known to be transmitted between generations.

With respect to the use of a rat model to assess lung physiology, a number of reagents that cause the human bronchi to constrict are not effective in the rat. These include histamine, which is broken down as soon as it enters the rat lung by histaminases (Rose 1940), and angiotensin II. However the rat bronchi did constrict in a concentration-dependent manner to the thromboxane mimetic U46619 and carbachol, mimicking the response seen in the human bronchi. The bronchodilators that were used did not result in significant relaxation of the bronchi once they had been constricted. A slight relaxation to salbutamol, isoprenaline and BRL37344 was observed and a total relaxation to the last dose of papaverine was also observed. These reagents do not seem to mimic the responses seen in the human lung. It could be that the receptors which cause the relaxation in the humans lung are not present in the rat, or that although they are present they are at low concentrations and so do not result in the same strength of reaction.

There is evidence of developmental programming of asthma in humans with several studies having linked a low birth weight, from both premature babies and those who suffered undernutrition, with increased risk of developing asthma in later life and BHR (Chan 1988). Thus the results of this study are significant, as they provide confirmatory evidence to support the concept that asthma susceptibility is, at least in part, determined by *in utero* environmental exposure. This raises the possibility of dietary intervention in pregnant women to reduce the risk of developing asthma in their children. As parental asthma is a significant risk factor for the development of asthma in the child (Arshad 2011), this would allow targeting of such intervention to those at highest risk.

5.1.3 Methylation and Gene Expression

In the antenatal period, it has been clearly demonstrated that maternal environmental exposures have the capacity to influence lung development. There is very strong evidence that maternal smoking in pregnancy has adverse effects on fetal lung development and asthma risk (Gilliland 2000; Hylkema 2009). This includes increased responsiveness to methacholine, smooth muscle layer thickness, and collagen deposition. Altered DNA methylation patterns have been observed in several genes in buccal cells from children exposed *in utero* to tobacco smoke, which may be a likely mechanism for increased risk of diseases (Hylkema 2009). Similarly, and perhaps more closely related with altered immune system development, maternal infections and the use of antibiotics have been associated with an increased risk for allergic airways disease (McKeever 2002), as have the level of specific micronutrients within the maternal diet (Chatzi 2008; Hollingsworth 2008), maternal stress levels (Gheorghe 2010), and exposure to pollutants (Liu 2008), all of which have been associated with epigenetic changes in a range of cell types and tissues (Martino 2011). Given the observation that surrogate markers of exposure to nutrient restriction in humans, e.g. birth weight and early growth, had been observed to be associated with asthma susceptibility in humans (Lopuhäa 2000) and that the Southampton PR model had previously been shown to result in changes in DNA methylation at specific gene promoters (Lillycrop 2007) we hypothesised that exposure to maternal PR would result in altered DNA methylation in the lungs of offspring exposed to maternal PR *in utero.*

This study found no difference in the global levels of methylation in DNA from the lungs or in the gene expression levels of the individual transcripts that were targeted. Previous investigation into this area using the PR model observed, in lungs from 120 day old rats, a difference in expression of Hsd11β2, IGF1, IGF2, Pcdh1 and Tp53 genes (increased expression levels) between PR and control groups (Rose-Zerilli 2010). This study did not find these alterations in gene expression levels. As previously stated, in section 4.3.6.2.4, this could be due to the age of the samples. The same lung tissue was used to extract the RNA and so it had been thawed again between the two extraction time points. This could have degraded the RNA, which is plausible as these results could not be replicated. Alternatively, the previous observation could have been the result of type I error (false positive).

Alteration of methylation and specific gene expression levels could be primed by the dietary challenge but, as asthma is a complex disease, require a postnatal environmental stimulus to initiate these changes. In animal studies, an asthma like phenotype can be induced by sensitisation to allergens, such as ovalbumin or the house dust mite allergen Der p 1 (either by intra-peritoneal injection with alum or repeated aerosol exposure), followed by allergen challenge by an inhaled route (intranasal or aerosol exposure) (Nabe 2005).

With assessment of global methylation levels, it is the methylation of repeated elements of DNA that dominate the measurement. This means that the individual methylation within the promoters of the gene involved in diseases processes are not closely examined. To do this candidate genes can be selected and their expression levels analysed, as done within this study. However, targeting of individual gene is difficult due to the many different genes that make up either A) a certain pathway of interest within a disease or B) a mechanism of regulation within the disease. There are other methods that could have been used such as whole genome gene expression, which can then mapped back to the gene involved to ascertain if there could be a link with the disease, or gene expression of entire pathways that are involved within the disease.

5.1.4 Southampton Model of Protein Restriction

The nutritional challenge that the mothers are exposed to during their pregnancy has been detailed in section 3.1. This challenge has no overall altered calorific intake; as a result no significant difference in the body weights of the offspring should occur. A non-significant difference in the body weights was seen that followed the same trend has been seen previously in other studies using this model (Torrens 2002; Rodford 2008). However several studies have shown that a more severe nutritional challenge can alter the body weights of the offspring (Chen 2004). These challenges almost always involve an extreme dietary challenge such as a total reduction in nutritional intake by 50%.

The Southampton model has been shown to have effects that are similar to those seen in a human population. The dietary exposure is also more common place in the human population as exhibited in mothers who are vegetarian and do not take the appropriate supplements to counter act the effect of a reduced level of protein within the diet. A complete reduction of 50% of nutritional intake during pregnancy is only seen in extreme famine conditions for which there is no comparable data and is unlikely to be seen in the general population.

5.2 Future Directions.

5.2.1 Protein Restriction Model

In the current study, a nutritional exposure involving a reduction in protein content of 50% in the maternal diet that is made up with carbohydrates making it a very mild restriction that has no overall calorie difference. This is a model that mimics exposures more commonly seen within a human population not exposed to extreme starvation.

The protein restriction model has been shown to have an effect on the responsiveness of the bronchi. In humans, repeated contraction of the bronchi in response to either methacholine or inflammation induced bronchoconstriction can cause airway remodelling to occur in non-asthmatic individual's bronchi (Grainge 2011). However, no remodelling was observed in the F1 or F2 offspring lungs in this study. It could be that the maternal dietary challenge has primed the asthmatic phenotype, but that due to a lack of postnatal exposure this phenotype has not been expressed. The introduction of number of challenges, such as those utilised in animal models of asthma, could shift the phenotype from one that is primed to one that is expressed within the structure of the lungs. The most commonly used asthmatic challenge within animal models is sensitisation with an interpreitoeal injection of ovalbumin, followed by aerosol challenge (Nabe 2005). It would be interesting to observe whether BHR responses *in vivo* using whole animal measurement of lung function would show an enhanced BHR phenotype in response to allergen challenge in the PR group in comparison to control and whether this results in increased airway remodelling. Alterations in the timing and type of dietary challenge could also be considered, see sections 5.1.1.2 and 5.1.1.3.

5.2.2 Humans

The use of animals models give an idea of what could be happening due to an alteration in the level of protein intake during pregnancy. However, a normal human diet would not suddenly have a reduction in a single food group, and data derived from animal models does not always fit when compared back to humans. The use of detailed dietary data during pregnancy and a closely followed up birth and life of a human child could either prove, or disprove, the ideas suggested within this thesis. Several cohorts have collected the data required to start an investigation into this area. A correlation between dietary intake of specific nutrients during pregnancy and bronchial hyperresponsiveness has already been identified in the Southampton Women's Survey cohort (Pike 2011). Other studies into the exact dietary program could highlight the more important food groups to be included for a reduction in the offspring risk of developing respiratory diseases later in life.

Other studies that have examined maternal diet during pregnancy have been undertaken retrospectively and the mothers are required to recall what they consumed during the nine months of their

pregnancy. There are several problems with this, mainly the effect of misreporting of food consumption. A nine month period is a long time to remember the information needed and the consumption of foods that are perceived to be bad may be under-reported by the mother concerned. Due to this fact, most studies have focused on a certain food group or a certain, regional, type of diet. For example, a study by Chatzi et al. looked at the effect of a Mediterranean diet. This diet is high in fruit and vegetables, as well as wholegrain bread and cereals, and low in dairy, eggs and red meat. This results in decreased levels of fatty acids and increased levels of carbohydrates, fibre and anti-oxidants. Children of mothers who consumed this diet during pregnancy were found to have a decreased risk of developing asthma and allergy (Chatzi 2007; Chatzi 2008). However, it is possible that this observation is confounded by high similarities between maternal diet during pregnancy and the child's diet postnatally.

5.2.3 Methodology

Alterations in DNA methylation, and the subsequent change in gene expression levels, could be investigated in a different manner that would allow a hypothesis independent approach. The use of genome-wide expression arrays could be used to identify differentially expressed genes followed by targeted DNA methylation analysis of those genes or an array based method to identify differentially methylated sections of DNA and the gene involved in those sections could then be assessed for expression levels. For example, a study by Weinhouse et al. of supplemental increase of 50mg bisphenol A (BPA), during gestation and continued through until weaning, utilised genome-wide gene expression analysis to identify differentially expressed genes in the liver of 22 day old male mouse offspring. In this study, 2 genes (Glcci1 repeat 1 and Glcci repeat 2) were identified as having metastable epialleles that are differentially expressed as a result of maternal BPA exposure and the authors went on to show that the differences in gene expression were due to alterations in DNA methylation of specific CpG sites in both of these genes (Weinhouse 2011).

This study has linked maternal diet during pregnancy with increased risk of BHR, a defining phenotype for asthma. Although there have been no other altered results in the offspring lungs this could be due to the lack of allergen challenge/environmental exposure needed by most respiratory diseases to express the disease phenotype.

Other questions that need to be considered in further work would be:

- Is the BHR observed sex specific or would it also be observed in female offspring?
- Would this also be shown in F2 and F3 generations, as has previously been observed for blood pressure?

- Would an allergen challenge produce a BHR phenotype at a younger time point or result in enhanced airway remodelling in PR offspring?
- Does an identical biochemical mechanism resulting in altered smooth muscle functionality underlie the observation of BHR in airways and raised blood pressure in vessels in response to maternal PR?
- Does the increased smooth muscle surrounding pulmonary vasculature lead to pulmonary hypertension as has been previously observed in rats in response to a more severe maternal nutritional restriction?

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Appendix 1: Nugget Effect for Smooth Muscle.

Sample data for the first grid trialled for used in stereology analysis.

	P_i	$P_i \times P_i$	$P_i \times P_{i(+1)}$	$P_i \times P_{i(+2)}$
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	55	3025	0	0
5	0	0	0	0
6	0	0	0	0
7	77	5929	231	0
8	3	9	0	0
9	0	0	0	0
10	0	0	0	0
11	13	169	0	26
12	0	0	0	0
13	2	4	0	2
14	0	0	0	0
15	1	1	16	-
16	16	256	-	-
Total	**167**	**9393**	**247**	**28**
		A	**B**	**C**

$A(p) = 8x9$

$= 72mm$

Correct for magnification = 72/30000

$= 0.72$

T = thickness of sections = 5um

Variance $= \sum Pi \times A(p) \times t$

$= 167 \times 0.72 \times 5$

$= 6.68 \times 10^{-5}\ mm^3$

$Nug = 0.0724 \times 10 \times \sqrt{(n \times \sum P_i)}$

$= 37.4$

Variance of sum of total area = (3(A-Nug)-4B+C)/12

$= (3(9393-37.4)-(4x247)+28)/12$

$= 2258.9$

Nug + Variance of total area = 37.8 + 2258.9 = 2296.3

CE = (√2296.3/167) x 100

= 28% (As this is above 10% a grid with closer points will have to be used)

Nug = (100 x 37.8) / 2296.3

= 1.63% (As this is below 5% the grid is fine for this effect)

As both the percentages were not below their respective values a smaller grid will be trialled to see if it is appropriate for this investigation.

Nugget effect for smooth muscle with second grid

Sample data for the second grid trialled for used in stereology analysis.

	P_i	$P_i \times P_i$	$P_i \times P_{i(+1)}$	$P_i \times P_{i(+2)}$
1	61	3712	1952	12566
2	32	1024	6592	5888
3	206	42436	37904	3502
4	184	33856	1972	21344
5	17	289	1472	1564
6	116	13456	17940	22620
7	92	8464	23790	11224
8	195	38025	10248	16380
9	122	14884	9744	1952
10	84	7056	20416	14784
11	116	13456	2816	14268
12	176	30976	21648	4576
13	123	15129	3198	13653
14	26	676	2886	6344
15	111	12321	27084	-
16	244	59536	-	-
Total	**1905**	**295305**	**189662**	**150665**
		A	**B**	**C**

A(p) and t are the same as above as the magnification and thickness of sections has not been altered.

Variance = $\sum$Pi x A(p) x t

= 1905 x 0.72 x 5

= 6858 mm^3

Nug = 0.0724 x 10 x √(n x $\sum P_i$)

= 126.4

Variance of sum of total area = (3(A-Nug)-4B+C)/12

= (3(295305-126.4)-(4x189662)+150665)/12

= 23129.4

Nug + Variance of total area = 126.4 + 23129.4 = 23255.8

CE = (√23255.8/1905) x 100

= 8% (As this is below 10% the grid is fine for this effect)

Nug = (100 x 126.4) / 23255.8

= 0.5% (As this is below 5% the grid is fine for this effect)

As both the percentages were below their respective values this grid is appropriate for this investigation and so will be used.

Appendix 2: Primer sets for gene expression assays

Gene name	Target or reference	Assay type	Intron spanning	Forward primer	Reverse primer
Adam33	Target (IUGR marker)	UPL	Yes	TAGTGGCCCTGCACAGTCT	TGGTAGTAGCACCAGGCTAGG
Atp1a1	Target (IUGR marker)	SYBR green	Yes	TGGATCAATGATGTGGAGGA	CAATATTCCTCTGCTCGTAGG
Atp1a2	Target (IUGR marker)	SYBR green	Yes	GACCTCATCATTTGCAAGACC	CTAGCAGCCCAAAAATCAGG
Gr	Target (IUGR marker)	TaqMan	Yes	TGCATGTATGACCAATGTAAACACA	CCTTCCTTAGGAACTGAGGAGAGAA
Hsd11b1	Target (IUGR marker)	SYBR green	Yes	TGTCCTCGGCTTCATAGACA	GCGCAGAACTGTGCCTTT
Hsd11b2	Target (IUGR marker)	SYBR green	Yes	GGGGTATCAAGGTCAGCATC	TCCCAGAGGTTCACATTAGTCA
Igf1	Target (IUGR marker)	SYBR green	Yes	CGGCCTCATAATACCCACTC	AAGACGACATGATGTGTATCTTTATTG
Igf2	Target (IUGR marker)	SYBR green	Yes	CGCTTCAGTTTGTCTGTTCG	GCAGCAACTCTTCCACGATG
MR	Target (IUGR marker)	SYBR green	Yes	CAAATACCCCAGCAAGCAGT	CTGTGCTGTGATGGATCCTG
Pcdh1	Target (IUGR marker)	UPL	Yes	GAGAGCACTGCCCACCAG	AACATCTCGAAGCGCTCAC
Tp53	Target (IUGR marker)	SYBR green	Yes	GACTCTGTAAAGCAGTTCTACACCAA	GGGCACCTGTGGTGCTAC
Ucp2	Target (IUGR marker)	SYBR green	Yes	TAGTGGCCCTGCACAGTCT	TGGTAGTAGCACCAGGCTAGG
HDAC2	Target (methylation marker)	SYBR green	Yes	ATGCTGAAGAGAGGGGTCCT	TCATAGAATTCATTGGGTGCTTC
HDAC3	Target (methylation marker)	SYBR green	Yes	CGCTGACTCCCTCTCTGGT	TCACTACTTCTACACATTTAGCGTGA

Gene name	Target or reference	Assay type	Intron spanning	Forward primer	Reverse primer
HDAC8	Target (methylation marker)	SYBR green	Yes	GTGGGAATTGGCAAGTGTCT	CCGTGTTGGCAAGGTTGTA
Actb	Reference (endogenous control)	TaqMan	Yes	CGTGAAAAGATGACCCAGATCA	CACAGCCTGGATGGCTACGT
B2m	Reference (endogenous control)	TaqMan	Yes	ACTCTGAAGGAGCCCAAAACC	TCCAGATGATTCAGAGCTCCATAG
Ubc	Reference (endogenous control)	TaqMan	No	CGTACCTTTCTCACCACAGTATCTAGA	GAAAACTAAGACACCTCCCCATCA