Nutrition, Epigenetics, Genetics: Impact on Health and Disease.
A summary of the lectures and discussion
by W Philip T James.

This analysis presents a summary of the main lectures given at the invitee only meeting in Stockholm in June 2015 and this is followed by a collation of the contributions during the discussion with appropriate references included where these are deemed helpful. The sequence of issues in the discussion has been rearranged to ensure a more coherent format than the wide-ranging spontaneous interactions which took place over period of up to 8 hours. I have taken into account each lecturer's presentation and very briefly summarised these as well as the discussants' contributions some of which also involved presenting some slides of their particular field of interest. Sometimes there were references to unpublished work and where these contributions have now been published since the meeting the corresponding references are given where possible. The main contributors to the meeting have now published their own overview – see[i] and this website.

Andrew Feinberg: Epigenetics at the cross roads of genetics and epigenetics leading to disease.

I am going to consider epigenetics as the crossroads between the impact of genetics and the environment on the development of disease and where we have to consider the genomic wide changes in epigenetics which Stephan Beck in the UK, staff at the Karolinska in Stockholm and our collaborators in Iceland, led by Vili Gudnason, have also been assessing.

We developed an array based assay scheme called CHARM as a way of looking at about a quarter of the potential sites of DNA methylation across the genome using an agnostic approach i.e. making no assumptions about which sites might be affected. This involves the crucial help of bio-mathematicians who can now even test for the alignment of the methylation. These mathematicians also need to work with us before we undertake the studies so that we can optimise the approach to assays that in practice are cheaper as well as better. We have also benefitted from mathematicians based in astronomy as they too have to look at a huge array of data and check for alignment and other relationships.

When looking at phenotypic differences we are used to assessing, for example, species differences but we are less well versed in explaining the differences between individual humans. Furthermore we need to understand what determines the distinctions between different organs. So the difference between a human stomach and a human eye is far greater than the differences between the stomachs of a gorilla and a human. This idea about the genetic programming of epigenetic differences between tissues and how the environment influenced these epigenetic patterns is actually what Conrad Waddington, the great embryologist at Cambridge University, defined as epigenetics. The process of tissue differentiation and function he saw as completely deterministic whereas now we see epigenetics in a more functional mode and where we now know that these changes can involve not

only methylation differences but changes in histone and chromatin structure - these last two processes are now under greater scrutiny and analysis.
If we consider the methylation of genes we know that this process leads to gene silencing but there are also crucial processes that involve demethylation. Most of the differences in DNA methylation are not at the densest regions where the cytosine, guanine (CG) dinucleotides occur (in what are called "islands") and where these very dense regions of many CGs cluster together but are nearby in an area referred to as "CpG island shores". They are regions of intermediate density and we now know they are also enriched in tissue specific enhancers. The methyl groups come from adenosyl – methionine derived from the essential amino acid methionine.

There is a long standing puzzle in human genetics relating to the so-called missing heritability of common diseases with the common variants explaining only 1% of the propensity to, for example, schizophrenia and no more than 20% for disorders like rheumatoid arthritis with 80% or more unexplained. So this issue has been a subject of a lot of literature including conferences that were published in Nature, and news stories. Then the Vogelsteins considered the predictive capacity of personal genome sequencing and their mathematical analyses from identical twins showed that even with complete genome sequences you cannot explain more than 20% of their disease risk. Yet we know that environmental factors such as smoking and too much sun exposure have often profound effects and many analyses show that dietary factors do too. So these effects combined with the recognised genetic factors may interplay through the effects of epigenomic changes.

Epigenetics and cancer Cancer is clearly an environmentally driven disease which may well arise from cycles of injury and repair and the real problem with this induced plasticity is that it can form the basis for the early stages of the transition to malignancy. Hepatocellular cancer is the leading cancer killer in the world with cirrhosis occurring following cycles of regeneration and cell death. Then the recurrent regeneration eventually leads to an increased risk of malignancy. Similar cycles of injury and repair seem to underlie skin cancer and cancer after inflammatory bowel disease. We also discovered that most types of tumour develop epigenetic changes before the tumour actually arises and these involve enormous regions of hypomethylated blocks of DNA. The most variable genes, showing variable gene expression in cancer, lie within those large blocks together with Large Organised Chromatin K-modifications (LOCKs) which are also associated with domain wide gene silencing. These also relate to lamins which link these changes to the nucleus. So we find that the overwhelming majority of mutations in cancer affect the epigenetic machinery. Localized hypomethylation of CpG island shores and hypermethylation of CpG islands are common in cancer with large scale decondensation of heterochromatin: there is a loss of LOCKs with hypomethylated DNA blocks up to a megabase in size. There is then an associated series of changes in the nucleosomes which become disorganized in cancer.

Then we found that this chromatin modification can occur in response to environmental exposures of different kinds and multiple exposures eventually

lead to permanent changes in the chromatin structure and this leads to hypervariable gene expression within that region. This involves in many tissues a sequence of repair and this is a widespread phenomenon whether it is in the skin or the liver. The disorganization of the chromatin leads to an array of changes with many of the inhibitory pathways no longer operating. In major European and US projects assessing the mutation changes in cancer we found that 90% of these changes are to the epigenome and involve both the DNA and the chromatin machinery. In fact with the huge array of methylation and demethylation changes we can think mathematically in terms of the entropy of the system and the extent of deviation from the normal. We can often see changes which are transient in the chromatin response to environmental factors but the DNA changes of hypomethylation often reflect permanent changes important for the development of cancer and fundamentally involving the epigenome[ii],[iii].

Other metabolic diseases associated with epigenome changes
Rheumatoid arthritis. Here we find changes in both the epitopes of HLA genes and in the MHC complexes i.e. the regulatory RNA that seems to regulate the expression of those genes and that itself is controlled by widespread sequence variants. We think that these epigenetic changes may explain 20% of the variance in the propensity to disease with genetic factors contributing another 20%[iv]. However, when one has the combination of differential methylation variance in both the gene and in the epigenome then this explains up to 80% of the likelihood of getting rheumatoid arthritis. Smoking also seems to exacerbate the problem.

Diabetes Again with collaborators here in Sweden we have shown that the development of diabetes in Black 6 mice on a high fat diet induces methylation changes in a particular region of the genome and these changes correspond exactly to what we then find in patients with diabetes. Only by analyzing both the GWAS and EWAS data sets and using the Bonferroni corrections for multiple analyses were we able to find the same features in both species associated with abnormalities in glucose metabolism. We then went on to show these effects are reversible when patients with diabetes have bariatric surgery with remission of their diabetes[v]. These differential methylation regions overlap with 27 genetic T2D risk loci, only one of which was deemed significant by GWAS alone. Functional analysis of genes associated with these regions revealed four genes with roles in insulin resistance.

Autism. We have now found epigenetic differences in the sperm of fathers of children with autism We examined genome-wide DNA methylation in the sperm of a selected group of fathers who already had one child with autism. We then assessed the next child at 1 year of age for their propensity to autism using a standardised Autism Observation Scale for Infants (AOSI). We analysed methylation data from 44 sperm samples run on the CHARM 3.0 array and also examined associated regions in an independent sample of post-mortem human brain from patients who had had autism. Using region-based statistical approaches, we identified 193 differentially methylated regions (DMRs) in paternal sperm associated with an increased propensity on

the AOSI score to autism in the children. The DMRs clustered near genes involved in developmental processes, including many genes in the SNORD family, within the Prader-Willi syndrome gene cluster. 24% of the array probes also showed consistent differences in the cerebella of autistic individuals compared with controls. So this suggests that epigenetic changes in paternal sperm may contribute to the risk of autism and that there are also epigenetic changes in the cerebellum of patients with autism[vi].

Joseph Nadeau: Epigenetics transgenerational dimensions

We currently work a lot on trans-generational inheritance involving behaviour, cancer[vii], and metabolic diseases. However, I am going to focus now more on the mechanism of epigenetic inheritance to which we have referred many times over the years[viii]. Mendel set out the rules for the inheritance of simple traits. The first rule is independent segregation whereby individuals have at each gene locus two alleles, one from the mother and one from the father. We know of some famous exceptions involving segregation distortion where the proportion of different alleles is skewed during gametogenesis resulting in preferential transmission of one genotype[ix]. This is observed in drosophila, mice and several other species.

Nutrients affecting the choice of genotypes at fertilisation. What we stumbled on recently was a different mechanism relating to the selection of particular genotypes not during gametogenesis but at fertilisation and which may well be affected by diet[x]. In looking at the problem of neural tube defects we were, of course, aware of its relationship to folate deficiency and that the condition could be exacerbated by other conditions e.g. the use of anticonvulsant drugs, by diabetes and obesity and that a lower socioeconomic status was a risk factor. But the specifics of the developmental features of this problem were unclear whilst recognising that different studies had shown that 50%-70% of the defects could be avoided by appropriate intakes of folic acid. So we were initially concerned to find out why some cases are not responsive to folic acid.

There are several hundred neural tube single gene mutations that lead to neural tube defects in mice but only a few mutations have been studied for their dietary responsiveness. Thus the Axd strain has been shown to be responsive to methionine but not folic acid whereas the Sp (Pax3) strain responds to either thymidine or folic acid and the ct strain of mice responds only to inositol but not to folic acid or methionine.

Then if we take mice which had had their Apo lipoprotein B gene knocked out. These mice have a recessive mutant and one therefore needs a mouse that is homozygous for the knock out before one sees the clinical picture which includes a much greater propensity to neural tube defects. So when we put them through a series of crosses with heterozygous parents then we can predict the proportion of the offspring with different genotypes. We then undertook this reproductive experiment to identify the genotype proportions with two different levels of folic acid intake i.e. 2ppm or 10 ppm provided in the diet throughout both their adult life and pregnancy. Although both levels of

folic acid intake are within the acceptable physiological range animals maintained on the higher folic acid intake showed a remarkable difference in the proportion of genotypes in their offspring from that predicted from Mendelian genetics. There was a far higher proportion of homozygous offspring than expected with fewer heterozygotes so instead of a 1:2 ratio of homozygotes to heterozygotes the ratio was close to 1:1. Now if one were losing particular genotypes as a result of the extra folic acid in the diet then one would expect the litters to be appreciably smaller but they are not. The number of ova produced determines the litter size so the same number of ova is clearly formed and they await fertilisation. So this in turn means that the choice of genotype of spermatozoa actually fertilising the ova is being affected by the prevailing level of folate in the tissue fluids. We went on to test 21 different genotypes where the mutations induced neural tube defects and found that in 5 of the 21 mutant strains the level of dietary folic acid in some way affected the proportions of homozygotes and heterozygotes in the litter.

The mechanisms by which dietary folic acid induces these changes in genotype selection for fertilisation is unclear but may well relate to polyamine metabolism to which folate metabolism is closely interconnected through the process by which S-adenosyl methionine, the major donor of methyl groups in the one carbon pool, helps to generate spermidine from putresine - see Fig 1 . Now the polyamines, 90% of which are attached to nucleic acid, are involved in a host of cellular processes including the acetylation and deacetylation of histones within the chromatin structure. A polyamine deficiency leads to infertility. So in some way the change in folate intermediate availability and processing and its close relationship to polyamine metabolism seem to be influencing the propensity of genetically different gametes to combine.

Although we do not yet understand the precise mechanism, this issue of the nutritional alteration of the selective combination of specific genotypes of sperm and ova is not just confined to the propensity to neural tube defects in our range of mutant mice. We have now found the same phenomenon in the propensity of mice or rats to develop testicular cancer. This is also dependent on the mutations in the Deadend1 gene[xi] with fertilisation experiments again showing that there is a propensity for particular genotypes of sperm and ova to combine and conceive a mouse which then goes on to develop the testicular cancer. So again we see non-Mendelian genotypes without loss of litter sizes indicating that again there must have been biased fertilisation.

Philip Collas: Dynamic associations of chromatin with the nuclear envelope during adipocyte differentiation.

I am presenting mainly unpublished work on the nuclear lamina i.e. the nuclear envelope. We know that during the differentiation of adipocytes the chromatin relates closely to the nuclear double membrane which separates the nuclear DNA from the rest of the cell. This nuclear membrane is crossed by 4 complexes which allow the import and export of molecules across the membrane. Between the inner nuclear membrane and the nuclear chromatin, lies a meshwork of intermediate filaments called nuclear lamins of two types, A and B. The type B lamin is solely located, we think, at the nuclear

periphery. The type A lamins are developmentally regulated so they are not expressed in gametes or in early embryos and are found in both the periphery of the nucleus and in the interior part of the nucleus. These lamins protect the nucleus in a mechanical sense and are crucial to the relaying and targeting of nuclear signalling molecules from the cell cytoplasm to the chromatin. Lamins interact with and are involved in chromatin organisation. We need to think about lamin associated domains (LADS) that were traditionally considered as only being in the periphery. But we now know that they are found throughout the nucleus and are composed of large chromatin chunks which in size are from 100kb to 10 megabases or more. This large mass involving the chromatin implies that the genome interacts with lamins but the LADs themselves tend to be gene poor especially those at the nuclear periphery. Where the genes are linked to the chromatin in LADs they are silenced. Several techniques have been used to assess the genome involvement with the LADS and the chromatin but they all suggest that there is appreciable interaction between the lamins and the genes[xii].

We and others have concluded that LADs are generally conserved between cell types but some cell types clearly show different lamin structures and this may be part of the differentially development of regulatory processes. For example we have studied adipose stem cells and found that lamin A is associated with nearly 5,000 promoters. Depending on where the lamin A interacts with the promoter, this lamin A may then either repress or promote the transcription of the promoter's gene. Lamin A is also heavily involved in the features of the developing adipocyte by interacting with distinct spatially restricted sub-promoter regions, both within and outside peripheral and intra-nuclear lamin-rich domains. These localized interactions are associated with distinct transcriptional outcomes in a manner dependent on local chromatin modifications[xiii] with the whole process being regulated during the phases of cell development.

It has now been shown that mutations in about 400 different places on the lamin A can lead to 15 different diseases including myopathies, partial lipodystrophies with insulin resistance and glucose intolerance, neuropathies and progeroid i.e. premature ageing syndromes. The lipodystrophies are caused by a single point mutation in the lamin A gene and the problem is associated with elevated glucose levels in the blood. This elevated glucose level in the human adipocytes then affects undifferentiated human adipose tissue stromal cells (ASCs) with a glucose dependent induction of histone methylation and an upregulation of the promoters of a subset of inflammation response (IR) genes and an alteration in the adipocytes promoter histone methylation patterns with transcriptional de-repression. Modelling of chromatin states from combinations of histone modifications in nearly 500 IR genes unveil three overarching chromatin configurations reflecting repressive, active, and potentially active states in promoter and enhancer elements with a predominant upregulation of the genes involved in the inflammatory response[xiv]. The glucose dependent processes modifying histone – lamin interactions involve the generation of β-*N*-acetylglucosamine (O-GlcNAc) which is highly controlled by O-GlcNAc transferase (OGT), which adds the sugar, and β-*N*-acetylglucosaminidase (O-GlcNAcase), which hydrolyzes it[xv].

Dynamic posttranslational modification of serine and threonine residues of nucleocytoplasmic proteins by β-*N*-acetylglucosamine (O-GlcNAc) regulates cellular processes such as transcription, signalling, and protein–protein interactions. It is the *O*-GlcNAcylation of histone 2B (H2B) on the Serine 112 which is particularly involved in epigenetic processing of metabolism and differentiation[xvi] and several mutations leading to lipodystrophies.

Paulo Sassoni – Corsi: The importance of cellular clocks in the epigenetic control of metabolism.

The circadian control of metabolism is important for our understanding of cyclic rhythms during the day and night. But these cyclic systems also allow adaptation to environmental influences and nutrition is especially important. The central clock in mammals is in the brain and it responds to light and darkness but has an intrinsic 24 hr cyclical rhythm which is located in the supra-chiasmic nucleus of the hypothalamus. But in addition about 15 years ago we realised that there was a clock in every cell of every tissue of the body and that the central cock connects in an ill defined way to all these other cellular clocks. This primordial system also allows the peripheral clocks to feed back signals to the central clock. Now there can be disturbances of the central clock and this can occur not only in depression and other mental disorders but is also routinely affected by the light /dark cycle and responds, for example to the much longer nights in the winter in the more Northern countries of the globe. The other major change in the physiology of clock related systems is in response to food and this differs depending on the time of day that the food is ingested[xvii]. The clocks in cells control the cyclic activation of a large proportion of the genome through connections to their promoter regions as well as multiple feed back repressor signalling systems which allow the whole process to be tightly controlled and fine tuned. Studies with targeted mutations of the clock gene induce many metabolic disorders and through involvement of the NAD-dependent deacetylase SIRT1, affect the control of cellular metabolism, inflammation and aging[xviii]. A similar deacetylase, SIRT6, which is chromatin bound, also contributes to circadian control, through its effects on a different group of cyclic genes other than SIRT1 and with particular interactions with fatty acid metabolism. So SIRT1 and SIRT6 partition the circadian epigenome, leading to a segregated control of cellular metabolism. A mitochondrial NAD+-dependent deacetylase SIRT3 is also implicated in the circadian control of mitochondrial function.

Andrew Prentice. Peri-conceptual nutritional factors influencing methylation

I would like to present a short account of my team's work in a remarkable place called MRC Keneba which is in the middle of the African bush in Gambia, West Africa, where the Medical Research Council(MRC) has been working since 1948. The Gambia lies just below the Sahara and has an incredible annual seasonality with the rains coming for only 3.5 months starting in late June at a time when the land looks just like a desert. Then the rain comes and the countryside is transformed and soon appears lush and prosperous. But in that early rainy season food insecurity is at its worst as the

crops are growing, the work demands are high but last year's food is fast running out. So one needs enough food grown in that short season to last the whole year with no further rain and a progressively more arid countryside as the annual cycle progresses.

Over several decades the cyclical pattern of eating has been recognised with different foods becoming available at different times but with annual periods of semi-starvation and then of plenty at harvest time. There is, perhaps unsurprisingly, also a seasonal pattern of disease which we have been studying in the village where we live. We conduct detailed studies in our village and in three neighbouring villages and also study 36 other villages in less detail.

We were fortunate that all births were recorded by Sir Ian MacGregor, Director of his MRC unit from 1948 onwards, and this allowed us to study the death rates of children born at different times of the year. We discovered[xix] that in these earlier times in the Gambia almost half of the under 5 year olds died but later, as things improved, it became clear that there was a persisting remarkable difference in the death rates of both boys and girls over the age of 15 years depending on which season in which they were born. If you are born in the dry season, you have a post 10-year-old mortality rate up to the age of 60 yrs. of just 3%, in spite of living in this rural area of the Gambia. But if you are born in the rainy season, then your mortality rate is 20% - so there is a 6-fold difference in mortality rate.

We then collaborated with Rob Waterland who had already shown in animals that you can modulate the phenotype by tweaking the mother's diet before she is mated[xx]. We found that the maternal diet did indeed affect the methylation potential in the women at different times of the year[xxi]. We then went on to show that these changes in the availability of factors relating to one carbon pool metabolism alter the DNA methylation of human epigenetically modifiable loci called metastable epialleles[xxii] i.e. they are modified soon after conception and then are maintained during differentiation and growth. We can see this in the lymphocytes and hair DNA taken from infants at birth and the pattern clearly relates to the maternal methyl metabolome[xxiii]. We have recently gone on to show[xxiv] that the genomically imprinted VTRNA2-1 is an environmentally responsive epiallele and with stochastic inter-individual variation in DNA methylation at the VTRNA2-1 and that the periconceptional environment affects the offspring VTRNA2-1 epigenotype. We showed the stability of these changes by analysing the DNA from the same children at a mean age 7 and at 17yrs. and observed that the changes remained hypermethylated across this 10-year span. We also found in this unbiased screening process more than 100 additional candidate metastable epialleles associated with cis genomic features including transposable elements. Children conceived in the dry season show a much greater variance in their methylation than those conceived in the rainy season. These changes seem to be associated with the highest levels of homocysteine in the mothers at that time and this marked variation in the methylation of the VTRNA2-1 epigenotype seems to relate to the time when the riboflavin levels in the blood of these mothers are the lowest. Given the marked variations in folate intake

by season we also assessed the relationship between folate levels and the variation in the methylation of the VTRNA2-1 epigenotype and found none whatsoever.

VTRNA2-1 expresses the non-coding RNA 886, which is 101 nucleotides long and is central in regulating RNA associated protein kinase and cell-cycling and appears to have a reciprocal effect on cancer susceptibility. Treppendahl et al[xxv] have already shown that the survival of acute myeloid leukaemia (AML) patients depends on the degree of methylation at the VTRNA2-1 with the group of patients with the unmethylated VTRNA2-1 having much longer survival times. There is also evidence that the degree of methylation will also affect individuals' susceptibility to viral infections. So given the evidence that the season of birth in Gambia relates to the propensity to die in early adult life from what seem to be a variety of infections there is now a need to assess the susceptibility to viral infections and responses to viral challenges in the Gambian population.

Anna Krook: Epigenetic regulation in skeletal muscle: impact of exercise, diabetes and obesity.

In our studies of muscle metabolism and insulin action we are now coming to the point of recognising that we also need to consider epigenetics. We know that there seem to be some epigenetic marks, perhaps left by our grandparents on the propensity to develop metabolic disease[xxvi]. We also know, for example, that different environmental effects such as exercise or early maternal dietary patterns e.g. high fat intakes can induce some kind of epigenetic change in the offspring, and that exercise can affect these changes[xxvii] and their longer term significance[xxviii]. We also know that epigenetic changes are being induced in early and later adult life as has been shown in identical twins[xxix]. So clearly postnatal events can markedly affect the epigenetic profile. Romain Barrés in our lab then showed that 44 genes in the DNA of skeletal muscle in the mitochondrial pathway of patients with diabetes are affected and that PGC-1α methylation in the DNA promoter is changed in people with type 2 diabetes compared with adults with normal glucose tolerance. He found cytosine hypermethylation of peroxisome proliferator-activated receptor gamma (PPAR gamma) coactivator-1 alpha (PGC-1alpha) in subjects with diabetes[xxx]. The highest proportion of cytosine methylation within PGC-1alpha was found within the non-CpG nucleotides. This did indeed seem to have some functional consequences because the mitochondrial numbers are reduced in skeletal muscle in these patients with type 2 diabetes. We then found that exposing human skeletal muscle cells in vitro to palmitate led to an increase in the methylation of the same promoter methylated PGC-1α.

Now we know that a bout of exercise increases the insulin sensitivity of muscle so we then assessed the effects of a bout of exercise on promoter methylation and observed that there was an acute demethylation of PGC-1α but 3 hours later it had returned to normal. So it is an acute wave of hypomethylation[xxxi] that we can observe in the whole genome in skeletal muscle biopsies obtained from healthy sedentary men and women after acute

exercise. Exercise induced a specific dose-dependent expression of PGC-1α, PDK4, and PPAR-δ, together with a marked hypomethylation on each respective promoter.

So It would seem that basically the exercise-induced promoter hypomethylation is not caused by circulating factors because it can be reproduced in a test-tube and seems to be intrinsic to the skeletal muscle tissue: there is no remodelling or a recruitment of other cells, or a loss of other cells. So if we can then change methylation dynamically in a matter of minutes or hours this then raises the question of whether we can actually change DNA methylation in people with insulin resistance?
We then looked at the well-known change in insulin resistance in obese patients after bariatric surgery[xxxii]. We already knew that obesity was associated with the altered expression of a subset of genes enriched in metabolic process and mitochondrial function and found that the promoters' low methylation of both PGC-1α and PDK4 was restored to non-obese levels after RYGB-induced weight loss. Indeed 11 of the 14 identified genes were normalized to levels observed in normal-weight, healthy controls. Then a genome-wide DNA methylation analysis of skeletal muscle revealed that obesity is associated with hypermethylation at CpG shores and exonic regions close to the start sites for transcription. So it was becoming clear that obesity and RYGB-induced weight loss have a dynamic effect on the epigenome There is also a concomitant effect on insulin resistance with greater PGC-1α methylation being a marker of insulin resistance. We then found that we could see similar changes in the PGC-1 methylation of circulating white blood cells.

PDK4 is a gatekeeper in the metabolism of lipids and glucose but the methylation state of PDK4 shows the opposite response to exercise from that of PGC-1 and so the dynamic regulation of methylation is both gene-specific and promoter-specific.

We then went on to study the methylome and methylation changes in the liver in patients who were obese or had diabetes and again compared them with controls[xxxiii]. We observed hypomethylation of CpG-sites within activating transcription factor (ATF)-4 motifs in four genes involved in hepatic glycolysis, lipogenesis and insulin resistance in the liver of severely obese non-diabetic and type 2 diabetic patients. These were associated with increased mRNA expression and protein levels of lipogenic enzymes suggesting that site specific DNA hypomethylation promotes pathological activation of glycolysis and subsequently de novo lipogenesis via increased stearate synthesis, which may be facilitated by ATF transcription factors activation and increased gene transcription. We are therefore moving to a position where we see the effects of epigenetic changes in explaining the alterations in insulin resistance in the liver.

MicroRNAs and epigenetic changes. MicroRNAs are considered, in some cases at least, to be part of the epigenome. Mice studies[xxxiv] have already shown that the Lin28/let-7 axis centrally regulates glucose metabolism. Let – 7 is a tumour suppressor microRNA but overexpression of let-7 or loss of another microRNA, Lin28, also results in insulin resistance and impaired

glucose tolerance. We have also found that miR-29a and 29c are over-expressed in type 2 diabetics and this expression can be increased in human muscle cells if you make them insulin resistant in vitro by exposing them to either palmitate or TNFα. So it is now clear that just having more miR-29 makes you insulin resistance and one can show this by overexpressing the miR-29 in one leg of an animal and comparing it with the other normal limb. When this is done the glucose uptake of the limb with overexpressed miR-29 is impaired with a drop in the corresponding muscle glycogen levels.

Bas Heijmans: Prospects for probing epigenetic mechanisms in human subjects.

I would like to discuss exclusively human studies as it seems to me that mice and humans may differ markedly in their responses to environmental stresses, First I will consider the Dutch famine which was a severe famine at the end of the 2nd World War affecting the Western part of the Netherlands. The daily rations were set by the local authorities at less than 700 kcals per day, on average. So it was a very severe famine affecting the whole population; 20,000 people died during this famine period.

Our design was prospective in the sense that my colleagues went through midwife clinics and hospitals, got the birth records from 1944/45/46 and traced back the individuals who were exposed to the famine and also recruited controls, namely the same sex siblings, So the sex was the same but they were born and conceived before or after the famine. They shared, of course, their genetic background and were raised in the same family environment but at different times in relation to their birth before, during or after the famine. We already knew that individuals exposed early in gestation had higher birth weights than individuals born before and after the famine in the same institutions. Replicated later-life outcomes include an unfavourable metabolic profile consisting of a higher BMI, an altered glucose response and elevated LDL and total cholesterol levels[xxxv].

Then we compared the genomic scans of a variety of groups e.g. individuals who were conceived before the famine, but exposed in late pregnancy to the famine. We focused on peri-conceptional exposure and took 24 individuals conceived at the time of the famine and compared with them 24 same-sex siblings not exposed to the famine. We combined CpG sites according to such genome annotations as those regions relating to enhancers or promoters in the hope that these CpG sites functionally relate to each other. Then if you find the DNA difference afterwards, then it is more interpretable, or more relevant. Of 27 observed genomic features we found that 5 of them, as a group, were associated with pre-natal famine exposure. We then went on in the next phase to see if we could identify individually differentially methylated regions. In those 5 associated genomic features there were 90,000 individual regions and 181 of them were associated with peri-conceptual famine exposure and relate to their expression during blastocyst formation. After multiple testing corrections we found there were both hyper- and hypo-methylation sites with no evidence of tissue-specific expression. This was reassuring as we were studying cells from whole blood.

We then investigated whether the differential methylations extended into biological pathways in which 6 identified genes were involved and found they were associated with the regulation of growth. Now we have to recognise that these individuals studied are the surviving embryos that we were then able to study and there is probably also considerable adaptation to the prevailing conditions. The changes are quite small but their significance may be marked.

We then went on from a genomic study to epigenomic scans. We collaborate with six other groups dealing with biobanks and again used whole blood as the tissue with a focus on blood lipid outcomes despite recognising that liver tissue might be a more valuable source for reference when considering blood lipids. We are, however, focussing on a Mendelian randomization approach and assessing factors affecting the levels of LDL cholesterol, triglycerides and HDL cholesterol. So we then compared the SNPs in relation to the lipid levels and found in this preliminary analysis that the aggregated SNPs seemed to explain about 5% of the variation in blood lipids. We then looked to see whether SNPs in the polygenic score were close to the relevant CpG for the control of these lipid levels and found greater effects.

The genes are those affecting carnitine palmitoyltransferase 1A, sterol regulatory element-binding transcription factor 1, 24-dehydrocholesterol reductase and ATP-binding cassette sub-family G member 1. However, there is increasing evidence that the blood lipid levels themselves can affect the epigenome[xxxvi].

Peter Gluckman: Applying epigenetics within nutritional biology: current utility and its future potential

We have to recognise that we are at the beginning of our studies of epigenetics as we have not discussed hydroxyl methylation of CpG sites nor the issue of RNA methylation, histone modifications and chromatin structural changes. With different pathways there may be very many different forms of adaptation to the evolutionary pressures. There is an incontrovertible body of experimental, clinical and epidemiological evidence linking the conditions of early life to later life outcomes including longevity, osteoporosis, allergy, mood disorders and some cancers. We also know that a child's risk of being overweight is affected by several risk factors e.g. the mother's pre-pregnancy BMI, excess pregnancy weight gain, maternal vitamin D status, smoking in late pregnancy and breastfeeding the babe for less than a month[xxxvii]. Yet the early effects on long-term health were not accepted for years because it was assumed that a single pathway was involved and this did not fit well with the empirical data. There was also a focus on low birth weight and a failure to recognize that these pathways operate under normative conditions. There was also no plausible biological mechanism, a confusion between disease risk and causation, a lack of a conceptual framework and no evidence as to its relative importance. Each of these objections has now been addressed, in part through epigenetic biology, and we now realise that early life exposures generally do not directly lead to pathophysiology but they change the sensitivity to subsequent environmental cues which can be pathogenic.

Mark Vickers and I showed many years ago[xxxviii] the results of taking animals that have been programmed by maternal undernutrition in utero and exposing them to signals of high nutrition when they are born and then studying their weight gain for the rest of life. These maternally undernourished animals, both male and female, when exposed post – natally to a high fat diet become hyperphagic, insulin and leptin resistant, obese and anxious. Our experiment then involved a group receiving leptin from day 3 to day 12 of life postnatally[xxxix]. Mark has more recently done it again with growth hormone and found that we can completely prevent the development of the thrifty phenotype so you can change the destiny of these animals at the right time by giving them different hormonal or nutritional signals. It was interesting that we completely reversed all the metabolic syndrome phenotypes of these animals by giving them leptin or growth hormone, and changed most of their hepatic and epigenetic changes in the liver. Interestingly enough, one of the few genes which is not reversed in the liver by neonatal leptin is Ter1 gene relating to terminating replication - it is one of the few genes that appears resistant to manipulation. Then Keith Goddrey with others looked at the genes in the umbilical chord of new born bases and related changes to their degree of adiposity when they were 6 and 9 years old and observed a change in the methylation of the CpG site of RXRAα[xl,xli]. I want to emphasise that we need to make sure that we are getting replicate data from both within cohorts and between cohorts to rely on this sort of data. We have then gone on to show in Singapore that a lower umbilical cord CDKN2A methylation is associated with higher child's adiposity at age 6 years and Karen Lillycrop has then shown that in adipose tissue biopsies from adults who are lean or fat, you see changed expression of this gene as well.

Early malnutrition. Jamaican babies were prone to suffer from either the marasmic or kwashiorkor forms of malnutrition – sometimes with the two types evident in the same families. The MRC Tropical Metabolism Unit staff were unable, originally, to separate out anything in the nutritional environment that distinguished the two types of malnutrition. So Terrence Forrester found the birth records of a 1000 children who developed either marasmus or kwashiorkor and much to our surprise we found that there was a 300g lower birth weight in children who later developed marasmus than those who developed kwashiorkor[xlii]. As Terrence and others have shown, after they have been rehabilitated for 6 months, children who had marasmus still have a different ability to regulate their metabolic control under starvation conditions compared to children who had kwashiorkor[xliii]. So it is the children who had had marasmus who are able to induce β oxidation better and better able to down-regulate protein turnover compared to children who had previously had kwashiorkor. So our hypothesis was that children who were born small anticipated living in a poor environment; they had therefore adapted their nutritional physiology better for a low nutritional environment as adults. We then discovered that the marasmic children when adult have a much higher incidence of stunting and obesity and they have impaired glucose tolerance as they go through the nutritional transition[xliv]. Terrence Forrester has taken muscle biopsies from nearly 300 of these survivors and seen profound epigenetic changes in their immune pathways. Survivors of

malnutrition also show cardiovascular changes as adults with smaller outflow tracts and a lower cardiac output than controls but markedly elevated peripheral resistance. Malnutrition survivors are thus likely to develop excessive rates of hypertension in later life, especially when exposed to obesity[xlv]. Forrester's group is also finding phenotypic differences in brain function between those who had previously had marasmus and kwashiorkor compared with controls. The kwashiorkor children are much closer in brain function as adults to the normal than are the marasmic children.

In all these analyses we need to replicate epigenetic findings in more than one cohort before we can begin to reach definitive conclusions as there have been discrepancies in the findings in some of the early studies. The analyses of methylation changes also are not always reliable when some of the mathematical and analytical assessment methods are used so we need to be careful. Array methodologies also have inherent abnormalities in the distribution of data around the edges. If you use least square approaches which is what most people do, you will end up with a lot of type 2 errors and say there is no effect when there really is an effect.

One of the many issues we need to think about - and it may be the most important of all - is the issue of genome / epigenome interactions, both in the cis and trans forms. I will illustrate this from our GUSTO studies.

We have been studying a cluster of children in Singapore in the GUSTO cohort and have monitored them from 10 weeks of gestation and assessed them sequentially after birth. We have 450k data from the umbilical cord DNA of 1,200 children as well as SNP data from the mothers and from about 300 of the fathers with studies on the children up to the age of 5 years. We looked in an unbiased way at variable methylated regions, regions in the DNA and which regions across the population of infants had a high variation in methylation. We found changes were largely confined to the shores. We then correlated each of those variable methylation regions with the SNPs we found on the Omniexpress array and looked to see where those SNPs are. The SNP that had the highest correlation with the variable methylation region was in the gene. Now, as you know there are three ethnicities in Singaoore and we observed distinct differences in the SNPs depending on their ethnicity but when you look at the 450k data on methylation patterns there are no ethnicity related differences. So we need to think about the interactions of the genome and the way in which the SNP changes interact and influence the effects of the differences in methylation.

To look at these interactions we have 40 measures of the maternal environment during pregnancy and we consider each of these measures in relation to the variation in SNPS and methylation variation. We recognize that inter-individual variation in methylation can be a consequence of DNA sequence polymorphisms that result in methylation quantitative trait loci (methQTLs) and, potentially, the interaction between fixed genetic variation and environmental influences. We surveyed the genotypes and DNA methylomes of 237 neonates and found 1423 punctuate regions of the methylome that were highly variable across individuals. These were termed

variably methylated regions (VMRs), against a backdrop of homogeneity. MethQTLs were readily detected in neonatal methylomes, and genotype alone best explained about 25% of the VMRs. This was true even if we only took one ethnic group e.g. the Chinese. We found that the best explanation for 75% of VMRs was the interaction of genotype with different in utero environments, including maternal smoking, maternal depression, maternal BMI, infant birth weight, gestational age, and birth order[xlvi].

Now one of the marked epigenetic effects identified by Ranjan Yajnik relates to the one carbon pool and the availability of methyl groups during development[xlvii] as shown by the ratios of S-adenosyl methionine(SAM) to S-adenosyl homocysteine (SAH). We took this concept and assessed the importance of the SAM/SAH ratios in vitro during the differentiation of murine adipocytes[xlviii]. We found that the expansion and differentiation of murine 3T3-L1 pre-adipocytes in the presence of SAH impaired both basal and induced glucose uptake as well as lipolysis compared with un-treated controls. SAH significantly reduced expression of CAAT enhancer-binding protein-a (Cebpa), Cebpb, and retinoid x receptor-a (RxRa) compared with untreated adipocytes. SAH increased RxRa methylation on a CpG unit relative to untreated adipocytes. Trimethylated histone H3-Lys27 occupancy was significantly increased on Cebpa and RxRa promoters in SAH-treated adipocytes, consistent with the reduction in gene expression. So the SAH did not affect adipogenesis as such but altered the function of the adipocytes through epigenetic mechanisms so that they showed altered glucose disposal and lipolysis. This means that micronutrient imbalances during early development can then influence the later responses of the adipocytes and these in vitro findings are in keeping with Yajnik's findings in Indian children[xlix].

We are still in the process of learning about the importance of epigenetics and how the changes may affect our therapeutic approaches to different diseases. However, we need to think about the public health implications. The Developmental Origins of Human Disease (DOHAD) model was largely ignored for 30-40 years until epigenetic science came along. And two months ago the World Health Organization released the report of which I am one of the co-authors which specifies for the first time that if you are going to address the issue of childhood obesity, you have to think about the mother before she conceives, the father (whom we have not talked about today), before he contributes sperm and the mother during pregnancy as well as the infant during lactation and infancy[l]. It is not just sufficient to focus on the obesogenic environment itself. And the entire logic for this WHO report focuses on the life-course and this approach has come from epigenetic biology. So it is quite clear that the interaction of developmental nutrition from before pregnancy, through infancy, mediated and perhaps monitored through epigenetic mechanisms, helps to explain childhood obesity and will need to be taken into account as we try to reduce childhood obesity through dietary measures and more exercise.

General Discussion
(grouped into different topics)

Analytical Methods: identifying genetic and epigenetic effects and their interactions

Peter Gluckman: There is no doubt that different methodologies have different uses in different contexts. There are discovery methodologies but when you come to clinical utility we need to make sure that the tools that we use for clinical utility have sufficient robustness so that they can be used in either public health or clinical medicine properly. I think there is a danger that people think you can move from discovery research to clinical application using the same methodology.

Keith Godfrey: Andrew Freiburg is exactly right that we must consider the whole range of epigenomic effects and I rather differ from Bas Heymans' approach which is to steer away from that towards more hypothesis-driven stuff. We need hypothesis-driven research, but we also need these agnostic approaches and some of our tools don't enable that: these 450K arrays are great, but we now have 6 replicated perinatal methylation marks associated with childhood obesity and of those only one of the 6 is covered by the 450K array. If we look at candidate genes - we have done loads of work looking at candidates – even with chosen candidates for scrutiny you are just guessing as to which region you should be looking at. Traditionally the focus has been on CpG islands but when you assess the inter-genic regions and the shores, you start discovering things that previously you would have ignored. So I think these wider coverage approaches have to be an important stepping-stone before we drill down and link specific changes with clinical outcomes.

Bas Heijmans: I don't think genome-scale assessments are wrong and we can prefer an agnostic approach and assess the whole genome. But that doesn't mean that you can't have an hypothesis. I agree that just doing candidate genes is not very useful. The hypothesis is much broader - the hypothesis is about whether my study design fits the question I want to answer and then what part of the methylome of the epigenome you measure is just one aspect of it. So it is really important in defining your hypothesis whether your study design is actually suitable to test the question you want to ask.

Leif Groop: I think you need both hypothesis-free discovery and hypothesis-based research, but I would also like to pay attention to resources. We heard a lot about intrauterine programming, but most of that work is done for obvious reasons in animal models, and researchers tend to study in humans those tissues that are available in adult subjects. But what we really need to ensure for future research is how we get access to human foetal tissues. We have many examples where you can have a gene like KCNQ1 - it is methylated and imprinted during the foetal period, but it is completely biallelically expressed during the adult period.

Andrew Feinberg: It really is important to integrate genetics and epigenetics. At least in the US, people are thinking that you can take just genetic studies and put them directly into some sort of clinical practice process. That is not going to work unless you combine these gene changes with information on epigenetics.

Peter Gluckman: I agree and we cannot just think about gene changes and methylation differences. We have to think about the different layers of epigenetics and RNA biology as well before we can integrate this whole story.

Bas Heijmans: We may have missing variants that account for a phenotype so that when we do GWAS analyses in relation to a disease we account for perhaps 20% of the variance. Meta-analyses are still producing more variants that contribute so there are a lot more genetic variants that contribute, but their contribution is so small that it is really painful to identify. This was convincingly shown by our looking at quantitative traits such as height where misdiagnosis, as with diseases, is not involved. And in height you know that only about 17% of the variation in height is accounted for by genetic variation. Yet height is highly heritable and it just means that probably 20,000 genes each contribute a little. So many people start to believe this approach is too cumbersome. Furthermore, if we think there must be a major effect from epigenetics then we find only one publication I am aware of that has reported an additional 13% effect size and this relates to 3 CpGs. So in general the effect sizes of epigenetic changes are very, very small.

Joseph Nadeau When the GWAS analyses are done the approach has usually been to test a specific hypothesis one gene at a time, as though the genes have additive effects. They are not looking at genetic interactions. Now I know that there are papers saying that the gene

interactions are small or rare, or negligible. But is not clear to me that the appropriate analytical methods have been used. Then, as Peter Gluckman discussed today, we see a lot of gene interactions as well as gene-environment interactions. So with so much heterogeneity we need a different approach from just scanning different individuals for specific changes.

Keith Godfrey: I think to a certain extent it is a false dichotomy, genetic versus epigenetic variance. There are some nice published data suggesting that from an evolutionary perspective you would expect epigenetics to have bigger effect sizes than genetic variance[li]. When we looked at perinatal epigenetic marks in association with later obesity in the child we have found that 25% of the variance is explained by a single methylation change[40]. I think that the figure of 25% may be reduced as many more studies assess this link but we are surprised by the amount of variance that we see explained - individual changes in isolation are predicting 10-15% of the observed variance and when we add them together a much greater - indeed substantial - part of the variance is explained. In terms of genetics itself McCarthy and others have highlighted the limited role for fixed genetic variance[lii]. So about 5- 10% is the aggregated maximum estimate for type 2 diabetes that can be explained by genetic variance. The current figure for obesity is about 2%. So we need to look gene/gene interactions, but I don't think we are going to predict anything like as much of the variance as we see with the epigenetic analyses.

John Stamatoyannopoulos: There has been a fairly substantial rethink on this in the last 6 months. The hypothesis was that a very small number of relatively high effect common variants would account for the disease phenotypes but coding variants across several common diseases typically account for a very small amount of the heritability – of the order of 1-2%. However, an artificial threshold has been applied to all of these studies i.e. assessing the genome-wide significance threshold. This was originally introduced as a quality measure with an attempt to determine a threshold for potentially significant variation. So when a study is done, a number of variants pass this arbitrary 10^{-8} threshold that is applied uniformly across the genome. We had published a couple of years ago in *Science*[liii] the concentration of variants associated with common disease in regulatory DNA, and one of the major findings was not just that the phenomenon exists but that the genome-wide significance threshold was essentially completely arbitrary because you have this concentration well below that threshold. Alkes Price[liv] has shown in 11 common diseases that you can reanalyse the GWAS data across many common diseases and you will find, with a variance component analysis of measures of regulatory DNA, that 10-15% of the SNPS account for 80-90% of the heritability, across 11 diseases. So this missing heritability is essentially a misperception about how to analyse the data. And I think that the GWAS and the whole genetic phenomenon in the last 10-12 years have a lot of lessons for this nascent field of EWAS. The problem has been that there are voluminous databases but they have been woefully under-analysed. The under-analysis has come partly because the data were put in a repository that is extremely difficult to work with. Therefore there has been a sort of combination of data hoarding and fragmentation that has prevented large scale reanalyses of what are in practice enormous data sets. The repository has genotype data on 600,000 individuals; 240,000 of them were collected ostensibly as controls for different studies and incorporate about 900 major phenotypes and over 2,800 minor phenotypes. But the number of those that have been analysed is fractionally small and I think that the wrong conclusions have been coming from the genetic data.

Leif Groop: I fully agree with this: I think that the definition of how to estimate heritability is still quite obscure. Most of the GWAS studies have been done in outbred populations where there is no information really on the clustering of disease. We have also never taken into account that you can inherit variants differently from mother and father and there are quite a few opposing SNPs from the mother and the father that neutralise any effect. It is also very important to know the precise phenotype that you are really studying. Type 2 diabetes is crudely specified when it is considered that the diabetes is not type 1 – all the other forms of diabetes are just lumped together as type 2 diabetes. However, a study in Southern Sweden has shown what they term "discordant diabetes" in Skoner[lv]. The youngest had diabetes on the first day of life, the eldest 97 years old and of 12,000 people with diabetes we realised we had five forms of phenotypic diabetes. Then we included genetics in these five clusters and found even the top SNPs associated with Type 2 - DCF702- were in two clusters with opposite effects. So I don't think we actually have known the appropriate phenotype and we have made far too many assumptions in the past[lvi].

Joseph Nadeau: A question for John Stamatoyannopoulos: if you take away an arbitrary

threshold then presumably you rank the profiles of genetic variation and start going down the list but where do you stop?

John Stamatoyannopoulos: The threshold for specifying the magnitude of an important genetic variance was formulated statistically by first specifying whether you assume it is a monogenic, oligogenic or polygenic phenomenon as this alters the approach to assessing genetic differences. But when one is trying to assess "epigenetically aware" p-values and thereby define how far down the list you should go, given the disorder, the cell type etc. then this issue is still unresolved. Take the GWAS data and model it as fully polygenic with every single variant making a potentially small contribution. Then, having controlled fully for the issue of multiple analyses with "random variation", throw away all SNIPs with a p value of $<10^{-4}$. So now you are dealing with what is universally agreed to be in the residual garbage. However, what Jonathan Prtchard showed was that you can still partition and get excluded signals that may play a useful functional role e.g. in immune cells[lvii]. So basically you are dealing with something that is massively polygenic. So, if one is dealing with environmental impacts on genetic factors, then one has to assume that there are multiple interactions of genes and one cannot just consider each variant on its own.

Stephen Beck: Originally, of course, the GWAS studies were based on a platform that took linkage disequilibrium into account. But with the majority of regulatory elements proving to be in non-coding regions, this requires a completely different approach. When the first GWAS studies were done they tested the hypothesis that the common variants cause common disease. Then this proved not to be the case, but I think it was a prudent approach and now we progress incrementally in our understanding.

Andrew Feinberg: By starting with epigenetic changes we can see that some processes are conserved across species. So epigenetic analysis substantially enhances what we call conventional GWAS. The other thing I emphasised briefly last night relates to what we rather ineptly call GeMes[lviii] - defined as potentially non-contiguous methylation clusters under the control of one or more methylation quantitative trait loci (MQTL). Those methylation sites could be really far away and they don't even necessarily have to be within the same LD block as the SNP and that is because the DNA has moved during evolution so filtering used in GWAS analyses do not work. So by just doing an epigenetic analysis in conjunction with a SNP analysis, you just see a lot more things that probably really are being driven by the genome itself.

Peter Gluckman: I think we need to work out to what extent a phenomenon that appears to be epigenetic inheritance is actually genetically driven - and to what extent is there truly epigenetic inheritance? The debate in Nature late last year[lix] was on whether there is epigenetic inheritance, independent of genomic variance, or not? My own bias is that there is a significant independent effect, at least over a small number of generations. You can then come into the evolutionary arguments over why that might be the case and why it might be held. But I think that we have got to be more open-minded on this.

Joseph Nadeau: I want to put it in a different way - maybe in a more applied way. If epigenetic changes depend on underlying DNA sequence-differences then can you think of an epigenetic change just as a mechanism by which the gene is doing what it does and it is really just the genetic effect? So the epigenetic effect is then just a marker, a modulator, just like a biochemical reaction or something encoded by the gene. If it is dependent on the DNA sequence variant, then is it anything different? So you would have to partition the epigenetic variation into that which is dependent on genetic variation from the epigenetic changes that occur in places that are independent of gene control.

Barry Keverne: I think demethylation is just as important - particularly demethylation in the germ line where we have germ cells in a state of toti-potency with demethylation crucial in the resetting of the genome. What we have been finding recently is that you get some genes escaping demethylation at that stage and they remain methylated into the next generation[lx]. In the mouse there are about five to six hundred of these, but in the work that has been done recently on the human genome, this number goes up into the thousands. So there is a large number of escapees and about half of these escapees are expressed in the brain. In a sense, that doesn't surprise me because the brain itself has the demethylating enzymes TET1, TET2, TET3 and these are continually on the go reprogramming the brain. So the brain very much epitomizes an epigenetic organ in its responses to its environment. The brain needs activity to generate connections and to advance the strength of connections.

Charlotte Ling: I think the MQTLs are currently underpowered and Feinberg has been testing whether the SNP affects methylation and thereby the phenotype. We have done

similar analyses[lxi,lxii,] but these studies need to be much bigger and include analyses of sex differences[lxiii]. We have shown that half of the SNPs in pancreatic cells in diabetes are differentially methylated. Genomic regions close to the transcription start site showed low degrees of methylation and regions further away from the transcription start site such as the gene body, 3'UTR and intergenic regions showed a higher degree of methylation. While CpG islands were hypomethylated, the surrounding 2 kb shores showed an intermediate degree of methylation, whereas regions further away (shelves and open sea) were hypermethylated in β- and α-cells of human islets from Type 2 diabetes patients. Then the problem is that you have multiple tissues so I think that when you say that you explain X amount of the variance with one tissue, then we need to realise we have not considered other tissues. So the genome-wide SNP analysis and the genome-wide methylation studies need to be expanded to include multiple tissues and also consider the environmental conditioning of these changes.

Ranjan Yajnik*:* OK but we also need to consider the life course evolution of a phenotype and distinguish, for example, between effects on pancreatic insulin secretion and tissue insulin resistance.

Robert Waterland: It seems to me that the one big advantage you have with some epigenetics is that this can be mitotically heritable and we can observe a stable change in gene expression and in specific genomic marks. So we can take a developmental perspective and look at an environmental perturbation during a critical period of development and then look to see if we can see a stable epigenetic change that is resulting from this perturbation. In that paradigm you can distinguish an environmental influence from genetic differences among individuals. There seem to be many different definitions of epigenetics that people are using even within this room.

Irv Rosenberg: Let us take nutritional factors as one of the environmental factors that can be modified experimentally. We are trying to understand the response in the incidence of neural tube defects to folic acid fortification and whether the environmental exposure is operating through a methylation process of some appropriate gene or pathway, and whether there is any difference between exposure to synthetic folic acid rather than the natural forms of folate working in the methylation pathway. It seems to me that the advantage of the epigenetic approach to some of these observations is that we can define experiments to examine the environmental exposure involved with epigenetic change that we can't do with genetics alone.

Keith Godfrey: Epigenetics can definitely have a utility beyond what we have talked about: the literature linking maternal smoking or smoking at any point in the life course with methylation measured by the 450K array is absolutely rock solid, and it is emerging as being a really valuable marker of maternal smoking in the mother/offspring cohorts[lxiv]. I am not a tremendous fan of the 450K array. There were data in the Lancet last year linking one particular methylation marker of HIF3A in obese adults to their body composition with a conclusion that the methylation mark was a consequence of the obesity rather than a driver of it[lxv]. The developmental cohorts can add something to this and from the GUSTO Singaporean cohort we have just published data showing a pretty much identical effect size that you can see between HIT3A methylation and adiposity that is present from birth. So there is a developmental component - probably a dominant developmental component but we have no idea what it is really affecting[lxvi]. The 450 k array only picks up one of our six replicated perinatal marks that are associated with later adiposity, so we do prefer wider approaches. We are also doing some candidate work and the PGC1α data that was presented this morning sits very nicely as the backdrop to some work we have been doing on a group of kids where we have blood samples longitudinally from age 5 years to 14 years. These methylation sites are further away from the promoter than those that were described this morning. And these are remarkably fixed in the methylation levels across childhood and I think, as Peter Gluckman was saying, we have got to be more sophisticated in our thinking of methylation. There are sites that are turning over rapidly, there are sites that are fixed from fertilisation to death, transgenerational sites - they are all variations here. Yet the methylation at age 5 predicted their adiposity in our studies: a 10% change in methylation was associated with around a 10% change in fat mass in the offspring.

Vardham Rakyan: So which approaches do you prefer over 450K for genome scale analyses?

Keith Godfrey: We are currently doing quite a lot of short select methyl searches and I think that the work that is being done on the whole genome bisulphite sequencing is better - it generates more data. I think the RRBS is good, but I think there are challenges because of

the biases that are induced in it. So none of the techniques are perfect, but to focus on a technique that covers around 2% of CPG sites and is biased to cover areas away from the regions where we think the developmental effects are operating is not the preferred approach. And in our hands to technique which doesn't cover 5LR6 replicated marks also doesn't make sense for our use. The preferred method that we are using at the moment in an affordable way and in large numbers of subjects is Sure Select methyl sequencing.

RNA as a vector of epigenetic heredity

Minoo Rassoulzadegan: In several rodent models[lxvii] and in human epidemiological analyses[lxviii] obesity seems to be transmitted from one generation to the next if the parents are first fed on a high fat diet to make them obese This is transmitted through at least two generations. So the question that we addressed was to look for the vector of the epigenetic effect. To test the role of RNA we prepared the RNA from sperm or from testes of the obese male mouse and then used micro-injection to put this RNA into the normal weight female mouse's eggs. So for this study we had completely genetically homogenous male and female mice and sought to see if the offspring were maintaining the normal food intake of a normal mouse or if they became obese or diabetic. The progeny proved to be obese and more prone to diabetes[lxix]. Controls were also undertaken, of course, to prove the effect. So the obese phenotype can be transmitted from the father and the RNA from testes transmits obesity into the normal eggs to induce obesity in the next generation. Given the presence also of RNA in human sperm[lxx] we can conclude that RNA can be a powerful vector of trans-generational signalling.
Anna Krook: That is just beautiful work. I have heard discussions suggesting it is the tRNA that is involved but what do you think?
Minoo Rassoulzadegan: TRNA was proposed and microarray analysis showed about 100 candidate genes and small RNA sequences. I don't think it is only one RNA which is responsible for this phenotype and the more experiments we do the more it seems that RNA by itself is not enough, but RNA modification is the key factor in the transmission of heredity. We are studying what seems to be a requirement for the expression of cytosine methyltransferase Dnmt2 to ensure that we have two of the recognised epigenetic features i.e. epigenetic modulation of the Kit gene, resulting in altered fur coloration, and the modulation of the Sox9 gene, resulting in an overgrowth phenotype[lxxi].
Vardham Rakyan How much of that RNA do you inject?
Minoo Rassoulzadegan: 2 picograms but 90% of it comes out immediately after the microinjection.
Robert Waterland I love your general point that RNA as well as DNA methylation clearly conveys information trans-generationally but when you show data on body weight do you have any data on body composition? In other words, could this just be an overgrowth syndrome, or is this actual obesity in these mice.
Minoo Rassoulzadegan: Both excess body fat and glucose intolerance are clear evidence that it is not just overgrowth.
Ragnhil Eskeland When you talk about the F1 progeny in the male, that is not trans-generational inheritance - that is direct impact. If the mother eats a high fat diet, that affects the mother and it then affects the foetus with its effects on the germ cells in the foetus. We have to go one generation further to actually claim trans-generational inheritance.
Minoo Rassoulzadegan: Yes, it is true that the epigenetic effect is then translated in a different way but we use the male and not female so we are not directly affecting the uterine environment.
Andrew Feinberg: I agree with this – it comes from the father so is epigenetic!
Barry Keverne: We also know that demethylation during early development is very different in the matriline compared with the patriline, so you can get the matrilineal genome reprogrammed in the mother's uterus. So I agree with you, Minoo, that you are focusing on the patrilineal genome which when reprogrammed in sperm affects the fertilized egg and then it undergoes a second phase of reprogramming, post-fertilisation in the zygote. The post-fertilisation demethylation is very much under matrilineal control, partly because when it starts to happen, the maternal genome has not been switched on. It is silent; it has got protamines there that are compacting the DNA. They need to be replaced by the histone so the genome is not available for transcription. Secondly there are expressed maternal genes which are

protective of demethylation. So there are lots of phases where things can go wrong. In the brain this involves a couple of examples of abnormal genes which, as it were, escape demethylation and are then expressed in the brain and come out as autism[lxxii,lxxiii] and also Parkinson's disease[lxxiv]. So there may well be pathologies there that are related to this demethylation and as the germ line gets older there are much greater chances of errors creeping in. The whole purpose of the brain is its continuous process of epigenetic change in response to its environment[lxxv]. It has millions of neurones that make billions of synapses and the making of those synapses is very much in response to what is happening in its environment. That is what learning and memory is about and in a sense that is transgenerational. It is one of the things that we as humans are so very good at: we can transmit transgenerational information through our brains to other people's brains[lxxvi], to other people's brains in different generations[lxxvii]. So the brain has got a complete environment of its own where exactly these same kinds of things are happening as occur in the germ line. So I think demethylation is an area that has not been looked at very carefully.

Histone modifications

Philip James. We have been focusing so far on methylation and demethylation – where are we with scanning techniques for assessing changes in histones?
Philippe Collas: It is great that you bring up the role of histones because the topic has been largely ignored. My view is simplistic – if you work on gene methylation then you tend to think that gene-methylation is the most important thing. But that is one out of 200 or 300 changes - including chromatin modifications. So the effect on gene methylation cannot be expected to explain all the changes. Even though a site is methylated or unmethylated the effect will still depend on the chromatin changes so I think we need to consider our DNA from a chromatin perspective. DNA is wrapped around 4 coding histones and they are all modified in many different ways. So we are just starting to appreciate these types of modifications. We know a lot about a few modifications but given there are so many changes we have to think of the community of effects they produce. Fortunately there are now bio-informatic tools that enable labs to look at combinations and scan modifications. Let's say we have over 200 base pairs in part of a genome and we find 15 - 20 different histone modifications. There are now modelling approaches and also machine learning approaches that allow you to run this through a programme which will actually model the different chromatin states. One chromatin state is one specific combination of multiple histone modifications. So you can actually narrow down all this to let's say 15 states and then we can assess the potential effect of each of these states.
Philip James*:* But have these states actually been in any way related to anything metabolic?
Philippe Collas: No not yet but we also have to realise that even if you do these chromatin state modelling exercises you only cover part of the genome and we cannot presently map 80% of the genome.
Peter Gluckman: I would actually argue that we might know more about the histone system than we know about the methylation system! We have glibly talked about all methylation is the same: it is not! We have methylation associated with cell-differentiation; we have methylation associated with developmental plasticity; we have methylation associated with homeostasis, as one of the speakers showed this morning, because it can change very rapidly. We know that there is a very complex architecture of the CpGs across the genome. We are only beginning to understand what that means. We have ignored totally hydroxymethylation which is an activation not a repressor mark. We have methylation that is not on CpGs, but is on other cytosines and we have RNA methylation. So to think that just because we have a tool that we can easily use - the 450K or the various methods that have followed - we still need to recognise that we are still a long way from understanding methylation. One of the dangers I think is that there is a kit you can use to report methylation but it doesn't mean that what it is producing is going to be as useful as we think. So the tool kits are appearing without the intellectual support and the informatics support that is needed to allow investigators to really make sense of the data. I think it is a really big issue.
Stephen Beck: The problem is that the methylation work is driven by the technology but we can't quantify currently the histone modifications and that is the limitation.
Paulo Sassone-Corsi: We do know that there are metabolites that are directly utilised in order to obtain for example histone acetylation and this may occur in a circadian manner. The

chromatin back-bone is a system for synchronising effects. So demethylases not only remove a mark but also change the levels of those metabolites in the nucleus and that can change physiology dramatically. We are using florescence labelling *in vivo* to look at some metabolites and other molecules e.g. NADH which are naturally fluorescent *in vivo* in a cell or in a tissue so we can study processes of activation etc. One can clearly see that there is a strong relationship between metabolic changes and histone modifications.

Leif Groop: I agree that histone modifications and chromatin change have been difficult to quantify and a lot of presentations really are just about correlations. There are really very few experiments that are relevant like a functional study where you inhibit something to show whether the mechanism is really operational. If you assume that histone modifications influence the expression of a gene, which is damaging for the tissue and you show that it is actually turned on by let's call it histone acetylation, and then you prevent the histone acetylation by genetic engineering or whatever, then you do know that at least that process involves that gene change.

***Robert Waterland*:** Yesterday evening Andy Feinberg made the point that we still don't have any evidence that specific combinations of histone marks or histone modifications convey information through mitosis, so are they truly acting epigenetically? And, on a related topic, in response to Peter's comment about 5-hydroxy methylation, my take on that is that I haven't seen any convincing data that 5-hydroxy is really functioning as an epigenetic regulator, but rather it appears to be just on the pathway to demethylation.

Peter Gluckman: I think there are several papers[lxxviii] and I can show you some of our own unpublished data that show, in genome-wide scans, that 5-hydroxy-methylation appears to correlate with activation of that particular gene as opposed to its inhibition. I am not saying that those are definitive data. People ignored for instance non-CpG methylation and just regarded it as noise so excluded it from their analyses for a long time when we now think, with the accessibility of new tools, it may be important. So I think just because we can measure something easily and can't measure other things so easily, we tend to focus on what we can do and this makes us biased in our thinking.

Stephen Beck: The hydroxy-methylation appears in higher concentration than you would expect if it were only to be in the demethylation pathway. So that implies that there is another function involved. A second indication in its favour for a biological role is that there is a good number of proteins that bind specifically to hydroxymethylcytosine and if you have proteins that specifically bind to it, you have a much higher chance that these are really functioning biologically[lxxix]. So I agree that we don't know, but the evidence for me is in favour of hydroxymethylation having an additional biological function rather than just being an intermediate.

John Stamatoyannopoulos: I just wanted to correct the record on the transmission of chromatin features. This was first demonstrated by Martin Groudine and Harold Weintraub in 1982[lxxx] when they showed that you could induce - this was shortly after DNAase-hypersensitive sites had been discovered - that you could induce a DNAase-hypersensitive site, take away the stimulus that induced it and propagate it for 20 generations. It was followed up by another paper in 1983, from Weintraub again[lxxxi], showing this with a different stimulus and this remained in the literature as the only demonstration of propagation of chromatin states that were induced until Gerry Crabtree[lxxxii] published a paper in Cell in 2012 in which he used an extremely clever system. It was a chemical system in which he could bring a histone-modifying enzyme into contact with a piece of chromatin, deposit a histone modification, take it away and then they could show that that was actually stably-inherited mitotically. However, I am not sure that Andy is right in thinking that the whole histone code hypothesis is reliant on this being a ubiquitous, prevalent mechanism. I think he is right that this is one mechanism but not a highly prevalent one.

Andrew Fineberg: What I said last night was that I think there is some mechanism for copying chromatin information - because of the studies you mentioned.

***Robert Waterland*:** John, just to follow up on your examples, was it ruled out in either of those examples that those processes were independent of changes in DNA methylation, or are there other types of changes like auto-regulatory transcription factors or RNA involved - that sort of thing?

John Stamatoyannopoulos: The question of whether histone modifications themselves convey information through mitosis is still unclear.

***Robert Waterland*:** If I were to state my general perception of the field, I would recap a debate that happened when Steve Henikoff, debated David Allis about the histone code. We

know that the modifications come from transcription factors that are recruiting the modifying enzymes - and the modifying enzymes modify the neighbouring chromatin. So then the idea is that it is reset in every cell cycle because there actually is a mechanism for the transcription factors to template themselves and then the histone modifications are reset. On the other hand it has been argued that the modifications were templating themselves.
Charlotte Ling: So I want to bring it back to the MQTLs but then the question is if the inheritance on the histone modifications could also come from the genetic code and thereby affect the inheritance.
John Stamatoyannopoulos: That has actually been studied recently, for example, by Mike Snyder's group in Science[lxxxiii] where people have looked at the histone modifications across individuals. What you can show is that there is variation in the histone-modification pattern, but they are always linked to SNPs that modify a transcription factor by any site so there definitely is a very important direct genetic link.
I think there are two different definitions of epigenetic inheritance. One is transgenerational which is really what is perhaps determining disease susceptibility and the second one that intersects with the nutritional field and involves mitosis affecting development and differentiation where there is a massive forward propagation of information that is highly canalised and that is informing particular phenotypic outcomes. Now that process we know also is susceptible to environmental influence, but it is not clear that the influences are playing out in the same way.
Andrew Feinberg Maxwell Lee, a former trainee of mine, produced a series of papers[lxxxiv], [lxxxv] showing that there was Mendelisation of chromatin modifications – they are familial and that is the first evidence for sequence-directed chromatin modifications. New papers don't cite that work.

Metabolic memory and histone modifications

Leif Groop: I am definitely no expert on epigenetics or histone modifications, but some of the evidence relating to supposed glucose toxicity in diabetes may help and this relates to what is termed "metabolic memory" which I think was first highlighted in a type 1 diabetes control and complications trial (DCCT) cohort[lxxxvi]. They had an intensively insulin treated group and a conventionally treated group at the early onset of diabetes. After 2 years they basically had the same control in both groups but still the intensively treated group 15 years later had many fewer complications and after 27 years of follow up a lower mortality[lxxxvii]. Michael Brownlee emphasised the concept of metabolic memory with multiple cellular changes[lxxxviii] and a group in our institute has shown that in both kidney and islets, glucose really is a strong activator of true histone acetylation[lxxxix] and the activity of histone-modifying enzymes and other histone modifications of differentially expressed genes. These changes, and the damage caused, can be reversibly altered by a histone acetylase inhibitor or other histone modifying techniques.
Philip James*:* When discussing metabolic memory there is, of course, the Chinese Da Qing study on diabetes prevention where they have a very prolonged follow up with a persistent reduction in the development of diabetes and some complications[xc]. The difference in diabetes rates is still there. Are you saying that their reduction in any complications relate to changes in histones?
Leif Groop: I wouldn't be surprised if there would be the same mechanisms operating. But the few data that we have actually come from patients with Type 1 and Type 2 – and these analyses, of course, are mostly just *in vitro* cell line studies.
Philip James*:* In terms of metabolic memory there is also a recent study from the UK showing that if people in the general population lose weight at some time and are then followed up over the next 30 years, then those people who lose weight have a much lower cardiovascular event rate over the following 20-30 years which again implies metabolic memory without our having any understanding of the mechanisms at all[xci].
Leif Groop: There have been quite a few studies done with GWAS on diabetic complications, but none of them has paid off and it clearly could be that none of them really has taken into account the role of glucose and its effects on histone changes
Philip James*:* So you are seeing glucose as absolutely fundamental, operating through a histone acetylation process, as well as the glycosylation - the standard story about automatic progressive glycosylation of proteins which in fact have their own intrinsic aging processes?

Leif Groop: We know that treatment with the lowering of glucose has not been very effective in preventing the diabetic complications, so we need to think of other approaches.

Nutritional impacts on embryo selection, fertilisation and neural tube defects.

Philip James*:* Now let us turn to Joseph Nadeau's demonstration that there can be gene selection arising from the differential selection of sperm/ova interactions relating to nutritional conditions. Is this dependent on maternal or paternal states or both?
Joseph Nadeau: It is an issue of both sperm and egg.
Andrew Feinberg: Jo you said in the later part of your lecture you were dealing with 129 SVEV mice and they are susceptible to testicular or other germ cell tumours. Yet the same mutation, is used for a huge amount of work with knockout mice, so the billions of dollars that were spent by the National Cancer Institute to model single gene, oncogene knockouts and over-expression knockouts of tumour suppressor genes - all that was done on a background that already has a strongly penetrant gene for cancer? That would be horribly confounding would it not?
Joseph Nadeau Every strain, just like every individual, has some vulnerabilities and every strain of mouse has multiple things wrong with them. The idiosyncrasy of this strain, this family of 129 strains, is that they show a low frequency of spontaneous testicular germ cell tumours. You can see this by 4 weeks of age. The frequency varies from 1% of the mice to maybe 10%. Even though they are inbred and homozygous, the penetrance is low. Stevens discovered this back in the '50s at Jackson Lab when he was trying to do classical genetics and looking at the inheritance of 129 strain crosses with other strains of mice[xcii]. He looked at 11,000 male offspring of those backcrosses and inter-crosses and found one strain that affected this susceptibility which means that it is genetic. It was so rare in the crosses that this meant it is highly multi-genic or the inheritance is unconventional. Then Roy Stevens and Barry Pierce realised the stem cell of these tumours looked like embryonic cells so Roy wondered if you could grow them in culture and they could. So they asked themselves whether embryonic cells could be grown in culture and so it proved. Then they and others went on to show that you can engineer them. So the low susceptibility to tumours doesn't negate anything: it just says it happens on that background. If you had chosen Black 6, you have age-related hearing loss and other problems. So it doesn't negate anything.
Anna Krook: We were wondering Joseph why would there be a selection pressure to somehow sense the genotype of the egg or sperm approaching you and therefore determine which gametes get fertilised? And why is it dependent on folate?
Ingemar Emberg**:** I just want to understand a few very basic things about embryo selection: first is there any type of evidence from any molecular studies showing ovum variability? I guess methylation is looking at variability between ova at some epigenetic or transcriptional level. Secondly what mechanism is going on whereby the sperm is selecting its partner? I only know that sperm have in its nose human olfactory receptor 17 which is probably actually the primary olfactory system - so sperms have an olfactory G-capped receptor number 17 - so that could be one source of a gradient that the sperm hits, but are there any thoughts around how sperms find an ovum?
Joseph Nadeau: I have no idea! I don't know how the gametes are signalling each other - the way that you phrased it is just right: it is when they are coming together so that they know in some way whether that egg and sperm combination is appropriate. It is like a chemo-attractant but I don't know that it is. How they recognise each other and distinguish them I don't know. Why it depends on folate is also unclear but it is nice that it does because it provides a nice control because at one folate level everything is fine; at another folate level there is a bias, so it gives me some confidence that what we are seeing is a real biological phenomenon. You can imagine that maybe the genome has a way of recognising these foreign elements that are left at targeted sites; then for some reason, based on whether it needs folate or doesn't need folate, it is recognising those sites and marking these gametes in some way. This would provide a molecular explanation, except that it doesn't explain why it would work only in an intercross and not in a backcross.
Ragnhild Eskeland: There are histones in sperm, marking developmental genes that are turned on early. There are histone marks and chromatin itself is flexible: we are flexible because chromatin is flexible. So it is not only DNA methylation - there is nuclear organisation but as yet we have no quantitative studies on chromatin marks.

***Ingemar Emberg*:** I was also curious to know whether there are any studies to establish if there is a clock function in the ova and if it matters if the ovum is fertilised in the night setting of the clock, or in the day setting of the clock, and if that would influence the whole of the developmental pattern of the baby? Secondly, it is quite conceivable that the fertilisation process of the egg is the most critical issue for evolution in this whole discussion here. I can't imagine that evolution hasn't involved a mechanism to select the right matching, optimising matching between sperm and ova. I also want to remind you of the very controversial experiments where the actual sequence of the genome of a single human ova has been done before fertilisation and then the same ovum has been fertilised at the 4 or 6 cell stage. So whole genome sequencing is done on single cells from the 4 cell stage and then he takes another cell at that 4 cell stage and fertilises it. Then the eggs that suffer from a monogenic heritable genetic deficiency are excluded just like what I think the sperm and the egg would do *in utero*, by selecting away from eggs with disorders. So one can get absolutely healthy babies from genetically ill parents and one expert now has two conceptions going through in Beijing successfully, with healthy babies, this last winter. So man's brain is doing what I am sure biology has experimented with for three billion years.

Andrew Feinberg: I want to commend Ingemar for his great questions! I don't anyone has the answers! So I think we need to understand something about chromosomal motility and about, for example, the role of Leydig, and Sertoli cells in terms of fertility and ageing - things like that as well as the sperm too, of course.

Peter Gluckman: I think we forget that relatively few sperm actually enter the fallopian tube and then they sit there at the beginning of the tube and are largely selected out by maternal glycan receptors which reduce the number of sperm that get up to the ovum to a very small number. Those pictures you see of an egg surrounded by hundreds of sperm don't occur in the real world, only in the test tube. The reality is that the mother selects on a basis which we don't fully understand, because the sperm sit for about 12 hours at the entrance of the fallopian tube where they are selected out, and less than ten probably actually get to the upper end of the fallopian tube.

Barry Keverne: Can I just add to that in terms of the mis-equivalence - i.e. where the sperm genome and the maternal genome are not equivalent because one of the things that you all know about sperm development is the large number of cell divisions that it undergoes and this introduces mutations so most of the mutations are occurring in the patriline. One of the things that happens post-fertilisation is that you get the mismatch repair process underway, with this repair going on at the same time as you are getting the reprogramming going on of the paternal genome by the matrilineal genome. So you get this post-fertilisation period of remodelling, or renovating of the male sperm to make it equivalent.

Philip James: A couple of years ago we were talking about micro RNA and heard about the capacity of an amazing array of cells to extrude micro RNA. Do sperm extrude micro RNA?

Joseph Nadeau: I don't know whether this could be the explanation. Semen is loaded with RNAs and exosomes floating with RNA from semen is a neglected aspect in all of this biology.

Stephen Beck: Ten years ago I remember a whole spate of papers that discussed selection for heterozygosity between sperm and eggs and involved MHC with the implication that if you select for heterozygosity, you obviously have a better defence. And I wonder if this type of mechanism plays a role here and that it is just a different read-out of the same mechanism?

Joseph Nadeau: We see half the number of heterozygotes that we expect so if we are selecting for heterozygocity it is not doing a very good job.

Stephen Beck: But that is obviously not in a wild-type scenario, so that is in a laboratory where that selection falls …

Joseph Nadeau: Right, and none of the genes involved are in chromosome 17 so it is not the MHC.

Philippe Collas: Maybe there is a potential role for some sperm-derived RNA bringing in something else such as instructions coded for in the DNA or derived from the fertilisation process itself. Whether it has been through the form of DNA methylation, or maybe transposons we do not know. We found that zebrafish sperm contains 10% histones which are post transitionally modified[xciii] and we did the same thing in early embryos and it is quite striking that we found the same type of modifications in the same developmental area of the gene in two different labs across the world in sperm and in the early embryo. So either these marks are removed and redeposited with something encoded by the sperm that tells the embryo, OK, you need to be remethylated or these marks are also - at least in zebrafish -

transmitted with the sperm.
Joseph Nadeau: It has also been shown that there are some histone marks that persist through that protamine histone transition so some of them have to be closely linked to mutant loci - otherwise they would segregate independently and be in both the wild type and the mutant sperm. They could be genome-wide but the critical one has to be closely linked because we are seeing the presence or absence of the changes at that particular locus. So if there is something like that going on, then it has to be near the mutant gene.
Keith Godfrey: A brief comment on the histone issue - there is quite a lot of published data from rodent studies showing that alterations in maternal diet, both low fat and high fat diets, are associated with histone modifications so you would expect to have downstream effects on chromatin function etc. So I think the histone and chromatin work is just slower to come through. There will also be cross-talk between those features, the non-coding RNAs and the methylation issues. Now to go back to the great question - there is quite a literature - some of which Tom Flemming[xciv] has contributed to in rodent models, and now Nick Macklon in human studies[xcv], that tubal fluid composition varies with aspects of maternal nutrition and that tubal fluid composition alters the metabolism of the fertilised zygote and then the early blastocyst. So I think that is an additional dimension that we need to bring in when we are talking about these effects on the gametes - and seminal plasma.
Cutberto Garza: The example you showed was folate-specific, but there is nothing in your data that suggests that other bioactive molecules could accomplish the same thing?
Joseph Nadeau: No there may be other factors just lost in the literature. We stumbled on folate because we were specifically investigating the effects of folate deficiency etc.
Cutberto Garza: The other assumption is perhaps that the change interferes with fertilisation in terms of sperm penetrance?
Joseph Nadeau: The zona pelucida of the egg hardens to prevent more than one sperm going in.
Cutberto Garza: What if the zona pelucida never developed because there is something defective in that sperm?
Joseph Nadeau: Then you would see it in the backcross and not the intercross.

Clocks and times of conception

Barry Keverne: Is there any way if things change peripherally in a subsidiary clock, e.g. through diet that you can bring them back into line by over-activating the master clock?
Paolo Sassone-Corsi: One level of complexity is that the supra-chiasmic nucleus (SCN) in the brain is not the only clock in the brain. There are more. There is for sure one food entrainable clock that is not in the SCN. We have done a study where we have mutated the clock by ablating cells only in the SF1 neurones of the ventromedial hypothalamus. Now this is **not** the SCN and we know that by mutating the clock in that system we have the SCN functioning in a perfectly normal state. Yet with this mutation we find a completely changed state of energy metabolism. You, of course, know that the mouse and human clocks are different - at least in the sense that the mouse is a nocturnal animal, so all their clocks are inverted.
***Ingemar Emberg*:** Do we know anything in any model system or human ova that involves the clock principle? And in that case, does fertilisation, when the clock is in day mode, or in night mode, matter? When the clock is switched to a daytime pattern of gene expression it should have dramatic consequences in terms of sperm reception I guess?
Philip James*:* Also is there any evidence that children conceived through the dark winters of the artic affects them?
Peter Gluckman: The problem at least in humans, is that there is a very variable time between intercourse and conception: it is 12-36 hrs. so it is a very broad window. Now whether that is regulated by day length or timing, I don't think anybody knows. But the literature will be there in the cow embryo IVF literature on gene expression in great detail. I don't know if anybody has looked to see whether the clock genes are turned on in a single-cell embryo, but it will be in the literature.
Leif Groop: I want to come back to melatonin. There are two receptors for the melatonin and one of them, the melatonin receptor 1b is actually associated with an impaired insulin secretion and increased risk of type 2 diabetes but this seems to be dependent upon the season, so the risk is higher during the dark season[xcvi]. Melatonin also inhibits insulin

secretion: we have actually done a study on that when we recruited individuals based upon having two copies of the risk alleles and two copies of the non-risk and they received melatonin for three months and we observed, as we expected, impairment in insulin secretion in those with the risk allele but it is quite clear that this is really dependent upon the light. Melatonin is the hormone of darkness - why do we have it? Its metabolic effects could simply be to prevent us from getting hypoglycaemia during the night, but I really don't think we understand what melatonin is there for.

***Philip James**:* Paolo - just sort us out!

Paolo Sassone-Corsi: Let me tell you a story about the English ferret. Lord Bissonnette[xcvii] in 1932 was studying the English ferret. The female ferret has only two hours of oestrus per year! So what he did was to take the English ferrets and then he went to South Africa and within 6 months, so half of the year's cycle, the 2 hours oestrus had moved to the Springtime of South Africa instead of the Springtime in England. So the key thing is that the length of the day and night affects the amount of activation of the retinal- hypothalamic tracts and thereby the gonadotrope activation.

***Philip James**:* But are you implying that melatonin has profound effects….

Paolo Sassone-Corsi: No, melatonin is a hypnotic hormone that allows you to sleep at night. That is why the very few elderly people in this room have a harder time to sleep because the amplitude of the melatonin oscillation decreases with aging.

***Philip James**:* But is there any effect on babies depending upon when they are conceived?

Paolo Sassone-Corsi: no - there is no clear relationship. Neither is there proof genetically that if you knock out the clock genes, you have decreased reproduction. So what we know is that the egg, even before fertilisation and thereafter, has the clock of the mother. So it is perfectly timed with the clock of the mother.

Leif Groop: I wonder if in the Gambia the season of conception is also reflected in the incidence of later diabetes?

Andrew Prentice: It may be but the people are still so lean, fit and frugal that there is no diabetes. So as you know, the whole Developmental Origins of Human Disease (DOHAD) story is that you have an early exposure linked to a later pathological environment. We don't have that later pathological environment so the insulin sensitivity and glucose tolerance of this population is in fantastic shape. We have zero cases of diabetes and only 3 with impaired glucose tolerance so we have got no variance to look at. We have not assessed glucose tolerance in pregnancy.

Vardham Rakyan: Would an *in vitro* fertilisation experiment help us to work out the signalling and choice of sperm and ova?

Joseph Nadeau: I agree we need to look at an *in vitro* fertilisation experiment. Now there is no guarantee that this will recapitulate with *in vitro* fertilisation. If it does then you are right and we could do a million things. But it is easy to imagine that there are factors present *in vivo* that we don't quite recapitulate *in vitro*, and we don't see.

Patrick Stover: I want to depress you a little bit more! A lot of what we heard this morning about missing heritability and being able to classify people, based on whatever signatures there are in the promoter of their DNA then leads to the next step: once you have classified them, how can you then treat them? Once you learn the mechanism to try to overcome whatever those liabilities are what do you do? In the case of neural tube defects we have clinical trials and we know that 70% of the problem will be resolved. Whether it is Jo's mechanism or whatever, you get a wonderful response rate from a single intervention (folic acid). But now there are on-going GWAS studies trying to identify the pathways and the genes involved and all the GWAS studies that have been reported to date are just showing that there are changes are all over the map. You are not seeing clustering of any of the genetic risk alleles around any given pathway that is discernible in any sort of network analysis.

Joseph Nadeau: For the non-syndromic neural tube defects?

Patrick Stover: Yes and most of these neural tube defects are folate responsive. So it is sort of discouraging in that you can have an intervention that you know rescues a phenotype but we are not able to identify any sort of a clustering of a genetic risk.

Juan Rivera: Joseph –my issue relates more to public health. You know 20% - 30% of these neural tube defects do not respond to folate so do you have any ideas about the type of research that is needed to identify which additional potential interventions are involved or other measures to increase the efficacy of the intervention.

Joseph Nadeau: This is the reason why we began these studies years ago - to try to

understand the basis for the failure of some mouse models to respond to the beneficial effects of the folate supplementation. I showed you a table of options but authorities are not interested in funding these other options partly because 70% of the cases work with folic acid in humans. So they say the problem is largely resolved. But it would be interesting to determine whether other treatments would work, and probably mouse-models are the place to do it because although epidemiology was the big breakthrough in identifying folate you can imagine that unless you are lucky, and know what other agents or combinations of metabolites and nutrients to look for it is exceptionally difficult to do meaningful epidemiological analyses. We might also need to consider some factors harming the process rather than being absent protective agents. There are probably 200 at least single gene mouse-mutants so the human possibilities seem endless. Furthermore the other caveat is that those are single gene mouse-mutants and not the kind that Patrick was thinking about. There are very few mouse models that are polygenic, neural tube defects - curly tail is one and was on our list.

Cutberto Garza: Is it correct, Jo, that in some areas in the United States, there are relatively high levels of fortification but the apparent efficacy of folate fortification is less than the 70% - for example, along the Texas-Mexico border? Or is it just because they don't access fortified food products?

Patrick Stover: Along the Texas-Mexico border it is thought that a large percentage of the non-folate responsive neural tube defects are due to defects in the polar cell clarity pathway. And elements in that pathway are inhibited by some of the aflatoxins and toxins that are in corn. So those are probably in a separate category, independent of folate and a vulnerable pathway i.e. not folate responsive.

Irv Rosenberg: I really would reinforce that conclusion: there are different populations that have different levels of risk for neural tube defects because of other factors. It is not only their responsiveness to folic acid, but I would also add that I think that your data, Jo, seem to have shown that dose is really potentially quite important and that you may have beneficial effects at one dose - whether one is talking about fertility and so forth - and you may have adverse effects at higher doses. It seems to me that one has, within the kind of experiments that you described, the possibility of looking even further at what the pathways are that may explain either the protective or the adverse effects, and something about the dose response.

Joseph Nadeau: So, two things: first all five of those mutants are resistant to the beneficial effects of folate at both levels of folic acid supplementation. So the dose of folate didn't fix the neural tube defects. Then there were some homozygous animals that respond to both levels of folic acid. Some years ago, we did expression profiles of maternal liver in models that were protected versus resistant. And we included in that a mutant called Ski that has a neural tube defect that was homozygous. That mutant had not been tested for responsiveness, but it fell into - by expression profile - a group that we would have said was resistant i.e. we predicted that it was resistant. And when we tested it, it was resistant so at least in that one case we were able to predict the responsiveness but it would be fun to do more mutants and see if we can predict the effects of folic acid supplementation.

Irv Rosenberg: In a human population, there will be a mix of those that are responsive, and those that are non-responsive, and there may be different pathways by which the non-responsive, and even some of the responsive, will have adverse effects with different doses of folic acid.

The role of vitamin B_{12} deficiency before and during pregnancy: its relationship to folate and its long-term effects.

Ranjan Yajnik: Related to this subject of multiple types of neural tube defect we had a paper relating to over 300 neural tube defect (NTD) cases in India[xcviii]. We found folate and vitamin B_{12} levels were not predictive but low levels of holo-transcabalamin were. The commonly associated maternal polymorphism 677C>T in the methyl tetrahydrofolate reductase (MTHFR) gene did not predict risk of NTDs in the offspring and 1298A>C and 1781G>A polymorphisms in MTHFR were protective. However, the maternal 776C>G polymorphism in the transcobalamin TCN2 gene relating to B_{12} was strongly predictive of NTD in the offspring So in a population which is predominantly vegetarian on a multi-generational basis, part of the apparent folate effect is in fact mediated through the B_{12} pathway deficiency. In Canada there are some reports that after the folate / folic acid fortification programme the biggest potential

contribution now to the neural tube defects comes from B_{12} deficiency[xcix]. Following David Barker's advice we surveyed more than 2,500 women who were married and not pregnant and followed them every month for their menstrual dates and every three months for their detailed anthropometrics as an indication of their nutrition. 814 of them became pregnant during this study and they were studied intensively during pregnancy 3 times, and on one occasion the husbands were also studied. Foetal growth was monitored by ultrasound, and 770 delivered a singleton, normal full-term baby. They have been followed up every 6 months for their growth up to 18 years i.e. up to 2013. Every 6 years both the children and parents have been studied intensively for their diabetes and cardiovascular risks, neurocognitive function and all their samples have been bio-banked. Our follow up rates are approaching 95% and I think this is one of the biggest success story we have - to be able to account for practically everyone. And after studying them for their first 18 years of life, we are now able to reflect back on what we call the life course history of the trajectories of either the growth or the metabolic endocrine phenotypes that evolved over that time. The characteristics of the cohort were that mothers were only 42 kg, with a BMI of 18 at the start of their pregnancy. They were really on the undernourished side by international criteria and we then described the thin/fat baby phenotype where the Indian babies were 800 grams lighter than babies born in Southampton but still showed a higher % body fat. This was confirmed by MRI to be largely abdominal or truncal, not only visceral but both subcutaneous and visceral. In addition the babies' cord bloods had higher insulin and leptin concentrations and lower adiponectin concentrations compared with the English babies. So all the risk factors for future diabetes and metabolic syndrome were present at birth[c]. We found an association with maternal nutrition in the form of high micronutrient-rich foods being predictive of foetal growth, namely green leafy vegetables, milk and fruit. And in subsequent analyses with help from Helga Refsum[ci] we were able to demonstrate that this population has substantial B_{12} deficiency, high homocysteine concentrations and this deficiency predicted intrauterine growth retardation. The red cell folate concentrations in the mother were a strong predictor of foetal size as was the green leafy vegetable consumption but high homocysteine levels predicted intrauterine growth retardation. When we followed up these children at 6 years of age the mothers who had the lowest B_{12} concentration and the highest folate concentration had produced the babies which were the most adipose and most insulin resistant. These are the two risk factors for future diabetes.

So we were linking the future risk of diabetes in the baby with an imbalance in B_{12} / folate nutrition in the mother during pregnancy. In our long-term follow-up study at 18 years we have now done a series of tests including glucose tolerance. At 18 years we found fasting glucose to be high in 25% of boys by the American Diabetic Association criteria, and 10% in girls. Total rates of pre-diabetes affect 25% of this population and this was associated with the various indices of glucose and insulin metabolism at 18 years. What was interesting is that the phenotype was evident when the children were both 6 and12 years of age – they had higher blood glucoses in the fasting state and a slower disposition of glucose after the glucose load. At birth, unexpectedly, we did not find any association with birth size, but those who had pre-diabetes at 18 years were born 4 days earlier than the controls. In pregnancy, their mothers had lower blood triglyceride concentrations with the implications that maternal under-nutrition was driving this phenotype. It was also the basis of so-called malnutrition-related diabetes to which we contributed our ideas 25 years ago.

The second phenotype is the MRI measured central fat distribution in the offspring. We were able to relate a larger birth size to later higher subcutaneous fat in the MRI when the children were 18 years of age. And contrary to expectations, this subcutaneous abdominal fat was directly related to all the cardiovascular risk factors whereas visceral fat mass was inversely related to these risk factors! This seems to be an age effect because a large study reported from the United States showed similar associations of subcutaneous but not visceral fat with risk factors in children whereas in the parents the associations are the other way round[cii]. So it seems to be an age related effect.

In pregnancy low vitamin D, low vitamin B_{12} and high folate in the mother were associated with higher adiposity in the subcutaneous tissue of the children so again indicating that probably genome active micronutrients are contributing to the distribution of the fat in the abdominal area during the intrauterine development of the child. Again maternal and paternal characteristics are involved. Based on this, three years ago we started an intervention in the adolescents in the cohort. When the children were 16 years of age we intervened with B_{12} - 2 microg. per day in one group; B_{12}+multimicronutrients and milk powder in another group, and

the usual standard of care in the 3rd group. We are now into the 3rd year of the intervention. Over 80 girls are married and now, last week, actually 45 have delivered from whom we have obtained cord blood samples + enteral samples for detailed OMIC studies and the cord blood measurements of the metabolome of the B_{12} and folate deficiency.
Another thing that I would like to bring to your notice is that the genotype / phenotype associations, which is what we have been talking about, could be different in different populations. We worked with Andrew Hattersley in a series of studies - so when Andrew described the FTO gene as the obesity gene, which was originally found on the GWAS of Type 2 diabetes, the European finding was that FTO increased the risk of type 2 diabetes by about 27%, but that became non-significant when it was adjusted for BMI. In the Indian study, which was part of our original big cohort, FTO predicted type 2 diabetes with a similar increased risk of 27%. But adjusting for BMI did not get rid of the association[ciii].
Subsequently, a larger analysis in South Asians confirmed our findings[civ]. So the genotype or phenotype associations might be influenced by population-specific factors and I think one of them could be 1-carbon metabolism and the methyl donors in the diet.
Philip James: Ranjan knows that I have had a question as to whether the supply of the essential amino acid methionine from a poor protein intake in these vegetarian societies is also a factor that contributes to the paucity of methyl groups and the lack of lean body mass in these children. My assessment of the Indian diet is that it is really very poor in essential amino acid input. I just wondered to what extent that might be contributing?
Ranjan Yajnik: Yes, I agree. Recently we measured the amino acids in the blood and the number of essential amino acids, including methionine, is lower in Indians compared with the contemporary American women, whereas serine and glycine are higher. And that is where Satish Kalhan (Cleveland Clinic) came in to help to sort this out[cv].
Keith Godfrey: Ranjan, thanks very much. I am interested in your current thinking on why high folate was apparently worse in the setting of low B_{12}?
Ranjan Yajnik: One explanation could be that what we are talking about is not high blood folate levels but the use of folic acid which the obstetricians are using, and we know that folic acid is different from folate. We are currently setting up an assay to measure folic acid as free folic acid to see whether that is contributing. Then we are trying to investigate the changes in metabolism that happens with a high folate and low B12 - one of the things suggested is that there is uracil misincorporation into the DNA which would lead to a whole series of issues. Do you have any other explanation?
Philip James: So you can have a metabolic issue because of diverted one carbon units with a reduction in nucleic acid synthesis. Is it then a question of competition between pathways?
Ranjan Yajnik: Yes indeed!.
Irv Rosenberg: I don't know why the effect of high folate would be related to high folic acid in this population, unless they are getting some synthetic forms of folic acid.
Philip James: My understanding is that physicians in India sprinkle folic acid by the gram-load into the diet of pregnant women!
Ranjan Yajnik: That's true! Each tablet of folic acid on the market is 5 mg of folic acid.
Irv Rosenberg: So these women are not getting the current WHO recommended dose- this measure was before they might be given the WHO recommended.
Ranjan Yajnik: No, the official National Programme recommends giving them iron and 500 micrograms folic acid.
Irv Rosenberg: OK! So this could be folic acid itself! I would remind us all that folic acid is not the natural form of folate - all the data that showed a potentially adverse effect of high folic acid in people with B_{12} deficiency was actually dealing with a different issue than the masking of pernicious anaemia - all those data came from people that had been treated with high doses of synthetic folic acid, not folate. So I think there is the possibility that high amounts of folic acid have a different metabolic fate and exert a different adverse effect at high doses. This is a very different effect from the effects seen with lower folic acid doses used in the original prevention of neural tube defects.
Charlotte Ling: I just want to comment on the fact that in people with diabetes we see - if we also take tissues from case controls where about 95% of all significant CpG sites, based on an EWAS, are actually hypermethylated. So there is decreased methylation and now our hypothesis is that that is due to decreased s-adenosyl methionine or folate levels. So can you comment on that in relation to this folic acid versus B_{12} effect and whether the hypermethylation we see is due to the nutrient supply of the methyl donors?

Ranjan Yajnik: I don't know about this diabetes situation, but in diabetic embryopathy Ericsson in Uppsala has worked on this in relation to foetal development and the same issue of methylation[cvi].
Patrick Stover: What is really missing in the field is to show that folic acid actually has some biological activity. There is no evidence that it has any specific activity itself i.e. it is inert in terms of the hypermethylation process. There are a couple of papers - and it hasn't been followed up rigorously - but lysine-specific demethylase (LSD1) i.e. a histone demethylase is a folic binding protein[cvii] that generates formaldehyde and it is not clear how much the activity of LST1 in vivo actually requires folate to demethylate histones and whether that changes DNA methylation. So folic acid may not only have an effect on the supply in terms of s-adenosyl methionine but also have an effect on rates of demethylation in specific situations.
Anna Krook: Keith Godfrey: your slide suggested that if you are born with 60% of your PDs, 1α promoter methylated then you stay that way for 10 years - is that correct?
Keith Godfrey: Of the nine different CpG sites there was variation between those 9 different CpG sites and the individual methylation levels were remarkably stable in the samples over that period of time. There was variability, not within the subjects, but between subjects and that variability between subjects predicted later adiposity; the methylation patterns were already there in cord blood at birth.
Anna Krook: So do you think that is epigenetic and it is fixed, or it is genetic, determining that this will be your level of PDs1α throughout methylation?
Keith Godfrey: From the work we have done with Peter Gluckman in Singapore, we are not yet seeing these as being meth QTLs, which are being genetically determined - we are starting to see maternal exposures that relate to these perinatal variations in methylation.
Philip James*:* So this is not a response to being fat? It is a predictor of children who become fat?
Keith Godfrey: Exactly, right, and there are broadly two groups of kids we see who become fat. There are those who are fat at birth by DEXA scanning, and stay fat at 4, 6 and 8 years, driven by the route of maternal obesity, gestational diabetes and excessive gestational weight gain. But there is another very important group that is perhaps analogous to Bass Heijmans' findings in his Dutch famine studies, where the babes are thin at birth and progressively put on adiposity during childhood. That is a different epigenetic signature reflecting different maternal drivers for example with maternal Vitamin D insufficiency in pregnancy. And that may be triggered by appetite or it may be triggered by metabolic changes.
Charlotte Ling: So Anna Krook touched on this in her talk emphasising that some methylated regions are very dynamic and change due to environmental exposures, while what you show here is stable and this is something puzzling. So do you think the methylation you see is stable, throughout different tissues.
Keith Godfrey: This is work in progress, but for some of the methylation sites we have been looking at, we see identical associations between umbilical cord tissue and cord blood. The buccal DNA work is still coming through. Undoubtedly there are going to be some marks where they have absolute tissue specificity. I think that there are some that are observable across a range of tissue types and others that aren't.
Peter Gluckman: We have been doing some repeated epigenetics on buccal smears in a longitudinal study of infants in Singapore - the same cohort we have been talking about. And we see some CpGs that are stable over the first 18 months of life but others change in the first few months of life in one direction, and others change in another direction in the first 6 months of life. Whether it is related to infant feeding or not is not clear, given the nature of breast-feeding in that population. Most of the population is partially breastfed from birth or soon after birth. But that is what we are trying to resolve at the moment - the effect to which some of these changes in early life are influenced by breast-feeding.
Philip James*:* As you know people talk about the 1000 days as being crucial so as you look at the flexibility of these markers, do you see that much greater flexibility during that first two years - so that you can say, "oh well, perhaps there are effects being induced by diet during that period?"
Peter Gluckman: I showed some of the famine data in Jamaica this morning where the effects have clearly been determined in later life by external events which happened in early life - there is no doubt that in that data set that's what we were seeing. But that is an extreme phenotype. I think what is becoming clear from the kind of work that Keith Godfrey talks about, the work that we do, some of the work from the Gambia, and even from Bass's work on the Dutch famine is that we are dealing with a normative range of ecological exposures in

early development. There are echoes that persist throughout life which are at least reflected in epigenetics. However, I think it is a big shift to establish whether the epigenetics is simply a phenotypic phenomenon or whether these changes are mediators of metabolic change. It is hard to prove the mediation effect from clinical studies but there are some experimental Japanese studies in the infant rat which clearly show that epigenetics are mediators of some of these lifelong effects.

***Robert Waterland*:** Just following up on Anna Krook's comment - Keith, you said that you had been searching for MQTL associated with these differences, but with such a small range of less than 10% methylation it is going to be very difficult to detect any intra-individual variance MQTL that might be explaining those differences.

Keith Godfrey: Just to clarify: these are the cut points for the thirds of the distribution. So across the whole distribution, you are seeing variation of 30% to 40% - and for the RXRA variation there is an even greater spread of methylation that we see. There are other areas - and we have looked at 100s of them where there is very little variability between individuals. This is using your sort of approach of focusing on those where there is the greatest variability between subjects.

The Dutch famine studies

Philip James. I have always been concerned about the finding of obese offspring in the mothers who conceived during the Dutch famine because if the women were heavier at the start of the famine they would be the group to maintain longest the capacity to conceive so perhaps the data reflect differential fertilisation of sub-groups of women with a greater or smaller propensity to familial obesity? But Bas Heijmans has now observed markers that relate to the early blastocyst which might imply a genuine epigenetic effect of the food deprivation

Bas Heijmans: It is moot point whether they are genetic signatures of selection but we don't have the data yet. What we did do is we measured GWAS SNPs associated with higher body mass index with the idea that if the offspring we are studying have higher frequencies of alleles that are predisposing to overweight - those alleles from their mothers could signify some kind of selection of mothers with higher obesity. We found no evidence of that. But we also had to admit that the power of that analysis and the sample size is so small, that it is not very strong evidence.

Andrew Feinberg: We actually have data on the same group of individuals that I didn't present that answers your question. We have been examining 780 patients who were exposed to food scarcity in the first trimester of foetal life and compared them with those who were exposed later in pregnancy and the controls who were conceived after the siege. Then we have their children and even the next generation. We have done genome-wide analyses and also methylation analysis which I did not present as I knew Bas was presenting on the Dutch famine. But I can tell you that we see epigenetic changes even adjusting for the weight of the mothers, so if there is a genetic predisposition to being obese, at least it wasn't reflected in the weight of those mothers during pregnancy.

Bas Heijmans: Actually Mats Rudling here has just had to remind me that our study design inherently corrects for this issue because we compare siblings. So we compare exposed and unexposed individuals from the same mother. So I think it is not perhaps the selection of the mother, but I can imagine that it is the selection of the embryo. So that it is not a DNA methylation change but might be a selection for an embryo with a certain epigenomic or genetic background.

Andrew Prentice: I used to be interested in 'thrifty genes' and was hopeful that we might be able to detect a predisposition amongst some mothers to be able to conceive in our hungry season. We get a highly seasonal difference in birth conceptions in the Gambia and we have done the same analysis with a quarter of a million babies in Bangladesh and found the same highly seasonal conception rates. So that begs the question that you, Philip, raised - is it a selective effect? Now because mothers in the Gambia have up to 10 to 12 babies, or they used to, we were able to ask the question, do specific mothers have a greater tendency to be able to conceive in the hungry season or not? At first I was very disappointed that we could find no evidence to support that at all, but now, of course, we are using the research for looking at the epigenetic season of conception. So our findings are rather similar to what Bas and others have just been saying.

***Philip James**:* But what proportion of conceptions are coming in the hungry season? You showed astonishing differences in mortality and therefore, logically, if that hungry season is part of a seasonal cycle persisting for thousands of years I expect you must have extraordinary genetic selection unless this is really a very small sub-group of society born during that period. Am I making any sense?
Andrew Prentice: As we all know the hypothalamic pituitary axis gets shut down by starvation, and this comes out very clearly, both from our huge Bangladeshi cohort, and from our Gambian data. Actually babies conceived in the rainy season when the mothers are hungry are bigger babies at the end of pregnancy because they have their second and third trimester in the harvest season. So there are some very complicated growth and developmental dynamics. And just to make it even more complicated, there is a huge difference in twinning rates, according to season as well. I want to have a chat with you Jo (Nadeau) and invite you to come and work with us, because we have had trouble getting this published because the evidence that we have is that we also have a seasonal variation in monozygotic twins and that is a real problem to get your head around i.e. how that occurs because these differences are so big. Our first thought was that it reflected differential mortality in the early foetus, but the sums just don't add up for that. So we have got some very intriguing issues in relation to the sorts of things I think, Jo, you were talking about.
Peter Gluckman: I think there are some issues we need to think about. Humans are unusual in that, unlike most species, we do not completely suppress ovulation during famine. Most species - completely reduce fertility but humans don't. So there is an evolutionary selection to maintain fertility to some level in humans during famine. At the best, it is reduced by about 60% - I mean if we look at Ethiopia, if we look the Chinese Great Leap Forward with good data - the numbers are that fertility rates are reduced by about half.
***Philip James**:* And actually you have to get pretty low in your BMI don't you before you shut ovulation off?
Peter Gluckman: Correct. Then the second point I want to raise is something which people forget. We pointed it out first, and then Andrew did a good study in the Gambia - gestational length varies a lot according to the season of conception, or the season of birth - nutritional state at conception - there is roughly a two week difference in gestational length - it might be 10 days, Andrew in your data[cviii]. Similarly, if you look across populations, the gestational length in India is less than the gestational length in Northern Europe.
***Philip James**:* And that gestational length is determined by early events?
Peter Gluckman: Well, we showed that in sheep it was affected by nutrition around conception, and Andrew came to the same conclusion based on his seasonal studies in the Gambia. So we tend to forget that we do not know the consequence of prematurity, but if you take the work of Wayne Cutfield[cix] and others, they have seen far bigger long-term effects of prematurity on epigenetic change and prematurity on expression changes in the offspring than that seen in relation to birth weight. And I think that there may be a set of phenomena that come from shorter gestation and the earlier appearance of a neonate which may be magnifying and changing what we see. So it may all be triggered by what happens at the beginning of pregnancy and the mechanisms may involve a cascade of events.
Stephen Beck: I would like to pick up on the theme that Andrew just raised: the possibility of a thrifty phenotype that might play a role here. The question is whether there is now evidence that there is some sort of hitchhiking whereby the advantage gained from being able to conceive in a starvation period might well bring added susceptibility to schizophrenia. So if what you actually observe here is a hitchhiking effect and whether in these individuals who have now been followed up for several generations - whether any advantage in metabolism has become apparent, if that is measurable?
Peter Gluckman: We published data to show that there is a selection advantage to responding to pre-natal malnutrition. The marasmic children don't die so readily whereas the kwashiorkor children do. They have an epigenetic difference although we haven't done the gene times the epigenome interaction yet. These marasmic children are born smaller, in response to pre-natal undernutrition and they have an advantage in famine - they don't die. In Ethiopia the children who die first in famine are the children with larger birth weights.

Early environmental exposure and later mental health.

Andrew Feinberg: There is one other thing I want to mention about phenotype which I don't think Bas mentioned i.e. that there is a double incidence of schizophrenia among those children who were conceived during the famine but we can't explain that and the sample size is too small and I don't think that he can either. But there was a similar exposure in China during the Great Famine i.e. Leap Forward when 30 million died. The survivors of that period have the same doubling in the incidence of schizophrenia and I guess it is possible that there is some genetic selection that survives through the exposure, but Ockham's Razor would say this is some kind of exposure-related increase in the frequency. And don't forget that the single leading contributing factor to psychiatric disease that we know - even for psychotic diseases like schizophrenia - is trauma, that is early life trauma, not necessarily pre-natal, but it is early life trauma.

Keith Godfrey: We have got to be clear that schizophrenia is a disease, not a normal variation. We have just published data suggesting that normal variations in pre-natal brain development have a much stronger influence on later brain function i.e. cognition, executive function and poor ability to learn because they don't engage with education as well as children. Previously it was thought that educational achievements to be largely driven by post-natal influences but using epigenetic markers, we have suggested that in fact there is a much greater pre-natal component which means that it might confer these selection advantages later[cx].

Philip James*:* OK, that is profoundly important because the Jamaican evidence was the first to also show that postnatally the combination of play therapy was actually somewhat more important, but when combined with good nutrition, there was a dramatic improvement in what we called the capacity to cope in different dimensions of sensory motor executive functions. Is that right?

Keith Godfrey: Yes that is Sally Grantham-McGregor's data[cxi]. We have got to be careful about extrapolating from the famine situation to the non-famine situation. There have been huge analyses done of season of conception. So if you look over the last 50 years, conception rates have completely flipped in the Northern and Southern hemispheres and are now being driven by social factors having been previously driven by biological factors. This applies to the entire populations of Australia and other Southern Hemisphere populations and European populations. The seasonal pattern has flipped over the last 50 years.

Peter Gluckman: I want to pick up on the point that Keith made. If Michael Meaney had been able to be here, he would explain better his work with Keith (Godfrey) and myself in this cohort in Singapore. We are finding that relatively subtle changes in maternal mood within the normative range in unselected cohorts are having effects on brain structure in the infants - hippocampal volume at birth, measured by MRI; on functional changes in attention and cognitive emotional function in the child and that these effects are influenced by things like the brain derived neurotrophic factor (BDNF) polymorph status of the child and influenced by epigenomic interaction. So we have mild changes in maternal mood (just picked up on the Edinburgh rating scale which is done on every pregnant women in Singapore) relating through to anatomical changes in the brain. The functional changes in the brain seem to be mediated by 6 genomic variations in the form of at least BDNF polymorphisms and are reflected in changes in epigenetic states. So while I am a great fan of epigenetics I think we are starting to realise that we can see a lot of things if we study well phenotyped normative populations.

Barry Keverne: Given these changes in utero on cognitive function and emotional behaviour how long do they last postnatally? Is it due to a slowing down of development, or is it a pathology in actual development?

Peter Gluckman: We think it is a pathology in active development - that doesn't mean that plasticity with play therapy and cognitive therapies cannot in time overcome it, but the children we have studied are only 5 years old now and the pathologies and the behavioural deviations remain. Now Keith has got some older data from Southampton and may be able to comment further.

Keith Godfrey: We have looked at 9 year old children and found different domains of function which relate back to different time periods of brain growth and development. By this I mean differences in language development, performance development - they all have their different associations with periods of brain development in different parts of the early life course.

***Philip James**:* But I understand Barry that you and Michael Meaney have done animal experiments showing the effect of early maternal behaviour on the pups - is that right?
Barry Keverne: Michael has done that far more than me; I have really been looking at brain development. I do look at behaviour as well in the adults. But I think that the important component about *in utero* development that we need to think about is the placenta. What a lot of people don't realise is that there is a coordinated function between the lower limbic areas of the brain, including the hypocanthus and hypothalamus and the placenta. We have been doing work with imprinted genes, genes which are expressed according to parental foraging, either maternally expressed and paternally silenced, or *vice versa*. One of the things we found is that, first of all, if we do a very global change - that is to say we change all of those genes by making cells either parthenogetic with only maternal alleles expressed or androgenetic, with only paternal alleles we find this is, of course, a gross and lethal manipulation. But you can make chimeras where just a few cells have the markers so you can track them as they go into the brain as well as into the placenta. The outcome of that is that if we look at what is happening in terms of brain development, with respect to the placenta, the parts of the brain - that we call the emotional brain that is developing primarily *in utero* - is developing at the same time that the placenta is talking to the mother's hypothalamus. Now the placenta talks to the mother's hypothalamus in quite a complex way; it actually gets the mother to think ahead, you might say, because what it does is it increases mother's feeding behaviour in animals, long before there is any energetic requirement for it; if you had to wait until there is an energetic demand, mother simply couldn't eat enough, so the foetal placenta tells the mother to eat. It shuts down the mother's fertility, stops ovulation - no point in being fertile if you have already got a baby *in utero* - so this is the foetus now that is talking to the mother. It also primes the mother's brain for maternal care so that what happens is the mother - before she has any offspring in most species - starts being maternal, starts building a nest before they have apparently become cognitively aware of being pregnant. It also primes the mother's brain so that as soon as the infant is born she is immediately maternal. And it does this by producing hormones that act on the mother's brain. Now what is interesting is that imprinted foetal genes are expressed at the same time as the placenta is talking to the adult hypothalamus - it is that same gene that is developing the foetal hypothalamus. So here you have an intergenerational effect that can be selected for, in evolutionary terms. In other words, if you ask the question "how does the mother's brain know to do these things in advance of actually seeing or being aware of an offspring the answer is that there is this co-adaptive process gong on. Now it could occur through infant genes but it could also occur just by natural selection i.e. by a Darwinian natural selection. But I think this is more appropriately termed what we might call Waddingtonian natural selection: it is an epigenetic process. If you think about it, you have got two separate things happening here: you have got a hypothalamus which is going to mature and have its affects when adult and you have got a placenta which is acting *in utero* when intergenerational processes are happening. How does the hypothalamus developing *in utero* know how it has got to develop to perform when an adult? The only way that can happen is by the coordination of the infant genes that are co-expressed in the placenta and in the developing hypothalamus. Now we have done knock-out studies: first of all some 90 imprinted genes are expressed in the placenta and a number of these are co-expressed in the developing hypothalamus. If you do reciprocal crosses, which you can do with imprinted genes, so that you can have the lesion in the brain and not in the placenta, or lesion in the placenta as a gene lesion and not in the brain. What you find is the phenotypes are remarkably similar. So you have got this co-adaptive process going on of the foetal brain developing at the same time as the placenta is talking to the mother's hypothalamus. If it gets it right, then it will get the development of the hypothalamus right as well, so you have got that link between the two. So, of course, we are talking about an area of the brain that is functioning in the early developmental process and in regulating emotional behaviour. But, of course, the brain is much more complex than that and most of the cortical development occurs post-natally where we also know that imprinted genes are involved.
Joseph Nadeau: Why is it necessary that the imprinted genes are involved - why do you need to involve imprinting?
Barry Keverne: It could occur without the imprinting genes, but it would take, in evolutionary terms, an incredibly long time to get it right. If you think about it - if something goes wrong in the placenta then of course you can lose the offspring - that is a big cost to the mother. So if

anything goes wrong with the placenta being able to communicate with the adult brain, things will go wrong.

Joseph Nadeau: But I don't see why imprinting still needs to be involved - you could imagine figuring out how to do this without imprinting. There are lot of communicating systems that involve imprinting, so I am not saying that it doesn't happen. I am just trying to understand how it does, why it does and what your thinking is.

Barry Keverne: You don't have to have imprinting, but the fact remains you have got it - it is there, you see this with your reciprocal crosses. The question is then why, what are the advantages of this should we say? My feeling is that the advantages of this, in evolutionary terms, is that it will evolve much faster. If you are thinking about errors occurring randomly, say, in the hypothalamus and placenta you are going to get a loss of the expensive foetus. It is going to take a longer time to get those events synchronised without this imprinting and so that you don't get any foetal loss.

Miguel Constancia: In marsupial studies how do you see your placental brain system working when the placenta is very primitive and there is a very short gestational period?

Barry Keverne: Marsupials actually have imprinted genes, but most of marsupial development occurs post-natally rather than *in utero*. I don't know whether you have seen the marsupials at birth - they are about as big as your thumbnail and they are extremely premature so a lot of that development occurs in the pouch - far longer in the pouch rather than it does in utero.

Peter Gluckman: In the marsupial the breast milk changes are much more dynamic than in the human. There are a lot of changes in milk composition across time in the marsupial – dramatic changes in the protein content in ways we don't see in humans which suggest that there is on-going signalling between the mother and the suckling marsupial by a different mechanism. But it is probably related to the same phenomenon that Barry is talking about.

Juan Rivera: In Mexican babies we have seen some examples of the effects of acute starvation during conception and gestation. But very relevant to developing growth now is the problem of chronic undernutrition - that is really the condition in which most countries live. So there are some data - and this is really epidemiological data without mechanistic information from, for example, the 5 birth cohorts being studied in Brazil, Guatemala, Philippines, South Africa & India. These cohorts are being followed up to adulthood[cxii]. They have been looking at the relationship between birth weight and the appearance of growth during infancy and then later outcomes. For example, the higher the birth weight the lower the risk of hyperglycaemia; the lower the risk of not finishing secondary school, but the higher the risk of obesity and hypertension. So there seem to be some trade-offs. And the same thing happens with length gain from birth to two years of age - that leads to improvements in human capital, for example in a reduced risk of dropping in height scores and preventing increased risks of obesity and hypertension.

Philip James*:* So that is post-natal?

Juan Rivera: This part is post-natal, but the higher birth weight reflects pre-natal events and leads to an increased risk of hyperglycaemia, but also a lower risk of obesity and chronic diseases e.g. hypertension.

Cutberto Garza: If I recall correctly, in that study, when they look at those trade-offs, the author is very clearly saying that there is no choice. In fact you want normal growth because the risks were minimal when they looked at size effects compared to the pathologies that you see with low birth weight and small length gain[cxiii].

Caroline Fall: In this COHORT group multi-country study a lot of the link between high birth weight and later obesity relates to an increased lean mass; a lot of other literature shows that birth weight is more strongly related to adult lean mass than to adult fat mass

Peter Gluckman: I think we are getting too caught up on birth weight. There is no doubt that there is a U-shaped relationship in birth weight in relation to risks. Larger babies tend to occur with diabetic mothers and infants of obese mothers are on one pathway to obesity that doesn't have an adaptive origin because I would argue that this is a modern, evolutionary, novel phenomenon. But the low birth weight is part of the continuum within the normative range, given that over time humans have largely lived in relatively known nutritional environments and maternal constraint has been part of the regulation of foetal growth anyhow. The explosion in the DOHaD field over the last decade has been in the recognition that most of these programming or epigenetic phenomena are effectively independent of birth weight. And in fact we are seeing lots of epigenetic changes that are entirely independent of

birth weight - their effects on cognitive function, cardiovascular function and so forth. There is also a lot subtler signalling with health consequences.
Now those of you who looked at me askance when I said gestational diabetes was non-adaptive let me explain: my suspicion is that gestational diabetes was relatively rare until recently. And the argument for that is, of course, that insulin resistance is the normative part of pregnancy, with placental growth hormone and lactogen inducing a level of insulin resistance to allow glucose to transfer across the placenta. But unlike other nutrients, in particular amino acids, there is no absolute limit to the amount of glucose that will pass the placenta, so the foetus will just get bigger and bigger and bigger. The more glucose that is circulating and crossing the placenta due to maternal adiposity the bigger the baby and these events can lead to the death of both the mother and the child if it is unrestrained. So I think evolution would have selected against gestational diabetes leading to foetal overgrowth, had it been a common problem in the past.
***Philip James**:* Let's come to that whole societal shift because that is a huge issue now in some parts of the world.

The development of the diabetes epidemic.

***Philip James**:* We discovered years ago in both Mexico and Asia that the development of diabetes is 4 to 5 times greater at any body mass index than in Caucasians brought up in the West. But now we have an even worse problem of phenomenal diabetes rates in pregnancy and we already know - from numerous studies around the world - that this has enormous implications for future generations.
Peter Gluckman: In Western populations the instance of gestational diabetes mellitus (GDM) is roughly between 7-8% and 10-12%, depending on the population. That is what it was traditionally said to have been. There have been some redefinitions based on what happens to the baby, as opposed to just using a biochemical test in the mother, and that incidence has now risen. But when one looks at Asian populations, we are now dealing with 30% of pregnancies with gestational diabetes in Shanghai and 30 or 35% in Hong Kong. In Singapore the rate has risen from 10% to over 25% in less than 20 years: these are dramatic changes. Now everybody assumes that it is just simply obesity. But it is not. If you look at Singapore, the highest rates are in Indians - at about 25-27% of the population; the lowest rates are in Malaysians at about 12-15% of the population, but the Malaysians are much more obese. Now admittedly there can be differences in the visceral obesity - as Ranjan has talked about before - but it is more complex than that. There do seem to be differences in the biology of Indians versus Malaysians and Chinese that may be playing a role in terms of muscle biology and so forth. The problem is that gestational diabetes, although relatively reasonably easy to manage in the short term from the maternal perspective, involve offspring that are prone to get both diabetes and obesity themselves. So we actually have a feed-forward intergenerational system in play. A calculation been made in the Canadian First Nations people that some enormous fraction of the high rate of type 2 diabetes in the adult population there has its origins inter-generationally. So I think that we in Asia - people like Ranjan who has done a lot of work on this and myself - are really worried that gestational diabetes and its consequences for the next generation is going to become a No. 1 public health issue.
***Ranjan Yajnik**:* All the studies in India show that gestational diabetes prevalence is almost equivalent to the prevalence of diabetes in younger adults. So it is not only gestational diabetes, it is diabetes in the young people. The problem is that we don't measure glucose before gestation. We measure glucose in gestation because it is now part of the standard of care.
***Philip James**:* So you are implying that young women before they become pregnant are pre-diabetic or actually have diabetes?
***Ranjan Yajnik**:* Yes, before gestation. I have now studied 200 of the 1000 women I have treated over the last 20 years within 6 months after delivery. Now gestational diabetes is usually thought to disappear after delivery and then increases the risk of diabetes in later life. The standard textbook figures state that by 15 years, 50% of women will have diabetes, and that is how the original O'Sullivan definition of gestational diabetes, although relating to the concern for the baby, was also concerned with future diabetes in the mothers. Now ten years ago, we published that at an average of 4.5 years after delivery, 70% of women in Pune India,

who were originally diagnosed with gestational diabetes, had already developed post-pregnancy either pre-diabetes or diabetes. That was within 4.5 years. Now I have studied them within 6 months of delivery and 42% of them have pre-diabetes or diabetes. This strongly suggests that almost half of what we are calling gestational diabetes is actually pre-gestational diabetes. Now this turns the story completely around because we have been talking the whole morning about pre-conceptional programming and gestational diabetes. But now this prime focus on pregnancy is a classic example of what we should **not** be doing: we diagnose at the beginning of the 3rd trimester because there is a pharma-industry driven awareness campaign amongst doctors and it increases the market for various anti-diabetic drugs. If it is post-conceptional, then the programming of the baby has already happened – the baby is moving on a higher trajectory of growth and in the 3rd trimester we introduce what provocatively I am going to say is a starvation treatment - and we reduce the growth rate which I can call intrauterine growth retardation. So we introduce iatrogenically one more risk factor and I think this has exacerbated the risk of diabetes in the children because we diagnose the problem at the wrong time and I suspect we are giving the wrong treatment. This is classically seen in the genetic model of glucokinase gene mutations - glucokinase is a mild, relatively benign diabetes. The girls usually get diagnosed first as having gestational diabetes. Treatment of gestational diabetes produces IUGR in pregnancy - Andrew Hattersley has a big study on this[cxiv].

Now, coming to treatment: Peter (Gluckman) said it can be effectively treated and he is hoping that it will reduce the risk of obesity and diabetes in the children. There are two major trials which have reported: one was in Australia, the ACHOIS Trial[cxv] and the second one is the Landon Trial in the US[cxvi]. Both have now partial follow-ups in childhood and show that the treatment as it was done has not reduced obesity in children - at the time it was measured. Whether it will do so in future, we don't know. So I am proposing an unpopular view that this is a case of mistaken diagnosis: we need to diagnose pre-diabetes before conception and prevention and treatment should be pre-conceptional rather than during pregnancy.

The average birth weight in India is 2.7 kg. The average birth weight in a gestational diabetic pregnancy in my unit is 2.85 kg. So all the problems related to large birth weight in the Western world, i.e. large for gestational age or macrosomia are absent in India to a large extent and therefore the Western statistics are not readily transferred to lower income countries, especially those which have low birth weights due to multigenerational under-nutrition.

Andrew Feinberg: I have a question for the public health experts, based on three really remarkable comments that people have made in the last half hour. One was Patrick who said that we are using food as medicine instead of medicine as medicine; one was from Villy (Vilmundur Gudnason) who said that there has been a fall in cardiovascular mortality I think by 80% mortality; and then one was by Bo (Angelin) who said that the cardiologists of all people who should understand the benefit of statins, refused to understand the benefit until they could see the arterial dimensions changing. So my question is, should people like us who are working on epigenetics be thinking about drugs for gestational diabetes? I mean real drugs that would be used on a population basis the way that statins are? I mean gestational diabetes is one of the classical examples of epigenetic effects, based on imprinting, evolutionary analyses and a lot of clinical evidence. I think that has not been on the radar screen - should that be something that our community should be thinking about?

Charlotte Ling: I have done experiments where we exposed beta cells to high levels of glucose and we can then show that we get increased DNA methylation of the PDX promoter and the insulin promoter. Then we also showed differential methylation of these genes in diabetic islets. I think also the work from Rebecca Simon's group suggests the role of epigenetics during development. I think we are thinking of Andrew Feinberg's issues and the potential of using epigenetic editing of these genes. So if you get a defect *in utero* that you bring with you, you potentially could use targeted epigenetic editing approaches to affect that. I also think that both the drug industry and several of us are thinking a lot about molecules affecting epigenetic enzymes that potentially could restore the defects that have been caused by early childhood.

Epigenetics and the Implications for Public health

Philip James: We have spent quite a lot of time on laboratory work and then moved on to issues such as the Dutch and Chinese famines with other data on malnutrition in Africa, India, Singapore, Mexico and Central America where we are confronting huge issues of public health with now a marvellous number of children cohorts being studied. There are also relatively new analyses of the global burden of disease highlighting the importance of nutrition. In the International Congress of Nutrition in Rome last November there were Ministers, and Prime Ministers emphasising the terrible problem of malnutrition in the world and the need to produce more food. Then there was another group highlighting the massive epidemic of obesity and diabetes and there wasn't much linkage, actually, between those two aspects of nutrition and public health in the discussion. So where does our new understanding fit with this complex picture?

Caroline Fall: We can't just talk about having enough food to prevent hunger. We need to be talking about the quality of the diet that the mothers are having.

Philip James*:* You just mean more fresh vegetables and so on?

We have heard about folate and then Ranjan Yajnik was insisting that we think about B_{12} Andrew (Prentice) talked about B_6 and choline. So we seem to need to think about the whole food system and have to produce a micronutrient-rich food system which is widely available, whatever that means?!

Patrick Stover: If you look at what is going on with cell phones and big data and these chip technologies, we are going to be able to measure your total micronutrient status for a couple of dollars so these data coupled with genetic information raises al sorts of new possibilities.

Philip James*:* Are you talking about for example in Africa and Mexico?

Patrick Stover: These developments in technology are meant for Africa and Mexico! This is where Abbott is putting a lot of money in - Abbott Nutritionals - these technologies that will collect big data rapidly at the level of the individual. Then the question is going to be what do you want? Do you want to prevent neural tube defects? What do you want to do to chromatin? We don't have those answers, but data are not going to be limiting and very soon it is going to be cheap and you are going to be able to collect it anywhere at the level of the individual. Yet we currently have terrible problems of poverty where, for example, in Africa the general public cannot afford simple and very cheap life-saving equipment. So there is a discrepancy in thinking about practical measures.

Per-Ole Iversen: There is a major problem of food insecurity in Africa and this will require a transformation of the political system which I think pessimistically will take a generation or two!

Keith Godfrey: The harsh reality is that the RCT evidence that your policy makers would like simply does not exist in this epigenetics area. We do not have in the food system field the equivalent of the pharmaceutical drivers which have enabled drug developments to be developed and the margins for the food industry partners are so small that they haven't got much involved. But there is sufficient evidence on the basis of the observational data that we have, if we put the case cogently, to inform policy-makers that there are steps that should be taken, particularly with adolescents, with pre-conception which probably have substantial benefits. There is also a whole raft of trials now coming through from pregnancy in attempts to prevent gestational diabetes but they are all failing! So the Limit Trial from South Australia[cxvii], the Upbeat Trial[cxviii] which we have just had accepted for publication in obese pregnant women in the UK, the EMPOWaR Trial of metformin in pregnancy[cxix] - all have signally failed to prevent gestational diabetes. Probably a large part of the reason is that the interventions haven't commenced pre-conception. Doing these pre-conception trials which Caroline (Fall) has done[cxx] – one of the very few trials- is a very big, expensive undertaking, but as a research community we need to be committed to doing those trials alongside informing the policy-makers that there are policy decisions which can be taken on the basis of the limited evidence that we have and that the epidemic is so catastrophic that we cannot just sit back and wait for those RCTs to come through.

Philip James*:* But I still want to know, on the basis of the limited evidence, what we should do?

Keith Godfrey: On the basis of what we know, educate teenagers together with a whole reorganisation of pre-conception health contacts with women and their partners - all these steps are wise steps to take. I think it is the sort of thing where Peter's (Gluckman's) WHO Commission and Mark Hanson's WHO Scientific Committee for the Commission have been

assembling the evidence about tangible steps that could be done. I think that we need to read those documents; we need to input into the process and we need to come up with a collective view of what can be done.

Leif Groop: Most of these interventions try to improve insulin sensitivity a little bit, because gestational diabetes really is also due to impaired insulin secretion - probably pre-programmed from early life, so it is much too late to start at that stage. From a public health perspective, 40-50% of those women with gestational diabetes will develop permanent diabetes within 5 years so that is what we can try to prevent, and there are means. But we also need to consider the micronutrients - zinc really improves insulin secretion.

Minoo Rassoulzadegan: Animals know what is good for them and they find and then eat suitable foods. So I think by education we can teach children at school the benefit of herbs and all of the little things that our great-grandparents have used the years.

Philip James: I'm very sceptical about the supposed role of health education – we have been preaching this message for 40 years and look at our new nutrition based epidemics!

Caroline Fall: I agree with you that if you sit people down and preach at them about what they should be doing, then that is very ineffective. But there have been huge changes in behaviour in the last couple of decades from people getting knowledge about smoking and about diet in our own country, so I disagree with you that education is ineffective. And the other thing, I just wanted to say is that in our pre-conception trial in Mumbai, gestational diabetes was halved. This was a pre-conceptional trial of green leafy vegetables, fruit and milk.

Boerge Nordestgaard: My background is in cardiovascular disease and a lot in the prevention of cardiovascular disease. What does it take to actually show that it helps preventing disease and what can you do? In these areas there have been lots of failures when the conclusions are based on observational data. Thus, for example, women were told to should take hormone replacement therapy after the menopause because the observational data were so convincing in suggesting a reduction in cardiovascular disease. But after this policy was implemented globally the trials then showed that this was not a straightforward policy as these women also had more breast cancer and perhaps more deep vein thrombosis. So I think if you have a causal factor like tobacco smoking then it is OK to do whatever you can to prevent it. But if you want to alter the diet, you have to be very cautious because you can make things worse.

Philip James*:* David Barker, whom many of you met was very much against trials of nutritional intervention in pregnancy because he felt that we might be doing harm. My response was that by not intervening, we are still making a positive decision to stay with the status quo and all its consequences so the issue is the balance of evidence.

Patrick Stover: I just want to make a comment about education. At NIH they funded 5 education intervention studies. One was on post-partum weight gain; another was on gestational weight gain; there were five rigorous trials (three involving nutrition) and these trials were all based on the latest technology of using social media interventions, intensive interventions with full engagement i.e. all around education and appropriate environmental exposures but they all failed!

Epigenetics and the WHO Commission on ending childhood obesity

Peter Gluckman: I should explain that my main job is as Chief Science Advisor to the Prime Minister of New Zealand but I am also Chair the WHO Commission on Childhood Obesity which has provided one interim report. Sadly there were only 20 submissions from the scientific community to that report despite it being widely publicised. Now that report is based on a committee of scientists on one hand with a working group's summary of the evidence[cxxi]; another committee is looking at implementation issues and my Commission is a mixture of scientists and lay people at the top. We were required to report to the World Health Assembly by January 2016 on an Action Plan for Childhood Obesity[cxxii].

Now, if I could just make a couple of general comments. The one phrase a policy-maker never wants to hear from the scientist is that "more research is needed"! I have just come from running a seminar on science advice to governments for young people dealing with the environment and the first thing I teach them is you never use those words: "more research is needed!" All politicians think is that you are simply making a case for more money!

The second thing is that policy-makers are not scientific referees, so if you cannot produce a scientific consensus around which the community of scientists will work, then you have a real problem. And the biggest problem in the childhood obesity area has been a fight that has gone on for a decade between those who think it is all just about dealing with the obesogenic environment - dealing with diet and exercise and axing or taxing foods, and those who think that there are biological determinants that play a role. Trying to get these two groups to actually talk and work with each other is a real challenge. The whole point of the Commission, which Dr Margaret Chan (Director General of WHO) set up, was actually to deal with that conflict and to try to reach a consensus. Now the reason why a consensus has been reached is because good biological evidence, largely from epigenetics and the cohorts from which those epigenetics were obtained, has proven that there are biological determinants in early life that change one's sensitivity to being obese and insulin resistant later in life.

If you read that report the point is made that there is no magic bullet! There is a whole lot of things that have to be done in parallel which is awkward because politicians actually like magic bullets. They want something that they can flag up and say we did that and it solved the problem. It is not just about food tax; it is not just about nutritional education. It is not about any one thing, it is about a whole lot of things that come together. But if I could just finish on this issue of nutritional education: there is compelling evidence that nutritional education of the right kind, given to young adolescents can have long-term effects. It probably doesn't work very much for adults - as the NIH found out - but it must not be done in a "thou shalt not; thou must do this…." sort of way, - it is best done, say embedded within science education - in a soft way. You can change the behaviour of adolescents - and it has been duplicated several times now - in terms of eating behaviours. It doesn't work so well for drug education; it probably does work - if you get them young enough - for smoking education - but for nutritional education there is good growing and peer-reviewed evidence now that early adolescence is when one can change their understanding. There is good work being done in India[cxxiii], and Southampton[cxxiv] in New Zealand[cxxv], in the Pacific Islands[cxxvi] now with adolescence-orientated lifestyle education to try and reduce the rates of obesity and improve healthy eating.

I think when you come to the point that was raised earlier about micronutrients - I think we underestimate how common micronutrient deficiencies are even in Western populations, as a group. But the way you are going to address it is by shifting people towards healthier eating habits as a whole - it is about food, it is not about pills. I think those are the issues we need to get through. It is complex, but the world can't wait and I think it has been sad that the scientific community has sometimes been too reductionist to actually understand that policy-makers want to act and need to act.

Andrew Feinberg: I am going to challenge you, Peter! I would suggest that we say to the policy-makers we need more research! I am hearing very conflicting information from the people who are the experts - really great experts in nutritional intervention. If we are talking about more subtle things like the long-term health effects of bad nutrition or effects from pregnancy - maybe we don't actually know enough and it is not unreasonable to think about this the same way that we think about it in our labs - there is genetics, there is environment, and there is gene-environment interaction. Maybe we need to start focusing some of the research we are currently doing in the Western developed world - the same kinds of studies that we have been hearing about in the last couple of days in the developing world. I will give you an example: Sarah Tishkoff is a wonderful investigator at the University of Pennsylvania and her Pioneer Award is to study the genetics and epigenetics in Africa where there is a much more diverse population. But she hasn't really been asking nutritional questions but you combine that with nutritional data and you start to collect information. That sort of agnostic but thorough approach might help a little bit maybe.

Leif Groop: We can do something during pregnancy about the anaemia that is quite common during the 3rd trimester; anaemia during the 1st and 2nd trimesters can actually influence foetal growth and body composition and anaemia is a problem we can treat. It is not difficult to treat.

Peter Gluckman: Of course more research is needed, but all I am saying is that there are political windows of opportunity and this is not the way to argue with them. I think we have an unusual opportunity - the one opportunity in perhaps a ten-year period when an issue can get to the top of the agenda. We managed to get NCDs to the top of the agenda through a lot of work in 2011. That flowed through to the WHO picking up childhood obesity and if we can't

get an agreement on a plan of attack - which will obviously involve us in more research - it will not be looked at for another decade. It is just the way the policy cycle works at both a global and a national level. It is not like an infectious pandemic which forces the world to respond.
***Philip James**:* Yes, that is actually true and if one just talks about the obesogenic environment and so on then the Ministers of most of the world are locked into the fact that they have what they think of as a big problem of **under**nutrition and specify that obesity is a Western disease, "but we have got to sort out malnutrition". So what Peter is describing is actually an important, appropriate biological link between those two domains. And it is beginning to come through: by November 2014 even the UN was recognising that there is interplay between malnutrition as they understood it and the development of obesity, diabetes, heart disease and cancer etc. Now we can progressively change societal behaviour by a number of measures including education in a very particular mode and you can affect food supplies through the education of caterers etc. But one of the top priorities in the world now is agriculture and food supplies. We won't go into it in detail but, for example, Ranjan yesterday talked about his B_{12} deficiency which is widespread. Ranjan and I independently calculated that B_{12} inadequacy affects about a third of the world's population.
***Ranjan Yajnik**:* A couple of weeks ago in the adolescent nutrition summit in Portland, Lindsay Allen said B_{12} could now be called the most prevalent micronutrient deficiency in the world with her new calculations - so it is certainly very big and requires different approaches[cxxvii].
Cutberto Garza: I think those of us who have been advising governments have really not forced them to ask the question about what exactly to do about these problems e.g. B_{12} deficiency. You ask what do we have to tell policy makers to shift all of their policies? What the policy-maker is looking for is not a proper solution - they are looking for a simple, quick and cheap solution! And the reality is that we are now in a stage of development where there may be no cheap, simple solutions. H.L. Mencken - a columnist for the Baltimore Sun back at the turn of the (19^{th}) century - had a very astute observation: he said for every complex problem, there is a simple solution that is wrong! But in practice we create healthy children all the time and we don't have obesity rampant in high-income groups. It is a very socio-economically determined phenomenon. The reality is that we know how to prevent childhood malnutrition and each of us does it in our families every day. But when we are asked for one of the different but simple cheap solutions e.g. choosing between toilets, good food, education in poor countries there are very few of us who would be wiling to trade any of those within our own families. Now there are a few instances where one change can make a very dramatic difference. We know those with some monogenic problems are a minority of cases. So the solution to these complex problems lies with those individuals advising governments and asking them first to reformulate their question. If what they are looking for are simple, cheap solutions, we need to just tell them that there are none! Every randomly controlled trial, for example, that has been done, trying to solve the stunting issue in very robust ways have come up with improvements of 3-4%. That is significant and not trivial but we are not going to be able to deal with these massive problems if we focus on one factor.
***Philip James**:* OK, that's fine, but a week ago I was chairing a three day conference for 22 Ministries of Health in Amman, Jordan. I was suddenly landed at 12 hours notice with a meeting seeking to make a major impact on maternal and child health. They identified the key problems as maternal anaemia with a major resulting mortality; small birth weight because low birth weight is something they can measure; under-5 mortality and stunting. We had already got the stunting locked into maternal wellbeing. I had to cope with the Sudan where 80% of mothers delivered outside any healthcare system at all. The same applies to the Yemen, Somalia and Djibouti not to mention Pakistan and Afghanistan! But the whole question of access to food and what appropriate foods are available, and what one does in a health system that barely exists, is exactly the same challenge for Tanzania and Uganda and Kenya, Central Africa and elsewhere. We actually came up with a number of key measures on the basis which are actually fundamentally locked into nutrition and it involves big policy measures and also involves, if you take Peter's (Gluckman's) challenge on childhood obesity, the status of young women before they become pregnant.
Peter Gluckman: And of fathers too!
Philip James: Yes, of course, but the problem in the Middle East and indeed in several different global regions is putting women right up the priority agenda. So the position of women in society becomes a fundamental societal issue.

Bo Angelin: I think we really underestimate the spread of information technology; and by accumulating big data get a much better picture of what is developing in the world. So we shouldn't underestimate the fact that the use of mobile phones is spreading extremely rapidly and that is obviously promoted by completely different forces where there is a lot of economic initiative. Through this access, we can indeed reach this early adolescent population. So you can actually spread knowledge if you want in a non-linear way. But if you want to do that, you also have to accept some of the problems with this situation. Nevertheless we could spread extremely good messages and then the issue is to make sure that we counter in different ways the spreading of a lot of bad messages too with the same system.
Keith Godfrey: The potential for using these apps and social media is immense, but we, the scientific community, are not doing our populations a service by not engaging with developing those messages with psychologists and subjecting them to proper randomised trials. In the UK the Economic and Social Research Council has effectively stopped funding trials of these approaches because the overall results have been so disappointing to date because they haven't been done on a sufficient scale, they haven't been done with the right conceptual framework and they haven't been evaluated properly. So if we are going to utilise this to move forwards, we must engage in these RCTs and we must utilise all the knowledge that comes from psychologists.
Juan Rivera: The model that we have been using in Mexico and other Latin American countries is to take the best available evidence – for example relating to under-nutrition where we have a lot of evidence on how to reduce stunting, anaemia and so on with micronutrient supplementation or fortification, measures within primary health care, using cash transfer programmes for improving living conditions and so on. The other very important thing that we have been doing is evaluating the impact of these programmes and learning from that evaluation because we cannot wait to implement policies until we have all the evidence. We are doing exactly the same with obesity - we are using the best available evidence and now we are evaluating the impact of food taxes, the impact of regulation in schools and regulating food marketing.
Andrew Prentice: I am very impressed by the fact that with the economic transition in South America most of the issues of stunting and anaemia have disappeared, or are rapidly disappearing. Now of course, the flip side to that is that the issues of obesity are rising! And if you draw a chart of the incidence of stunting versus obesity in different countries across South America you get this beautiful reciprocal relationship. There are only a few countries that lie at the nadir and that may be temporary. Cuba, for instance, and Jamaica actually is in good shape at the moment but probably they won't be there for very long.
Nevertheless I am very much in favour of some of the comments that Andy and Cutberto have made that we really have to continue our research - despite what you say about policy-makers hating this issue of "we need more research". With the epigenetics field I feel we are on the threshold of understanding something completely different. At last we are beginning to understand the mechanisms and if we can get that then we have a really big story to take to the policy-makers and big stories sell!
I feel I am on the verge of having really good evidence and that within a few years we can get some very much better answers. My answer to policy-makers at the moment is all about motherhood and apple pie! You can tell them sensible things about eating a varied diet, maintaining a healthy body weight etc. which is what, as Cutberto said, all that the higher socioeconomic groups do - we can propagate those messages and we should be propagating those messages, but in this epigenetics meeting I think we are on the verge of something really special where we can make a quantum leap.
Kaare Norum: The successful practical policies cannot be applied in every country, but in Norway and Scandinavia and so on, we have some rules that help us. You can prohibit people smoking and the smoking has gone down. You can tax alcohol and alcoholism is going down. When you subsidise the cost of fruit more fruit is eaten, and you could tax sugar and saturated fat and put that into policy. And this could be a nil cost effect because you use the tax to subsidise other things combined with school education for children between 9 and 14 years old when you can really influence them on diet. When you teach them in school … you give them free fruits and so on. So we can do lots of things in Scandinavia and we should be trying some of these systems all over the world.
Charlotte Ling: I must come back to the question of randomised controlled trials but if we have good evidence of real changes e.g. molecular proofs then this is a powerful message. So, for instance, we did this exercise intervention study that showed that in adipose tissue,

one third of all genes changed epigenetically and I just published the paper and thought that's fine[cxxviii]. Then I went on vacation and the whole thing blew up with extraordinary global interest in the fact that 6 months exercise changed one third of your genes epigenetically! So I don't think the same has been done for dietary factors. So I am now part of some trials of genome-wide epigenetic analysis in randomised controlled trials on for instance saturated versus polyunsaturated fats. So if we have molecular mechanisms, maybe we have a stronger proof for the policy-makers and even with our children!

Joseph Nadeau: Let me be difficult! Imagine that we could learn everything that we ever wanted about epigenetic changes, histone changes, methylation changes, whatever. Fine but I am not sure that that will translate nicely or effectively into anything relating to public policy that changes the obesity rates or disease rates in the world. I am not saying that we shouldn't do the research we do but we also need to know how it helps so we can make a case that knowing that will make a difference.

Keith Godfrey: Jo's statement is poignant - knowledge of mechanisms can help us to know where we should be investing our resources to effect change. And I mean if you can use epigenetics, for example, to look longitudinally from pre-natal studies with then analyses of foetal circulating DNA in the mother and changes found at birth then we can start to say whether the interventions before conception, pregnancy, early childhood, later childhood etc. are likely to be effective. And you can use the epigenetic marks potentially as outcome measures for intervention studies that can bring down the time scale for informative policy to be derived from those RCTs. You can also identify new interventions - and that is exactly what we have done with a new pre-conception intervention - from the knowledge of the transcription factors that are binding to these particular sites.

There is another policy area that involves working with the food industry and it is something that nutritionists have typically shied away from. But there are data that the food industry has reduced the sugar content of toddler foods in the National Diet and Nutrition Survey of the UK from 17% to 12% over a ten-year period by the action of food companies. It hasn't been associated with a reduction in childhood obesity at all, but it has effected a huge change in intakes.

Joseph Nadeau: Was the change in sugar consumed motivated by public policies or the social conscience of the food industry or arising from an understanding of the underlining molecular biochemical, physiological mechanisms? .

Vilmundur Gudnason: it has been interesting to listen to all these questions about policies. We actually have - not only in Iceland but also in most of the Western world - wonderful examples of success by influencing the lifestyle of whole populations. I am referring to the cardiovascular mortality drop with in Iceland a drop of 80% in mortality between 1981 and 2006. Two to three quarters of that could be attributed to changes in lifestyle, particularly a drop in cholesterol, probably due to less consumption of saturated fat. Smoking also dramatically changed. And one of the characteristics of all this was a close coalition between professionals - like us, scientists and laymen - people who got interested in the cardiovascular problem and diet and influenced their population, and the politicians to change policies. And I think that this is something that was surely seen in Finland, in the UK and most of the Western world. I think we should recognise the value of coalitions of the professionals, laymen and politicians.

Peter Gluckman: I want to remind you that 9 of the 10 fattest countries in the world are poor countries: they are the Pacific Island countries with a rate of adult obesity in the order of 50%. This is not always a first world problem that we are dealing with.

Philip James*:* In Egypt, the obesity rates are somewhere close to 40% in women on a national basis.

Peter Gluckman: If you look at the rise in obesity in sub-Saharan Africa and the rates of women's obesity and women's insulin resistance, it is dramatic. So I think to just focus on the Western world, where there are all sorts of easy things to do. To simply try to change behaviours does not tackle the global problems. I am reminded that when I was Chair of the WHO Workforce on optimising the outcomes of pregnancy in 2004 we had the startling figure that less than one third of children were weighed at birth across the globe! That is what we are dealing with - the reality of the most basic thing, of actually weighing a baby at birth which if undertaken means that there is some sort of minimal health care at birth which is currently needed for half the planet's population!

I think Keith (Godfrey) has made a couple of points that I wanted to make and I will just repeat them very briefly. What the mechanistic work has done is to enable us to make a strong

argument for periconceptual, early pregnancy, pregnancy care and infancy care, rather than just focusing on adult obesity. Without the experimental work - whether molecular or epidemiological - but particularly because of the epigenetic data - we could give the problem a plausible biological mechanism. So the argument has shifted. So I think that the mechanistic stuff allowed for a major change and allowed the policy argument to occur between the public health professionals.

What I think it has now done is to open up a new set of questions, critical windows, when is the right time, and what are the stimuli that matter in an intervention? You have all assumed that it is "nutrition" but famine is also associated with stress; there is a lot of data about the relationship between stress and the induction of Cocaine and Amphetamine Regulate Transcript (CART) that then inhibits leptin signalling in the brain so you get this relationship between eating behaviour and stress. Where the research will now go is using epigenetic biomarkers: if they can be validated as we talked about yesterday, and other systems to actually understand the key windows for intervention. What are the key windows in which reversibility is possible, as has been done frequently now with leptin, with growth hormone, with folate, with a number of other different mechanisms. And most importantly which stimuli matter? And this is all where the basic research will ultimately inform public policy.

Bo Angelin: The need for proof is important. Take something like the development of statins: Experts who were interested in intermediate metabolism were soon convinced that there was a mechanism for reducing cholesterol levels that made sense! The cardiologists were completely impossible to convince until you could show that the size of the pipes in the body could be influenced by treatment and the public health people wanted to have data on survival and actually, in the long run, you also need data on safety. But we got on with the development of statins without all this evidence as we had a convincing mechanism. So, as someone said, we won't convince a politician or a policy-maker by just having a very nice molecular mechanism - but with really important mechanistic observations we can then develop and set out the other requirements needed to make a convincing case.

Irv Rosenberg: Well I want to reinforce the observation that Jo Nadeau just made earlier - questioning how often it is that we are able to use an understanding of mechanisms as a basis for influencing action in policy? Let us refer to the issue of neural tube defects where national, and now international policies used essentially one randomised controlled trial[cxxix] of folate - as well as testing separately some other vitamins to effect a certain important outcome. Policies were then initiated - and I was involved with the Food and Drug Administration in our fortification recommendations - without any understanding of the mechanism of interaction of folate. And we still, despite a lot of good work from Patrick Stover and Jo Nadeau etc., we still don't know what that mechanism is. So I think it is an interesting case study for us because, in the epigenetic sphere, I share Andrew Prentice's view that with this kind of case, maybe the techniques that we are developing in epigenetics will help us and tell us how folic acid is working in the amelioration of that problem. I certainly look forward to further work with that. But I would also say that within this case study, we also must be sure in the recommendations that we make to our policy makers that making recommendations are not harmful. And the extension of the recommendations from one country for controlling a problem like neural tube defects may not be the appropriate ones for another. So I think I agree, we certainly need to push forward with research on mechanisms. And I think we have a few case studies that can help us make the appropriate link with policy.

Caroline Fall: I think this group has come to a very strong consensus already that the events around the time of conception are very important. We may not know all the ins and outs of nutritional needs at that time, or other environmental effects, but we know that they are important and that is it not good enough to start maternal care 12 weeks into pregnancy which is when it is generally started at the moment. So the need to consider pre-pregnancy care is something that the general public is pretty unaware of given all the beautiful and complex things that happen around the time of conception - now we have got to somehow get that across to the general public.

Charlotte Ling: I am actually want to move away a little bit from conception and go to early childhood and behaviour and the fact that food intake at early childhood has been shown in animal models to imprint across the cortical nuclei and genes that affect behaviour. So the community has a responsibility to dissect out that this feature more clearly in relation to food intake behaviour.

Patrick Stover: What a policy maker wants to know is that something will work. They don't necessarily have to care about the mechanism. So the clinical trials show that folic acid

prevents neural tube defects and we have no idea why - it is a black box! So give people folic acid and the neural tube defects are prevented. The problem has been that we are using food as medicine but when you use food as medicine there are going to be side effects and there is going to be tons of uncertainty. And it is the uncertainty that has caused the quagmire around fortification. So where mechanism comes in is trying to predict: you can't test for every possible side effect. Since the fortification programmes started there has been a surge in asthma, insulin resistance, in a zillion things so we have to ask whether these problems related to the fortification? That is where the science is absolutely critical: dealing with the uncertainty. As we continue to medicalise the food supply, I think the example of folate is just going to extend into all sorts of spheres.

***Philip James**:* OK, thank you very much. What we have had is a panorama of research relating to the development of changes in pregnancy and their long-term consequences. We have actually come a long way since we last discussed this about 20 years ago and I think that the research community, both in a molecular and a physiological sense - but also particularly in epidemiological ways - have really advanced the agenda, such that Peter (Gluckman) and others amongst us who are involved in policy are in a much better position now to try to cope with what is happening. But please remember that your research is becoming even more critical because there is a rampaging change in society going on, with enormous accelerating problems of obesity - which as we have just heard - stem from before pregnancy and evolve throughout the gestational period.
So on that basis we are now at the point of having to make reasoned policy proposals, as well as really needing to continue with our research. Thank you again for coming and this discussion.

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