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 3.5.3:

Proteomics

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

Much of this work package has utilised 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. Whilst much of this partnership has been designed around genomic studies many of the new statistical and bioinformatics expertise can be applied to proteomic studies. Proteomic analysis of placentas from rodent models of maternal micronutrient deficiency will be complementary to other studies in this workpackage. In addition to giving us information on protein expression levels, proteomic analysis can also detect whether the post-translational modification of the proteins is effected by nutritional stress.

As in activities 3.5.1 and 3.5.2 are target tissue for analysis is the placenta. The placenta regulates all transfer between mother and fetus. To do this, it expresses a very wide variety of transporters, regulators, channels and receptors. Any and all of these can be differentially affected by nutritional disorders. Additionally, the placenta expresses many hormones and cytokines, and these too can be altered by changes in the nutritional environment. In this workpackage we will continue the studies of the rodent micronutrient deficiency models, maternal copper deficiency and maternal iron deficiency.

RESULTS

Two dimensional gel analysis (Deliverables 50, 87 and 88)

Maternal Copper Deficiency

The samples from the copper deficient model were used to establish the protocols. Membrane and soluble protein fraction extracts were prepared from one placenta in each litter. Two-dimensional gel electrophoresis was carried about by initial separation on pH 3-10 gradient strips, followed by isoelectric focussing. Spots were visualised by staining with a Coomassie blue solution. Spot patterns were analyzed using PDQuest software and spots with densities that significantly differed between treatments ($p<0.05$, Student's t-test) were excised from the SDS-PAGE gels using the BioRad spot cutter. In our hands, the detection limit is about 1 μg protein per spot. Proteins were sequenced by LC/MS/MS (Liquid chromatography tandem mass spectrometry) and identified using the MASCOT database.

PD quest analysis comparing the membrane fraction from control and copper deficient placenta identified 573 spots. Spot intensity between individuals within the same treatment group was quite variable but statistically analysis identified 72 spots differentially expressed as a consequence of treatment. 322 proteins represented the proteome for the soluble fraction of the placenta. As was observed for the membrane fraction, there was variation in spot intensity between individuals of the same treatment groups. 11 proteins were identified as differentially expressed. Of these differentially expressed spots 72 were identifiable proteins. 47 proteins were identified, 12 of which were identified by multiple spots. Four of these proteins showed both up- and down-regulation, presumably as a consequence of altered post-translational modification. 12 proteins showed higher levels of expression and 31 proteins had lower levels during the Cu-deficient conditions compared to the controls. The proteins have been categorized according to function as reported in the current literature. Most of the proteins included chaperones or chaperone-like proteins, proteins involved in calcium handling, proteases and enzymes, extracellular matrix proteins, signal transduction proteins, acute phase proteins and proteins involved in iron metabolism. Interestingly, none of the proteins identified were Cu-proteins.

Principal component analysis of the spot density values revealed that 24% of all variance in the dataset was accounted for by the first principal component (PC1), and an additional 15% was accounted for by the second principal component (PC2) (Fig. 1). There

was a clear separation of the spots into two groups corresponding to the two treatments. Since the protein spot components were calculated without reference to the treatment group from which the sample was derived, the fact that the groups are well separated indicates that the treatment is a major source of variation in the protein expression patterns observed. There were no obvious reasons for the one outlier observed in the control group, and it remained included in all further analysis.

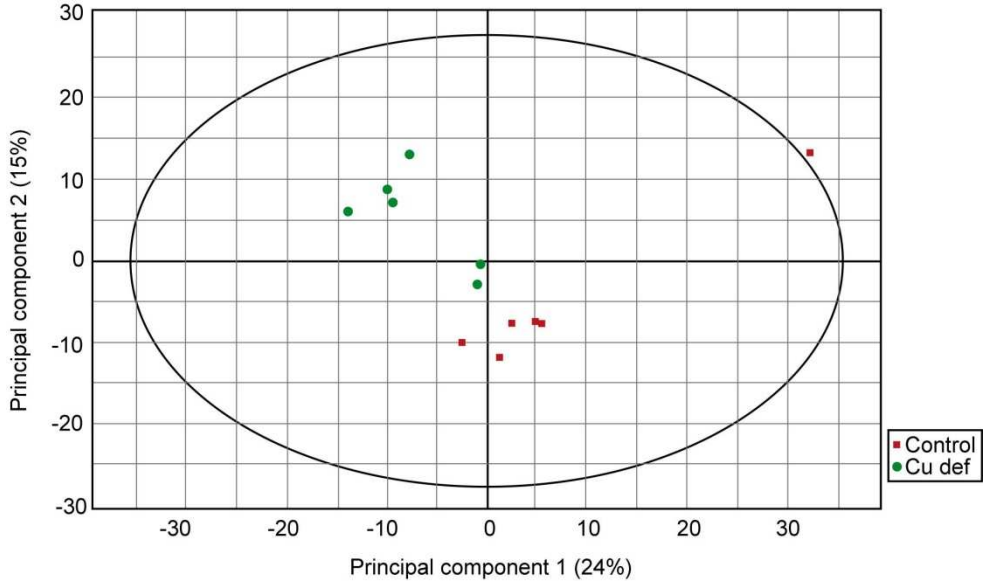

Figure 1. Score plot of principal component analysis of spot density of the total number of spots detected in the placenta from control and copper deficient rats.

Protein clustering was analyzed by correlation analysis for the different dietary treatments. A network plot of all pair-wise interactions of proteins with a correlation coefficient > 0.6 (corresponding approximately to 5% significance), prepared using the software tool Cytoscape is shown in Figure 2. Such analysis shows which proteins vary in a similar way throughout the different treatments. Two clusters of proteins were identified in the data set. One cluster (A) (Fig. 2), comprised calreticulin, cytokeratin 8, delta-aminolevulinic acid dehydratase, contrapsin-like protease inhibitor 1 and 3, alpha-1 antitrypsin precursor, alpha 1B-glycoprotein and annexin A2. These proteins were all down regulated in the placenta of rats fed copper deficient diet compared to the placenta from control fed animals.

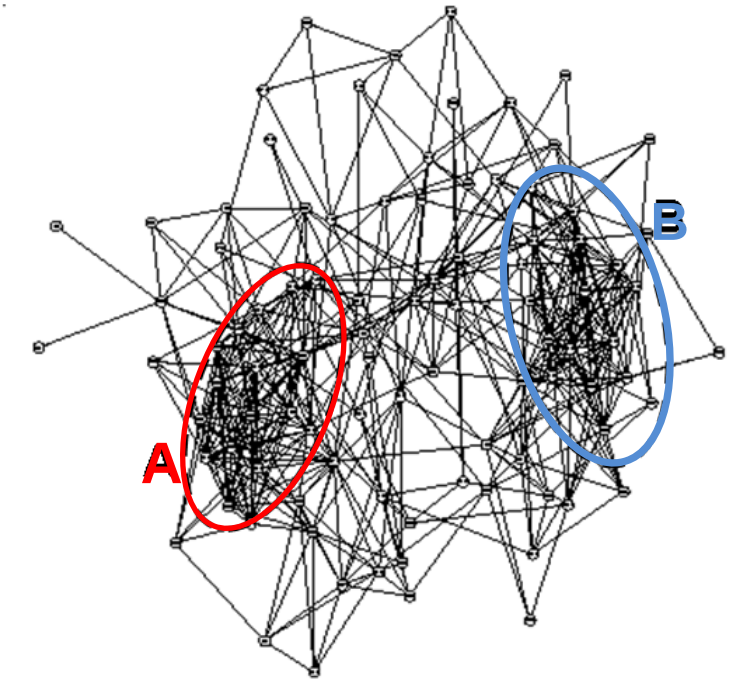

Figure 2. A network plot of all pair-wise interactions of proteins. Two clusters of proteins were identified in the data set: (A) comprised mainly of secreted proteins and (B) included acute phase response proteins, and proteins involved in iron metabolism, cell proliferation and differentiation.

Another cluster of proteins identified (cluster B) (Fig. 2) included acute phase alpha-1 protein, transferrin and serotransferrin, liver regeneration related protein LRRG03, aconitase hydratase, 3-alpha-hydroxysteroid dehydrogenase, tropomyosin 1, myosin regulatory light chain 2-A, 26 protease regulatory subunit S10B, ribosomal protein RS,40K, voltage-dependent anion-selective channel protein 1, beta actin, keratin type II, proliferating cell nuclear antigen and guanine nucleotide-binding protein beta-2 chain. Of these, 7 proteins were over expressed and the rest were down regulated in the placenta of copper deficient animals as compared to the controls.

Maternal Iron Deficiency

Following the failure of the SELDI technique, see below, all proteomic analysis of the placentas from the maternal iron deficiency model were carried out by 2-D gel analysis. The protocols and analysis were repeated for placental samples from the iron deficiency model, with the exception that the protein extracts were not separated into membrane and soluble fraction, but treated as a total protein extract.

PD quest analysis comparing control and iron deficient placenta identified 660 spots. Following statistical analysis 28 of these spots showed differential expression. All 28 differentially expressed spots were identified as proteins, with 2 proteins being identified from multiple spots. 11 proteins showed higher levels of expression and 15 proteins had lower levels in placentas from fetus subjected to iron deficiency. Most of the proteins were identified as enzymes, structural proteins and proteins involved in signal transduction and calcium signalling.

Pathway analysis was under taken on the 26 identified differentially regulated proteins, using the pathway analysis program ‘Metacore’ (Fig 3). Enrichment analysis consists of matching IDs of differentially expressed proteins to gene IDs in functional ontologies in MetaCore. The probability of a random intersection between a set of IDs the size of target list with ontology entities is estimated as the p value of hypergeometric

intersection. The lower p value the higher relevance of the entity to the dataset, which shows in higher rating for the entity. The GeneGo Pathway Maps represent a set of about 650 signalling and metabolic maps covering human biology (signalling and metabolism) in a comprehensive way. The height of the histogram corresponds to the relative expression value for a particular gene/protein.

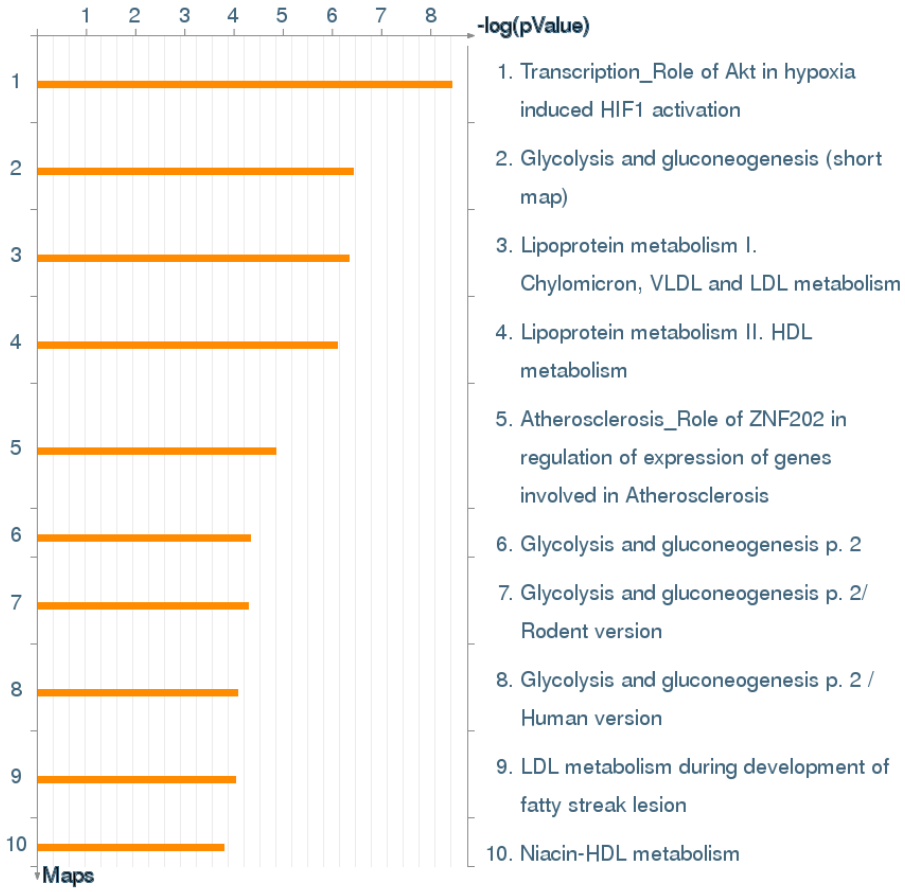

Figure 3 The ten GeneGo Pathway Maps most significantly affected by maternal iron deficiency.

SELDI analysis (Deliverables 51)

The objective of this deliverable was to examine whether using SELDI was possible as a way of identifying differential protein expression in placentas of fetus of iron deficient rats. SELDI is the acronym for Surface Enhanced Laser Desorption Interferometry. We have used this technology in a different format to examine the difference in serum between normal and cancer patients, and control and copper deficient subjects with a significant degree of success. We were therefore confidant that in this deliverable, we would be able to optimise the system for placenta.

The SELDI is a fairly simple instrument in concept. Proteins are bound to a plate using a variety of chemistries almost exactly analogous to those used for column chromatography. The proteins are then eluted by a laser hitting the plate and are separated using a Time of Flight mass spectrometer and are identified by their size and charge. The molecular weight is actually calculated as an m/z ratio. Depending on the strength of the laser pulse, proteins of different m/z ratios are released. The size of peaks is proportional to the amount of any particular protein. One can then use a variety of normalisation and analytical approaches to identify changes induced by an experimental approach.

Our problems have arisen, we believe, with regard to our particular instrument. In initial experiments, we had problems with the controlling computer and plate drives. These were completely replaced and the machine recalibrated. However, we then ran into problems with the laser. During testing to try and get this working properly, the laser finally burnt out completely. This is apparently “normal” since the lasers have only a limited life, but our usage was much less than average. Additionally, it appeared that the lasers were not firing uniformly, so that peak size of the spectra that we obtained was not very reliable. Currently, the instrument is still not working properly, and we have decided our samples are too valuable to risk until and unless we can get it operating reliably. This may not happen. The costs are very high (of the order of more than £35,000) and the guarantee of success not certain. We are currently negotiating with BioRad to try and resolve the problem.

CONCLUSIONS

The results generated by the proteomic analysis of the placentas from copper and iron deficient pregnancies will form the basis for future study. By using the whole proteome approach, as opposed to studying the expression of target proteins individually, we have been able to identify proteins and pathways that would never have been on our initial target list. For example, the pathway analysis carried out on the iron deficient placenta indicates that five of the most significantly affected pathways involve lipid metabolism; indicating that the placental lipid metabolism/transport could be significantly affected by maternal iron deficiency. This in turn could have significant effects on the growth and development of the fetus. Therefore this deliverable has provided us with a testable hypothesis for determining the link between maternal diet and offspring health.

Interestingly, when you compare the proteins which show differential expression in the copper deficient and iron deficient models, four proteins are common to both lists. The regulation of these proteins is also consistent between the two models. These proteins are alpha-enolase, alpha-fetoprotein, transferrin and the tubulin beta chain. Studies are now planned to verify the differential expression of these four proteins.

Undertaking proteomic analysis via 2-D gels is both labour intensive and time consuming. It also has limited sensitivity. Therefore one deliverable in this workpackage was to investigate the use of other technologies available for proteomic analysis. We had used the SELDI technology previously with a significant degree of success; therefore we believed it would be ideal for our placental studies. However it proved not to be very successful despite having put a significant amount of time, effort and money into this aspect of the project. We would probably have persevered further, except that the separation of protein samples on the 2D gel electrophoresis was proving more successful and we were getting good and reliable results using this technology. However, it does not seem to have any value in pursuing this approach, since the data are so unreliable and we have now concluded that we will not pursue this avenue of enquiry any further.