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EARNEST

EARly Nutrition programming- long term follow up of Efficacy and Safety Trials and integrated epidemiological, genetic, animal, consumer and economic research

Instrument: Integrated Project

Thematic Priority 5.4.3.1: Food Quality and Safety

Final public report on activity 3.1.2

The timing of protein imbalance and later consequences; are they reversible by drug treatment?

Start date of project: 15.04.2005

Organisation Name of Lead Contractor for this report: University of Nottingham

Principal Investigator: Professor Simon Langley-Evans

Staff funded through EARNEST contribution: Andrea Haase, Ruth Austin.

PhD students who contributed to this work: Sarah Engeham, Matthew Harrison, Aml Erhuma, Christina Lilley.

Objectives for this workpackage.

There were two principal goals for workpackage 3.1.2. These were to:

1. Examine whether appropriate treatments, either through administration of anti-hypertensive drugs, or using dietary supplements can reverse or prevent the programming effects of a low protein diet, in the rat.
2. Explore the mechanisms through which maternal undernutrition programmes long-term cardiovascular, renal and metabolic disturbance in the fetus.

Principal findings.

1. Interventions to prevent or reverse the programming effect of protein restriction

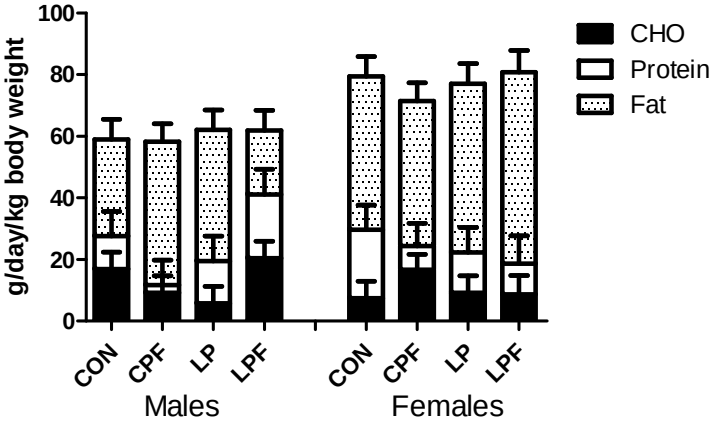
1.1 The project examined two possible means of intervening to prevent or reverse the effects of prenatal exposure to a maternal low protein diet in the rat. The first approach was to exploit knowledge of the involvement of the renin-angiotensin system in programming of blood pressure and renal phenotypes by utilizing a pharmacological blockade of angiotensin II receptors. Blockade was targeted to the early postnatal period, with offspring from control or protein restricted dams being treated with an AT1R antagonist in postnatal weeks 3, 4, or 3 and 4. The intervention was successful in preventing the expected programmed increase in blood pressure in the low protein exposed offspring, but was associated with an exaggerated renal deficit (lower nephron number) in the same animals. In control offspring the AT1R blockade promoted an increase in blood pressure and a reduction in nephron number. **We conclude that although partially successful, the use of angiotensin II receptor antagonists in early postnatal life is not a suitable intervention for reversal of programmed cardiovascular phenotype.**

1.2 The second approach to intervention focused upon the use of dietary supplementation during pregnancy. The work drew from earlier reports in the literature which suggested that maternal supplements of either folic acid or glycine may be beneficial. The rationale for this focused upon a potential mechanistic link between protein restriction, the methionine-homocysteine and folate cycles and DNA methylation. The latter was of particular importance due to the putative role of changes to epigenetic marks as drivers of the programming of disease. Supplementation strategies were targeted at the whole of pregnancy, and whilst low protein fed mothers were supplemented with high doses of folate (5 mg/kg diet) or glycine (3% of diet w/w), control dams were provided only with folate supplements.

1.3 Supplementation of the maternal diet with folate had contrasting effects depending upon the protein intake of the animals. In low protein fed dams, folate supplementation generally reversed the effect of protein restriction upon programmed phenotypes in the offspring. As shown in Figure 1, as adults LP exposed males had a preference for fat-rich foodstuffs when offered a self-selection diet. Folate supplementation prevented the programming of this food preference. Interestingly, folate supplementation of a control protein diet was

frequently observed to have a programming influence that was almost as potent as protein restriction. **We conclude that folate supplementation in pregnancy can prevent some of the programming effects of a maternal low protein diet. However, as excessive folate can in itself have a detrimental programming effect, blanket supplementation may not be a suitable preventive strategy for humans.**

Figure 1: Food selection



Self-selection of high fat, high CHO or high protein foods was programmed in males. LP males had a significantly greater intake of high fat compared to CON. CPF males consumed more fat than all other groups ($P<0.05$)

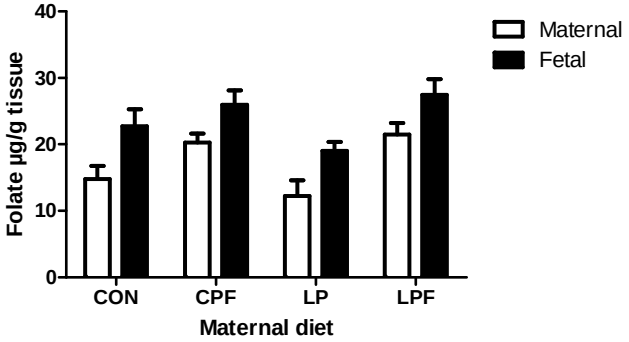
1.4 Consistent with earlier reports, protein restriction was found to alter the fetal:placental weight ratio in day 20 gestation pregnancies. Low protein diets tend to increase fetal growth to this point and limit the growth of the placenta. Folate supplementation did not prevent this pattern of growth. The addition of glycine to the maternal diet had a detrimental effect on both fetal and placental growth, resulting in a growth retarded fetus.

1.5 It is widely assumed that folate and glycine may prevent or reverse programming effects of a low protein diet through effects on methionine-homocysteine and folate cycles. We investigated this possibility by measuring maternal and fetal circulating and hepatic concentrations of key components of these pathways, and the mRNA expression of key rate-limiting enzymes. In maternal circulation and liver we found that there were no significant effects of low protein diet upon homocysteine, folate, choline or phosphocholine. When folate or glycine were added to the maternal diet there was little impact upon the selected endpoints. Folate supplementation resulted in heavy storage of total and polyglutamated folate in the liver (Figure 2). Similar findings were noted in fetal tissues.

Table 1. mRNA expression of key genes of the methionine-homocysteine and folate cycles in livers of day 20 gestation fetuses. CON-control, CPF- control + folate supplement, LP- low protein, LPF- low protein + folate supplement.

	Dietary group							
	CON		CPF		LP		LPF	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Male fetuses								
DNA methyltransferase 1	5·74	2·07	4·15	0·51	3·38	0·48	6·66	3·67
Met synthase	5·35	2·93	2·84	0·47	5·31	2·53	8·05	2·67
Methyltetrahydrofolate reductase	14·02	4·77	9·45	2·67	12·81	4·30	8·05	2·67
Female fetuses								
DNA methyltransferase 1	4·12	0·44	3·64	0·80	4·20	0·55	3·18	0·81
Met synthase	2·45	0·48	2·61	0·44	3·59	0·79	3·95	0·87
Methyltetrahydrofolate reductase	6·69	1·90	7·88	2·59	7·11	1·07	7·01	1·63

Figure 2: Hepatic folate



There was no effect of LP diet upon hepatic folate in either maternal or fetal tissue at d20 gestation. Folate supplements markedly increased hepatic folate storage.

1.6 We determined the mRNA expression of methionine synthase, methyltetrahydrofolate reductase and DNA methyltransferase 1 in maternal liver and found no significant effect of maternal diet upon expression of any these genes. As shown in Table 1, there was no evidence of any effect of either protein restriction or folate supplementation on the mRNA expression of these genes. When global DNA methylation was assessed using a cytosine extension assay there were no differences between groups in day 20 fetuses. **We conclude that maternal protein restriction does not bring about gross disturbances of the methionine-homocysteine and folate cycles. We have excluded these processes as contributors to the alteration of epigenetic marks in protein restricted fetuses.**

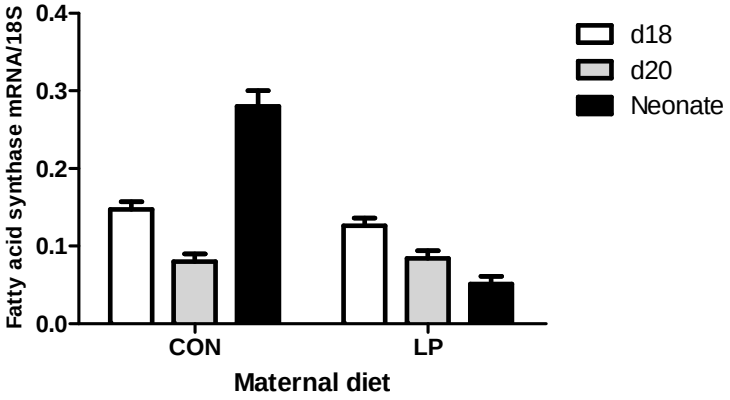
2. Mechanisms of nutritional programming

2.1 The basic premise for our approach to this objective was that maternal undernutrition impacts initially upon the expression of key genes and pathways in fetal tissues. This will result in modification to tissue morphology and hence later function. Some of the differentially regulated genes may remain under- or over-expressed beyond the period of nutritional insult, but many genes found to be differentially expressed in adults may be "programmed" as part of the secondary response to the initiating mechanism.

2.2 We collected fetal tissue from protein restricted pregnancies at different stages of development. mRNA expression of a number of putative targets was determined in fetal liver. Candidate genes were selected on the basis of studies of postnatal animals. We noted

few significant changes at days 18 and 20 gestation, although DNA methyltransferase was up-regulated by protein restriction in female offspring at day 18. However, in newborn animals we observed a significant suppression of lipogenic pathways, which was absent at earlier time points (Figure 3). **We conclude that the pathways that are seen to be programmed by undernutrition in older offspring are not necessarily the primary drivers of whole tissue/whole body programming responses.**

Figure 3: Expression of lipogenic pathway



Hepatic expression of lipogenic enzymes and associated transcription factors was suppressed by maternal protein restriction but only post-partum ($P<0.001$).

2.3 Studies from our laboratory had identified evidence of intergenerational programming of blood pressure and renal development. Offspring from mothers fed a low protein diet in pregnancy had high systolic pressure and lower nephron number as adults. F2 offspring derived from these animals showed the same phenotypes, regardless of whether a male or female LP-exposed animal was their parent. F3 generation offspring did not show the programmed phenotype. As such data is consistent with epigenetic inheritance of traits, we examined global DNA methylation in renal tissue across F1, F2 and F3 generations. We found no evidence of any effects of maternal, grand-maternal or great-grand maternal nutrition. However, analysis of the renal transcriptome using a targeted microarray showed that there were 6 genes out of 113 on the array whose expression in kidney was consistently influenced by the original maternal protein restriction, across both F1 and F2 generations. These included matrix metalloproteinase 9 which was down-regulated and endothelial cell growth factor 1, which was up-regulated. **We conclude that intergenerational programming of blood pressure and renal development may involve the inheritance of differential patterns of gene expression. This process must involve some diet-induced changes to epigenetic marks on specific gene loci which can be passed on by both male and female animals to their offspring. The nature of these processes remains to be determined.**

2.4 There is an extensive literature to indicate that some of the effects of maternal undernutrition on offspring blood pressure and renal development may be mediated by disruption of the placental barrier to glucocorticoid transfer from mother to fetus. We performed two experiments to investigate the impact of glucocorticoids upon these phenotypes and the expression and methylation of the adrenal angiotensin II receptor 1b.

2.5 In the first experiment we administered dexamethasone to chow diet fed, pregnant rats in the final week of gestation. Control animals were pair fed to the intakes of treated dams in order to remove any influence of the appetite suppressive effects of glucocorticoids. Offspring were studied at weaning and at 12 weeks of age. It was noted that dexamethasone treatment resulted in higher blood pressure and lower nephron number, a finding identical to the effects of maternal protein restriction. **We conclude that fetal exposure to glucocorticoids can programme blood pressure and renal development independently of any effect of hormone treatment upon maternal food intake.**

2.6 In the second glucocorticoid experiment pregnant rats were fed either control or low protein diets. Low protein fed dams were treated (over the first 14 days of pregnancy) with metyrapone to inhibit endogenous glucocorticoid synthesis. This intervention blocked the programming effect of protein restriction upon offspring blood pressure and nephron number. This was consistent with the hypothesis that glucocorticoids mediate some of the programming response to protein restriction. In collaboration with Professor Adrian Clark at Queen Mary, University of London, we examined the expression and promoter methylation of adrenal AT1b. As shown in Figure 4, there was strong evidence that, for this gene, protein restriction resulted in hypomethylation and over-expression. These effects were reversed by metyrapone (LPM) treatment. **We conclude that glucocorticoids of maternal origin can determine epigenetic marks at specific gene loci and that this may mediate the programming response to maternal protein restriction.**

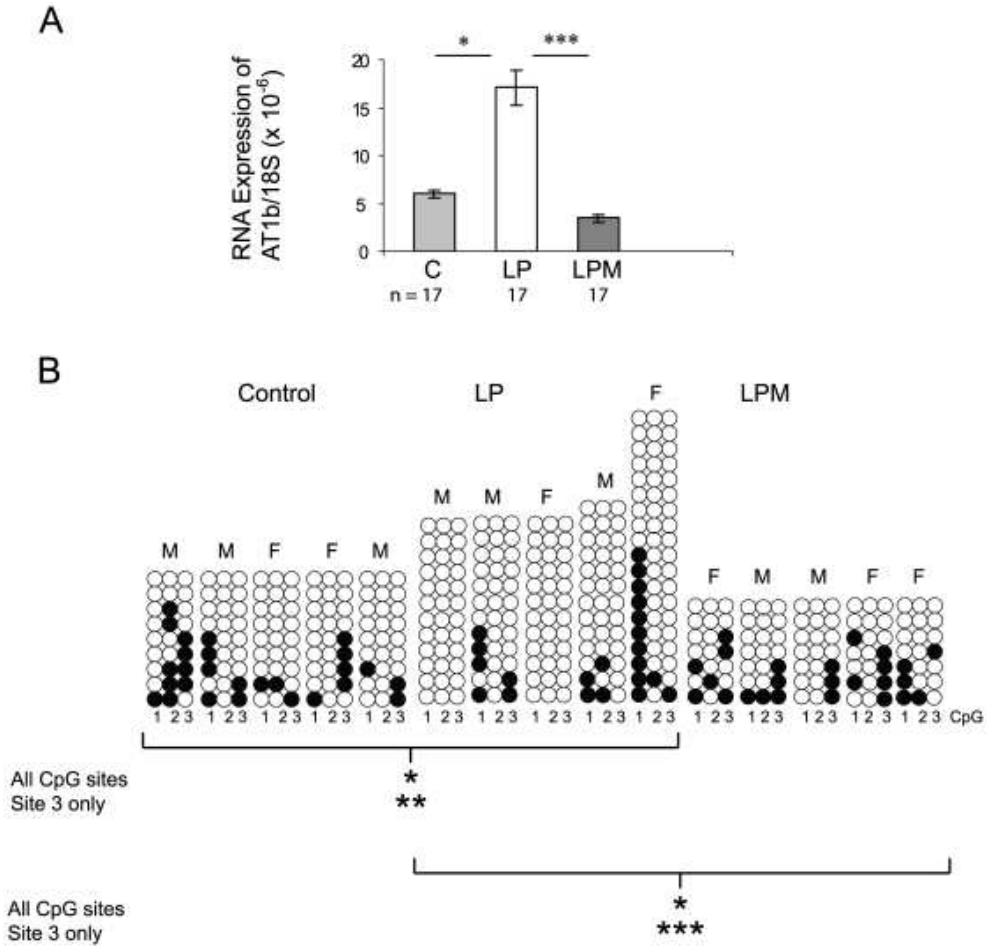
<u>Overall conclusions from this work package</u>

Data outputs from work package 3.1.2 represent the results of a complex series of experiments designed to explore the hypothesis that programming may be reversed or prevented by suitable interventions and to add to understanding of the mechanisms through which exposure to maternal undernutrition determines risk of disease in later life.

The overall conclusions arising from this work are:

1. Programming effects of maternal protein restriction are reversible or preventable using both dietary supplementation and pharmacological approaches. However, any intervention performed in early life may have independent detrimental programming effects in offspring of well-nourished mothers.
2. The hypothesis that dietary perturbation of the methionine-homocysteine or folate cycles is a principal driver of the rewriting of epigenetic marks is not supported by the available evidence.
3. Changes in gene expression in response to maternal undernutrition that are observed postnatally, are not necessarily present in fetal life and may not explain the programmed response to dietary restriction. The primary drivers of programming may be transient changes in the expression of yet to be identified genes, proteins and pathways.
4. Glucocorticoids appear to mediate at least some of the fetal programming response to maternal protein restriction. There is evidence that protein restriction establishes heritable changes in gene expression that may be mediated by alterations to DNA methylation at specific loci, but not across the whole genome. Glucocorticoids of maternal origin appear to play a role in this resetting of epigenetic marks.

Figure 4: Expression and DNA methylation of adrenal AT1b.



Collaborations

Researchers involved with this work package developed a number of important collaborative activities under the auspices of EARNEST. Professor Langley-Evans and colleagues published findings of work produced with Prof Jaap Joles, an EARNEST Theme 3 partner. Data generated within activity 3.1.2 included results from material shared with Dr Ozanne at the University of Cambridge. EARNEST interactions facilitated discussions which led to successful funding bids based on collaborations with Prof Joles, and Prof McArdle.

Publications

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