FOOD-CT-2005-007036

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 3.5.2:

Gene discovery

Period covered from 15.04.2005 to 14.10.2010

Start date of project: 15.04.2005 Duration: 5,5 Years

Organisation Name of Lead Contractor for this report: RRI

ACTIVITY OBJECTIVES AND MAIN TASKS

This work package aimed to utilise the link between the European Nutriogenomics Network (NuGO) and the EARNEST consortium in order to identify genes and gene families whose expression is altered by maternal micronutrient deficiency. Collaboration with NuGO has allowed EARNEST partners access to up to the minute technologies and essential statistical and bioinformatics expertise.

The initial objective of this activity was to use the placental gene array developed in activity 3.5.1, to aid in gene discovery. However a stated in the report for 3.5.1 this plan was dismissed in favour of using the whole genome arrays available via a commercial partner within NuGO. Access to bioinformatics support via NuGO has allowed us to widen our target from the identification of genes and gene families to include biochemical pathways. The nutritional stressor that this activity was targeting has also changed. Do to the unique access to multiple nutritional models that EARNEST has brought about we were able to test a fundamental hypothesis of fetal programming.

In experimental systems, notably rats and mice, a wide variety of nutritional stresses have been used to model the fetal programming. These include, for example, different micronutrient deficiencies, low or high protein and high fat diets. Despite the fact that there are so many different approaches, the effects generated are remarkably similar and mimic metabolic syndrome to a greater or lesser degree. This has given rise to the concept of "gatekeeper" genes or "gatekeeper" pathways (Fig 1), where the diverse stresses all act at one point or pathway which is critical for optimal development.

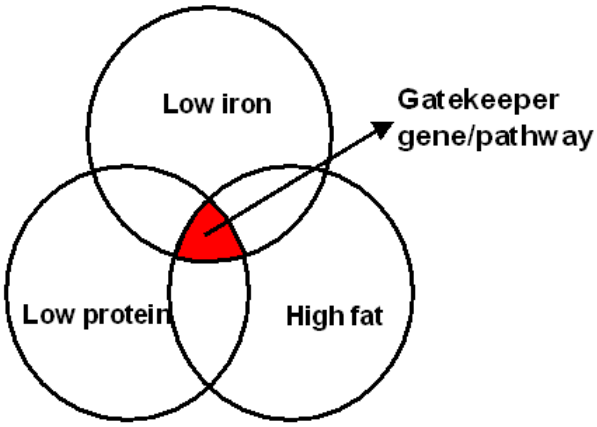

Fig. 1. The Gatekeeper Hypothesis. By comparing the different nutritional stresses directly with each other identifies commonly affected genes/pathways. The darkened central point contains these genes in common.

Within the EARNEST consortium we had rodent models for fetal programming based on micronutrient deficient, low protein and high fat diets. In this activity, using placenta as our first target tissue, we aimed to begin the search for this "gatekeeper" by combining our different models and identifying altered genes, gene families and pathways. This would be best accomplished by high throughput methods such as DNA array or proteomics. During the life span of this activity deliverables were amended and added to keep pace with the data as it was generated, for example as well as using genomics and proteomics we also began to carry out lipidomics to aid with the identification of critical genes and pathways.

RESULTS

Gene identification (Deliverables 84-85)

This deliverable combines the microarray data set generated in Activity 3.5.1, with newly generated data sets. The protocols and procedure established in Activity 3.5.1 where followed throughout these deliverables. Further microarray analysis was used to generate data sets of

gene expression changes in the placenta as a result of a maternal diet low in protein or high in fat. The placental samples for the low protein and iron deficient models were taken at day 21 of gestation, whilst in the high fat model the samples had been taken at day 17 of gestation. Placental samples were taken from male fetuses only. The data sets from these three different dietary treatments where compared (Fig 2).

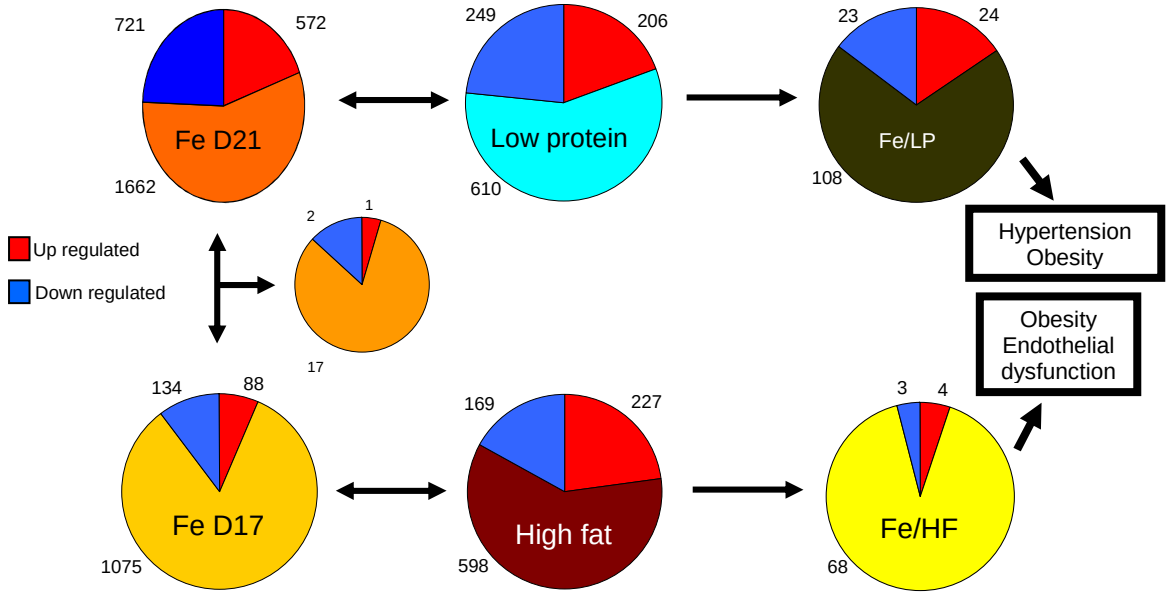

Fig. 2. Common genes identified following comparison of the different fetal programming model. Numbers next to each chart section refer to the number of genes up-regulated, down-regulated or unchanged. For further descriptions and abbreviations see section below.

Within each group gene expression was compared between control and experimental animals, the resulting list of differentially expressed genes were compared with that of the other models. For example, in Fe deficient placenta (sampled at day 21 gestation, Fe D21), 721 genes were significantly down-regulated, while 572 were significantly up-regulated, whilst 1662 genes were expressed above background levels but were unaffected by the dietary treatment. When comparing the differentially expressed genes in the low-protein and low Fe diets (Fe/LP) there were 23 genes down-regulated and 24 up-regulated in both models, whilst the expression of the remaining common genes was not consistent between the models. Comparison with high fat diet was more complex, since the samples were taken at D17.5 of pregnancy rather than D21.5. In order to overcome this problem, placental samples from low-Fe animals at D17.5 were generated. This had the added advantage of allowing us to monitor developmental gene expression and how it was affected by maternal iron deficiency. Unfortunately during the course of this deliverable it became clear that when considering the male offspring only the high fat treatment gave us some differences in phenotype (Fig 2), which meant that we could not completely carry out a gatekeeper comparison with the low protein and low Fe diets.

Comparison of differential expression in the placenta between the iron deficient and low protein models has continued. Through EARNESTs partnership with the European Union's Network of Excellence, NuGO, we have access to pathway analysis tools Metacore^{TM}. In this deliverable we have used this software to help to identify the common genes and pathways between the iron deficient and low protein models (Fig. 3). The placental genes expressed above background levels are split into three groups, unique, similar and common. There were 457 genes unique to the low protein model, 1631 genes expressed only in the iron deficient and 110 genes were expressed in the placentas from both models. Of the

Fig. 3. Common placental genes identified following comparison of the iron deficient and low protein models using the pathway analysis programme 'Metacore™'. The light blue bar indicates genes which are unique to the low protein model, the orange bar those unique to the iron deficient model, whilst the hashed par represents the gene common to both models.

stated 110 common genes, only 92 have confirmed IDs, of those 6 are uncharacterised or hypothetical proteins. Of the 86 identified genes, 32 genes were regulated in opposing directions by the two dietary conditions and therefore cannot be consider as potential gatekeepers. There were 54 similarly regulated under both dietary treatments, 25 genes are down-regulated and 29 genes are up-regulated. We are now in the process of creating a priority list for these genes. The differential expression of each of these genes has to be confirmed by either RT-PCR or an increase in their expression of the corresponding protein. This is the area of the research which is taking the most time as the expression levels of many of these genes are barely above background, and as we are working with nutritional and not pharmacological stresses the fold changes are small.

Lipidomics in fetal programming - initial data set of lipidomic profiles (Deliverables 49)

This deliverable aimed to take a slightly different approach to gene discovery. DNA microarray and proteomic analysis of placenta (Activities 3.5.1, 3.5.2, 3.5.3) and microarray analysis on the fetal liver, demonstrated that several genes involved lipid metabolism were altered by maternal iron deficiency. This together with the evidence in the literature that many rodent models of fetal programming show obesity and/or dyslipidema in the offspring indicates that maternal dietary imbalance may alter lipid metabolism in their offspring.
Previous data from our lab, showing that animals born to iron deficient mothers had increased adiposity and dyslipidema, in keeping with our new 'EARNEST 'data indicates that maternal iron deficiency may alter lipid metabolism in the fetus. Therefore the aim of this deliverable was to study in greater detail the effect of maternal deficiency on lipid metabolism in the mother, fetus and offspring.

Fatty acid analysis and lipid profiling were carried out on liver and serum from control and iron deficient dams and fetuses at day 21.5 of gestation, and 38 week old male offspring, for materials and methods please see deliverable report. Triglycerides, cholesterol and NEFAs increased significantly in the iron deficient maternal liver (Table 1; p=0.02, 0.02, 0.01), however no change was observed between the control and Fe deficient fetal and offspring livers. A more complex pattern of changes was seen in serum. An increase in triglycerides was seen in maternal and offspring serum (p=0.002 and 0.02 respectively), whilst fetal levels were unaffected. Although maternal cholesterol were unchanged, both the fetal (p>0.01) and offspring (p=0.05) serum, interestingly in opposing fashion. The levels of free fatty acids were unchanged in both maternal and offspring serum, however a decrease in levels was seen in the fetus (p>0.01).

Liver	Maternal	Fetal	Offspring
Cholesterol	↑ (70%)	↔	↔
Triglycerides	↑ (237%)	↔	↔
Free Fatty Acids	↑ (54%)	↔	↔

Serum	Maternal	Fetal	Offspring
Cholesterol	↔	↓ (33%)	↑ (18%)
Triglycerides	↑ (56%)	↔	↑ (19%)
Free Fatty Acids	↔	↓ (23%)	↔

Table 1. Summary of altered fatty acid profiles in the mother, fetus and offspring as a result of maternal iron deficiency. (↔ No change, ↓ significant decrease, ↑ significant increase in levels)

Further analysis was carried out in order to obtain lipid profiles for mother, fetus and offspring. In serum, the levels of docosapenticoic acid (C22:5n3) were higher in both the iron deficient mother and the fetus (Fig 2). Docosahexinoic acid (C22:6n3) was found to increase in both maternal and 38 week serum however, it was undetected in the fetal serum. Linoleic acid (C18:2n6) was found in increased amount in the fetal and 38 week serum but did not change in the mothers (Fig 2). The maternal liver lipid profile showed increases in phosphatidylcholine (PC) Palmitic acid (C16:0) and decreases in PC steric acid (C18:0), oleic acid (C18:1n9c) and arachidonic acid (C20:4n6). This data is consistent with total NEFA analysis demonstrating the iron deficiency is having a significant effect on the maternal lipid metabolism. As expected the liver lipid profiles showed no significant change in the fetal and 38 week offspring. However, when the fetal serum NEFA profiles were analysed for the delta 5/6 ratio there was a significant change (p=0.03) (Fig 4).

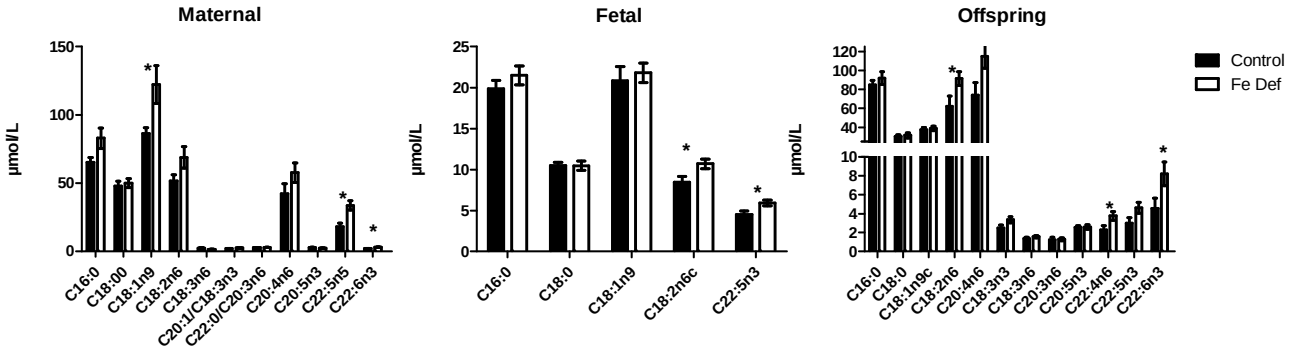

Figure 4. Maternal iron deficiency leads to altered lipid profiles in the mother, fetus and offspring

CONCLUSION

Gene identification

This activity has led to greater and sustained interaction between EARNEST partners. The individual array data sets provide the basis of many years of study for each of the rodent models. In fact much of the data has and will be used as pilot data for a number of grant applications. The difficulties encountered by this activity, for example different rat strains and sampling time points has contributed to a better understanding of how the 'ideal study' showed be planned. Despite this, this approach has provided many testable hypotheses for understanding the link between maternal diet and offspring health. Studies are under way in order to verify the array results and map the physiological changes caused by altered target gene expression

Lipidomics in fetal programming - initial data set of lipidomic profiles

The results obtained in the lipidomics study show that iron deficient dams have an altered liver lipid profile, increased levels of total triglyceride, cholesterol and NEFA. Maternal serum also has an altered fatty acid profile and increased triglyceride. The maternal serum increases maybe the result of increased transport of triglycerides out of the maternal liver. It is unlikely that the serum increases were due to a block in placental transport as both the fetal and maternal serum show increased DPA, indicating transport across the placenta was functional. Fetal serum showed no change in triglycerides however, cholesterol and free fatty acids did significantly decrease. These results were not surprising as it is known that triglycerides are broken down into their fatty acids components for transport across the placenta. The decrease in fetal NEFA may be due to several reasons; the fetal liver may have an altered lipid metabolism, transport of fatty acids across the placenta may be affected, or the altered maternal lipid metabolism may affect the lipid metabolism of the fetus. In the offspring the liver showed no change in any of the lipid profile however, changes found in the serum profile were that were reflected in both the mothers and the fetus.

Late gestation is an important period for the fetus to store fats to use for growth after birth. In the iron deficient model alteration in the fetal serum profile would suggest that different types of fatty acids may be stored compared to the controls. This implies that in later life when the offspring need access these fats they have the access to inappropriate fatty acids. An example of this would be the increase levels of linoleic acid seen in both the fetus and offspring. The fatty acids are not stored in the liver, but in adipose tissue, therefore you would not expect to see any changes in the offspring liver. This alteration in stored fatty acids may also contribute to the dyslipidema and obesity in the offspring of iron deficient mothers and forwards a hypothesis to be tested in the other rodent models of fetal programming which display the dyslipidema and obesity phenotype.

Objectively verifiable indicators

Meeting Abstracts

Richmond et al (2008) ‘Gene expression changes observed in the placenta from different maternal diets have identified possible Gatekeeper gene candidates’ NuGO annual meeting and Physiological Society Summer meeting

Grants

The comparitive data between the iron deficient and low protein models generated in this workpackage was part of pilot data presented in a BBSRC grant application. This joint application between two EARNEST partners, University of Nottingham and the Rowett Institute of Nutrition and Health has been awarded and this will allow us to continue the work begun with the support of the European Union. The NuGO network also awarded a Focus Team grant, "Identification of gatekeeper genes in development" to further study the array data