FOLATES AND NEURAL TUBE DEFECTS IN MARRIED FEMALE HOSPITAL POPULATION

By

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A thesis submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy

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بِسْمِ اللّٰهِ الرَّحْمٰنِ الرَّحِيْمِ

In the name of Allah,
the Most Beneficent,
the Most Merciful

In loving memory of my parents Dr. Abdul Rashid and Razia Begum, who gave me the best education and taught me to live honestly, work hard and never give up.

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List of abbreviations

CA	Congenital Anomaly
CDC	Center for disease control
CNS	Central nervous system
CSHMT	Cytoplasmic serine hydroxymethyl transferase
DHFR	Dihydrofolate reductase
DHLP	Dorsolateral hinge point
DM	Diabetes mellitis
DSH	Dishevelled
EDTA	Ethylenediaminetetraacetic acid
ETS	Environmental tobacco smoke
FA	Folic Acid
FR	Folate receptors
FTHFc	Formyl tetrahydrofolate synthetase
5-Methyl THF	5-Methyl Tetrahydrofolate
MPH	Median hinge point
MTHFc	Methylene tetra hydrofolate cyclo hydrolase.
MTHFD	Methylene tetrahydro folate dehydrogenase
MTHFR	Methyltetrahydrofolate reductase
MS	Methionine synthase
MTRR	Methionine synthase reductase
NTD	Neural tube defect
OCM	One-carbon metabolism
PAH	Polycyclic aromatic hydrocarbons
PCFT	proton-coupled folate transporter

PCP	Planer cell polarity
PCR	Polymerase chain reaction
RFC	Reduced folate carrier
RFLP	Restriction fragment length polymerism
SAM	Sal adenosyl metionine
Shh	Sonic hedgehog
SB	Spina bifida
SES	Socioeconomic status
SHS	Second hand smoke
SNP	Single nucleotide polymorphism
Hcy	Homocysteine
THF	Tetrahydrofolate
TS	Thymidylate synthase

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ABSTRACT

Abstract

Neural tube defects (NTDs) are severe, distressing, congenital abnormalities of central nervous system which result in either death of infant and in those who survive suffer from lifelong disabilities. Folic acid has been proven to prevent most cases of spina bifida and anencephaly. Neural tube defects (NTDs) have been associated with maternal folate deficiency and the mutation C677T in the methylenetetrahydrofolate reductase gene (MTHFR), a key enzyme in folate metabolism. Mothers who delivered NTD infant or had come for termination were identified in Obstetrics Department of Holy Family Hospital, Rawalpindi and matched mothers who gave birth to healthy babies were selected from same ward as control. Maternal demographic data was computed after direct interviews. Among the 190 neural tube defects identified myelomeningocele (30%), meningocele (42.11%) and anencephaly (18.95%) were the commonest. The rare NTDs identified were lipomeningocele (2.11%), Dandy Walker syndrome (1.05%), Spina bifida+Arnold –Chiari syndrome and syringomyelia. Hydrocephalous along with NTDs was present in 35.79%. Mean maternal age of case mothers was 27.51 ± 0.38 years. Significantly more females were present in the NTD offspring (P=<0.005). Highest percentage of NTDs was observed in age range between 25-29 years. Majority of NTDs were present in first parity (26.31%) with a steady decline in subsequent parities. Consanguinity was a risk factor in this study. Frequency of consanguineous marriages among case mothers (60%) were significantly higher than in control mothers(45%) (P=<0.015). Mothers with NTD births from rural areas were significantly more than from urban areas (P=<0.0001). Case mothers exposed to passive smoke were significantly more than control mothers (P= <0.0066) and gave birth to babies with both cranial NTDs(10%) as well as spinal NTDs (31.05%). Case mothers exposed to environmental hazards (chemical waste and garbage dumps) were significantly more than control mothers exposed to same hazards (P=<0.0413). High temperatures were reported by 11.05% mothers with NTD births and diabetes was present in 13.68%. Case mothers who took diet inadequate in fruits and vegetables were greater than control mothers and the difference was significant (P= < 0.0026). None of the case mothers had taken folic acid in periconceptional period and alarmingly low knowledge of folic acid was present in case mothers as well as control mothers.

Awareness regarding importance of folate intake and its relevance in preventing NTDs was lacking in case mothers (0%), only 25% of control mothers had heard of folic acid and only 5% taken it in periconceptional period. Adverse reproductive history in previous pregnancy was present in 61.59% case mothers which was significantly higher than control mothers (P= <0.0001). Majority of case mothers had not gone to school (45.26%) and belonged to low socio economic group (44.74%). In control mothers (58%) and case mothers (75.79%) were house wives. Occupational status of fathers with NTD births were mostly unskilled (35.79%) and skilled laborers (33.68%) with similar results in fathers with normal babies. Folate status was assessed by estimation of serum and RBC folate levels. Mean Serum and RBC folate (P=<0.0001) were significantly low compared to control mothers. Compared to control group in all the age cohorts case mothers showed significantly low RBC folate levels (P=<0.001). Serum folate was non-significantly low in case mothers compared to controls. Case mothers exposed to passive smoke had significantly low mean RBC folate levels (P=<0.0001) compared to control mothers, while mean serum folate levels (P=0.07) were not significantly different in case and control mothers. RBC folate was significantly low in control (P=<0.0001) and case mothers (P=<0.0139) residing in rural areas, whereas serum folate levels showed no significant difference. Both serum folate and RBC folate levels were higher in control and case mothers who reported intake of folate rich diet compared to those who reported diet insufficient in fruits and vegetables and the difference was significant. The levels of RBC folate and serum folate were higher with increase in levels of education and a better socioeconomic status. Genotyping of MTHFR C677T mutation was carried out by polymerase chain reaction and restriction fragment length polymorphism studies(PCR-RFLP). Comparison of polymorphism in the 2 groups was done using the chi-square test. Genotyping results showed MTHFR 677TT genotype significantly more prevalent in case mothers as compared to control mothers (P= <0.039).

The study showed consanguinity, diet poor in folate, low folate status with MTHFR 677TT genotype were risk factors for occurrence of NTDs in Pakistani hospital population. In Pakistan, with meager health resources and high poverty level, much needs to be done in order to increase consumption of folic acid in women of child-bearing age and for a developing country like Pakistan with high rate of illiteracy the optimal solution is flour fortification with folic acid.

INTRODUCTION

Introduction

Background

Congenital anomalies are structural defects acquired during the intrauterine development. These may be noticeable at birth or shortly afterwards. Among the most distressing congenital defects in humans are structural defects of central nervous system like Neural tube defects (NTDs) (Czeizel et al.,2011;Copp et al.,2013). These congenital anomalies result from the failure of neurulation (De Marco et al.,2006). The common NTDs are spina bifida, anencephaly and encephaloceles (Detrait et al., 2005; Kondo et al., 2009). Formation of neural tube is a complex and synchronized process completed by 28^{th} day post conception. Genetic susceptibility and exposure to environmental factors can result in an NTD (Copp, 2003; Detrait et al., 2005; Copp et al., 2013). Identification of the underlying genetic factors is imperative for determining the interactions between genes and the environmental factors that can alter their expression. Deciphering these interactions could form the basis for designing preventive strategies and their implementation.

It was two decades ago that a strong association between the occurrence of NTDs and a folate deficient diet in the periconceptional period was established (Hibbard and Smithells, 1965; Smithells et al., 1976). In a retrospective study, Hibbard (1964) observed that women with fetal malformation affected pregnancies had higher incidence of disturbed folate metabolism. In early 1990s two randomized clinical trials indicated that high dose of folic acid (MRC: Vitamin Study Research Group, 1991) or folic acid-containing multivitamin supplementation during the periconceptional period was effective in the prevention of occurrence and recurrence of NTDs. Even though association between adequate folate status at the time of conception and a risk reduction of NTDs has been established, NTDs have a complex and imperfectly understood etiopathogenesis with involvement of genetic, nutritional and environmental factors (Cabrera et al., 2004; Kondo et al., 2009; Copp et al.,2013). The mechanism whereby folate deficiency might interact with genetic vulnerability is still poorly understood. NTDs are viewed as preventable congenital disorders with folate rich diet and

supplementation (Botto et al., 1999; Berry et al., 1999; Suzuki, 2007; Bestwick et al., 2014).

In the non-pregnant adult daily requirement of 100 micrograms is normally met with but the average Pakistani diet does not contain the 400 micrograms a day of folic acid required by the pregnant mother for protection from NTDs in the developing fetus. Important factors such as a diet low in folate, poor intestinal absorption and loss through unhealthy cooking leads to folate deficiency in majority of women of reproductive age. Neural tube closure occurs by the 28^{th} post conception day, before women are aware of their pregnancy. This excludes the efficacy of folic acid given after the diagnosis of pregnancy for prevention of NTDs (Heseker, 2011; Safi et al., 2012).

Since more than fifty percent of pregnancies are unplanned it is important for women of reproductive age to have an adequate folate status before conception. This requires women of child bearing age to consume food with high folate content or take 0.4 mg of folic acid each day. Although multiple factors are implicated in etiology of NTDs, folic acid supplementation (0.4mg/day) given at preconception and in the first few weeks after conception can measurably reduce the occurrence of these disabling malformations (Northrup et al., 2000; Lumley, 2001; Ross, 2010).

In a notable study conducted on Chinese population by Berry et al (1999) where there was high NTD prevalence half the population (geographically confined) was given folic acid supplementation whereas the other half received normal prenatal care. There were fewer offspring with NTDs born to mothers who took folic acid supplementation. Folate levels were found to be low in women with history of NTD pregnancy than women who had normal offspring. Subsequent case control intervention studies have inferred that periconceptional use of folic acid exerts a protective effect on fetal development. In countries where folic acid supplementation of food was implemented a reduction in the incidence of neural tube defects has been observed (Smithells et al., 1980; MRC: Vitamin Study Research Group, 1991; Blencowe et al., 2010).

Neural tube defects (NTDs) arise from intricate relationship among genes, metabolic needs and nutritional adequacy. Genetic polymorphisms in enzymes of one carbon

metabolism have been documented. Folate plays a key role as a donor and acceptor of one-carbon units in various enzymatic reactions associated with one-carbon metabolism (Appling, 1991; Greene et al., 2011).

Genetic and environmental factors together alter gene expression as NTDs occur in different frequencies in different geographical regions more in lower socioeconomic group with inadequate nutrition. Improving nutrition alters gene expression. NTDs can be prevented by good diet in periconceptional period. NTDs occur in early embryological period hence public health intervention must target all women of child bearing age (Carmichael et al., 2006; Safi et al., 2012). High dose of folic acid (FA) intake (400 mg /day) taken in periconception period prevents neural tube defects (NTD) is considered a major nutritional discovery during the last 50 years (Katan et al., 2009). Folate deficiency can be reduced by taking FA or eating fortified foods (Berry et al., 1999), and is the most efficacious intervention to prevent occurrence of NTDs (Honein, 2001; De Wals et al., 2007; Crider and Bailey, 2011). Other environmental factors implicated in increasing the risk for NTDs include geographical regions, socio-economic class, age of mother, diet of mother especially in periconception period, history of Diabetes in mother, history of high temperatures in early pregnancy, consanguinity, smoking, and drug exposure mainly to antiepileptic drugs (Frey and Hauser, 2003; Mitchell, 2005; Padmanabhan,2006;Kondo et al.,2013).

Mechanism of Neurulation

Formation of the neural tube is a cascade of multiple tightly regulated complex processes involving the coordinated growth and morphogenesis of multiple tissues including the neural tissue, ectoderm and surrounding mesoderm. Imbalances in growth or differentiation in any of the tissues involved can result in a NTD. In early embryogenesis, neurulation in the mammalian embryo takes place in two phases: primary and secondary neurulation (Purves and Lichtman, 1985; Copp et al., 2003). Both phases develop in distinct areas along the rostro-caudal axis of the developing embryo. Secondary neurulation occurs in the more caudal area of the tail bud (caudal eminence) located beyond the caudal neuropore. Secondary neurulation occurs by proliferation and condensation of mesenchymal stem cells. It then cavitates and transforms into a tube, the

lumen being in continuity with the lumen of the tube formed during primary neurulation (Copp et al., 2003; Bassuk and Kibar, 2009).

Primary neurulation results in formation of the central nervous system rostral to caudal neuropore, while secondary neurulation results in formation of spine, caudal to the sacral vertebrae. Primary neurulation results in transformation of ectodermal cells over the notochord into a hollow tube whereas in secondary neurulation there is no formation of a neural plate. Cells of the tail bud aggregate into a medullary cord that undergoes cavitation to form the epithelial tube which joins with central canal formed in primary neurulation (Schoenwolf,1984; Colas and Schoenwolf,2001; Joó,2009;Zohn,2012). Neurulation is a fundamental component of embryonic development. The genetic risk factors in embryonic development need to be elucidated if prevention strategies by folic acid are to be enhanced and precise embryonic mechanisms need to be understood to form basis of therapeutic intervention treatment in-utero (Fleming et al., 1997; Copp et al., 2013).

Primary Neurulation

This highly complex and intricate process comprises the conversion of flat ectodermal plate into a cylindrical neural tube (Copp et al., 1990; Schoenwolf and Smith, 1990; Bassuk and Kibar, 2009). There are four stages in the process of primary neurulation:

Formation of Neural Plate: The neural plate is formed with differentiation of the dorsal midline ectoderm into the neuroepithelium. The process of neural induction takes place by suppression of epidermal fate by bone morphogenetic proteins antagonists, including chordin, noggin, and follistatin, which emanate from the primitive node and result in the formation of neuroectoderm. Signaling pathways namely fibroblast growth factors, canonical wing less Wnt signaling, and insulin-like growth factor influence neural induction (Bainter et al., 2001; De Robertis et al., 2004; Stern, 2006).

Shaping of Neural Plate: Shaping of the neural plate results in the conversion of the neural plate into an elongated structure which is broader in the cranial region and narrow at the spinal end. The mechanism of this morphogenetic phase is termed convergent extension (CE). Convergent extension cell movements result in narrowing and

lengthening of the cells of the neural plate (Keller, 2002;Bassuk and Kibar,2009) and initiates closure of mammalian neural tube (Copp et al.,2003; Ueno and Greene, 2003). Convergent extension depends on a noncanonical Wnt signaling pathway, termed the planar cell polarity (PCP) pathway (Mlodzik, 2002; Zohn et al., 2003; Simons and Mlodzik., 2008). PCP genes required for PCP signaling in all tissues include *Frizzled* (*Fz*), *Disheveled* (*Dsh*), *Strabismus*/*Van Gogh(Stbm/Vang), Flamingo* (*Fmi*), *Prickle (Pk),* and *Diego* (*Dgo*) (Simons and Mlodzik, 2008).

Bending of Neural Plate: Shaping of the neural plate is followed by bending. This involves formation of hinge points at two sites: the median hinge point (MHP) overlying the notochord and extending along the rostrocaudal axis, and the paired dorsolateral hinge points (DLHP) at the lateral sides of the folds, predominantly at the future brain levels. Bending of the neural plate involves both furrowing and folding. The forces causing furrowing are generated by the wedging of neuroepithelial cells within the hinge points, a process driven by both apical narrowing and basal expansion (Schoenwolf and Smith, 1990). This differential bending at different axial levels is controlled by signals emanating from the notochord, including the signal transduction protein sonic hedgehog(Shh) (Greene and Copp, 2009). After hinge points are formed, the neural plate elevates by rotation around the MHP and convergence around the paired DLHP. The morphogenetic process of bending of the neural plate with formation of neural folds is termed apical constriction whereby columnar cells in the neural plate are converted into wedge-shaped cells (Wallingford, 2006). Apical constriction is regulated by expression of two actin-related genes: *p190RhoGap,* a negative regulator of Rho GTPase involved in regulating actin dynamics (Brouns et al., 2000) and Shroom that codes for an actin-binding protein (Hildebrand and Soriano, 1999; Haigo et al.,2003). Shroom localizes in cells that constrict during cranial neural tube closure and regulates these apical constrictions that drive epithelial folding in vertebrate embryos (Martin, 2004). Shroom knockout mice exhibit anencephaly with their brain bulging out like a wild mushroom and with spina bifida in some cases (Hildebrand and Soriano, 1999). Bending and formation of neural folds, with elevation and convergence of the folds towards the dorsal midline forms a trough like space, the neural groove which is the lumen of primitive neural tube (Copp et al., 2003). The signaling forces that result in furrowing emanate

from underlying notochord and are mediated by a secreted protein Sonic Hedgehog (Shh). Shh drives the formation of median hinge point and the floor plate. Dishevelled (DSH), a member of the non-canonical Wnt signaling pathway is required for the process of convergent extension and in animal models mutation of this gene results in NTDs (Wallingford and Harland, 2001; 2002a,b).

Closure of neural tube: Neural tube closure is the final event of neurulation, in which the pair of neural folds appose in the dorsal midline, adhere to one another and fuse forming the roof of neural tube. This separates it from overlying epidermal ectoderm which forms skin on back of embryo (Colas and Schoenwolf ,2001). Research on this subject carried out over past 30 years state that NTDs are not a monogenic disorder but an interaction of multiple genes and associated environmental factors (Ross,2010). Fusion of the neural folds requires changes in cell adhesion, including N-cadherin and E-cadherin. Their exact roles in this process are still unclear. The neural folds approach each other in center and cellular protrusions expand from apical cells interdigitate, the process followed by formation of permanent cell contacts (Lawson and England, 1998; Bassuk and Kibar, 2009; Pyrgaki et al.,2010).

Theories of Neural Tube Closure: Neural tube closure is initiated as a discontinuous process and is initiated at discrete points in mammals along the rostro–caudal axis (Van Straaten et al., 1996 ;Copp et al., 1990). Three sites of closure have been described in the mouse (Greene and Copp, 2009). Events similar (as in mice) to closure 1 and 3 have been described in humans (O'Rahilly and Muller, 1994, 2002). During primary neurulation failure of neural tube closure during primary neurulation at any level of the body axis from the brain to the sacral spine leads to "open" NTDs (Kibar et al .,2007;Rossi et al.,2004). The site of initial closure (equivalent to mouse closure1) is postulated to occur at a slightly more rostral level in humans than in mouse, and is located in the rhombencephalon as opposed to the rhombencephalon/cervical boundary (O'Rahilly and Muller, 2002). Failure of closure 1 results in a condition where neural tube remains open throughout the brain and spinal cord and is termed craniorachischisis. A more caudal failure of fusion from closure 1leads to open myelomeningocele (Bassuk and Kibar, 2009). Closure 3 occurs at the extreme rostral end of the neural plate in humans as in the

mouse (O'Rahilly and Muller, 2002). Closure 2 is controversial in humans as described in some studies (Van Allen et al., 1993; Golden and Chernoff, 1995; Seller, 1995) but not others (O'Rahilly and Muller, 2002). The presence of closure 2 site was studied on late stage anencephalic fetuses (Van Allen *et al*., 1993; Seller, 1995), and directly on early human embryos with the suggestion that closure 2 occurs either more caudally than in mice, in the hindbrain (Nakatsu et al., 2000), or may be non- existent (O'Rahilly and Muller, 2002). The failure of closure 2 leads to exencephaly which develops into anencephaly. In anencephaly the skull vault is missing and the exposed brain tissue undergoes degeneration. Failure of closure 3 leads to anencephaly in the forebrain region. Closure in the cranial region is completed on 25^{th} day and closure of the posterior neuropore which completes primary neurulation occurs at 26^{th} to 28^{th} day post fertilization (Bassuk and Kibar,2009). Closure of neural tube involves the neural folds coming in contact in the dorsal midline with adhesion at points of contact associated with epithelial breakdown and fusion. Two separate epithelial layers are formed, the epidermal ectoderm and neuroepithelium, with intervening mesenchymal cells of the neural crest. The neural folds secrete a cell surface coat (Sadler, 1978: 2005), which aids in these events, but the exact composition and function of this cell coat remains unknown. The importance of ephrins A1, A3 and A4 (Cell surface glycosyl phosphatidylinositol (GPI)-anchored proteins) in neural fold fusion has been demonstrated (Holmberg et al., 2000;Abdul-Aziz ,2009). Failure of the process of neurulation results in neural tube defects. The open NTDs (including open spina bifida and anencephaly in mammals) result from failure of opposition and fusion of the neural folds during primary neurulation. Neuronal damage with degeneration may occur due to exposure of the unfused neuroepithelium to amniotic fluid. Open NTDs can occur at different levels of the body axis, in mutant mouse strains as well as in humans, variation in axial levels of these defects reflects multiple closure sites (Greene and Copp, 2009).

Role of folic acid in neural tube closure: On the neurulation-stage embryo folic acid exerts a direct effect. Animal experiments have shown that treatment of genetically predisposed mouse embryos with folic acid in vitro can result in normal neural tube closure (Marean et al., 2011). The role of folic acid in one-carbon metabolism, results in production of pyrimidines and purines required for DNA replication during cell

proliferation. Folic acid acts as a methyl group donor to DNA, proteins, and lipids. In neural tube closure cell multiplication has an important role (Smith and Schoenwolf.,1987; Copp et al.,1988; Greene et al.,2011) hence the hypothesis that increased cell proliferation could be an important effect of folic acid. The role of methylation of genomic DNA and histones are being increasingly implicated in the epigenetic regulation of gene expression（Greene et al., 2011; Imbard et al.,2013). Low maternal folate levels could be the cause of impaired cellular remodeling leading to apoptosis and abnormalities of neural tube closure (Bassuk and Kibar, 2009).

Secondary Neurulation

Secondary neurulation commences after closure of the posterior neuropore and completion of primary neurulation (Dias and Partington, 2004). The secondary neural tube arises from the pluripotent cells derived from the regressing primitive streak located at the caudal end of the embryo termed the caudal eminence (Colas and Schoenwolf, 2001). Cells of the caudel eminence undergo proliferation followed by condensation, cavitation and fusion with the central canal of the neural tube formed by primary neurulation (Catala, 2002).

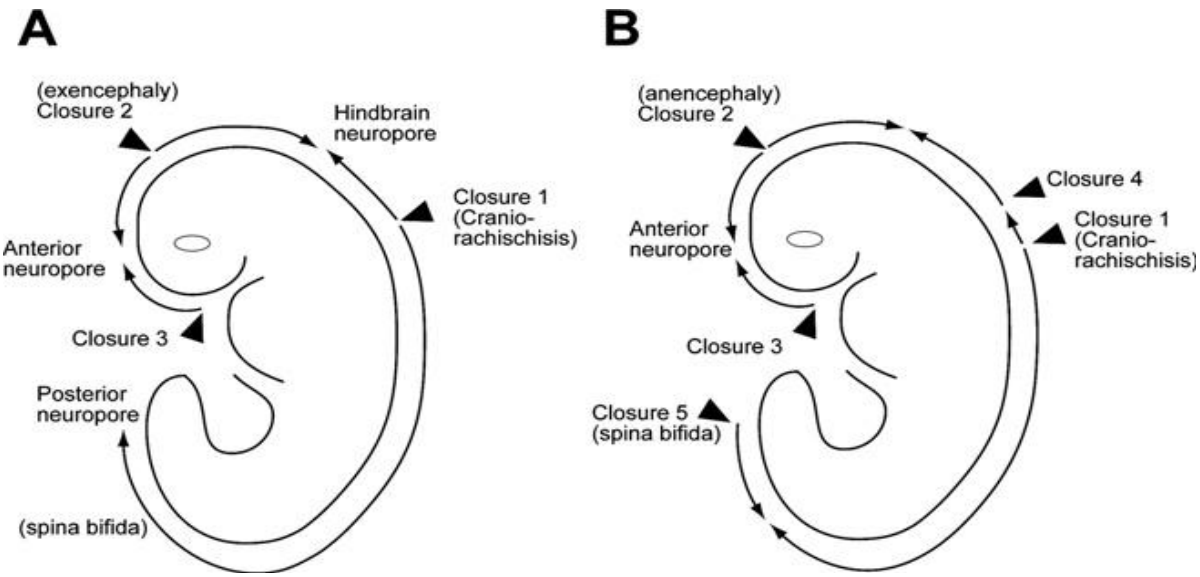


Figure 1. Diagrammatic **s**ites of neural tube closure in mouse (A) and human (B) embryos.

A: side view of a **mouse embryo** with the initiation sites of neural tube closure (arrowheads): closure 1 at the hindbrain/cervical boundary; closure 2 at the forebrain/midbrain boundary and closure 3 at the rostral extremity of the forebrain.

B :side view of a **human embryo** with the initiation sites of neural tube closure (arrowheads). In human with closures 1, 2 and 3 as described in mice, closure 4 in humans occurs at the caudal end of the hindbrain and closure 5 in the lumbar region. Closure occurs bi directionally from closure 1 and 2 and uni directionally from closures 3, 4 and 5.

The different NTDs that result from failure of closure at these sites are shown in parentheses. Van Allen's model (Van et al.,1993)

Neural tube defects (NTDs)

NTDs are congenital malformations of CNS that result from failure of the neural tube to close during embryogenesis (De Marco et al.,2006). Infants born with NTDs have increased risk of mortality within the first year of life and those who survive face life-long morbidities including neurologic, cognitive, urologic, and gastrointestinal complications. NTDs are devastating abnormalities as most of the defects are always associated with neurological deficits producing varying degree of limb paralysis and incontinence bladder and bowel (Digra, 2004; Pitkin, 2007).

Types of Neural tube defects (NTDs)

NTDs can be classified, on embryological basis and the presence or absence of exposed neural tissue, as open or closed types (Van Der Put et al., 2001; Sadler,2005 ; Greene et al., 2009).

The different forms of NTDs are characterized by failure of the neural tube to close during the fourth week of embryogenesis. The nature and severity of NTDs is determined by the stage and axial level at which closure fails (Greene and Copp, 2009). Disturbance of neural induction (neural folding) involved in primary neurulation at the anterior and posterior neuropores results in an range of errors of neural tube closure accompanied by alterations of axial skeleton. These defects manifest primarily as anencephaly, encephalocele or spina bifida (myelomeningocele and meningocele) (O'Rahilly and Muller, 1994; Copp et al., 2003).

Most common forms of neural-tube defects are described as open defects including anencephaly and myelomeningocele that results from the failure of the neural tube to fuse in the cranial and spinal region of the neural tube, respectively. Less common open dysraphisms include myeloschisis, hemimyelomeningocele,and hemimyelocele are sometimes associated with a Chiari II malformation. Closed NTDs are covered with skin, categorized on the presence of a subcutaneous mass lipomyeloschisis, lipomyelomeningocele, meningocele, and myelocystocele or the absence of such a mass complex dysraphic states, including split cord malformations, dermal sinus, caudal

regression, and segmental spinal dysgenesis. Infants with anencephaly die shortly after birth or are stillborn, where infants with spina bifida survive with severe disabilities (Copp et al.,2003; Tortori-Donati et al.,2000).

Open Neural Tube Defects;

In open NTDs defect overlying skin and the neural tissue is exposed to the environment. **Craniorachischisis** is a severe lethal open NTD in which the brain and spinal cord are exposed to the surface. It is the most severe NTD, results from failure of closure 1. It extends across midbrain, hindbrain and entire spinal region (Inagaki, 2000).

Anencephaly is cranial open neural tube defect and exists when brain tissue is exposed to the surface through a defect in the scalp and skull but in contrast to craniorachischisis, the spinal cord remains intact. Anencephaly is a lethal defect occurs as a result of non - closure of rostral neuropore. Many conceptions result in spontaneous abortion and those who survive till term, expire soon after birth (Jaquier, 2006). Human anencephaly can be categorized in cases mainly affecting the rostral brain and skull (meroacrania) and affecting the posterior brain and skull (holoacrania) as well (Seller,1995).

Meningomyelocele and Myeloceles (open spina bifida) commonly occur in the lumbar region but can occur anywhere along the spinal axis. These are caused by defective closure of the primary neural tube and clinically characterized by exposure of the neural placode through a midline skin defect on the back. Meningomyeloceles account for more than 98% of open spinal dysraphisms while myeloceles are rare (Tortori-Donati et al., 2000). Hemimyelomeningoceles and hemimyeloceles can also occur but are extremely rare. Degeneration of the persistently open neural tube in utero leads to loss of neurological function below the lesion level. These conditions occur when a myelomeningocele or myelocele is associated with diastematomyelia (cord splitting) and one hemicord fails to neurulate (Parmar et al., 2003).

Closed Neural tube defects (postneurulation NTDs).

The embryonic ectoderm covers the surface of the embryo. Examples of closed NTDs comprise of meningocele, lipomyelomeningocele, lipomeningocele, and tethered cord (Lemire, 1988; Brody and Shane ,2001).

Iniencephalus: is a rare craniospinal lesion, which involves abnormalities of brain, spinal cord, skull and vertebrae and is covered with skin. It has a low prevalence and is always fatal (Inagaki, 2000;Tortori-Donati et al., 2000).

Encephalocele: is a cranial, closed, post neurulation defect (Campbell et al.,1986; Dias and Partington ,2004). Encephalocele is a defect in development of cranial mesoderm. The defect is an opening in the skull, at occipital, parietal, or frontoethmoidal regions. The meninges herniate through the opening creating an extra cranial mass. The brain may herniate in severe cases. Pathological analysis of the exposed brain tissue reveals a relatively normal morphology. There is no evidence of failed neural tube closure. Majority of encephaloceles (70–80%) occur in the occipital region whereas nasal and parietal ones are rare. (Bozinov et al., 2005; Harding and Copp, 2008). These are classified according to presence of brain within skin covered sac (encephalocele) or only cerebrospinal fluid (cranial meningocele). In United States occipital encephalocele are prevalent (75%) whereas in Burma, Thailand and India anterior encephaloceles are more frequent and may be associated with syndromes (Cohen and Lemire ,1982).

Meningocele :occurs when meningeal tissue herniates through a skeletal opening (i.e., in the vertebral column). About two-thirds of Spinal NTDs occur in caudal region. Etiopathogenesis of meningocele is not known and more research on animal models is required for deciphering the cause of this defect. It can cause serious disabilities, though not always fatal, such as impaired bladder and bowel function and paraplegia, with paralysis of the lower extremities (Pitkin, 2007).

Lipomeningocele: Lipoma is a benign tumor of fat cells**.** It can occur at any level along the spinal cord. Lipomeningocele is a lipoma with a caudal cyst which is expansion of the dural sac that contains cerebrospinal fluid.

These are covered by skin and are closed NTDs (Tortori-Donati et al.,2000).

Arnold-Chiari syndrome: Four types of Arnold-Chiari syndrome are recognized.

Type I is minor herniation of the cerebellar tonsils and may be accompanied with hydromyelia and syringomyelia. Hydromyelia is dilation of central canal of the spinal cord and syringomyelia is characterized by presence of fluid filled cavities within spinal cord tissue.

Type II malformations are more extensive with descent of both cerebellar tonsils and vermis below the level of the foramen magnum with caudal displacement of cervical cord by herniated tissue. Type II malformations may be associated with myelomeningoceles and meningoceles.

In Type III malformations there is herniation of the cerebellum and the medulla into the spinal canal and may be associated with low occipital/high cervical encephaloceles. In Type IV malformations, the cerebellum is hypoplastic (Inagaki, 2000).

Hydrocephalus: There is dilation of cerebral ventricles with excess cerebrospinal fluid with blockade in the flow of cerebrospinal fluid from the ventricles to subarachnoid space. Hydrocephalus is associated with myelomeningocele and meningocele (Copp and Greene, 2013).

Incidence and prevalence of NTD

The incidence of Neural tube defects varies in different geographic regions and within the countries is dependent on ethnicity, gender of the affected infants and socioeconomic status of the parents, parity and maternal age (Berry ,1999;Hendricks, 1999;Kondo et al., 2013). A decrease in NTD frequency has been reported from some areas, while the incidence remains stable in some areas (McDonnell et al.,1999 ; Chan et al.,2008). It is proposed that decrease in the incidence may be effects of prenatal diagnosis, genetic counseling, nutritional supplementation and termination of NTD pregnancies, during the periconceptional period and pregnancy (Lemire, 1988; EUROCAT Working Group

1991:2003; Stevenson et al., 2000; Chan et al., 2001; Williams et al., 2002). Anencephaly prevalence is reported to be lower in Europe as compared to the British Isles. NTD rates in Australia and New Zealand are similar to the reports from North America and Europe (Little and Elwood, 1991). NTD prevalence in northern China has been reported to be among the highest in the world. A decline has been seen in the prevalence of anencephaly and spina bifida in Europe, North America, Australia, and New Zealand (Campbell,1986; Little and Elwood,1991;Yen,1992;McDonnell, 1999).

Etiology of neural tube defects

Neural tube development is a multi-step complex process which is strictly controlled by genes and environmental factors modulate it. This complex morphogenesis comprises of gene–gene, gene–environment and gene–nutrient interactions. Etiology of NTD remains complex and poorly understood (Volcik et al., 2002; Frey and Hauser, 2003:Copp et al 2013). The importance of environmental factors is evident from several studies. The risk for NTDs is higher among families of lower socio-economic status (Little and Elwood, 1992). Other environmental factors that are recognized in the past literature include maternal diabetes (Becerra et al.,1990) hyperthermia (Graham et al.,1998; Edwards et al.,2006) and obesity (Shaw et al.,1996; Agopian et al.,2013). Factors that may associate with NTDs include maternal age, intake of alcohol, excessive use of Vitamin A and exposure to lead, heat exposure (Au et al.,2010) and high intake of tea during the first trimester of pregnancy (Ye et al., 2011). History of abortion and parity have been implicated as factors that contribute to the occurrence of NTDs (Frey and Hauser, 2003). A variation in micronutrient status of the mother is an important environmental factor associated with NTDs. Preconceptional intake of zinc is found to decrease the risk of NTDs (Velie et al.,1999). Administration of periconceptional folate has been observed to reduce the incidence of NTDs. The protective effect of folate varies between different populations and indicates an association between genes and environment in the prevention of NTDs (Pulikkunnel and Thomas, 2005).

Risk factors in etiology of Neural Tube defects

Research indicates a complex etiology to NTDs, which involve environmental and genetic factors. Environmental factors include geographical location, epidemic trends, socio-economic class (poverty), maternal diet, maternal age, maternal diabetes and obesity (Frey and Hauser, 2003: Mitchell , 2004:2005).

Maternal Age and Birth Order

Maternal age is an important factor in relationship of NTDs with folic acid. In USA, women in the age of 18-24 years account for one third of all births and this age group had the least awareness and knowledge about folic acid and the lowest reported daily use of supplements (Morin et al., 2001; CDC, 2008; Timmermans et al., 2008). Women in this age group have multiple risk factors for inadequate folic acid consumption. Majority of pregnancies are unintended, reaching 80% of all pregnancies in this age group (Finer and Henshaw, 2006) and birth control was reported by 1 in 5 women (Chandra et al., 2005). In this age group, women have the lowest median annual household income and education level is low. Younger women tend to have unhealthy dietary habits that fail to meet required recommendations (Anding et al., 2001). These behavioral, economical, educational and nutritional factors contribute to the low level of folic acid consumption in this age group and to the increased risk of pregnancies affected by NTDs. Reefhuis and Honein (2004) in their study found women aged 14-19 years to be twice as likely to have a pregnancy with NTD as compared to women 25-29 years of age. Maternal age above 40 years is a risk factor for having an offspring with neural tube defects (Au et al., 2010). This effect is stronger for spina bifida than for anencephaly (Vieira and Taucher, 2005). Related to maternal age, birth order could be a potential risk factor for NTDs. It was observed in a study by Vieira (2003) spina bifida (SB) was likely to occur in children with higher birth order.

Consanguinity

Consanguineous marriages have been practiced since the early existence of modern humans. The term consanguinity is derived from the Latin words "con" meaning

common and "sanguineus" meaning blood, hence, refers to a bond between two people who share a common ancestor (Alwan and Modell, 1997; Modell and Darr, 2002; Bittles,2008). Consanguineous marriages include unions termed first cousins, first cousins once removed and second cousins. Consanguinity preferred social trend and deep rooted in around one-fifth of the world population mostly in Middle East, Asia and North Africa, and also among emigrants from these communities now residing in America, Europe and Australia (Hamamy, 2011). In consanguineous marriages the inbreeding coefficient (*F*) equals or is higher than 0.0156, where (*F*) is representative of a measure of the fraction of loci at which the offspring of a consanguineous union is expected to inherit identical gene copies from each parent (Bittles, 2001). There is growing public awareness on prevention of congenital disorders in highly consanguineous communities and couples are ready to seek advice on health risk for the new born (Hamamy, 2011). Consanguinity allows clustering of susceptible genes, the expression of which could possibly contribute to development of NTDs (Carter et al., 1968). A number of researches on Middle East and Saudi Arabian population reported high rates reported high rates of consanguineous marriages (89%) in parents of spina bifida and other congenital anomalies in children (Rajab et al., 1998; Murshid ,2000; Sawardekar,2005). Iran has a reported high incidence of anencephaly with a high rate of consanguinity suggesting role of a major recessive gene (Zlotogra, 1995). Latin American population studies reported parental consanguinity as a risk factor for congenital anomalies including NTDs (Liascovich et al., 2001). Consanguinity (44.74 percent) was reported as a major risk factor in occurrence of NTDs. A study on congenital anomalies in Pakistani population reported consanguineous marriages (44.74%) as a major risk factor with NTDs as most common (65.8%) type of anomaly (Parveen and Tayab, 2007).

Diabetes Mellitus (DM)

There is a global rise in Diabetes mellitus, the incidence estimated to be 6.4% as of 2010 (Shaw et al., 2010). About 3%-5% of pregnancies are reported to be complicated by diabetes mellitus which carries a high degree of mortality and morbidity (Gabbe and Graves, 2003; Dheen et al., 2009). Maternal DM during pregnancy is associated with a higher risk of CAs in offspring (Moore et al., 2004). Experimental studies have indicated that diabetic embryopathy is associated with altered expression of genes controlling

embryonic development (Zabihi and Loeken, 2010) . Maternal diabetes induces hypoxia, oxidative stress and other metabolic disturbances in the embryo (Li et al.,2005a). These changes lead to aberrations in signaling pathways and have been implicated as major causative factors in diabetes-induced embryopathy. Oxidative stress caused by hyperglycemia disrupts the expression PAX3 genes which are involved in closure of neural tube leading to NTD (Loeken, 2006). The interplay between epigenetic mechanisms and environmental factors such as diet modify the response of the body to teratogenic effects of Diabetes mellitus(Salbaum and Kappen, 2011). Variations in gene expression due to hyperglycemia comprising DNA methylation, noncoding RNA expression, transcription factor activities, and histone modifications (Salbaum and Kappen, 2012). In experiments on diabetic rodents incidence of NTDs was less in embryos that were supplemented with folic acid (Gareskog et al., 2006; Oyama et al.,2009). A study carried out in Hungary on diabetic women observed reduction in maternal teratogenic effect of diabetes after folic acid supplementation during pregnancy (Bánhidy et al., 2011).

Physical and chemical environment

Occupational exposure, chemical waste from industrial installations and pesticides, paints, X-radiation are associated with a high risk for NTD (Matte et al., 1993; Sever, 1995; Blanco Munoz et al., 2005; Brender et al., 2010). Exposure to chemicals released from landfill sites into air, water and soil in vicinity have been implicated in a number of diseases and also increase risk of congenital anomalies (Vrijheid,2000 ; Padula et al.,2013). In a research study conducted in Poland (Perera et al.,1999) found a significant transplacental transfer of polycyclic aromatic hydrocarbons (PAH) and environmental tobacco smoke (ETS) constituents from mother to fetus affecting development of fetus in utero. Benzene is one of the most widespread Hazardous air pollutants (HAPs) found in urban areas (Mohamed et al.,2002) and increased incidence of spina bifida was found in American women exposed to this organic solvent (Lupo et al.,2011). More research is required to assess effects on developing fetus of air pollutants and other environmental toxicants.

Hyperthermia during Early Pregnancy

In humans an oral temperature of 37°C and body core temperature of 38°C is considered normal (Edwards, 2006). An elevation of maternal temperature from 38°C to 40°C is embryotoxic as seen in experimental animals. In humans, high temperatures are associated with NTDs (Shaw et al., 1998; Suarez., et al 2004). During embryogenesis, the neural tube is sensitive to heat stress. Increased body core temperature appears to interfere with critical developmental processes such as cell proliferation, migration, differentiation and apoptosis (Edwards, 2003). Animal studies have shown the effect of heat on embryo to be dependent on the dose and duration of exposure and species specific (Edwards et al., 1995, 2003). Maternal hyperthermia in early pregnancy and treatment with is a risk factor for development of NTDs and FA reduces the teratogenic effect. **(**Moretti et al., 2005; Czeizel et al., 2011). The duration of hyperthermia and the timing during early pregnancy is important as its teratogenic risk is dependent on the critical period of exposure and is different for specific congenital anomalies (CAs) (Czeizel et al., 2008). Folic Acid reduces the high-fever-related CA risk (Botto et al., 2001). Shaw and colleagues (1998) reported a 7.4-fold increased risk for NTDs in mothers with high fever who did not take folic-acid-containing multivitamins.

Smoking/passive smoking and Neural tube defects (NTDs)

Maternal smoking has been identified as a risk factor for NTDs (Suarez et al., 2008). Cigarette smoke comprises of numerous chemicals like carbon monoxide, polycyclic aromatic hydrocarbons and nicotine. In the early stages of pregnancy, the chemicals in cigarette smoke pass through the placenta and can be detected in urine samples from newborns (Windham et al., 1992; Li et al.,2012). The relationship between maternal secondhand smoke (SHS) or passive smoking and NTDs was investigated in human and animal models and found to be toxic to developing embryo (Suarez, 2008; Wasserman., et al. 1996; Suarez, 2011). Animal studies have shown that SHS causes excessive apoptosis in neural epithelium, and could be the etiological factor in the occurrence of NTDs (Wang and Yu, 2004;Yu et al.,2005). Low levels of folate were found in women who never smoked but were exposed to passive smoke and folate

deficiency in periconceptional period has been implicated in causation of fetal NTD (Kim et al.,2010).) Exposure of mothers to secondhand smoke from their partners during the periconceptional period was linked with a 1.7-fold increase in the risk of NTDs. This link showed a marked dose-response trend (Li et al., 2011). No effects of smoking were observed with a California sample (Wasserman et al., 1996). Pregnant women exposed to secondhand smoke inhale most of the compounds taken in by smokers. Various studies have shown that smokers (active and passive) have significant low levels of folate in their serum and red blood cells, as compared to people not exposed to cigarette smoke. The decreased levels were not entirely because of variations in folate intake through diet or supplements (Cafolla et al., 2000; Vardavas et al., 2008). A meta-analysis performed by Wang et al (2014) suggested maternal passive smoking during pregnancy and in periconceptional period was associated with an increased risk of total NTDs and specifically the three type's anencephaly, spina bifida and encephalocele.

Adverse reproductive history in prior pregnancy and risk of NTDs.

Women with history of reproductive loss may be at an increased risk of having an adverse outcome in subsequent pregnancies. In a case-control study, Mexican-American women who had a history of miscarriage faced 4.58 times higher risk of anencephalous fetuses than those who did not (Blanco-Muñoz et al.,2006). Two hypothesis were put forward by Lawerence and Roberts (1977). The first hypothesis states that the presence of a trophoblastic cell rest left over from the previous aborted pregnancy could interfere with normal embryogenesis. The second hypothesis states that the previous aborted fetus was affected by NTD which remained undetected. An investigation by Carmi et al (1994) on Jewish population reported significantly higher spontaneous abortion rate (48%) in the preceding pregnancy in the NTD group compared to the group with other birth defects (20%). Hence, a history of spontaneous abortion indicates a high risk of NTD, and such women should be counseled to wait longer before trying for another pregnancy and advised periconceptional folic acid treatment.

Nutritional status

Diet and maternal nutritional status is a major environmental factor that influences development of embryo, pregnancy outcome and maternal health (Keen et al., 2003). In maternal nutrition folic acid has been focus of much attention. Biologic derivatives of folates play a pivotal role in the folate and methionine cycles that form basis of one-carbon metabolism (Blom et al.,2006; Beaudin and Stover, 2007). Folic acid supplementation in periconceptional period reduces the risk of NTDs (Blom et al., 2006 ; Molloy et al., 2009). Women on folate rich diet and who took folic acid were at a lower risk of a child with NTD as compared to those who did not take multivitamins (Czeizel et al., 2011). Research has shown that folate supplements need to be started about a month before conception and during the first month following conception (Carmichael et al., 2006). Closure of the neural tube takes place (or tragically fails to close) by 28th day post-conception, when many women are unaware of their pregnancy (Kadir, 2002). Folates protect against neural tube defects and have been shown in some studies to prevent other types of birth defects (Rosano et al.,1999; Berry et al.,1999). Nutritional state of the mothers can alter the epigenetic state (stable alterations of gene expression through DNA methylation and histone modifications) of the fetal genome and an inadequate diet can subsequently result in adverse pregnancy outcome (Greene et al., 2011).

Education and Socioeconomic factors

Among the most serious and common congenital malformations, neural tube defects have been found more frequently among children born to women with a lower socioeconomic status (SES). Low-SES measured by parental occupation and education and household income has been reported in various studies (Brender and Suarez., 1990; Canfield et al., 1996; Farley et al., 2002; Meyer and Siega-Riz, 2002; Rosano et al.,2008) but not others (Strassburg and Greenland,1983;Vrijheid et al., 2000). A significantly higher risk for NTD pregnancy were found in women with low socio-economic status as compared to women with higher education, living in the same area (Grewal et al., 2009). A similar correlation of maternal education status and NTDs among economically deprived Hispanic Americans showed that they were at higher risk of having offspring affected with NTDs (Canfield et al., 2009). Use folic acid was likely in mothers

belonging to higher socioeconomic groups and a higher education level during the periconceptional period and the period of neural tube closure. Women, less likely to take folic acid everyday were young, non-Caucasians who reported no health insurance, less education, and low income. Based upon these data, multi-level education campaigns that specifically target lower-SES women need to be considered (Brough et al., 2009).

Folate

The term folate is representative of all forms of this B vitamin. Folic acid (pteroylmonoglutamic acid) is the synthetic form found in dietary supplements and fortified foods (Stanger, 2002). Folic acid (pteroylmonoglutamic acid) is the synthetic form found in dietary supplements and fortified foods. Folate was first discovered in 1931 for its effects in curing megaloblastic anemia observed in late pregnancy. In 1931 Lucy Wells used a yeast extract to treat macrocytic anemia in pregnant women in India. This extract was later identified as folate (Hoffbrand, 2002). It was first isolated in 1941 from spinach leaves and termed it folic acid (Mitchell et al., 1988) and later synthesized successfully by Bob Stokstad in 1943 (Hoffbrand and Weir, 2001) and by Angier and colleagues in 1945(Angier et al., 1945). The term Folic acid comes from "folium" (Latin for leaf) since the first extraction was from spinach leaves.

Structure and function

Folate is a generic term for all water soluble B-vitamin compound with common vitamin activity centered on the parent structure pteroyl-L-monoglutamic acid (Finglas, 2003). Folate occurs naturally in foods and is now recognized as a major component of the periconceptional care of women in the childbearing age (Combs , 2008). Folates are the essential cofactors in metabolic pathways that facilitate methylation reactions, with formation and transfer of "one-carbon units" to nucleotides purines and pyrimidines in the DNA synthesis. Their role is now recognized in transcription, chromatin structure, genomic repair and genomic stability and the regulation of gene expression (Lucock, 2000; Stanger, 2002).

Folate is the water-soluble B vitamin consisting of pteroic acid attached to a glutamic acid tail. Derivatives of folate differ in the oxidation state of the pteridine ring,

the one-carbon unit at the N5 and N10 positions and in the number of glutamate residues in the glutamic acid chain (Lucock, 2000). Folate is also available as a supplement in a more stable synthetic form, folic acid (pteroylmonoglutamic acid). It is a monoglutamate, oxidized form and does not require hydrolysis after ingestion. Folic acid is absorbed directly by the cells of the small intestine where it is reduced to 5-methylTHF (Mason et al .,1990;Lindzon and O'Connors, 2007). Folate can also be obtained from micro-flora in the colon that are able to synthesize small amounts of this vitamin, particularly under conditions of folate deficiency (Rong et al.,1991; Said et al., 2000; Kim et al ., 2004).

Inadequate folate intake is associated with adverse health outcomes in humans including neural tube defects (NTDs), cleft lip and/or palate, low infant birth weight, abruptio placenta, preeclampsia, spontaneous abortion, stillbirth, macrocytic anemia, cardiovascular disease and neuropsychiatric disorders (Lucock, 2000; Tamura and Picciano, 2006). Suboptimal intakes have also been shown in some studies to play a role in carcinogenesis including cancer of the colorectum, breast, cervix, lung, pancreas, esophagus, and stomach as well as neuroblastoma and leukemia (French et al., 2003; Kim, 2008).

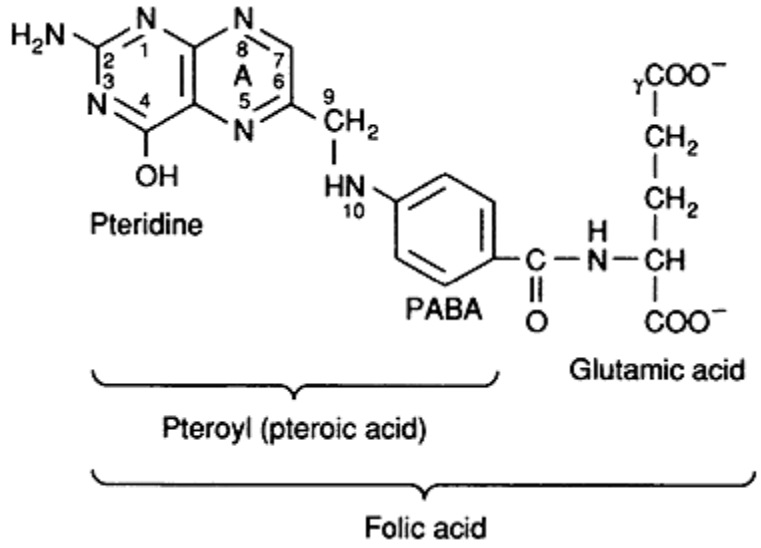


Figure 2: The structure of folic acid illustrating the three groups of the molecule (Lucock, 2000).

Sources of natural food folates

Rich sources of folate in nature are green leafy vegetables and citrus fruits. Within organic foods liver is a rich source of folate. Only about 50% of food folate is absorbed. Most of cooking practices as of stewing, processing and storage destroy folates in natural foods (Talaulikar and Arulkumaran , 2011).

Absorption, Transport, Excretion and Storage

Dietary folates exist as polyglutamate in nature, which are converted to the monoglutamate form and absorbed in the proximal small intestine (Tamura and Stokstad, 1973). Dietary polyglutamyl folates are broken down to monoglutamate via folate hydrolase in the brush border membrane of the jejunum and then absorbed through a carrier-mediated system or through passive diffusion at saturating concentrations (Shafizadeh and Halsted, 2007). Folic acid (pteroylmonoglutamic acid) is the more stable synthetic form. It is the oxidized form and does not require hydrolysis after ingestion but is absorbed directly by the intestinal cells where it is reduced to 5-methyl THF. This is the main circulating form of folate that is transported through the cell plasma membrane and the main form of folate supplied to the tissues (Shane and Stokstad, 1983). Folate transport systems across the cellular membranes include transport in placenta, renal tubular cells, and the blood brain barrier. At high concentrations of folic acid, when reducing enzymes are saturated in the enterocyte, un-metabolized folic acid can enter the circulation (Mason et al., 1990). Dietary folates are hydrolyzed to the folate monoglutamate form in the small intestine lumen by the action of a brush-border Zn dependent γ-glutamyl hydrolase or conjugase (folylpolyglutamate carboxypeptidase) encoded by the gene Glutamate carboxy peptidase II (GCP II) (Reisenauer et al., 1977; Halsted, 1980; Chandler et al., 1991). These monoglutamates are taken up by the mucosal cells in jejunum at physiological concentrations via a saturable energy dependent active carrier-mediated process, receptor mediated and by passive diffusion at high concentration (Selhub et al., 1984; Matherly and Goldman, 2003).

There are three principal mechanisms for intestinal absorption. At higher intraluminal concentrations (such as >10 μ mol/L) a nonsaturable ion-mediated process

of passive diffusion transports deconjugated folates through the enterocytes without modification (Selhub et al.,1984; Gregory ,1995; Bailey et al.,2001, Matherly and Goldman, 2003). Reduced folate forms are transported more quickly than folic acid by this route but plays a minimal role in absorption because of anionic, lipophobic nature of folates at physiological pH (Mason,1990; Zhao et al.,2009). Three mechanisms for folate transport into the cells have been identified; carrier-mediated, receptor-mediated, and transport-mediated process (Matherly and Goldman, 2003). Carrier mediated transport includes the reduced folate carrier1 (RFC1); receptor mediated occurs through folate receptors 1,2 and 3 (Folr1, Folr2, and Folr3 in mice); and the third occurs via proton-coupled folate transporter (PCFT). These three systems differ from each other in tissue expression, specific affinity for reduced or oxidized folates, and mechanism of transport. RFC1 is main route of transport across mammalian cell (Sirotnak and Tolner, 1999 ; Matherly and Goldman, 2003).

At lower luminal concentrations, but maximally between 10 and 20 μmol/L, monoglutamyl folates may be absorbed by active transport in the small intestine (Halsted,1990). This process is saturable, pH and energy dependent with an optimal pH between 5.0 and 6.0 (Steinberg, 1984). Reduced folate carrier (RFC) mediated absorption occurs primarily in the proximal jejunum and functions at neutral pH. The optimal activity for the RFC is around pH 7.4 with negligible activity below 6-6.5 (Zhao et al., 2009). Two genetically unrelated folate carrier proteins, the RFC and PCFT are present in the human intestine (Matherly and Goldman, 2003). The PCFT, a high affinity folate transporter protein, is expressed along the length of the human intestine as well as in other tissues, particularly in the small intestine, kidney, liver, placenta, retina and brain (Zhao and Goldman.,2007). Folate transport by PCFT is proton coupled, Na^+-independent and has a low pH (5.5) (Qiu et al., 2006; Wani et al., 2012). PCFT is expressed more in the duodenum, less so in the jejunum, and at low levels in the ileum, cecum, colon and the rectum. PCFT exhibits a high affinity for folates and folic acid (Qiu et al., 2006; Zhao and Goldman, 2007). RFC transports folates in reduced form and displays higher affinity for 5-methyltetrahydrofolate and a very low affinity for folic acid (Matherly and Goldman, 2003).

RFC functions as an importer or an exporter of folate from cells and is expressed in the apical membrane along the length of the intestine, highest in the duodenum and including the colon (Subramanian et al., 2008). Folate binding proteins (FBPs) are also associated with intestinal expression and transport (Zhao and Goldman, 2007). There are three high affinity folate binding proteins, coded by three separate genes, also expressed in humans. These are the folate receptors FR α and FR β , proteins anchored on the cell surface by a glycosylphosphatidylinositol anchor and FR γ , a soluble receptor (Elkanat and Ratnam, 2004). FRs are crucial to the assimilation, distribution, and retention of food folates. Soluble FBPs concentrate, store and protect folates from oxidation, while membrane-associated FBPs are involved in transport of folate between cells (Selhub, 1994; Antony,1992 ;1996). The FRs bind physiological levels of folate with a high affinity for folic acid than for reduced folates 5 MethylTHF or 5 formyl THF. FRα is glycophosphatidyle inositol-linked glycoprotein with high affinity for 5methyl THF expressed on renal tubular epithelium, choroid plexus and placenta. FR γ is soluble secretory form of FR. FRγ'is truncated form of FRγ (Wang et al., 1992; Kamen and Smith, 2004).

There is an inverse relationship between FR expression and extracellular folate concentration. The receptor mediated transport of 5-MeTHF occurs via endocytic vacuoles (Hjelle et al.,1991) or via caveolae. The receptor internalizes and recycles within the caveolae without dissociating from the plasma membrane (Rothberg et al., 1990). Caveolae form a membrane-bound compartment that protects FRs both from acid treatment and from antifolate receptor IpG. The FRs concentrate 5-MeTHF at the cell surface and deliver the vitamin to a vesicle (Kamen et al.,1988). They are clustered on the cell surface of folate-dependent tissue cells. FRs are encoded by a family of genes on chromosome 11q13.3 through 11q13.5, where four FR genes and a pseudo gene were located within a 140-kb region (Ragoussis et al., 1992).

In man folate is mainly stored in the liver (Whitehead, 1973; Hoppner and Lampi, 1980; Higdon, 2003), which is assumed to contain 50% of the normal total body folate content of 5–20mg. Folate absorbed from the intestine is cleared from the circulation within minutes and is taken up by various tissues and the liver. Hepatocytes store 10-20%

of the folate, the greater part released after metabolism, mainly into the bile. Folate undergoes enterohepatic circulation, with a daily turnover rate of approximately 90 μg (Steinberg et al., 1979; Weir et al., 1985; Herbert and Das, 1993; Finglas, 2003) with folate concentrated tenfold in bile compared to serum levels. Folate in bile is the readily available endogenous source of monoglutamate compounds. Tetra hydrofolate (THF) in the human organism is mainly present as 5-methyl-THF, 5,10- methylene-THF and 10-formyl-THF. 5-methyl-THF makes up approximately 40-50% of the total folate polyglutamates in red blood cells. 5-methyl-THF is present in the serum as monoglutamate, and intracellularly as polyglutamate. Polyglutamates are transported across cellular membranes, ensuring their cellular retention and are the preferred form in one-carbon metabolism. Total body content of folate has been estimated to be 38–96 mg (86–165) μmol. Small amounts are excreted in the feces and urine and additional amounts are metabolized and lost by desquamating cells in scales from body surfaces (Birn, 2006). Folate can be found in human milk. Folate is mainly required by organs or systems involved on the rapid proliferation of new cells. Folates are essential for normal cell division and growth. Folic acid deficiency causes megaloblastic anemia and besides bone marrow tissue, the immune system, mucous membranes, hair and fingernails are affected as well (Shils et al., 2006).

Transplacental transport of folate

Folate is essential to the growth and viability of the developing embryo. Since folate is an essential vitamin, the embryo relies on folate transported across the placenta from the mother. The placenta is one of few tissues that is rich in the α-isoform of FRs, a receptor that has high affinity for folic acid and 5-methylTHF. A unique, bidirectional mechanism for transporting folate across the placenta, against a concentration gradient occurs by a two-step process (Henderson et al., 1995; Antony, 1996). 5-methylTHF in the maternal circulation is rapidly bound by high affinity placental FR-α and becomes concentrated in the intervillous blood and is then slowly released into the fetal circulation down a concentration gradient (Chancy et al., 2000). The more recently discovered folate transporter, heme carrier protein 1(HCP1), plays a role in folate uptake into the placenta. The saturable level of 5-methylTHF transfer across the placenta is very high, above

physiological concentrations. Fetal blood has a much higher folate concentration (~3 times) than in the mother (Yasuda et al., 2008).

Blood folate levels and risk of neural tube defects

Folic acid reduces risk of neural tube defects. An adequate folate status has been defined as a serum or plasma folate concentration above 7 nmol/ml (3 ng/ml) and a red blood cell concentration above 305 nmol/ml (140 ng/ml) (Institute of Medicine, Food and Nutrition Board: "Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline." Washington, DC: National Academy Press, 1998). Serum folate concentrations fluctuate and reflect only recent consumption. RBC folate is a better marker of tissue stores (Suarez et al., 2003). A number of case–control studies have reported significantly lower RBC folate concentrations among women carrying a fetus with an NTD (Kirke et al.,1993; Wald et al 1996; Candito et al.,2008). Suarez et al (2003) failed to find a significant difference in serum or plasma folate concentrations between women affected with an NTD pregnancy and women carrying a normal fetus in Texas, America. Although the role of periconceptional folic acid supplementation has been the focus of research since the last two decades still many women remain sub optimally protected against folate-sensitive NTDs (Tam, 2009). In Chinese population Ren et al (2007) compared folate concentrations in areas with a high and low prevalence of NTDs. The mean plasma and RBC folate concentrations among pregnant women in the low-prevalence area were double the concentrations when compared with women in the high-prevalence area. Epidemiological data from Canada, the United States, and Chile also show that fortification was associated with population-wide increases in blood folate concentrations with a significant decreases in the prevalence of NTDs (Honein et al., 2001; Centers for Disease Control and Prevention (CDC,2007; De Wals et al., 2007; Hertrampf and Cortes, 2008). A case–control analysis of folate status by Daly et al (1995) showed that NTD risk is inversely associated with maternal RBC folate concentration with not much difference in serum folate levels in case and control mothers. The observed NTD risk was highest for women with RBC folate concentrations below 340 nmol/L (6.6 per 1000 live births) and the risk decreased to 0.8 per 1000 births for women with RBC folate concentrations of 906 nmol/L or higher. Women of reproductive age should aim for an

RBC folate concentration of at least 906 nmol/L (400ng/ml) at which risk for NTD is low (Daly et al., 1995). This target concentration is supported by Ren et al (2007).They reported a mean RBC folate concentration of 910 nmol/L (400ng/ml) among pregnant women in an area of China with low NTD prevalence (0.46 per 1000 births).The most effective intervention method to increase folate status among women of reproductive age are vitamin supplementation and ingestion of fortified foods (McNulty et al.,2000). Folate status is affected by alcohol consumption (Halsted,1995) and smoking. Some pharmaceuticals, including anticonvulsants such as Dilantin, antifolates such as methotrexate, anti-inflammatory medications, some diuretics, and sulfa antibiotics also alter folate levels (Institute of Medicine, Food and Nutrition Board: "Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline." Washington, DC: National Academy Press, 1998; CDC. Vital and Health Statistics: Blood Folate and Vitamin B12:United States,1988-94. US Department of Health and Human Services;1998; Alonso-Aperte and Varela-Moreiras,2000; Pagan et al.,2001). Consumption from natural food sources does not achieve adequate folate levels and compliance with supplementation is poor even in developed countries, the FDA mandated folate fortification of all grain products in 1996 to increase folate intake in women. This was associated with a significant increase in folate status, decreasing the prevalence of low folate concentrations (less than 3 ng/ML) and increasing mean serum folate concentrations from 4.6 to 10.0 ng/mL (Jacques et al., 1999).The target goal of health policy in developed countries was to bring median RBC folate level among this group from 160ng/mL to 220ng/ml. This would reduce the incidence of neural tube defects by 50% in the same time period. An increase in folate levels was observed by CDC (2004) in American population when they compared serum folate and RBC folate in 1988-1994 to levels in 1999-2000, after the fortification of food products by the FDA. They found an increase in both Mean RBC folate from 181mg/mL to 315 ng/mL and mean serum folate increased from 6.3 to 16.2 ng/mL. Pfeiffer et al (2007) in their report on American population stated that people with low serum folate (<3 ng/mL) declined from 21% in the period before fortification (1988–1994) to <1% of the total population in the period following fortification (1999–2000) . NTD prevalence decreased by 36% after fortification, from 10.8 per 10,000 population during 1995–1996 to 6.9 at the end of 2006 (Daly et al., 1997; CDC, 2000;CDC, 2010). It is predicted that

there is a 60% decrease in the risk for NTD when RBC folate levels are raised from 150 ng/ml to 400 ng/ml. Food fortification programs in countries, including Canada, Costa Rica, Chile, and South Africa have successfully resulted in significant increases in blood folate concentrations and a consequent 25%–50% decline in the prevalence of NTD-affected pregnancies (Berry et al., 2010). Chile fortified wheat flour for bread at 220 μg folic acid/100g with the resultant 43% reduction in NTDs from 17.1 per 10,000 population in 1999–2000 to 9.7 in 2001–2002 (Hertrampf and Cortes, 2008).

Periconceptional use of folic acid and NTDs

Almost four decades ago, Smithells et al (1976) suggested that folate deficiency was an important factor in the etiology of NTDs as women who had given birth to an NTD infant had low blood folate levels. The same group in 1983 observed that periconceptional vitamin supplementation, including folic acid, reduced the recurrence of NTDs (Smithells et al., 1983). MRC study (Wald et al., 1991) and trial concluded that a daily dosage of 4,000 mcg of folic acid, besides folate in the diet, before and during early pregnancy, resulted in a 71% recurrence reduction of NTDs whereas use of multivitamins without folic acid did not result in a reduced risk for NTDs. Carmichael et al (2010) reported a lowered risk of NTDs in mothers' taking vitamin supplements. They also stated that lower risk of NTD was associated with regular dietary intake of nutrients contributing to one-carbon metabolism and oxidative stress. It is important for women of child bearing age to have an adequate folate status before conception and to achieve this women need to consume a folate rich diet or take a multivitamin with 0.4 mg of folic acid each day (Lumley, 2001; Ross, 2010).

Women's knowledge and awareness concerning folic acid

Awareness concerning folic acid is usually evaluated in response to the question "have you ever heard or read anything about folic acid?" levels of awareness reported in the literature vary in different populations. In the USA, the March of Dimes Birth Defects Foundation reported national levels of awareness of 52% in 1995, increasing to 84% in 2005 (March of Dimes Birth Defects Foundation, 2006). CDC(2008) report showed that from 2003 to 2007, levels of awareness were stagnant around 80% (79% in 2003 and

81% in 2007). Level of awareness is 95% in Canada (French et al., 2003). In countries where folic acid intake is very low, Women may have high levels of awareness but intake in periconceptional period is less. In Lebanon where 60% of women knew about folic acid but only 7.5% of them were using supplements adequately (Nasr Hage et al, 2011). In Turkish population, investigation report showed 18% level of awareness (Turgul et al., 2009). Even though women may be aware of FA, knowledge concerning its importance in NTDs prevention, sources of natural and synthetic folic acid and right period of folic acid intake is still lacking. In USA, studies by March of Dimes Birth Defects Foundation, (2006) reported in 1995 only 4% of American women knew that folic acid help reduce the risk of birth defects. These numbers reached 24% in 2004 before decreasing to 19% in 2005. The same report stated that only 2% of American women identified the adequate period for folic acid intake, percentages reaching 12% in 2004 and dropping to 7% in 2005 (CDC, 2008). In Canada where the levels of awareness and intake are relatively high, only 25% of women in Vancouver were aware of the benefits of folic acid especially prevention of NTDs (French et al., 2003). Likewise low levels of knowledge were reported from countries with less intake of folic acid such as Thailand (25%), Lebanon(60%) of surveyed women knew that folic acid was something important and knew about the role of folic acid in NTD prevention and about the adequate period for supplementation (Nasr Hage et al., 2012; Nawapun and Phupong, 2007).

Genetic factors in etiology of neural tube defects

Neural tube defects (NTDs) have a multifactorial etiology such as nutritional, environmental, and genetic (Botto et al., 1999). Role of Genetic factors in etiology of NTDs is evident from familial aggregation of the defect although there is no strict Mendelian pattern of inheritance. There is a 2 to 5% recurrence risk for NTD in siblings of patients with myelomeningocele (Sebold et al., 2005). In siblings of offspring with myelomeningocele, recurrence risk for NTD is 2 to 5%. A significantly higher incidence of NTDs occurs among first and second degree relatives of the affected babies as compared to the general population. NTDs particularly appear in females and monozygotic twins (Windham and Edmonds, 1982; Kondo et al., 2009).

Genetic defects of folate transport

Reduced folate carrier (RFC) polymorphism: In mammalian cells the transport of reduced form of folate occurs by a carrier-mediated mechanism. The reduced folate carrier (RFC) is a membrane protein responsible for this mechanism of transport. A common polymorphism in the RFC-1 gene, 80A_G, may contribute to NTD susceptibility. When this polymorphism is associated with other gene defects the risk for NTDs is increased (De Marco et al., 2003).

Defects in receptor mediated folate uptake: Receptor mediated folate transport is a mechanism of folate transport across mammalian cell membranes (Elnakat and Ratnam , 2004). Folate receptors (FR) are important mediators for the absorption, distribution and retention of food folates. Animal studies have shown mouse fetuses without folate binding protein-1(FBP-1) gene die during gestation and show defects in neural tube closure. Mice homozygous and heterozygous for polymorphism in FBP-1gene have lower folate levels. Genetic defects in folate receptor genes could be important in the etiology of NTDs (Heil et al.,1999; Blom et al., 2006). Folate receptors are important in embryonic development and mutation in the genes coding for these receptors result in lethal abnormalities. Studies on mice have confirmed that mice homozygous for the FBP-1 null allele failed to close their neural tube and developed lethal abnormalities (Barber et al.,1998;Gos and Szpecht-Potocka, 2002). Folate receptor antibodies measured in maternal serum have been associated with increased risk to NTDs (Rothenberg et al., 2004; Cabrera et al., 2008; Molloy et al., 2009). The maternal autoantibodies to folate receptors also impair folic acid binding to receptors (Boyles et al., 2006; 2011).

Genetic defects in metabolic pathways

Methylene tetrahydrofolate reductase (MTHFR)

Methylenetetrahydrofolate reductase (MTHFR)is the key enzyme in folate metabolism. It is a flavoprotein and catalyzes the reduction of 5,10 methylenetetrahydrofolate (5,10-CH2-H4 folate) to 5 methyltetrahydrofolate (5, CH3-H4folate) which is the main circulatory form of folate. Methyl THF is the methyl donor for methylation of homocysteine to methionine. The common C677T is a missense mutation in the MTHFR

gene and is implicated as a risk factor in the etiology of NTDs (Shields et al.,1999). Other forms of MTHFR polymorphism include A1298C, T1317C and T1068C but these have not been proven to be clinically important (Parle-McDermott et al., 2003). The frequency of the allele 677 T allele varies in different geographical areas and ethnic groups and roughly correlates with the incidence of NTDs (Botto et al.,1998;1999).

Methionine synthase

A common polymorphism in the methionine synthase gene is substitution A2756G, which leads to a substitution of aspartic acid with glycine D919G (Chen et al.,1997). The D919G polymorphism is associated with improper cofactor oxidation level, which may decrease methionine synthase activity with an increase homocysteine level in cell (Harmon et al., 1999). Cell methylation reactions are affected with decrease in the levels of SAM and result in defective neurulation (Pulikkunnel and Thomas, 2005).

Methionine synthase reductase (MTRR) gene defects

Methylated cobalamine levels are regenerated by Methionine synthase reductase (MTRR) from the oxidized cobalamin (II) form and maintain the active state of methionine synthase (MTR). The essential enzyme MTR catalyzes the conversion of homocysteine to methionine. Single nucleotide polymorphisms (SNPs) in the MTRR gene lead to a reduction in MTR activity with an increase in levels of homocysteine. Increase levels of homocysteine are a known risk factor in the etiology of (NTDs). A mutation in MTRR leads to decrease enzyme activity of methionine synthase (MTR) and a decrease in methylation of homocysteine to methionine. A common polymorphism in methionine synthase reductase gene is A66G substitution leading to isoleucine replaced by methionine (Leclerc et al., 1998). The prevalence of 66GG genotype was found to be higher in NTDs affected children and their mothers than in the control group and the deleterious effects of this mutation are important in association with low Vitamin B12 levels (Wilson et al., 1999).

Methylene tetrahydrofolate dehydrogenase (MTHFD) gene defects

This trifunctional enzyme MTHFD has three activities: Methylene tetrahydrofolate dehydrogenase(NADP dependent), Formyl tetrahydrofolate synthase (ATP dependent) and methylene tetrahydrofolate cyclohydrolase. Mutation analysis of MTHFD gene in NTDs patients has led to the identification of G878A substitution in a patient with familial NTDs. In patients with isolated NTDs, a substitution G1958A was seen but there was no difference in the incidence of this mutation in control and patient groups. Hence its association with etiology of NTDs could not be recognized (Hol et al., 1998).

Cystathionine beta synthase (CβS) gene defects

Cystathionine beta synthase gene (CβS) is an enzyme that catalysis the irreversible synthesis of cystathionine from homocysteine and serine. SNPs in the gene coding for CβS could lead to decreased enzymatic activity and a consequent increase in cellular homocysteine level and homocysteinuria. CβS forms cystathionine by the transsulfuration pathway and catalyzes the condensation of homocysteine with serine. Decreased CβS enzyme activity and an aberrant folic acid metabolism increases the risk factor for neural tube defects (Kraus,1999; Saxena et al.,2011).

The mechanism of folate deficiency and NTDs

Folate-mediated one-carbon metabolism

Beaudin and Stover (2007) put forward pathway linking low folate levels and the occurrence of NTDs. They linked aberrations in cellular metabolism of folate with increased levels of homocysteine, impairment in nucleotide biosynthesis, and reduced cellular methylation resulting in altered gene expression with a decrease in mitosis and impaired DNA repair (Greene et al.,2011). Cellular responses during neural tube closure including cell proliferation, differentiation and migration were also reduced. SNPs in folate-related genes influence the maternal folate status and metabolism resulting in the reduced synthesis of nucleotide precursors, DNA, RNA, and other proteins (Mithal et al., 2013).

At cellular level folates function as cofactors referred to as one –carbon units. These are used in the metabolic reactions that constitute folate-dependent one-carbon metabolism (OCM). The metabolically active form of folate, Tetrahydrofolate (THF) carries one-carbon units at three different oxidation states and these forms of folate can be interchanged by action of enzymes (Appling, 1991; Greene et al., 2011).

At cellular level folate-mediated OCM is sorted in the mitochondria, the cytoplasm, and the nucleus. In mitochondria folate metabolism generates formate and glycine from the enzymatic cleavage of serine. Glycine is catabolized to generate formate through mitochondrial folate metabolism that is initiated by the mitochondrial isoform of serine hydoxymethyltransferase, which catalyzes the conversion of serine and THF and forms methylene-THF and glycine (Christensen and MacKenzie, 2008; Fox and Stover, 2008). Methylenetetrahydrofolate dehydrogenase (MTHFD) catalyzes the oxidation reaction of Methylene-THF to produce methenyl-THF which undergoes hydrolyzation and results in generation of 10-formyl THF by the enzyme methenyltetrahydrofolate cyclohydrolase (MTHFC). Free formate and THF is generated when formyl group of 10-formyl-THF is hydrolyzed. The reaction is catalyzed by formyl tetrahydrofolate synthetase (FTHFS). Formate then traverses into the cytoplasm and provides the one-carbon units for cytoplasmic OCM (Christensen and MacKenzie, 2008).

Folate –mediated OCM in the cytoplasm is essential for de novo purine and thymidylate biosynthesis, and in the remethylation of homocysteine to methionine and synthesis of S adenosylmethionine (Adomet) a universal one carbon donor for methylation of chromatin, proteins and lipids (Shane,1995). Folate deficiency leads to impairment in folate-dependent thymidylate biosynthesis resulting in depletion of dTTP levels with increased uracil incorporation in DNA synthesis with genomic instability. The enzymes which are folate-dependent and involved in the de novo thymidylate synthesis include cytoplasmic serine hydroxymethyltransferase (cSHMT), thymidylate synthase (TS) and dihydrofolate reductase (DHFR) (Anderson et al., 2007; Woeller et al.,2007; Anderson et al.,2012). With low folate levels, micronutrient deficiencies and polymorphisms in genes that encode folate metabolizing enzymes folate mediated OCM is impaired (Bailey, 2001; Stover,2004). The biomarkers of impaired OCM include reduced synthesis of

thymidylate de novo leading to increased uracil content into DNA (Blount et al.,1997) elevated levels of homocysteine (Selhub,1999) and hypomethylation of DNA (Rampersaud et al., 2003).

Methylenetetrahydrofolate Reductase (MTHFR) and neural tube defects.

Methylenetetrahydrofolate Reductase (MTHFR) is an important enzyme in folate metabolism. Its position in the folate pathway regulates the distribution of one carbon units in nucleotide synthesis and other methylation reactions in the cell. MTHFR catalyses the conversion of 5,10-methyleneTHF, a carbon donor for nucleotide synthesis, to 5-methylTHF by using NAD(P)H as a reducing agent (Yamada et al.,2001). 5-MethylTHF is essential for the remethylation of homocysteine to methionine with generation of universal methyl donor, Sal adenosyl methionine (SAM). In 1990, Rozen, Kang, and coworkers purified MTHFR 30,000-fold from human cadaver liver (Zhou et al., 1990; Goyette et al.,1994).

Gene Structure and Regulation

The gene that has been most extensively studied is 5, 10-methylene-tetrahydrofolate reductase (MTHFR) as a risk factor for NTD (Aneji et al., 2012). MTHFR regulates the levels of 5 methyl tetrahydrofolate. 5 methylTHF converts homocysteine (hcy) to methionine (Bassuk and Kibar, 2009) and utilizes NADPH as a reducing agent and flavin adenine dinucleotide (FAD) as a co-factor. Mammalian MTHFR is a dimeric protein of 70kDa or 77kDa subunits containing a catalytic domain at N-terminal and a regulatory domain C-terminal (Daubner et al., 1982; Frosst et al., 1995). MTHFR can be regulated in a number of ways. Increased levels of DHF and SAM are thought to inhibit its activity (Matthews and Daubner, 1982). The MTHFR gene has been mapped at chromosome 1p36.3 and is 2.2 kb in length. Goyette et al (1998) initially reported 11 exons but later studies reported >11 exons and the existence of multiple transcripts in the exons (Tran et al., 2002; Homberger et al., 2000).

In the MTHFR gene several single nucleotide polymorphisms have been characterized, but the most widely studied is the C677T polymorphism (Greene et al., 2009; Sameer et al., 2011). In exon 4 the C-to-T transition at nucleotide 677 is a

missense point mutation that converts a cytosine (C) to a thymine (T), with an amino acid substitution of alanine to valine (677C-T, Ala222Val, rs1801133) (Frosst et al., 1995;Sharp and Little, 2004). This mutation creates a Hinf I site, which allows for easy screening of this missense mutation by PCR-RFLP (Frosst et al., 1995). The missense mutation results in increase in thermolability and a reduction in enzyme activity, with a decrease in folate concentration in serum, plasma, and red blood cells and an increase in plasma homocysteine concentrations (van der Put and Blom, 2000).

MTHFR (C677T) polymorphism and Neural Tube Defects.

The association of 677C/T thermolabile isoform of MTHFR polymorphism and NTD susceptibility has been the focus of much research and studies have been carried out globally in different populations (Sharp and Little, 2004). MTHFR reduces 5, 10-methylenetetrahydrofolate to 5-methylenetetrahydrofolate, the principal circulatory form of folate utilized as a carbon donor for the re-methylation of homocysteine to methionine (Frosst et al., 1995). The low MTHFR activity in 677 TT individuals is associated with low plasma folate and high homocysteine levels resulting in DNA hypomethylation affecting gene regulation and differentiation (Robertson and Wolffe , 2000). MTHFR missense mutation 677C/T renders enzyme thermo labile with reduced enzyme activity(Frosst et al., 1995). Low levels of folate with the MTHFR T mutant allele have been associated with higher levels of homocysteine (Hcy). This association between the MTHFR polymorphism and folate levels is the hypothesized link between the C677T polymorphism and neural tube defects (van der Put et al 1995; Botto and Yang, 2000).

The RBCs of 677TT mutants have low concentration of methylated tetrahydrofolates and formylated folate compared to cells from the wild-type 677CC individuals which contain only methylated folate derivatives (Bagley and Selhub, 1998).

Individuals homozygous for the MTHFR C677T mutation exhibit a lower grade of DNA methylation with lowered folate status (Lathrop et al., 2000; Friso et al., 2002). In a study on Dutch population association of 677C-T polymorphism with increased risk of spina bifida was reported (van der Put et al., 1995). Studies from Europe and the USA that included spina bifida and other NTDs also showed similar results (Whitehead et al.,

1995;Possey et al., 1996; van der Put et al., 1997;Botto and Yang, 2000; Shaw et al.,2009). Naushad et al (2010) and Harisha et al (2010) reported an increase risk of MTHFR 677 TT genotype with occurrence of NTDs in Indian population. The 677TT homozygosity for 677C-T was associated with a 7.2 fold increase in the risk for NTDs (Ou et al., 1996; Volcik et al., 2000). Many subsequent studies had similar findings (Martínez de Villareal et al., 2001; Richter et al., 2001; García-Fragoso et al., 2002; Zhang et al., 2013). Mutation in the MTHFR (677TT) gene with low maternal folate status puts the developing embryo at risk, increasing incidence of NTDs (Shields et al., 1999; Botto and Yang, 2000; Obeid et al., 2013). The MTHFR C677T polymorphism is a risk factor low income countries and poor folate nutrition, which could explain the difference in results from various studies on different populations and regions (Shields et al., 1999).

In a study by Rampersaud et al (2003) no association was found between NTDs and MTHFR polymorphism. In Brazilian population Perez et al (2003) and in Japanese population (Kondo et al., 2013) compared the prevalence of TT homozygosity in mothers with NTD offspring with those of the control mothers and did not find a significant difference. Ou et al (1996) in their study did find an association of NTDs to this gene but only 11%–19%. The 677C>T variant of the enzyme shows 50%–60% lower activity than the wild type CC but homozygotes (TT) with a good diet rich in folate and normal folate levels could overcome deleterious effects of this mutation (Botto and Yang, 2000). Individuals heterozygous for this MTHFR 677C/T polymorphism have enzyme levels intermediate between the low and high activity of those homozygous for the variant and normal allele, respectively (Kang et al., 1991; Frosst et al., 1995; van der Put et al., 1995; Jacques et al., 1996; Rozen, 1996). A second common polymorphism in the MTHFR gene is the A1298C transition, involving a glutamate-to-alanine amino acid substitution in exon 7 of the gene. The polymorphism results in decreased activity of MTHFR in the homozygous but not in heterozygous state, but the effect are less than the C677T variation. In individuals heterozygous for both the C677T and A1298C polymorphisms exhibit a phenotype similar to the homozygous C677T variant with elevated homocysteine levels and low folate status. No association has been observed with A1298C polymorphism elevated plasma homocysteine or low plasma folate levels and

increased risk of NTDs (van der Put et al., 1998; Weisberg et al., 1998; Zhang et al., 2013).

Population studies of MTHFR

The worldwide distribution of frequency of MTHFR genotype is heterogeneous (Rodriguez et al., 2006). Studies from different countries revealed that the frequency of the C677T variant allele varies between different ethnic populations in different geographical locations with highest T allele reported in Mexico and lowest in Sub-Saharan Africa (Mutchinick et al., 1999; Amouzou et al., 2004).

The 677C-Tpolymorphism was found in relatively high frequency throughout the world (Schneider et al., 1998; Relton et al., 2004), even in admixed populations like in the island of Puerto Rico (García-Fragoso et al., 2010).The 677C-T polymorphism has been reported to be more frequent among Caucasians than in African Americans (McAndrew, 1996). In Mexico, CC genotype was reported to be (17.6%), CT (47.6%), and TT (34. 8%) genotype which is very high with gene frequencies of 0.414and 0.586% for the C and T alleles, respectively (Mutchinick et al., 1999). In Malaysian Malay population MTHFR 677TT genotype was absent in both patient and control groups. In Japanese population Kondo et al (2013) did not find significant difference in T allele between mothers with spina bifida offspring and the control group. In European population a north-to-south gradient has been reported of T allele prevalence has been observed in Western Europe, and the highest frequency occurs in Sicily. The hypothesis for the variation being low folate rich food intake in North compared to South (Bollander-Gouaille, 2002). The frequency of *C677T* homozygosity in Europe varies from 8 percent in Germany to 18 percent in Italy (de Franchis et al., 1998; Candito et al., 2008). In Ireland and Britain, two areas with historically high rates of neural tube defects, the frequencies of homozygosity were 11% and 13%, respectively. The frequency of homozygosity among Whites outside of Europe (e.g., in Canada, the United States, Brazil, and Australia) ranged from 10 % to 14 % (Relton et al., 2004).

Hispanic populations in the United States have the highest frequencies of this polymorphic allele (40%), whereas the lowest frequencies of the allele were found in

African- Americans in the United States and in sub-Saharan African populations (6–14%) (Botto and Yang, 2000). In European populations, allelic frequencies for the C677T polymorphism is between 30% and 38%, with the lowest frequencies reported among Germans and Dutch with homozygosity occurring in less than 11% of these populations. Botto and Yang (2000) performed a meta-analysis on *MTHFR* data from a number of different countries and ethnic groups, The thermolabile MTHFR 677C→T (A222 V) variant is a risk factor for neural tube defects (NTDs) in some but not all populations and is associated with low folate and elevated homocysteine levels (Botto and Yang, 2000).

Studies focusing on maternal rather than infant MTHFR genotype have suggested that folate deficiency and/or 677T/677T MTHFR genotype in mothers are important risk factors for NTDs and more so when folate status is low (Martinez de Villarreal et al., 2001; Volcik et al., 2000). In Chinese population Liu et al (2014) reported MTHFR mutation 677TT to be a risk factor in development of NTDs.

Data regarding prevalence of MTHFR C677T polymorphism in Pakistan are almost nonexistent except a few reports describing association of this mutation with primary closed angle glaucoma (Michael et al., 2009) and coronary artery disease (Iqbal et al., 2005). Pakistan has a high geographic and social diversity (Ahmed and Sirageldin, 1993). Study on different populations in Pakistan showed a varied distribution of MTHFR C677T allele in various ethnic groups residing in diverse geographical regions of the country. T allele was found to be more frequent in Northern Areas as compared to South and also varied in different ethnic groups residing in the country (Mansoor et al, .2009).

The case control study will be carried out in Holy Family Hospital, Rawalpindi, Quaid-i-Azam University and Life and Brain center, Institute of Human Genetics, University of Bonn. Meager information regarding NTDs in Pakistani is available in Pakistani women. From the few observational studies available, it appears to be on higher side. Also there is sparse available published literature on folate status and MTHFR 677 C→T mutation in Pakistani women with NTD affected pregnancy. This study will decipher some of the environmental, nutritional and genetic risk factors for NTDs. The aim of the study is to evaluate the role of *MTHFR* 677 C→T mutation as a risk factor for NTD in the Pakistani female population with a history of NTD pregnancy. The plan is to

determine the relative importance of the genotypes in the mother and correlate with RBC and serum folate levels. The mothers with NTD affected pregnancy and control mothers with normal offspring will be identified from the Gynecology and Obstetrics Department, Holy Family Hospital, Rawalpindi. Folate studies (RBC and serum folate) will be carried out in the Pathology Laboratory and genotypes will be determined at Institute of Human Genetics, Life and Brain center, University of Bonn. Germany.

SUBJECTS AND METHODS

Subjects and Methods

This study was conducted at Holy Family Hospital (HFH) Rawalpindi, Quaid-i-Azam University (QAU), Islamabad and Institute of Human Genetics, Life and Brain Center, University of Bonn, Germany. Holy Family Hospital is a tertiary care teaching hospital of Rawalpindi Medical College, Rawalpindi, Pakistan. The Department of Gynecology and Obstetrics of HFH caters for the need of pregnant women in the twin cities of Rawalpindi and Islamabad. It is a referral center for small towns and nearby rural areas along with the referred cases from Northern areas and Azad Kashmir.

Case and control groups

Case Definition

For this study women who had come to Department of Gynecology and Obstetrics with previously diagnosed pregnancy with Neural tube defect on antenatal anomaly scan or delivered a baby with Neural tube defect with no prior antenatal record were identified. Such cases were referred from nearby rural areas or small towns where health facilities are either not available or cannot cater a complicated pregnancy. Neural tube defects (NTDs) included anencephaly, encephalocele and spina bifida. Spina bifida includes meningocele and myelomeningocele. A small number of rare cases were identified in this study including lipomeningocele, spina bifida+Arnold-Chiari syndrome and syringomyelia. The diagnosis of sub types of neural tube defects was confirmed in Department of Pediatrics and Department of Neurosurgery. The terminations were confirmed on Ultrasonography in Department of Radiology. For this study the woman who delivered a baby with NTD will be referred to as a case mother.

Case mothers

- **Inclusion criteria**
    - Whatever the outcome of conception, all were considered as a case whether it was a miscarriage, live birth, neonatal death, or a stillbirth with NTD.
- **Exclusion criteria**
    - Epileptics and those on antiepileptic or anti-folate drugs were not included.
    - Women with severe liver disease or renal disease or any chronic illness such as tuberculosis were not included.

Control group: Women who had delivered a normal baby were included in this group. These will be referred to as control mothers.

- **Inclusion criteria**
    - Healthy women who had delivered a normal healthy baby.
- **Exclusion criteria**
    - Healthy women with history of liver disease or kidney disease
    - Women diagnosed for epilepsy and on anti -epileptics were not included.
    - Women with chronic illness like tuberculosis were not included.

Data collection.

After obtaining an informed consent from the mother with NTD pregnancy a direct interview was conducted with mothers after delivery and before hospital discharge. A

specially structured Performa comprising socio-demographic and obstetric data was filled.

After noting the name of mother, husbands' name, and contact number, details were first noted regarding the demographic data.

Demographic data: This included residential area of case and control mothers whether from rural or urban area. The case mother was inquired regarding proximity of living area to a chemical factory area for risk of exposure to hazardous chemical waste which was polluting the air or soil. Information was taken whether there was a radiation exposure or pollution due to presence of garbage dumps nearby. Maternal age at presentation and age at marriage was recorded. Consanguinity in case and control mothers was noted. History was taken regarding smoking habits or partner smoking (or anybody else smoking in house). Breathing in Second hand smoke (SHS) or Passive smoke is equally hazardous as active smoke as combustion products are also embryotoxic(Li et al.,2013).

Educational status of case mother was noted and categorized as those who did not receive school education, college and university education.

Occupational status of case mother and that of her husband was categorized in five classes:

Category 1: Professional

Category II: Business class

Category III: Clerk and peons

Category IV: Skilled labor (painters, electricians, drivers etc.)

Category V: Unskilled labor.

Economic status: Assessment of economic status was based on monthly income of husband and was divided into four classes, first between Rs 5000-10000, second between Rs 11000-15000, third between Rs 16000-20,000 and fourth >20,000. Holy Family Hospital is a public sector hospital and majority of patients availing health facilities from

this hospital fall in lower socioeconomic bracket with no schooling and poor educational status.

Nutritional status: was assessed by semi - quantitative food intake questionaire on average intake of fresh fruits/nuts, eggs, milk and vegetables by recall method. They were divided into two groups, one who were taking fruits and vegetables adequately in their meals per week and the other group included those control and case mothers whose diet was deficient in fruits and vegetables per week. Diet history questionnaire modified from Laurence et al (1980). Meat does not contain folic acid except liver so it was not included while assessing intake of folate rich foods. Lack of knowledge and low awareness of the importance of folate were the two most common reasons reported for not consuming a folate rich diet especially in periconceptional period.

History of Periconceptional intake of folic acid or a vitamin containing folic acid was recorded. Periconceptional period was defined as period one month before and three months after conception. A woman was defined as a folic acid user if she reported use of Supplements containing folic acid more than once a week during a 4-week period (Nilsen et al., 2006).

Obstetric history: regarding parity, live births, abortions, miscarriages and still births were recorded. The patients' past history of an NTD pregnancy was inquired and noted. Family history of NTD in family was also noted.

History was taken regarding congenital abnormality in previous pregnancy, or its occurrence in family with type of congenital anomaly.

Included in the Performa were the details of the offspring, sex of fetus, the birth order, whether alive or dead or mother had come for termination.

Knowledge of folic acid: Patients were asked about their level of awareness, knowledge and use of folic acid. Awareness regarding folic acid and knowledge of its importance in preventing NTDs was assessed. Awareness was assessed by simple question "have you heard of folic acid". Knowledge of the importance of folic acid was defined as being able to answer the role in preventing NTDs and the importance of the correct time to take the

supplement.. Women were asked if they had ever used Folic acid or a multivitamin pill with folic acid in periconceptional period and during the current pregnancy (Ren et al., 2006a).

Collection and processing of blood samples for serum folate, red blood cell (RBC) folate, hemoglobin and hematocrit.

Collection of Blood samples

After obtaining informed consent from the subjects, blood samples were collected in morning following an 8 hour fast as serum folate concentrations increase shortly after consumption of folate containing foods. For Serum and RBC folate analysis blood samples were collected aseptically in k3EDTA-Vacutainer tubes and serum separator tube (SST) red cap tube (Becton Dickinson, Franklin Lakes, and NJ) centrifuged within 1 hour of collection. 10 ml blood was drawn from anti-cubital vein, and divided into three tubes, 5 ml was placed in EDTA (ethylenediaminetetraacetic acid) lined tube for DNA analysis, 2.5 ml was placed in another EDTA lined tube for RBC folate analysis and 2.5 ml was placed in was placed in serum separator tube (SST) red cap tube for serum folate estimation and kept at -20ºC till analysis.

Serum Specimens

After complete clot formation, sample was centrifuged (centrifuge 3000/Rev/min; Rotofix 32 A, Hettich Lab Technology, Germany). If the specimen is centrifuged before a complete clot forms, fibrin may cause erroneous results. Serum is separated from the clot within 24 hours of draw. Serum specimens were stored at-20°C till testing.

Whole Blood Specimens

The whole blood sample for DNA Analysis was permanently stored at -20ºC prior to analysis. Whole blood specimens collected in tri potassium EDTA tubes for RBC folate analysis were stored at -20°C till testing. Complete blood picture was performed on Sysmex XP-300™ Automated Hematology Analyzer for hemoglobin and hematocrit before storage.

Ax SYM System for Serum folate and RBC folate analysis.

The principle of AxSYM Folate system is an ion capture assay for the quantitative estimation of folate in human serum, plasma or red blood cells on the AxSYM System Unoccupied folate specific binding sites are bound to matrix using a conjugate of pteroic acid (a folate analog) and alkaline phosphatase as the signal-generating molecule, and a substrate, 4-methylumbelliferyl phosphate (An immunoassay analyzer AxSYM, Abbot Laboratories, Abbot Park, Ill).

Biological principles of the procedure

The principle of AxSYM Folate is ion capture with Ion Capture solution (Bulk solution 2), a high molecular weight quaternary ammonium compound is dispensed on the glass fiber matrix of the matrix cell which gives a positive charge to the matrix and the capture of negatively charged analyte complexes is completed. In this procedure there is formation of negatively charged polyanionanalyte complexes. The complexes are captured through electrostatic interaction of the negatively charged polyanion analyte complexes with the glass fiber matrix which is positively charged. The assay machine AxSYM Folate assay uses a soluble affinity reagent which contains folate binding protein(FBP) affinity and is coupled with monoclonal antibodies. These monoclonal antibodies are covalently bound to carboxymethylamylose (a polyanion). Folate is quantified by measuring the population of unoccupied FBP sites bound to the matrix using a conjugate of pteroic acid, a folate analog and a signal-generating molecule alkaline phosphatase.

RBC folate and serum folate analysis was carried out according to manufacturer's protocol.(Abbot Diagnostic Division,2010)

Genotyping for Methyl Tetrahydrofolate reductase (MTHFR) C677T mutation by PCR-RFLP (Polymerase chain reaction; Restriction Fragment Length Polymorphism) method.

MTHFR 677 C/T Genotyping.

For the MTHFR 677 C/T mutation analysis PCR and *Hinf* I digestion was carried out according to protocol by Frosst et al (1995). The RFLP digestion products and the Allele Specific products of PCR are run on agarose gel electrophoresis and separation of bands is according to size. Gel electrophoresis gives different bands subsequent to RFLP digestion and the banding patterns are used to identify whether an individual is homozygous normal, heterozygous or homozygous mutant for a specific mutation.

DNA Extraction

DNA extractions were performed from whole blood by using magnetic bead technology with the Chemagic Magnetic Separation Module I and the Chemagic DNA Kit (Chemagen, Baesweiler, Germany) according to manufacturers' protocol. DNA concentrations were determined.

Principle: The basis of chemagen chemistry for DNA extraction from whole blood is the use of paramagnetic beads. In the initial step, Lysis of the white and red blood cells is done by addition of protease for degradation of proteins. Isolation of DNA is achieved following its capture by magnetic polyvinyl alcohol beads (M-PVA Magnetic beads): The coating of these beads specifically binds the DNA. When an electromagnetic field is applied the beads and the bound DNA are attracted to magnetised metal rods. DNA is transferred by the metal rods from one wash buffer into the other wash buffer. After electromagnet is deactivated at the end of each transfer step, the rods begin to rotate leading to a homogenous re-suspension of particles. In the final step,the beads are transferred into elution buffer which then inactivates the contact between the beads and DNA. After the magnetic beads are removed isolated DNA in suspension is acquired.

Procedure

The DNA extractions were performed in 50 ml falcon tubes arranged in 4 × 3(=12) arrangement and placed in special metal racks that would fit into the track system of chemagic Magnetic separation module 1. The module consist of an electromagnet with a metal head with 12 and 96 rods. The rods which are magnetizable dip in the falcon tubes containing the sample with suspension of magnet beads. When the electromagnet is switched on the rods become magnetic with separation of the beads.

DNA purification Protocol for 2ml of whole blood using the chemagic Magnetic Separation Module 1 with 12 metal head.

Protocol name: A: Lysate mixing blood 2k.txt

B: Chemagic Blood 2k elution 200µl 4ml tubes v050921.txt

Positioning racks on the tracking system (Integrated robotic system)

Position 1	Rack with disposable tips
Position 2	50ml tubes containing 2ml blood,10µl protease, 3ml lysis buffer, 8ml binding buffer 2, 240µlMagnetic beads.
Position 3	5 ml wash buffer3
Position 4	5 ml wash buffer 4
Position 5	5 ml wash buffer 5

Position 6 5 ml wash buffer 6

Position 7 4 ml tubes prefilled with 0.2 ml TE-4

TE-4 elution buffer is 10 mM Tris-HCl ph 8.0

Binding buffer; wash buffer 3,4,5,6 contain ethanol.

Procedure

Preparing the Blood Lysate

- The disposable tips are placed in position 1 on the tracking system
- 10µl Protease was added to each tube containing 2ml of blood
- 3ml Lysis Buffer 1 dispensed into the tube containing blood and Protease
- Tubes placed in position 2 on the tracking system
- On computer screen the protocol "A – Lysate mixing Blood2k.txt"was selected and the automated lysate mixing was started by pressing the **Start** button; the lysate was mixed for 10 minutes.
- After the first protocol is finished all racks with prefilled tubes were placed in the positions given above.
- The racks and tubes were checked for correct fitting on the tracking system
- 8 ml Binding Buffer 2 was added to the lysate and then 240µl Magnetic Beads were added.

Resuspend the beads carefully before adding to the lysate.

- The tubes were returned with the lysate to position 2 on the tracking system
- The protocol "B – chemagic Blood 2k elution 200µl 4ml tubes V050921.txt" was selected and to start the automated isolation process the **Start** button was pressed.

(DNA Purification Protocol taken from the Chemagen user manual for the chemagic Magnetic Separation Module I, Version no. 050127)

Figure 3: Chemagic Magnetic Separation Module I

(Image courtesy: Tech Dragon Limited)

DNA quantification by NanoDrop®

Nanodrop technology is used for measuring nucleic acid concentrations in sample volumes of one microliter. The sample is directly placed on top of the detection surface and the surface tension is used to create a column between the ends of optical fibers. Quantification of nucleic acid is done. This is based on measurement of the spectrum in the defined pathway of 1 mm and its absorbance is measured at 230 nm, 260 nm and 280 nm. The attached computer displays the absorbance values, concentrations and the ratios at 230/260 nm and 260/280 nm (for the evaluation of purity of DNA). These are calculated by the NanoDrop ND-1000 software.

(High concentrations of DNA were diluted to get a uniform concentration of 23ng/ul by adding Millipore water)

DNA Amplification and Genotyping

Primer design

The programs Restriction Mate v1.0 and Ensembl (http://www.ensembl.org/index. html) were used to design the primers. Primer design is important for successful reactions and the following parameters were considered:

- The primer length should be 18 – 20 nucleotides in length,
- GC content should be 40 – 60 %,
- Primer pair should not be complementary to each other especially at the 3' end,
- They should not bind to any region containing SNPs, and
- Both primers should have a similar melting point.

DNA Amplification (Polymerase Chain Reaction)

Polymerase chain reaction is a sensitive method in which a specific or target fragment of DNA is amplified making a large number of DNA copies. It uses repeated cycles, each of which consists of three steps:

1. Denaturation step: The double stranded DNA melts open to single stranded DNA due to the breakage of weak hydrogen bonds between the bases whereas the bonds between deoxyribose and phosphates, which are stronger covalent bonds, remain intact.

2. Annealing step: In the second step, the temperature is lowered to allow primers to anneal to the single strand of DNA. The optimal annealing temperature is dependent on the melting temperature of the primer-template hybrid. At very high temperatures, the primers do not anneal well, and at low temperatures, the primers annealing may be non - specific. As a simple rule of thumb, the Tm of the primers can be estimated by adding 2 ^{o}C for each Adenine or Thymine and 4^{o}C for each Guanine (G) or Cytosine (C).

3. Elongation step: this step requires DNA synthesis by a thermostable DNA polymerase. Using the original strands as templates, DNA polymerase synthesizes two new strands of DNA. In this process there is resultant duplication of the original DNA, with each of the new molecules containing one old and one new strand. With the

continuous repeated steps, around 10^7 copies of the original DNA segment can be obtained.

PCR-reaction and conditions

To analyse the MTHFR variant 677C/ T in the MTHFR gene, amplification of exon four of the gene by polymerase chain reaction (PCR) was carried out with standard conditions and the use of modified primers

4F:5'-TCTTCATCCCTCGCCTTGAAC-3';

4R:5'-AGGACGGTGCGGTGAGAGTG-3' according to Frosst et al. (1995).

PCR was accomplished in 25 µL reactions containing of 2 µL DNA (20 ng μL^{-1}), 2.5 µL buffer (containing $MgCl_2$ 15 mM), 0.5 µL dNTP (dNTP Mix, (Labomedic GmbH)Mix containing 10 mM each of dATP, dCTP, dGTP, and dTTP (1 x 200 µl), 1 µL each of forward and reverse primers, and Millipore water (Merck) to make up the final volume of 25 µl. The PCR reactions were conducted in a 25 ul reaction mixture containing 10 x buffer, 100 mM Tris-HCl, pH 8.3 (Qiagen GmbH);10 mM of each dNTP;10 pmol 677F; 10 pmol 677R; DNA Taq Polymerase(5M/ul) (MBI Fermentas, Hanover, USA) and Millipore water. All reactions were prepared on ice.

Table 1: Protocol for master mix for DNA amplification

Buffer (10x)	2.5ul
d-NTPs (10mM)	0.5ul
Primer F (10pmol/µl)	1ul
Primer R (10pmol/µl)	1ul
Taq (5M/µl)	0.2ul
H2O	17.8ul
DNA (20-100µg/µl)	2ul
Total	25ul

Thermocycler conditions for PCR

DNA was amplified by using a PCR thermal cycler with the Standard PCR programme TD-100

Cycling conditions for the 677C>T polymorphism were set at an initial denaturation step at 95°C for 10 min, followed by a shorter one for 30 seconds. Annealing temperature was set at 63ºC for 30 sec and elongation at 72ºC for 1 minute.The PCR product was cooled down to 12 °C.

Table 2: Standard PCR Program-TD 100

Step	Temperature	Time	Cycles
1	95º C	5 min	1x
2	95º C	30 sec	
3	63º C	30 sec*	38 times
4	72º C	1 min	
Go to Step 2,38 times			
5	72º C	10 min	
6	12º C	forever	

* Initial annealing temperature is 63ºC then in every cycle the temperature is decreased by 1ºC, and ends 15 cycles at 55ºC as the final annealing temperature.

Agarose-gel electrophoresis for PCR products

Gel electrophoresis is a method used for analysis of macromolecules (DNA, RNA and proteins) and their fragments, based on their molecular size and charge. The negatively charged DNAs and RNAs are pulled to the positively charged end of gel by an electrical field through a gel that contains small pores. Small DNA molecules migrate through the pores in the gel faster than larger DNA molecules. The bands formed by DNA molecules can be visualized by a fluorescent dye, ethidium bromide, under ultraviolet light.

For the experiment, the sizes of all the amplified PCR products were confirmed by resolving the products on a 2 % (w/v) agarose gel, stained with ethidium bromide, which was suspended in 1XTBE buffer(1x TRIS-Borate-EDTA(TBE)-buffer, 0,01%)(Sigma Aldrich). For each PCR reaction 5 µl of PCR product were mixed with 5 µl of Blue buffer before the samples were loaded into the wells of the agarose gel with a multichannel 10µl pipette. Gel electrophoresis of the PCR product was carried out for 30 minutes at 120 V and 400 mA. Subsequently, the PCR products were visualized and documented using a Gel Documentation system (BIO-RAD). The sizes of visualized bands in the gel were approximated using a 100 bp DNA ladder.

Detection of mutation C677T by enzymic digestion of the PCR products by Restriction Fragment Length Polymorphism (RFLP)

The detection of the C677T mutation in the MTHFR gene was performed by PCR

Amplification followed by digestion with restriction enzyme. Restriction enzymes are isolated from bacteria and recognize specific sequences in DNA and then cut the DNA to produce fragments. These are called restriction fragments. The amplified PCR products (MTHFR) were subjected to Hinf 1restriction enzyme digestion at 37°C for 24 hour. The PCR products subjected to enzyme digestion were visualized on 3% agarose gel stained with ethidium bromide.

Table 3: Protocol for the Enzymic digestion of PCR products.

	Amount (µl)
PCR product	10 ul
Water (nuclease–free)	18 ul
10x buffer R	2 ul
Hinfl	2ul

Procedure:

All reactions were prepared on ice.

- Mix gently and centrifuge for a few seconds
- Incubate for 24 hours at 37º C
- Thermal inactivation: Hinfl is inactivated by incubation at 65º C for 20 min.
- Cooling at 12ºC for 10 minutes.

The MTHFR mutation was determined by enzymatic digestion of the initial PCR product with HinfI enzyme (Thermoscientific, MA; USA) at 37ºC for 24 hours followed by inactivation of the enzyme by incubating at 65º C for 20 minutes and cooling at 12º C for 10 minutes. 3% agarose gel in 1x Tris borate EDTA buffer was used to separate the digested DNA ethidium bromide staining. The PCR Digest product was electrophoresed for 60 minutes at 90 V and 400 mA. The digested products were finally visualized by means of a Gel Documentation system (BIO-RAD). A 100 bp DNA ladder was used to assess the size of the bands in the gel.

Sequencing by capillary electrophoresis.

Six samples (CC, CT and TT genotypes in two; respectively) detected by the PCR-Enzymic digestion were also confirmed with Sequencing by Capillary Electrophoresis (Applied Biosystems). Sequencing is preceded by purification of amplified DNA sample followed by amplification by cycle sec and a clean sec to purify the cycle sec product.

Paramagnetic bead purification of PCR product (Agentcourt® AMPure® XP system)

The PCR product contains many contaminants like unincorporated dNTPs, primers, primer dimers, salts etc. to be removed before performing a Sanger sequencing reaction (cycle sequencing). For the removal of these contaminants, the Agentcourt® AMPure® XP system was used. The AMPure solution, containing paramagnetic beads, binds the amplified DNA. The tubes with the respective PCR product were kept in a magnetic plate, which drew the paramagnetic beads with DNA to the walls of the reaction tubes. The remaining solution was then discarded and washed twice with 70 % ethanol. Finally, the purified PCR product was eluted into a final volume of 40 µL of TE^{-4} buffer.

Steps of procedure;

1. 1.8ul AMPure XP is added per 1.0 ul of PCR product

2. PCR products bind to paramagnetic beads

3. Contaminants separated from beads + PCR

4. To remove contaminants, beads washed + PCR product 2x with 70% Ethanol.

5. Elute purified PCR product from beads.

6. The purified product is then transferred to a new plate.

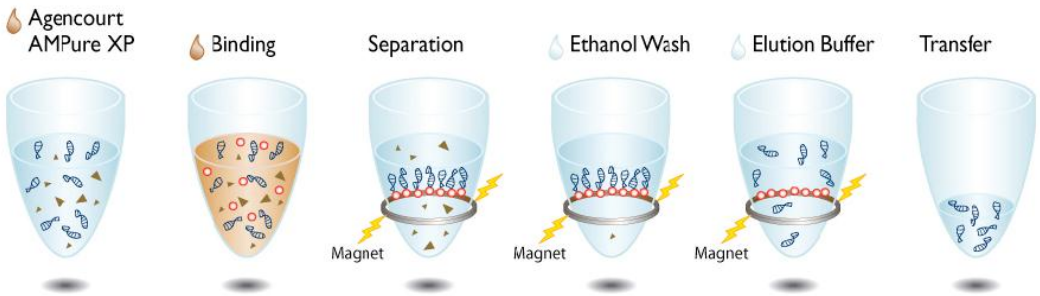


Figure 4: Agencourt AMPure XP process.

Sanger sequencing

Sanger sequencing, based on the chain termination method, was used to determine the nucleotide sequence of DNA (Sanger et al., 1977). It utilizes dideoxy-ribonucleotide triphosphates (ddNTPs) to terminate DNA chain elongation because of having a hydrogen atom attached to the 3' carbon rather than an OH group. Hence, they are unable form a phosphodiester bond with the next deoxynucleotide, thus ending the reaction. Each of the four ddNTPs is labeled with a different fluorescent dye. The dye emits light at different wavelength.

Sanger sequencing reactions and conditions

A special PCR reaction was performed for this purpose. The primer used was forward primer 4F:5'-TCTTCATCCCTCGCCTTGAAC.

PCR was performed in 20 µL reactions, consisting of amplified PCR product of 2 µl, 1 µl forward primer, 3.75 µL of buffer, 0.5 µL of BigDye, 1 µl of DMSO and Millipore water to make the volume up to 20 µL as shown in Table 4.

Table 4: Sanger-Sequencing Reaction

	Amount (µl)
Amplified PCR Product	1µl
Primer Forward or Reverse	1µl
5x Sequencing Buffer	3.75µl
BigDye 3.1	0.5µl
DMSO	1µl
Water	Upto 20µl

Table 5: Thermocycler conditions for cycle sec.

Step	**Temperature**	**Time**	**Cycles**
Initial denaturation	96º C	1 min	1x
2. Denaturation	96º C	12 sec	30 x
3. Annealing	50º C	20 sec	30x
4. Extension	60º C	40 sec	30x
5. Hold	4º C	forever	

Paramagnetic bead purification of cycle sequencing product (Agentcourt® CleanSEQ® System)

After the completion of the Sanger sequencing reaction, the Agentcourt® CleanSEQ® system was used to remove the excess dye terminators and contaminants as they could interfere with the interpretation of the sequence in the Genetic Analyzer. The kit is based on the same principle as the Agentcourt® AMPure® XP system as described above. 85% ethanol was used and the final sample is eluted in HPLC water instead of TE^{-4} buffer.

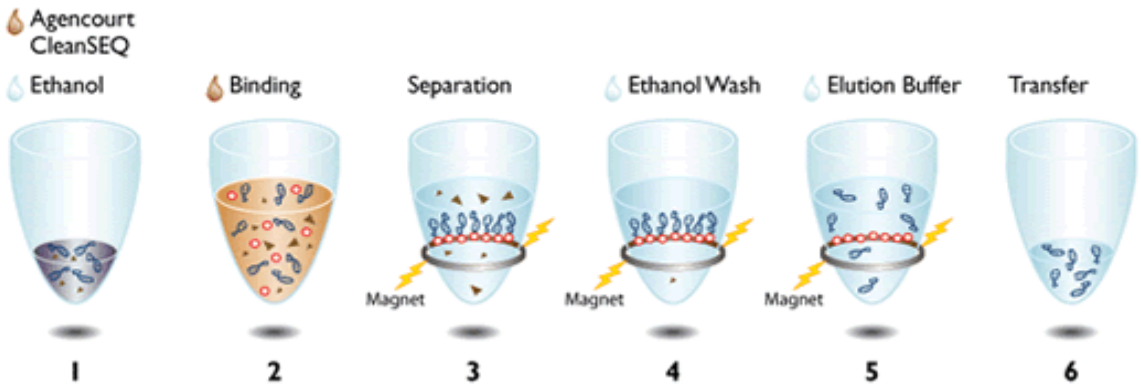


Figure 5: The Agencourt CleanSEQ system

Automated Sequencer by Capillary Gel Electrophoresis

The automated sequencer, 3130xl Genetic Analyzer, was used to separate Sanger sequencing product fragments by size. The principle is similar to gel electrophoresis. In this technology, matrix is located within the capillaries through which DNA fragments, each ending with a fluorescently labeled ddNTP, are separated according to their size. The fluorescence labeled fragments migrate through the gel, pass a laser beam at the end of the capillary that excites fluorescent molecules and emits different colored lights, representing each base with different colors. The results are then depicted in a computer in the form of a coloured chromatogram, showing the location of nucleotides in the sequence.

Mutation Analysis of the sequences

The sequences, obtained by capillary gel electrophoresis, were transferred and analyzed using the DNA Star Seqman II software package. Each electropherogram could be viewed in Seqman II, which was aligned with a reference sequence in order to determine if a variant was present.

If a variant was found, it was checked in the Single Nucleotide Polymorphism database (dbSNP, Build 136) to check if it has been already reported and to compare the MAF of the respective variant. dbSNP is the world's largest database of variations and contains information on all variants that have been reported. Variants submitted to dbSNP receive a reference SNP number, which allows the variant to be tracked.

Statistical analysis

All the data was analyzed by using Statistical analysis package, Graph pad prism version 5. Summary statistics are presented as mean and SEM. Chi-square test (for numbers) was used for univariate analysis for the significance of association between categorical variables. Differences were considered statistically significant P-value of <0.05 for all tests. Comparisons were analyzed applying students t test with P value of <0.05 considered as statistically significant

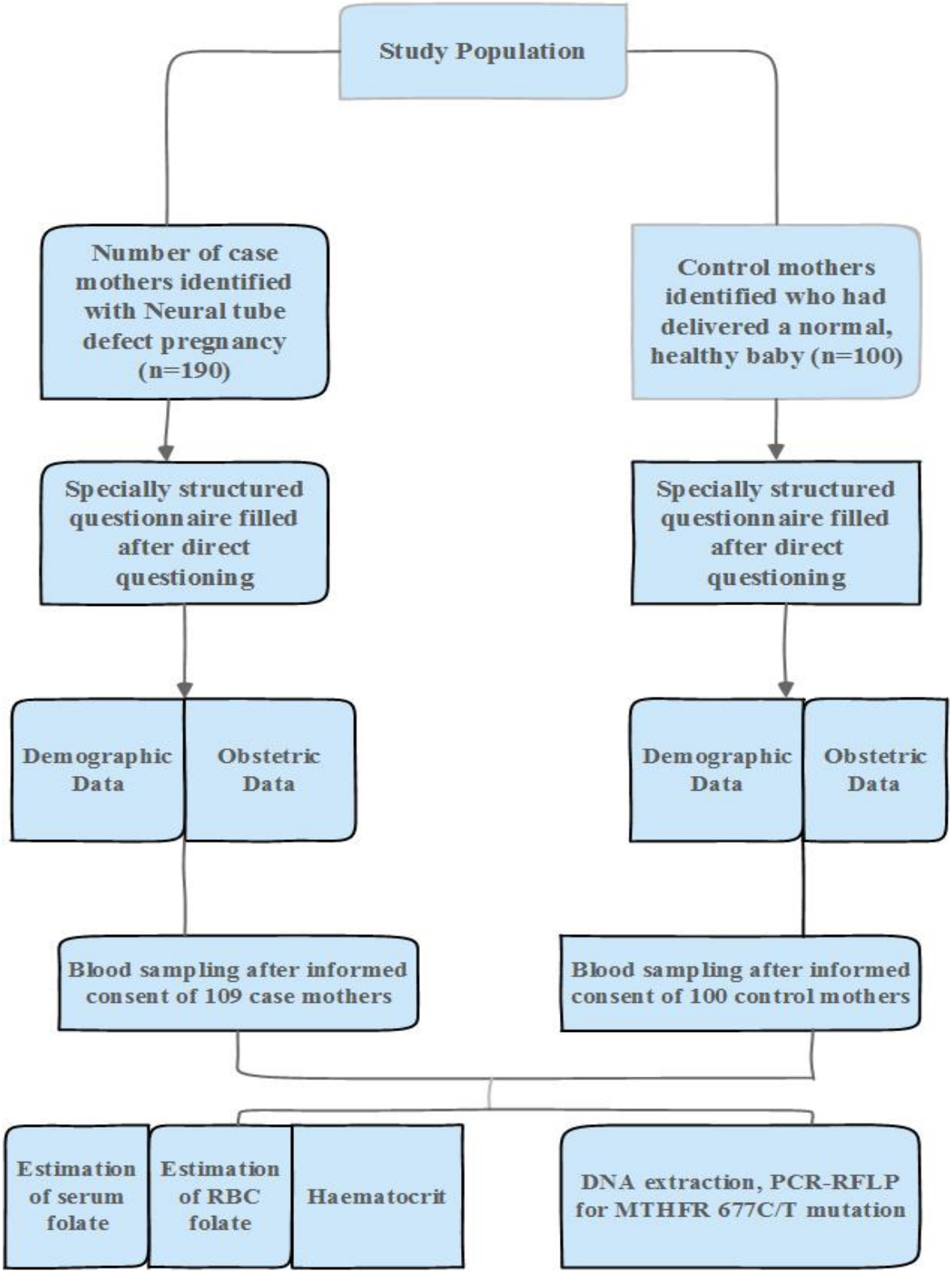


Figure 6: Study Plan

RESULTS

RESULTS

Study population comprised of 100 Control mothers and190 mothers who gave birth to offspring with Neural tube defects (NTDs). These 190 cases included 114(60%) live births, 62(32.63%) still births and 14(7.36%) terminations (Fig 4). Termination of pregnancy (TOP) refers to culmination of pregnancy after confirmation of neural tube defect in fetus on ultrasound examination by using pharmacological or surgical means.

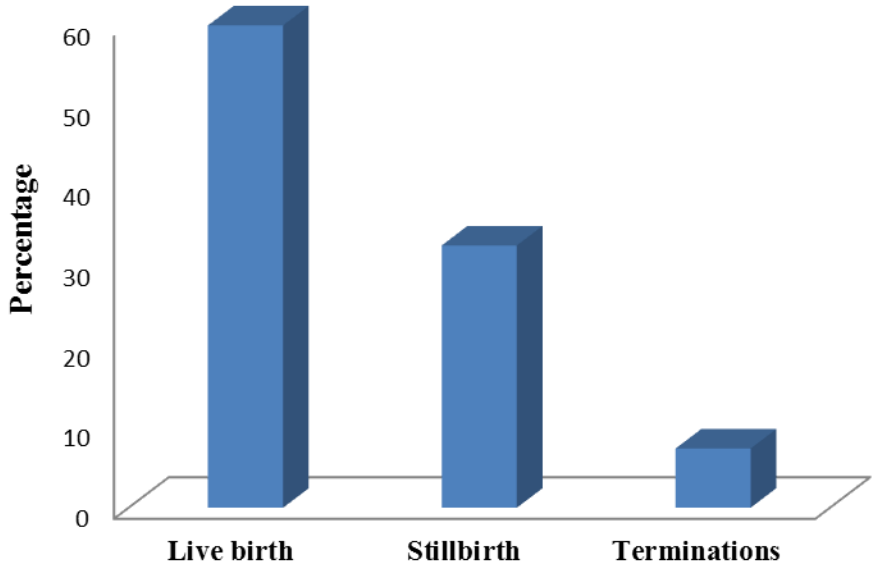


Figure 7: Percentage of Live births, still birth and terminations in off spring of study population with neural tube defects.

Distribution of the total number of neural tube defects (NTDs) identified in this study are presented in Table 6 along with their number and percentage. Two types of Neural tube defects were diagnosed (1) Cranial neural tube defects (2) Spinal neural tube defects. Both types of neural tube defects were further categorized as open neural tube defects (neural tube exposed to surface) and closed neural tube defects (covered with skin). In cranial NTD anencephaly is open NTD comprising 18.95% (n=36) and encephalocele is closed NTD 7.4% (n=9). In spinal NTDs myelomeningocele is an open defect and meningocele is closed NTD. Also included in closed spinal NTDs are some not so common defects like; lipomeningocele, Dandy Walker syndrome, Spina bifida+ Arnold –

Chiari syndrome and syringomyelia. There were 45(23.68%) cranial or upper neural tube defects and 145(76.32%) spinal or lower neural Tube defects.

In spinal NTDs maximum number and percentage of cases 80(42.11%) were meningocoele, then myelomeningocele 57(30%) and lipomeningocele were 04(2.11%). There were two cases of Dandy Walker syndrome (1.05 %) and two cases of which one case (0.53%) is Spina bifida with Arnold-Chiari syndrome and the other is syringomyelia (0.53%).

Total number of neural tube defects with hydrocephalous condition and other defects were 68(35.79%) Table7. There were 24(12.36%) cases of myelomeningocele with hydrocephaly and meninogocele in combination with hydrocephalous were 39 (20.53%). There were two cases of myelomeningocele in combination with hydrocephalous as well as esophageal atresia and another two cases were diagnosed of myelomeningocele in combination with hydrocephaly+ hydronephrosis. Dandy-Walker syndrome appeared in combination with hydrocephaly and was observed only in one case (0.53%).

In addition to hydrocephalous, there were 12 (6.32%) cases of neural tube defects which appeared in combination with other congenital anomalies. There were two cases each of esophageal atresia, hydronephrosis, polycystic kidneys and multiple anomalies. There were three cases of talipes and a single case of hydrocele.

Figure 8 shows pictures of babies born with NTDs in Holy Family Hospital, Rawalpindi, Pakistan.

Table 6: Distribution of types of Neural tube Defects(n=190)in cranial and spinal neural tube defects.

Neural Tube Defects(NTDs)Total number of cases=190						
Cranial Neural tube Defect (n=45)				**Spinal Neural tube Defects (n=145)**		
	n	%			n	%
Open cranial NTDs				**Open spinal NTDs**		
Anencephaly	36	18.95	Spina bifida	Myelomeningocele	57	30.00
Closed cranial NTDs				**Closed spinal NTDs**		
Encephalocele	9	4.74		Meningocele	80	42.11
				Lipomeningocele	4	2.11
				Dandy Walker Syndrome	2	1.05
				Spina bifida+Arnold Chairi Syndrome	1	0.53
				Syringomyelia	1	0.53
Total	45	23.68			145	76.32

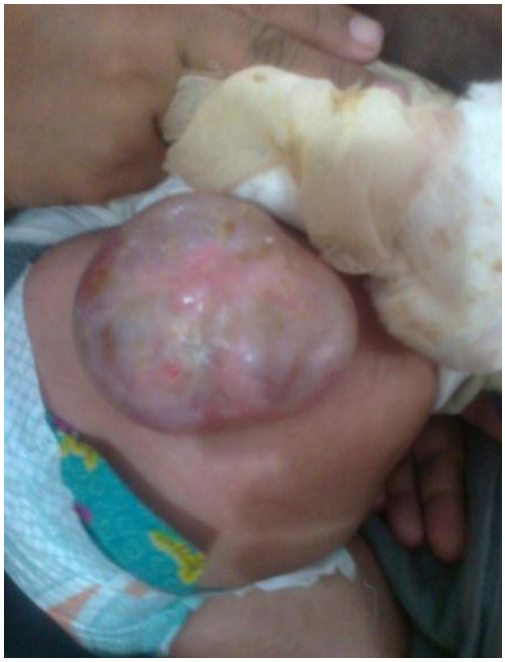
All percentages were calculated based on 190 NTDs

Table 7 : Distribution of Neural tube defects in combination with hydrocephalous and other defects

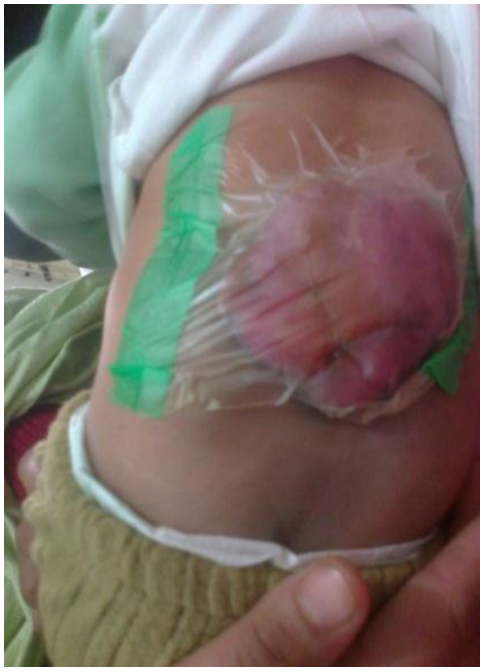
Neural Tube Defects (n=190)		
	n	%
NTDs with no hydrocephalous	110	57.89
NTDs with hydrocephalous	68	35.79
NTDs with other congenital anomalies	12	6.32
Total	**190**	**100**
NTDs with no hydrocephalous		
Anencephaly	36	18.95
Encephalocele	9	4.74
Myelomeningocele	22	11.57
Meningocele	36	18.94
Lipomeningocele	4	2.11
Dandy Walker Syndrome	1	0.53
Spina bifida+Arnold Chairi Syndrome	1	0.53
Syringomyelia	1	0.53
Total	**110**	**57.89**
NTDs with hydrocephalous		
Myelomeningocele with hydrocephalous	24	12.63
Myelomeningocele with hydrocephalous +esophageal	2	1.05
Myelomeningocele with hydrocephalous + hydronephrosis	2	1.05
Meningocele with hydrocephalous	39	20.53
Dandy-Walker syndrome + hydrocephalous	1	0.53
Total	**68**	**35.79**
NTDs with other congenital anomalies		
Meningomyelocele with Hydronephrosis+hydrocephalous	2	1.05
Meningomyelocele with Multiple anomalies	2	1.05
Meningomyelocele with Talipes	3	1.58
Meningocele with Esophageal atresia+hydrcephalous	2	1.05
Meningocele with Polycystic kidneys	2	1.05
Meningocele with Hydrocele	1	0.53
Total	**12**	**6.31**

A

B



C



D

Figure 8: **A:** Occipital encephalocele **B:** Frontal encephalocele **C:** Myelomeningocele **D:** Ruptured myelomeningocele

Sex of live births, still births and terminated fetuses with Neural Tube Defects

There is no statistical difference in female and male offspring from control mothers (100♀♀:132.6♂♂; $\Sigma \chi^2_{(1)} = 1.96$; P= < 0.10). In the total defective births sex ratio (100♀♀:65.49♂♂; $\Sigma \chi^2_{(1)} = 8.14$; P= < 0.005) shows significantly less male births with NTDs than females. Table: 8

Cranial NTDs: In cranial NTDs females are significantly higher in number than the males (100♀♀; 40.62♂♂; $\Sigma \chi^2_{(1)} = 8.02$; P=<0.005) (Table 8). Among the Cranial NTDs, anencephaly females appear highly significantly more than the males (100♀♀; 28.57♂♂; $\Sigma \chi^2_{(1)} = 11.12$; P=< 0.001) Table 9. Encephaloceles show no significant difference in sex ratio.

Spinal NTDs: In spinal neural tube defects there is no significant difference in the two sexes (100♀♀; 75.31♂♂; $\Sigma \chi^2_{(1)} = 2.81$; P= 0.10) Table 8. In meningocele, sex ratio is (100♀♀; 83.72♂♂; $\Sigma \chi^2_{(1)} = 0.62$; P=>0.20) and in myelomeningocele offspring it is (100♀♀; 71.80♂♂; $\Sigma \chi^2_{(1)} = 1.48$; P=>0.20) Table 9.

Other spinal neural tube defects like lipomeningocele, spina bifida+Arnold-Chiari syndrome and syringomyelia were recorded only in females in this study. Dandy Walker was, however, recorded only in males.

Both still birth (100♀♀; 46.34♂♂; $\Sigma \chi^2_{(1)} = 8.06$; P= < 0.005) as well as live births (100♀♀; 61.43♂♂; $\Sigma \chi^2_{(1)} = 6.45$; P=<0.01) show that females with neural tube defects were significantly higher in number than the males (Table 10). The sex of the terminated fetuses was identified at birth by the obstetrician and recorded as they were carried out after the 22weeks anomaly scan. There was no significant difference among both sexes.

Table 8: Sex ratio of study population(Control offspring and NTD affected births with cranial and spinal neural tube defects.)

Sex		Control mothers offspring (n=100)	Case mothers offspring (n=190)	Neural tube defects(n=190) Cranial NTD (n=45)	Neural tube defects(n=190) Spinal NTD (n=145)
Female	n	43	113	32	81
	(%)	(43)	(59.47)	(71.11)	(55.86)
Male	n	57	74	13	61
	(%)	(57)	(38.94)	(28.89)	(42.07)
Ambiguous genitalia		—	3 (2.07)	—	3 (2.07)
Sex ratio		100♀♀:132.6♂♂ $\Sigma \chi^2_{(1)} = 1.96$; P= < 0.10	100♀♀:65.49♂♂ $\Sigma \chi^2_{(1)} = 8.14$; P= < 0.005**	100♀♀:40.62♂♂ $\Sigma \chi^2_{(1)} = 8.02$; P=< 0.005**	100♀♀:75.31♂♂ $\Sigma \chi^2_{(1)} = 2.81$; P= 0.10

Table 9: Sex ratio of female and male babies in different Neural tube defects

Sex		Total number of cases (n=190)							
		Cranial NTD(n=45)		Spinal NTD(n=145)					
		Anencephaly	Encephalocele	Meningocele	Myelomeningocele	Lipomeningocele	Spina bifida + Arnold - Chiari Syndrome	Dandy-Walker Syndrome	Syringomyelia
Female (1)	n	28	4	43	32	4	1		1
	(%)	(14.74)	(2.11)	(22.63)	(16.84)	(2.11)	(0.53)		(0.53)
Male	n	8	5	36	23			2	
	(%)	(4.21)	(2.63)	(18.95)	(12.11)			(1.05)	
Ambiguous genitalia	n			1	2				
	(%)			(0.53)	(1.05)				

Anencephaly (100♀♀;28.57♂♂; $\Sigma \chi^2_{(1)} = 11.12$; P= 0.001**).

Encephalocele (100♀♀;125♂♂; $\Sigma \chi^2_{(1)} = 0.1101$; P=0.40).

Meningocele (100♀♀;83.72♂♂; $\Sigma \chi^2_{(1)} = 0.62$; P= >0.20)

Myelomeningocele (100♀♀;71.80♂♂; $\Sigma \chi^2_{(1)} = 1.48$; P= >0.20)

Table 10 : Sex of live births, still births and terminated fetuses with Neural Tube Defects

Sex	Total number of NTD births (n=190)			
	Total cases	Live birth	Still Birth	Terminations
Female	119 (62.63)	70 (36.84)	41 (21.58)	8 (4.21)
Male	68 (35.79)	43 (22.63)	19 (10)	6 (3.16)
		100♀♀;61.43♂♂ ; $\Sigma \chi^2$ (1) = 6.45; P= <0.01**	100♀♀;46.34♂♂; $\Sigma \chi^2$ (1) = 8.06; P= < 0.005**	100♀♀;75♂♂; $\Sigma \chi^2$ (1) = 1; P= 0.1
Ambiguous genitalia	3 (1.58)	1 (0.53)	2 (1.05)	

Maternal age at presentation.

The Mean age at presentation of mothers who had Neural Tube Defect birth was 27.51 ± 0.38 years (Range16-40 years; n=190). In control mothers the mean age was 27.17 ± 0.50 years (Range16-40 years; n=100).

Mean age of case mothers in different age groups and NTDs

The distribution of number and mean age (years) of case mothers with cranial and spinal NTDs births is shown in Table 11. In case mothers the highest NTD births were observed in age range between 25-29 years (n=65:34.21%; mean age: 29.51 ± 0.17years). The lowest number and percentage of NTDs was observed in the lowest maternal age group between 15-19 years. Increase in NTDs started from age group 20-24 years (n=50; 26.32%) but the decline in NTDs started from 30-34years (n=40; 21.05%) and further declined in age group>35 years (n=28; 14.74%). Majority of cranial NTD (n=18; 9, 47%) and spinal NTDs (n=47; 24.74%) were noted in age range from 25-29 years. Decline in NTD number was seen from 30 to >35years of maternal age (Figure 5).

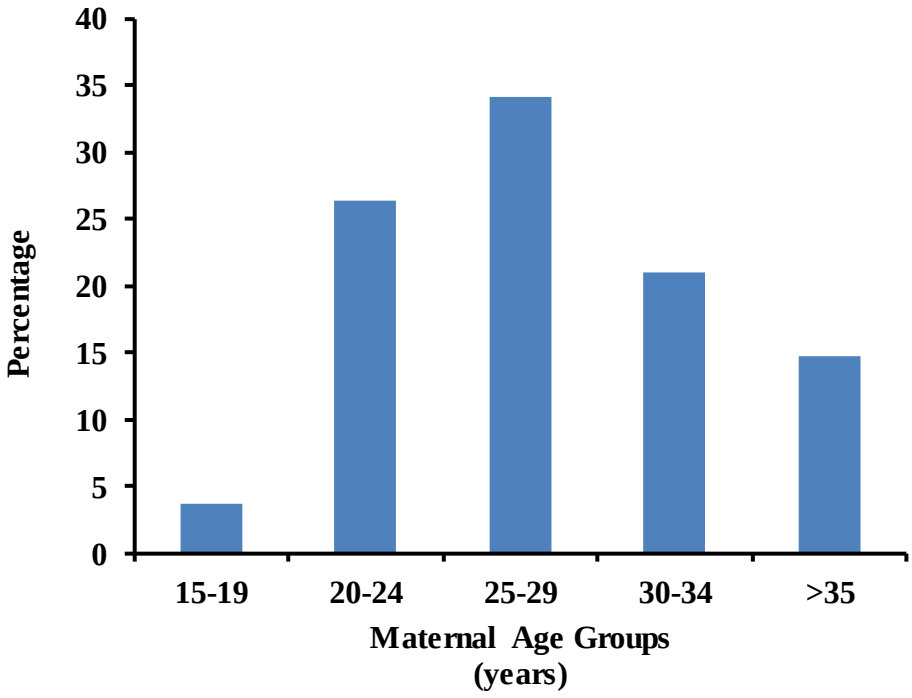


Figure 9: Percentage of case mothers in different age groups.

Table 11 : Distribution of number of case mothers with Cranial and Spinal NTD affected births in different age groups with Mean age(in years).

Maternal age			Neural tube defects	
Maternal age range (years)	Total no of cases(n=190)		Cranial NTDs	Spinal NTDs(n=145)
	n (%)	Mean Age (years)	n (%)	n (%)
15-19	7 (3.68)	17.57 ± 0.37	2 (1.05)	5 (2.63)
20-24	50 (26.32)	22.34 ± 0.19	9 (4.74)	41 (21.58)
25-29	65 (34.21)	26.51 ± 0.17	18 (9.47)	47 (24.74)
30-34	40 (21.05)	30.65 ± 0.18	8 (4.21)	32 (16.84)
>35	28 (14.74)	37.07 ± 0.43	8 (4.21)	20 (10.53)

Types of NTDs in offspring according to parity of case mothers

The number and percentage of NTDs (cranial and spinal) according to parity of case mothers is given in Table12. Majority of NTDs were present in first parity (n=51, 26.31%) and the number of case mothers becomes less with increase in parity. In cranial NTDs maximum number was observed in first parity (n=13; 6.84%) of which 8 were anencephaly and 5 encephalocele. Among the spinal NTDs in Parity 1, meningocele and myelomeningocele were 14 and 21 in number respectively and together they represent majority of defects (18.42%; n=35) of total sample. Among the less common NTDs one case of lipomeningocele was seen in each of parity 1, 3, 5 and >8. Similarly one case each of Dandy Walker Syndrome and Spina Bifida with Arnold –Chairi syndrome appeared in parity 1 and 2. A single case of syringomyelia was observed in parity 2. In parity 2 there were total 40 cases which represent 21.05% of total sample. In this parity an equal number of myelomeningocele and meningocele(n=15; 7.89%) was observed.

The cranial NTDs included 3.16% (n=6) anencephalic, 2 cases of encephalocele (1.05%) and one case each of Dandy Walker Syndrome and syringomyelia. In parity 3 the total number of cases were 14.74 % (n=28). In this parity majority of NTDs were meningocele (n=15;7.89%) followed by 7 anencephalics 3.68%, four cases (2.11%) of myelomeningocele and one case (0.53%) of lipomeningocele. The number of cases decreased with increase in parity. In parity 4, number of cases were 24(12.63percent).In this parity the number of meningocele were 12(6.32%), 5(2.63%) anencephalic and 6(3.16%) myelomeningocele. There was one case (0.53%) of encephalocele. In parity 5 total number of cases were 19(10%) with majority of cases being meningocele (n=10; 5.26percent). There were five anencephalic (2.11%) and 3(1.58%) myelomeningocele. There was one case of lipomeningocele in this parity. In parity 6 number of case mothers were 12 which were 6.34% of whole sample. There were 2(1.05%) anencephalic and 5(2.63%) cases each of meningocele and myelomeningocele. There were 8(4.21%) NTDs each in parity7 and 8. In parity 7, there were 5(2.11%) meningocele, one myelomeningocele (0.53%) and 2 anencephalic. Majority of cases were meningocele (n=9; 4.74%). There were 3(1.58%) anencephalic, 2(1.05%) myelomeningocele and one case (0.53%) of lipomeningocele. Meningocele and myelomeningocele predominate in this

Table 12 : Number and percentage of types of NTDs births according to parity of case mothers

Parity	Total number of NTDs(n=190)	Types of Neural Tube defects							
		Cranial NTDs		Spinal NTDs					
		n=45(23.68)		n=145(76.32%)					
	n (%)	Anencephaly	Encephalocele	Meningocele	Myelomeningocele	Lipomeningocele	Dandy Walker Syndrome	Spina Bifida+Arnold Chiari syndrome	Syringomyelia
1	51 (26.31)	8	5	14	21	1	1	1	–
2	40 (21.05)	6	2	15	15	–	1	–	1
3	28 (14.74)	7	1	15	4	1	–	–	–
4	24 (12.63)	6	1	11	6	–	–	–	–
5	19 (10)	4	–	10	4	1	–	–	–
6	12 (6.34)	2	–	5	5	–	–	–	–
7	8 (4.21)	2	–	5	1	–	–	–	–
>8	8 (4.21)	1	–	5	1	1	–	–	–

study population. Patients with these defects were observed in all the parities (1->8). The highest percentage of these patients (n=35 and 30; 18.42% and 15.79%) were seen in parity 1 and 2 respectively. {Percentages are based on total number of NTDs: n= 190}

Consanguinity

The Study sample data were classified according to genetic relationship of parents of NTD affected babies and that of control group. Marriages were grouped as double first cousins, first cousins, second cousin, baradari, distant relations and unrelated. In distant relationship and baradari (clan), the exact genetic relationship was difficult to ascertain. These were, therefore, grouped under non consanguineous marriages. In baradari (clan) group people marry in families having the same surname disregarding whether they are genetically related or not.

Consanguineous and non-consanguineous marriages among control and case mothers are shown in Table13; Figure 6. Frequency of consanguineous marriages among case mothers were significantly higher than in control mothers ($\Sigma\chi^2_{(1)}$ =5.95; P=<0.015). The marriages contracted among different genetic relationships in case and control mothers are given in Table 14. In case mothers majority of marriages contracted are between first cousins (46.84%) than in control mothers (25%). A detailed distribution of cranial and spinal NTDs among different genetic relationships is given in Table 15; Figure 7. Majority of NTDs were observed in case mothers who had contracted first cousin marriages. Prominent NTDs among first cousin marriages were anencephalic (n=16) meningoceles (n=37) and myelomeningoceles (n=28). In unrelated marriage types anencephalic (n=10), meningoceles (n=23) and myelomeningoceles (n=15) were the prominent NTDs. All NTDs appearing in consanguineous and non- consanguineous together showed that NTDs appeared in highly significantly greater number in consanguineous marriages than in non- consanguineous ($\Sigma\chi^2_{(1)}$ =7.60; P=<0.005).The calculated coefficient of inbreeding (F) in control mothers is (F= 0.0286).This is comparable to that in general population (Shami et al., 1989).In the case mothers the coefficient of inbreeding (F) is higher (F=0.342)than control mothers which indicates that in case mothers compared to control mothers more loci show homozygosity.

Table 13 : Consanguinity in control and Case mothers

Consanguinity		Control mothers (n=100)	Case mothers (n=190)
Consanguinous marraiges	n (%)	45 (45)	114 (60)
Non-consanguinous marraiges	n (%)	55 (55)	76 (40)

$\Sigma\chi^2_{(1)} = 5.95$; P= <0.015*

Table 14 : Consanguinity and marriage types in control and Case mothers

Consanguinity	Marriage Type	Control mothers		Case mothers	
		Number	Percentage	Number	Percentage
Consanguinous marraiges	First cousins	25	25	89	46.84
	Second cousins	18	18	20	10.53
	Double first cousin	2	2	5	2.63
Non-consanguinous marraiges	Distant relation	8	8	13	6.84
	Baradari	12	12	8	4.21
	Unrelated	35	35	55	28.95
Coefficient of in breeding (F)		0.0208		0.0342	

Table 15 : Distribution of different neural tube defects among different marriage types of case mothers.

		Marriage Types						
Neural Tube Defect		Consanguineous marriages			Non- consanguineous marriages			
Total no of cases=190	n (%)	First cousin	Second cousin	Double first cousin	Baradari	Distant	Unrelated	Total
Anencephaly	36 (18.75)	16	4	2	1	3	10	36
Encephalocele	9 (4.74)	4	1	–	–	–	4	9
Meningocele	80 (42.11)	37	8	1	6	5	23	80
Myelomeningocele	57 (30)	28	7	2	1	4	15	57
Lipomeningocele	4 (2.11)	1	–	–	–	1	2	4
Dandy Walker Syndrome	2 (1.05)	1	–	–	–	–	1	2
Spina bifida+ Arnold-Chiari syndrome	1 (0.53)	1	–	–	–	–	–	1
Syringomyelia	1 (0.53)	1	–	–	–	–	–	1
Total		89	20	5	8	13	55	190

Consanguineous marriages vs non- consanguineous marriages : $\Sigma\chi^2_{(1)} = 7.60$; P=0.005**

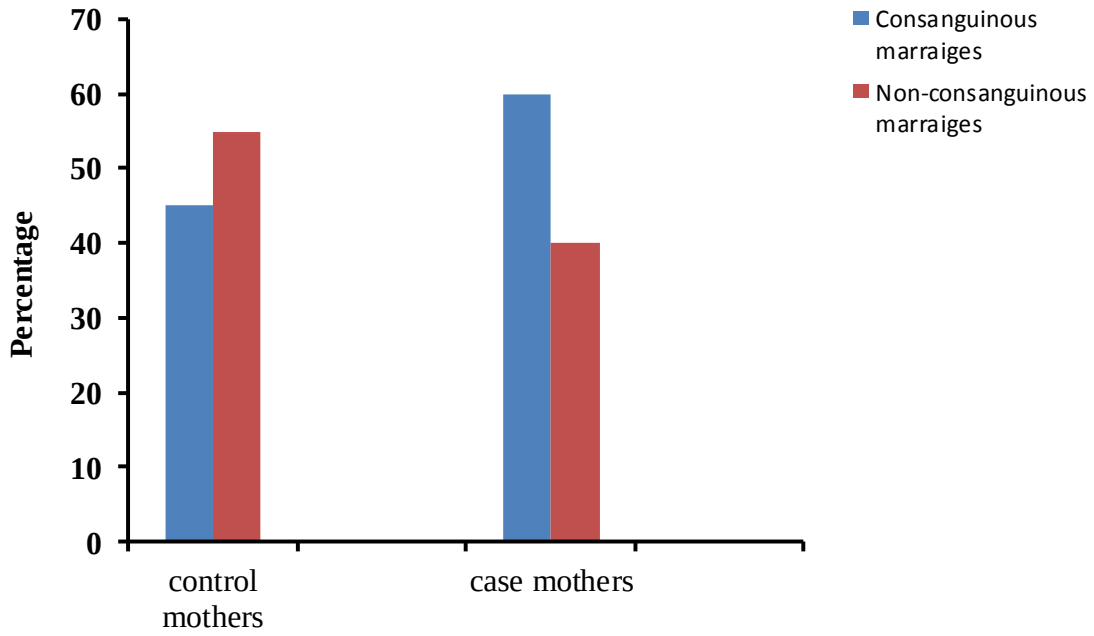


Figure 10: Percentage of consanguineous and non-consanguineous marriages in control and case mothers.

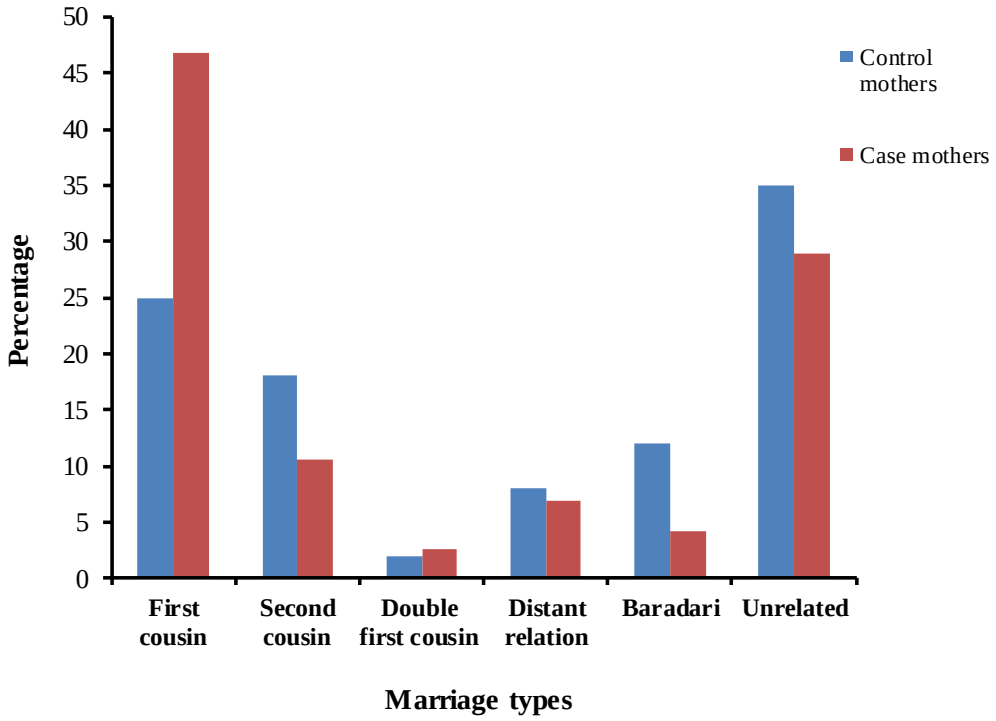


Figure 11: Percentage of study population in different marriage types

Exposure to passive smoke

In the present study all control mothers and case mothers did not smoke (Table16). Case mothers who reported exposure to passive smoke delivered NTD babies of which 19(10%) were with cranial and 59(31-05%) were with spinal NTDs whereas case mothers who were not exposed to passive smoke gave birth to affected babies which were 26 (13.68%) with cranial NTDs and 86(45.26%) were with spinal NTDs. The difference was not significant in the distribution of cranial NTDs and spinal NTDs in exposed and not exposed to passive smoke condition ($\Sigma\chi^2_{(1)}$ =0.033; P=0.86). However, the distribution of case and control mothers in the two conditions of exposure to smoke was highly significant ($\Sigma\chi^2_{(1)}$ =7.37; P=<0.0066).

Residential origin (rural/urban) of control and case mothers.

Distribution of control and case mothers according to their place of residence are shown in Table 17. Among the 190 case mothers 37.89 % (n=72) were from urban areas whereas 62.11% (n=118) were from rural areas. In the control group majority of mothers lived in urban areas (75%) and those from rural areas were 25 %. Case mothers are highly significantly more from rural areas compared to control mothers ($\Sigma\ \chi^2_{(1)}$ = 28.07; P= < 0.0001).The number of cranial NTDs from case mothers who lived in urban areas were 5.79 %(n=11) and those from rural areas were 17.89% (n=34) whereas spinal NTDs were 32.11 % (n=61) from mothers living in urban areas compared to 44.21% (n=84) from rural areas. The distribution of cranial and spinal NTD babies from rural and urban mothers showed significantly higher cranial and spinal NTDs from rural areas as compared to urban areas ($\Sigma\chi^2_{(1)}$ = 4.53; P = <0.033)

The distribution of different cranial and spinal NTDs in rural and urban areas is given in Table18.

Sub-types of NTDs: The study of cranial NTDs showed that the greater number of anencephalic (14.74%) and encephalocele (3.16%) were from the rural areas. In spinal NTDs the number of meningocele (25.26%) and myelomeningocele (17.37%) were seen more in case mothers belonging to rural areas. The four cases of lipomeningocele (2.11%) and two cases of Dandy walker syndrome (1.05%) were from urban areas

Table 16 : Number and percentage of control and case mothers exposed and not-exposed to passive smoke;Neural tube defects.

History of exposure to passive smoke		Mothers		Neural Tube Defects(n=190)	
		Control n=100	Case n=190	Cranial NTDs n=45	Spinal NTDs n=145
Mothers exposed to passive smoke	n (%)	25 (25)	78 (41.05)	19 (10)	59 (31.05)
Mothers not exposed to passive smoke	n (%)	75 (75)	112 (58.95)	26 (13.68)	86 (45.26)
		$\Sigma\chi^2_{(1)}$ =7.37; P=0.0066*		$\Sigma\chi^2_{(1)}$ =0.033; P=0.86 ^{ns}	

Table 17 : Number of control, case mothers and cranial and spinal NTDs with respect to living area.

Residential area		Mothers		Neural tube Defects(n=190)	
		Control n=100	Case n=190	Cranial NTDs n=45	Spinal NTDs n=145
Urban	n	72	72	11	61
	(%)	(72)	(37.89)	(5.79)	(32.11)
Rural	n	28	118	34	84
	(%)	(28)	(62.11)	(17.89)	(44.21)
		$\Sigma \chi^2_{(1)} = 28.07$; P= < 0.0001***		$\Sigma \chi^2_{(1)} = 4.53$; P= < 0.033*	

Table 18 : Number and percentage of types of cranial and spinal NTDs with respect to living area.

Residential area		Neural tube Defects(n=190)							
		Cranial NTDs		Spinal NTDs					
		Anencephalic	Encephalocele	Meningocele	Myelomeningocele	Lipomeningocele	DandyWalker syndrome	Spina bifida+Arnold Chairi syndrome	Syringomyelia
Urban	n	8	3	32	24	4	2		
	(%)	(4.21)	(1.58)	(16.84)	(12.63)	(2.11)	(1.05)		
Rural	n	28	6	48	33			1	1
	(%)	(14.74)	(3.16)	(25.26)	(17.37)			(0.53)	(0.53)

whereas one case each of spina bifida+Arnold Chairi syndrome and syringomyelia belonged to rural areas.

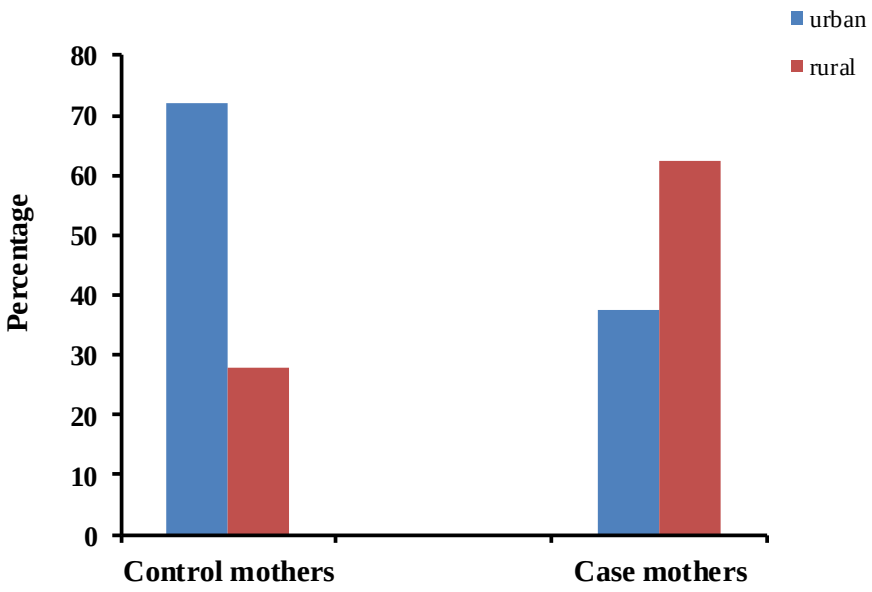


Figure 12: Percentage of control mothers and case mothers in rural and urban areas.

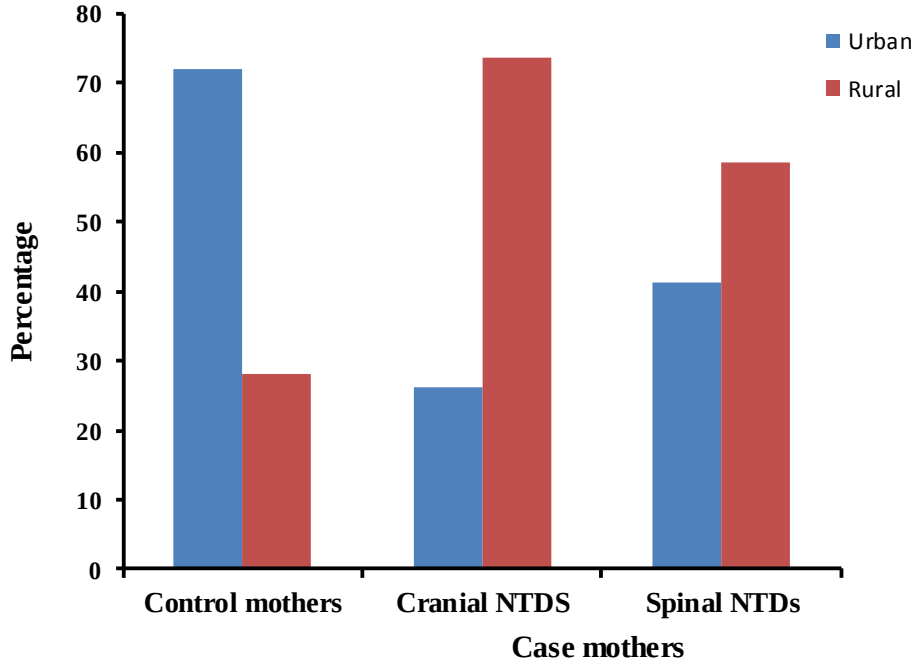


Figure 13: Percentage of case mothers with Cranial and Spinal NTDs in urban and rural areas.

Exposure to occupational and environmental hazards

In this study population, majority of case mothers had no schooling, and belonged to the low income group, with little access to clean and better housing facilities. There were 89% control and 57.37% case mothers who did not give history of exposure to either chemical hazards or pollution from garbage dumps. Case mothers living close to factories were 17.36% (n=33) and those residing in polluted areas (close to garbage dumps) were 25.26 % (n=48) . Those women who reported being exposed to chemical waste could not specify the nature of chemical waste. (Table 19). Area of residence close to chemical waste and garbage dumps may have an effect on the birth of a defective (NTD) baby. A comparison of control and case mothers from hazardous areas and non- hazardous areas was carried out which showed significantly greater number of case mothers exposed to environmental hazards (n=81;42.63%) ($\Sigma\chi^2_{(1)}$ =4.16, P=<0.0413). Cranial NTDs from mothers exposed to chemical waste were 9(4.74%) and those exposed to pollution from garbage dumps were 7(3.68%). Spinal NTDs exposed to chemical waste were 24(12.63%) and those exposed to pollution from garbage dumps were 41(21.58%). The difference between cranial and spinal NTDs exposed to chemicals and garbage dumps was not significant ($\Sigma\chi^2_{(1)}$ =1.99; P=0.1587). Table 20 shows distribution of cranial and spinal NTDs with both environmental hazards. Cranial NTDs from case mothers exposed to environmental hazards were16 (8.42%) whereas with spinal NTDs they were 65 (34.21%). Comparison between cranial NTDs exposed to environmental hazards compared with spinal NTDs from mothers exposed to environmental hazards was not significant($\Sigma\chi^2_{(1)}$ =1.21; P=0.271).

Maternal risk factors for neural tube defects

History of high temperatures in first trimester and Diabetes are risk factors for neural tube defects (Cabrera et al., 2004).Case mothers who reported history of high temperatures were 21(11.05%) whereas control mothers were 13%. The types of NTDs in case mothers reporting high temperatures were anencephalic 4(2.10),encephalocele1(0.53%) meningocele 10(5.26%)and myelomeningocele 6(3.16%). The high temperatures reported were non -specific. There were 13.68% (n=26) diabetics among case mothers and 7% (n=7) in control mothers (Table 21). The types of NTDs in these diabetic women were

Table 19 : Exposure to occupational and environmental hazards of control and case mothers and appearance of NTDs in these environments.

Environmental Hazard		Mothers		Neural Tube Defects(n=190)	
		Control (n=100)	Case (n=190)	Cranial NTDs n=45	Spinal NTDs n=145
No exposure to envirnmental hazards	n	89	109	29	80
	(%)	(89)	(57.37)	(15.26)	(42.11)
Reported Proximity to chemicals	n	1	33	9	24
(Hazardous waste from Factories)	(%)	(1)	(17.37)	(4.74)	(12.63)
Reported exposure to Pollution	n	10	48	7	41
(Garbage Dumps in vicinity to residential area)	(%)	(10)	(25.26)	(3.68)	(21.58)
		$\Sigma\chi^2_{(1)}$ =4.16; P=0.0413*		$\Sigma\chi^2_{(1)}$ =1.99; P=0.1587	

Table 20 : Effect of exposure to environmental hazards.

Environmental Hazard		Neural tube defects		
		Cranial NTDs n=45	Spinal NTDs n=145	Total n=190
Exposed to environmental hazards	n	16	65	81
	(%)	(8.42)	(34.21)	(42.63)
Not Exposed to environmental hazards	n	29	80	109
	(%)	(15.26)	(42.11)	(57.37)
		$\Sigma\chi^2_{(1)}$ =1.21; P=0.271		

Table 21 : Maternal risk factors for neural tube defects in case mothers compared to control mothers

Factor		Control mothers n=100		Case mothers n=190	
		n	%	n	%
History of high temperature					
	yes	5	5	21	11.05
	no	87	87	169	88.95
Diabetes					
	yes	7	7	26	13.68
	no	93	93	164	86.32

($\Sigma\chi^2_{(1)}$ =0.035, P=0.851)

meningocele (n=14; 7.37%) myelomeningocele 4.74 %(n=9), anencephalic 1.05 %(n=2) and encephalocele 0.53 %(n=1). The number of case mothers reporting history of high fever and diabetes were non-significantly higher than in control mothers ($\Sigma\chi^2_{(1)}$ =0.035, P=0.851).

Nutritional status

Case mothers were asked about nutritional intakes especially in periconceptional period. They were divided into two groups, one who were taking fruits and vegetables adequately in their meals per week and the other group included those control and case mothers whose diet was deficient in fruits and vegetables per week (Laurence et al., 1980) Table 22. The number of cranial NTDs from case mothers with who took adequate diet were 22(11.58%) while with spinal NTDs they were 53(27.89%). On the other hand to 23(12.11%) cranial NTDs were from case mothers taking inadequate diet and 48.42% (n=92) with spinal NTDs. The number of case mothers who took diet inadequate in fruits and vegetables is greater than control mothers and the difference was significant ($\Sigma\ \chi^2_{(1)}$ = 9.057; P= < 0.0026). However the difference between cranial NTDs and spinal NTDs according to the diet history of case mothers was not significant ($\Sigma\ \chi^2_{(1)}$ = 1.331; P= < 0.2487).

Use of folic acid, iron and multivitamins in periconceptional period and after confirmation of pregnancy by control and case mothers.

Case and control mothers were inquired about intake of folic acid, iron and multivitamins during periconceptional period defined as three months before and one month after last menstrual period (LMP). All case mothers did not take folic acid, iron and multivitamins during this period. However, 5% control mothers had taken folic acid, 40% multivitamins and 15% iron during this period. Their number and percentage is given in Table 23. Once pregnancy was confirmed by HCG levels and ultrasound on first antenatal visit after first missed menstrual period, case mothers did take folic acid (n=75;39.47%), multivitamins (n=78;41.05%) and iron (n=122;64.21%) and control mothers who took folic acid were (68%), multivitamins (57%) and who took iron were (69%).

Table 22 : Nutritional status of control and case mothers and NTDs according to reported diet history.

Reported Diet History		Mothers		Neural tube defects(n=190)	
	n (%)	Control (n=100)	Case (n=190)	Cranial NTDs (n=45)	Spinal NTDs (n=145)
Reported diet adequate in fruit, vegetable and meat.	n	58	75	22	53
	(%)	(58)	(39.47)	(11.58)	(27.89)
Reported diet inadequate in fruit, vegetable and meat.	n	42	115	23	92
	(%)	(42)	(60.53)	(12.11)	(48.42)
		$\Sigma \chi^2_{(1)} = 9.057$; P= < 0.0026**		$\Sigma \chi^2_{(1)} = 1.331$; P= < 0.2487	

Table 23: Reported use of folic acid multivitamins and iron before and during pregnancy by control and case mothers.

		Control mothers n=100		Case mothers n=190	
Intake during periconceptional period		n	%	n	%
Folic acid	Yes	5	5	none	
	no	95	95	190	100
Multivitamins	yes	40	40	none	
	no	60	60	190	100
Iron	yes	15	15	none	
	no	85	85	190	100
Intake after confirmation of pregnancy (2-6 months gestational period):					
Folic acid	Yes	68	68	75	39.47
	no	32	32	115	60.53
Multivitamins	yes	57	57	78	41.05
	no	43	43	112	58.95
Iron	yes	69	69	122	64.21
	no	31	31	68	35.78

Periconceptional period:3months before and one month after last menstrual period (LMP).

Adverse reproductive history in control and case mothers prior to index pregnancy

Adverse reproductive history regarding spontaneous abortion, still birth, neonatal death and NTD birth in previous pregnancy was noted. The total number of case mothers who reported adverse pregnancy outcome in previous pregnancy (n=117; 61.59%) was significantly higher than control mothers (n=14; 14%) ($\Sigma \chi^2_{(1)} = 59.88$; P= <0.0001). Table 24. The adverse pregnancy outcomes reported by case and control mothers are shown in Table 25. Among the100 control mothers a very small percentage gave history of previous spontaneous abortion (7%), stillbirth (4%) and neonatal death (3%) and none gave history of NTD in prior pregnancies. On the other hand, in case mothers previous history of spontaneous abortions was present in 36.32% (n=69).The number of reported stillbirths was 16(8.42%) and neonatal deaths 21(11.05%). There were 11(4.21%) case mothers who gave history of NTD in previous pregnancy. History of spontaneous abortion and miscarriage in the previous pregnancies is implicated as a risk factor for NTD (Todoroff and Shaw, 1999).

Educational status of case mothers

Control and case mothers were categorized into four groups according to their educational level (no schooling, schooling, college education and university education). The distribution of control and case mothers in different levels of education is shown in Table 26. Majority of case mothers (n=86, 45.26%) and control mothers (46%) had no school education. There were 75 (39.47%) case mothers and 27% control mothers who attained school education. Only 25(13.16%) case mothers had acquired college education and 4(2.11%) attained a university level degree whereas in control mothers 16 % had college education and 11% a university level degree. In mothers with no school education births with cranial NTDs were 18(9.47%) mothers, with school education 21(11.05%) and with a college degree cranial NTDs were 6 (3.16%). Spinal NTDs were maximum 35.79% (n=68) in the offspring of mothers who had never been to school. The number decreased with school, college and university education (n=54(15.26%), 19(10%), 4(2.11%) respectively). The number and percentage of control and case mothers with no schooling and school education were similar in control and case mothers but chi square

Table 24: Adverse pregnancy history of control and case mothers prior to index pregnancy

Previous Pregnancy	n (%)	Mothers: Control (n=100)	Mothers: Case n=190
No history of adverse pregnancy history	n (%)	86 (86)	73 (38.42)
Adverse pregnancy history	n (%)	14 (14)	117 (61.59)

$\Sigma \chi^2_{(1)} = 59.88$; P= <0.0001***

Table 25: Adverse pregnancy outcomes of control and case mothers prior to index pregnancy

Previous pregnancy		Control mothers	Case mothers
Spontaneous abortion	n (%)	7 (7)	69 (36.32)
Still birth	n (%)	4 (4)	16 (8.42)
Neonatal death	n (%)	3 (3)	21 (11.05)
Born with NTD	n (%)	—	11 (4.21)

Table 26: Educational status of Control and Case mothers (Cranial NTDs and Spinal NTDs)

Educational Status		Mothers		Neural tube defects	
	n %	Control n=100	Case n=190	Cranial NTDs n=45	Spinal NTDs n=145
No schooling	n %	46 (46)	86 (45.26)	18 (9.47)	68 (35.79)
School education	n %	27 (27)	75 (39.47)	21 (11.05)	54 (15.26)
College education	n %	16 (16)	25 (13.16)	6 (3.16)	19 (10)
University education	n %	11 (11)	4 (2.11)	—	4 (2.11)

Control vs case mothers

College and university education ($\Sigma \chi^2_{(1)} = 5.177$; P= < 0.0229*)

analysis regarding college and university showed a significant difference ($\Sigma \chi^2_{(1)} = 5.177$; $P = < 0.0229$).

Socioeconomic Status

Socio economic status (SES) was rated on parental monthly income into Rs 5000-10,000; Rs 11000-15,000; 16,000-20,000 and >Rs 20,000 cohorts. The distribution of control and case mothers in these cohorts is given in Table 27. Majority of case mothers (n=85; 44.74%) had a very low monthly income in the cohort Rupees 5000- 10,000. There were 24.21 % (n=46) case mothers in monthly income cohort between Rs11,000 to 15,000 whereas in the income group Rs 16,000-20,000 case mothers were 33 in number (17.37%).In the income cohort >Rs 20,000 there were only 13.68 % (n=26) case mothers and 43% control mothers. There was a significant difference in economic status between control and case mothers ($\Sigma\chi^2_{(3)} = 35.35$; $P = <0.0001$). Case mothers in low income group had the highest percentage of cranial NTDs (n=21; 11.05%) and spinal NTDs (n=64; 33.68%).With the increase in monthly income there was a systematic reduction in the number of cranial as well as spinal NTDs (Table 27). It was also observed that educational status within the lowest to highest monthly income group had an effect on appearance of cranial and spinal NTDs. The present data indicates that respective educational status in the high income group gave awareness about health and neural tube defects (Table 28).

Occupational Status of control and case mothers

Distribution of control and case mothers according to occupational status of mother is given in Table 29. In this study majority of control (58%; n=58) and case mothers (75.79 %; n=144) were house wives. In the housewives group there were 24% control mothers who were educated and among the case mothers there were 36.32% (n=69) who had either been to school or college. Case mothers who did skilled labor were mostly tailors (11.58%) and a similar percentage was observed in control mothers (12%). Unskilled labor was described as house maids and they were 12% in control mothers and 7.89 % in case mothers. Professional women were teachers in school and college. These were 4.74% in case mothers and 18% in control mothers.

Table 27 : Socio-economic status(monthly income) of husbands' of mothers with cranial and spinal neural tube defect

Monthly income (Ruppees)		Mothers		Neural tube defects	
	n (%)	Control (n=100)	Case n=190	Cranial NTDs (n=45)	Spinal NTDs (n=145)
5000-10000	n	39	85	21	64
	(%)	(39)	(44.74)	(11.05)	(33.68)
11000-15000	n	12	46	9	37
	(%)	(12)	(24.21)	(4.74)	(19.47)
16000-20000	n	6	33	8	25
	(%)	(6)	(17.37)	(4.21)	(19.41)
>20,000	n	(43)	(26)	(7)	(19)
	(%)	43	13.68	3.68	10

Control vs case mothers: $\Sigma\chi^2_{(3)} = 35.35$; P=<0.0001***

Table 28 : Relation between economic status & educations status of case mothers and control group

Socio-economic status	Educational status		Control mothers (n=100)		Case mothers (n=190)			
					Cranial NTDs		Spinal NTDs	
			n=100		n=45		n=145	
			n	%	n	%	n	%
5000-10000	No schooling		29	29	12	11.01	33	30.28
	School education	middle/Matric	9	9	8	7.34	30	27.52
	College education	Graduate	1	1	1	0.92	1	0.92
	University education	Post graduate	—	—	—	—	—	—
11000-15000	No schooling		6	6	4	3.67	19	17.43
	School education	middle/Matric	2	2	5	4.59	15	13.76
	College education	Graduate	3	3	—	—	3	2.75
	University education	Post graduate	1	1	—	—	—	—
16000-20000	No schooling		3	3	—	—	8	7.34
	School education	middle/Matric	2	2	4	3.67	7	6.42
	College education	Graduate	1	1	4	3.67	7	6.42
	University education	Post graduate	—	—	—	—	3	2.75
>20000	No schooling		6	6	2	1.83	8	7.34
	School education	middle/Matric	14	14	4	3.67	2	1.83
	College education	Graduate	11	11	1	0.92	8	7.34
	University education	Post graduate	12	12	—	—	1	0.92

Table 29: Occupational status of Control and Case mothers

Occupational status	Control mothers (n=100)		Case mothers (n=190)	
	n	%	n	%
Professional	18	18	9	4.74
Skilled labor	12	12	22	11.58
Unskilled labor	12	12	15	7.89
Housewife educated	24	24	69	36.32
Housewife uneducated	34	34	75	39.47

Table 30 : Occupational status of fathers of normal neonates and those born with NTD

Occupational status	Control fathers (n=100)		Case fathers (n=190)	
	n	%	n	%
Professional	16	16	25	13.15
Business	18	18	19	10
Clerk	5	5	14	7.37
Skilled labor	31	31	64	33.68
Unskilled labor	30	30	68	35.79

Occupational Status of fathers of normal neonates and those born with NTDs

In the present study majority of fathers of NTD affected neonates were unskilled (35.79%) and skilled laborers (33.68%) Table 30. Similar results were observed in control fathers with 31% skilled laborers and 30% unskilled laborers. In business group there were 18% control fathers and 10% case fathers. Professional fathers are 16% in control group and 13.15% in case fathers.

Folate awareness

Women were inquired about use of folic acid supplements. Those who had not taken folic acid were probed as to if they were aware of the importance of folic acid and if so, the reason for not taking the supplement. None of the case mothers had heard of folic acid and were unaware of its importance in prevention of NTDs. In control mothers the percentage of women who had heard of folic acid was very low (25%) and among these only 5% took folic acid in periconceptional period. Knowledge regarding folic acid, its effect on developing embryo, and its use in prevention of NTDs is alarmingly low and could be due to low level of education and economic status of this study population.

Outcome of pregnancy in case mothers after index pregnancy

All the case mothers were counseled at the time of interview on regular intake of folic acid regularly if they planned to have a baby. They were also apprised of the importance of folic acid regarding prevention of recurrence of a subsequent NTD baby. In the follow up, all the 190 case mothers were contacted to get information about further pregnancies and intake of folic acid (Table 31).Of these, 86 case mothers did not respond. Out of 104 case mothers 44(23.15%) did not take folic acid and did not produce further offspring. Sixty case mothers regularly took folic acid and out of these 38 produced normal babies, 4 gave history of spontaneous abortions and 18(9.47%) did not go for further pregnancy.

Table 31: Incidence of the outcome of pregnancy in case mothers after the Index pregnancy

	Case mothers (n=190)	
	n	%
Case mothers who did not respond	86	45.26
Case mothers who did respond	104	54.74
Case mothers who did respond	**104**	**54.74**
i) Case mothers who did not take folic acid	44	23.15
ii) Case mothers who took folic acid regularly	60	31.58
Case mothers who took folic acid regularly	**60**	**26.32**
a)Normal live birth	38	20
b)Spontaneous abortion	4	2.11
c)Did not concieve	18	9.47

Folate Status of study population

Of the 190 case mothers only 109 agreed to follow up for folate studies and genotyping. All the control mothers agreed for this study.

Serum and RBC Folate levels were determined in control and case mothers with neural tube defect births. Although fetus accesses folate through maternal plasma folate, RBC folate is a better indicator of folate stores of body and sampling was done after delivery.

RBC Folate levels:

The Mean RBC Folate levels in control mothers (n=100) was 337.2 ± 18.42 ng/ml and 104.1 ± 9.17ng/ml in case mothers (n=109). In case mothers RBC Folate levels were highly significantly low ($t_{(207)}$ =11.57; P=<0.0001) compared to that of control mothers. RBC Folate levels were arranged in different groups starting from the lowest to the highest levels both in control and case mothers (Table 32, Figure 10). RBC Folate levels in case mothers were highly significantly low in the lowest group of levels (0-150ng/ml) compared to control mothers ($t_{(91)}$ =5.40; P=<0.0001). In the next two groups with higher levels of RBC Folate there is no significant difference between case and control mothers. Maximum number of case mothers are in the lowest level group (n=77; 70.64%). The number of case mothers is less with higher levels of RBC Folate with 28(25.69%) in 150-300ng/ml group and only 4(3.67%) in the 301-450ng/ml group. None of the case mothers had RBC folate levels above 440ng/ml. In the control mothers there were 16% who had levels in the 0-150ng/ml group depicting folate deficiency and 84% had levels above 150ng/ml.

Serum Folate levels:

Overall mean serum folate levels were 10.27 ± 0.56 ng/ml in control mothers (n=100) and 6.75±0.42 ng/ml in case mothers (n=109). Serum folate levels were highly significantly low in case mothers compared to that of control mothers ($t_{(207)}$=5.03;P=<0.0001).As with RBC Folate levels, serum folate levels were also divided into groups from the lowest to the highest levels (Table 33, Figure 11). There was not

Table 32 : Mean RBC Folate levels(ng/ml) of control and case mothers

RBC Folate (ng/ml)	n	%	Control mothers(n=100) Mean RBC Folate (ng/ml)	n	%	Case mothers(n=109) Mean RBC Folate (ng/ml)
0-150	16	16	105.4 ±6.31	\	70.64	52.13 ± 4.29[a***]
151-300	34	34	238.3 ± 8.14	28	25.69	205.2 ± 7.38
301-450	23	23	352.5 ± 8.43	4	3.67	395.5 ± 23.51
451-600+	27	27	586.2 ± 23.95	—	—	—

Control vs case mothers a P=<0.0001***

Table 33 :Mean Serum Folate levels(ng/ml) of control and case mothers

Serum Folate ng/ml	Control mothers(n=100)			Case mothers(n=109)		
	n	%	Serum Folate (ng/ml)	n	%	Serum Folate (ng/ml)
0-2.9	8	8	1.98 ± 0.24	27	24.77	2.18 ± 0.10
3-4.9	4	4	4.30 ± 0.25	18	16.51	4.04 ± 0.14
5-6.9	22	22	5.92 ± 0.14	20	18.35	5.88 ± 0.14
7-8.9	19	19	7.79 ± 0.14	18	16.51	7.94 ± 0.12
>9	47	47	15.23 ± 0.60	26	23.85	13.93 ± 0.65

much appreciable difference in serum folate levels in control and case mothers in each group. Case mothers with levels below 3ng/ml were 27(24.77%) showing severe deficiency. In 82(75.23%) case mothers levels were above 3ng/ml. In the control mothers 8% were in the severe deficiency group<3ng/ml and 92% had levels above 3ng/ml.

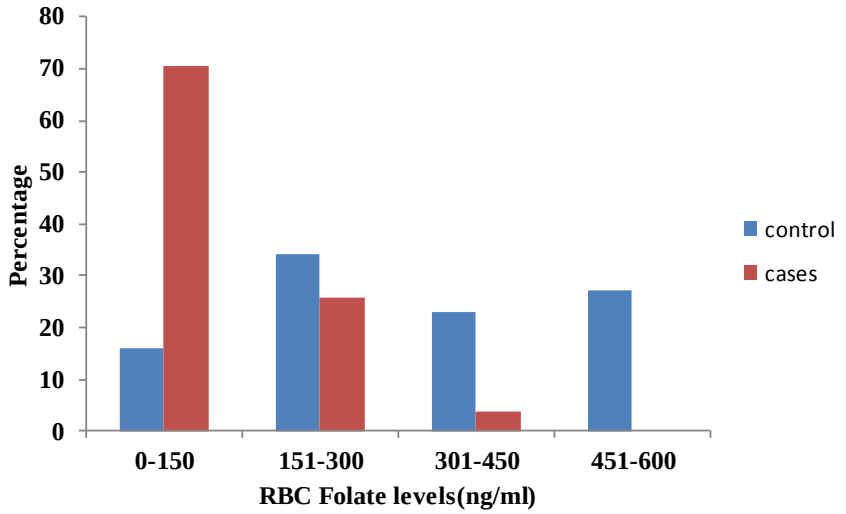


Figure 14: Percentage of control and case mothers in different ranges of RBC folate levels.

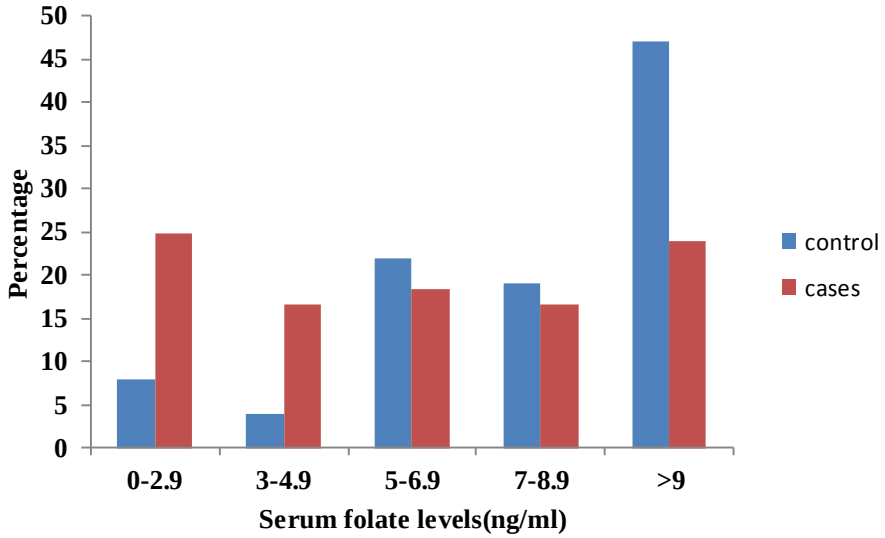


Figure 15: Percentage of control and case mothers in different ranges of Serum folate levels.

RBC and Serum Folate levels (ng/ml) in control and case mothers in relation to Age Groups.

Mean age of control mothers (n=100) and case mothers (n=109) was 27.17 ± 0.50 years and 27.73 ± 0.53 respectively. Ages of both set of mothers were divided into groups with an age interval of 5 years.

RBC Folate: Mean RBC folate levels in control mothers were 337.2 ± 18.42 ng/ml and in case mothers were 104.1 ± 9.17ng/ml. Both control and case mothers were arranged in different age groups with an interval of five years (Table 34).Compared to control mothers in all age groups, the case mothers show highly significantly low RBC folate levels(P=<0.001). In case mothers the lowest age group showed the lowest RBC folate levels (71.98±26.62ng/ml). On the other hand, in control mothers from the lowest age group, RBC folate were highest (395.5 ± 97.77ng/ml) compared to that in all other age groups. In case mothers as well as control mothers there is no systematic increase or decrease in mean RBC folate levels rather they fluctuate in different age groups. In all the age cohorts compared to control mothers the case mothers had significantly low RBC folate levels, age cohort 15-19 years ($t_{(8)}$=3.19;P=<0.013);age cohort 20-24 years ($t_{(52)}$=5.46;P=<0.0001);age cohort 25-29 years ($t_{(63)}$=6.14;P=<0.001);age cohort 30-34years ($t_{(52)}$=5.76;P=<0.0001);age cohort >35years ($t_{(24)}$=5.08;P=<0.0001).

Serum Folate: Mean serum folate levels in control mothers were 10.27 ± 0.56 ng/ml and in case mothers it was 6.75 ± 0.42ng/ml. As with RBC Folate levels, maternal age were divided into different age groups. In case mothers the lowest serum folate levels were observed in the lowest age groups (15-19years) (Table 35) but in control mothers the lowest age group had comparatively higher serum folate levels compared to other age groups. Like RBC folate levels, serum folate levels also show fluctuation in different age groups. In the age cohort 15-19 years, serum folate levels in case mothers were lower than in control mothers, the difference being non- significant ($t_{(8)}$=2.11;P=<0.068). In the age group 20-24 years it was observed that the difference in serum folate levels between control and case mothers was significant ($t_{(52)}$=2.47;P=<0.017). In the age cohort 25-29 years the difference in serum folate levels between control and case mothers was non-significant ($t_{(63)}$=1.67;P=<0.102) whereas this difference was highly significant in age

Table 34 : Age groups and mean RBC folate level(ng/ml) of control and case mothers

Age groups	Control mothers (n=100)			Case mothers (n=109)		
	No of cases		Mean RBC Folate level ng/ml	No of cases		Mean RBC Folate level ng/ml
	n	%		n	%	
15-19	5	5	395.5 ± 97.77	5	4.59	71.98 ± 26.62[a*]
20-24	24	24	364.5 ± 47.59	30	27.52	115.10 ± 15.12[b***]
25-29	36	36	293.0 ± 25.48	29	26.61	90.78 ± 16.22[c**]
30-34	27	27	358.5 ± 34.71	27	24.77	115.1 ± 24.03[d***]
>35	8	8	345.8 ± 55.58	18	16.51	99.31 ± 21.38[d***]

Control mothers vs Case mothers

	Age groups	
a	15-19	P=<0.013*
b	20-24	P=<0.0001***
c	25-29	P=<0.001**
d	30-34	P=<0.0001***
e	>35	P=<0.0001***

Table : 35 Age groups and mean serum folate levels(ng/ml) of control and case mothers

Age Groups	Control mothers(n=100)			Case mothers(n=109)		
	No of cases		Mean Serum folate level ng/ml	No of cases		Mean Serum folate level ng/ml
	n	%		n	%	
15-19	5	5	12.32 ± 3.25	5	4.59	5.20 ± 0.91[a]
20-24	24	24	10.68 ± 1.19	30	27.52	6.99 ± 0.93[b*]
25-29	36	36	9.01 ± 0.88	29	26.61	7.04 ± 0.74[c]
30-34	27	27	10.77 ± 1.06	27	24.77	6.76 ± 0.85[d**]
>35	8	8	11.78 ± 2.24	18	16.51	6.31 ± 1.21

Control mothers vs Case mothers

	Age groups	
a	15-19	P=<0.068
b	20-24	P=<0.017*
c	25-29	P=<0.102
d	30-34	P=0.005**
e	>35	P=0.023*

cohort 30-34 years ($t_{(52)}$=2.45;P=<0.005) and also significant in age cohort >35years ($t_{(24)}$=2.45;P=<0.023).

Neural tube defects and RBC Folate levels (ng/ml) in case mothers.

In case mothers RBC folate levels were arranged in different groups. Against each RBC folate level group respective mean RBC folate levels as well as number of neural tube defects have been given in (Table 36).The neural tube defects are presented as Cranial NTDs and Spinal NTDs with their sub-types. Among cranial NTDs, anencephalic were in the highest number (n=8) where mean RBC levels were the lowest. It was observed that as the RBC folate levels increased the number of anencephalic decreased. A similar picture was observed with encephalocele. In the case of Spinal NTDs, meningocele (n=39) and myelomeningocele (n=25) their highest number was observed where the case mothers had the lowest mean RBC folate levels(ng/ml) and number decreased as the levels of RBC folate increased. Other spinal NTDs given in the table are of rare appearance and these cannot be analyzed in detail. The lower the RBC folate levels, the greater number of NTDs were observed and vice versa.

Neural tube defects in relation to Serum folate levels (ng/ml) in case mothers.

Serum folate levels (ng/ml) of case mothers in relation to different NTDs are given in Table 37. Serum folate levels were arranged in different groups from their lowest to highest range. Among cranial NTDs, anencephalic (n=2) appeared when maternal serum folate levels ranged between 0-2.9 ng/ml and 3 in 5-6.9ng/ml range. Increase number of anencephalic (n=5) appeared when maternal serum folate levels increased to 7-8.9 ng/ml and >9ng/ml. Two encephaloceles were seen in serum folate range 7-8.9 ng/ml and one in the range>9ng/ml. In the case of spinal NTDs, myelomeningoceles (n=10) and meningoceles (n=15) appeared in the highest number when maternal serum folate levels ranged between 0-2.9ng/ml. The number of myelomeningoceles fluctuated with the increase in serum folate level ranges. The increase and decrease in the number of meningoceles did not show relationship with the increase in maternal serum folate level range. Meningoceles also showed fluctuation in number with the increase in maternal

Table 36: Neural tube defects in relation to Mean RBC Folate levels(ng/ml) of Case mothers.
Total number of cases=109

RBC Folate ng/ml	Neural Tube Defects(n=109)						
	Cranial NTDs (n=18)		Spinal NTDs (n=91)				
	Anencephaly	Encephalocele	Meningocele	Myelomeningocele	Spina Bifida+Arnold Chiari syndrome	Lipomeningocele	Syringomyelia
0-149.9	45.34 ± 13.74 (8)	30 (2)	56.67 ± 6.13 (39)	42.13 ± 6.7 (25)	–	91 (2)	145.5 (1)
150-300	198.0 ± 19.22 (6)	190.89 (1)	203.9 ± 10.97 (12)	212.1 ± 18.76 (6)	201.6 (1)	222.8 (2)	–
301-450	350.78 (1)	–	359.2 (1)	436 (2)	–	–	–

Number of NTDs ( )
No cases present in this bracket. –

Table 37 : Neural tube defects in relation to Mean Serum Folate levels(ng/ml) of Case mothers .
Total number of cases=109

Serum Folate ng/ml	Neural Tube Defects(n=109)						
	Cranial NTDs(n=18)		Spinal NTDs(n=91)				
	Anenc ephaly	Encep halocele	Myelo meningocele	Mening ocele	Spina Bifida+Arnold Chiari syndrome	Lipo meningocele	Syring omyelia
0-2.9	2.08 (2)	–	2.31 ± 0.17 (10)	2.12 ± 0.14 (15)	–	–	–
3-4.9	–	–	3.98 ± 0.15 (9)	4.16 ± 0.26 (8)	–	3.6 (1)	–
5-6.9	5.33 ± 0.24 (3)	–	6.13 ± 0.12 (5)	5.97 ± 0.21 (11)	5.22 (1)	–	–
7-8.9	7.86 ± 0.26 (5)	7.7 (2)	7.6 (2)	8.1 ± 0.18 (7)	–	8.9 (1)	7.4 (1)
>9	13.72 ± 1.44 (5)	9.4 (1)	12.97 ± 1.34 (7)	13.62 ± 1.10 (11)	–	12.7 (2)	–

Number of NTDs ( )
No cases present in this bracket –

serum folate levels. Other rare spinal NTDs are given in the Table along with serum folate levels.

RBC and serum folate (ng/ml) levels of control and case mothers in relation to exposure to passive smoke.

Mean serum and RBC folate levels in control and case mothers exposed to passive smoke and not exposed to passive smoke are given in Table 38. The mean serum folate levels of control mothers exposed to passive smoke were highly significantly low compared to those not exposed to passive smoke ($t_{(98)}$=2.87;P=<0.005). The mean RBC folate levels in those exposed to passive smoke were not significantly different from those not exposed to passive smoke ($t_{(98)}$=1.18;P=0.24). In case mothers, like control mothers, mean RBC folate levels show no statistical difference in case mothers exposed and not exposed to passive smoke ($t_{(107)}$ =1.33; P=0.185). On the other hand, mean serum folate levels in case mothers exposed to passive smoke are highly significantly low than in those not exposed to passive smoke ($t_{(107)}$ =3.38; P=<0.0001). Case mothers exposed to passive smoke had significantly low mean RBC folate levels ($t_{(90)}$=7.08;P=<0.0001) compared to control mothers, while mean serum folate levels ($t_{(90)}$=1.83;P=0.07) were not significantly different in case and control mothers. The case mothers who were not exposed to smoke showed significantly low folate levels compared to control mothers both in RBC ($t_{(115)}$ = 7.47; P=<0.0001) and serum ($t_{(115)}$ = 2.71; P=<0.0079).

RBC and serum folate (ng/ml) levels of control and case mothers in relation to urban and rural residential areas.

RBC and serum folate levels in relation to urban and rural residential areas of control and case mothers are given in Table 39.In control mothers serum folate levels were not significantly different in urban and rural residents ($t_{(98)}$=1.60;P=<0.112) but RBC folate levels were significantly low in rural residents than in urban ones($t_{(92)}$=6.91;P=<0.0001) . Similarly, in case mothers serum folate levels in urban and rural residents were not significantly different ($t_{(107)}$ = 0.961; P=<0.339), but RBC folate were significantly low in rural residents than in those living in urban areas ($t_{(107)}$ =2.501; P=<0.0139).

Table 38: RBC and Serum Folate levels of control and case mothers in relation to exposure to passive smoke.

History of exposure to passive smoke	Control mothers(n=100)			Case mothers (n=109)		
	n (%)	Serum folate ng/ml	RBC folate ng/ml	n %	Serum folate ng/ml	RBC folate ng/ml
Exposure to passive smoke	25 (25)	7.56 ± 1.05^{a}	299.3 ± 36.77^{c}	67 (61.47)	5.67 ± 0.50^{b}	94.41 ± 11.28^{d}
No exposure to passive smoke	75 (75)	11.17 ± 0.64^{e}	349.9 ± 21.37^{g}	42 (38.53)	8.49 ± 0.68^{f}	119.4 ± 15.44^{h}

Exposure to passive smoke vs No exposure to passive smoke

Control mothers	**a vs e**	Serum Folate	P=<0.005**
	c vs g	RBC Folate	P=0.24
Case mothers	**b vs f**	Serum Folate	P=<0.001**
	d vs h	RBC Folate	P=0.185

Control mothers vs case mothers

Exposure to passive smoke	**a vs b**	Serum Folate	P=0.07
	c vs d	RBC Folate	;P=<0.0001***
No exposure to passive smoke	**e vs f**	Serum Folate	P=<0.0079**
	g vs h	RBC Folate	P=<0.0001***

Table 39: RBC and Serum Folate levels(ng/ml) of control and case mothers according to rural/urban areas.

Residential origin	Control mothers(n=100)			Case mothers(n=109)		
	n (%)	Serum folate ng/ml	RBC folate ng/ml	n (%)	Serum folate ng/ml	RBC folate ng/ml
Rural	32 (32)	9.08 ± 1.09[a]	300.4 ± 35.53[b]	62 (59.6)	7.26 ± 0.58[c]	94.92 ± 11.42[d]
Urban	68 (68)	10.74 ± 0.64[e]	359.0 ± 21.21[f]	47 (43.1)	7.05 ± 0.64[g]	131.9 ± 19.10[h]

Control mothers			
Rural vs urban	Serum folate	P=<0.112***	**a**
	RBC folate	P=<0.0514	**b**
Case mothers			
Rural vs urban	Serum folate	P=<0.339	**c**
	RBC folate	P=<0.0139*	**d**
Rural			
Control vs case mothers:	Serum folate	P=0.02*	**e**
	RBC folate	P=<0.0001***	**f**
Urban			
Control vs case mothers:	Serum folate	P=0.0002***	**g**
	RBC folate	P=<0.0001***	**h**

Table 40: RBC and Serum Folate levels(ng/ml) in case mothers with cranial and spinal NTD offspring according to rural/urban areas.

Residential origin	Case mothers(n=109)					
	n (%)	Rural case mothers(n=62) Serum folate ng/ml	RBC folate ng/ml	n (%)	Urban case mothers(n=47) Serum folate ng/ml	RBC folate ng/ml
Cranial NTDs						
Anencephaly	11 (10.09)	7.21 ± 1.26	88.56 ± 25.98	4 (3.67)	12.18 ± 2.03	231.90 ± 41.68
Encephalocele	1 (0.92)	9.4 ± 0.64	42.4	2 (1.83)	7.6	104.25
Spinal NTDs						
Meningocele	32 (29.36)	6.42 ± 0.88	83.57 ± 11.57	20 (18.35)	6.53 ± 0.86	117.1 ± 22.61
Myelomeningocele	16 (14.68)	5.61 ± 0.91	75.33 ± 26.48	17 (15.59)	6.22 ± 1.21	117.3 ± 29.26
Lipomeningocele		—	—	4 (3.67)	9.48 ± 2.20	156.9 ± 46.00
Spina bifida+Arnold Chairi syndrome	1 (0.92)	5.22	201.6		—	—
Syringomyelia	1 (0.92)	7.4	145.5		—	—

Control and case mothers' RBC and serum folate levels were also compared in relation to their urban and rural residences. In rural case mothers both serum folate levels ($t_{(92)}$=2.13;P=<0.02) as well as RBC folate levels($t_{(92)}$=6.91;P=<0.0001) were highly significantly low compared to that of control mothers. Similarly in urban case mothers serum folate levels ($t_{(113)}$=3.89;P=<0.0002) and RBC folate levels ($t_{(113)}$=8.12;P=<0.0001) were highly significantly low compared to that in urban control mothers. The above observation indicate that case mothers living in rural areas show very low serum folate and RBC folate levels compared to those living in urban areas. The effect of low folate levels has been seen in the appearance of higher number of cranial and spinal NTDs compared to those living in urban areas (Table 40).

Folate status of case and control mothers in relation to reported intake of folic acid after pregnancy

RBC and serum folate levels of case mothers (n=109) and that of control mothers (n=100) who reported having taken folic acid after confirmation of pregnancy and those who never took folic acid are given in Table 41. In the control mothers who took folic acid after confirmation of pregnancy, the increased levels of serum and RBC folate were significant compared to control mothers who did not take folic acid. For serum folate ($t_{(98)}$ =2.82; P=0.006) and for RBC folate ($t_{(98)}$ =2.97; P=0.004). Serum folate levels and RBC folate levels were higher in case mothers who reported use of folic acid after confirmation of pregnancy than those who did not but the difference was not significant ($t_{(107)}$=0.968;P=<0.335;($t_{(107)}$= 1.38;P=<0.16) respectively. The intake of folic acid after confirmation of pregnancy does not have an effect on the occurrence of NTDs in case mothers yet it is required in normal growth of fetus and placenta.

Reported Diet history and Folate status (ng/ml) of case and control mothers

In this study population 66 (60.55%) case mothers and 42% control mothers reported intake of diet inadequate in fresh fruits and vegetables (Table 42). A better diet history was reported by 58% control mothers and in this group the serum folate and RBC folate levels were higher than in those who reported diet insufficient in fruits and vegetables and the difference was significant for serum folate levels ($t_{(98)}$=3.22;P=0.<006)

Table 41 : Reported intake of Folic acid and Serum and RBC folate levels(ng/ml) of case and control mothers.

Reported history of folic acid intake	Control mothers(n=100)				Case mothers(n=109)			
			Serum folate	RBC folate			Serum folate	RBC folate
	n	%	ng/ml	ng/ml	n	%	ng/ml	ng/ml
Reported intake of folic acid after confirmation of pregnancy	52	52	11.75 ± 0.88[a]	388.1 ± 26.58[b]	45	41.28	7.24 ± 0.71[c]	119.2 ± 13.49[d]
No history of folic acid use after confirmation of pregnancy	48	48	8.67 ± 0.62	282.1 ± 23.44	64	58.72	6.41 ± 0.52	93.43 ± 12.32

Control mothers

Folic acid intake vs No folic acid intake

a Serum Folate P=<0.006**

b RBC Folate P=<0.004**

Case mothers

Folic acid intake vs No folic acid intake

c Serum Folate P=<0.335

d RBC Folate P=<0.16

Table: 42 RBC and serum folate levels (ng/ml) of case and control mothers according to reported diet history.

Reported Diet History	Control mothers(n=100)				Case mothers(n=109)			
	n	%	Serum folate ng/ml	RBC folate ng/ml	n	%	Serum folate ng/ml	RBC folate ng/ml
Reported diet adequate in fruit, vegetable and meat.	58	58	11.16 ± 0.72^{a}	382.8 ± 25.51^{b}	43	39.45	8.26 ± 0.81^{c}	143.2 ± 16.71^{d}
Reported diet inadequate in fruit, vegetable and meat.	42	42	8.75 ± 0.82^{e}	274.3 ± 23.57^{f}	66	60.55	5.53 ± 0.40^{g}	79.86 ± 9.74^{h}

Control mothers

Reported adequate diet vs Reported inadequate diet

a vs e	Serum Folate	P=0.006**
b vs f	RBC Folate	P=0.003**

Case mothers

Reported adequate diet vs Reported inadequate diet

c vs g	Serum Folate	P=0.0003***
d vs h	RBC Folate	P=0.0009***

Control mothers vs case mothers

Reported adequate diet	**a vs c**	Serum Folate	P=0.0049**
	b vs d	RBC Folate	P=<0.0001***
Control mothers vs case mothers			
Reported inadequate diet	**e vs g**	Serum Folate	P=0.0014**
	f vs h	RBC Folate	P=<0.0001***

and for RBC folate levels ($t_{(98)}$=3.006;P=<0.003). Case mothers who reported adequate diet intake had higher Serum and RBC folate level vs those who reported diet inadequate in fresh fruits and vegetables and the difference was significant ($t_{(107)}$=3.77; P=0.0003: $t_{(107)}$=3.43;P=<0.0009) for serum and RBC folate levels respectively. Serum folate and RBC folate levels were higher in control mothers receiving adequate diet when compared with case mothers and the difference was significant both for RBC folate and serum folate ($t_{(99)}$=2.88;P=0.<0049:$t_{(99)}$=7.36;P=<0.0001). In control and case mothers who reported an inadequate diet, the serum folate and RBC folate levels were again higher in control mothers compared to case mothers and the difference was significant ($t_{(106)}$=3.28;P= <0.0014; $t_{(106)}$=8.69;P=<0.0001) for serum and RBC folate respectively.

RBC and serum folate levels (ng/ml) of control and case mothers according to their educational status.

RBC and serum folate levels of control and case mothers according to educational status are given in Table 43.

In this study population 50 case mothers (45.87%) out of total 109 cases and 45% control mothers tested for folate levels had never attended school. In the no schooling group the mean serum folate level of control mothers was significantly higher compared to case mothers ($t_{(90)}$=3.78;P=<0.0003). Also the control mothers had significantly high levels of RBC Folate levels compared to case mothers ($t_{(90)}$=7.98;P=<0.0001). There were 28% control mothers and 36.69% case mothers who received school education. In this cohort the serum folate levels in control mothers were higher as compared case mothers ($t_{(66)}$ =2.91; P=<0.005) and the RBC folate levels in control mothers were significantly high as compared to case mothers ($t_{(66)}$ =7.31;P=<0.0001). In control mothers (16%) and case mothers (14.69%) with college education, the serum folate levels were higher than in case mothers but the difference was not significant ($t_{(30)}$= 0.91;P=0.37) whereas in control mothers RBC folate levels as compared to those in case mothers were significantly high ($t_{(30)}$=3.52;P=<0.001). In the University educated control mothers the serum folate levels and RBC folate levels were not significantly high compared to case mothers($t_{(12)}$= 0.35;P=0.74;$t_{(12)}$=1.93;P=<0.07) respectively. The results

Table 43 : RBC and Serum Folate levels of control and case mothers according to their educational status.

Educational Status	Control mothers n=100				Case mothers n=109			
			Mean serum folate	Mean RBC folate			Mean serum folate	Mean RBC folate
	n	%	ng/ml	ng/ml	n	%	ng/ml	ng/ml
No schooling	45	45	9.39 ± 0.95	328.5 ± 29.35	50	45.87	5.55 ± 0.52	86.48 ± 13.32
School Education	28	28	9.31 ± 0.99	337 ± 36.32	40	36.69	6.99 ± 0.77	95.26 ± 13.41
College education	16	16	11.22 ±1.54	339.1 ± 51.29	16	14.69	9.44 ± 1.21	138.5 ± 25.04
University education	11	11	11.06 ± 0.86	350.1 ± 24.90	3	2.75	9.64 ± 1.47	162.2 ± 42.87

Control mothers vs case mothers

No schooling

Serum Folate P=<0.0003***

RBC Folate P=<0.0001***

School education

Serum Folate P=<0.005**

RBC Folate P=<0.0001***

College education

Serum Folate P=0.37

RBC Folate P=<0.001**

University education

Serum Folate P=0.74

RBC Folate P=<0.07

show increase serum folate and RBC folate levels in control mothers compared to case mothers in the educational groups.

RBC and Serum folate levels (ng/ml) of control and case mothers according to economic status.

Control and case mothers were grouped according to economic status and corresponding Serum and RBC folate levels (ng/ml) Table 44.

In case mothers very low levels of RBC Folate (< 150ng/ml) were observed in economic bracket Rs5000-10000, 11,000-15,000 and 16,000-20,000. In the economic bracket Rs 5000-10,000 the serum folate and RBC folate levels in control mothers were significantly high compared to case mothers ($t_{(81)}$=3.88;P=<0.0002:$t_{(81)}$=6.86;P=<0.0001) respectively. In the economic bracket Rs 11,000-15,000 the serum folate of control mothers was non-significantly high compared to case mothers ($t_{(43)}$=1.03;P=<0.31) whereas the RBC folate levels were significantly low in case mothers compared to control mothers ($t_{(43)}$=6.89;P=<0.0001). There were no control mothers in economic bracket Rs16,000-20,000. In the economic bracket >Rs 20,000 serum and RBC folate levels in control mothers was higher compared to case mothers but the difference was not significant ($t_{(50)}$=1.19;P=0.24; $t_{(50)}$=1.79;P=0.08) respectively.

Table 44: Mean serum and RBC folate levels of control and case mothers according to their economic status.

Monthly Income(Rupees)	Control mothers n=100				Case mothers n=109			
			Serum folate	RBC folate			Serum folate	RBC folate
	n	%	ng/ml	ng/ml	n	%	ng/ml	ng/ml
5000-10000	40	40	9.01 ± 0.92	320.9 ± 34.65	43	39.45	5.02 ± 0.51	76.38 ± 12.03
11000-15000	14	14	8.59 ± 0.78	315.6 ± 37.51	31	28.44	7.25 ± 0.80	95.26 ± 13.41
16000-20000			-	-	29	26.61	8.33 ± 0.96	127.4 ± 21.77
>20000	46	46	11.88 ± 0.85	358.1 ± 24.36	6	5.5	8.97 ± 1.55	235.1 ± 26.79

Control mothers vs case mothers

Rs 5000-10,000

Serum Folate P=<0.0002***

RBC Folate P=<0.0001***

Rs 11,000-15000

Serum Folate P=<0.31

RBC Folate P=<0.0001***

Rs > 20,000

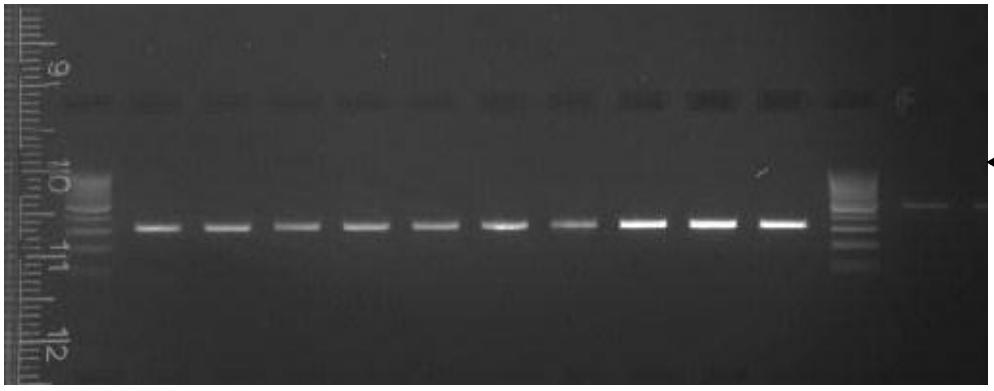
Serum Folate P=0.24

RBC Folate P=0.08

Results of PCR-RFLP (Polymerase chain reaction; Restriction Fragment Length Polymorphism) genotyping

DNA extraction was carried out from blood samples of 100 control mothers and 109 case mothers. Genomic DNA of the blood samples was amplified by PCR and the product imaged by 2% agarose gel electrophoresis. The target gene of 315bp nucleotide sequences are presented in (Figure 16).

Genotyping was carried out by PCR-RFLP. Figure 13: A few genotyping results after gel electrophoresis on 3% agarose gel and ethidium bromide staining . C677T in the MTHFR gene is a missense mutation in exon 4 of the MTHFR gene. At nucleotide 677 a cytosine to thymine substitution converts alanine to a valine codon. Identification of the genotypes were named according to the presence or absence of the enzyme restriction sites. This C to T trans version at nucleotide position 677 of the MTHFR gene gives a cutting site, which if present indicates the T allele, while if it is absent indicates the C allele. Thus, the CC genotype is homozygote for the absence of the site (single band at 314 bp),CT genotype is heterozygote for the absence and presence of the site (bands at 314, 165 and 139bp), and TT genotype is homozygote for the presence of the site(bands at 165 and 139 bp(Figure 17). Two samples each of CC,CT and TTgenotype were confirmed by Sanger Sequencing (Figure 18)



Lane 1

Figure16: Electrophoresis on 2% agarose gel of amplified PCR products of the samples. Lane 1, 100 bp marker ladder; lanes 1-10, samples. The 314 bp bands are the target gene.

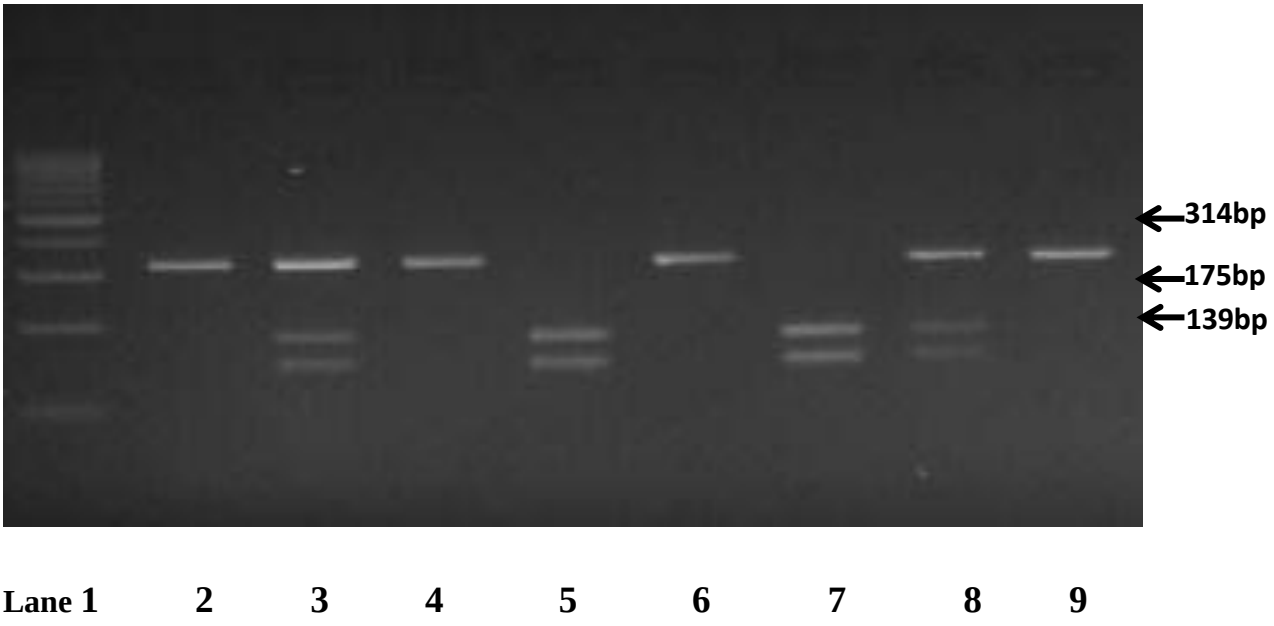


Figure 17: DNA fragments on Agarose gel 3% electrophoresis after enzyme digestion with Hinfl; Genotyping of NTD case mothers and control mothers. Lane 1: 100bp ladder. Lane: 2,4,6,9 represent 677C wild type(C/C) homozygous genotype. Lane3,8: show C/T heterozygous genotype and Lane 5,7: represent 677T (T/T) allele.

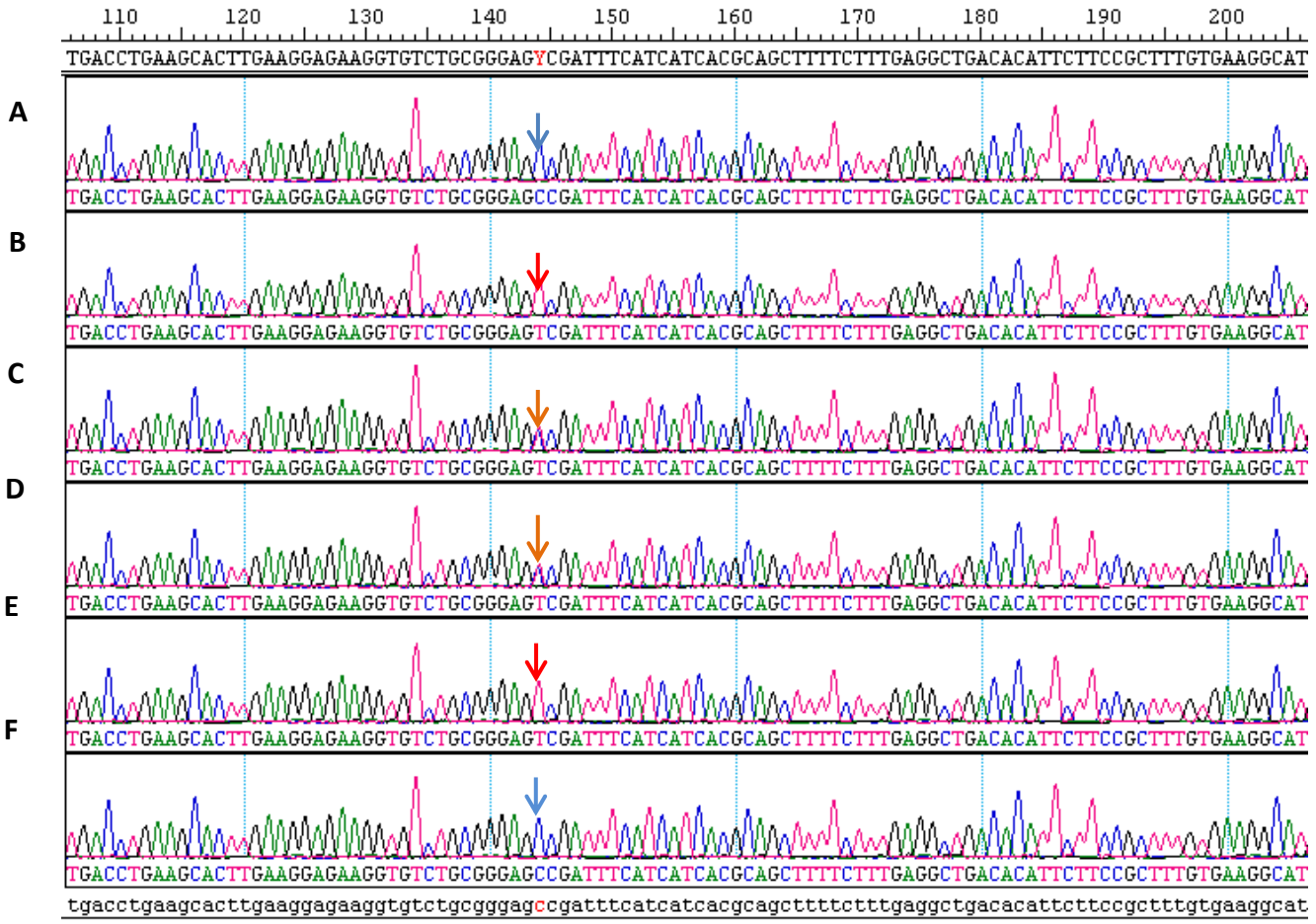


Figure 18: Results of sequencing

The results were shown as CC, CT and TT genotypes by PCR-RFLP

A and **F** CC genotype

C and **D** CT genotype

B and **E** TT genotype

Genotypes and allelic frequencies

The frequency of MTHFR C677T genotypes and alleles is shown in Table 45.The frequency of CC, CT, TT genotypes in case mothers was 61.47, 28.44 and 10.09 % respectively, while in control mothers it was 72%, 26% and 2% respectively. The frequency of C and T alleles was 75.69% and 24.31 % respectively in case mothers. In control mothers, the incidence of C and T allele was 85 and 15 % respectively. There was highly significant difference in the distribution of the three genotypes i.e. CC, CT, and TT ($\Sigma\chi^2_{(21)} = 6.47$;P=<0.0393) respectively in case mothers compared to control mothers. The allele frequency C and T in case mothers and control mothers also showed a highly significant difference in present sample ($\Sigma\ \chi^2_{(1)} = 5.68$; P= <0.017). This indicates that allele T shows an association with the appearance of NTDs in case mothers.

Folate analysis and MTHFR genotype

Table 46 shows the distribution of Mean serum folate levels and Mean RBC folate levels (ng/ml) among control and case mothers corresponding with the MTHFR genotype. Red blood cell folate levels in the 677CC and TT homozygous groups in case mothers was significantly low compared to that of 677CC and TT group in control mothers. ($\Sigma\chi^2_{(1)}$ = 1191; P<0.0001). In 677C/T heterozygotes + TT homozygotes significantly low levels of serum folate ($\Sigma\chi^2_{(1)} = 21.46$;P=<0.0001) and RBC folate ($\Sigma\chi^2_{(1)} = 1078$; P= <0.0001) were observed when case and control mothers were compared.

Maternal age groups and MTHFR genotype

Table 47 shows distribution of maternal genotypes in different age groups and in cranial and spinal NTDs in case mothers. There is one 677T/T homozygous polymorphism in age group 15-19 years with a meningocele affected baby. In age group 20-24 years there were two 677T/T mutations with one meningocele and one myelomeningocele affected birth. Majority of 677T/T mutation (n=5) were present in age cohort 25-29 years. In this age group also appeared spinal NTDs (3 meningocele and 2 myelomeningocele). Less number of the 677T/T allele were seen with increasing age, two in age cohort 30-34

Table 45: Genotypic and allelic frequencies of the MTHFR C677T in case mothers and control mothers (n,%).

		Genotypes			Alleles	
		CC	**CT**	**TT**	**C**	**T**
Case mothers (n=109)	n	67	31	11	165	53
	(%)	(61.47)	(28.44)	(10.09)	(75.69)	(24.31)
Control mothers (n=100)	n	72	26	2	170	30
	(%)	(72)	(26)	(2)	(85)	(15)

CC vs TT= $\Sigma \chi^2_{(2)} = 6.31$; P= <0.0393 * C vs T $\Sigma \chi^2_{(1)} = 5.68$; P= <0.017 *

CC vs CT vs TT= $\Sigma \chi^2_{(2)} = 6.47$; P= <0.0210 *

n =number

( )=percentage

Table 46 : MTHFR C677T genotypes in case and control mothers with mean RBC and Serum Folate level(ng/ml)

	MTHFR Genotypes			RBC Folate (ng/ml)	Alleles	Serum Folate (ng/ml)	Alleles
Control mothers		n	%				
(n=100)	CC	72	72	328.6 ± 20.81	(C)170	9.94 ± 0.64	(C)170
	CT	26	26	378.6 ± 39.78		11.55 ± 1.19	
	TT	2	2	111.4 ± 12.47	(T)30	5.6 ± 3.10	(T)30
Case mothers							
(n=109)	CC	67	61.46	112.5 ± 12.40	(C)165	7.25 ± 0.58	(C)165
	CT	31	28.44	100.5 ± 16.87		6.36 ± 0.72	
	TT	11	10.09	62. 65 ± 13.70	(T)53	4.86 ± 0.83	(T)53

Control vs case mothers CC/TT	Serum folate levels	$\Sigma \chi^2_{(1)} = 32.77$; P= <0.0001***
	RBC folate levels	$\Sigma \chi^2_{(1)} = 1191$; P= <0.0001***
Control vs case mothers CT/ TT	Serum folate levels	$\Sigma \chi^2_{(1)} = 21.46$; P= <0.0001***
	RBC folate levels	$\Sigma \chi^2_{(1)} = 1078$; P= <0.0001***

Table 47: Distribution of Maternal Genotype C677T mutation in maternal age groups and Cranial and Spinal neural tube defects.

Maternal Age groups	Genotype	Total number of cases		Case mothers(n=109)						
				Neural tube defect(109)						
				Cranial NTD		Spinal NTD				
		n	%	Anencephaly	Encephalocele	Meningocele	Myelomeningocele	Lipomeningocele	Spina bifida+Arnold Chairi syndrome	Syringomyelia
15-19	CC	3	2.75	1			2			
	CT	1	0.53			1				
	TT	1	0.53			1				
20-24	CC	20	10.53	2		9	8	1		
	CT	8	7.34	2		3	3			
	TT	2	1.83			1	1			
25-29	CC	16	14.68	1	3	4	7	1		
	CT	8	7.34			6	2			
	TT	5	4.59			3	2			
30-34	CC	15	13.76	3		8	2	1	1	
	CT	10	9.17	1		5	3			1
	TT	2	1.83	1		1				
>35	CC	13		2		8	3			
	CT	4		2		1			1	
	TT	1				1				

years(one anencephaly and one meningocele) and one in >35 years age of mother that produced one meningocele baby (Figure 15).

The results show that of the 11 NTDs produced due to TT mutation ten were spinal NTDs and one was cranial NTD. This indicates that T/T mutation affects more the spinal neural tube region.

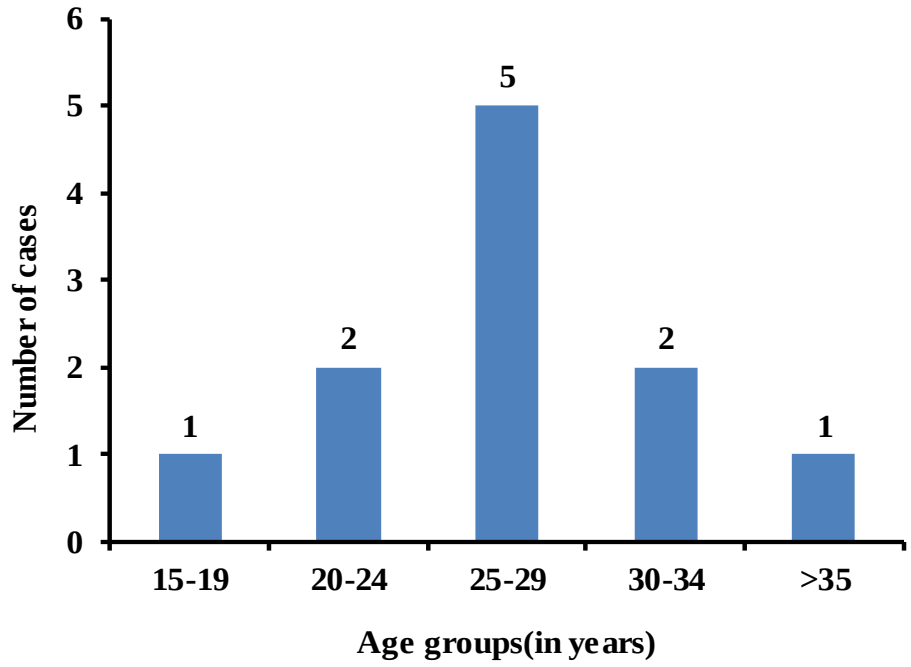


Figure 19: Distribution of MTHFR (C677T) TT homozygous polymorphism in different age groups in case mothers.

DISCUSSION

Discussion

The present study included 190 case mothers who had delivered babies with neural tube defects and 100 control mothers who had delivered healthy babies. Both case and control mothers were identified from Department of Obstetrics, Holy Family Hospital. The neural tube defects (NTDs) were confirmed by Department of Pediatrics and Department of Neurosurgery. The terminations were confirmed on Ultrasonography in Department of Radiology. Holy Family Hospital is a public sector hospital and majority of patients coming for health care are the less educated (no schooling) and those belonging to lower socioeconomic group. The mean age of case mothers was 27.51 ± 0.38 years and that of the control mothers was 27.17 ± 0.50 years.

Neural tube defects

Neural tube defects(NTDs) are heterogeneous group of severe and disabling structural congenital anomalies of the central nervous system which are formed in early embryogenic period between the 20th and 28th day after conception. The cells of the neural plate fold back forming the neural groove and neural folds which appose and fuse in midline forming the neural tube. If the neural tube fails to close completely, exposure of the brain and spinal cord results in NTDs (Lemire, 1988; Botto et al., 1999; Kibar et al., 2007).

Neural tube defects (NTDs) affect 1 in every 1000 pregnancies (van der Put NM et al., 2001; Copp et al., 2013). They carry a high degree of mortality and morbidity and are a leading cause of life long disability (Carmona, 2005). There is dearth of data available for the incidence of NTD's in Pakistan but observational reports point toward the higher end of the world range (0.5 to 12 per 1000 live birth (Jooma, 2004). In the present study anencephaly and spina bifida (meningocele and myelomeningocele) were the most common abnormalities (72.10%). Anencephaly is fatal, most of the offspring are stillborn or die shortly after birth but children born with spina bifida (meningocele and myelomeningocele) have a better survival rate but may suffer from severe lifelong disabilities including motor weakness in legs, loss of bowel and bladder control and

associated psycho-social maladjustment(Pitkin, 2007 ; van der Linden et al., 2006).

Of the 190 NTDs, spinal defects were the most common of NTDs (n=145; 76.31%) whereas cranial defects were 24.71% (n=45). Higher frequency of NTDs (65.8%) were reported by Parveen and Tayab (2007) among all congenital anomalies in Pakistani population born in Sindh province. In another study on Pakistan Sind population Rehman et al (2006) observed 56.6% NTDs among the congenital anomalies identified. Qazi (2010) found hydrocephaly and anencephaly were the main defects in Pakistani population from northern areas. In South Indian population frequency of spina bifida was 58.4% followed by anencephaly (31.6%), and encephalocele (11.6%) Mahadevan and Bhat,2005). In Sub -Saharan African population de Paul et al (2008) the most common defect seen was myelomeningocele (68.11%) followed by encephalocele (27.54%), meningocele (4.35%) and associated hydrocephalus was present in 40.02% of cases. In another study from Sub-Saharan Africa Njamnshi et al (2008) reported spina bifida (myelomeningocele and meningocele) were 71% , encephalocele 21.1% and anencephaly 5.4%. In the present study after spina bifida (72.11%), anencephaly was (20%) and encephalocele (4.71%). Rare cases of syringomyelia and spina bifida +Arnold –Chairi syndrome were observed, however, there were two cases of Dandy Walker syndrome and four of lipomeningocele. McDonnell et al (2014) in their results on Irish population reported 45% anencephalic, 49% with spina bifida and 6% were diagnosed with an encephalocoele. In Algerian population Houcher et al (2008) reported 56.7% spina bifida cases, 32.1% anencephaly, 1(0.5%) encephalocele and 10.7% with spina bifida + anencephaly. Of the 215 NTD cases in the Algerian study, there were 64 (29.8%) with associated hydrocephalus. Pacheco et al (2009) in their study on Brazilian population reported spina bifida to be the most common type of anomaly (45.4%), followed by anencephaly (36.1%) and encephalocele (18.5%).

In this Pakistani study population meningocele NTDs were 42.11%, myelomeningocele 30% followed by the cranial NTDs with anencephaly 18.95% and encephalocele 4.74%. Hydrocephalous was present in 35.79% patients in combination with the neural tube defects. The pattern of NTDs differs from the Sub-Saharan African population where

there is predominance of myelomeningocele and encephalocele NTDs. In Jordanian population myelomeningocele (66%) was predominant followed by anencephaly (Masri, 2009). Associated hydrocephalous with NTD is comparable with African population which was 40.02% (de Paul et al ., 2008). The results of present study are comparable with data analysis from Iran, Golalipour et al (2007) with increased number of spina bifida cases were followed by anencephaly and then encephalocele. McDonnell et al (2014) in a recent study on Irish population reported results comparable with the present study population with 49% spina bifida, 45% anencephaly and 6% had an encephalocoele.

Sex of fetus

The present investigation shows female offspring affected with neural tube defects were significantly higher in number than males (100♀♀:65.49♂♂; $\Sigma \chi^2_{(1)} = 8.14$; P= < 0.005). In the types of NTDs a significant female predominance was observed in anencephalics (P=0.001).In meningocele and myelomeningocele females were more than males but difference was not significant. In Chinese population, Li et al (2006) reported less prevalence of males in total NTDs and a significant preponderance of females in anencephalic. Lary and Paulozzi (2001) and Rittler et al (2004) also observed a significant female majority in NTDs. Houcher et al (2008) reported on Algerian population significantly more females (58.6%) as compared to males (40.9%). They also found significant differences between the types of NTD with regard to the female to male sex ratio with females significantly higher among anencephalics and in spina bifida + anencephalic but not in spina bifida where number of males and females were equal. Regarding sex differences, our results indicate that the rate of NTD was higher in females than males (male to female ratio = 0.70). Sex ratio from research reports by Daoud et al (1996) in Jordanian population was 0.73 and Golalipour et al (2007) 0.78 in Iranian population. Wasant and Sathienkijkanchai (2005) in their study on Thai population and de Paul (2008) on African population reported a male predominance 1.07. In this investigation in contrast to studies on various populations female NTDs are predominant with a non-significant female predominance with spina bifida births (Little and Elwood, 1991;Canfield et al.,1996;McDonnell et al.,1999; Hendricks et al.,1999;Berry et al., 1999;

Lary and Paulozzi, 2001).In a report from South African populationTeckie et al (2013) reported significantly more female infants affected with NTDs (M:F 0.82) and this observation was for both anencephaly (M:F 0.84) and Spina bifida (M:F 0.76).

Sex of infant influences the risk for NTDs. Females as compared to males are more likely to have anencephaly and spina bifida. Female preponderance is influenced by other factors like the presence of additional birth defects and regional distribution (McDonnell et al., 1999; Forrester and Merz, 2004). Preponderance among females also depends on differences between the sexes in embryonic development and teratogenic susceptibility (Little and Elwood, 1991).

Maternal Age

Epidemiological data from various studies hypothesize that maternal age is an important risk factor. In the study population mean age at presentation of case mothers who had delivered child with NTD was 27.48 ± 0.42 years (Range15-40 years) with a similar presentation in control mothers. Prevalence of all NTDs was highest in the age groups 20-24years and 25-29 years (n=58; 34.12%). This is the common childbearing age in Pakistani population. A linear decline was observed with least number of cases at 30years of age (21 cases (12.35%). Similar age group distribution was observed by McDonnell et al (1999) in Irish population with a decreasing prevalence of NTDs (spina bifida and anencephaly) with the increase in mothers' age at delivery. A low folate diet and meager knowledge of folate supplementation could be the contributing factors for NTDs in lower age groups. In Swiss population reported age at delivery (Poretti et al., 2008) was 30 years(range 15-45 years) in NTD affected pregnancies. In Pakistan majority of marriages take place at early age as compared to European population and consequently start family at early age. A different trend than this study was observed in Algerian population (Houcher et al., 2008) where majority of women with NTD pregnancies were observed in age cohort 31-35 years (21.9%) and in age cohort 36-40 years (9.7%). In Chinese population (Li et al.,2006; Ren et al.,2006) NTD prevalence was higher in mothers less than 20 years of age and greater than 30 years of age. Similar findings were reported by in other studies that NTD prevalence rates were higher among infants born to mothers in younger and older age groups (Sipek et al., 2002; Forrester and Merz, 2004).

Maternal Parity

Studies on Neural tube defects show increased incidence in first parity with decreasing incidence in second and third parity and then a raised incidence at higher parities (Buccimazza et al., 1994; Vieira, 2004). In the present study there were 51(26.31%) cases in first parity and the number systematically decreased with increase in parity. Teckie et al (2013) in their study on spina bifida and anencephaly in South African population showed a significantly higher occurrence of NTDs in primiparas than in subsequent pregnancies (43% v. 22% in the general population; $p<0.005$). Also observed in this study was an increased occurrence of NTDs in the fifth or higher pregnancies ($P<0.005$).

Consanguinity

Consanguineous marriages have been in practice and preferred since the emergence of contemporary human society and more widely practiced in several global communities (Shawky et al., 2013). Consanguineous marriages continue to be extremely common in Pakistan and much of South East Asia (Hussain et al.,2001). In Pakistani society cultural, social, and family pressures compel families to arrange marriages of their children within family. Consanguineous marriages among case mothers were significantly higher than non-consanguineous marriages in this study population ($P=<0.015$). Among the consanguineous unions the most frequent type were first cousin marriages (46.84%). In control mothers consanguineous marriages were 45% and among these first cousin unions were 25 %. In consanguineous unions children are at a greater risk of inheriting genetic disorders as autosomal recessive gene mutations could be inherited from a common ancestor. There is increased probability of expression of mutated recessive genes in close biological relationship (first cousins) between parents(Shawky et al., 2013). In a study on Pakistani population Qazi(2010) reported 21% consanguinity in couples with children affected with congenital anomalies. Another study on congenital anomalies (Parveen and Tayab, 2007) reported 44.74% marriages were among close relations. The rates of consanguinity are dependent on socio-cultural, geographical and religious diversities (Tadmouri et al., 2009). South East Asia and Middle East Arab countries reveal some of the highest rates of consanguineous marriages in the world with first cousin marriages being more prevalent (25-30%) of all marriages. Among Arabs and other Middle East

countries, due to consanguinity there are adverse reproductive outcomes and increase in rates of congenital malformations (Hamamy et al., 2005; Al-Ani et al., 2010). Consanguinity increases the rate of homozygosity which is one factor for such malformations. In Pakistan consanguineous marriages are favored because of economic and cultural reasons particularly social security for the females. In Middle East and Saudi Arabia increased consanguinity resulted in the appearance of spina bifida, anencephaly and hydrocephalous offspring (Rajab et al., 1998; Murshid et al., 2000). A similar picture was reported from Palestinian population by Zlotogora, (1997) regarding higher incidence of malformations among children coming from consanguineous marriages.

The coefficient of inbreeding (F) in this study group in case mothers is F=0.0342 and in control mothers F= 0.0208 which indicates more homozygosity at certain loci but not in non-non-consanguineous marriages (F=0.021). Analysis between genetic diseases with different modes of inheritance has shown high values of inbreeding coefficients (F= 0.021) as compared to controls (F=0.019) in recessive disorders(Shawky et al., 2013). Mokhtar et al (1998) reported in a study on Egyptian children with autosomal recessive disorders, a high incidence of consanguinity (60% with 48% first cousins) and the average inbreeding coefficient was higher F=0.03 compared with Egyptian population in general F=0.01. In Indian population Jain et al (1993) reported commonest types of consanguineous marriages were between first cousins (50.6%) and uncle and niece (42.4%). The mean coefficient of inbreeding was F= 0.056 which very high compared to that reported in this study.

Maternal cigarette smoking/Exposure to passive smoke

Although in this study population none of the case mothers were smokers yet there were 64(37.65%) fathers of affected babies who were smokers. Hence these case mothers were exposed to harmful effects of tobacco smoke. Case mothers exposed to passive smoke were significantly greater in number as compared to control mothers exposed to passive smoke(P=< 0.0066). Pregnant women who are exposed to passive smoke inhale the harmful constituents and metabolic products contained in smoke of active smokers and these chemical by- products such as nicotine, polycyclic aromatic hydrocarbons and

carbon monoxide pass through placenta with harmful effects on fetus (Windham et al., 1992; Li et al., 2013). Animal studies have reported teratogenic effects of cigarette smoke on developing embryo and neural tube closure (Alexander and Tuan ., 2003;Wang,2004; Zhao and Reece ,2005). It was reported by Suarez et al (2011) in a case control study that maternal exposure to passive smoke is a risk factor for NTDs. Hence women of reproductive age and planning a pregnancy should not only refrain from active smoking but avoid exposure to passive smoke. In a previous case control study on Mexican American Women, Suarez et al (2008) stated a 2.6 fold increased NTD risk in women exposed to passive smoke. Most of women in the study population were unaware of harmful effects of passive smoke to which they were exposed due to smoker partners. Li et al (2013), reported increased NTD risks among women exposed to partner smoking, compared to those who were not exposed. They further suggested if pregnant women avoid inhalation of smoke, it could effectively prevent the injurious effects of second hand smoke (SHS) on developing embryo. Counseling of pregnant women and their partners to create awareness regarding SHS exposure is essential (Li et al., 2013; Wang et al., 2014). Majority of women in study may be unaware of harmful effects of SHS to developing fetus. Hence counseling of pregnant women is required to not to expose themselves to passive smoke to avoid any risk of NTDs.

Hyperthermia

Maternal exposure to high temperatures which may be due to fever or practice of hot tub and sauna first few weeks of pregnancy is related with an greater risk for NTDs (Milunsky et al., 1992). Hot tub and sauna baths or use of electric blankets were not a cultural part of study population but history of high fever was reported by 11.05% case mothers and 5% control mothers. In South African population Teckie et al (2013) reported 5.8 %(n=34) of case mothers had febrile illness during early pregnancy in their study sample. Moretti et al (2005) after meta-analysis of fifteen studies concluded maternal hyperthermia in early pregnancy increases the risk for development of neural tube defects and teratogenic for human embryos. Previous study by Suarez et al (2004) on Mexican-American women with NTD-affected pregnancies and history of high fever in early embryonic period, reported higher risk in the offspring. Furthermore, the risk of

NTD with high fever can be reduced by intake of multivitamins with folic acid of folic acid alone (Botto et al., 2001). In their study on American women Shaw and colleagues (1998) reported a 7.4-fold increased risk for NTDs in mothers who suffered from high fever with no intake of folic-acid-containing multivitamins. Hence, women planning a pregnancy should be counseled to avoid heat exposures, get prompt treatment for any febrile illness occurring in first trimester of pregnancy and to regularly take folic acid supplements.

Diabetes mellitus

Maternal diabetes mellitus during pregnancy is recognized as one of well-known risk factor for birth defects, such as neural tube defects (NTDs) and its role as a human teratogen has been known since early 1900's (White,1937;Castori,2013).Other complications associated with Diabetes during pregnancy are spontaneous abortions, still birth, and congenital malformations (Greene,1999;Moore et al.,2000;Salbaum and Kappen, 2010). In this study among the case mothers there were 26 (13.68%) and 7% in control mothers who were diagnosed for diabetes. The types of NTDs in these diabetic women were meningocele (n=14; 7.37%) myelomeningocele 4.74%(n=9),anencephalic 1.05%(n=2) and encephalocele 0.53%(n=1). In South African population Teckie et al (2013) reported 1%(n=6) of case mothers had Diabetes. Neural tube defects (NTDs) are the second commonest birth anomaly associated with maternal diabetes. Research experiments by Loeken (2006) have implicated increased level of glucose in maternal blood and an increased glucose transport to developing embryo as a possible cause of NTDs. Studies on animals have shown that NTDs in embryos of diabetic mice result from altered expression of genes which control neural tube development and closure (Dheen et al., 2009). In diabetic women planning a pregnancy antenatal counseling on good blood glucose control to essential to reduce the risk of having NTD affected pregnancy. Folic acid supplementation is advised to diabetic women planning a pregnancy as folic acid reduces the teratogenic effect of high glucose levels in developing embryo and the dose is much higher as compared to non-diabetic women (Allen et al., 2007)

Rural/urban residential area

There is an urban–rural disparity in the occurrence of NTDs and is evident in this study. In rural areas limited access to education, health awareness, and compromised maternal nutritional status as compared to urban areas are factors that could contribute to this difference. Case mothers (62.11%) of this study belonged to rural areas (P= < 0.0001). Mothers with cranial NTDs affected births (17.89%) and spinal NTDs (44.21%) residing in rural areas were significantly more than those residing in urban areas (P= < 0.033). An increase prevalence of NTDs in women from rural areas has been reported in Chinese population (Lian et al., 1987; Li et al., 2013).

In a study on Texan American population, the urban-rural difference in the types of NTDs showed no association of urban-rural residence variations in the frequency of anencephaly or spina bifida whereas rates of encephalocele in this population were significantly higher in rural areas as compared to urban areas (Luben et al., 2009). In Chinese population low educational level was significantly associated with high risk for an NTD pregnancy (Li et al., 2006) and with 90% of illiterate peoples living in rural areas. Moreover folic acid awareness and intake among women in the 6 northern province of China revealed that proportion of women who had knowledge of folic acid and who used folic acid supplements was 73.0% and 10.5%, respectively, in the urban areas, compared with 50.0% and 7.6% in the rural areas respectively (Zeng et al., 2011). In China increased number of rural women(40%) had deficient red blood cell folate levels in the north, compared to 20% in the south (Ren et al., 2007). In rural areas exposure to pesticides and chemical fertilizers during pregnancy could be associated with a relative high prevalence of NTDs (Li et al., 2005). An increase number of rural mothers with NTD affected births in present study population could be attributed as in the Chinese and American population to illiteracy with lack of awareness and use of folic acid supplements. In American population Luben et al (2009) implicated use of pesticides in rural agricultural land to be a significant variable but in present investigation case mothers could not recall exposure to chemical fertilizers and pesticides in periconceptional period.

Exposure to chemicals and garbage dumps

Exposure to chemicals released from landfill sites into air, water and soil in vicinity have been implicated in a number of diseases and also increase risk of congenital anomalies. (Vrijheid, 2002; Padula et al., 2013). This study includes 17.37% case mothers living in close proximity to chemical exposures emanating from industrial installations. Exposure to pollution from garbage dumps was reported by 25.26% mothers of NTD affected children. Studies by Vrijheid et al (2002) and Morris et al (2003) reported no risk to pregnant mothers from landfill sites situated at distance of over 3 km. The landfill sites contain a range of chemicals which can contaminate surface and ground water, plants and cattle bred in the areas close by. Garbage wastes dumps are an inevitable by- product of developed societies. Disposal of this waste still remains a problem. There are no proper land fill sites for hazardous and non-hazardous sites in Rawalpindi and the few landfill sites created by public themselves are open resulting in chemicals being released in air as well as contaminating the soil and underground water. No statistical data are available on landfill sites in this population. Elliott and colleagues (2001) in a study on UK population reported large sections of population being exposed to hazardous waste or its by-products. Only a slight increase in risk of congenital anomalies was seen in populations living near landfill sites. The EUROHAZCON study (Dolk, 1998) reported 80% of population living within 2 km of landfill sites and showed a 33% increase in risk of congenital anomalies.

Adverse reproductive history prior to index pregnancy

Adverse reproductive history in a previous pregnancy is a risk factor for NTDs (Todoroff and Shaw, 2000). In the present study, significantly greater number of case mothers (61.59%) as compared to control mothers (14%) reported spontaneous abortions, still births, neonatal death, and NTD in a previous pregnancy (P=<0.0001). NTD risk was reported to be high in women with a previous spontaneous abortion, still birth or neonatal death even if infant or fetus did not have NTD (Little and Elwood,1991;Canfield 1996;Rivas et al.,2000) but Todoroff and Shaw (2000) in American women and Lu et al (2011) in Chinese population did not find any association with previous history of abortions and NTDs. Bianca et al (2002) in their study on Italian population found

multi parity and spontaneous abortion in previous pregnancies a risk factor for NTDs. One hypothesis put forward thirty years ago by Knox (1970) and later worked on by Clarke et al (1975) stated that residual trophoblastic tissue ("cell rest") from prior spontaneous abortion may alter uterine environment resulting in NTD. Previous spontaneous abortions could lead to depletion of folate stores and an increased risk of NTD in subsequent pregnancy. Hence mothers with history of adverse pregnancy outcomes and planning a pregnancy need to be counseled on use of folic acid and its role in prevention of NTDs.

Education and Socioeconomic factors

Women belonging to low socioeconomic strata are at a higher risk for development of NTDs (Little and Elwood, 1992). The nutritional status of women in their reproductive age is associated with their socio-economic state with diet low in folate observed in less privileged class. More over socio-economically deprived women were less likely to use folic acid during the early embryonic period (Relton, 2004; CDC, 2008; Grewal et al., 2009).

In present study population there were 44.74% women who belonged to the socio economically deprived class and there was a declining trend with less number of cases observed in case mothers at higher range of socioeconomic status. A statistically significant difference was observed between socio economic status (SES) of case and control mothers (P=<0.0001). In the past several studies have reported low SES linked with an increased risk of NTDs (Wasserman et al., 1998; Vrijheid et al. 2000; Farley et al. 2002; Meyer and Siega-Riz, 2002; Blanco et al., 2005;Li et al., 2006; Yang et al., 2008).

In an Australian study low income levels were associated with poor diet and less folate supplements intake (Forster et al, 2009). Similar results were reported on Dutch and Irish populations (Timmermans et al., 2008; McGuire et al., 2010) with low intake of folic acid associated with low socioeconomic status (SES).

In this study only 13.68% of case mothers were in the economic cohort >Rs 20,000 as compared to 43% in control mothers. Majority of case mothers (44.74%) were in low

income group (Rs 5000-10,000) and had either no schooling (n=45) or only school education (n-38). In present investigation both low SES and meager education are risk factors for NTDs. In their study on American population, Grewal et al (2009) reported school and less than school education to be associated with increased risk NTD affected pregnancy.

Study on intake of folic acid in Thai population showed high level of education in women of childbearing age was associated an increased folic acid intake (Nawapun and Phupong, 2007). Educational level and household income of mothers have been shown to be associated with high risk of having a baby with congenital abnormality (Farley et al.,2002; Yang et al., 2008;Olesen et al.,2009). There is three- fold increase risk of congenital anomalies in American women with less education as compared with women with more than four years of higher education (Wasserman et al.,1998).

In this study only 13.16% case mothers had college education and 2.11% attained university education. A comparable picture is observed in this study population with American (Grewal et al., 2008), Australian(Forster et al., 2009) and Chinese population studies (Li et al., 2006) with higher risk of NTDs appearing in the less educated and those belonging to lower socio-economic status. Hence prevention strategies need to target the less privileged socio- economic class with folic acid awareness education, folate supplementation and food fortification.

Diet and Nutritional status

Malnutrition is a common social problem in Pakistani pregnant women as in other developing countries. Adequate maternal nutrition is an important factor that can result in alteration of fetal gene expression with adverse pregnancy outcomes (Greene et al., 2011) and no intake of folic acid (Shaw et al., 1995; Werler et al., 1993). Maternal diet inadequate in dietary folate and other micronutrients can alter fetal genome with reduction in methylation cycles altering the genotype expression and an abnormal phenotype outcome (Khulan et al., 2012; Safi et al., 2012). In present investigation diet inadequate in fruits and vegetables was reported by 60.53 % (n=115) mothers with NTD affected babies significantly high than in control mothers (P=<0.0029). Folate is essential

for the developing embryo and gets it from the mother via placenta. Folate supply to fetus depends on maternal folate intake, absorption, metabolism; folate transport across the placenta, and embryonic folate uptake (Prasad et al., 1994; Tamura and Picciano, 2006). Higher intakes of folate was associated with a decrease in risk of neural tube defects by 72%, according to a randomized, double-blind vitamin supplementation trial including 1,817 women of reproductive age from seven countries (MRC, 1991) but the observed reduction of 26% is much less than the predicted 50-70% (Cena et al.,2008). Results of this study support the correlation between economic status, level of education and diet rich in folate as was put forward by Lawrence et al., (1980). Women belonging to low income group do not take folic acid supplementation or diet rich in fruits and vegetables (Cena et al., 2008). Findings of this study also suggest there is strong need to implement dietary counseling programs for women of childbearing age and apprise them of the significance of proper diet rich in fruits and vegetables before conception. Achieving a high folate diet requires change in dietary pattern (Brouwer et al.,1999). Women in low socioeconomic group do not have the financial means to acquire folate rich diet and since folate supplementation compliance is low even in developed countries (Chako et al., 2003) hence food fortification of staple food like flour and cereals need to be implemented to reduce risk of NTDs in Pakistan.

Periconceptional use of Folic acid

Intake of folic acid in periconceptional period can reduce the incidence of NTDs and is one of the foremost strategies for prevention of these serious, disabling, preventable congenital abnormalities of CNS (MRC, 1991; Czeizel and Dudás, 1992). None of the case mothers and only 6.67% of control mothers had used folic acid in periconceptional period. A 100% lack of folic acid use in periconceptional period was reported by Al-Ani et al., (2010) in Iraqi mothers with NTD affected babies. Women from developing countries in Asia, Africa and South America were less likely to use folic acid supplements than those from Western Europe with the highest intake observed among women from North America, Australia and New Zealand (McGuire et al., 2010). Cultural background influenced the uptake of folic acid supplements in the Netherlands,

where non-western women took less folate supplements than western women (Timmermans et al., 2008).

In present study after pregnancy intake of folic acid was (39.47% in case and 68% in control mothers) as most of public sector antenatal clinics provide free folic and iron to pregnant women. Although intake of folic acid during pregnancy is required for optimal growth of fetus but it has no effect on formation of neural tube. Most of pregnancies in developing countries are unplanned and the recommendations to take folic acid for prevention of NTDs often cannot be implemented. Because of poor compliance to public health campaigns on use of folic acid supplements, now mandatory fortification of foods is being considered as an optimal solution. There is an urgent need in our country for a specific policy to supplement basic staple food with folic acid that would improve folate status of women of child bearing age and thereby reducing the frequency of NTDs and other congenital anomalies.

Awareness of folic acid for prevention of neural tube defects

In this study population, there was no awareness regarding use of folic acid and the significance of its role in prevention of neural tube defects. None of case mothers and only 5% control mothers were taking folic acid before pregnancy. Timmermans et al (2008) in study on Dutch population, Safi et al (2008) in American population and McGuire et al (2010) in Irish population observed low economic and educational status and lack of knowledge on their importance of folate were important contributing factors towards poor intake of vitamins and folic acid. Studies have been conducted to assess levels of awareness and knowledge in women of childbearing age and its association with low folic acid intake with variable results from different countries.

In Turkey awareness level in pregnant woman was 42.8% and was dependent on level of education and socioeconomic status (Köken et al.,2013). In Nepalese women, 40% had heard of folic acid supplementation, 16% knew it was important for health but only 5% knew it should be taken pre conception (Paudel et al., 2012). Health awareness programs in the West have helped to increase level of awareness from 60% to 72% and knowledge from 21% to 45%. Increase in awareness caused a simultaneous increase in folic acid

consumption but only from 14% to 23% showing a positive but insufficient impact (Chivu et al., 2008).

In the present study both awareness of folic acid and knowledge of its importance in prevention of NTDs is alarmingly low. In Pakistani population health education programs need to be conducted in antenatal clinics or via media with the aim of creating awareness in women of reproductive age on the importance of taking folic acid supplements in the periconceptional period and their role in occurrence and recurrence of NTDs and other congenital anomalies.

Folate Status

Folate status is determined by measurement of RBC folate concentrations and plasma/serum levels. Red cell folate is better indicator of long term status over the previous 180 days, while serum folate reflects a more recent dietary intake (McNulty and Scott, 2008). Folate deficiency is defined as a serum folate concentration <7nmol/l (3ng/ml) or a red blood cell folate concentration <315nmol/l (140ng/ml) (Crider et al., 2011). Deficiency of Folic acid has been indicated as an important environmental factor in the pathogenesis of NTDs (de Villareal et al., 2001). This study provides information on folate status of women who had delivered an offspring with NTD (n=109) and compared it with control group (n=100). Not many studies have been conducted to determine folate status of women in their reproductive age in Pakistan. This study determined folate status of women with an NTD affected pregnancy. The Mean RBC Folate levels in control mothers was 337.2 ± 18.42 ng/ml and 104.1 ± 9.17ng/ml in case mothers which were highly significantly low as compared to control mothers (P=<0.0001). The number of case mothers 77 (70.64%) were significantly high as compared to control mothers (16%) where RBC folate levels were less than 150ng/ml (folate deficiency cohort). The results are consistent with those of de Villareal et al (2001) in Mexican women. This case control study also found significantly higher number of case mothers in RBC folate less than 150ng/ml cohort (75 vs51.2% ;P=<0.05). Similarly number of case mothers with serum folate levels < 2.9ng/ml were higher in this study(27 vs 8) and in study by de Villareal et al (2001) it was 16 vs 0%(P=0.01). Daly et al (1995) reported an increase risk of NTDs with a decrease in RBC folate levels. Red cell folate levels have been reported

low in women with NTD affected pregnancy as compared to those carrying a normal baby (Kirke et al.,1993; Malloy et al.,1999).

The lower limit of the normal range for red cell folate is 317 nmol/L (140 ng/mL), but levels above 905nmol/ml(400ng/ml) are needed for protection against NTDs. Red cell folate levels higher than 150 µg/L, could prevent anemia were associated with an increased risk of NTDs (Daly, 1995; 1997). Data from Irish researchers (Kirke et al., 1993) in a large case-control study determined folate levels in women on first ante natal visit and reported an increased risk of NTDs associated with decreasing Red Cell Folate levels. With a RCF level of <150µg/l the risk of NTD is 6.6/1000 but if the RCF level is increased beyond 400ng/ml the risk falls to 0.8/1000. The RCF levels can be increased over 6 month's period with doses of 400µg of folic acid which can reduce the risk of NTDs by 47% (Daly, 1995). In the present study there were no case mothers with RBC folate levels above 440ng/ml which is optimum level needed for protection against occurrence of NTDs (Daly, 1995).

Czeizel and Dudas, (1992) and MRC, (1991) were two important epidemiological studies conducted in USA and Hungary. They learnt from the research that adequate maternal folate status is critical for normal closure of neural tube during embryogenesis. Their findings supported the hypothesis that by raising maternal folate levels, the occurrence and recurrence of NTDs could be significantly reduced .Plasma folate concentrations vary with recent dietary intake and are therefore, not informative about long-term folate status commonly derived from RBCs folate concentrations (Eichholzer and Zimmermann, 2006). Research report by Beaudin and Stover (2007) showed low folate status disrupts folate metabolism with epigenetic alteration of gene expression. Eskes, (2002) reported low serum folate and RBC folate concentrations in early pregnancy in women with NTD-affected pregnancy compared to controls. Maternal age is an important determinant of folate intake (CDC, 2008). In present investigation case mothers in younger age group (15-19 years) had low levels of RBC folate as compared to higher age groups and this could be due to lower intake of folic acid in younger age group.

Folate levels in pregnant women who smoke are significantly lower than pregnant women who do not smoke Mcdonald (2002). Although in this study none of the women

smoked but there were 61.47% case mothers and 25% control mothers exposed to deleterious effects of passive smoke. In present investigation RBC folate levels in mothers who were exposed to passive smoke were low as compared to those not exposed to passive smoke but the difference was not significant. Serum folate levels were statistically low in case and control mothers exposed to passive smoke (P=0.001; P=0.005) respectively. Suarez et al (2011) in their study on US population showed decreased RBC folate and serum folate in women exposed to passive smoke and thus supporting the hypothesis of increased risk of NTDs in women who smoked tobacco or were exposed to passive smoke. In this study case mothers were unaware of harmful effects of exposure to passive smoke, hence women planning a pregnancy should be counseled to avoid passive smoke and thus reduce the risk of NTDs and other adverse pregnancy outcomes (Mcdonald, 2002).

Ren et al (2006) reported in Chinese women less awareness and use of folic acid in rural residents compared with women living in urban areas and these results correlate with other studies showing that pregnant women in rural areas had lower concentrations of blood folate and higher NTD prevalence rates than urban dwellers (Lian et al., 1987). In present study majority of case mothers 62.11% were rural residents and their mean RBC folate was significantly low as compared to those residing in urban areas (P=0.0139). These results are comparable with report by Ren et al (2007) in Chinese rural population whence low RBC Folate levels in women of reproductive age were observed.

Research has shown that correct use of folic acid in early pregnancy could prevent 50-70% of neural-tube defects (Botto et al., 2001). There is an increased risk to women belonging to lower socioeconomic (SES) and since these women have little access to folate rich food with low folic acid supplement intake (Relton et al., 2004). In American population Caudill et al (2001) observed in low socioeconomically placed women, RBC folate levels were 16% lower and serum folate levels 24% lower than women in higher socioeconomic group. In the present study case mothers in lower socioeconomic cohort (Rs 5000-10,000) also had significantly low RBC and serum folate levels as compared to control mothers (P=<0.0001). The Folate levels increased with higher socioeconomic status.

Folate levels are alarmingly low in this study population as mean RBC folate levels in highest economic cohort (235.1 ± 26.79ng/ml) were present in only 5.5% of total number of case mothers and 94.5% of case mothers had RBC folate levels below the deficiency mark. In current investigation folate levels were stratified according to educational status. Analysis showed low folate levels in no schooling group and a systematic increase in levels was observed with higher education levels. Socio-economically deprived and less educated women were unaware of importance of use folic acid in periconceptional period and because compliance to proper use of folic acid was poor even in the developed countries food fortification is the most optimal solution to raise folate levels of women in reproductive age whereby reducing incidence of NTDs (Relton et al., 2004; Blanco Muñoz et al., 2005).

Methylenetetrahydrofolate reductase (MTHFR) and Neural tube defects

Methylenetetrahydrofolate reductase (MTHFR) a flavoprotein is a crucial enzyme in folate metabolism. Folate metabolic pathway plays an central role in DNA methylation, DNA synthesis, and cell replication (Morrison et al. 1998; Imbard et al 2011). Aberrations in folate metabolism and impaired DNA synthesis could affect the neurulation process. Research has been conducted on a number of polymorphisms in the MTHFR gene and the C677T mutation has been most widely described (Goyette et al., 1998).

In this study population wild type CC homozygotes were more in numbers in control mothers (72%) as compared to case mothers (67%). The CT heterozygotes and TT homozygotes were significantly more in case mothers compared to control mothers (P=<0.0393). The differences in distribution of the allele frequency C and T in case mothers and control mothers is also highly significant (P= < 0. 0.017). The T allele has higher frequency in case mothers compared to control mothers. In heterozygote (CT) polymorphisms normal enzyme activity is 65% with a 10% decrease in RBC Level. The homozygous (TT) variant has low(30%) of normal enzyme activity and lower(18%) red blood cell folate levels (Malloy et al., 1997; Rozen,1997). Significantly low RBC folate concentrations were present in TT variant in case and control mothers of this study population (P= <0.0001). de Villarreal et al (2001) in their case control study on Mexican population showed in genotyping results TT genotype significantly more in case

compared to control mothers (P=<0.05). In Mexican residents RBC and serum folate were markedly low in women who had delivered an NTD baby as compared to control mothers both in C and T genotype. In present investigation on Pakistani population significantly low RBC and serum folate levels were associated with TT genotype as compared to CC genotype. Christensen et al., (1999) showed similar results with TT 677 genotype and low folate status synergistically increasing risk of NTDs. An investigation on Algerian population did not identify MTHFR polymorphism (Houcher et al., 2009). Likewise in Italian and Irish population (de Marco,2002; Kirke et al.,2004) the TT genotype were not in higher frequency as compared to control mothers. The 677 CT and TT variants show heterogeneity in different populations and with a low folate status increase the risk for severe NTDs (Botto and Yang, 2000). In Asian and Caucasian population the variant CT and TT are associated with significantly increased NTD risk but no association was found in African studies (Yan et al.,2012). Several researchers (Whitehead et al., 1995; Ou et al., 1996; Kirke et al., 2004; Carter et al., 2011; Cadenas-Benitez et al., 2014; Liu et al., 2014) observed that TT and CT 677 genotype are risk factors for the development of NTDs. The effect of low folate levels associated with CT and TT genotype can be overcome by folic acid supplementation and food fortification. Hence more studies need to be conducted to elucidate the mechanisms underlying the gene-nutrient interactions in these preventable congenital malformations.

The present study provides important data implicating TT MTHFR genotype and low folate status with occurrence of NTDs. The fact that 50-70% of NTDs can be corrected by folic acid intake lays stress on the importance of public health intervention programs to create awareness and promote use of folic acid supplementation and food fortification in women of reproductive age to prevent neural tube defects.

Conclusions

The present study showed spina bifida (myelomenigocele and meningocele) and anencephaly the most common NTDs in Pakistani population. Female NTD births predominated. Majority of case mothers were in age group 20-29 years. Consanguinity, exposure to passive smoke and diet inadequate in folate were risk factors. There were more rural than urban case mothers. Rural clustering needs to be investigated further to decipher more environmental hazards in etiology of NTDs. Data from this study showed an association between inadequate nutritional status, low folate levels and presence of MTHFR genotype 677TT to be risk factors in etiology of NTDs. Both serum and RBC folate levels were alarmingly low in case mothers of this study population and in majority of women these levels were much below the cut off value required for protection against the occurrence of neural tube defects (WHO/NMH/NHD/EPG/12.1.2012). MTHFR genotyping showed number of MTHFR 677TT genotype significantly high in case mothers as compared to control mothers Genetic counseling need to be geared up to apprise mothers of importance of diet rich in fruits and vegetables especially in periconceptional period and of the deleterious effects of micronutrient deficiencies on the developing embryo. Frequency of consanguineous marriages was higher among parents of offspring with NTDs compared to control group, hence the need for genetic counseling on risks cousin marriages and adverse reproductive outcome.

This study was carried out to assess folate levels and MTHFR 677 C/T genotype in mothers with NTD offspring. More large scale studies are required to assess folate status of women in their reproductive age as such data is lacking on Pakistani population. These studies would help in policy making on folate supplementation and flour fortification.

Recommendations

- Neural tube defects (NTDs) are one of the most common, serious, disabling, preventable congenital defects of the central nervous system. Prevention strategies by both public and private sector need to be implemented to prevent the occurrence and recurrence of these tragic congenital anomalies.
- There is need to educate the community, health professionals, policy makers, and the media, about neural tube defects and the opportunities for effective care and prevention. Target population are women in their reproductive age belonging to socioeconomically deprived class with no or little education. Educational campaigns need to be carried out to apprise women on folate rich food and their role in prevention of neural tube defects.
- Folic acid supplementation: All women of reproductive age should take 4mg/day of folic acid such that when she conceives there is enough folate in her system to afford protection against neural tube defects. The limitation to this proposal is poor compliance even in developed countries and in underdeveloped countries the dilemma is that many may still remain deprived because of poor delivery of health services.
- Food fortification with folic acid is more likely to reach all women before conception with the disadvantage of exposing an untargeted population added dose of folic acid. Reports from many countries who have fortified their staple food claim to have reduced the incidence of NTDs like USA, Canada, Chile. Government policies need to undertake this problem on urgent basis and mandatory fortification of flour with folic acid should be undertaken as primary public health intervention in reducing the prevalence of NTD-affected pregnancies. Food fortification has the potential to successfully improve folate status of women in their reproductive age and thus help to reduce and even eliminate most of the folic acid-preventable NTDs.
- Folate awareness in women: There is lack of folate awareness among women in reproductive age group regarding its role in preventing birth defects. Lack of awareness of folate was most common reason for not using folic acid supplements. Intense media campaigns are needed in our country to apprise

women of reproductive age about folate and role in preventing birth defects. Unfortunately, folate awareness seems to lack in health care professionals. Hence folate awareness programs need to be extended to doctors, nurses, and paramedics.

- Secondary prevention by early diagnosis and termination of selected cases: All women coming for antenatal care should be screened by ultrasound and MSAFP screening to identify NTDs. Ultrasound examination can visualize the lesion, while maternal alpha fetoprotein (MSAFP) levels, if elevated, will require a detailed fetal ultrasound survey and/or amniocentesis to confirm the diagnosis. Screening for NTD, early diagnosis proper management and termination of selected cases will help reduce incidence of disease.
- The presence of MTHFR 677TT mutation indicates the need for more research as presence of this mutation increases folic acid requirement. Adequate intake of folic acid in women with 677TT can reduce the deleterious effects of this mutation reducing the occurrence and recurrence of neural tube defects.

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