Details of Research Work

Gene-gene and gene-environment (micronutrients) interactions in type 2 diabetes: An approach towards primary prevention using Genotype-Epigenotype interface

Type 2 diabetes (T2D) is a common multifactorial complex non-communicable disorder (NCD) of enormous public health concern (1). India is known as the diabetes Capital of the world, competing with China for the top position. Compared to 2-4% in Europeans, the prevalence of type 2 diabetes in Indians ranges between 16-18%. Type 2 diabetes burden is huge in India and is projected to reach epidemic proportions. The disease has strong heritability and many intermediate traits like obesity, insulin resistance and secretion are established risk factors for T2D. Further, a precise diagnosis of type of diabetes is important for appropriate management since the treatment approaches differ significantly between different subtypes. Several candidate-based genetic studies and genome-wide association studies (GWASs) worldwide have provided significant insights into the genetic basis of T2D and potential mechanisms involved in its pathogenesis (3,4). Most of the T2D loci have been discovered in individuals of European descent and the few GWASs that were carried out in subjects of other ancestry including South, East and Southeast Asians have uncovered new loci without replicating all common European signals (2,5,6).

Given a different phenotype of T2D patients and the high ethnic and genetic diversity of Indians in comparison to Europeans and East Asians (7), my colleagues and I have made systematic attempts to understand whether there are different genetic loci that predict susceptibility to type 2 diabetes in Indians and if they can explain the phenotypic differences between patients belonging to other ethnicities. Further, since obesity and insulin resistance are two important intermediate traits for development of type 2 diabetes and Indians are known to be centrally obese and at least 1.5 times more insulin resistant than the Europeans, we put specific focus in investigating the genetic basis of these two important intermediate susceptibility traits. In addition, obesity [measured by body mass index (BMI) and DEXA] and insulin resistance are important risk factors for cardiovascular diseases, for breast cancer, neurological disorders and many other non-communicable diseases. In this regard, we took lessons from our earlier studies on chronic pancreatitis, another example of NCD, having a very different phenotype and clinical course and complications in Indians compared to Europeans. My team for the first time provided evidence of genetic and mutational heterogeneity in susceptibility to chronic pancreatitis in Indians as compared to Europeans and correlated them to the different pattern of mutations in specific genes (8). Briefly, we failed to identify in Indians, any of the reported or novel mutations in both cationic and anionic trypsinogen genes (*PRSS1* and *PRSS2*), the commonest cause of chronic pancreatitis identified in the Europeans. Mutations in *PRSS1* were proposed as a screening tool for the risk of chronic (Hereditary) pancreatitis, which became redundant and useless for Indians in the background of our study results (8,9). In fact, we identified two novel genes Serine Protease Inhibitor, Kazal Type-1 (*SPINK1*) and Cathepsin B (*CTSB)* (both included in Online Mendelian Inheritance in Man; OMIM; Figure 1) where specific mutations and an interaction of mutations in these genes was observed to be associated with risk of chronic pancreatitis and its clinical course in Indians (10). Further, we reported lack of common European-specific mutations and a repertoire of different mutations in other candidate genes like chymotrypsin C (*CTRC*) (11) and Carboxypeptidase I (*CPA1*) (12) in

OMIM GR Chandak

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1. #608189 - TROPICAL CALCIFIC PANCREATITIS
   Cytogenetic locations: 5q32
   OMIM: 608189

2. *116810 - CATHEPSIN B; CTSB
   Cytogenetic locations: 8p23.1
   OMIM: 116810

3. *167790 - SERINE PROTEASE INHIBITOR, KAZAL-TYPE, 1; SPINK1
   Cytogenetic locations: 5q32
   OMIM: 167790

Figure 1: OMIM Catalogue of genes related to tropical calcific pancreatitis, a type of chronic pancreatitis

Indian chronic pancreatitis patients. These novel mutations identified in Indians were confirmed to be causal in nature through in vitro functional characterisation (13). Overall, our studies on chronic pancreatitis established a different genetic basis and mutational heterogeneity likely due to ethnic diversity and provided clues that the genetic and mutational heterogeneity may be responsible for a different clinical presentation and clinical course for chronic pancreatitis in Indians. These observations led to development of specific genetic screening test for Indian Chronic pancreatitis patients and is now available at various places. In addition to all the above, they provided motivation for investigating the genetic basis of other non-communicable diseases like type 2 diabetes which has a different phenotype and disease course in Indians.

As mentioned earlier, type 2 diabetes is no different than chronic pancreatitis in terms of a different clinical phenotype. Indians develop type 2 diabetes a decade earlier than Europeans and at lower body mass index, possibly because they are centrally obese and more insulin resistant (2). Over last few years, my colleagues and I have conducted single candidate gene-based, multiple candidate gene-based and agnostic GWAS study on a well-characterised cohort of type 2 diabetes and normal subjects. While we confirmed the association of *TCF7L2* variants (the top GWAS associated locus) (14) in Indians with a similar effect size as in Europeans, we also identified ethnicity-specific differences in genetic susceptibility to type 2 diabetes and showed a comparatively larger effect size in eight GWAS significant genes (from the initial GWAS studies) in Indians than in Europeans (15). Interestingly, we observed that *FTO* gene predicted independent risk to both obesity and type 2 diabetes in Indians which was in contrast to the fact that *FTO* influenced the risk of type 2 diabetes in Europeans through obesity (16) (Figure 2). Further, using a weighted genetic risk score comprising of then identified 33 T2D-associated loci (in 2011), we showed that the combined effect of the genes (now better known as polygenetic risk score) had a higher predictability of T2D disease risk (17). This indicated the probability of genetic heterogeneity in the background of different ethnicities as observed in chronic pancreatitis.

Type 2 diabetes in general is considered a success story for genome-wide association studies since close to ~400 genetic loci have been identified which have also helped in understanding various functional abnormalities in diabetes. It is yet a matter of concern that together these many loci explain only 18-20% of heritability leaving a large vacuum of missing heritability for type 2 diabetes. However, through participation in the whole genome and whole exome sequencing of thousands of type 2 diabetes patients and normal subjects, we have concluded that the missing heritability of type 2 diabetes is not likely explained by rare variants with large effect sizes (18). Through our own GWAS analysis in well-characterized type 2 diabetes patients and normal subjects and through our participation in various International cohort studies (DIAGRAM, DIAMANTE, etc.) that includes other South Asian cohorts, the conclusions made above were found to be robust (18). A combination of the observations made in the above studies indicated that the risk genetic loci for type 2 diabetes may be largely similar between Indians and Europeans. However, there may be trait specific genetic differences as we have shown earlier for *FTO* gene (16). Based on the above observations, we hypothesised that the missing heritability and the phenotypic variability may be due to differential regulation of

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A Common Variant in the *FTO* Gene Is Associated with Body Mass Index and Predisposes to Childhood and Adult Obesity
Timothy M. Frayling, *et al.*
Science **316**, 889 (2007);
DOI: 10.1126/science.1141634

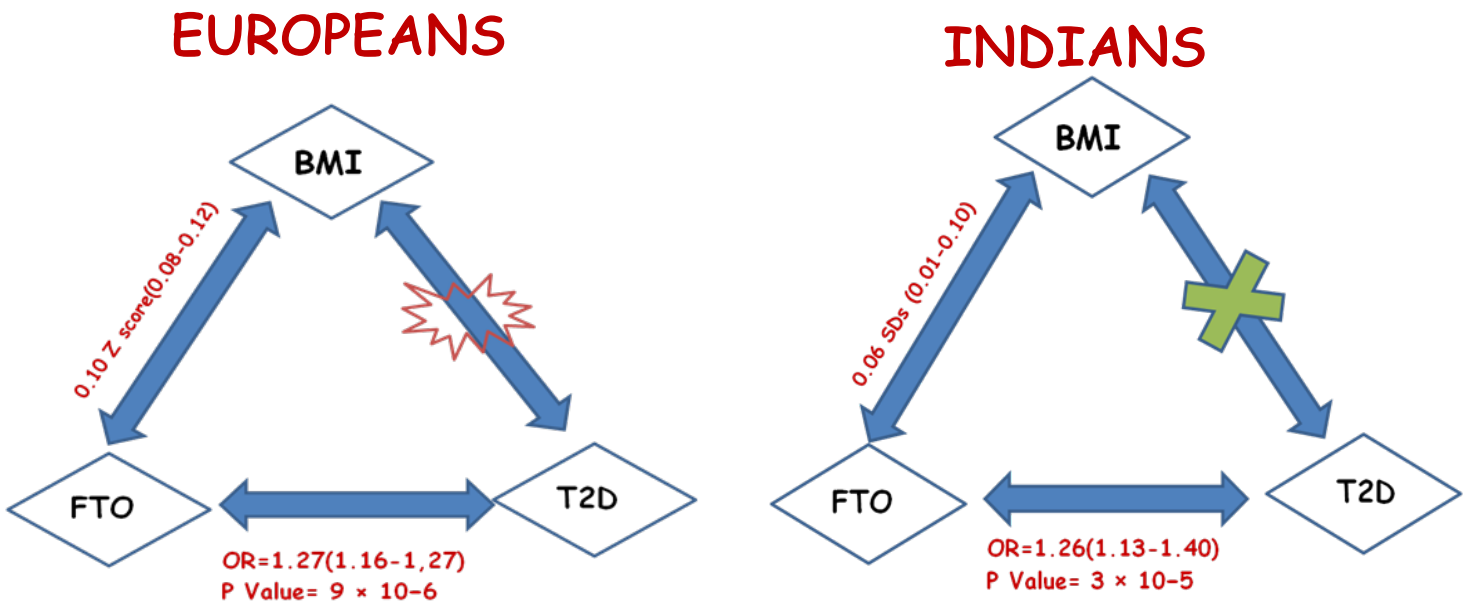


Figure 2: FTO gene variants independently predict susceptibility to obesity and type 2 diabetes in Indians

genes where common variants in the type 2 diabetes susceptibility loci are located (12). The above observations shifted the focus back to common variants common disease hypothesis; however, exploration of other possible mechanisms also started in full swing in my group.

Our interest in conducting studies on type 2 diabetes was to identify risk-associated genetic loci and possible differences in genetic susceptibility in Indians and use them for predictive diagnosis so that at-risk individuals can modify the risk through life-style modifications; a common paradigm for prevention of complex non-communicable diseases. This was further prudent since there are large number of T2D loci with small effect sizes and the genetic risk is fixed at birth and cannot be structurally modified for so many loci. Hence, we decided to focus on identification of other non-genetic factors (especially modifiable) that influence the risk of type 2 diabetes. Simultaneously, we also made specific attempts to understand the life stage at which the risk for diabetes or related intermediate traits is determined and various prevailing factor(s) that may determine the T2D risk. Overall, this plan was intended to identify not only the different factors but also the ways that the risk of T2D could be modified by intervening at an early stage. We developed a disease cohort comprising of type 2 diabetes patients and normal subjects for genetic studies which was on regular follow-up. Hence, this cohort was available for investigating the genetic association and their influence on the clinical course in the background of the individual's environment during their lifetime.

We further focussed our attention to Barker's hypothesis (David Barker) which suggests that early life environment (including maternal nutrition) determines the birth size including birth weight and other anthropometric parameters and influences the future risk of cardiometabolic disorders (19). This is now known as "Developmental Origins of Health and Disease (DOHaD)". It is well established that individuals born with low birthweight are at a higher risk of cardiometabolic disorders. We hypothesised that it is no coincidence that India is the joint capital of low birth weight (close to 1/3rd of the newborns are LBW) as well as type 2 diabetes and hence aimed to understand this relationship. Further, it had been demonstrated in one of the birth cohorts, Pune Maternal Nutrition Study (PMNS) that Indians have low muscle mass but higher fat mass at birth, although Indian babies have 20% lower birthweight compared to Europeans (Thin-Fat Phenotype) suggesting that apart from the LBW phenotype, it is the fat mass and its relative excess compared to the muscle mass which predict the future risk of metabolic syndrome (20). This motivated me to collaborate with a number of birth cohorts [for example PMNS, Mysore Parthenon Cohort (MPC), etc.] throughout the country (and abroad) that had phenotype data and biological samples on mothers and their children from early life and serial measures of various anthropometric and biochemical parameters during pre-pregnancy, pregnancy and at regular intervals in children. Birth weight (a surrogate of size at birth) is known to be influenced by various fetal and maternal factors including genetic effects. In a GWAS study including seven Indian and Bangladeshi cohorts, we replicated the association of a weighted fetal genetic risk score (calculated from ~200 genetic variants identified in the Europeans) with their birth weight having a similar effect size in South Asians (21). We further demonstrated the association of these genetic variants with early childhood body size and with fasting glucose and triglycerides levels in adults, suggesting that common genetic variants explain part of the association between birth size and adult metabolic

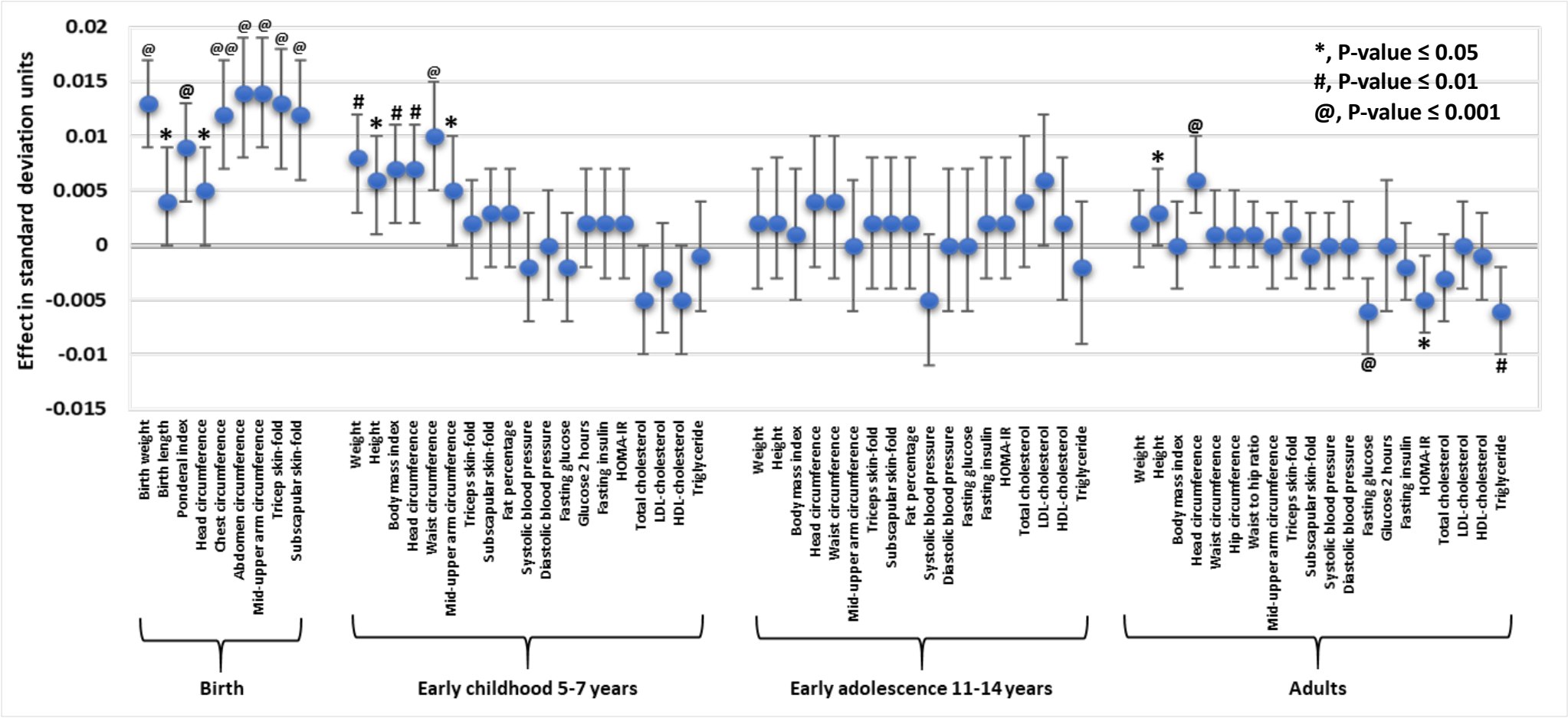


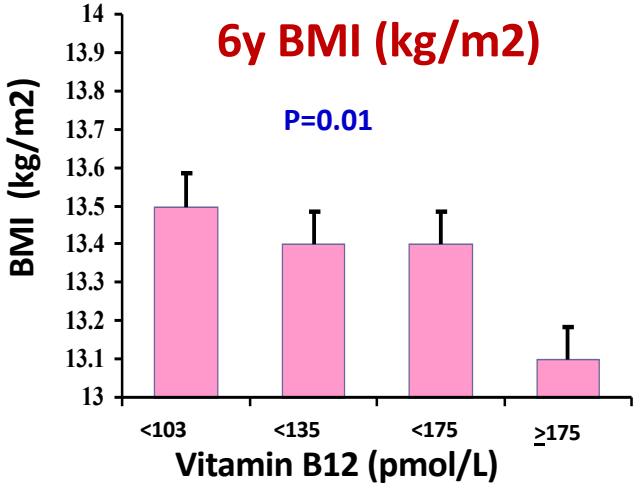
Figure 3: Association of fetal genetic score with various anthropometric and cardiometabolic traits at different follow-up stages in the Indian subjects (From PMNS and MPC birth cohorts)

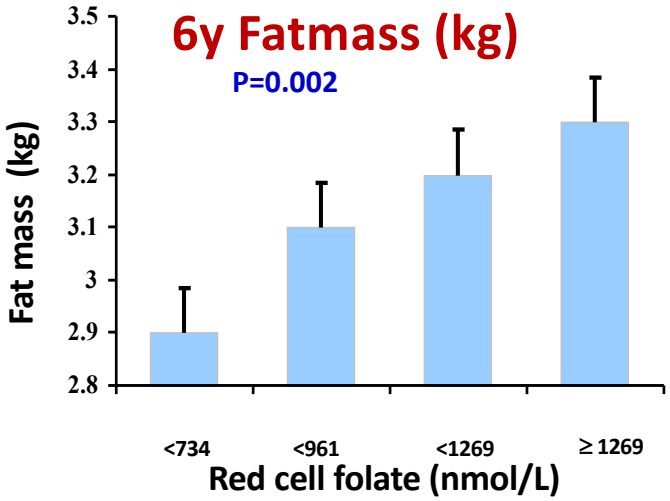
syndrome (Figure 3) (21). Despite similar association of fetal genetic scores with birthweight as in Europeans, South Asians showed a considerably lower birthweight. The substantially smaller newborn size in South Asians with comparable fetal genetic effect to Europeans on birthweight suggested a significant role of factors related to fetal growth that are not captured by the present genetic scores. These factors may include different environmental exposures, maternal body size, health and nutritional status etc. Thus, we decided to investigate the molecular mechanism of association of early life exposure with low birth size and future risk of cardiometabolic disorders. The premise was that if such factors are identified, then appropriate intervention/supplementation can nip/modify the future risk of cardiometabolic disorders in future and thus aid in primary and/or primordial prevention.

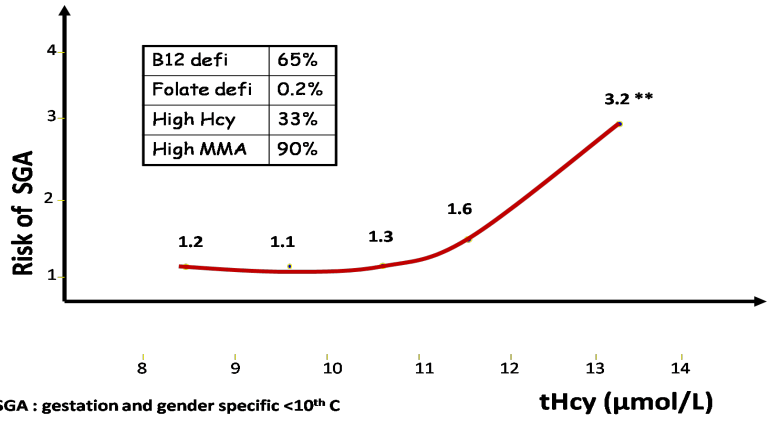
Through further follow-up studies in the PMNS cohort, it was observed that children born to mothers with raised plasma homocysteine levels were obese and insulin resistant at the age of six years and those born to mothers with lowest tertile of vitamin B12 (B12) and highest tertile of folate were most obese and insulin resistant. This suggested an influence of folate/B12 imbalance on two important intermediate traits (obesity and insulin resistance) of cardiometabolic disorders (22) (Figure 4). This provided one of the best evidence of "Nutrient Mediated Teratogenesis (NMT)" where early life exposure of low B12 and/or B12+folate imbalance could programme the fetal phenotype and future risk of metabolic syndrome. It was also noted in another cohort Mysore Parthenon Cohort (of Dravidian ethnicity) that children born to gestational diabetes mellitus (GDM) mothers were big, obese and insulin resistant providing an evidence of "Fuel Mediated Teratogenesis (FMT)" (23), It was further noted that the GDM mothers in MPC who had persistent low B12 levels were themselves more obese and had higher risk of continuing or developing type 2 diabetes within 5 years of the delivery (24). These observations suggest an important role for vitamin B12 and B12/folate imbalance in fetal programming of future risk of cardiometabolic disorders through a "Dual Teratogenesis" model overlapping both NMT and FMT (Figure 5).

In population-based studies, we identified that B12 contributes ~65% of population attributable risk to homocysteine levels, in contrast to folate levels which accounted for 2-3% only. This is in contrast to the observations made in Europeans where folates are majorly known to influence the homocysteine levels (22). However, reverse causality can confound this association since the plasma levels of B12, folates or homocysteine are influenced by many other factors such as gender, gestational age, diet, ethnicity, etc. We performed whole genome genotyping (using Illumina GSA microarray chip) of the mother and children in the PMNS and MPC cohorts and performed Mendelian Randomization analysis using *MTHFR* gene variant (as the surrogate of homocysteine levels) and *FUT2* and *TCN2* genetic variants (as surrogate of plasma and tissue B12 levels respectively) (25). We demonstrated that children born to mothers carrying the risk allele at *MTHFR* 677C>T variant had lower birth weight (26) (Figure 6). Similarly, mothers carrying B12 lowering alleles at *FUT2* and *TCN2* gave birth to obese and insulin resistant children. We also generated a weighted genetic risk score using polymorphisms in eight genes in one-carbon metabolism influencing the homocysteine levels and again through Mendelian Randomization confirmed the observations made through single gene genetic variants (26). Since the genotypes are fixed at birth and there is no question of reverse causality in Mendelian

Maternal homocysteine levels due to folate-B_{12} imbalance Programs childhood Adiposity & Insulin Resistance







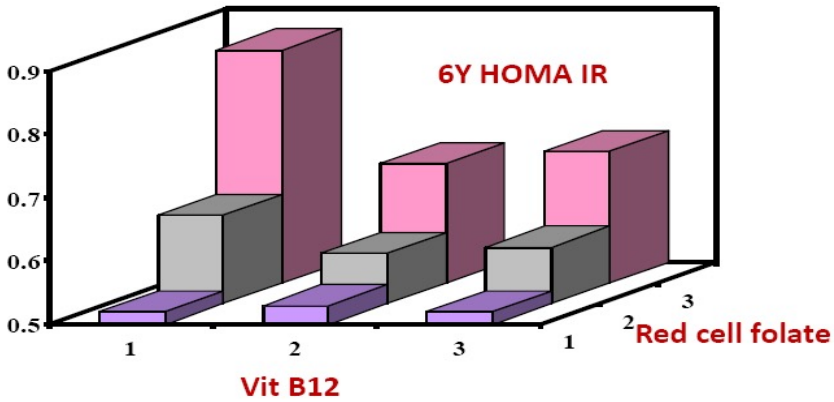


Figure 4: Kids born to mothers with low levels of B12 and high folate levels are obese Diabetologia 2008
And the kids born to mothers with lowest tertile of B12 and highest tertile of folate are most insulin resistant

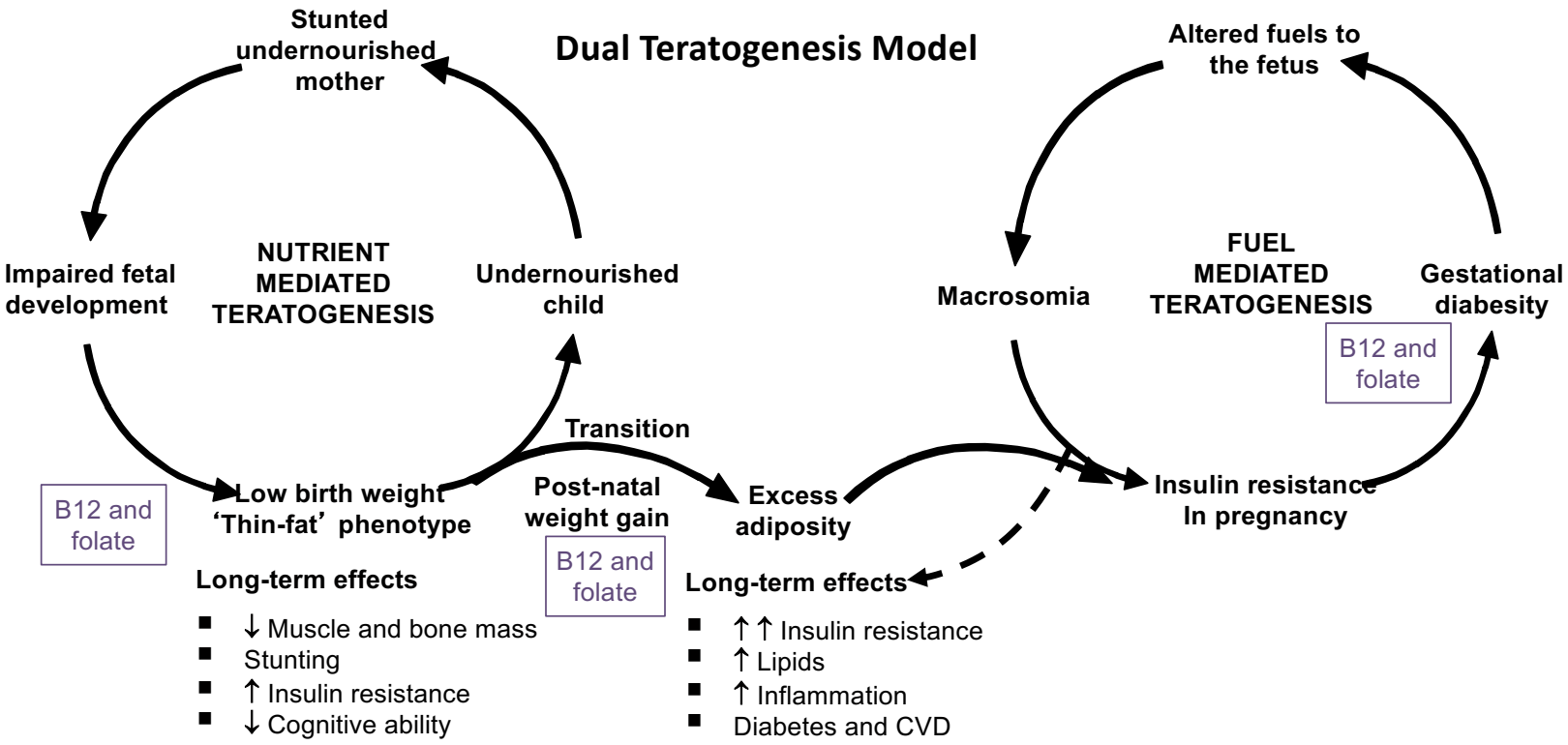
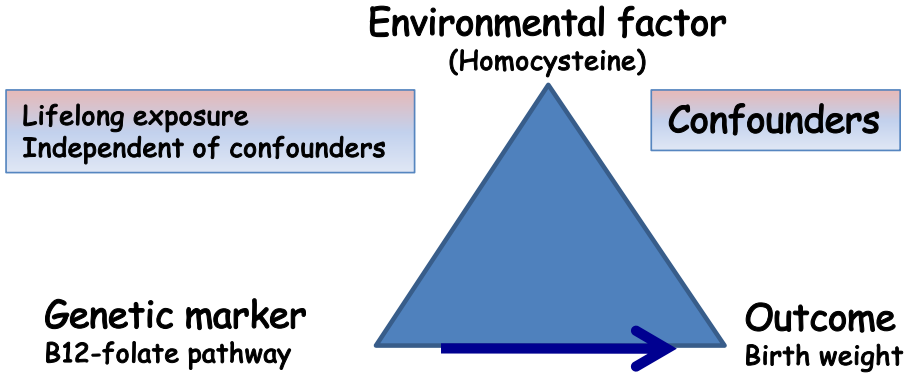


Figure 5: Nutritional transitions causing inter-generational disease

Mendelian Randomization Confirms Causal Role of Maternal Homocysteine in Programming of Diabesity in their Children



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Commentary: One-carbon metabolism has major implications for fetal growth and development beyond neural tube defects

Sarah J Lewis

Int. J Epidemiology, 2014

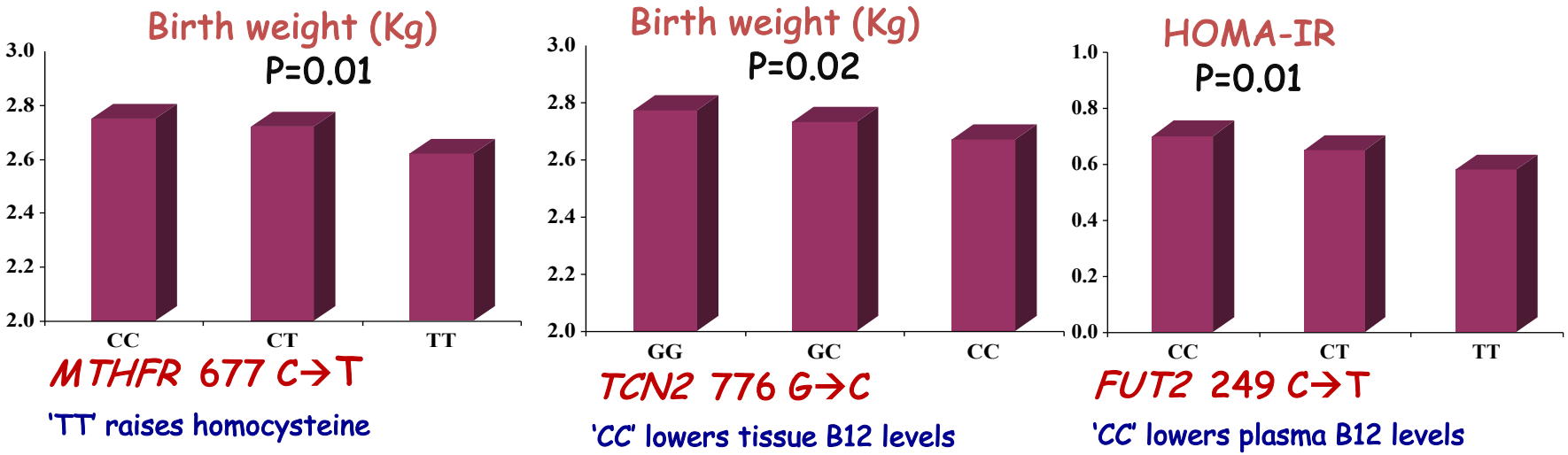


Figure 6: Kids born to mothers with risk allele at MTHFR and TCN2 variant are born with lower birth weight and The kids born to mothers with B12 lowering allele of FUT2 are insulin resistant

Randomization, we proved the causality of maternal homocysteine levels (mediated through low B12 levels confirmed through *FUT2* and *TCN2* variants) leading to birth of obese and insulin resistant children (Thin-Fat Phenotype). Through an intervention with vitamin B12 and/or folate, we further showed that only vitamin B12 supplementation can significantly reduce homocysteine levels whereas folates have very minor influence on homocysteine levels (26). Thus, we could establish two facts (1) vitamin B12 deficiency (in the presence of normal or higher folate levels) causes high homocysteine levels in the mothers during pregnancy which leads to birth of obese and insulin resistant baby and (2) that vitamin B12 supplementation at any age can reduce the homocysteine levels. This provided an excellent opportunity to explore the possibility of reducing the future risk of cardiometabolic disorders through targeted B12 and or multiple micronutrient supplementation at appropriate time points.

It is well established that both B12 and folate are major players in one-carbon metabolism cycle which determines the methylation potential of the cell. Hence we decided to explore the DNA methylation differences, one of the major epigenetic regulation mechanism, using a multi-pronged approach and understand the molecular mechanism of B12/folate mediated future programming of metabolic syndrome (Figure 7). First, we confirmed the observations (made in Human cohorts) in rat models of B12 and/or folate deficiency where pups born to B12 deficient dams had lower birthweight and dysregulated glucose and lipid metabolism with evidence of obesity by the age of 12 months (27). We also demonstrated reversal of adverse phenotypes like obesity and metabolic derangements by B12 supplementation at various stages of gestation (27,28). Further we showed differential methylation and expression of *PPAR* group of genes in the rat liver as a result of B12 deficiency and reversal of the molecular changes through B12 supplementation at pre- and peri-conceptional stages (29). This provided hint that differential methylation of genes associated with type 2 diabetes and its risk traits like obesity and insulin resistance may be related to B12 deficiency and the molecular changes can be reversed through its supplementation during crucial gestational stages.

We then moved onto human subjects from two abovementioned cohorts (MPC and PMNS) and investigated whole-genome DNA methylation changes using Methylated DNA Immunoprecipitation sequencing (MeDIP-seq) approach. We compared the methylome in 6 years old children born to mothers from the 1) lowest decile of B12 and highest decile folate and were most insulin resistant and 2) highest decile of B12 and lowest decile of folate and were least insulin resistant. We identified several differentially methylated regions (DMRs) in several genes associated with type 2 diabetes, related intermediate traits and their regulatory regions. Furthermore, using in vitro techniques, we functionally characterized a specific DMR in the peroxisome proliferator-activated receptor delta (*PPARD*) locus as an insulator/enhancer blocker element (30). We also characterized a repressor element downstream to the characterized insulator element in the *PPARD* gene and demonstrated that when present upstream to the minimal promoter, it suppresses the promoter activity. Further, we demonstrated an interaction between these two regulatory elements which may be perturbed in an altered methylation state of the insulator element. We hypothesised that abnormal methylation pattern of the insulator element may result in dysregulation of *PPARD* gene leading to higher adiposity and insulin resistance in children born to mothers with an imbalance

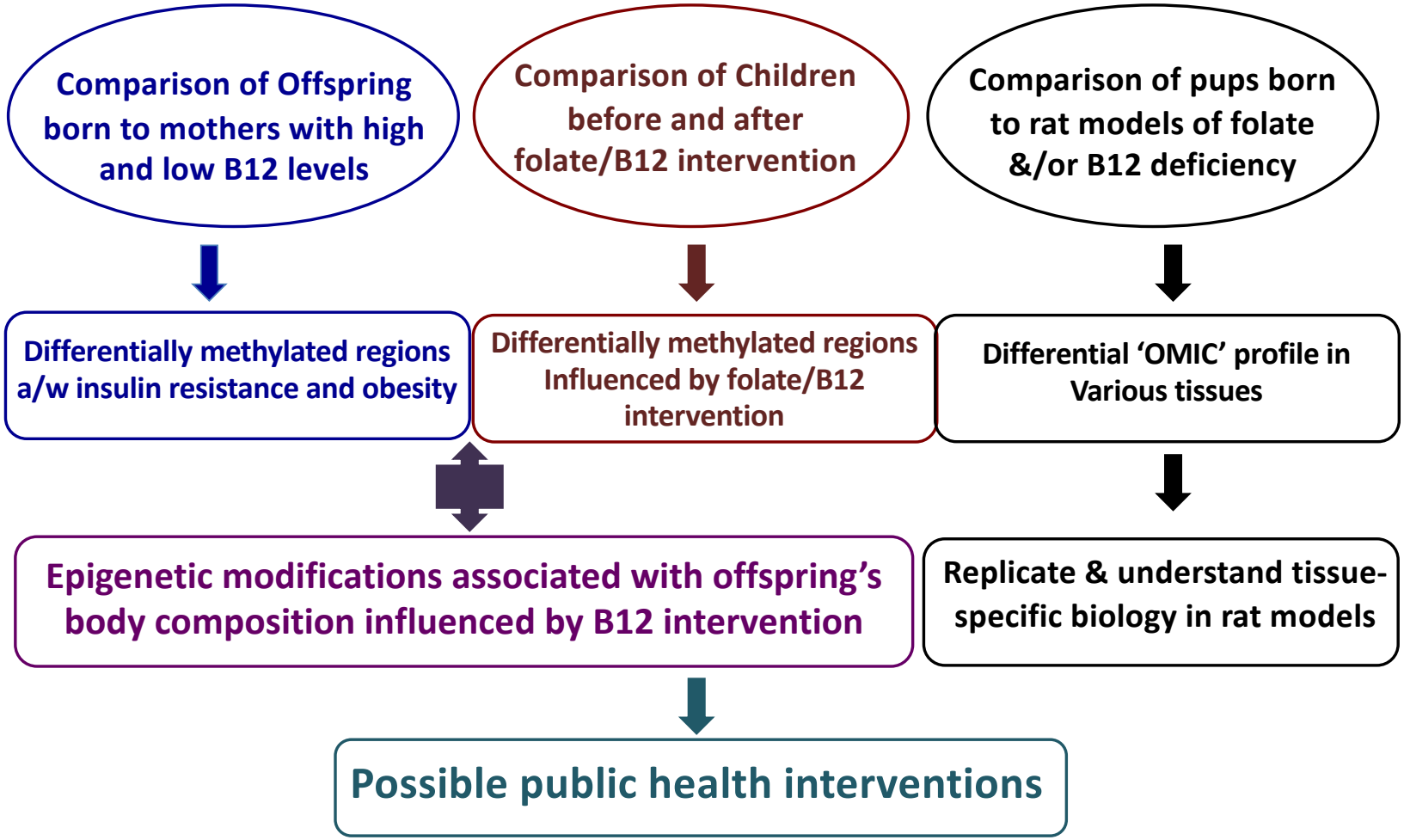


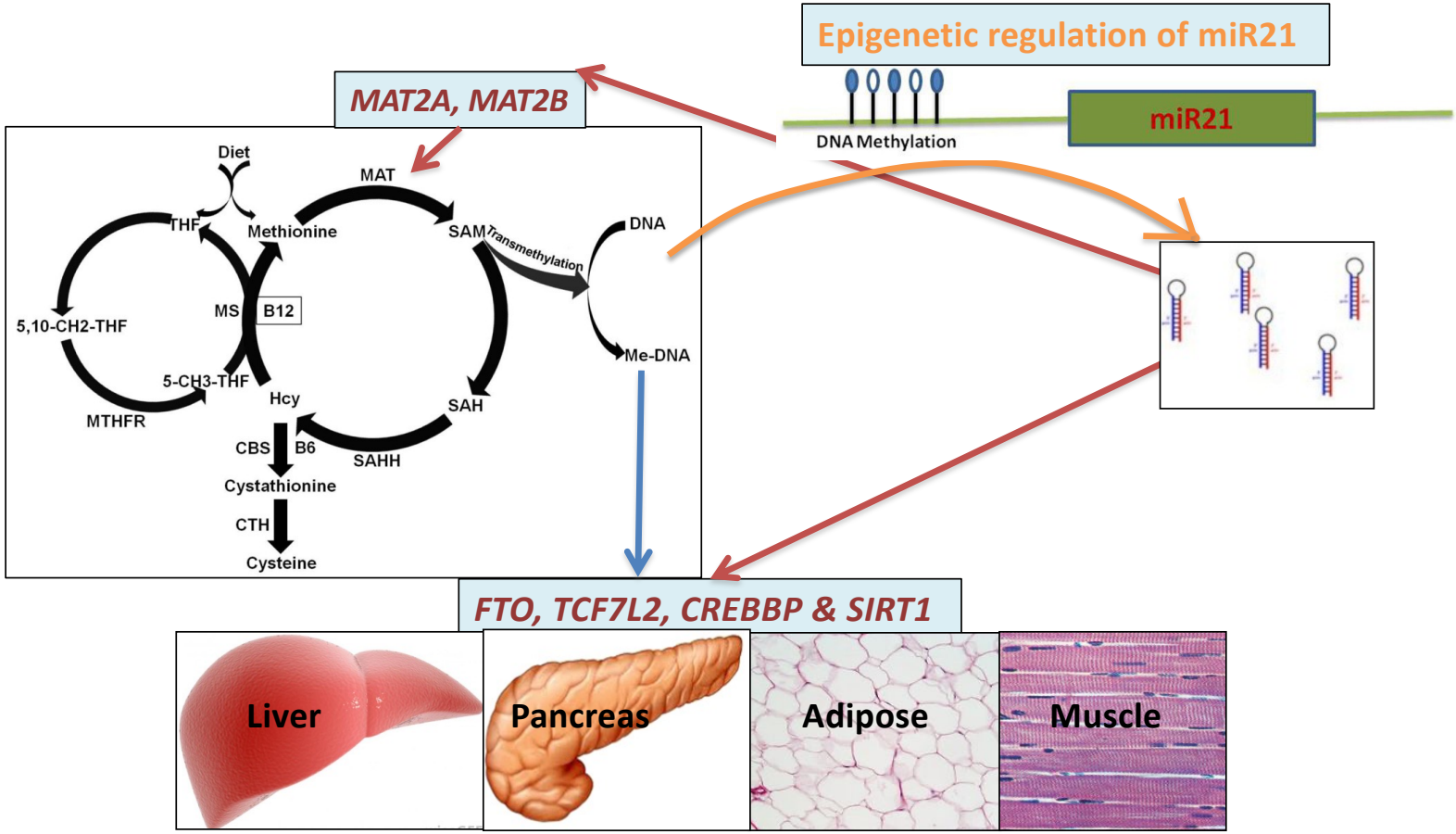
Figure 7: Multi-pronged strategy for studying epigenetic basis of programming of Diabesity as a result of intrauterine exposure

of B12 and folate levels in Indians (30). We performed an independent candidate-based methylation analysis in 5 year old children in Mysore Parthenon Cohort and showed a statistically significant association of *PPARD* gene methylation (CpGs in the DMR region) with Insulin sensitivity (Unpublished data; manuscript under preparation). Since PPARD has an established role in T2D, lipid and glucose metabolism and insulin sensitization, our results aid in understanding the link between B12 mediated risk of T2D and its related intermediate traits such as insulin resistance and adiposity.

Finally, we investigated the influence of vitamin B12 and/or folate supplementation on whole genome methylation using Illumina 450K Methylation Chip. For the first time, we demonstrated that vitamin B12 supplementation regulated methylation of key genes associated with type 2 diabetes such as *TCF7L2* and *FTO*, which are two major genes predicting the risk of insulin resistance & secretion and obesity, respectively (31). In addition, we also identified a differentially methylated microRNA, miRNA21 whose targets are key enzymes, Methionine Adenosyl Transferase (MAT-A and MAT-B) which catalyse the conversion of methionine to S-adenosyl methionine in one-carbon methylation cycle thus decide the methylation potential of the cell (21) (Figure 8). It was further interesting to note that top T2D genes like *TCF7L2* and *FTO* were also the bioinformatically predicted targets of miRNA21. We provided the evidence of their interaction using in vitro experiments. We, therefore propose that B12 plays a crucial role in deciding the methylation potential of the cell which then differentially methylated key genes in one-carbon metabolism cycle as well as key genes predicting the risk of type 2 diabetes (31). In conclusion, we have demonstrated that B12 supplementation in Indian adolescents alters the methylation state of genes influencing intermediate traits of type 2 diabetes such as obesity, insulin resistance & secretion and a regulatory key microRNA, and thus regulate their transcription and interaction with downstream targets.

Overall, my studies over last several years have covered details of phenotypes, their follow-up in various cohorts followed by directed as well as agnostic genetic and epigenetic studies. Further, I have combined the approach of linking the molecular changes with various clinical details including the follow-up data at various stages. This approach has allowed to link them in a way that they can be utilised for the human benefits. For example, while we identified a different genetic basis for chronic pancreatitis, we continued till the full evidence of genetic and mutational heterogeneity in the Indians was fully established and got translated in the genetic testing of chronic patients of Indian origin. We expanded this model further in researching diabetes and identified a largely similar genetic susceptibility as in Europeans but also show trait-specific genetic differences in Indians which guided towards investigating the nutrition and other modifiable factors. We took a multi-pronged approach to not only understand these modifiable factors but also established their causality by using genetics as a tool and further through intervention/supplementation trials, understand the molecular mechanism. Combined with the approach of Fetal Programming where we demonstrated biological evidence of role of vitamin B12 (and altered one carbon metabolic pathway) in the causal pathway of obesity and insulin resistance, two important traits related to risk of diabetes and as a link to both NMT and FMT in a dual teratogenesis model. Investigating the epigenetic basis of diabesity (diabetes+obesity) using a multi-pronged strategy, we identified dysregulation of type 2 diabetes associated genetic loci and further dissected the pathways

Link between One-Carbon Metabolism and type 2 diabetes



Gluconeogenesis, Glycogenesis, Beta cell mass function Insulin production and secretion, Glucose uptake, lipid metabolism

Figure 8: B12 supplementation influences methylation of key type 2 diabetes genes, both directly as well as Through methylation of a specific microRNA, miR21. It also influences methylation of key genes in one-carbon metabolism

Susceptibility

Genetic

+

Precipitating Factors

Obesity
Diet
Physical inactivity
Stress

DOHaD (Programming)

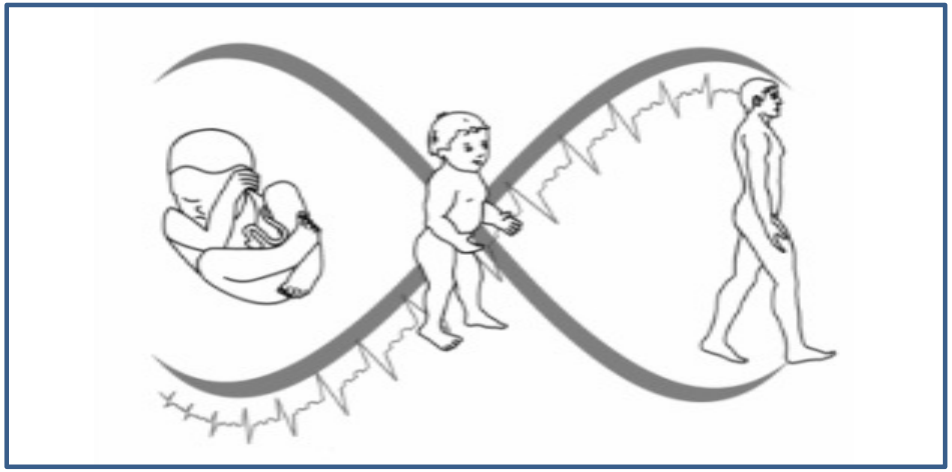


Figure 9: The Dogma of type 2 diabetes – Gene-gene (Genotype) and Gene-environment interaction (Epigenotype)

through which this risk is medicated. Thus, these observations provide a novel 'epigenetic' basis for the association between deregulation of one-carbon metabolism, risk of adiposity, insulin resistance and diabetes (Figure 9). One of the observation about the folate and B12 levels in the Indian population led us to study the biochemistry and genetics of neural tube defects which is associated with high maternal homocysteine levels and shown to be due to low folate levels in Europeans and Americans. Through a systematic multi-centre study, we demonstrated that rather than folates, low B12 levels predicted high homocysteine levels and maternal vitamin B12 deficiency during pre- and peri-conceptional period was associated with high risk of neural tube defect in their children. This was proven by an enrichment of B12 lowering alleles in the *TCN2* gene and a low allele frequency of the *MTHFR* genetic variant. This was another evidence of important differences between Europeans and Indians and had immense translational values suggesting inclusion of vitamin B12 in the peri-conceptional plan for women planning for a child. In combination, these observations make crucial addition to the understanding of the B12 and related one-carbon metabolism disturbances in Indian population. It may not be inappropriate to infer that as we understand these better, there is scope of intervening with B12 since a large majority of Indian population is B12 deficient and influence the risk of cardiometabolic disorders.

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