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Association of *MTHFR* Gene Polymorphism and Serum Folate Level with Neural Tube Defects Among Neonates in Duhok City

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بِسْمِ اللهِ الرَّحْمنِ الرَّحِيمِ

وَقُل رَّبِّ زِدْنِي عِلْمًا

سورة ابراهيم الاية 114

DEDICATION

To

**** My respectful mother***

**** My husband for his endless help and support***

**** My son***

**** My dear brothers and sisters***

Acknowledgement

Before all great thanks to Allah"; the Glorious, the merciful, the compassionate…

Thanks to Duhok Directorate of Health for helping and supporting me to study master.

I would like to thank the staff of Duhok Medical Research Center (DMRC) at the College of Medicine / University of Duhok and specially Ahmed M. Salih the head of DMRC, M.Sc. Soleen Sardar Zudi the reporter of DMRC and Deendar Abdulrahman the technician of DMRC for giving me the permission and hand to do my practical part of my study.

Thanks and appreciation for my both supervisor prof. Dr. Nedzvet skyi Viktor From Bingol University and Assistant Prof Dr. Ahmed Mohammed Salih from college of medicine /Duhok University.

Special thanks for my brother Dr. Amir Ibrahim Ahmed & Assistant lecturer Deldar Murad to statistical analysis for his efforts to analyze the study result in Nursing College / University of Duhok and Shreen zuhair for her frequent help.

Special thanks for Emergency, Acorn, Hivi, Gynecology & Maternity Teaching Hospital for giving me the approval of sample collection. And thanks for all patients whom concern to participants in percent study.

Thanks to all friends who helped and supported me.

ABSTRACT

Background: Neural tube defects are a group of conditions in which an opening in the spinal cord or brain remains from early in human development. Besides the role of folic acid, a number of maternal factors have also been found implicated in the etiology of NTD, particularly hyperthermia, diabetes, hyperinsulinemia, obesity. Some mutations in MTHFR gene are associated with methylene tetrahydrofolatereductase deficiency. The best-characterized MTHFR genetic polymorphism is a common missense mutation consisting of 677 C→T transition, resulting in thermolabile enzyme variant that has reduced catalytic activity. **Material and methods:** A total of 32 neonates with neural tube defects, 32 Mothers and 30 fathers attending Emergency, Hivi, Acoren and Gynecology and Maternity Teaching Hospitals were enrolled in the study. Ten of age and sex matched subjects with no history of Neural tube defects were recruited to the current study as control. A questionnaire form has been used to take the family information of each subject. All the patients and controls had their folic acid levels examined. The methylenetetrahydrofolatereductase (MTHFR) C677T mutation polymorphism was studied using RFLP method. The PCR amplified products were cut using polymerase chain reaction-restriction fragment length polymorphism with specific primers and digestion of the amplified products with *HinfI* restriction enzyme.

Results: The current study recruited (31) Neural tube defects patients (infants/child), with their corresponding (30) fathers and (32) mothers in Duhok City. The majority of the infants/child involved in the study were female (23, 56.1%). Twenty nine (72.5%) of the Neural tube defects patients were positive methylenetetrahydrofolatereductase (*MTHFR*) 677C→T mutation, most of them were heterozygous type (23, 79.3%). With respect to their parents, the rate of methylenetetrahydrofolatereductase 677 C→T mutation was (22, 73.3% and 26, 81.3% for fathers and mothers, respectively) and heterozygous (21, 95.5%, 21, 80.8% for fathers and mothers, respectively).

Conclusions: According to the results extracted based on the available data it was found that the Neural Tube Defect disease in infants/children could not be attributed to *MTHFR* 677 C→T mutation neither to the estimated levels of the serum folate. (Fisher's Exact Test, p=0.227)

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List of Abbreviations

1298A/C	Adenine replaced with Cytosine at nucleotide position 1298
bp	base pairs
BSA	Bovine Serum Albumin
C677T	Cytosine to Thymine substitution at nucleotide position677
CT	heterozygous of *MTHFR* gene mutation
CC	wild-type of *MTHFR* gene mutation
D.W	Distilled Water
ds	double-stranded
EDTA	Ethylenediaminetetraacetic Acid
MTHFR	Metylenetetrahydrofolate Reductase
PCR	Polymerase Chain Reaction
RFLP	Restriction fragment length polymorphism
SDS	Sodium Dodecyl Sulfate
Taq	*Thermus aquaticus*
TBE buffer	Tris-EDTA-Boric acid buffer
TT	homozygous of *MTHFR* Gene mutation
UV	UV Ultraviolet
WHO	World Health Organization
NTDs	Neural Tube Defects

Chapter One

Introduction

1. Introduction

1.1. Neural tube defects (**NTDs**) are a group of conditions in which an opening in the spinal cord or brain remains from early in human development. In the 3rd week of pregnancy called gastrulation, specialized cells on the dorsal side of the embryo begin to change shape and form the neural tube.(Friday, 2012). There are several types of neural tube defects; spinal bifida is neural tube defect which results due to failure of closure of neural tube. It is characterized by incomplete closure of spinal column. (Iqbal *et al.*). Anencephaly (pronounced an-en-SEF-uh-lee) is a more severe, but less common, type of neural tube defect (Hachinski *et al.*, 2006). Encephalocele is another rare type of neural tube defect, occurs when the tube fails to close near the brain and there is an opening in the skull (Hachinski *et al.*, 2006).

Iniencephaly, another rare but severe type of neural tube defect, is diagnosed when the infant's head is bent severely backward (Health, 2013).

In a study done in Al-Anbar, it has been revealed that the rate of NTDs was 8.5 birth defects per 1000 births. In Basrah The same rates were reported in 1998 (7.76 per 1000 births) *(Al-Sadoon et al., 1999),*and in 1994 (8.7 per 1000 births) *(Al-Hadithi et al., 2012).* Recently, a higher rate of NTDs was reported (12.36 per 1000 births) in Baghdad, Iraq in 2007 *(Hameed, 2007).* Most of these reported rates are generally lower than those reported in Turkey (11.1 per 1000 live births during 1988–1995) *(Himmetoglu et al., 1996)* and Iran (16.55 per 1000 total births) which increased from 10.46 in 2000 to 17.01 per 1000 births in 2004) *(Dastgiri et al., 2007).*

Over the years, epidemiologic studies have been instrumental in elucidating the causes of neural-tube defects in humans. Overall, these studies have suggested that genetic and environmental factors have a joint role in the causation of NTDs. (Elwood *et al.*, 1992). In particular, maternal nutritional factors have been identified as important contributors (Kirke *et al.*, 1993). The best known of these factors is the preventive role of supplemental folic acid (Group, 1991),(Czeizel and Dudas, 1992). Besides the role of folic acid, a number of maternal factors have also been found implicated in the etiology of NTD, particularly hyperthermia, diabetes, hyperinsulinemia, obesity, (Shaw *et al.*, 2003; Loeken, 2005). Another maternal factor is Maternal stress**,** Recently,

it is assumed that the health effects of maternal stress may include increased risk of certain birth defects *(Carmichael et al., 2007), (Tegethoff et al., 2011).*Also Maternal age is strongly associated with chromosomal anomalies and the rising proportion of older mothers is likely to contribute to increase in prevalence of anomalies *(Kalter and Warkany, 1983).* Maternal epilepsy and medications used to treat epilepsy, valproic acid and carbamazepine, have been also found to increase the risk of spina bifida.(Delport *et al.*, 1989) , (Hernández-Díaz *et al.*, 2001)Other factors are still mostly speculative and need to be evaluated further (Correa *et al.*, 2000), (Czeizel *et al.*, 2001).

Methylene tetrahydrofolatereductase (MTHFR) is the rate-limiting enzyme in the methyl cycle, and it is encoded by the *MTHFR* gene. *(Goyette et al., 1994).* MTHFR catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a cosubstrate for homocysteineremethylation to methionine. Some mutations in this gene are associated with methylene tetrahydrofolatereductase deficiency. (*(E Trimmer, 2013).* The best-characterized MTHFR genetic polymorphism is a common missense mutation consisting of 677C→T transition, resulting in thermolabile enzyme variant that has reduced catalytic activity (Frosst *et al.*, 1995). Another common genetic polymorphism of MTHFR is a missense mutation consisting of nucleotide 1298A→C transition, resulting in decreased MTHFR activity, which is more pronounced in homozygous than heterozygous state(van der Put *et al.*, 1998)

1.2. Aims of the study:

- The current study aims at investigating the association MTHFR 677C→T mutation polymorphism in parents and NTDs neonates.
- Also, the study aims at finding the correlation between the level of folic acid and the rate of NTDs among neonates.

Chapter Tow

Review of literature

2. Review of literature

2.1. Congenital disorders:

Although is called as **birth defect, congenital disease**, or **anomaly. Pooler (2009)** stated congenital diseases are the foremost cause of infant mortality in developed countries and a key cause of health problems. (Harris and Juriloff, 2007). It is predictable that around 10-15% of the structural congenital anomalies are the consequence of the negative influence of environmental reasons on the growth before birth (Brent, 2001). Thus about 1 in 250 newborns and structural defects caused by exposure to environmental causes, and presumably, the biggest number of children have a developmental delay or functional abnormalities resulting from the causes of nongenetic, in other words, the effects of teratogens. (AbuRuz *et al.*, 2015). A teratogen is well-defined as any environmental factor that can produce a permanent abnormality in structure or function(Al-Sadoon *et al.*, 1999), restriction of growth, or death of the embryo or fetus. A dose-response relationship should be demonstrated in animals or humans as the greater the exposure throughout pregnancy, the additional severe the phenotypic effects on the fetus (Frías and Gilbert-Barness, 2008).

Genetic causes of congenital anomalies include inheritance of abnormal genes from the mother or the father, in addition to the new mutations in one of the germ cells that provided rise to the fetus. Male germ cells mutate at a

greatly faster amount than female germ cells, and as the father ages, the DNA of the germ cells mutates quickly (Sartorius and Nieschlag, 2009) *(Zhu et al., 2005)*

In condition, an egg is fertilized with sperm that has damaged DNA, there is a opportunity that the fetus could progress abnormally *(Sartorius and Nieschlag, 2009)*. Animal studies designate that the mother's (and likely the father's) diet, vitamin intake, and glucose levels *preceding* to ovulation and conception have long-term effects on fetal development and adolescent and adult illness (Rutecki, 2010)

Besides, in some data shown, congenital anomalies resulted in about 632,000 deaths each year in 2013 down from 751,000 in 1990. Congenital heart disease are the type with the greatest numbers of deaths with (323,000), followed by neural tube defects (69,000) *(Naghavi* et al., *2015)*.

2.2. Types of Congenital Abnormalities

A human being has twenty-three pairs of chromosomes. One member of each pair is taken from the father, and the other member from the mother. Chromosomes carry genes, the basic units of heredity. Genes are self-possessed of DNA base pairs, which work through coding for the making of proteins. (Lewis, 2007).

2.2.1.Chromosomal disorders

Ascend from faults in the packing of the genetic material. (Moraczewski and Atkinson, 1980).Either one, excessively chromosomal material is present, or not

enough is present, or the chromosomal material has been misplaced and reordered (States, 1990). In a small number of cases, these disorders are hereditary from one of the parents who are a carrier, but in the massive mainstream of cases they ascend when a defective germ cell (the sperm or the ovum) is formed or when the cell is subjected to some alteration. Furthermost pregnancies with chromosomal disorders are spontaneously aborted, but some go to term ((Moraczewski and Atkinson, 1980).

2.2.2. Single-Gene Abnormalities

A single-gene defects can arise from abnormalities of both copies of an autosomal gene (a recessive disorder) or of only one of the two copies (a dominant disorder). Some circumstances result from deletions or abnormalities of a small number of genes placed contiguously on a chromosome.

Furthermore, Single-gene disorders can be passed on to subsequent generations in numerous ways. Genomic imprinting and uniparental disomy, however, may affect inheritance patterns. (Williams and Obaro, 2011)

2.2.3. Autosomal dominant inheritance:

Merely one mutated copy of the gene will be necessary for a person to be affected by an autosomal main disorder. Each affected person usually has one affected parent(Raman *et al.*, 2009). The chance a child will receive the mutated gene is 50%. Autosomal main circumstances occasionally have reduced penetrance, which means although only one mutated copy is needed, not all individuals who inherit that mutation go on to develop the disease. Examples of this sort of disorder are Huntington's disease. (Contreras de Vera, 2015)

2.2.4. An autosomal recessive disorder

This type is caused by abnormalities in both members of a pair of genes. Both parents may carry a single dose of the gene on one chromosome but have a normal gene on the other chromosome, which prevents the appearance of the disease in the carrier parent. A child must get one abnormal gene from each parent in order to be afffected by the disease. ((Lebacqz, 1983)).

2.2.5. An X-linked recessive disorder

When the abnormal gene is carried on the X chromosome this disorder occurs. A female with an abnormal gene on one chromosome will usually not have symptoms because of the normal partner gene on the other X. On the other hand, if a gene on the X chromosome of the male is abnormal, there is no partner gene on the Y to compensate, and the symptoms of the disorder will occur. Hence, X-linked disorders are carried by females, but mainly affect males. (States, 1990).

2.2.6. X-linked dominant inheritance:

Occasionally stated to as X-linked dominance is a mode of genetic inheritance by which a dominant gene is carried on the X chromosome. As an inheritance form, it is fewer common than the X-linked recessive sort. In medicine, X-linked dominant inheritance indicates that a gene responsible for a g/'enetic disorder is located on the X chromosome, and only one copy of the allele is adequate to cause the disorder when inherited from a parent who has the disorder *(Dobyns et al., 2004)*

2.2.7. Multifactorial disorders

Are caused by a combination of genes inherited from parents and environmental factors. In different meanings, defective genes predispose individuals to a condition, but further causes seem essential for the existence of the sickness. ((Moraczewski and Atkinson, 1980). Defects due to multifactorial

inheritance include congenital heart disease, furthermost sorts of cleft lip/palate, club foot, and neural tube defect (e.g., anencephaly and spina bifida). ((Atkinson *et al.*, 1980).

2.3. Conditions During Pregnancy That Affect the fetus development:

Certain diseases during pregnancy: like **Diabetes,** It is important for women with diabetes to manage their blood sugar levels before getting pregnant. Elevated blood sugar levels can cause birth defects during the first few weeks of pregnancy, often before women even know they are pregnant (Gee and Corry, 2012)

In the case of Alcohol consumption : The mother's consumption of alcohol during pregnancy can cause a continuum of various permanent birth defects : cranofacial abnormalities,(Jones and Smith, 1975) brain damage "(Clarren et al., 1978) intellectual disability "(Abel and Sokol, 1986) heart disease, kidney abnormality, skeletal anomalies, ocular abnormalities.(Strömland and Pinazo-Durán, 2002). Very few studies have investigated the links between paternal alcohol use and offspring health.(De Santis et al., 2008).

Smoking during pregnancy:

Generally smoking and more particularly the carbon monoxide (CO) and nicotine from cigarettes, has negative impacts on both maternal and foetal health. Nicotine and CO reduces foetal oxygen supply. In addition, nicotine increases foetal blood pressure. Moreover, due to placental characteristics, nicotine and CO levels in the foetus are significantly higher than those found in the mother .(Matters).

Lack of nutrients:

For example, a lack of folic acid, a vitamin B, in the diet of a mother can cause cellular neural tube deformities that result in spina bifida. Congenital disorders such as a neural tube deformity (NTD) can be prevented by 72% if the mother consumes 4 milligrams of folic acid before the conception and after 12 weeks of pregnancy (Raats *et al.*, 1998) Folic acid, or vitamin B_{12}, aids the development of the foetal nervous system (Raats *et al.*, 1998).

Medications and supplements

The most notorious teratogenic drug is the thalidomide, developed at the end of 1950 by Chemie Grűnenthal as a hypnotic and antiemetic and therefore frequently prescribed to pregnant women in almost 50 countries worldwide between 1956–1962 (Raats *et al.*, 1998)\

2.3.4. Unknown Causes

Even though important development has been made in recognizing the etiology of some birth defects, approximately 65% have no known or identifiable reason (O'rahilly and Müller, 1996) . These are mentioned to such as sporadic, a term that indicates an unknown cause, random occurrence nevertheless of maternal existing conditions, *(Bezerra et al., 1999)* and a low recurrence risk for children in future. For 20-25% of anomalies there appears to be a "multifactorial" cause, meaning a complex interaction of multiple minor genetic anomalies with environmental risk causes. Additional 10-13% of anomalies have a purely environmental reason (e.g. infections, illness, or drug abuse in the mother). Only 12-25% of anomalies have a purely genetic cause. Of these, the majority are chromosomal anomalies. (Kumar et al., 2014).

2.4. Neurulation and Neural Tube Defects

Basically, Neurulation is a fundamental event in embryogenesis that culminates in the foundation of the neural tube, which is the precursor of the brain and spinal cord (Wallingford, 2005). Essentially, Neurulation arises with the foundation of the neural plate as a thickening of the dorsal ectoderm. Then, Neural plate shapes, with the procedures including convergent extension. After that, the neural plate bends, elevates and begins to move towards the midline. The next phase is extremities come into contact and fuse to create the neural tube, which, thereafter, becomes covered by epidermal ectoderm. To bear in mind, Closure of the cranial neural tube is essential not only for maintenance of brain development but also for initial formation of much of the skull (Copp, 2005).

It is worth mentioning, that the fusion of neural folds, is subject to debate concerning the number of initiation sites of fusion and their location. Neural tube closure depends upon the cooperation of several mechanisms: convergent extension of the neural plate, neuroepithelial apoptosis, neural crest cell migration, proliferation and differentiation. The development and closure of the neural tube is completed 28 days after conception. In condition, neural tube closure fails, the embryo develops an NTD. However, some authors also support the possibility of some NTD resulting from a closed neural tube secondarily reopening (Padmanabhan, 2006).

2.5. Neural Tube Defects:

Neural tube defects (NTDs) are a common group of central nervous system anomalies affecting 0.5–2 per 1000 pregnancies worldwide. NTDs arise when the neural tube, the embryonic precursor of the brain and spinal cord, fails to close during neurulation. (Detrait *et al.*, 2005) . Neural tube closure takes place between the 3rd and 4th week of in utero development and is influenced by a complex multifactorial etiology including both genetic and environmental factors. (Au *et al.*, 2010)

In other researches it is been indicated that the NTDs can be classified in "open" NTDs in which the neural tissue is exposed and "closed" NTDs with the neural tissue covered by tissue (Sadler, 2005) .The clinical features of NTDs vary greatly. The two foremost frequent sorts of NTDs are anencephaly and myelomeningocele which seem about 40 and 50 percent of NTDs detected in established pregnancies, respectively (Copp *et al.*, 2013). Besides, the clinical features of anencephaly are the lack of brain and cranial vault which are associated with fetal loss or stillbirth.

Furthermore, Myelomeningocele is associated with an open spinal cord protected only with the meningeal sac (SB cystica) or completely exposed (known as spina bifida aperta). Myelomeningocele is usually associated with live births, with symptoms including hydrocephalus which can cause increased intracranial pressure inside the skull and progressive enlargement of the head and mental disability [10]. Craniorachischisis is the most severe disorder of primary neurulation, clinically characterized by the complete absence of skull and extensive defects in the vertebrae and skin. Supreme cases are related with spontaneous abortion early in pregnancy (Puvirajesinghe and Borg, 2015).

Finally, "Open" NTDs include craniorachischisis consequential from a total failure of neurulation with most of the brain and the entire spinal cord remaining open, anencephaly once the defect occurs in the cranial region and spina bifida cystica when the defect is localized in the lumbosacral area. In this last defect, if meninges and cerebrospinal fluid herniates through the defect, it is referred as meningocele, while a myelomeningocele directly involves spinal cord and/or nerve roots (Thompson, 2009).

2.5.1. Open" NTDs include:

2.5.1.1. Anencephaly

Anencephaly (pronounced an-en-SEF-uh-lee) is a supplementary severe, but less common, type of neural tube defect. This condition occurs when the neural tube fails to close at the top. The fetus has little or no brain matter and also may be lacking part of its skull. Infants born with this condition are usually unconscious as well as deaf and blind and unable to feel pain. They may have reflex actions, such as breathing and responding to touch. All infants with anencephaly are stillborn or die soon after birth (Health, 2013).

2.5.1.2. Encephaloceles

Additional rare sort of neural tube defect, happens once the tube fails to close near the brain and there is an opening in the skull. The brain and membranes that cover it can protrude through the skull, forming a sac-like bulge. Some cases, there is only a small opening in the nasal or forehead area that i s not noticeable (Hachinski *et al.*, 2006).

2.5.1.3. Hydranencephaly

Hydranencephaly or **hydrancephaly** is a circumstance wherein the brain's cerebral hemispheres are absent to varying degrees and the lasting cranial cavity is filled with cerebrospinal fluid (Kandel *et al.*, 2000).

2.5.1.4. Iniencephaly

Iniencephaly (pronounced *in-ee-ehn-SEF-ah-lee*), is another unusual but severe sort of neural tube defect, is diagnosed once the infant's head is bent severely backward. The spine is exceptionally distorted. Habitually, the infant lacks a neck, with the skin of the face attached to the chest and the scalp attached

to the back. In other abnormalities may occur, as a cleft lip and palate, cardiovascular irregularities, anencephaly, and malformed intestines. It is worth mentioning, infants born with this condition frequently do not live longer than a few hours (Grinnon *et al.*, 2012). \

2.5.1.5. Spina bifida

This sort which is called 'Spina bifida' is neural tube defect which consequences due to failure of closure of neural tube. Although, it is characterized by incomplete closure of spinal column. In SB spinal cord, its coverings and vertebral arches progress abnormally throughout gestation (Iqbal *et al.*).

2.5.1.5.1. Spina bifida cystica

This sort is it is obvious a cystic swelling is present at the site of lesion. The cystic swelling may contain meninges or both meninges and spinal cord. Spina bifida cystica has two sub-variants:

- Spina bifida cystica with meningocele
- Spina bifida cystica with myelomeningocele (Treble *et al.*, 2013)**.**

2.5.1.5.1.1.Meningocele:

This sort is fewer severe and is categorized by herniation of the meninges, nevertheless not the spinal cord, over the opening in the spinal canal. *(Le Tao and Neil, 2010)*

2.5.1.5.1.2. Myelomeningocele

This sort includes herniation of the meninges as well as the spinal cord through the opening *(Le Tao and Neil, 2010).*

2.5.1.6.2. Spina bifida occulta

In this type of neural tube defect, the meninges do not herniate through the opening in the spinal canal.(Le Tao and Neil, 2010) .It is a common condition, occurring in 10–20% of otherwise healthy people By definition, spina bifida occulta means hidden split spine. (Kidega, 2012). The most frequently seen form of spina bifida occulta is when parts of the bones of the spine, called the spinous process, and the neural arch appear abnormal on a radiogram, and is generally harmless. Usually the spinal cord and spinal nerves are not involved. (Pittman, 2008)

"Closed" NTDs, encompass encephalocele and spina bifida occulta. Encephalocele is a defect of the bony skull through which part of the brain herniates. Spina bifida occulta, results from a gap in one or more vertebral arches in the lumbosacral area, but the spinal cord and meninges remain entirely within the vertebral canal. In these types of defects the folds may have come together, but the normal fusion process was disrupted. Unlike the cranial defects, which are usually lethal at or before birth, spina bifida is compatible with postnatal survival. Spina bifida cystica is more severe; these patients being at increased risk for morbidity and mortality throughout their life. Only 1% of children born with an open NTD are free from disability. Affected patients usually have anesthesia of the skin, abnormalities of the hips, knees, and feet, reduced ability to walk or need a wheelchair, have little or no bowel and/or bladder control, and require frequent surgical interventions to minimize the effects of hydrocephalus (Thompson, 2009). Thus, the lifetime medical costs of spina bifida affected patients are considerable. (Yi *et al.*, 2011)

2.5.2. Epidemiology of NTD

The prevalence of birth defects are reported in Iraq, (AbuRuz *et al.*, 2015), Al-Anbar study detected a rate of 8.5 birth defects per 1000 births. *(Al-Sadoon et al., 1991)* reported Identical rates in Basrah in 1998 (7.76 per 1000 births) and 1994 (8.7 per 1000 births) *(Habeeb and Al-Sadoon, 1995)* .Recently in 2007 a high rate of 12.36 per 1000 births was reported from Baghdad, Iraq . *(Hameed, 2007)* .

The most common kinds of birth defects between live births were central nervous system defects *(Al-Sadoon et al., 1991).* The rate of neural tube defects (NTDs) that was reported from Duhok, Iraq was (4.7 per 1000 live births) *(Abdurrahman, 2007).*

But in Baghdad the higher rates of NTDs were reported (5.95 per 1000 births) (Hameed, 2007) and Diwaniyah (8.4 per 1000 total births in 2000) (Al-Shammosy, 2002) of Iraq.

In Turkey, NTDs are one of the most acute congenital dosorders; epidemiological findings suggest that predominance levels change with demographic and regional features. However, this information is actually not available in the medical registry system of Turky. (Tunçbilek, 2004). According to data gained from different cities of Turkey (Himmetoglu *et al.*, 1996) (Tuncbilek *et al.*, 1998) the spread of NTD ranges from 3-5.8 per 1000 births (Tunçbilek, 2004) Rates as high as 8.9 in Izmir while in Mustafa Kemal Pasa of Bursa 20.0 per 1000 births have been listed following the Chernobyl disaster (Hoffmann, 2001). A university of clinical study demonstrated that in Northern and Eastern Anatolia the frequency of NTD is highest, with an incidence of 4.32 and 4.54 per 1000 live births, but in Western Anatolia is lowest, with an incidence of 2.17 per 1000 live births. This rate was 9.1 per 1000 live births in uneducated mothers who were estimated based on education level of mothers. (Tunçbilek, 2004).

In some parts of the Islamic Republic of Iran previous studies on Neural Tube Defects have been carried out out. In the capital, Tehran a study on 13 037 births (1969–78), 17.6/10 000 newnates had NTD (Farhud *et al.*, 1986).In Hamadan in another study of 8585 deliveries in (1991–97) (a north-west province), the prevalence of NTD was 50.1/10 000 (Farhud *et al.*, 2000) and out of 14 915 births, 55.0/10 000 newborns was affected by NTD in Cannadajh–Kordestan (another north-west province), (Mohammadbegi and Rahimi, 2002).

Many studies, about the epidemiology of NTDs, have been reported from different areas of Saudi Arabia (Ali et al., 2011). All of these studies have been fundamentally directed toward estimating the happening of the NTDs.A study (Dawodu et al., 1988) From King Fahad Hospital at Al Khobar in the Eastern province detect an incidence, over a 10 year period, of 1.83 per 1000 live births. Magbool et al, (Magbool et al., 1989). reported an happening of 1.04 per 1000 live births, and also Khaliji et al, (Thaliji et al., 1986). from the Eastern Province, found an elevate incidence of 1.6/1000. Murshid (Murshid, 2000) in Al Madina (1.09/1000) and (Safdar et al., 2007). in Jeddah (1.3/1000) reported the incidence which is much higher than those reported from the Southwest Asir area in 1992 (0.82/1000) (El Awad, 1991) and in 2001 (0.78/1000). (Asindi and Al-Shehri, 2001).

China, India and Ukraine are areas having the highest cases, (Yuskiv *et al.*, 2004). In Middle East and Jordan there are little reports concerning to the recurrence of NTDs *(Masri, 2006)*.Jordan doesn't have an ongoing surveillance system for congenital disorders. However, in Jordan the happening of NTDs is notified to be as high as 6.5 per 1000 live births based on a study done at King Hussein Medical Centre. *(Aqrabawi, 2005).*

2.5.3 Causes of neural tube defect

NTDs have multifactorial and complex etiologies which involve life style,genetic, and environmental factors. Chromosomal abnormalities can be related to NTDs, but only appear in 2% to 16% of isolated NTDs (Lynch, 2005). Many observations support that the genetic factors are related to the formation of NTDs: first,an elevated danger in some ethnic groups (e.g., Irish and Mexican) and second, the high repetition risk for siblings of infected individuals. Environmental influences like maternal obesity ,maternal nutritional and parental occupation case have been associated to NTDs in addition to genetic factors. (Au *et al.*, 2010).

2.5.3.1. Genetic Factors:

The series of the coding places detected that patients who are affected by neural tube defects (NTDs) have missense mutation (i.e. of amino acid). **(Netto *et al.*, 2009).**

The function of folate metabolism is done by enzymes which are encoded by NTD related genes. Among these groups of enzyme, one enzyme is 5, 10 methylene tetrahydrofolate reductase (MTHFR),(Jelsma and Ploetner, 1953)

2.5.3.2. Non-Genetic factors:

When the pregnant women taking the anti-convulsant valproic acid (Depakin) during pregnancy, the fetus will be infected with NTDs. (Iqbal *et al.*) The valproic acid's action is to prevent histone decarboxylase, Which changes the way the proteins leading to NTDs (GARDNER, 1960). Intaking of Periconceptional of folic acid decreases neural tube defects by 70 percent. Drugs of Antiepileptic are related to NTDs. anther factors related to neural tube defects include maternal obesity ,maternal pyrexia, poor nutritional status , maternal diabetes, vitamin B12 and folate insufficiency. (Bauer *et al.*, 1977)

2.5.3.3. Environmental factors:

The environmental factors includ following:

• Organic solvents
• Indoor air pollution
• Pesticides
• Polycyclic aromatic hydrocarbons
• Air pollution
• Nitrates related compounds. (Copp *et al.*, 2015)

Following factors participate in the epidemiology of NTDs:

- Maternal hyperthermia is one of the beginning factors of pregnancy resulting in NTDs with drug usage and maternal caffeine during pregnancy. **(Müller and O'Rahilly, 1987) (McLone and Knepper, 1989)**

- There is raised risk of NTDs in the families of low socioeconomic conditions, (Iqbal *et al.*)

- In mothers with age less than 19 years and more than 40 years , the risk of NTDs is increased risk . **(Drake *et al.*, 1998).**

Particularly, before more than 40 years, it has been proposed that maternal folate case is related to the risk of NTD (Hibbard, 1967). A fundamental number of reviews have been published on folic acid and NTDs (Blom, 2009).

2.6. Association of folic acid with Neural Tube Defects

Folate is a water soluble B-vitamin (vitamin B9) that is fundamental for the synthesis of DNA and RNA and various methylation reactions.Normal diet have polyglutamates and folate monoglutamates which have to be hydrolyzed to monoglutamates for the absorption intestines. The monoglutamate form, 5- methyltetrahydrofolate (5-methyl THF), is the dominant folate in the circulation if blood. 5-methyl THF is taken up into cells by tissue-specific folate receptors

and is isolated after polyglutamylation. Polyglutamated folates do not go through cellular membranes and are thus kept inside the cell (Imbard *et al.*, 2013) .Within the cell, folate acts as an acceptor and a donor of one-carbon units in many critical lanes. These pathways include catabolism or synthesis of amino acids (glycine ,serine, methionine and histidine,) and production of nucleotides (thymidine and purines).

The maternal folate case was related to Neural Tube Defects danger before 40 years ago (Botto *et al.*, 2005) .In the early 1990s two researches suplied unequivocal guide that maternal supplementation with folic acid, the more constant synthetic shape of folate, through pregnancy stopped both Neural Tube Defects repetition (Group, 1991) and appearance (Czeizel and Dudas, 1992) recommend the pregnant women to take 400 micrograms of folic acid of childbearing age. Up to 70 percent of neural tube defects can be prevented by the concentration of folic acid in the red blood cell optimized mothers. Therefore, the exact mechanism of how can folic acid prevent neural tube defects is not clear (Blom *et al.*, 2006)

The advantage of folate supplementation at the periconception in decreasing the danger of NTDs in offspring has been demonstrated both in observational and experimental studies. (Intakes, 1998).

The first neural tube defects translational research study investigated the supplementation of folic acid for recurrence prevention of neural tube defects in a randomized double-blind clinical trial including 1195 completed high danger pregnant women from 33 centres .(Sayed *et al.*, 2008) .The NTD repetition level lowered from 3.5% in a non-supplemented group to 1% for women randomized to the group taking an oral 4 mg folic acid supplementation every day before to pregnancy and during the first 6 weeks of pregnancy. The second NTD translational research study was a randomized controlled trial for the primary prevention of NTD happiness. (Czeizel and Dudas, 1992) In 2471 women taking 0.8 mg per day of folic acid the recurrence of NTDs was zero compared with 6 cases in 2391 women not taking folic acid. This RCT study supported previous case–control studies that had supplied evidence that pregnant women using multivitamins having folic acid or dietary folic acid had a lower risk of occurrence NTDs than women not recieving supplements.(Bower and Stanley, 1989).

2.7. Genetic of Neural Tube Defects:

2.7.1Gene mutations associated with NTDs:

(**Methylene tetrahydrofolate reductase** (**MTHFR**) :

The 5, 10- *MTHFR* gene is located on chromosome 1 at 1p36.3. The complementary DNA sequence is 2.2 kilobases long and consists of 11 exons (Figure 2.1.).

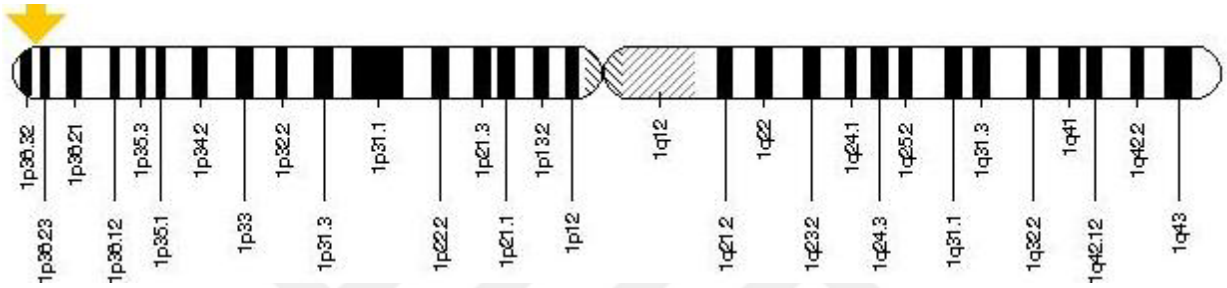


Figure (2.1.): The *MTHFR* gene is existing on the short (p) arm of chromosome 1 at 36.3 position. More exactly, the *MTHFR* gene is located from base pair 11,785,729 to base pair 11,806,102 on chromosome 1 (Camp and Trujillo, 2014).

In humans, the main product of the *MTHFR* gene is a catalytically active 77-kilodaltons protein, though a minimal isoform of approximately 70- kilodaltons has been observed in some tissues (Rozen, 1997).

MTHFR stimulates the transformation of 5, 10-MTHF into 5-MTHF which is the major circulating form of folate . (Chango *et al.*, 2000) .Genetic polymorphisms in the MTHFR gene are well established, the most extensively studied of which are C677T and A1298C single-nucleotide polymorphisms (SNPs). (Brattström *et al.*, 1998) .

2.7.1.1. C677T allele:

Mutation (C677T) arises from a point mutation at position 677 of the MTHFR gene that converts a cytosine (C) into a thymine (T); this mutation results in an amino acid substitution (alanine to valine) in the enzyme . (Frosst *et al.*, 1995) .At 37°C or more the activity of the encoded enzyme is reduced hence the C677T allele is commonly called "thermolabile" (Kang *et al.*, 1991). Accordingly, among C677T homozygotes the activity of MTHFR is 50-60 percent

lower at 37°C and approximately 65 percent lower at 46°C than in similarly treated controls. Heterozygotes are in the intermediate range. Moreover, people who are homozygous for the C677T allele tend to have mildly elevated blood homocysteine levels if their folate taking is insufficient but normal blood levels if their folate intake is adequate .(Rozen, 1997). Persons with a homozygous 677TT genotype have an activity of the MTHFR enzyme of 30% compared to the wild variant while 677CT heterozygotes have a 65% activity of the enzyme. (Frosst *et al.*, 1995) Homozygosity for the T allele of the C677T polymorphism of the gene encoding the folate dependent enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) is a risk factor for NTDs (Botto and Yang, 2000) Both the heterozygous (CT) and the homozygous (TT) and genotypes are related with higher homocysteine concentrations, lower tissue levels of folate and lower enzyme activity than the wild type (CC) genotype; these effects are more marked in homozygotes. Low folate and increased homocysteine levels in the beginning of pregnancy are risk factors for NTDs. (Mills *et al.*, 1995)

2.7.1.2. A1298C allele (C1289A)

It has been demonstrated that in the A1298C allele, a point mutation in exon 7 causes the substitution of glutamate for an alanine residue. (De Marco *et al.*, 2002) and has also been related to a mild reduction in enzymatic activity (van der Put *et al.*, 1998).

Chapter Three
Material and Methods

3.1. Materials

Table (3.1): Chemicals

NO	Chemical	Chompany	Origion
1-	Agarose (Analytical Grade)	Promega	ESP
2-	BSA (Bovine Serum Albumin)	Promega	USA
3-	TBE Buffer10X	Promega	USA
4-	DNA Ladder 100 bp	Promega	USA
5-	Ethanol Absolute 70%	Scharlau	Spain
6-	Ethidium Bromide	Promega	USA

7-	EDTA (Di sodium salt)	BDH	England
8-	Loading Dye- Blue/Orange 6X	Promega	USA
9-	Sodium Dodecyl Sulfate (SDS)	Promega	USA
10-	Tris-Base	Sigma	USA
11-	Protinase– k	Promega	

3.1.2. Equipment's and apparatus:

Table (3.2), shows the aparatuses used for Performing the practical part of the current study.

Table (3.2): Equipments

NO	Equipment	Company	Origin
1-	Automated pipettes (Different Sizes)	Physio Care Concept	Germany
2-	Electrophoresis Power Supply	GE Healthcare	China
3-	Enduro Gel XL	Labnet	USA
4-	Electronic Balance	Kern	Germany
5-	Milli-Q-Ultrapure Water	Millipore	France
6-	Microfuge 18 Centrifuge	Beckman Coulter	Germany
7-	Mini Gel Compact Electrophoresis	Labnet	USA
8-	Revolver (Mixer)	Labnet	USA
9-	Thermal cycler	Applied Biosystems	Singapore
10-	Vortex V-1 plus	Biosan	EU
11-	Water Bath	Ycw	USA
12-	Autoclave	Labtech	Korea
13-	PH- meter (Ecotester PH2)	Oakton	Malaysia
14-	Multi-spin	Biosan	EU
15-	Minifuge	Labnet	Korea
16-	Proxima 10 phi	Isogin	Holland

3.1.3. Enzymes and primers:

In the current study one kind of primers with one restriction enzyme were used. This enzyme and primers with their concentrations, production companies and country of origin are shown in table (3.3).

Table (3.3): Enzyme and the primers

Restriction **Enzyme**	**Concentration**	**Company(origin)**
Hinf I	10 u/µl	Jena Bioscience Germany

Oligo- nucleotides Targeting *MTHFR* gene	**Sequence (5'→3')**	**Company(origin)**
F	**GCCTCTCCTGACTGTCATCC**	Jena Bioscience Germany
R	**GGAGCTTATGGGCTCTCCTG**	Jena Bioscience Germany

3.1.4. PCR master mix kit:

The HotStarTaq Master Mix kit (QIAGEN) has been used to prepare the PCR Mix for each sample according to the manufacture thesis.

3.1.5. Subjects:

In current study, was conducted in Duhok Medical Research Center (DMRC) at the college of medicine, university of Duhok. Between (February - October 2016).

A total of 32 neonates with neural tube defects, 32 mothers and 30 fathers from attending emergrncy, Hivi, Acoren and Gynecology and maternity hospitals were enrolled in the study. Ten of different age and sex matched subjects with no
history of Neural tube defects were recruited to the current study as control. After getting the consent, a questionnaire form has been used to take the family information of each subject.

3.2. Methods

3.2.1. Sampling and sample handling:

Three milliliter (ml) of peripheral venous blood was collected by venipuncture from each subject involved in the study. Each taken sample was then divided in to two tubes as follow:

- One and a half mL of blood was placed in sterilized tube containing anti-coagulant (EDTA), mixed well, then stored at (- 20°C) to be used for isolation of genomic DNA.
- The remaining 1.5 ml of blood was left at room temperature centrifuged within 20 to 30 minutes to obtain serum and kept in freezer to be used later for folic acid estimation.

3.2.2: Folic acid measurement

The (Folate III kit, Cobas, Roche) was used to estimate the folic acid level in the sera of all NTD patients with their mothers and controls according to the manufacturer's instruction.

3.2.3. Molecular methods

3.2.3.1. Solutions and buffers preparation:

- **NaCl (5M) :**

It was prepared by dissolving (146.1) g of NaCl in (350) ml of D.W, then brought up to 500 ml with D.W. and the volume was sterilized by autoclaving at 15 p.s.i. for 15 min.

- **Ethanol (75%%): 75 ml** of Ethanol was added to 30 ml of deionized water then prechilled to -20°C.
- **TBE buffer:**

It is prepared by adding 54g of Tris base with 27.5 g of boric acid and 20 ml of 0.5 M EDTA, then adjust pH to 8.3 by HCL.

- **SDS :** 200 g SDS in 800 mL H2O
- **TES :**
-

- **Primers:**

According to the manufacturer instruction, in order to prepare 23.7 n.mol of the forward primer, it has been dissolved in 237 ul of d.d H2O, and in order to obtain the 20.4 n.mol of the R primer, it has been dissolved in 204 ul of d.H2O.

- **Ethidium bromide staining:**

This was prepared by dissolving 50 µl of Ethidium bromide in 1 Liter D.W.

3.3. Procedure of DNA extraction:

The salting – out DNA extraction procedure from whole blood was used as follows:

1- 0.3 ml of mixing blood (fresh or frozen) was taked to sterile 1.5 ml ependrof tubes.

2- 0.9 ml of cold DW were added to the blood and vortex mixing (washing steps).

The tubes were then centrifuged for 10 minutes at 13500 r.p.m using a Minor bench centrifuge, after centrifugation 0.9 ml of the supernatant was removed and this step another 2 times. In the last washing >0.9 ml was removed and only little of DW with the setteleddoen material were kept.

3- 0.3 ml of TES(NaCl/EDTA: Ph 7.5) were added . Also 50 µl of proteinase K 1%, 24 µl of 10 % SDS were added and the mixture is vortex mixing to get Homogeneity.

4-Incubating for 1 hour or even more on dry hotplate 54 °C.

5-After incubation 120 µl of NACL (-6 M) were added,then vortex to get homogeneity; centerifuged for 10 minutes at 13500 rpm using a Minor bench centrifuge, after centrifugation most of the supernatant were taked to anew 1.5 ml ependrof tubesand the old tube were discarded with its content (debris and NaCl).

6- 300 µl of Isopropanolol were added and mixed gently until DNA has to be seen.

7- Then it was centrifuged for – 20 sec at 13500 rpm using a Minor bench centrifuge, after centrifugation (the DNAsettled down in the bottom) as possible as of the supernatant were removed carefully (be caution of DNA).

8-Then1 ml of 75% Ethanol were added and mixed gently then centrifuged for 1-2 min. at 13500 using a Minor bench centrifuge, after centrifugation (the DNA setelled down in the bottom) as possible as of the supernatant were removed carefully(be caution of DNA) and the tubewere kept open for 10-20 min.The DNA were left to dry and the remaining alcohol to evaporate (avoiding the excessive dryness).

9- 20-100 µl of DW were added (water for injection) according to the amount of DNA extracted.

10- The number and the date were written on the cup of the tube and the side also.

11- DNA is ready to use after 2 hrs. or so.

3.4. Polymerase chain reaction (PCR)

The method of (Frosst P., Blom H., Milos P., Sheppard C., Matthews R., Boers G. (1995). A candidate genetic risk factor for vascular disease: A common mutation in methylenetetrahydrofolate reductase. Nature Genetics ; 10: 111-113.) was followed for investigating *MTHFR* C677T mutation, amplification accomplished by using genomic DNA and the primers 5'TGAAGGAGAAGGTGTCTGCGGGA- 3'
and 5'-AGGACGGTGC GGTGAGAGTG-3' to produce a 254 bp fragment.

- **Procedure for amplification of *MTHFR* gene:**

The components of master mix for *MTHFR* gene amplification and reaction mixture and are detailed as follow:

Table (3.4): Components of reaction mixture for *MTHFR* gene.

Ingredients	**1X reaction**	***n x* reaction**
Master Mix (µl)	10	*n* x 10
Primer MTHRF-R (µl)	0.8	*n* x 0.8
Primer MTHRF-F (µl)	0.8	*n* x 0.8
dH2O (µl)	2	*n* x 2
Enhancer solution*	2	*n* x 2
coraload	2	*n* x 2

The enhancer (Peqlab) was used to promote the PCR.

- The extracted DNA samples were allowed to thaw and the PCR master mix Kit (Qiagen) was used to prepare the reaction mixture . Then 17.6 µl of reaction mixture was poured

into each 0.5ml Eppendorf tube and finally 2.8µlof the extracted DNA sample was added to each tube.

- Each tube then labeled, smoothly mixed and spun down for few seconds for avoiding air bubble formation.
- The Eppendorf tubes were then placed in the thermo-cycler to

perform amplification. The Amplification program was run as follows:

Thermal Cycling:

Initial denaturation 94°C for 5 minutes.

Thermo cycling (35 cycles)**:**

- ❖ Denaturation 95°C for 20 sec
- ❖ Annealing 63°C for 1 min
- ❖ Extension 72°C for 30 sec
- ❖ Final Extension 72°C for 7 minutes.

Then the PCR products were electrophoresed on (0.8) % agarose gel to confirm amplification and UV imaged for documentation.

3.5.RLFP of the *MTHFR* gene:

3.5.1 Digestion of the amplified products:

The amplified product was digested with *Hinf*I restriction enzyme to identify the *MTHFR* C677T mutation. This enzyme cleaves only the mutant *MTHFR* allele (C677T) into further fragments .The components of digestion master reaction are detailed below:

Table (3.5): Components of MTHFR PCR product digestion by *Hinf* I, the reaction mix is shown below.

Ingredients	**1 X reaction**	***n x* reaction**

Buffer B (Promega-U.S.A)	1.0µl	*n* x 1.0
BSA	0.12µl	*n* x 0.12
*Hinf*I restriction enzyme	0.2µl	*n* x 0.2

After preparation of master reaction, 1.3µl aliquots were added to each tube that contains 20.4µl of amplified PCR product of each sample separately, then by using shaking water bath the samples (final volume 21.7µl) were incubated at 37ºC overnight .

Then the tubes were removed from the water bath at the end of enzyme digestion and by using electrophoresis in 2% agarose gel the digestion products were analyzed followed by UV light photography.

3.5.2 Agarose gel electrophoresis:

The method of Maniatis *et al.* was used with few modifications for the optimization.

3.5.3 Preparation of agarose gel:

The agarose gel was prepared in concentration of 2% for PCR-RFLP product of the amplified and digested amplicons. Agarose gel was made by adding (1%) g of agarose by using (Electronic Balance) to 100 mL of 1X TBE buffer.

Microwave oven was used to solubilize the mixture by heating at boiling temperature for 2 mintes, then left it to cool to 55°C. Afterward, 7 µl of ethedium bromide were added. Later, it was gradually poured into a plastic tray and with combs placed close to cathode end. The gel was allowed for 30 min to harden at room temperature and then the comb removed gently from the tray. 1X TBE buffer was powered into the selected submerged electrophoresis tank and the gel tray was placed horizontally in it, in order that the buffer was allowed to cover the gel to the depth of 3-5 mm.

3.5.4. Loading the gel: On paraffin film, 7µl of the PCR products were smoothly loaded to the respective well in the gel cautiously (spillover to next wells was not permitted). One lane was left for loading a DNA Ladder .For the ladder, mix 7µl of ladder with 3 µl of of blue/orange 6X loading dye, The ladder was producing 11 bands of DNA in different molecular weights ranging between 100-1500 bp.

3.5.5. Running the gel:

After securing the lid and the electrodes of the electrophoresis tank**,** ninety volts were applied for 45 Minutes, until the DNA left the wells and moved in the direction of the positive electrode. Then after that, the gel was examined with the UV-transilluminator followed by documentation of the results by photography using (Proxima 10 phi).

3.6. Serum Folic Acid Estimation:

The level of serum folic acid was estimated in the sera samples of the subjects. Thirty microliters of the serum sample was used in each run, the Cobas system was used based on (folate III, Cobas, Rpche) according to the manufacturer instructions.

Chapter Four

Results

4. Results

The current study recruited (31) NTDs patients (infants/child), with their corresponding (30) fathers and (32) mothers in Duhok city. The data taken from the NTDs patients and their corresponding parents are shown in (Table 1), the majority of the infants/child involved in the study were female (23, 56.1%), (29, 72.5%) of the NTD patients were positive *MTHFR* 677C→T mutation, most of them were heterozygous type (23, 79.3%). With respect to their parents, the rate of *MTHFR* 677C→T mutation was (22, 73.3% and 26, 81.3% for fathers and mothers, respectively) and heterozygous (21, 95.5%, 21, 80.8% for fathers and mothers, respectively)

Table 4.1: Partipants Infants/ children characteristics

Characteristics		Patient or Control		Total
		Patient	Control	
Gender of Infant/children	Male	15	3	18
		83.3%	16.7%	100.0%
	Female	16	7	23
		69.6%	30.4%	100.0%
MTHFR of Infants/children	Positive	24	5	29
		82.8%	17.2%	100.0%
	Negative	7	4	11
		63.6%	36.4%	100.0%
Genotypes of Infants/children	Homozygous	6	0	6
		100.0%	0.0%	100.0%
	Heterozygous	18	5	23
		78.3%	21.7%	100.0%

4.1 Clinical history of infants and their parents

With respect to the clinical history of the infants/child and their parents, a total of 31 **infants/child** were involved in the study with the age range of **1 day to 8 years**, every patient was presented with apparent NTD and 10 healthy people also were involved in the study as controls. The control group had ages ranging between (14 months – 35 years).

The parents of every infant/child were involved in the study except 2 of them, they are involved in the study for personal reasons. The range of fathers' age was between **22 and 59 years** and mother's age ranged between 23 and 39 year old.

With respect to folic acid of infants and mothers, the range of serum folic acid concentration was 7.35-115.77 (nmol/L) for 31 infants with the 48.38% NTDs and the range of serum folic acid concentration for mothers 7.67-184.39 (nmol/L), (Table 2).

Table 4.2: Subjects involved in the study and their ages

Number of participant		**Age range of the participants**	**Folic Acid concentration Range (nmol/L)**
Children	32	1 day-8 years	7.35-115.77 (48.38% NTDs)
Fathers	30	22-59	N.A
Mothers	32	23-39	7.67-184.39 (no defect)

The distribution of the folic acid concentration among the infants/Children and the corresponding mothers is shown in figures 1 and 2.

Figure 4.1.: The distribution of the folic acid concentration among the infants/Children

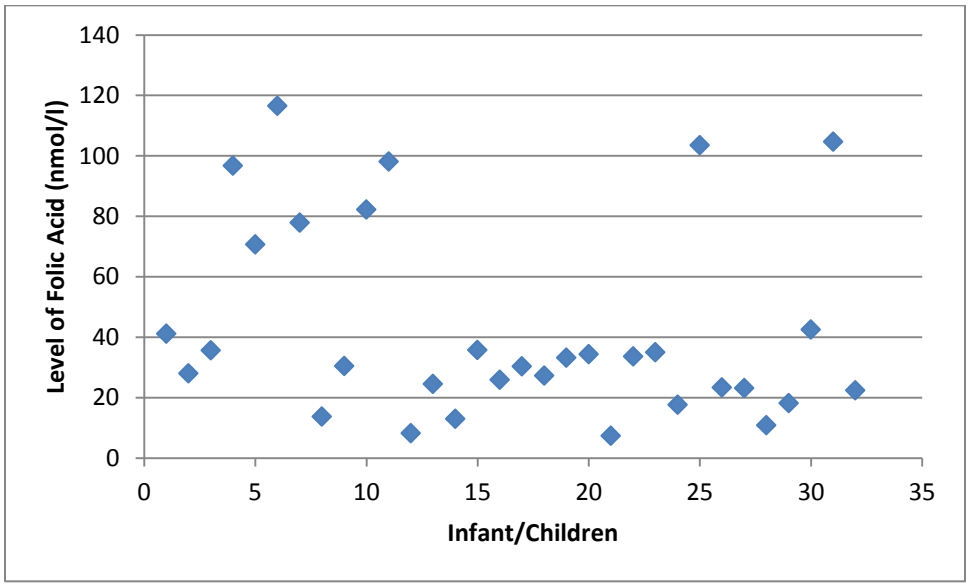
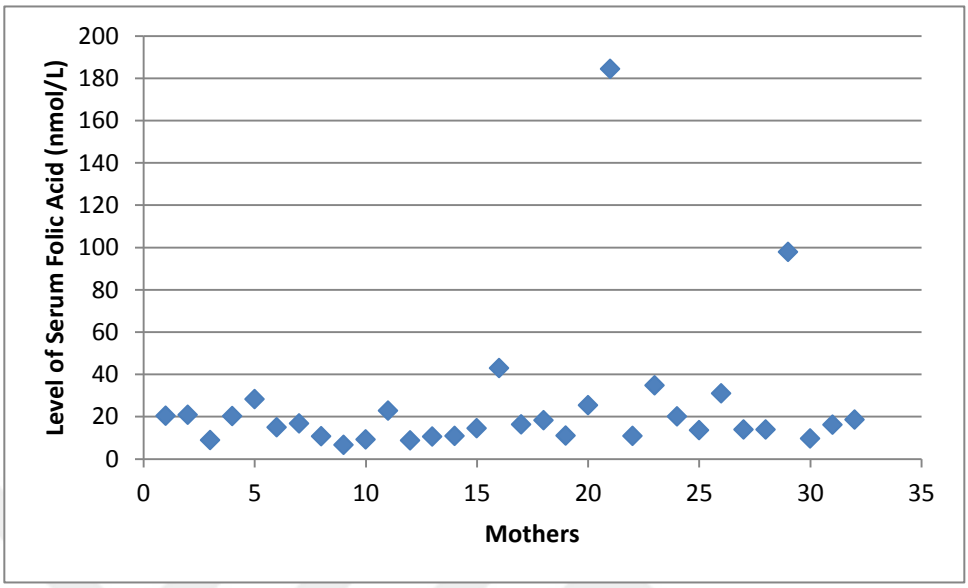


Figure 4.2.: The distribution of the folic acid concentration among the mothers



4.2 Family History

With respect to family history, 20 mothers (64.51%) received the folic acid during pregnancy in which 17 of them (54.83%) had one abortion or more, 3 of them (9.645%) received prescribed medication for some other health conditions, and also 24 of mothers (75%) did the prenatal investigations (Table 3).

Table 4.3: Mother's medical history

History of mothers	**Number, %**
Folic acid	20 (64.51%)
Number of abortion	17 (54.83%)
Other medication during pregnancy	3 (9.645%)
Mothers informed with NTDs perinatally	24 (75%)

4.3. Molecular studies:

4.3.1. DNA extraction:

Appropriate yield of genomic DNA (31 patients, 32 mothers and 30 fathers) were obtained from repeated experiments with an average yield of 1 to 100µg/µl.

4.3.2. PCR –RFLP analysis:

Genotyping for *MTHFR* 677C→T single nucleotide polymorphism in *MTHFR* gene was accomplished on leukocyte genomic DNA samples by using RFLP as a PCR-based molecular method (Forsst, 1995; Aleyasin andMirakhorli, 2012). There are two steps in this method; the first was the amplification of the genomic DNA by PCR, while the second step was the digestion of the amplified products with *Hinf*I the restriction enzyme.

4.3.3. MTHFR gene PCR Amplification:

The amplification of the *MTHFR* gene fragment sized 254bp (which includes position 677 of the MTHFRgene) was the aim of this step with specific oligoneuclutide primer set. The achievement of this step was completed by resolving the amplicon on 2% agarose gel electrophoresis, the electrophoresis was run at 95-100 volt/cm for 45 minute as shown in (Figure 1).

Figure 4.3: PCR amplification of the *MTHFR* gene fragment sized 254bp using specific oligoneulutide primers

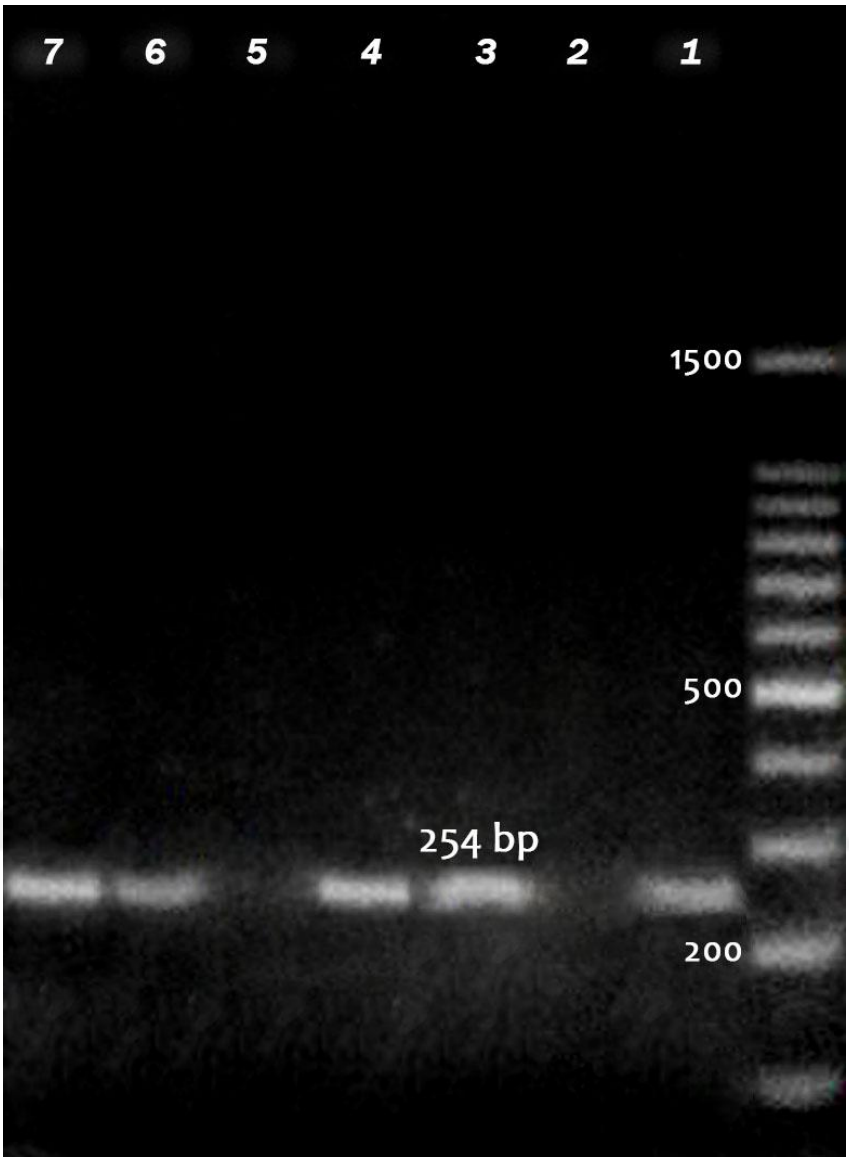


Figure (4.3): Gel electrophoresis (2%) showing PCR amplification products using specific primers to targeting 677 region of the *MTHFR* gene. lane L indicates DNA Marker of (100-1500bp) other lanes indicates the PCR products of 254bp size each.

4.3.4 PCR products restriction analysis by *Hinf*I enzyme:

Figure 2 shows the PCR products of the 254bp size of 677 *MTHFR* gene region were treated with *Hinf*I restriction enzyme at 37 C^{o} for 24 hours. Two fragments (147 and 107 bp) was resulted from the restriction of 254 bp PCR product of the MTHFR.The *Hinf*I cuts at C677T

region which helps to detect the *MTHFR* C677T mutation. After that the restriction mix were electrophoresed on 2% agarose gel, (Figure 2).

Figure 4.4: MTHFR 254bp fragment PCR products restricted with *Hinf*I for the detection of *MTHFR* C677T mutation

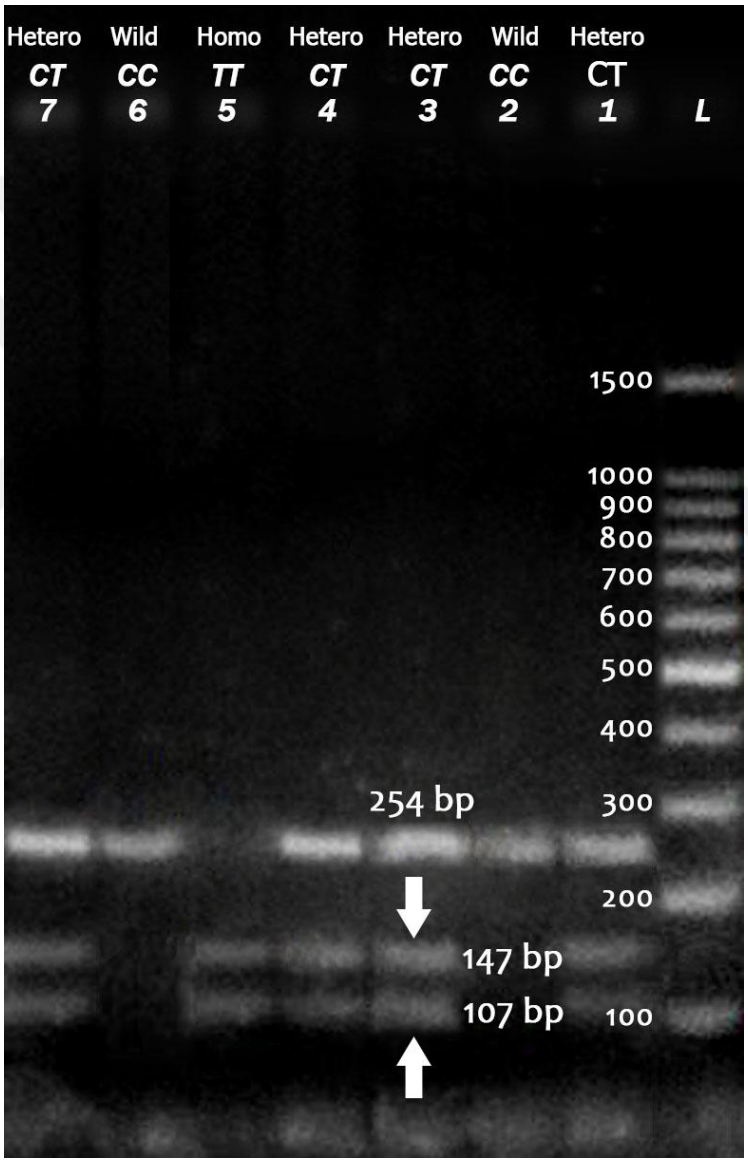


Figure (4.4): Restriction of the *MTHFR* 254 bp size containing the 677 fragment detecting the C677T mutation with *Hinf*I). in heterozygous (CT genotype) genotype the restriction produce three bands (254bp, 147 and 107 bp), while in homozygous the restriction produce two bands

(147 and 107 bp) (TT genotype) the persistence of an uncut (254bp) fragment coins a diagnosis of the wild state (not mutated CC genotype).

4.3.5 MTHFR Gene Polymorphism

Out of the 31 infants/children, 24 (77.41%0 of them had the *MTHFR* 677C→T gene mutation, 6 (25%) of them were heterozygous, while 18 (75%) were homozygous for the *MTHFR* 677C→T mutation, 22(73.33%) of fathers had *MTHFR* 677C→T mutation, 1 (4.54%) of them was heterozygous, while 21(995.45%) of them were homozygous for the mutation, among the mothers, 26(81.25%) of them had the *MTHFR* 677C→T mutation, 5(19.235) of them were heterozygous, 21(80.76%) of them were homozygous for *MTHFR* 677C→T mutation, (table 4).

Table 4.4.: The rates of *MTHFR* genotypes among participants

Participant	**No. of participant**	**No. of *MTHFR* 677C→T mutation**	**(C/T) heterozygous**	**(T/T) homozygous**
Infants/ children	31	24(77.41%)	18(75%)	6(25%)
Fathers	30	22(73.33%)	1(4.54%)	21(995.45%)
Mothers	32	26(81.25%)	5(19.235)	21(80.76%)

4.4. Attributions of NTDs in infants/children

According to the results extracted based on the available data and shown in the Table 5, it was confirmed that the Neural Tube Defect disease in infants/children could not be attributed to *MTHFR* 677C→T mutation (Fisher's Exact Test, p=0.227), and genotypes (Fisher's Exact Test, p=0.553), MTHFR of mothers and fathers (Fisher's Exact Test, p= 1.000), folic acid of patients and controls (Fisher's Exact Test, p=1.000), folic acid of patients with controls in *MTHFR* 677C→T positive (Independent t-test, p=0.196), folic acid of patients with controls in *MTHFR*

677C→T negative (Independent t-test, p=0.079), and folic acid of mothers and patients (Independent t-test, p=0.145) (table 5). However, the difference between folic acid of NTDs patients and the healthy samples in *MTHFR* 677C→T negative (Independent t-test, p=0.051) was very close to be significant statistically (p=0.051), the statistics could gives a completely different interpretation if the sample size is increased.

Table 4.5: Attribute factors of NTDs in infants/children

NTDs Attribute Variables	Two-sided Significance **(**Fisher's Exact Test)
Association of *MTHFR* 677C→T with NTDs and controls	0.227
Association of *MTHFR* Genotypes with NTDs and controls	0.553
Mothers' *MTHFR* 677C→T compared to NTDs	1.000
Fathers' *MTHFR* 677C→T compared to NTDs	1.000
Differences of folic acid level of NTDs and controls	0.196
Association of Folic Acid level of infants/ children with controls in *MTHFR* 677C→T positive	0.079
Association of Folic Acid level of infants/children with controls in *MTHFR* 677C→T negative	**0.051**
Association of Folic Acid level of Mothers with NTDs patients	0.145

Chapter Five

Discussion

5. Discussion

Neural tube defects (NTDs) are defined as a complex group of neonates defects that are not resulted from a single cause.(Wallingford *et al.*, 2013).

Every year about 300 000 or more pregnancies are affected by neural tube defects. (Shibuya and Murray, 1998). Neural tube defects (NTDs) are congenital anomalies of the central nervous system and rank amongst the most common birth defects alongside congenital heart anomalies and genito-urinary defects(Dolk *et al.*, 2010), those anomalies caused by the failure of the neural tube to close during neurulation between 21 and 28 days after conception *(Botto et al., 1999).* In the United States the prevalence statistics show 5 per 10,000 in 2001–2004 and in Western Australia 10 to 15 per 10,000 in 2001–2006 individuals were affected. In other countries like the China, NTDs are more prevalent by studies showing 20 per 1000 in 2002–2004 (Moore *et al.*, 1997)

At the same time, the rates of NTD change highly across time and among various geographic areas. The elevated incidences have been reported between, Irish, Mexican, Northern China , and Indian, populations (Moore *et al.*, 1997). The mutations of *MTHFR* gen is one of the strongest risk factor of NTDs. The current study recruited (31) NTDs patients (infants/child) with the age ranged between (1 day to 8 years), with their corresponding (30) fathers and (32) mothers in Duhok city. 24 (77.41%0 of them had the *MTHFR* 677C→T gene mutation. According to the results extracted based on the available data and shown in the Table 5, it was confirmed that the Neural Tube Defect disease in infants/children could not be attributed to *MTHFR* 677C→T mutation (Fisher's Exact Test, p=0.227), since there was no significant difference between the rate of *MTHFR* 677C→T mutation in the NTDs and controls. The findings in current study are

in accordance with other data obtained from similar studies. ((De Marco *et al.*, 2002) in their study showed that there was no relation or even a protective effectiveness in other populations. In another study done by (Shaw *et al.*, 1998), on a larger studies they found either a smaller or no association with NTDs.

But in contrast, a meta-analysis (Amorim *et al.*, 2007) has found that there was aspositive association only in non-latin groups, principally the Irish population.

Also (van der Put *et al.*, 1998) in another large-scale meta-analysis study including 2429 cases and 3570 controls proposed that maternal MTHFR C677T polymorphisms are a genetic risk factor for NTDs. In many studies that have been done by (Amorim *et al.*, 2007; Yan *et al.*, 2012)and (Blom *et al.*, 2006), all have found a significantly elevated risk of NTDs related with *MTHFR* 677C > T either in maternal and cases and even paternal genotypes. Another large study that done by (Gu *et al.*, 2007) they obtained neural tube defects (subjects) from an area in Northern China with a NTDs spread of 199.38/10,000 depended on the local epidemiologic observation data obtained between January 2002 and December 2004, they decided that there was a significant connection between the SNP sites rs1801133 in MTHFR gene and Neural Tube Defects. The GG genotype,G allele of rs1801133 in MTHFR significantly reduced the probability of NTDs happening. The relation of polymorphisms and NTDs danger may also be various depending on the kind or the localization of NTD (Volcik *et al.*, 2000; Wenstrom *et al.*, 2000; Shang *et al.*, 2008). After the latest previous years, some various groups have reported between a threefold and a sevenfold rise NTD danger related with this MTHFR mutation, particularly if the state of mutation is the homozygous, in Holland and Ireland (Frosst *et al.*, 1995; Dean *et al.*, 1999).

In the current study according to the homozygosity and heterozygosity, 18(75%) of the patients were heterozygous, while 6 (25%) were homozygous for the *MTHFR* 677C→T mutation. (table 3.4),these results was unlike that done by(Botto and Yang, 2000) who fulfill a meta-analysis on MTHFR data from various countries and racial groups for concerning to the frequency of the C677T allele. A pooled odds ratio of 1.7 for NTD risk among infants with the 677T/677T homozygous geno- type have been reported by them, with alittle lower odds ratio for infants who were heterozygous for the variant C677T allele, proposing a connection between the danger for NTDs and the number of variant 677T alleles. On the other hand, they counted a

gathered attributable danger of 6% for infants who were homozygous for the variant 677T allele. This calculation suposed that there is a causative relations between the development of NTDs and the MTHFR gene and . According to homozygosity for the MTHFR 677T variant allele, it showes that it is only related with an eleveted danger for spina bifida in extremely choosed populations, and only in doubtless studies within those populations.

Recently there was shown (Crider et al., 2011; Yan et al., 2012; Zhang et al., 2013) that *MTHFR* 677 genotype isrelated to danger of NTDs during studies .Likely to the published meta-analyses,*(Yan et al., 2012; Zhang et al., 2013)* we evaluated the comoarative risk of a NTDs affected pregnancy to be raised for the *MTHFR* TT genotype compared with CC (relative risk 1.49, 95% uncertainty interval 1.33 to 1.70) and for TT compared with CT (1.28, 1.17 to 1.39) genotypes. The inconsistency of the data in the previous studies could give an idea that mutations of gene other than *MTHFR* mutation could be implicated in the defects and reduction level of the neural tube growth. Larger scale studies are needed to investigate more genes mutations that are potential factors in the NT development.

Regarding to folic acid scales in the sera of the studied subjects, in the current study and according to the results extracted based on the available data and shown in the Table 5, it been seen that the Neural Tube Defect disease in infants/children could not be attributed to folic acid of patients and controls (Fisher's Exact Test, p=1.000),), folic acid of patients with controls in *MTHFR* 677C→T positive (Independent t-test, p=0.196), folic acid of patients with controls in *MTHFR* 677C→T negative (Independent t-test, p=0.079), and folic acid of mothers and patients (Independent t-test, p=0.145) (table 5). In addition to the genotype, several studies have calculated the maternal vitamin taking within the periconceptional time. While the homozygous 677T/677T variant genotype was noticed in an infant whose mother concumed multivitamins, there was only a modest tendency toward an raised odds ratio [OR=1.2; 95% cartainly interval (CI), 0.4–4.0] related to the reference group, who were infants with the homozygous 677C/677C genotype whose mothers took multivitamins. For conbitions that the mother did not eat multivitamins and the infant had the 677TT (homozygous) genotype, there was a little higher odds ratio (OR=1.6; 95% CI, 0.8–3.1). These outcoms are coordiant with an interaction between maternal vitamin use and the genotype, as the risk for NTDs become clearer to be higher among the offspring of women who did not take folic acid, but the variance between the two odds ratios

is statistically not significant. Therefore, this interaction does not become clear to have a main biological ffect on Neural Tube Deefects danger.(Volcik *et al.*, 2000; Zhang *et al.*, 2013).

Daly and colleagues notified a very high danger of NTDs at lower concentrations of red blood cell folate (<340 nmol/L: risk of 66 per 10 000 births) and basically lessen danger at concentrations over a cut-off concentration of 906 nmol/L (8 neural tube defects per 10 000 births; mean 1292 nmol/L).*(Daly et al., 1995)*, also they declared that women with plasma folate concentrations over 15.9 nmol/L had an related to the danger of 9 (95% confidence interval 5 to 17) NTDs per 10 000. (Kirke *et al.*, 1993). Notice that folate reduction with vitamin B12 reduction are danger factors for neural tube defects. (Laurence *et al.*, 1980) noticed that nutritional folate insufficiency and, in generally, a poor diet in the beginning months of pregnancy is related to neural tube defects offspring. The Italian women who did not take the foods which are rich with folate through the preconceptional deuration have a raised risk compared with users aid a reverse relationship between happening of NTD and Mediterranean diet. With reference to the over all rating of the global burden of NTDs, Italy is a low-incidence place, and investigater thing that tha cause could be the Mediterranean diet . (Kushi *et al.*, 1995)

According to (Mulinare *et al.*, 1988; Bower and Stanley, 1989) declared that the frequency of NTDs among 2471 women receiving 0.8 mg per day of folic acid was zero compared to 2391 women not receiving folic acid was 6 cases.This RCT study confirm previous case–control studies that had supply proof that pregnant women taking multivitamins including dietary folic acid or folic acid had a lower risk of the appearance of NTDs than that whose not receiving supplements.

In the non-fortified countries, including the whole of Europe, from no recommendations to dietary recommendations the health policies differ with or without vitamins either for selected at-risk women or all women of childbearing age (Botto *et al.*, 2005)..(Group, 2003).
Inspite of these activities,obvious lowering in NTDs rates have not found in any area of these countries (Stoll *et al.*, 2006). The controversy of the results concerning to the inflouence of the levels of the folate on NTDs at the first place could be caused by the size of the sample of which differs in all studies that were mentioned above, as well in our community, the socio-economic status might raise some factors that have impact on the development of the NT. More studies

including larger sample size could come out with more precise figure about the real association between the NT development and defects and the level of the folate in the serum, also the study of other vitamins could help to explore more potential attributes to the NTDs.

Chapter Six

Conclusions and Recommendation

6.1. Conclusions

1. The frequency of *MTHFR* C677T genotype polymorphism in the present study population shown non- significant difference between patients and controls, indicating that this polymorphism does not contribute to the etiology of NTDs in the population of Duhok city.
2. In contrast to most other studies, there is no significant deference between NTDs and folic acid of the patients and their mothers.
3. The difference between folic acid of NTDs patients and the healthy samples in MTHFR 677C→T negative (Independent t-test, p=0.051) was very close to be significant statistically (p=0.051).

6.2. Recommendations

1. Other studies involving other mutations in *MTHFR* gene (A1298C) as well as other genes that interfere with NTDs metabolism are recommended in a larger sample size.
2. Other studies are recommended to establish the relation of the level of folat to other environmental factors such as homocysteine, B12, alcohol consumption and other life styles to gain better insight into the pathogenesis of NTDs.
3. Folic acid is crucially recommended be measured and followed up during the period of pregnancy to know its effect on many congenital disorders including Neural Tube Defects.