

Review Article

Nutritional Modulation of Gene Function in Disease Susceptibility: Homocysteine-Folate Metabolism Pathway

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In this paper the interaction of genetic and nutritional variables in the homocysteine (Hcy) metabolic pathway genes and its cofactors have been reviewed to assess their influence on general health. Hcy metabolism leads to the synthesis of 2 amino acids- methionine that gives rise to S-Adenosyl-methionine (SAM), the main methyl donor to cellular biomolecules, and cysteine. Hyperhomocysteinemia (hypHcy) is a potential risk condition for various disorders like Neural Tube Defects (NTD), Congenital Heart Disease (CHD). It is shown how mutations in certain Hcy-pathway genes, especially *MTHFR* C677T, become a risk for NTD and CHD and other diseases in one population but not in the other, exemplifying the importance of gene-nutrition interaction in disease manifestation. Deficiency of micronutrients folates and vitamin B12, which are important constituents of the Hcy-pathway, can independently act as strong risk factors for the same diseases. A survey in the eastern part of India records that almost 50% population is deficient in vitamin B12, 11% in folates, and nearly 30% is hyperhomocysteinemic, indicating high disease predisposition. Globally the highest incidence of undernutrition and low birth weight occurs in this region. A high proportion of individuals afflicted with cleft lip±palate, male infertility, recurrent pregnancy failures and the chromosomal disorder, Down syndrome, have *MTHFR* 677T homozygosity as a risk factor but the extent of risk is modulated depending upon the levels of vitamin B12, folates and Hcy. Thus the genetic disease burden is influenced by the level of micronutrients. It is recommended that supplementation of both folates and vitamin B12 should be considered for prospective mothers during pregnancy.

Keywords: Nutrition; Metabolism; Disease Susceptibility

Introduction

Despite a reasonably good economic progress, India continues to have a high incidence of undernutrition and low birth weight. Health indices in the eastern region of India which includes eastern U.P., Bihar and Jharkhand, are even poorer than the rest of India (James, 2011). There is ample evidence to conclude that the children afflicted with mal- or under-nourishment neonatally, run greater risk of suffering from cognitive and other organic impairment in later life (see Ames, 2006). This article proposes to elucidate how nutrition influences genetic constitution of an individual in determining its phenotype, particularly in the manifestation of disease, and finally suggest that intake of adequate amounts of micronutrients could ensure proper maternal health, good pregnancy outcome and enduring child health

even in cases of genetic susceptibility. Based on our own research work in the eastern regions of India, this paper focuses on the Homocysteine-1-Carbon metabolic pathway that is vital for diverse biological functions to demonstrate how Hcy levels could vary due to modulation of gene function by micronutrients.

Homocysteine Metabolic Pathways

Homocysteine (Hcy) is a thiol-group-containing amino acid which is the progenitor of two amino acids, methionine and cysteine. Cyclical metabolism of Hcy into methionine (Met) and S-adenosylmethionine (SAM) paves the way to its recovery through S-Adenosylhomocysteine. The sulfonation pathway of Hcy, however, is a one way route in which Hcy synthesises cysteine (Fig. 1). The normal range of Hcy in plasma is between 3 and 12 $\mu\text{mol/l}$ but a level

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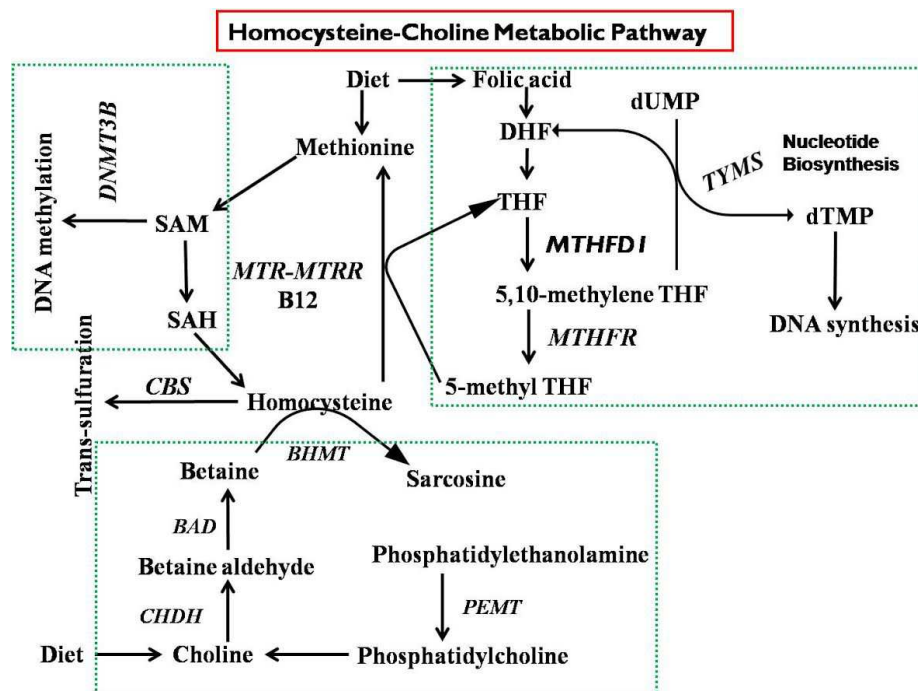


Fig. 1: The pathway of Homocysteine metabolism (modified from Selhub, 1999). Overview of the human folate- and vitamin B12-dependent homocysteine metabolism indicating its one-carbon donors and acceptors involved in methyl group biogenesis and DNA synthesis. MTHFR-methylenetetrahydrofolate reductase; MSR-methionine synthase reductase; dUMP-deoxyuridylate, dTMP-deoxythymidylate

greater than $15\mu\text{mol/l}$ is considered moderately high (hyper-homocysteinemia; hypHcy) while its level in excess of $60\mu\text{mol/l}$ is potentially a disease condition, homocysteinuria (see Sullivan *et al.*, 2015). Though not a disease by itself, individuals having hypHcy show susceptibility to various disorders. As illustrated in Fig. 1, conversion of Hcy into methionine requires addition of a methyl group (1-carbon) culled from 5-Methyltetrahydrofolate (M-THF) and catalysed by the enzymes MTR (methionine synthase) and MTRR (methionine synthase reductase). While addition of $-\text{CH}_3$ to Hcy converts it to Met, $\text{CH}_3\text{-THF}$ after losing the methyl group is converted to tetrahydrofolate (THF). $\text{CH}_3\text{-THF}$ is a reduced form of 5,10 methylenetetrahydrofolate, catalysed by the enzyme Methylene-tetrahydrofolate reductase (MTHFR). Sulfonation of Hcy leading to the formation of Cysteine, has an intervening step of cystathionine formation prior to conversion to Cys, the enzyme involved being cystathionine beta synthase (CBS). The catalytic reactions required for conversion of Hcy in either direction need vitamins as cofactors; vitamin B12 for the 1-C and vitamin B6 for the trans-sulphuration pathways. In addition, supply of folate as substrate

for the Hcy-Met reaction is vital. This paper covers some of the reasons for hypHcy and its predisposition to multifactorial effects as well as Down syndrome—a chromosomal disorder.

Folates and Vitamin B12 and Gene Polymorphisms as Disease Risk Factors – a Glimpse into History

The importance of Hcy-pathway related micronutrients in disease management came into light in the decade of 1920s, when George Minot, an American haematologist, tried “weird” nutritional regimes—raw liver, half-cooked hamburgers, raw hog stomach, even regurgitated gastric juices—as dietary treatment for pernicious anaemia. In 1926 he along with Dr William Murphy concluded that the cause of this anaemia was dearth of an important nutrient present in liver extract—a finding that fetched them Nobel Prize in 1934 (Murphy, 1934). There was another kind of anaemia, detected particularly in pregnant mothers in India that was as “pernicious” as the other one but non-responsive to Minot’s recipes. Lucy Wills, a British physician, travelled to India in 1928, and found its cure by feeding marmite (extracted

from yeast) (see Wordsworth 1988). It took more than a decade to discover that the “active principles” in the nutritional supplements used by Minot and Murphy in pernicious anaemia, and by Wills in tropical macrocytic anaemia were respectively, vitamin B12 (cobalamine) and folic acid (folates). However, use of folate as dietary supplement in the treatment of macrocytic anaemia and other disorders also brought into focus its interaction with vitamin B12, and the concept of folate trap which suggested that supplementation of folates to individuals deficient in vitamin B12 impaired Hcy-methionine-SAM cycle, rather than augmenting it (Rosenberg and Sehlub, 2006). More recently, a study has shown the contribution of folates and vitamin B12 in normocytic anaemia in Indians (Sukla *et al.*, 2014). Understanding of their clinical significance in several other diseases soon followed.

One of the most striking revelations of the human genome sequencing is that despite being more than 99% identical, human genomes differ from each other in hundreds of thousands of nucleotides. Individually, most of these variations are phenotypically innocuous. Therefore they survive in the population undetected, and are called polymorphisms (majority of them Single Nucleotide Polymorphism, SNP) rather than mutations. However, combinations of SNPs and other changes affect the phenotype under certain environmental conditions, highlighting the inevitability of the gene-gene and gene-environment interaction in the development of phenotypes, particularly the disease phenotype. The disorders caused due to interaction of one or more than one gene with the environment are collectively defined as the Multifactorial disorders (MD). One of the most obvious environmental factors that influence health and disease conditions is nutrition.

Hyperhomocysteinemia, Folate Deficiency, Hcy Pathway Enzymes as Risk Factors for Neural Tube Defects (NTD) and Congenital Heart Disease (CHD)

NTD and CHD are among the most common developmental disorders. In case of the former, children are born with incompletely closed neural tube. In population-specific manner its incidence ranges from 1 in 500 to 1 in 1000 live-births. Smithells *et al.* (1976) were among the first to show that mothers of

the NTD children were deficient in several vitamins, more importantly folates. This observation led them to offer women periconceptionally with a dose of multivitamins having 0.36mg folic acid (Smithells *et al.*, 1981). This was one of the first clear evidence of association of micronutrients with a developmental disorder. Literature is now replete with papers supporting this piece of observation (see Hesecker, 2011). CHD is a group of congenital heart ailments manifesting at birth or later in life. Studies from different parts of the world have shown maternal hypHcy and folate deficiency as important risk factors for CHD (see Huhta *et al.*, 2005). In response to this overwhelming body of evidence, governments in North America and Europe, have fortified flour and other grain products with folates, making it an integral part of diet. Population surveys in U.S.A., Canada and Europe after more than 10 years of introduction of folate supplementation have shown a striking drop in frequency of NTD (especially spina bifida) and CHDs (MRC 1991; CDC 2009; Zinck *et al.*, 2015). It should be noted, however, that the decline in the frequency is only up to 20%. Therefore, analysis of genetic proclivity of individuals to specific disease is intuitively a good possibility.

Study on the genetic susceptibility of multifactorial disorders has been greatly facilitated by the vast repertoire of DNA polymorphisms recorded from human genomes. One of the simpler approaches is the “case-control” study in which frequency of certain polymorphisms of gene in a cohort of randomly collected disease individuals is compared with that of a group of unaffected, matched (control) individuals from the same geographic location. Statistically significant difference in frequency of the normal (major) and mutant (minor) alleles between the diseased and control samples suggests association of the allele with the disease. Similar approach was adopted in the case of NTD and CHD in which polymorphisms in the genes for enzymes in the Hcy-pathway were compared in the case-control format. One of the genes chosen for this study was for the enzyme *Methylene tetrahydrofolate reductase (MTHFR)* which catalyses conversion of 5,10 methylene THF to 5methylTHF, the substrate contributing $-\text{CH}_3$ group to Hcy for the synthesis of methionine. A transition from Cytosine to Thymine at position 677(exon 4) of cDNA (*MTHFR* C677T) leading to replacement of

valine with alanine lowers the enzyme activity by 35% in heterozygous (CT) and by 70% in homozygous TT condition (Frost *et al.*, 1995) (Fig. 2). Several studies from different parts of the world, have shown its association with several diseases including NTD, CHD and also with hypHcy. However, the results are not consistent with most of the diseases. While in certain reports 677TT homozygotes show strong association with the disease, in others no association is detected. In fact, a survey of the frequency of this SNP in normal populations from different parts of the world shows significant difference in the incidence of TT, ranging from >30% in Mexicans and North Americans to 2-3% in Asians (Indian subcontinent) and <1% in Africans (McAndrew *et al.*, 1996; Stevenson *et al.*, 1997; Schneider *et al.*, 1998, Mutchinik *et al.*, 1999) (Table 1). Clearly if 30% of the 677TT individuals in a population are normal, unaffected by a disease, TT homozygous would be an unlikely candidate as a risk allele. On the other hand, less than 3% of this genotype in parts of Asia and Africa indicates a low selective value of this mutant in this region. It turns out that T allele (CT/TT) leads to elevation of Hcy

level which also correlates with the level of folates and vitamin B12 (Refsum, 2001). In American and European populations where non vegetarian diet and flour fortification maintain good vitamin B12 and folate levels, the Hcy level remains in check even in the presence of *MTHFR* 677TT genotype. In contrast, in populations poor in folate and/or vitamin B12 levels, TT homozygosity is a serious risk factor that elevates Hcy levels, leading to hypHcy (Sukla and Raman, 2012). Thus the selective potential of this variant is dependent on the nutritional profile of the population, and therefore genetic contribution to the manifestation of the disease phenotype is modulated by the intake of micronutrients. Another *MTHFR* SNP, A1298C that occurs downstream of C677T has also been detected as a risk factor for several disorders. More importantly compound heterozygotes of both the variants prove to be a greater risk than an individual variant. Other genes of the Hcy-pathway (*MTR*, *MTRR*, *RFC1*, *CBS* etc.) have been tested as risk factors for NTD and CHD and other diseases (Table 2), each of them showing an association in some populations but not in others. Generally this association is population specific and shows a broad correlation with the diet of the individuals in that population.

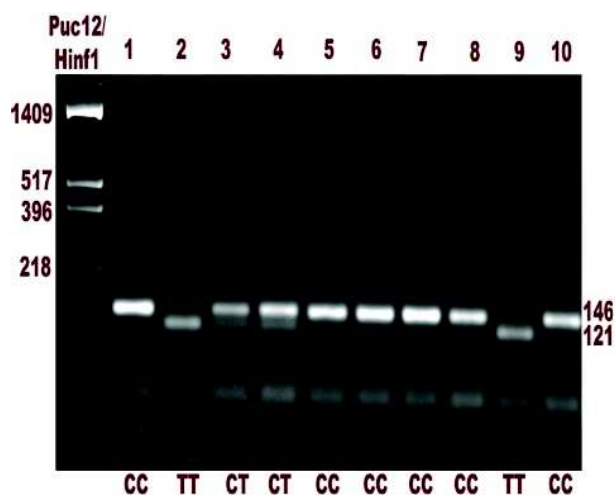


Fig. 2: RFLP-based analysis of SNPs of the *MTHFR* genes. Following PCR amplification of the desired fragment of the gene, the amplicon is digested with the enzyme *HinfI*. The DNA with CC will not be digested by the enzyme and it will yield a DNA amplicon of 146bp. If mutated to T, it creates a site for the enzyme at the position 121, yielding 2 fragments of 121 and 25bp (which is often not visible). The picture above shows the electrophoretically resolved *MTHFR* C677T on a 4% agarose gel. Lane 1 has Puc12/*HinfI* marker while other lanes have C677T amplicons from different individuals. The genotype is indicated below each lane

Gene-Micronutrient Interaction in Hyperhomocysteinemia (An Indian Study)

Lakshmi *et al.* (2001) and Refsum *et al.* (2001) working in the Southern and Western regions of India were among the first to show that in these populations Hcy level was generally higher; and folate and vitamin

Table 1: An overview of the frequency of *MTHFR* 677TT homozygosity in different populations

Population	Incidence (%)	Study reference
Mexicans	27-35	Mutchinick <i>et al.</i> , 1999
Brazilians	7	Schneider <i>et al.</i> , 1998
Subsahara Africans	0-1.5	McAndrew <i>et al.</i> , 1996
African Americans	2	McAndrew <i>et al.</i> , 1996
Yeminite Jews	2	Schneider <i>et al.</i> , 1998
Muslim Arab, Israelis	16	Schneider <i>et al.</i> , 1998
Italian	30	Schneider <i>et al.</i> , 1998
European	17	Schneider <i>et al.</i> , 1998
Japanese	19	Schneider <i>et al.</i> , 1998
Chinese	16	Schneider <i>et al.</i> , 1998
Pakistanis	4	Schneider <i>et al.</i> , 1998

Table 2: List of some of the Hcy-pathway genes and their polymorphism and associated diseases

Gene & Location	Function	SNPs	Associated Disorder
<i>MTHFR</i> 1p36.3	5,10 methylene THF to 5 methylTHF	C677T (exon 4) A1298C (exon 7)	Cardio vascular disease (CVD), Neural Tube Defect (NTD)
<i>MTR</i> 1q43	Methylation of HCy to form methionine	A2756G (exon 11)	CVD, colon cancer
<i>MTRR</i> 5p 15.3	Regeneration of methylcobalamina, cofactor for MTR	A66G (exon 1)	CVD, colon cancer
<i>CBS</i> 21q 22.3	Hcy to Cysteine	T833C, 844ins78bp (exon 8)	Hyperhomocysteinemia
<i>RFC1 (SLC19A1)</i> 21q22.2	Cellular uptake of folate	G80A (exon 2)	Down syndrome, colon cancer
<i>NNMT</i> 11q23	N-methylation of nicotinamide & other pyridines	A815G (intron 1)	Congenital heart disease

B12 levels lower than the W.H.O. standards. Two other studies from southern region (Rama Devi *et al.*, 2004); and Delhi and northern region of India (Kumar *et al.*, 2005) confirmed low frequency of *MTHFR*677T allele, and that the mutant 1298C was associated with hypHcy. Some of the studies also showed association of these SNPs with CHD as well as NTD, confirming what was already known globally (Sharma *et al.*, 1994; Kumar *et al.*, 2009; Godbole *et al.*, 2011).

In the light of the sporadic studies showing that the frequency of *MTHFR* 677T was rather low in India compared with those of European, North American and Japanese populations, we carried out a systematic population-based study on the Hcy-pathway gene polymorphism, status of vitamin B12 and folate and their impact, individually and interactively, on Hcy level (Sukla and Raman, 2012; Sukla *et al.*, 2013). The results and their import are summarised below.

The study was conducted on blood samples of 1426 (820 males and 606 females) “normal” and “healthy” adult individuals from different regions of Varanasi in eastern Uttar Pradesh (n=387), western Bihar (n=214), Jharkhand (n=561) and Chhattisgarh (n=264) to make an appraisal of their contribution to hypHcy and disease susceptibility. More than 36% individuals were vegetarians, and most others were occasional non-vegetarians. Keeping WHO values as standard, nearly 30% of the population was hypHcy ($\geq 15\mu\text{mol/l}$; optimum range 3-12 $\mu\text{mol/l}$), 11% was folate deficient (optimum range 3-24ng/ml plasma) and 50% was deficient in vitamin B12 (optimum range

220-1200pg/ml plasma). While average Hcy level was higher in males than in females, frequency of hypHcy was also greater in males as well as in vegetarians. Vitamin B12 deficiency, on the other hand, was independent of gender but strongly correlated with vegetarians which was true for other regional populations also. What was intriguing was that the frequency of individuals with vitamin B12 deficiency was much greater than those with folate deficiency, possibly because Vitamin B12 is primarily derived through animal foods, and Indians largely depend on vegetarian foods. That folates played smaller role in Hcy level was confirmed by the fact that among those having only folate deficiency and Vitamin B12 being optimum, 22% were hypHcy while in those having only Vitamin B12 deficiency 41% suffered from hypHcy. Of course in the individuals where both nutrients were optimum or deficient the frequency of hypHcy was 11% and 63%, respectively. Clearly, the Hcy level varied under the influence of both folate and Vitamin B12, impact of the latter being more severe.

When hypHcy level was viewed in the genomic context, polymorphisms in *CBS* and *MTR* did not influence Hcy levels. In contrast, both the mutant alleles of *MTHFR* 677T and 1298C, showed strong association with Hcy level, the median being 20.8 $\mu\text{mol/l}$ in 677TT and 13.1 $\mu\text{mol/l}$ in 1298CC individuals. The minor allele of *RFC1* (now called *SLC19A1*) 80A, on the other hand, proved to be protective against hypHcy. The group homozygous for *RFC1*80AA had Hcy value of 9.9, lowest among all the groups.

We now present evidence of how nutrition

affects the genetic constitution in the development of phenotype. As an example, we take the genotype *MTHFR* C677T and look at their Hcy levels in four groups: those (i) having both folate and Vitamin B12 deficiency, (ii) having low Vitamin B12 but optimum folate, (iii) optimum Vitamin B12 but low folate and (iv) both folate and Vitamin B12 levels optimum. The lowest Hcy was seen in individuals with *MTHFR* CC677 and group iv (median 9.3 $\mu\text{mol/l}$) while highest Hcy was seen in *MTHFR* 677TT and group I (43.2 $\mu\text{mol/l}$). In *MTHFR* 677TT with group iv, Hcy level was 16.9. That is, 677TT is a genetic risk for hypHcy even when micronutrients are optimally present. Studies from Yajnik's lab in Pune (Refsum *et al.*, 2001; Yajnik *et al.*, 2005) and of Sengupta (Kumar *et al.*, 2005, 2010) from northern India provide comparable picture.

Hcy-Pathway and Reproduction

Raman and Narayan (1995) and later Tamara *et al* (2003) demonstrated that inhibition of DNA methylation in mouse spermatogonia inhibited their transition into primary spermatocytes, suggesting important role of DNA methylation in spermatogenesis. Hypomethylation of the DMR2 (differential methylation Region) of *Igf2* has been shown to cause infertility in rat (Murphy *et al.*, 2011). Therefore when association of *MTHFR* C677T and A1298C in cases of idiopathic male infertility in an Indian cohort was studied, it showed extremely low frequency of T allele in *MTHFR* C677T (9% in control and 17% in the cases), especially TT homozygosity (4% in cases and 0.0% in controls) but also that T allele was a risk factor in male infertility, more so in the homozygous condition (Singh *et al.*, 2005). A meta-analysis on results from different parts of the world overwhelmingly shows the T allele to be a risk factor in male infertility, especially in homozygous condition (Liu *et al.*, 2015). Majority of the studies (more than 10) from different regions of India, considered in the meta-analysis, demonstrate *MTHFR* 677T allele to be a risk factor for infertility. The meta-analysis showed no association of A1298C from *MTHFR*, with infertility though the report from eastern India did indicate association albeit much less than with 677T (Singh *et al.*, 2010). There are no studies on other Hcy-pathway genes from India. Global data find *MTRR* as a mild risk factor for male infertility (see Liu *et al.*, 2015). Ebisch *et al.* (2006a, b), whose earlier

study from Europe did not find an association of *MTHFR* 677T with infertility (Ebisch *et al.*, 2003), found both folate deficiency and Hcy elevation as risk factors for male infertility, and recommended folate supplementation as a therapy.

There is no clear report on Hcy-pathway genes as risk factor in female infertility. However, in a case-control study, association of *MTHFR* 677T and 1298C with recurrent pregnancy failure has been documented (Nair *et al.*, 2012, 2013) in cases drawn from eastern India. There are several other studies from different parts of the world which corroborate the role of *MTHFR* variants and early pregnancy failures (see Cao *et al.*, 2013). Through a detailed study on early development, Enciso *et al.* (2016) have demonstrated strong association of *MTHFR* variants (677T and 1298C) on failure of blastocyst implantation and chromosomal aneuploidy as cause of early and recurrent pregnancy failures. They observed that while 25% of embryos having failed implantation were homozygous for 677T, only about 5% (1 out of 19 embryos) with successful pregnancy had 677TT homozygosity. Thus the role of *MTHFR* variants as risk factors for poor pregnancy outcome seems well established, and periconceptional supplementation of folates for mitigation has been internationally practiced. In India whether folate alone or in combination with vitamin B12 is a better alternative needs to be tested.

Nonsyndromic Cleft lip±Palate

Clefting of lip or palate is one of the most common congenital disorders, globally. The majority of cases occur sporadically but nearly 10-15% cases show a familial trend suggesting a genetic predisposition, which in interaction with the prevailing environmental condition, may result in the disorder. It being a congenital disease, quite naturally maternal environment during gestation period, parental genotype as well as the gestational environment and genetic constitution of the affected foetus, all could contribute to the development of the NSCL±P. Association of the Hcy pathway genes and Hcy level and folates has been analysed in cases and/or case-mothers from different parts of the world. In this case too, the results are quite mixed: with respect to *MTHFR* 677TT homozygosity, genotypes of either the mother or the probands or both or none of them was associated

with NSCL±P (Ali *et al.*, 2009; see Sullivan *et al.*, 2015). In our case-control study, conducted on more than 500 subjects (cases 323, controls 214), TT homozygosity was associated as risk factor both for the case as well as case-mothers (Ali *et al.*, 2009). *MTHFR* A1298C did not show an association. In *RFC1* G80A, the gene involved in cellular folate absorption, the G allele was also a risk allele and 677TT/GG80 together were a greater risk than any of them individually, but, as stated earlier, *RFC1* 80A proved to be protective against NSCL±P even in the presence of 677TT homozygosity (Kumari *et al.*, 2013). However, a recent study on a relatively smaller sample size in southern India (Murthy *et al.*, 2014) does not show the association with either of the risk SNPs. It finds, on the other hand, that in *RFC1*, the minor allele A, is associated with the disease (Lakkakula *et al.*, 2015).

An intriguing feature of NSCL±P is that its frequency is much higher in males than in females, the general ratio is 2:1, and this pattern is seen globally. While assaying Hcy level in the case-children in a case-control format, we found Hcy level to be elevated in cases than in controls, and the incidence of hypHcy was strikingly higher in the cases. The most revealing observation was that the level of Hcy was greater in the female case-children than in the males. This was striking because in general population (control) the level of Hcy is significantly higher in males than in females, while in NSCL±P, the levels were the opposite. It is also to be noted that nearly 60% of affected female children showed hypHcy even if they carried 677CC genotype which is not a risk genotype. Most likely these children suffered from Vitamin B12 and/or folate deficiency during pregnancy. This leads us to surmise that a higher level of Hcy threshold is necessary for expression of NSCL±P in females than in males, and that could be one of the reasons for lower incidence of the disease in females (Kumari *et al.*, 2013).

Low Birth Weight (LBW) and Neonatal Hyperbilirubinemia (NNH)

The frequency of neonatal deaths and low birth weight (LBW) are the highest in India, and one of the obvious reasons for it is the poor maternal health during pregnancy that affects foetal growth. More than 35% children born are clinically LBW (body weight <2.5kg),

and they are generally born to undernourished mothers many of whom are also deficient in folic acid and vitamin B12 and consequently high Hcy (Yajnik *et al.*, 2005). Taneja *et al.* (2007) also reported deficiency of these micronutrients in children within an age group of 6 to 30 months from northern India. We carried out a study on the cord blood of more than 400 just-born children in the University hospital to assay their Vitamin B12, folate and Hcy levels along with the genotype of the Hcy-pathway genes. Sixteen percent of the children were prematurely born while 38% showed LBW, and a subgroup of 25% children suffered from NNH. Surprisingly, their nutritional parameters were even poorer than those of adults from the same region: 25% children were hypHcy, and Vitamin B12 and folate deficiency was seen respectively in 65% and 27% children. This is the poorest statement on neonatal health, world-wide. All the three nutritional parameters individually are putative risk factors for LBW. This scenario is exacerbated by the fact that *MTHFR* 677T and *RFC1* A80G are the genomic risk factors for this trait. Within this cohort, 25% children developed jaundice (NNH) within 72h of birth. *MTHFR* 677T and folate deficiency proved to be clear risk factors for this transient malady (Sukla *et al.*, 2013).

These independent studies conducted in different parts of India allude to the fact that extreme paucity of micronutrients and their genetic predisposition may lead to diverse neonatal disorders, leading to high child mortality and even later age effect on their health. There is evidence that children having Vitamin B12 deficiency during foetal and neonatal stage run a high risk of coronary artery disease and neurological disorders, more specifically cognitive impairment (Huxley 2006).

Down Syndrome (DS)

On an average one out of 700 new-borns is afflicted with Down syndrome, a disorder leading to intellectual disability and multiple organ malfunctions. DS is a chromosomal disorder caused due to trisomy of chromosome 21 arising from error in chromosome distribution during parental meiosis. More than 85% occur due to nondisjunction in maternal meiosis (see Hassold and Hunt, 2001). The only risk factor associated with DS is the age of the mother, particularly mothers older than 35 years at the time

of conception (Penrose, 1933). Efforts to identify genetic risk factors were not successful until James *et al.* (1999) and Hobbs *et al.* (2000) showed in two independent case-control studies from America that *MTHFR* 677T is strongly associated with case-mothers. Over 60 studies have so far been conducted on association of Hcy-pathway genes with DS in different parts of the world, including Indian subcontinent. The broad pattern is that, *MTHFR*-DS association is more pronounced in Asian than in the European populations. In Europe, for instance, majority of the studies show no association of *MTHFR*/Hcy with DS, excepting 5 reports from Italy in which association of various Hcy pathway genes was apparent (for review, see Rai V *et al.*, 2014; Coppede *et al.*, 2015). In Europe, the consensus of opinion is that the lack of association is due to adequate micronutrient supplementation both through folates in the flour and Vitamin B12 through non-vegetarian diet. However, numerous independent genotype, studies from across the world have shown that nutritional deficiency and high Hcy level are uniformly risk factors for DS in case mothers (see Sukla *et al.*, 2015).

In India the first systematic study on association of DS with *MTHFR* 677T showed *MTHFR* 677T to be a risk factor for DS (Rai A. *et al.*, 2006). More importantly, 677TT homozygosity was seen predominantly in younger case mothers (<30 years) implying that those young mothers may be genetically predisposed to have DS children (Rai A. *et al.*, 2006). Since then, a number of studies from different regions of India have been published, and while some of them do show this polymorphism as a risk factor (Rai A *et al.*, 2006; Cyril *et al.*, 2009; Pandey *et al.*, 2013, Sukla *et al.*, 2015), there are others that do not find any association (Kohli *et al.*, 2008; Mohanty *et al.*, 2012; Kaur and Kaur, 2013). Jaiswal *et al.* (2015) working in the eastern region of India showed that SNPs -579G>T and -149C>T in a DNA methyl-transferase gene, DNMT3b, does not show any association with DS. Such discrepancies point to the role of other Hcy-pathway genes as well as the role of Hcy-pathway micronutrients in this chromosomal disorder.

In a rather detailed, integrated triad study, Sukla *et al.* (2015) have analysed genotypes of 7 variants in 6 Hcy pathway genes as well as levels of micronutrients and Hcy in DS children as well as their parents. The study, conducted on 151 case-triads and

200 controls each for male, female and the young, confirmed the role of *MTHFR* 677T as risk factor in mother, father and DS children. In *RFC1* G80A, the A allele turns out to be protective but only in DS children, both in homo- as well as heterozygous condition. The other variants viz., *MTR* A2756G, *MTHFR* A1298C, *MTRR* A66G, *CBS* 844 ins84bp and *TCN2* C776G do not show significant association. However, when the variant combinations were genotyped, 56% of DS mothers had 4 or more variants (out of the 7 analysed) as against 10% control mothers. Among the DS and control children the difference was even more staggering (65% in DS vs 13% in controls) while among fathers there was no difference. This result is comparable to some of the studies from Italy, Spain and Brazil (da Silva *et al.*, 2005; Coppede *et al.*, 2006; Biselli *et al.*, 2008). By implication, other variants also contribute to the risk of DS in small measures, and the paternal genotype (except *MTHFR* 677T) does not seem to have a bearing on the risk.

Sukla *et al.* (2015) also showed that Vitamin B12 and folate levels are uniformly much lower in the case group (case mother, father and DS) than the controls. Hcy level was higher in both the parents but lower in the DS children. In fact DS children had the lowest Hcy among all the different groups. This lowering is explained by the strikingly high level of cysteine in the DS (see Sukla *et al.*, 2015). Since *CBS* gene, that catalyses Hcy in the sulfonation pathway, is present on chromosome 21, its 3 copies must drive Hcy towards cysteine synthesis, the pathway that leads to loss of Hcy. Thus elevated level of cysteine and lowered level of Hcy are expected in DS children which could also be a survival advantage for them. More importantly, as earlier shown for LBW cases, the combination of *MTHFR* 677T and Vitamin B12 and/or folate deficiency and elevated Hcy is a far greater risk in DS mothers.

Accumulation of Hcy is a manifestation of lowered synthesis of methionine, which in turn lowers SAM level that would globally affect methylation of target sites, causing hypomethylation of a number of sites and their expression pattern. There are also reports that the pericentromeric heterochromatin is highly methylated. Both James *et al.* (1999) and Hobbs *et al.* (2000) had conjectured that hypomethylation of the centromeric region would create inadequate

condensation of the centromeric region causing malsegregation of chromosomes. There is sufficient evidence showing that nutritional deficiency of the vitamins, exacerbated by the genetic constitution, creates a condition for chromosomal aneuploidy (Hollis *et al.*, 2013; Encise *et al.*, 2016).

In this rather personalised review, I have presented evidence to establish the role of Hcy-pathway genes in the development of several disorders at all levels: congenital, late age or even chromosomal. It is also shown that the possible adverse effect of the genic variants could substantially be modified by the nutritional environment of the individual. The matter of particular concern is that it is reflective of the poor maternal health during pregnancy, and that a better management during those months could reduce disease burden to a large extent. In India, supplementation of iron, and also folic acid, to prospective mothers during pregnancy since last 40 years, has not brought down the incidence of anaemia in any significant way. There is a need to rethink on

the supplementation regime, both in terms of their timing, content and implementation. The limited scope that our work provides leads us to advocate for (i) the need for planned pregnancy and use of the supplementation months before its start, (ii) assessing the folate levels individually before deciding its dose and (iii) inclusion of Vitamin B12 in the recipe as an essential ingredient. Minor changes in nutritional course could make a big change in the health scenario of the population.

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