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Preface

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**Abbreviations**

- **THF**: tetrahydrofolate
- **dTMP**: 2’-deoxythymidine-5’-phosphate
- **dUMP**: 2’-deoxyuridine-5’-phosphate
- **DHF**: dihydrofolate
- **SAM**: S- adenosylmethionine
- **NTDs**: Neural Tube Defects
- **MUT**: methylmalonyl Coenzyme A mutase
- **MMA**: Methyl Malonic Acid
- **MTR**: Methyltetrahydrofolate – Homocysteine methyltransferas
- **UL**: Upper Level
- **SBO**: Spina Bifida Occulta
- **5-MeTHF**: 5- methyltetrahydrofolate
- **5-FOTHF**: 5-formyltetrahydrofolate
- **MCV**: Mean Corpuscular Volume
- **CBC**: Complete Blood Count
- **EDTA**: ethylenediaminetetraacetic
- **HPLC**: High Performance Liquid Chromatography
- **UV**: Ultra Violet
- **RR**: Rate Ratio
- **CI**: Confidence Interval
- **SD**: Standard Deviation
- **HeY**: Homocysteine
- **RDA**: Recommended Dietary Allowance
- **DEF**: Dietary Folate Equivalent
- **NICU**: Neonatal Intensive Care Unit
- **PA**: Pernicious Anemia
- **MTHFR**: Methylenetetrahydrofolate Reductase
- **Holo TC**: Holotranscobalamin
FAD  Flavin Adenine Dinucleotide
FAO  Food and Agriculture Organization
RNA  Ribonucleic Acid
DNA  Deoxyribonucleic Acid
1. Introduction and objectives of the study

Folic acid is an important nutrient for women in childbearing age. Adequate folate intake during the periconceptional period, the time right before and just after a woman becomes pregnant, helps protect against a number of congenital malformations including Neural Tube Defects (NTDs) which are the most notable birth defects that occur from folate deficiency. Folic acid fortification has been considered as a main strategy to control and reduce the prevalence of NTDs.

Micronutrient deficiencies, especially Iron, Zinc, Vitamin A and vitamin D were reported as one of the most common nutritional problems in Iran (Ministry of Health & Medical Education, 2001). In addition, there are evidence of low intake of calcium and Riboflavin at the national level (National Nutrition Institute, 2001).

Food fortification along with the other strategies including supplementation, nutrition education and public health measures are being implemented by Ministry of Health and Medical Education to prevent and control micronutrient deficiencies, but still the prevalence rate of these deficiencies is high.

Along with the other strategies, mandatory flour fortification with iron and folic acid has been considered to improve the micronutrient status of the Iranian population. Wheat flour is highly subsidized by the government and it is quite accessible to the whole population in urban and rural areas. Bread is an appropriate vehicle for adding iron and folic acid in Iran. It is a staple commodity. It is low cost as flour is highly subsidies by the Government and is readily available. In overpopulated and poor families that have few choices for foods, bread is a staple food.

For adding folic acid to flour, although, there is not any large scale study on folate status in Iran, considering the evidences on the role of folic acid to reduce the occurrence of Neural Tube Defects (NTDs) and also available experience on fortification with folic acid, it has been decided to add folic acid to wheat flour simultaneously with iron.

Based on the World Health Organization (WHO) recommendation, 1.5 ppm of folic acid is added to the wheat flour that is 150 µg per 100g of flour.
No interventional trial on the efficacy and safety of folic acid fortification acid has been conducted in Iran. It is necessary to study the efficacy of folic acid fortification with regard to folate status, NTDs prevalence and possible risk of masking vitamin B12 deficiency.

Recently, the possibility of masking vitamin B12 deficiency due to folate fortification has been raised. The reason is that vitamin B12 and folic acid have the same metabolic pathway and additional folic acid could mask the hematological signs of vitamin B12 deficiency postpones clinical diagnosis which could lead to neurological damage. Epidemiological studies showed a prevalence of vitamin B12 deficiency of around 20% in the general population of industrialized countries (Andres et al, 2004). Globally, the prevalence of vitamin B12 deficiency is likely to be higher in developing countries.

In Iran, based on the latest national food consumption survey (National Nutrition Institute, 2001), the average per capita consumption of meat and dairy products as rich sources of vitamin B12 is lower than the recommended food basket for Iranian population due to high cost or low physical accessibility in deprived areas. So, the possibility of vitamin B12 deficiency has to be explored before implementing flour fortification with folic acid nationally.
1.1. Aims and objectives of the study

Mandatory flour fortification has been considered a priority to improve the micronutrient status of the Iranian population. There was no interventional trial on the efficacy and safety of folic acid fortification by adding 1.5 ppm of folic acid to wheat flour in Iran. In the presented research, we studied the efficacy of folic acid fortification on NTDs prevalence and possible risk of masking vitamin B12 deficiency in child bearing age women with the following objectives:

1. To determine the prevalence rate of serum folate deficiency before and after flour fortification
2. To determine the Mean serum folate before and after flour fortification
3. To determine the prevalence rate of serum Vitamin B12 deficiency before and after flour fortification
4. To determine the Mean serum Vitamin B12 before and after flour fortification
5. To determine the prevalence rate of the high homocysteine levels before and after flour fortification
6. To determine the mean serum homocysteine before and after flour fortification
7. To determine the prevalence rate of low serum vitamin B12 without macrocytosis before and after flour fortification
8. To determine the incidence rate of NTD before and after flour fortification
9. To determine the prevalence rate of folate intake deficiency before and after flour fortification.
2. Literature review

2.1. Folic acid (Pteroylmonoglutamate)

2.1.1. Biochemistry and metabolism

Folic acid, the more stable form, rarely is founded in foods or the human body but is the form most often used in vitamin supplements and fortified foods. Folates are found in foods as well as metabolically active forms in the human body (National Academy of Sciences, 2000).

Folate is a general term related to a family of substances containing a pteridine ring joined to both $p$-aminobenzoic acid and glutamic acid (Goh and Koren, 2008). Reduced forms of this molecule are called dihydrofolate and tetrahydrofolate.

In the form of a series of tetrahydrofolate (THF) compounds, folate derivatives are substrates in a number of single-carbon-transfer reactions, and also are involved in the synthesis of dTMP (2'-deoxythymidine-5'-phosphate) from dUMP (2'-deoxyuridine-5'-phosphate). It is a substrate for an important reaction that involves vitamin B$_{12}$ and it is necessary for the synthesis of DNA, and so required for all dividing cells (Goh and Koren, 2008). The pathway leading to the formation of tetrahydrofolate (FH$_4$) begins when folate (F) is reduced to dihydrofolate (DHF) (FH$_2$), which is then reduced to THF. Dihydrofolate reductase catalyses the last step (Arinz, 2005). As the active form, THF, folate plays a role in a variety of reactions include:

2.1.1.1 one-carbon metabolism

The only function of folate coenzymes in the body appears to be in mediating the transfer of one carbon unites (Choi and Mason 2000). Folate coenzymes act as acceptors and donors of one-carbon units in a variety of reactions critical to the metabolism of nucleic acids (RNA and DNA), cell division and certain amino acids (Baily et al., 2003).

2.1.1.2. Nucleic acid metabolism

Folate coenzymes play a vital role in DNA metabolism through two different pathways. 1) The synthesis of DNA from its precursors (thymidine and purines) is dependent on folate coenzymes. 2) A folate coenzyme is required for the synthesis of methionine, and methionine is required for the synthesis of S-adenosylmethionine (SAM). SAM is a
methyl group (one-carbon unit) donor used in many biological methylation reactions, including the methylation of a number of sites within DNA and RNA (Arinze, 2005). Some of the important reactions in which SAM is involved are: 1) Methylation of DNA and RNA. DNA- and RNA-methylases use SAM as a source of methyl groups. A major target of methylases is the 5 position of cytosine of DNA. The degree of methylation correlates with transcriptional activity. 2) The conversion of epinephrine to norepinephrine is also catalyzed by an N-methyl transferase that uses SAM. The product after the methyl group is transferred is S-adenosyl-homocysteine. Hydrolysis gives homocysteine and adenosine (Figure 1).

1.1.2.3. Amino acid metabolism
Folate coenzymes are required for the metabolism of several important amino acids such as methionine, histidine, serine, and glycine. The synthesis of methionine from homocysteine requires a folate coenzyme as well as a vitamin B₁₂-dependent enzyme (Baily et al., 2003).

Figure 1: Folate metabolic cycle.
In vitamin B12 deficiency there is a buildup of the substrate, N5-methyl-THF which cannot be recycled. This causes a deficiency of the other forms of THF which are needed for other reactions, particularly in DNA synthesis. The loss of THF forms due to the inability to use methyl-THF is referred to as "the methyl trap."

2.1.2. Functions
2.1.2.1. Human Reproduction
Adequate folate intake during the periconceptional period, the time just before and just after a woman becomes pregnant, helps protect against a number of congenital malformations including Neural Tube Defects (NTDs) (Stevenson, 2000).

The protective effect of folate during pregnancy goes beyond NTDs. Supplementation with folic acid has been shown to reduce the risk of congenital heart defects, cleft palate, limb defects and urinary tract anomalies (Goh and Koren, 2008). A statistically significant association between high folate intake and lower sperm aneuploidy has been explored (Young et al., 2008).

2.1.2.2. DNA and cell division
Folate is needed to synthesize DNA bases (most notably thymine, but also purine bases) and also needed for DNA replication. Thus, folate deficiency hinders DNA synthesis and cell division. Since folate deficiency limits cell division, erythropoiesis, production of red blood cells is hindered and leads to megaloblastic anemia which is characterized by large immature red blood cells.

2.1.3. Dietary recommendations
The Dietary Reference Intake (DRI) for folate are expressed in a term called the Dietary Folate Equivalent. The Dietary Folate Equivalent (DFE) was developed to help account for the differences in absorption of naturally occurring dietary folate and the more bioavailable synthetic folic acid. 1 μg of DFE is equivalent to 1 μg food folate that is equivalent to 0.5 μg of folic acid (pteroylmonoglutamate) taken on an empty stomach. It is equal to 0.6 μg folic acid from supplements and fortified foods. DRI for women in childbearing age has been suggested equal to 400 μg/day, while for pregnant women is 600 μg/day and for lactating women is equal to 500 μg/day (National Academy of Sciences, 2000).
2.1.4. Absorption, Transport, and Storage

Different forms of food folate including pteroylmonoglutamate and pteroylpolyglutamate and their absorption are depended on different conjugates and oxidation states. The monoglutamate form of folate is actively transported across the proximal small intestine. Monoglutamates, mainly 5-methyl-tetrahydrofolate, are present in the portal circulation. Much of this folate can be taken up by the liver, where it is metabolized to polyglutamate derivatives and retained or released into the blood or bile. Folate concentrations in the liver were reported up to 4.5 μg/g after liver biopsies. Because the adult male liver weighs approximately 1,400 g, the total quantity of folate in the liver would be approximately 6 to 14 mg. If the liver is assumed to contain 50 percent of the body stores of folate, the estimated total body folate store would be 12 to 28 mg (National Academy of Sciences, 2000).

2.1.5. Bioavailability

When consumed under fasting conditions, supplements of folic acid are nearly 100 percent bioavailable (Gregory, 1997). Overall, different studies indicate that folate added to cereal-grain foods is highly available and efficacious. From these experimental data the bioavailability of folic acid consumed with food is estimated to be 85 percent. Studies have not been conducted to define the bioavailability of folic acid consumed with entire meals. It is assumed that the bioavailability would be somewhat lower than that observed with folic acid alone or with a small portion of food. Bioavailability of food folate has been reported no more than 50 percent that of folic acid (National Academy of Sciences, 2000).

2.1.6. Clinical effects of folate deficiency

Inadequate folate intake first leads to a decrease in serum folate concentration, then to a decrease in erythrocyte folate concentration, a rise in homocysteine concentration, and megaloblastic changes in the bone marrow and other tissues with rapidly dividing cells. When folate supply to the bone marrow becomes rate limiting for erythropoiesis, macrocytic cells are produced. However, because of the 120-day lifespan of normal erythrocytes, macrocytosis is not evident in the early stages of folate-deficient
megaloblastosis. As folate depletion progresses further, the mean cell volume increases above normal.

2.1.7. Causes of deficiency

2.1.7.1. Inadequate ingestion of folate-containing foods

Poor nutrition is prevalent among people with alcoholism (Allen 2008) and patients with psychiatric morbidities (Edeh and Toone, 2007) as well as elderly people (due to conditions such as ill-fitting dentures, physical disabilities, and social isolation). Moreover, for patients with renal (Karaku et al., 2004) and liver failure, anorexia and restriction of foods rich in protein, potassium, and phosphate contribute to decreased folate intake.

2.1.7.2. Impaired absorption

Celiac disease and tropical Sprue cause villous atrophy. The process of aging causes shorter and broader villi in 25% of the elderly population. Achlorhydria leads to elevation of gastric PH above the optimal level (i.e., PH of 5) for folate absorption. Anticonvulsant drugs, such as Dilantin, interfere with mucosal conjugates, hence impairing folate absorption. Other nutrient deficiencies such as zinc, riboflavin (vitamin B2), niacin (vitamin B3) and vitamin B12 may affect folate absorption and metabolism (Tapan et al., 2003).

2.1.7.3. Impaired metabolism

Antimetabolites that are structurally analogous to the folate molecule can competitively antagonize folate utilization. Methotrexate and trimethoprim both are folate antagonists that inhibit dihydrofolate reductase (National Academy of Sciences 2000). Hypothyroidism has been known to decrease hepatic levels of dihydrofolate reductase as well as methylene THFA reductase. People with alcoholism can have very active alcohol dehydrogenase that binds up folate and thus interferes with folate utilization (Tapan et al., 2003).
2.1.7.4. Increased excretion /loss

During vitamin B-12 deficiency, methylene THFA is known to accumulate in the serum, which is known as the folate trap phenomenon. In turn, large amounts of folate filter through the glomerulus, and urine excretion occurs.

2.1.7.5. Increased destruction

Superoxide, an active metabolite of ethanol metabolism, is known to inactivate folate by splitting the folate molecule in half between the C9 and N10 position. The relationship between cigarette smoking and low folate levels has been noted as possibly due to folate inactivation in exposed tissue (Allen 2008).

2.1.8. Folic acid deficiency Anemia

Megaloblastic anemia is usually caused by a deficiency of folic acid or vitamin B12 because both of these nutrients are essential to the synthesis of nucleoproteins. Folate deficiency anemia develops in four stages (Kathleen Mahan and Scott-Stump, 2003).

Stage 1: In this stage, a serum folate level is less than 3ng/ml which is named serum folate depletion. In fact, in this stage, an early negative folate balance is occurred.

Stage 2: This stage is characterized by a negative folate balance that means the cell has been depleted and a reduction in red blood cell folate levels to less than 140ng/ml has been occurred.

Stage 3: This stage is associated with a damage of folate metabolism and folate deficiency characterized by slowed DNA synthesis.

Stage 4: This stage is manifested by an elevated mean corpuscular Volume (MCV) and anemia that is named folate deficiency anemia.

Both deficiencies of folic acid and vitamin B12 will result to damage of DNA synthesis with the same clinical sign which is megaloblastic anemia.

2.1.9. Current issues and controversies

2.1.9.1. Neurological disorders

Several studies suggested that folate deficiency may occur in up to one third of patients with severe depression, and that treatment with folic acid may enhance recovery of the mental state (Gilbody, 2007 and Botteiglieri et al., 2000). Other studies suggest a link
between folate deficiency and impaired metabolism of serotonin, dopamine and noradrenalin (norepinefrine) which have been implicated in mood disorders (Botteiglieri et al., 2000). One study has shown that higher dietary intakes of folate, vitamin B12, and vitamin B6 might decrease the risk of Parkinson Disease through decreasing plasma homocysteine (Lau et al., 2006).

2.1.9.2. Cardio Vascular Disease
Some investigators have hypothesized that increased folate intake will reduce mortality from vascular disease and have proposed increases in the fortification level to achieve a maximal reduction in serum homocysteine levels (Wald et al., 2002). One study suggested that folic acid supplementation may do more harm than good (Lonn et al., 2006). As of 2006, studies have shown that giving folic acid to reduce levels of homocysteine does not result in clinical benefit. These studies suggests that folic acid in combination with B12 may even increase some cardiovascular risks (Zoungas et al., 2006, Bonna et al., 2006).

2.1.9.3. Cancer
A relationship between folate intake or status and several types of cancers including colorectal, breast, cervical, pancreatic, brain and lung cancers has been observed in several population based studies (Choi and Mason, 2000). Epidemiologic studies using large cohort groups support an inverse association between folate and risk of colorectal dysplasia or neoplasia (Baily et al., 2003). A 2006 prospective study of Swedish adults found that diets high in folate from foods, but not from supplements, were associated with a reduced risk of pancreatic cancer (Larsson et al., 2006). Most epidemiologic studies suggest that diets high in folate are associated with decreased risk of breast cancer, but results are not uniformly consistent: one large cancer screening trial reported a potential harmful effect of high folate intake on breast cancer risk, suggesting that routine folate supplementation should not be recommended as a breast cancer preventive (Kim., 2006) .One study in 2008 has shown no significant effect of folic acid on overall risk of total invasive cancer or breast cancer among women (Zhang et al., 2008). More over, the results of the population – based observations from two data sets from the
United States and Canada have shown an increase in Colorectal Cancer (CRC) incidence after flour fortification with folic acid and hypothesized that the institution of folic acid fortification may have been wholly or partly responsible for the observed increase in CRC rates. The theoretical concern is that, the pivotal role of folate in nucleotide synthesis, including its role as a cofactor in a rate limiting step for DNA synthesis (Wagner, 1995), also makes it a potential growth factor for neoplastic cells (Mason et al., 2007).

2.1.9.4. Fertility

In men, folate contributes to spermatogenesis. In women, on the other hand, it contributes to oocyte quality and maturation, implantation, placentation, in addition to the general effects of folic acid on fetal growth and organ development (Ebisch et al., 2007). Wong and colleagues found that folic acid administration (5 mg) to sub fertile and fertile men resulted in a significant increase of folate concentrations in seminal plasma, but no effect of this intervention was observed on sperm count or motility of spermatozoa, however, a 74% increase in total normal sperm count after intervention with a combination of folic acid and zinc sulphate was observed (Wong et al., 2002).

A study on homocysteine concentrations in follicular fluid in women opting for IVF treatment reported that women receiving folic acid supplementation had significantly lower homocysteine concentrations in their follicular fluid (the microenvironment surrounding the oocyte), a better quality of oocytes and, a higher degree of mature oocytes compared with women who did not receive folic acid supplementation (Szymanski et al., 2003).

2.1.9.5. Macular degeneration

The possible effect of folic acid on macular degeneration has been raised recently. A recent sub study of the Women's Antioxidant and Folic Acid Cardiovascular Study reported that use of a nutritional supplement that contains folic acid, pyridoxine, and cyanocobalamin decreased the risk of developing age–related macular degeneration by 34% (Christen et al., 2009).
2.1.9.6. Folic acid supplements and masking of B\textsubscript{12} deficiency

Reduction in the methylation cycle has multiple effects less easy to identify. One such effect is certainly on the nerve cells, because interruption of the methylation cycle causing neuropathy can also happen in vitamin B12 deficiency due to reduced activity of the vitamin B12-dependent enzyme methionine synthase.

Folic acid supplements can correct the anemia associated with vitamin B\textsubscript{12} deficiency. But it will not correct changes in the nervous system that result from vitamin B\textsubscript{12} deficiency. Permanent nerve damage could theoretically occur if vitamin B\textsubscript{12} deficiency is not treated (Morris et al., 2007).
2.2. Vitamin B12
2.2.1. Terminology

vitamin B₁₂ refers to a group of cobalt-containing compounds known as cobalamins - cyanocobalamin, hydroxocobalamin and the two coenzyme forms of B₁₂, methylcobalamin (MeB₁₂) and 5-deoxyadenosylcobalamin (adenosylcobalamin - AdoB₁₂). In a more specific way, the term B₁₂ is used to refer to cyanocobalamin, which is the principal B₁₂ form used for foods and in nutritional supplements (Mcdowell, 2000).

2.2.2. Structure

The structure of B₁₂ is based on a corrin ring, which is similar to the porphyrin ring found in heme, and cytochrome. The central metal ion is cobalt. Four of the six coordination sites are provided by the corrin ring, and a fifth by a dimethylbenzimidazole group. The sixth coordination site, the center of reactivity, is variable, being a cyano group (-CN), a hydroxyl group (-OH), a methyl group (-CH₃) or a 5’-deoxy adenosyl group, respectively, to yield the four B₁₂ forms mentioned above.
Historically, the covalent C-Co bond is one of first examples of carbon-metal bonds to be discovered in biology. The hydrogenases and, by necessity, enzymes associated with cobalt utilization, involve metal-carbon bonds (Lieberman et al, 2007).

2.2.3. Function

2.2.3.1. Metabolism function

Vitamin B12 is normally involved in the metabolism of every cell of the body, especially affecting the DNA synthesis and regulation but also fatty acid synthesis and energy production. In humans, only two corresponding coenzyme B12-dependent enzymes are known:

1) Methylmalonyl Coenzyme A mutase (MUT) which requires adenosylcobalamin to convert L-methylmalonyl-CoA to succinyl-CoA to an isomerization reaction. This is an important step in the extraction of energy from proteins and fats. This functionality is lost in vitamin B12 deficiency, and can be measured clinically as an increased methylmalonic acid (MMA) level (National Academy of Sciences, 2000).

2) 5-methyltetrahydrofolate – homocysteine methyltransferase (MTR), also known as methionine synthase. This is a methyl transfer enzyme, which requires methylcobalamin as a cofactor for the methyl transfer from methyltetrahydrofolate to homocysteine to form methionine and tetrahydrofolate (Ferrer 2004). This functionality is lost in vitamin B12 deficiency, and can be measured clinically as an increased homocysteine level in vitro. Increased homocysteine can also be caused by a folic acid deficiency, since B12 helps to regenerate the tetrahydrofolate (THF) active form of folic acid. Without B12, folate is trapped as 5-methyl-folate, from which THF cannot be recovered unless a MTR process reacts the 5-methyl-folate with homocysteine to produce methionine and THF, thus decreasing the need for fresh sources of THF from the diet (Scalabrino 2006).

2.2.3.2. Neurological function

Methylation cycle provide S-adenosyl-methionine (SAM) as a methyl donor to synthesis DNA, protein, hormones and lipids. The conversion of epinephrine to norepinephrine is also catalyzed by an N-methyl transferase that uses SAM. Interruption of methylation via
B12 deficiency leads to demyelination of nerve resulting in a neuropathy (Finkelstein and Martin, 2000). Evidence from several studies has demonstrated associations among vitamin B-12 status and many aspects of cognition. Clarke found a significant associated between low serum concentrations of holo TC and high MMA and a more rapid cognitive decline among persons aged ≥65 y during a 10-years period (Clarke et al., 2007).

2.2.4. Absorption, transport, storage

Absorption of food vitamin B12 requires an intact and functioning stomach, exocrine pancreas, intrinsic factor, and small bowel. Problems with any one of these organs makes a vitamin B12 deficiency possible. It is assumed that 50 percent of dietary B12 is absorbed by healthy adults with normal gastric function. A smaller fractional absorption would apply, however, if a person consumed a large portion of foods rich in vitamin B12.

Around 60% of vitamin B12 is stored in the liver (National Academi of Sciences, 2000). Due to the extremely efficient enterohepatic circulation of B12, the liver can store several years’ worth of vitamin B12. Vitamin B12 deficiency may arise in a year if initial stores are low (Zengin et al, 2009).

2.2.5. Causes of deficiency

2.2.5.1. Inadequate Consumption

Restriction or exclusion of all animal foods may result in low intake of certain micronutrients such as vitamin B-12, thereby affecting vitamin B-12 status. Overall, the studies have shown reduced mean serum vitamin B-12 and elevated mean homocysteine concentrations in vegetarians, particularly among vegans (Elmadfa et al, 2009). Inadequate intake, due to low consumption of animal-source foods, is the main cause of low serum vitamin B-12 in younger adults and likely the main cause in poor populations worldwide. In most studies, serum vitamin B-12 concentration is correlated with intake of this vitamin (Allen, 2009).

2.2.5.2. Inadequate Absorption

Overgrowth of bacteria in part of the small intestine can lead to inadequate absorption.
Patients with bacterial overgrowth that is longstanding can develop complications of their illness as a result of malabsorption of nutrients including vitamin B12 (McPhee et al., 2007).

2.2.5.3. Malabsorption disorders

*Food-cobalamin malabsorption syndrome* is characterized by the inability to release cobalamin from food or from intestinal transport proteins, particularly in the presence of hypochlorhydria, where the absorption of “unbound” cobalamin remains normal. A statistically significant relation between *H pylori* infection and serum vitamin B12 levels among boys and girls 5-18 years who underwent upper gastrointestinal endoscopy because of dyspeptic symptoms has been reported (Akam et al., 2007).

*Inflammatory bowel disease*: Deficiencies of both macronutrients, as well as micronutrients such as iron, cobalt, chromium, copper, manganese, selenium, zinc and vitamin B12 is a common feature of inflammatory bowel disease (Headstorm et al., 2008). Individuals with stomach and small intestinal surgeries may be unable to absorb enough vitamin B12.

*Drugs*: Acid suppression therapy, including H2-receptor antagonists and proton pump inhibitors (Andres et al., 2003) may inhibit B12 absorption. Metformin can interfere with vitamin B12 absorption. The probable mechanism is that the B12–intrinsic factor complex uptake by ileal cell membrane receptors is known to be calcium–dependent, and Metformin affects calcium-dependent membrane action. The result in B12 deficiency can be reversed by administering calcium, and it seems to be the clearest mechanism (Ting et al., 2006).

*Lack of intrinsic factor*: Pernicious anaemia is the most common cause of vitamin B12 deficiency. It is caused by an autoimmune gastritis that affects the gastric parietal cells, leading to the destruction of the gastric mucosa, reduced or absent gastric acid production and a lack of intrinsic factor. Intrinsic factor may be lacking because the part of the stomach where it is produced was surgically removed (Day, 2006).

2.2.6. Dietary recommendations

The Dietary Reference Intake (DRI) for adults ranges from 2 to 3 µg per day. The DRI for vitamin B12 in pregnant women is 2.6 µg per day and 2.8 µg during lactation periods.
There is insufficient reliable information available about the safety of consuming greater amounts of Vitamin B12 during pregnancy (National Academy of Sciences, 2000).

2.2.7. Toxicity

No toxic or adverse effects have been associated with large intakes of vitamin B₁₂ from food or supplements in healthy people. Doses as high as 1 mg daily by mouth or 1 mg monthly by intramuscular injection have been used to treat pernicious anemia without significant side effects. When high doses of vitamin B₁₂ are given orally, only a small percentage can be absorbed, which may explain the low toxicity. Because of the low toxicity of vitamin B₁₂, no tolerable upper intake levels (UL) have been set (National Academy of Sciences, 2000).

2.2.8. Epidemiology of Vitamin B₁₂ deficiency

Traditionally the problem was assumed to be due to strict vegetarianism, however it is now apparent that it is also an issue in lacto-ovo vegetarians (Herman et al., 2001, Majchrzak et al., 2006), aged population (Floodd et al., 2006) and in those with low meat consumption (Allen, 2009).

Epidemiological studies show a prevalence of cobalamin deficiency of around 20% (between 5% and 60%, depending on the definition of cobalamin deficiency used in the study) in the general population of industrialized countries (Andres et al., 2004).

A high prevalence of impaired vitamin B₁₂ status in pregnant Nepalese women has been reported. While 61% of the women had elevated s-MMA, and low s-cobalamin values were observed in 49% of the women, (Bondevik et al., 2001).

The Framingham Offspring Study found up to 39% of U.S. adults at risk for vitamin B₁₂ deficiency (Tucker et al., 2000). Globally, the prevalence of vitamin B₁₂ deficiency is likely to be higher in developing countries. Yajnik found 67% of low vitamin B₁₂ concentration among middle-aged men of the South Asian Indians (Yajnik et al., 2006).

According to the Allen study, more than 40% of the subjects of all ages in Latin America had low plasma vitamin B₁₂ and also in Mexico, the prevalence of deficient and marginal plasma B₁₂ values respectively was 41% and 16% in preschoolers (18-36 months), 22% and 25% in school children (7-10 years), 19% and 19.5% in non-pregnant
women, 19% and 43% in pregnant women, 30% and 25% in lactating women, and 27% and 15% in adult men (Allen 2004). Ling Hao, found that 11% of healthy Chinese men and women 35-64 years old in the southerners and 39% of the northerners were vitamin B-12 deficient (Hao et al., 2007).

### 2.2.9. Symptoms and damages of vitamin B12 deficiency

Vitamin B12 deficiency can potentially cause severe and irreversible damage, especially to the brain and nervous system. At levels only slightly lower than normal, a range of symptoms such as fatigue, depression, and poor memory may be experienced (Sethi et al., 2005). However, these symptoms by themselves are too nonspecific to diagnose deficiency of the vitamin. Vitamin B12 deficiency can also cause symptoms of mania and psychosis (Bernal et al., 2007).

Megaloblastic anemia (pernicious anemia) which results from inhibition of DNA synthesis in red blood cell production is often due to deficiency of vitamin B12 and/or folic acid. It is characterized by many large immature and dysfunctional red blood cells (megaloblasts) in the bone marrow, and also by hypersegmented or multihypersegmented neutrophils.

The neurological complex, defined as myelosis funicularis, consists of the symptoms such as impaired perception of deep touch, pressure and vibration, abolishment of sense of touch, irritability, focus/concentration problems, depressive state with suicidal tendencies and Paraphernia complex. These symptoms may not reverse after correction of hematological abnormalities, and the chance of complete reversal decreases with the length of time the neurological symptoms have been presented (Sethi et al. 2005).
2.3. Neural Tube Defects

2.3.1. Definition

Birth defects (congenital anomalies) are the leading cause of death in babies under 1 year of age. Neural Tube Defects (NTDs) are the second most common type of birth defect after congenital heart defects which arise during the process of neurulation, between the 17th and 30th post fertilization days. Depending on the size and the location of the defect, the patient can suffer either no physical handicap or lifelong disabilities (Detrait et al. 2005). Interruption of DNA biosynthesis or methylation reactions could prevent the proper closure of the neural tube. Such inhibition could be caused by simple deficiency of either folic acid or vitamin B12.

2.3.2. Types of Neural Tube Defects (NTDs)

There are two types of NTDs. The most common are called the open NTDs. Open NTDs occur when the brain and/or spinal cord are exposed at birth through a defect in the skull or vertebrae (back bones). Examples of open NTDs are spina bifida (myelomeningocele), anencephaly, and encephalocele. Rarer types of NTDs are called closed NTDs. Closed NTDs occur when the spinal defect is covered by skin. Common examples of closed NTDs are lipomyelomeningocele and lipomeningocele (Brand, 2007). Spina Bifida Occulta (SBO) is potentially another form of an NTD in which there is a typically benign (or non-symptom-causing) bony change in one or more vertebrae, but not involving the nerves within the spinal column. The incidence of SBO is not well defined; however, it is more common than the NTDs described above (Kaufman 2004).

2.3.3. Causes of NTDs

Genetic and environmental factors contribute to NTDs. However, it is generally agreed that most NTDs cases are of multifactor origin, having a significant genetic component to their etiology that interacts with a number of environmental risk factors (Padmanabhan 2006). NTDs are not only disorders of embryologic induction but also disorders of cellular migration and include the secondary mechanical complications that occur with an unprotected nervous system (Kashani 2001).
2.3.3.1. Genetic causes of NTDs

Several chromosomal and single-gene disorders have been reported to be associated with NTDs. Spina bifida occurs more frequently in autosomal trisomies. However, no single gene, which is solely responsible for NTDs in humans has been defined (Padmanabhan 2006).

2.3.3.2. Physical and chemical environment

Moretti reported significant association between the risk of NTDs and maternal hyperthermia exposure (Moretti, 2005). Maternal infections (e.g. rubella, cytomegalovirus, Toxoplasmo gondii, syphilis) (Padmanabhan 2006) and maternal metabolic conditions (e.g. phenylketonuria, diabetes mellitus, endemic cretinism) enhance the risk for NTDs (Ray et al., 2004). An association between maternal obesity and an increased risk of NTDs-affected pregnancy has been reported (Rasmussen and Sonja, 2008). Drugs such as Valproic acid have been identified as teratogens. Valproic acid is a known folate antagonist and its association with NTDs may be through that action (Duncan et al., 2001).

2.3.3.3. Nutritional factors

Zhang found significantly lower serum concentrations of 5- methyltetrahydrofolate (5-MeTHF), 5-formyltetrahydrofolate (5-FoTHF), total folate and vitamin B12 in NTDs affected pregnancies compared to the controls and concluded that these compounds could be identified as potential risk factors for NTDs early diagnosis (Zhang et al., 2008). In addition to low folate levels or polymorphisms in folate metabolizing enzymes, lower vitamin B12 concentrations during pregnancy may also be independently contributing to an increased risk for NTDs (Ray, 2003). A tripling in the risk for NTDs in the presence of low maternal vitamin B12 status, has been explored (Ray et al., 2007).

An association between the presence of NTDs and low level of serum zinc levels of mothers after delivery has been reported (Golalipour et al., 2009). An association between presence of NTDs and low zinc levels among newborns also has been found (Carrillo – Ponce, et al., 2004).
2.3.4. Epidemiology of NTDs

Geographic and temporal variation in the prevalence of anencephaly and spina bifida is well documented. In California, the prevalence of NTDs was reported to be highest in Hispanics (1.12 per 1,000), lowest in Blacks and Asians (0.75 per 1,000), and intermediate in non-Hispanic Caucasian (0.96 per 1,000) (Mitchell, 2005).

Anencephaly and spina bifida occur at frequencies ranging from 0.9 in Canada to 7.7 in the United Arab Emirates and 0.7 in central France to 11.7 in South America per 1,000 births (Detrait et al. 2005).

Gu and colleagues reported the overall NTDs rate for the 3 year study period equal to 199.38 per 10,000 pregnancies in Shanxi province in China (Gu, et al., 2007). In the United Kingdom and Ireland, yearly prevalence of NTDs has been 45 per 10,000 births before any preconception folic acid supplementation policy initiatives, and declined to 10 to 15 per 10,000 in the 1990s. In the rest of Europe the prevalence during the 1980s and thereafter was close to 10 per 10,000 births (Busby 2005).

In Australia, the prevalence of NTDs among births of 4.6 per 10,000 was reported (Abeywardana 2008). The Australian researchers showed that teenage women had the highest rate and women aged 30–34 years the lowest. The rate of pregnancies affected with NTDs was higher for women living in remote areas than for women living in major cities. Multiple pregnancies were more likely to have NTDs than singleton pregnancies (Abeywardana 2008).
2.4. Food fortification

The term “food fortification” refers to the addition of one or more essential nutrients to a food, regardless of whether they occur naturally in the food. The purpose of fortification is to correct a recognized population-wide micronutrient deficiency or to add micronutrients lost in processing back to their original levels (restoration) or even higher. In many situations, this strategy can lead to relatively rapid improvements in the micronutrient status of a population, and at a very reasonable cost, especially if advantage can be taken of existing technology and local distribution networks. Since the benefits are potentially large, food fortification can be a very cost-effective public health intervention. However, an obvious requirement is that the fortified food needs to be consumed in adequate amounts by a large proportion of the target population. It is also necessary to use food vehicles that are centrally processed. Food fortification reinforces and supports ongoing nutrition improvement programmes and should be regarded as part of a broader, integrated approach to improve micronutrient status at the population level.

2.4.1 Rational for folic acid fortification

A peri-conceptional increase in the intake of folic acid by women of childbearing age reduces the risk of having an infant with a neural tube defects (Ray 2004). Many countries are considering folic acid fortification to ensure sufficient intake of folic acid for women of childbearing age, because many women who plan their pregnancies do not take folic acid supplements in the advised period and many other pregnancies are unplanned. Food fortification with folic acid as the intervention seems likely to succeed in increasing folate intake among populations throughout the world, especially in less-developed countries. Dietary changes and prophylactic supplementation appear as unfeasible strategies in these countries, due to the lack of means for educational campaigns, lack of compliance, and the fact that most pregnancies are unplanned.

2.4.2. Experiences of folic acid fortification

About 75% of the folate in whole wheat is lost during milling, but folic acid has been included in cereal fortification programmes only relatively recently. In 1998, it became mandatory to fortify grain products with folic acid in the United States, the rationale
being that it would lower the prevalence of neural tube defect births. The required fortification level is 154μg/100 g flour. In addition to the United States, some 30 countries now add folic acid to flour, including Canada (150μg/100 g), Chile (220μg/100g wheat flour), Costa Rica (180μg/100 g), Dominican Republic (180μg/100 g), El Salvador (180μg/100 g), Guatemala (180μg/100g), Honduras (180μg/100g), Indonesia (200μg/100g wheat flour), Mexico (200μg/100g wheat flour), Nicaragua (180μg/100 g) and Panama (180μg/100 g) (Allen, et al., 2006).

2.4.3. Stability
Folic acid is added in the form of petroylmonoglutamic acid that is moderately stable to heat. There is some loss of the vitamin on exposure to light, and during cooking and baking. The biggest loose tend to occur from biscuits and pasta, but even these are probably no more than 20-30% (Allen, et al., 2006).

2.4.4. Safety of flour fortification with folic acid
The consumption of folic acid in amounts normally found in fortified foods has not been associated with adverse health effects. However, there has been some concern that high folic acid intakes could mask or exacerbate neurological problems, such as pernicious anaemia, in people with low intakes of vitamin B12. This has led to reluctance to fortify with folic acid in some countries. This concern is particularly pertinent to those individuals who derive folic acid from both supplements and a range of fortified foods, as it is the case in many industrialized countries. In this situation, some people may exceed the UL for folic acid, which has been set at 1000μg/day (National Academi of Sciences, 2000). To avoid any possible risk of adverse effects, folic acid fortification programmes should be designed so as to limit regular daily intakes to a maximum of 1mg (Allen, et al., 2006).

2.4.5. Flour fortification in Iran
Mandatory flour fortification with iron and folic acid has been implemented from 2005 and expanded simultaneously. There is evidence on the magnitude of iron deficiency and anemia among different age groups in Iran that shows the nessisity of adding iron to wheat flour. For adding folic acid to flour, although, there is not any large scale study on folic status in Iran, considering the importance of folic acid in health and available
experience on fortification with folic acid, the National scientific Committee of flour fortification in the Ministry of Health and Medical Education approved to fortified wheat flour with folic acid simultaneously with iron.

The premix that is used for fortifying wheat flour is consisted of 30 PPM iron in the form of ferrous sulfate and 1.5 ppm folic acid that should be added to the flour in proper proportion (200 gr per each ton of flour).

It is pretended that this amount of added folic acid to wheat flour obtain 150 μg folic acid per 100 g wheat flour.

2.4.6. Quality Control of fortified flour in Iran

2.4.6.1. Semi quantitative test

Semi quantitative test is being used at the mills level to control the fortified flour. Semi quantitative test or spot test (AACC method 40-40, Iron- Qualitative method) is universally used in the mills to check whether flour were properly fortified (Nalubola and Nestel, 2000). This test is the identification of ferrous sulfate by using thiosinate and 3% hydrogen peroxide solutions which is inexpensive and easy to teach to the laboratory technicians of the flour factories due its simplicity. Semi quantitative test give a rough indication of the level of an added Iron. The appearance of red-colored spots indicates the presence of iron. The number of spots is a rough estimate of the amount and homogeneity of Iron in the sample. For a more accurate estimation, test samples are compared with known concentrations of iron (30, 60 and 90 ppm). At least one spot test has to be done per 8 hr. shift of the mill.

2.4.6.2. Quantitative test

Quantitative test is being used by applying the spectrophotometric method to measure the amounts of iron in fortified flour in the Provincial Food Laboratory. Once a week, during the visit of the mills, 2 random sample of fortified flour which have already been tested by the spot test in the factory is collected and noted down their properties on a label and is sent along with the sample to the Provincial Food Laboratory. In the Provincial Laboratory, the proportion of iron in the sample is determined by spectorophotometric
method (Nalubola and Nestel, 2000) and finally the amount of iron is determined on ppm bases. In case the amount of iron is not acceptable, the problem is announced to the factory by phone and the Province’s Health center is also informed.

2.4.6.3. Indicator nutrient

The use of a single nutrient in flour as an index of all the micronutrients added by a fortification premix has been advised. Given that the indicator nutrient is within specification, it follows that all the other micronutrients should be as well providing that the fortification premix is correct. Iron is the most likely nutrient to be used as an indicator of adequate fortification in government control (Johnson et al., 2004).

Analysis of folate is not easy due to its multiple forms, lower stability, presence in lower concentration in biological systems, and complex extraction and detection techniques. Because of the difficulty and high cost of measuring the amount of folic acid in fortified flour in a routine way, it is decided to monitor iron as an indicator nutrient in the flour fortification program in Iran.
3. Methodology

3.1. Study location

This study was conducted before and after mandatory flour fortification with iron and folic acid in the Golestan province located in the north of Iran. Golestan province has a population of about 1.8 million and covers an area of about 20460 kilometer. In this province, the dietary pattern is plants based. Per capita supply of energy and protein are more than requirement, provide 65, 25, and 11% of total energy from carbohydrate, fat and protein, respectively. Cereals (bread and rice) are the main staple foods.

3.2. Study design

This evaluative study was designed as a population based study and included three components as follow:

1. A longitudinal hospital based study was conducted 12 months before and 12 months after flour fortification to determine the trend of NTDs.
2. A cross sectional survey was carried out in the pre-fortification period (2006). Biochemical indicators of folate status, socio-demographic, health characteristics and dietary intake were studied as baseline data in 580 women of child bearing age as representative sample of Golestan Province.
3. A cross sectional survey was carried out in the post-fortification period (2008). Biochemical indicators of folate status, socio demographic, health characteristics and dietary intake were studied in 600 women of child bearing age and compared to the results of baseline data.

3.3. Sample size

Sample size calculation is based on the study, before and after intervention. The sample size was 580 women at baseline and was rounded up to 600 in the final survey.

This sample size was chosen according to the following formula

\[ n = \frac{2 \times (z_{1-\alpha} + z_{1-\beta})^2 \times p(1-p)}{(p_0 - p_1)^2} \]

Design effect = 1.25

\[ N = n \times \text{Design effect} \]

\[ N = 580 \]
3.4. Variables

The dependent variables were including low serum folate level, low serum vitamin B12 level, high plasma homocysteine level, high MVC level, as well as mean of serum folate, mean of serum vitamin B12, mean of homocysteine level, mean of MCV level and prevalence rate of NTDs. As an independent variable, we collected data regarding the characteristics considered to be potential confounding variables. These were included age, level of education, residency (urban / rural), job, household size, history for stomach and intestine surgery, smoking, passive smoking, husband’s job and marriage status.

3.5. Study criteria

We included all healthy women aged 15-49 years from urban and rural areas. We excluded women from participation if they were pregnant or consuming supplements containing folic acid within the past one month or taking medication known to interfere with folate and vitamin B12 metabolism or had a history of chronic disease.

3.6. Sampling technique

Since the aim of this study was not to assess the variables separately in urban and rural areas, the sample size estimated for Golestan Province was only proportioned in the districts of the province. Then according to the sampling frame in rural and urban areas, main cluster households were selected. In each cluster 10 women aged 15 to 49 years were studied. There was a gap of at least one household between the two women in each cluster and if there was not a woman in this household, the nearest house was selected to find a woman in this age group. In each selected household only one woman 15-49 years old was studied (The first woman who the interviewer contacted her in the household). The sampling frame in each urban area was the list of households from the latest census. The sampling frame in each rural area was the latest list of households prepared by Provincial Health Center. Distribution of the clusters is shown in table 1.
### Table 1: Distribution of the clusters by districts in Golestan province

<table>
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<tr>
<th>District</th>
<th>Total population*</th>
<th>Total number of clusters</th>
<th>Urban population†</th>
<th>Number of urban clusters</th>
<th>Rural population∞</th>
<th>Number of rural clusters</th>
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<tr>
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<td><strong>30</strong></td>
<td><strong>245658</strong></td>
<td><strong>30</strong></td>
</tr>
</tbody>
</table>

* Total population of women 15-49 years in Golestan province

† Population of women 15-49 years in urban areas

∞ Population of women 15-49 years in rural areas

### 3.7. Data collection technique

#### 3.7.1. Socio-demographic data gathering

At the beginning of interview, the selected woman was provided with explanations about the study. After getting the written consent, the interview was conducted and then she was invited for the next morning to come in the rural or urban health center which was selected for collecting of blood samples. Interviews were conducted in a private place.
All the women were advised to be overnight fasting (at least 12 hours) for taking blood samples. Investigation teams started work at 8 to 8:30 AM, so that the interval between the first blood sampling and its transfer to the laboratory had to be no more than 3 hours. Each day, after the end of blood sampling procedure, samples were placed in cold boxes and transferred to the provincial laboratory.

3.7.2. Dietary assessment

Diet was assessed by a single 24-h-recall. Trained nutritionists from the Provincial Health Center interviewed each subject individually about their food and beverage intake of the previous day, processed food items (if known), and the type and names of manufacturers. The field work covered all days of the week, including weekends. The portion sizes were described in household measures and for processed food items by using the manufacturer’s information.

Three nutrition officers from the Ministry of Health supervised the study at the field level. The diet records were coded by one of the principal investigators using an Iranian food composition database (Dorosty, 2003), modified, using the new version of the UK food composition tables (Maccance and Widdowson, 2004) for the folate contents of those food items which were not included in the Iranian food composition software. The final dietary intake data were validated in the Department of Nutritional Sciences, University of Vienna, Austria, by comparing the nutrient content of selected food items, representative of the average Iranian diet, with the food composition data of the Austrian food composition database. The selected 42 food items included major food sources of folate (i.e. green leafy vegetables, fruits, pulses, cereals, dairy/egg products, and nuts/seeds). The observed variation in the nutrient content was mostly 10-15% for 27 of them such as vegetables, fruits, dairy products, and bread. In some kinds of pluses (i.e. lentils and beans), the observed variation was up to 30%, but within the expected limits.

3.7.3. Blood sample collection

From each woman, about 10 ml overnight fasting blood sample were collected. Before testing the blood samples, the cell counter in the provincial laboratory was calibrated and
the laboratory technicians were trained by the technical expert of the National Reference Laboratory.

To measure serum concentrations of folate and vitamin B12, 5 ml of blood sample were collected in a test tube, transferred to the Province Laboratory in cold boxes, and centrifuged (2000 RPM for 15 minutes) immediately.

For homocysteine determination in plasma, 2 ml of blood samples were collected in a test tube which contained 50 μL of EDTA. Those samples collected in rural areas were immediately centrifuged by using a mobile centrifuge (1700 RPM for 15 minutes) and transferred in cold boxes to the Province Laboratory, while the other blood samples were centrifuged in the Provincial Laboratory. Test tubes containing plasma were stored at -20 °C and transferred to the National Reference Laboratory in Tehran to measure folate, vitamin B12, and homocysteine concentrations.

For testing Complete Blood Count (CBC), 2 milliliter of whole blood was put in covered tubes contains 50 micro liters of EDTA and thoroughly stirred. These were transferred as fast as possible (in 3 hours’ time) to the provincial laboratory.

3.7.4. Bread sample collection

Samples of bread were collected during the final evaluation in 2008.

Bread samples were collected at the household level and during interview with the random selected woman. One sample of bread was collected from the home of the first women in each cluster, totally 60 bread samples were gathered from 60 selected clusters. Bread samples were collected during the final evaluation that was expected to be fortified with folic acid.

3.7.5. NTD data gathering technique

Since the Iranian Primary Health Care system does not have a national birth defect registry, we established a hospital – based surveillance system to register NTDs. This is a referral hospital with an annual rate of around 6500 deliveries, accounting for 25% of annual birth in Golestan province and the largest portion of deliveries (80%) in Gorgan district (capital city). Patients are usually from moderate to low socioeconomic
class families. In this hospital, two members of the staff (registered nurse) have been recruited and trained to revise all births, to register and to describe NTD. Types of NTD registered were anencephaly, encephalocele, and spina bifida associated or not with other malformations. If two neural tube defects in a newborn occur concomitantly, the anatomically higher defect was recorded. A specially trained clinical geneticist monitored the correct registration of NTD.

Rate of NTDs was defined as the number of NTDs cases, divided by the total number of live births, stillbirths, and pregnancy terminations for an NTDs. Total prevalence rates were calculated as a total number of NTD per 1000 births. Time trend analysis of monthly NTD rates was performed.

We included all live births and stillbirths (a gestational age of ≥20 weeks and ≥500 g who were admitted in neonatal intensive care unit (NICU) of Dezyani teaching hospital, from September 2006 (before flour fortification) to December 2008 (after flour fortification).

We defined NTDs according to the International Classification of Diseases (ICD, 2007). The design was based on a sample of 13361 postpartum women after admission for childbirth in Deziani hospital which is a referral center for obstetrics and gynecologic problems. Data were collected through interviews with mothers in the immediate postpartum, as well as by reviewing the patient records of both the mothers and newborn infants. The clinical notes of the babies and their mothers were reviewed, and the following data were recorded; antenatal diagnosis and care, age, level of education, usage of folic acid supplement, smoking and type of the NTD.

3.8. Analytical methods

3.8.1. Biochemical tests

The Complete Blood Count (CBC) was performed using a KX-21N (Sysmex) cell counter, in order to obtain MCV measure. A control solution (Labex) was used as a blood reference, and device and quality control checks were performed before and after daily analysis of the collected blood samples.
Folate and vitamin B12 levels were measured using the SimulTRAC-SNB Radioassay Kit vitamin B12 / Folate and the results were validated by the National Reference Laboratory. The same method for measuring serum folate and vitamin B12 was used in both before and after studies.

Determination of homocysteine concentration in overnight fasting blood plasma was made by High Performance Liquid Chromatography and fluorescence detection (Ubbink et al., 1991).

Reference values of Sauberlich were used to define lower limits of adequate serum concentrations of folate and vitamin B12, i.e. for folate < 6.7 nmol/L and for vitamin B12 < 110 pmol/L (Sauberlich 1999). An elevated plasma homocysteine concentration was defined as > 12 μmol/L (moderate hyperhomocysteinemia) (Stanger et al., 2003). The recommended dietary allowance (RDA) of 2.4 μg for vitamin B12 and 400 μg for food folate were used to define adequacy of intakes (National Academy of Sciences, 2000).

3.8.2. Bread testing method

Reversed – phase ion – pair high – performance liquid chromatography (HPLC) was coupled with detection by UV absorption (280nm) for separation and quantitation of added folic acid in fortified bread (Ossey et al., 1998).

3.9. Statistical Analysis of Data

Collected data on NTDs were analyzed by STATA version 10 and were compared with the chi-square test. The rate ratio (RR) and 95% CI was estimated for effect of fortification. A P-value of 0.05 or less was considered statistically significant.

SPSS for Windows (Version 13) was used for all statistical procedures of socio-demographic, blood tests and measures of folic acid in the bread samples and intake data. Summaries of numerical variables were presented as mean, standard deviation (SD), and 95% confidence interval (95% CI). Changes in hematological indices and intake data from base line before and after flour fortification were assessed using t-test.

Logistic and linear regression (for numeric variables) was used to adjust for confounders. Statistical significance was set at p<0.05.
4. Results

4.1. Socio-demographic characteristics

4.1.1. Completeness of data

At baseline (pre-fortification period), the total number of samples assessed was 579. One participant was excluded because of uncompleted questionnaire. The final numbers analyzed for different types of variables were as follow: women for dietary assessment, 557 (22 subjects were excluded due to incompleteness of dietary data); serum samples for folate and vitamin B12, 572 (7 samples were excluded due to clotting); plasma samples for homocysteine, 561 (18 samples excluded due to clotting).

At the post-fortification period we rounded up the sample to 600 women. We analyzed the results of 600 blood samples for serum folate, serum B12 and plasma homocysteine. Dietary intake data was analyzed for 594 completed questionnaires.

4.1.2. Subjects

We compared the socio-demographic characteristics of the studied women in order to find whether there are significant differences between the characteristics of the studied women in the pre-fortification and post-fortification period.

As table 2 shows the proportion of the studied women in rural and urban areas was not significantly different at baseline and final study (p = 0.907).

Table 2: Distribution of the studied women by residence before and after flour fortification

<table>
<thead>
<tr>
<th>Residence</th>
<th>Pre-fortification Period *</th>
<th>Post –fortification Period†</th>
<th>Total</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urban</td>
<td>Number</td>
<td>Percentage</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>292</td>
<td>49.3</td>
<td>300</td>
<td>50.7</td>
</tr>
<tr>
<td>Rural</td>
<td>Number</td>
<td>Percentage</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>287</td>
<td>48.9</td>
<td>300</td>
<td>51.1</td>
</tr>
<tr>
<td>Total</td>
<td>Number</td>
<td>Percentage</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>579</td>
<td>100</td>
<td>600</td>
<td>100</td>
</tr>
</tbody>
</table>

*December 2006  † December 2008
Table 3 shows that the distribution of the studied women by district was not significantly different at baseline and post-fortification period ($P = 0.847$).

Table 3: Distribution of the studied women by district before and after flour fortification

<table>
<thead>
<tr>
<th>District</th>
<th>Pre-fortification Period *</th>
<th>Post –fortification Period †</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Kalaleh</td>
<td>47</td>
<td>8.1</td>
<td>107</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>10</td>
<td>9.1</td>
</tr>
<tr>
<td>Minodasht</td>
<td>50</td>
<td>8.6</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>6.7</td>
<td>7.6</td>
</tr>
<tr>
<td>Gonbad</td>
<td>99</td>
<td>17.1</td>
<td>209</td>
</tr>
<tr>
<td></td>
<td>110</td>
<td>18.3</td>
<td>17.7</td>
</tr>
<tr>
<td>Azadshahr</td>
<td>40</td>
<td>6.9</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>5</td>
<td>5.9</td>
</tr>
<tr>
<td>Ramian</td>
<td>30</td>
<td>5.2</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>5</td>
<td>5.1</td>
</tr>
<tr>
<td>Aliabad</td>
<td>40</td>
<td>6.9</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>8.3</td>
<td>7.6</td>
</tr>
<tr>
<td>Aghghala</td>
<td>40</td>
<td>6.9</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>6.7</td>
<td>6.8</td>
</tr>
<tr>
<td>Gorgan</td>
<td>143</td>
<td>24.7</td>
<td>293</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>25</td>
<td>24.9</td>
</tr>
<tr>
<td>Bandartorkaman</td>
<td>50</td>
<td>8.6</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>8.3</td>
<td>8.5</td>
</tr>
<tr>
<td>Kordkoy</td>
<td>20</td>
<td>3.5</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>3.3</td>
<td>3.4</td>
</tr>
<tr>
<td>Bandargaz</td>
<td>20</td>
<td>3.5</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>3.3</td>
<td>3.4</td>
</tr>
<tr>
<td>Total</td>
<td>579</td>
<td>100</td>
<td>1179</td>
</tr>
</tbody>
</table>

*December 2006 † December 2008
### Table 4: Distribution of the studied women by education level before and after flour fortification

<table>
<thead>
<tr>
<th>Education</th>
<th>Pre-fortification Period *</th>
<th>Post –fortification Period†</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>Number</td>
<td>Number</td>
</tr>
<tr>
<td>Illiterate</td>
<td>58</td>
<td>80</td>
<td>138</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Primary level</td>
<td>207</td>
<td>214</td>
<td>421</td>
</tr>
<tr>
<td>Secondary level</td>
<td>135</td>
<td>124</td>
<td>259</td>
</tr>
<tr>
<td>Diploma</td>
<td>136</td>
<td>137</td>
<td>273</td>
</tr>
<tr>
<td>University degree</td>
<td>41</td>
<td>45</td>
<td>86</td>
</tr>
<tr>
<td>Total</td>
<td>577</td>
<td>600</td>
<td>1177</td>
</tr>
</tbody>
</table>

*December 2006  † December 2008

Table 4 shows that the education level of the studied women was not significantly different at baseline and post-fortification period (P = 0.429).
Table 5: Distribution of the studied women by job status before and after flour fortification

<table>
<thead>
<tr>
<th>Job status</th>
<th>Pre-fortification Period *</th>
<th>Post –fortification Period†</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Housewife</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td>477</td>
<td>511</td>
<td>988</td>
</tr>
<tr>
<td>%</td>
<td>82.4</td>
<td>85.2</td>
<td>83.8</td>
</tr>
<tr>
<td>Employed in home</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td>34</td>
<td>35</td>
<td>69</td>
</tr>
<tr>
<td>%</td>
<td>5.9</td>
<td>5.8</td>
<td>5.9</td>
</tr>
<tr>
<td>Employed out of home</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td>36</td>
<td>36</td>
<td>72</td>
</tr>
<tr>
<td>%</td>
<td>6.2</td>
<td>6</td>
<td>6.1</td>
</tr>
<tr>
<td>Student</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td>32</td>
<td>18</td>
<td>50</td>
</tr>
<tr>
<td>%</td>
<td>5.5</td>
<td>3</td>
<td>4.2</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td>579</td>
<td>600</td>
<td>1179</td>
</tr>
<tr>
<td>%</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

*December 2006  † December 2008

From the results presented in table 5, it can be observed that there was not a significant difference between distribution of women by job status in the pre –fortification and post-fortification period (P = 0.193).

Table 6 shows the distribution of the women by age groups. A significant difference between age distribution of the studied women at base line (before fortification) and final evaluation (after fortification) was not found (p = 474).
### Table 6: Distribution of the studied women by age groups, before and after flour fortification

<table>
<thead>
<tr>
<th>Age(year)</th>
<th>Pre-fortification Period *</th>
<th>Post-fortification Period†</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>15-20</td>
<td>72</td>
<td>12.4</td>
<td>69</td>
</tr>
<tr>
<td>21-30</td>
<td>212</td>
<td>36.6</td>
<td>201</td>
</tr>
<tr>
<td>31-40</td>
<td>180</td>
<td>31.1</td>
<td>211</td>
</tr>
<tr>
<td>&gt;40</td>
<td>115</td>
<td>19.9</td>
<td>119</td>
</tr>
<tr>
<td>Total</td>
<td>579</td>
<td>100</td>
<td>600</td>
</tr>
</tbody>
</table>

*December 2006  †December 2008

Comparing the other confounding variables (table 7) showed that there were not significant difference between distribution of the studied women by the mean of age (p = 0.129), smoking (p = 0.147), passive smoking (p = 0.396), history of stomach surgery (p = 0.474), contraceptive using (p = 0.336) and lactation (p = 0.323) and marriage status (p = 0.614). In contrast, there were significant difference at baseline and after flour fortification in the household’s size (p = 0.000), and husband’s job (% of unemployed) (p = 0.02).

In the logistic regression model, all biochemical indicators including serum folate, serum vitamin B12 and plasma homocysteine (Hcy) were adjusted for those socio-demographic characteristics of the studied women that were found to be significant.
Table 7: Other characteristics of the studied women before and after flour fortification

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pre- fortification Period*</th>
<th>Post- fortification Period†</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age ±SD (year)</td>
<td>31.4±9.1</td>
<td>32.2±8.9</td>
<td>0.129</td>
</tr>
<tr>
<td>Smoking</td>
<td>7 (1.2)</td>
<td>13(2.2)</td>
<td>0.261</td>
</tr>
<tr>
<td>Passive smoking</td>
<td>145 (25)</td>
<td>136 (22.7)</td>
<td>0.396</td>
</tr>
<tr>
<td>History of gastric surgery</td>
<td>7(1.2)</td>
<td>6(1.0)</td>
<td>0.786</td>
</tr>
<tr>
<td>Contraceptive</td>
<td>109(22.8)</td>
<td>126(24.1)</td>
<td>0.336</td>
</tr>
<tr>
<td>Lactation</td>
<td>84(17.5)</td>
<td>85(16.3)</td>
<td>0.613</td>
</tr>
<tr>
<td>Household’s size (mean ±SD)</td>
<td>4.89±1.9</td>
<td>4.51±1.6</td>
<td>0.000</td>
</tr>
<tr>
<td>Husband’s job (Unemployed)</td>
<td>62(10.7)</td>
<td>92(13.1)</td>
<td>0.020</td>
</tr>
<tr>
<td>Married</td>
<td>477(82.4)</td>
<td>499(83.6)</td>
<td>0.614</td>
</tr>
</tbody>
</table>

*December 2006
† December 2008
Figures in brackets are percentage
Total number of women in pre-fortification period = 579
Total number of women in pre-fortification period = 600

4.2. Biochemical indicators of folate status

Table 8 shows the mean (CI 95%) for serum folate and plasma homocysteine concentrations in the studied women at base line and after flour fortification with folic acid. As this table shows, the mean serum folate concentration significantly increased
from 13.6 nmol/L to 18.1 nmol/L, after the initiation of flour fortification with folic acid ( \( p = 0.000 \)) . Also, the mean plasma Homocysteine concentrations significantly declined from 12.6 \( \mu \)mol/L to 7.1 \( \mu \)mol/L after flour fortification (\( p = 0.000 \)).

**Table 8: Serum Folate and plasma homocysteine concentrations of the studied women before and after fortification**

<table>
<thead>
<tr>
<th>Indices</th>
<th>n</th>
<th>Mean</th>
<th>95% Confidence Interval</th>
<th>2.5 percentile</th>
<th>97.5 percentile</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum folate (nmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-fortification period *</td>
<td>572</td>
<td>13.6</td>
<td>12.8 14.3</td>
<td>4.1</td>
<td>36.6</td>
<td>0.000</td>
</tr>
<tr>
<td>Post-fortification Period†</td>
<td>600</td>
<td>18.1</td>
<td>17.4 18.8</td>
<td>6.8</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1172</td>
<td>15.9</td>
<td>15.4 16.4</td>
<td>4.7</td>
<td>37.2</td>
<td></td>
</tr>
<tr>
<td>Hcys (( \mu )mol/L)∞</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-fortification Period *</td>
<td>561</td>
<td>12.6</td>
<td>12.1 13.2</td>
<td>5.5</td>
<td>32.6</td>
<td>0.000</td>
</tr>
<tr>
<td>Post-fortification Period†</td>
<td>600</td>
<td>7.1</td>
<td>6.7 7.5</td>
<td>2.8</td>
<td>21.7</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1161</td>
<td>9.8</td>
<td>12.8 10.2</td>
<td>3</td>
<td>28.1</td>
<td></td>
</tr>
</tbody>
</table>

*December 2006
†December 2008
∞ Homocysteine concentrations

From the results presented in table 9, it can be observed that low serum folate has decreased significantly from 14.3% at baseline to 2.3% after flour fortification (\( p=0.000 \)).

Also, a significant reduction in the prevalence of hyperhomocysteinemia, from 38.3% in the pre-fortification period to 7.3% in the post – fortification period was found.
Table 9: Prevalence rate of low serum folate and hyperhomocysteinemia before and after flour fortification

<table>
<thead>
<tr>
<th>Indices</th>
<th>Pre-fortification Period *</th>
<th>Post-fortification Period†</th>
<th>Total</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>Folate (nmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>490</td>
<td>85.7</td>
<td>584</td>
<td>97.7</td>
</tr>
<tr>
<td>Low</td>
<td>82</td>
<td>14.3</td>
<td>14</td>
<td>2.3</td>
</tr>
<tr>
<td>Total</td>
<td>572</td>
<td>100</td>
<td>600</td>
<td>100</td>
</tr>
<tr>
<td>Hcys (μmol/L)∞</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>346</td>
<td>61.7</td>
<td>556</td>
<td>92.7</td>
</tr>
<tr>
<td>High</td>
<td>215</td>
<td>38.3</td>
<td>44</td>
<td>7.3</td>
</tr>
<tr>
<td>Total</td>
<td>561</td>
<td>100</td>
<td>600</td>
<td>100</td>
</tr>
</tbody>
</table>

*December 2006
†December 2008
∞Homocysteine concentrations

Figure 1 shows the distribution curves of serum folate concentrations before and after fortification. This figure shows the clear upward shift in the distribution of serum folate from baseline (before fortification in 2006) to the final evaluation (after fortification in 2008).
Figure 1: Decline in the prevalence of low serum folate after flour fortification with folic acid in the studied women (vertical line shows serum folate<6.7 nmol/L).

From the results present in the table 9, it can be observed that a significant reduction in the prevalence of hyperhomocysteinemia has been achieved, that is from 38.3% to 7.3% (P=0.000) in the pre- and post-fortification period respectively.

Figure 2 shows the distribution curves of plasma Hcy concentrations in the pre-fortification and post-fortification period. This figure shows a clear downward shift in the distribution of plasma Hcy concentrations from baseline to the final evaluation.
Figure 2: Decline in the prevalence of hyperhomocysteinemia after flour fortification with folic acid among the studied women (vertical line shows Hcy>12μmol/ L).

The distributions of the women by serum folate and plasma homocystein concentrations, before and after flour fortification are shown in figure 3.
**Figure 3**: Distribution of the studied women by serum folate and plasma Homocysteine before and after flour fortification

Figure 4 shows that there was a significant negative correlation between serum folate and plasma homocysteine concentrations in the pre-fortification period (r = -0.252, P < 0.01) and post-fortification period (r = -0.171; p = 0.000). 

---

**Figure 3**: Distribution of the studied women by serum folate and plasma Homocysteine before and after flour fortification

Figure 4 shows that there was a significant negative correlation between serum folate and plasma homocysteine concentrations in the pre-fortification period (r = -0.252, P < 0.01) and post-fortification period (r = -0.171; p = 0.000).
Figure 4: correlation between serum folate and Homocysteine before and after flour fortification with folic acid.

Figure 5 shows that there was a significant negative correlation between serum vitamin B12 levels and plasma homocysteine concentrations in the pre-fortification (r = -0.213, P<0.01) and post-fortification period (r = -0.208; p = 0.000). Our results show that after flour fortification with folic acid, homocysteine correlated better with serum vitamin B12 levels (r = -0.208; p = 0.000) than with serum folate levels (r = -0.171, p = 0.000).
As figure 6 shows, we observed a direct association between serum vitamin B12 and serum folate before flour fortification ($r = 0.322$, $p = 0.01$), and after flour fortification ($r = 0.093$, $p = 0.05$).
Figure 6: correlation between serum folate and vitamin B12 before and after flour fortification with folate acid

From the results presented in table 10, it can be observed that the mean serum folate was 11.3 nmol/L (95% CI 10.3-12.4 nmol/L) and 17.3 nmol/L (95% CI 16.1-18.5 nmol/L) for women classified as having a low serum Vitamin B12, in the pre-fortification and post-fortification periods respectively. Among the women classified as having a normal serum Vitamin B12, the mean serum folate were 14.3 nmol/L (95% CI: 13.3-15.2nmol/L) and 18.5 nmol/L (95% CI 17.8-19.3nmol/L), in the pre-fortification and post-fortification periods respectively.
Table 10: Serum folate status in the studied women according to serum vitamin B12 status, before and after flour fortification

<table>
<thead>
<tr>
<th>Serum vitamin B12</th>
<th>n</th>
<th>serum folate</th>
<th>95% Confidence Interval</th>
<th>Minimum</th>
<th>Maximum</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lower Bound</td>
<td>Upper Bound</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>130</td>
<td>11.3</td>
<td>10.3</td>
<td>12.4</td>
<td>2</td>
<td>40.6</td>
</tr>
<tr>
<td>Pre-fortification</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Period *</td>
<td>205</td>
<td>17.3</td>
<td>16.1</td>
<td>18.5</td>
<td>5.9</td>
<td>67.7</td>
</tr>
<tr>
<td>Post-fortification</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Period †</td>
<td>335</td>
<td>15</td>
<td>14.1</td>
<td>15.9</td>
<td>2</td>
<td>67.7</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>442</td>
<td>14.3</td>
<td>13.3</td>
<td>15.2</td>
<td>2.2</td>
<td>86.5</td>
</tr>
<tr>
<td>Pre-fortification</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Period *</td>
<td>395</td>
<td>18.5</td>
<td>17.8</td>
<td>19.3</td>
<td>5</td>
<td>51.4</td>
</tr>
<tr>
<td>Post-fortification</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Period †</td>
<td>837</td>
<td>16.3</td>
<td>15.6</td>
<td>16.9</td>
<td>2.2</td>
<td>86.5</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*December 2006
†December 2008
∞ Mean serum folate (nmol/L)
P mol / L

From the results presented in table 11, it can be observed that the mean serum vitamin B12 of the studied women has declined significantly in the post–fortification period (p = 0.000).
Table 11: Serum vitamin B12 status of the studied women, before and after flour fortification

<table>
<thead>
<tr>
<th>Serum vitamin B12 (pmol/L)</th>
<th>n</th>
<th>Mean</th>
<th>95% Confidence Interval</th>
<th>2.5 percentile</th>
<th>97.5 percentile</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lower Bound</td>
<td>Upper Bound</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-fortification Period *</td>
<td>572</td>
<td>194.4</td>
<td>183.8</td>
<td>205</td>
<td>48</td>
<td>490.8</td>
</tr>
<tr>
<td>Post-fortification Period †</td>
<td>600</td>
<td>163.5</td>
<td>153.4</td>
<td>173.6</td>
<td>17.6</td>
<td>413</td>
</tr>
<tr>
<td>Total</td>
<td>1172</td>
<td>178.6</td>
<td>1712</td>
<td>186</td>
<td>22.7</td>
<td>453.1</td>
</tr>
</tbody>
</table>

*December 2006  
†December 2008

The results of our study showed that the mean serum vitamin B12 significantly decreased by 30.9 Pmol/l, that is from 194.4 Pmol/L to 163.5 Pmol/L. As table 12 shows, the percentage of low serum vitamin B12 among the studied women significantly increased from 22.7 % before flour fortification to 34.2 % after flour fortification with folate acid (p = 0.000).

Table 12: Prevalence rate of low serum vitamin B12 in the studied women, before and after flour fortification

<table>
<thead>
<tr>
<th>Serum vitamin B12 (Pmol/L)</th>
<th>Pre-fortification Period *</th>
<th>Post-fortification Period †</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>Normal</td>
<td>442</td>
<td>77.3</td>
<td>395</td>
</tr>
<tr>
<td>Low</td>
<td>130</td>
<td>22.7</td>
<td>205</td>
</tr>
<tr>
<td>Total</td>
<td>572</td>
<td>100</td>
<td>600</td>
</tr>
</tbody>
</table>

*December 2006  
†December 2008
In order to assess the possibility of masking vitamin B12 deficiency, we investigated whether or not the proportion of the studied women with low serum vitamin B12 concentrations without macrocytosis has increased in the post – folic acid fortification period.

Table 13 presents the association between the proportion of women with low serum vitamin B12 concentrations (<110 Pmol/L) without macrocytosis (MCV>96fl). The proportion of women without macrocytosis was higher after flour fortification (82.9%) than before flour fortification (80%), and the difference was not significant (p = 0.370).

**Table 13: Distribution of MCV in the studied women with low serum vitamin B12, before and after flour fortification**

<table>
<thead>
<tr>
<th>MCV(fl)</th>
<th>Serum vitamin B12 Low (&lt;110Pmol/L)</th>
<th>Total</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-fortification Period*</td>
<td>Post-fortification Period†</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>Number 104</td>
<td>170</td>
<td>274</td>
</tr>
<tr>
<td></td>
<td>% 80</td>
<td>82.9</td>
<td>81.8</td>
</tr>
<tr>
<td>High MCV &gt;96fl</td>
<td>Number 21</td>
<td>32</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>% 16.2</td>
<td>15.6</td>
<td>15.8</td>
</tr>
<tr>
<td>Total</td>
<td>Number 130</td>
<td>205</td>
<td>335</td>
</tr>
<tr>
<td></td>
<td>% 100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

*December 2006  †December 2008  ∞ MCV>96fl

From the results present in the table 13, it can be observed that women with a low serum vitamin B12 concentration were no more likely to be without anemia after flour fortification with folic acid.

Table 14 shows the mean MCV (fl) among the studied women. Overall, mean MCV in the post -fortification period (83.5fl) was significantly lower than the mean of MCV.
(84.3fl) in the pre-fortification period (p = 0.000).

**Table 14: MCV status of the studied women before and after flour fortification with folic acid**

<table>
<thead>
<tr>
<th>MCV(fl)</th>
<th>n</th>
<th>Mean</th>
<th>95% Confidence Interval</th>
<th>2.5 percentile</th>
<th>97.5 percentile</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lower Bound</td>
<td>Upper Bound</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-fortification Period *</td>
<td>572</td>
<td>84.3</td>
<td>83.7</td>
<td>84.8</td>
<td>63.8</td>
<td>93.7</td>
</tr>
<tr>
<td>Post-fortification Period †</td>
<td>600</td>
<td>83.5</td>
<td>82.9</td>
<td>94</td>
<td>64</td>
<td>92.6</td>
</tr>
<tr>
<td>Total</td>
<td>1172</td>
<td>83.9</td>
<td>83.5</td>
<td>84.2</td>
<td>63.8</td>
<td>93.2</td>
</tr>
</tbody>
</table>

*December 2006  †December 2008

To determine whether those who had the lowest vitamin B12 concentrations were more likely than others to have higher MCV levels, we examined the correlation between serum vitamin B12 and MCV. A negative correlation was found between serum vitamin B12 and MCV before fortification that was not significant (r = -0.03; p = 0.485). However, after flour fortification a significant negative association between MCV and serum vitamin B12 was found (r = -0.139; p<0.01).
Figure 7: correlation between serum vitamin B12 and MCV before and after flour fortification with folic acid

4.3. Dietary intake’s Findings

Table 15 shows the mean intake of natural folate before and after flour fortification. The mean daily intake of naturally occurring folate for the studied women between pre-fortification and post-fortification period were 198.3μg/day (95% CI 185.4μg/day - 211.3μg/day) and 200.8μg/day (95%CI 191.9μg/day -209.6μg/day) respectively. A significant difference between natural folate intake in the pre-fortification and post-fortification period was not found (p = 0.741).
Table 15: The mean of natural folate intake of the studied women before and after flour fortification

<table>
<thead>
<tr>
<th>Folate (µg/day)</th>
<th>n</th>
<th>Mean</th>
<th>95% Confidence Interval</th>
<th>2.5 percentile</th>
<th>97.5 percentile</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lower Bound</td>
<td>Upper Bound</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-fortification *</td>
<td>557</td>
<td>198.3</td>
<td>185.4</td>
<td>211.3</td>
<td>32.4</td>
<td>519.8</td>
</tr>
<tr>
<td>Post-fortification †</td>
<td>594</td>
<td>200.8</td>
<td>191.9</td>
<td>209.6</td>
<td>65.5</td>
<td>461</td>
</tr>
<tr>
<td>Total</td>
<td>1151</td>
<td>200.3</td>
<td>189.1</td>
<td>210.4</td>
<td>49.2</td>
<td>491</td>
</tr>
</tbody>
</table>

*December 2006  †December 2008

In the post–fortification period, 6 uncompleted questionnaires were omitted.

Table 16 shows the mean total folate intake of the studied women in the pre-fortification period (natural folate) and post- fortification period (natural folate + folic acid added to bread). The folate intake of the studied women has increased significantly from 198.3 µg/day, before flour fortification to 413.7 µg/day after flour fortification (p = 0.000).

Table 16: The mean of total folate intake in the studied women before and after flour fortification

<table>
<thead>
<tr>
<th>Folate (µg/day)</th>
<th>n</th>
<th>Mean</th>
<th>95% Confidence Interval</th>
<th>2.5 percentile</th>
<th>97.5 percentile</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lower Bound</td>
<td>Upper Bound</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-fortification *∞</td>
<td>557</td>
<td>198.3</td>
<td>185.4</td>
<td>211.3</td>
<td>32.4</td>
<td>519.8</td>
</tr>
<tr>
<td>Post-fortification †∞</td>
<td>594</td>
<td>413.7</td>
<td>399.2</td>
<td>428.1</td>
<td>45.4</td>
<td>794.2</td>
</tr>
<tr>
<td>Total</td>
<td>1151</td>
<td>309.5</td>
<td>298</td>
<td>321</td>
<td>39.1</td>
<td>658.1</td>
</tr>
</tbody>
</table>

*∞December 2006, natural food folate  †∞December 2008, natural food folate + folic acid added to bread
We assessed the maximum amount of total folate intake after flour fortification. As it can be observed in table 16, the percentile of 97.5 for total folate intake was 794.2 μg/day. We also determined the percentile of 99 for total folate intake, after flour fortification and it was 919.5 μg/day.

We assessed the mean consumption of bread in the studied women. From the results presented in table 17, it can be observed that the mean (95% CI) of bread consumption was 246.2 g/day (234.2 g/day - 259.1 g/day) in the pre-fortification period, and 253.8 g/day (242.3 g/day - 265.4 g/day) in the post-fortification period respectively. There was not a significant difference between the amount of bread consumption before and after flour fortification (p = 0.821).

**Table 17 : Mean consumption of bread in the studied women before and after flour fortification**

<table>
<thead>
<tr>
<th>Bread (g/day)</th>
<th>n</th>
<th>Mean</th>
<th>2.5 percentile</th>
<th>97.5 percentile</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lower Bound</td>
<td>Upper Bound</td>
<td></td>
</tr>
<tr>
<td>Pre-fortification *</td>
<td>557</td>
<td>246.2</td>
<td>234.2</td>
<td>259.1</td>
<td>51</td>
</tr>
<tr>
<td>Post-fortification †</td>
<td>594</td>
<td>253.8</td>
<td>242.3</td>
<td>265.4</td>
<td>57</td>
</tr>
<tr>
<td>Total</td>
<td>1151</td>
<td>249.1</td>
<td>239.6</td>
<td>261.7</td>
<td>55</td>
</tr>
</tbody>
</table>

*December 2006 †December 2008

We measured the amount of folic acid in the bread samples that were collected in the post-fortification period. Analysis were performed using a high-performance liquid chromatography (HPLC) method. The mean folic acid content for 60 bread samples collected in the post fortification period, was 89 ±10 μg / 100g of bread (range49 -110 μg / 100g). Therefore, considering the average amount of bread consumption (253.8 g/day) in the post fortification period, the implementation of mandatory flour fortification resulted in an average additional dietary intake of 226 μg/day of folic acid from fortified
bread in the studied women. The maximum dietary intake of folic acid due to flour fortification was 570μg /day.

As the figure 8 shows, the percentage of the women having low folate intake (< 400μg/day) decreased from 93.8% before flour fortification to 52.9% after flour fortification. On the other hand, the percentage of women with folate intake above 400μg/day increased from 5.6% to 46.8% after flour fortification. Only, 0.3% of the studied women in the post-fortification period had a folate intake above 1000μg/day, compared to 0.6% of the women in the pre-fortification period.

**Figure 8**: Percentage of folate intake (μg/day) among studied women before and after flour fortification with folic acid

Figure 9 present the distribution curves of the studied women by folate intake before and after flour fortification. After flour fortification with folic acid, almost half of the women received 400 μg folic acid per day.
Figure 9: Distribution curves of the studied women by folate intake before and after flour fortification. The vertical line shows folate intake equal to RDA (400 μg/day).

Figure 10 shows the correlation between folate intake and serum folate in the studied women. Folate intake was positively correlated with serum folate concentrations in the pre- and post-fortification period (r = 0.084; p<0.05). As this figure shows by increasing the folate intake, serum folate has increased significantly.
From the result presented in table 18, we can observe a significant decline in the mean intake of vitamin B12 after flour fortification that was from 2.6μg/day in the pre-fortification period to 1.7μg/day in the post-fortification period (p=0.009).
Table 18: Mean of vitamin B12 intake of the studied women before and after flour fortification

<table>
<thead>
<tr>
<th>Vitamin B12 (μg / day)</th>
<th>n</th>
<th>Mean</th>
<th>95% Confidence Interval</th>
<th>2.5 percentile</th>
<th>97.5 percentile</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lower Bound</td>
<td>Upper Bound</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-fortification *</td>
<td>557</td>
<td>2.6</td>
<td>1.9</td>
<td>3.2</td>
<td>0.0∞</td>
<td>7.9</td>
</tr>
<tr>
<td>Post-fortification †</td>
<td>594</td>
<td>1.7</td>
<td>1.5</td>
<td>1.8</td>
<td>0.0</td>
<td>5.9</td>
</tr>
<tr>
<td>Total</td>
<td>1151</td>
<td>2.1</td>
<td>1.8</td>
<td>2.4</td>
<td>0.0</td>
<td>6.7</td>
</tr>
</tbody>
</table>

*December 2006
†December 2008
∞Due to assessment method (single 24-h-recall) and assigned zero-values for all plant foods in the food composition database considering that all plant foods consumed contained no cobalamin

As table 19 shows, vitamin B12 intake in 48.1% and 51.9% of the women was lower than the RDA (2.4 μg /day) in the periods of before and after flour fortification, respectively. The difference between the prevalence of low vitamin B12 intake in the pre-fortification and post-fortification period was not significant (p=0.4).

Table 19: Distribution of the studied women by vitamin B12 intake, before and after fortification

<table>
<thead>
<tr>
<th>Vitamin B12 (μg / day)</th>
<th>Pre-fortification *</th>
<th>Post-fortification †</th>
<th>Total</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>Low ∞</td>
<td>351</td>
<td>48.1</td>
<td>379</td>
<td>51.9</td>
</tr>
<tr>
<td>Normal</td>
<td>206</td>
<td>49</td>
<td>214</td>
<td>51</td>
</tr>
<tr>
<td>Total</td>
<td>557</td>
<td>48.4</td>
<td>593</td>
<td>51.6</td>
</tr>
</tbody>
</table>

*December 2006
†December 2008
∞<2.4 μg / day
We examined whether low serum vitamin B12 in the studied women was correlated to vitamin B12 intake, but did not find a significant correlation.

4.3. Trend of NTDs prevalence before and after flour fortification

Between September 2006 (pre-fortification period) and December 2008 (post-fortification period), 35 NTD cases were recorded, of which spina bifida was the most common type of anomaly (51.4%), followed by anencephaly (48.6%).

As the results presented in table 20, it can be observed that the overall prevalence of NTDs decreased from 3.16 per 1000 births including live births and stillbirths, before flour fortification (September 2006-July 2007) to 2.19 per 1000 births during the full-fortification (December 2007-December 2008) period (RR 0.09, 95% CI 0.042 - 0.21) that was statistically significant (P-value<0.01).

The total annual rate of NTDs significantly declined by 31% (95%CI 26%-35%) after the implementation of folic acid fortification (P <0.01). As table 19 shows, although the prevalence rate of NTDs has been significantly declined after flour fortification with folic acid, still there is a high prevalence rate of NTDs in Golestan province (2.19 per 1,000 births).

Our finding shows that the prevalence rate of spina bifida declined from 1.75 per 1000 birth before fortification to 1.09 per 1000 birth after fortification (38%). At the same time, the prevalence of anencephaly decreased from 1.68 per 1000 birth to 1.09 per 1000 birth (35%). These differences were not significant.
We examined association between the selected socio-demographic characteristics of mothers and NTD. There was not a significant difference between mother's age (p = 0.579), level of education (p = 0.346), taking of folic acid supplement (p = 0.481), smoking (p = 0.721) and the incidence of NTDs, before and after flour fortification periods.
Figure 11. Rates of NTDs, September 2006 (before flour fortification) – December 2008 (after flour fortification), 28 months moving average rate.
5. Discussion

In this population based study the effectiveness of the public health strategy of flour fortification with folic acid was evaluated and possible adverse effects resulting from fortification were determined in women of child bearing age.

This study was undertaken in Golestan province in the north of Iran, due to the historically high rates of NTDs. We assessed whether the response in serum folate and plasma homocysteine as the markers of folate status were associated with fortified bread consumption.

The mean daily intake of naturally occurring folate was calculated by using a 24-h recall questionnaire, in the periods before and after flour fortification. For assessing the additional folic acid intake, the mean consumption of bread as the main source of folic acid was calculated. Furthermore, the average additional daily intake of folic acid from fortified breads was determined by measuring folic acid in the fortified bread, for the period after flour fortification.

The results of the present study demonstrate that the mean intake of natural food folate is almost half of the recommended levels, indicates that the women in childbearing age can not reach to the optimal folate intake through diet. The mean daily intake of naturally occurring folate among studied women in the present study (during pre and post flour fortification periods) is higher than the folate intake in Dutch women (Konings, et al., 2001) but lower as compared to Austrian (Elmadfa and Freisling ,2004) and Canadian women (Shiliang, et al, 2004) . However this finding is similar to intake data of Finnish women (Alfthan, et al,2003).

The mean folate intake of the studied women after fortification (413.7μg/day) is consistent with the mean folate intake (427μg/day) of women in Chili after implementing flour fortification with folic acid (Hertrampf and Cortes, 2004).

The results of the present study indicate that flour fortification with folic acid has increased typical folic acid by an average 226μg/day, due to consumption of fortified bread in women of childbearing age. The maximum dietary intake of folic acid due to flour fortification was 570μg / day, based on the maximum amounts of consumed bread.

When we considered the percentiles of 97.5 and 99 for the total folate intake (794.2
μg/day and 919.5 μg/day, respectively). The possible risk of high folate intake (> 1000μg/day) due to flour fortification with folic acid would be at the maximum level of 1% of this population.

The current study shows that there was no significant difference in folate intake from naturally occurring sources in the pre and post fortification period and suggests that any change in folate status is the result of folic acid added to bread as part of the mandatory flour fortification policy in Iran. On the other hand, there was not a significant difference between the amount of bread consumption before and after flour fortification (p = 0.821). It indicates that the amount of bread consumed was not changed significantly in the pre-fortification and post-fortification periods and increase in the folate intake is because of fortified bread with folic acid. Additionally due to the non availability of any other fortified food products with folic acid in Iran and, no folic acid supplements were taken by any of the studied women, we can conclude that the wheat bread fortified with folic acid was the main source of this nutrient in the studied population. Considering that folic acid fortification is restricted to only two kinds of flour which are used for baking flat breads, the possibility of high folic acid intake due to consuming fortified bread is low.

The results of this study are consistent with those who found the potential of a mandatory fortification policy to improve folate intake and folate status of women in child bearing age ((Ray et al, 2002, Hirsch et al, 2002, Ganji and Kafai, 2005) to a similar degree as achieved in this study.

In this study serum folate and plasma homocysteine as biomarkers of folate intake and folate status are used. The results show that due to flour fortification with folic acid, the prevalence rate of low serum folate (<6.7nmol/L) decreased by 84% in the studied women.

The results are similar to the finding by Ganji who showed 91% reduction in the low serum folate in the United States (Ganji and Kafai, 2005). Hickling reported 56% decline in the subjects with low serum folate (Hickling et al, 2005).

We found 25% increase in the mean serum folate in the studied women after flour fortification. Although this findings disagrees from Jacques who reported 57% increase in the mean serum folate in a population of middle aged (Jacques et al, 1999) and Lawrence who reported 71% increase in median serum folate values in Kaiser clients residing in
Southern California (Lawrence et al, 1999), however consistent with Hickling who showed 38% increase in the mean serum folate after introduction of voluntary folate fortification policy in Australia (Hickling, 2005). Different degrees of improvement observed in these studies may be due to the length of exposure to folic acid.

The results of this study show the increase of 4.5 μg/l in serum folate under mandatory folic acid fortification in Golestan province, which is higher than the measured increase in serum folate (3.8 μg/l) under mandatory folate fortification policy reported by Ray in Canada (Ray et al, 2002) and voluntary folate fortification in Australia (2.8 μg/l) (Hickling et al, 2005), and lower than the measured increase in serum folate in USA (5.4 μg/l) (Jacques et al, 1999) and in Chile that was 7.2 μg/l (Hirsch et al, 2002). Only those subjects who had fasted ≥ 12 h was included for the reason that folate concentration measured in a fasted state is a better indicator of folate status (Ganji and Kafai, 2005), and there is evidence that serum folate in fasting blood sample is good for assessing folate status as red blood cell folate at the population level. A good correlation between serum folate and red cell folate has been reported (Galloway and Rushworth, 2003). The advantage of measuring serum folate is that, it is easier to perform and less expensive.

Folate intake was positively correlated with serum folate concentrations in the pre- and post-fortification period. Serum folate is an indicator of recent folate intake (National academy of Sciences, 2000) and the food intake data were collected through a 24-hour-recall, also representing recent intake.

Findings of the present study show a significant reduction in the mean Hcy concentrations of the studied women by 56% (p = 0.000), which is in accordance with the Jacques who reported a reduction by 52% in the mean Hcy concentrations in the studied women (Jacques et al, 1999). On contrary, Hickling found 21% reduction in mean Hcy in the studied population after introduction of the voluntary folate fortification policy in Australia (Hickling et al, 2005) and Ganji reported 16.1% reduction in the mean Hcy concentrations after folic acid fortification in the United States (Ganji and Kafai, 2005). The results reflect that after flour fortification with folic acid, homocysteine correlated better with serum vitamin B12 levels (r = -0.208; p = 0.000) than with serum folate levels (r = -0.171, p = 0.000). Similarly, Hirsch reported a better correlation between
homocysteine and serum vitamin B12 levels than with serum folate levels, 6 months after
flour fortification with folic acid (Hirsch et al, 2002). Results of this study support the
finding by the other researchers that, since folic acid fortification vitamin B12 has
become the main determinants of plasma homocysteine in postfortification populations (Robertson et al, 2005) or, in subjects with optimized folate status (Quinlivan et al, 2002).
A mandatory folate fortification policy, which can reach a greater proportion of the
population, is likely to result in a wider spread increase in serum folate and a greater
decrease in Hcy concentrations.

Findings show a considerable reduction in the prevalence rate of hyperhomocysteinemia,
from 38.3% to 7.3% in the studied population (88%).

This result is consistent with other findings. Hirsch reported a significant reduction in
hyperhomocysteinemia, from 31% to 17%, 6 months after flour fortification with folic
acid (Hirsch et al, 2001). Jacques also reported a reduction of high Hcy concentrations
from 18.7% to 9.8% (Jacques et al, 1999).

In our study, the decrease in the mean plasma homocysteine concentrations that is 5.5μ
mol/l is higher than the measure decrease in the mean plasma homocysteine in Australia
that is 2.1μmol/L (Hickling et al, 2005). Results of the present study indicate that the
mean Hcy concentrations of the studied women (7.1mol/L) fell within the lower end of
normal range (Ueland et al, 1993) and reflected the positive folate balance of these
women.

This improved folate status in current study, remained significant after adjusting the
analysis for socio-demographic characteristics of the women.

Assessing serum folate concentrations, demonstrate that with increasing folate intake due
to fortified bread, there was a significant increase in the proportion of women achieving
optimal folate status (i.e., a serum folate value > 6.7 nmol/l; Sauberlich, 1999).

It is not possible that the observed differences in serum folate status resulted from
variations in folate intake that was unrelated to fortification. However, we adjusted serum
folate values for folate dietary intake and therefore we are confident that the changes seen
in serum folate status can be ascribed to folic acid fortification.

Blood folate levels are still an intermediate outcome, and the real measure of the impact
of increased folic acid consumption is the reduction of NTDs rates.
The current study shows 31% reduction in the prevalence rate of NTDs after flour fortification.

This observation is similar to reports from other countries where fortification is taking place. The reduction in the NTDs rate after implementation of fortification in our study is greater than the 19% reduction in the prevalence of NTDs in the US after mandatory fortification (Honein et al. 2001) but lower than the 78% reduction in Newfoundland (Liu et al., 2004) and 54% reduction in rate of NTDs reported in Nova Scotia after fortification (Persad et al., 2002). Similar to our results, 32% reduction in NTDs in Quebec was reported (De Wals et al., 2003). Also, Bower has explored 30% reduction in NTDs after folic acid fortification in Western Australia (Bower et al., 2002). Similar to our study, Honein et al., reported a non significant reduction in spina bifida and anencephaly after mandatory flour fortification in US. Evidence from several European populations shows that, in the absence of mandatory folic acid fortification, public health strategies to prevent NTDs have been ineffective (Botto et al., 2005, Busby et al., 2005).

Findings, therefore, support the view expressed by many that mandatory fortification with folic acid is the only means of ensuring that all women of reproductive age can benefit in terms of reduced risk of NTDs. Decline in NTDs prevalence rate in our study was also consistent with the observed increase in serum folate levels and folic acid intake in the women of reproductive age after flour fortification with folic acid.

Our Findings confirm that, mandatory flour fortification in Iran would be effective in reducing NTDs risk in women who regularly consume fortified bread.

According to our findings, we believe that a significant decrease in prevalence rates of NTDs is corresponding to the implementation of flour fortification with folic acid. The limitation of our study is that it was carried out in a referral center and we did not include spontaneous abortion caused by NTDs as it was difficult to obtain the data.

Although the prevalence rate of NTDs has been significantly declined after flour fortification with folic acid, still there is a high prevalence rate of NTDs in Golestan province (2.19 per 1,000 births). We found 31% reduction in the NTDs prevalence after folic acid fortification, it means that not all NTDs are related to folic acid, and that other genetic and nutritional factors such as vitamin B12 and Zinc may play an important role.
Researchers have shown the role of maternal vitamin B12 deficiency (Ray et al., 2007) as a potential risk factor for NTDs.

In theory, the potential role of vitamin B12 in preventing NTDs and other congenital anomalies is explicable by its metabolism. Vitamin B12 interacts with folic acid in the remethylation of homocysteine to methionine, a key metabolic reaction in the function of folic acid. More recent studies have made use of better biomarkers of vitamin B-12 status, including methylmalonic acid and holotranscobalamin (holoTC).

By assessing Holo TC that represents the bioavailable fraction of circulating vitamin B-12, Thompson in a large Canadian cohort accrued before and after folic acid fortification, found a 3-fold increase in the risk of NTDs in mothers who had vitamin B-12 status in the lower quartile, regardless of folic acid fortification (Thompson et al., 2009).

High plasma Homocysteine may play an independent role in the development of NTDs. Felkner suggested that high homocysteine levels have a detrimental effect on NTD-risk even when serum B12 or red blood cell folate levels are high (Felkner et al., 2009). Three B vitamins are involved in homocysteine metabolism: the biological active form of vitamin B6 as a cofactor for the enzyme cystathionine β synthase, vitamin B12 as a cofactor for methionine synthase, and 5- methyltetrahydrofolate a substrate. The formation of methyltetrahydrofolate is catalyzed by methylenetetrahydrofolate reductase (MTHFR), a vitamin B2-dependent enzyme (Finkelstein, 1990). Homocysteine metabolism plays an important role in so-called one-carbon metabolism providing methyl groups to all kinds of substance, including the synthesis and regulation of DNA and mRNA, which are essential for embryonic cells (Massaro and Rogers, 2002). A possible derangement of folate-dependent homocysteine metabolism in women who had NTD offspring has been reported (Steegers-Theunissen et al., 1991). The derangement of homocysteine metabolism in mothers with NTD offspring is based on gene mutations of the MTHFR enzyme. A change of alanine to valine at the 225th amino acid of MTHFR decrease the activity of this enzyme by 35% (Engbersen et al., 1995). The mutation predisposes to mild hyperhomocysteinemia in the presence of a low folate status (Kluijtmans et al., 1996). A shortage of MTHFR as a result of the C677T mutation located on chromosome 1 in families with NTD has been found (Van der Put et al., 1995).
There are different ethnic groups in Golestan province and it is necessary to determine the prevalence of the genetic mutation C677T in these ethnic groups. The C677T mutation is associated with an elevated plasma homocysteine and a low folate status (Massaro and Rogers, 2002). Also, other genes involved in folate-homocysteine metabolism and their interaction should be investigated in this province.

Zinc deficiency is another factor that may result NTDs. Animal model studies have shown that hepatic methionine synthase activities were increased in zinc deficient rats. This increased resulted in a decreased 5- methyltetrahydrofolate in the liver of zinc deficient rats. Furthermore, plasma folate and homocysteine were lower in zinc deficient rats (Hong et al., 2000). Also, folate and Homocysteine metabolism in copper deficient rats, has shown that hepatic methionine synthase activity in the copper deficient group was significantly lower. The percentage of 5- methyltetrahydrofolate in total hepatic folate in the copper deficient group was significantly higher (Tamura et al., 1999). All of these changes in folate metabolism in zinc or copper deficiency are likely to be secondary to the alterations in methionine synthase activity (Massaro and Rogers, 2002).

In humans, it may be reasonable to that decreased folate concentrations in the maternal circulation with zinc deficiency, result in decreased placental folate transfer; thus, compromised folate may lead to fetal growth retardation or malformations of the central nervous system (Massaro and Rogers, 2002). Association between presence of NTDs and zinc deficiency in newborns affected with NTDs also, has been reported (Carrillo-Ponce et al., 2004).

In Golestan province, Golalipour reported the association between NTDs and maternal zinc deficiency (Golalipour et al., 2009). Due to using small sample size in this study, further researches with large enough sample size is needed to confirm the role of zinc deficiency in the prevalence of NTDs in this province.

The other factor that may have a role in the NTDs prevalence in Golestan province, could be vitamin B2 (riboflavin) deficiency, since, a high prevalence of low vitamin B2 intake, has been reported in Iran (Ministry of Health & Medical Education, 2001). Studies demonstrated that low plasma riboflavin concentrations are associated with higher plasma total homocysteine concentrations. The relationship is confined to individuals who have both lower circulating folate concentrations and are homozygote for
the MTHFR C677T polymorphism. MTHFR converts methylenetetrahydrofolate to methyltetrahydrofolate, which is required for the methylation of homocysteine to methionine; riboflavin, in the form of flavin adenine dinucleotide (FAD), is a cofactor for MTHFR (Jacques et al, 2002).

In those countries where, enriched flour and grain products include riboflavin, riboflavin status would not appear to be an important determinant of circulating Hcy concentration. However, the importance of riboflavin as a determinant of homocysteine should not be underestimated in other countries, particularly where the prevalence of inadequate folate and riboflavin intakes is more common.

Current study examined whether folic acid fortification would mask vitamin B12 deficiency. The reason is that vitamin B12 and folic acid have the same metabolic pathway and additional folic acid could correct the hematological signs of vitamin B12 deficiency and postpone clinical diagnosis, which could lead to neurological damage.

The metabolic interrelationship between folic acid and vitamin B12 suggests that when the folate cofactor 5- methyltetrahydrofolate (THF) is formed, the enzyme 5,10-methyleneTHF reductase that forms the cofactor cannot use it in the back reaction in vivo (the methyl trap hypothesis). Thus, the only way for this folate cofactor to be recycled to THF (and thus participate in DNA biosynthesis and allow cell division), is through the vitamin B12-dependent enzyme methionine synthase. When the activity of the enzyme is compromised, as it would be in Pernicious Anaemia (PA), the cellular folates will become progressively trapped as 5-methylTHF. The consequence will be an anemia identical to that seen in true folate deficiency. Thus, in PA there will be a neuropathy and/or anaemia. Treatment with vitamin B12, if given intramuscularly, will reactivate methionine synthase, allowing myelination to restart. The trapped folate will also be released, thus allowing DNA synthesis and generation of erythrocytes, redressing the anaemia. It is known that treatment with high concentrations of folic acid will also treat the anaemia but not the neuropathy in PA, because when folic acid enters the cell it is first converted via dihydrofolate to THF. However, administration of folic acid cannot do anything to restart the methylation cycle, blocked as it is at the vitamin B12-dependent enzyme methionine synthase. Thus, the neuropathy will continue (masking vitamin B12
deficiency), only to be diagnosed later when treatment with vitamin B12 may only partly reverse the nerve damage (Cuskelly et al, 2007).

The present study did not find the evidence of masking vitamin B12 deficiency after folic fortification.

This finding is in agreement with Mills findings which showed no increase in the number of subjects with a combination of low vitamin B12 concentrations and no macrocytosis after the introduction of folic acid fortification (Mills et al, 2003). On the other hand, Wyckoff reported that the proportion of the subjects with low serum vitamin B12 concentration but without macrocytosis increased after flour fortification with folic acid (Wyckoff, 2007).

Our results provide important reassurance that folic acid exposure due to flour fortification has not resulted in an increase in the proportion of women with a low serum vitamin B12 concentration but normal MCV values.

Due to the non-availability of any data on vitamin B12 status of the women of childbearing age in this province, we assessed vitamin B12 status of the women in the present study. We found a high prevalence of low serum B12 among the women of childbearing age both in the pre-fortification and post-fortification period. The result demonstrates that the prevalence rate of vitamin B12 deficiency was higher even after flour fortification.

A significant increase in the mean serum vitamin B12 levels in Canadian women aged 19-44 was reported one year after folic fortification (Liu et al, 2004). It is disagree with those of (Hertrampf and Cortes, 2004), who reported no changes in the vitamin B12 distribution curves of Chilean women of childbearing age after flour fortification with folic acid.

A high rate of vitamin B12 deficiency was reported 9 years after the implementation of Canadian folic acid flour fortification (Ray et al, 2008). High prevalence rate of vitamin B12 deficiency, as we found in our study, shows that Vitamin B12 status of population has to be considered when a folic acid fortification program is being implemented.

This study was unable to demonstrate a significant correlation between vitamin B12 intake and serum B12, whereas other studies have reported a strong positive association between serum B12 and a dietary pattern that included frequent intake of animal food.
sources (Villamor et al, 2008). The results of this study show that vitamin B12 intake in almost 50% of the women was lower than the recommended level in the periods of before and after flour fortification, respectively. A possible explanation for vitamin B12 deficiency in this studied population might be low consumption of Vitamin B12 rich food sources. There are, however, other possible explanations such as H. pillory infection, giardiasis (Olivares et al, 2002) and self treatment by anti acid drugs.

The mean daily (µg) vitamin B12 intake of Iranian women in the present study was less as compared to the women in European countries, such as Finland (7.4 µg), Denmark (4.3 µg), United Kingdom (5.4 µg), Ireland (3.6µg), Netherlands (4.3 µg), Germany (4.7 µg), France (6.3 µg), and Spain (4.9 µg) (Dhonukshe- Rutten, et al, 2007).

Higher dietary intake of vitamin B12 in European women as compared to Iranian women may be explained by higher socio-economic status which leads to increase consumption of animal protein in European populations. Serum vitamin B12 concentrations in these countries are also higher (Dhonukshe- Rutten, et al, 2007).

It is assumed that vitamin B12 deficiency is unlikely to occur except under special circumstances, such as vegetarianism, elderly period, food-cobalamin malabsorption syndrome, Helicobacter Pylori infection, and intestinal microbial proliferation, which can be caused by antibiotic treatment. Nevertheless, recent studies have shown a high prevalence of serum vitamin B12 deficiency in different population groups such as school children (Rogers et al, 2003) and both adult men and women (Allen 2004).

In developing countries, low serum vitamin B12 concentrations could be a consequence of reduced consumption of animal protein due to its high cost. According to the Food and Agriculture Organization of the United Nations (FAO), per capita supply of animal products in developing countries constitutes less than one third of the documented supply in industrialized countries (Jetzi, 2003).

Our findings suggest that vitamin B12 status of general population should be monitored, during folic acid fortification in developing countries, where the probability of vitamin B12 deficiency is high. Vitamin B12 is a risk factor, independent of folate status, in NTDs occurrence especially in the era after folic acid flour fortification. Further work is required to establish this, and possibly, considering adding vitamin B12 to folic acid fortified flour.
We may have underestimated nutrient intakes because of the known fact of underreporting in self-reported dietary intake measurements. A particular disadvantage of the 24-hour-recall method, which was used in this study, is the difficulty of determining whether the day being recalled represents an individual's typical intake because of day-to-day variation in intakes of individuals. However, mean intake of energy and nutrients of a population should be unaffected against individual day-to-day variation of dietary intake. One the other hand, advantages of a single recall are ease in administration at the population level, minimization of biases associated with altering food intake because of knowledge that one is being observed, and the low interview burden. Regarding to vitamin B12 intake, because vegetarianism in our study region is uncommon, we consider gross underestimation to be unlikely.

Although this was an observational study and therefore can not confirm a causal relation, the higher serum folate status and lower Hcy concentrations observed in the studied women were the direct result of fortification rather than a generalized positive dietary pattern. In support of this, we found no difference in natural food folate intakes at baseline and after flour fortification. Only folic acid intakes were found to increase and fortified bread was the only source of the latter.

**Limitations**

An important limitation of this study lies in the use of serum B12, although the indicators such as methylmalonic acid (MMA) are more sensitive (Ubbnik, etal, 1991). However, the high cost of this test at the population level constrained the use of this test. On the other hand, MMA can be elevated by intestinal bacterial overgrowth (Lindenbaum,etal, 2006) resulted over estimation of B12 deficiency. More over, there is an increasing interest in the diagnostic value of plasma holotranscobalamin concentrations. However, there is usually a strong correlation between total serum B12 and holo TC in populations (Clark etal, 2007 and Shahab –Ferdows etal, 2008). Thus, considering the higher cost of the holo TC assay; it was more practical for this study to rely on serum B12 for the estimation prevalence of vitamin B12 deficiency in the studied population.

Another limitation of the current study is that the dietary data was derived from a single 24-hour-recall, which has limitations regarding the assessment of the prevalence of
nutrient inadequacy in a population group. Food consumption data were obtained from a single day. There are possibilities that quantitative assessment of folate intake may not be accurate enough and/or underestimation of folate intake, because, assessing of dietary folate is complicated by data gaps in food composition tables, variations between methods used for folate analysis, and limited understanding of the bioavailability of food folate.

The studied population could have changed over the course of the study. There were, however, no major changes in the socio demographic and health status of the studied population and no significant difference in confounding variables were found.
6. Summary

Folic acid is an important nutrient for women in childbearing age. Adequate folate intake during the peri-conceptional period, the time right before and just after a woman becomes pregnant, helps protect against a number of congenital malformations including Neural Tube Defects (NTDs) which are the most notable birth defects that occur from folate deficiency.

Folic acid fortification has been suggested as a main strategy to control and reduce the prevalence of NTDs. The aim of this research was to study the effects of folic acid fortification on reduction of NTDs and improve folate status of Iranian women in childbearing age. This study were conducted before and after mandatory flour fortification (2006-2008) with folic acid in the Golestan province located in the north of Iran, which has a high prevalence of NTDs. Two cross sectional study as base line (pre-fortification period) and final evaluation (post –fortification period) were conducted. In each phase, biochemical indicators of folate status, socio-demographic, health characteristics and dietary intake were studied in 600 women of child bearing age as representative sample of women in Golestan province and, the results were compared. Diet was assessed by a single 24-h-recall, covered all days of the week, including weekends. The final dietary intake data were validated in the Department of Nutritional Sciences, University of Vienna, Austria, by comparing the nutrient content of selected food items, representative of the average Iranian diet, with the food composition data of the Austrian food composition database.

Additionally, a longitudinal hospital based study 12 months before and 12 months after flour fortification was conducted to determine the trend of NTDs. The results show that due to flour fortification with folic acid, prevalence of low serum folate decreased by 84% and the mean serum folate showed 25% improvement after fortification (from 13.6nmol/L to 18.1nmol/L; p=0.000). The findings of this study show 88% reduction in the prevalence of hyperhomocysteinemia (from 38.8% to 7.3%; p = 0.000). This improved folate status remained significant after adjusting the analysis for socio-demographic characteristics of the studied women.
Assessing serum folate concentrations, demonstrate that with increasing folate intake due to fortified bread, there was a significant increase in the proportion of women achieving optimal folate status.

In the present study, the trend of NTDs was determined, before and after flour fortification with folic acid, as the impact of folic acid fortification in Golestan province. Total prevalence rates were calculated as a total number of NTD per 1000 births. Time trend analysis of monthly NTD rates was performed. All live births and stillbirths (a gestational age of ≥20 weeks and ≥500 g who were admitted in neonatal intensive care unit (NICU) of Dezyani teaching hospital were included from September 2006 (before flour fortification) to December 2008 (after flour fortification).

The current study shows 31% reduction in the prevalence rate of NTDs after flour fortification from 3.16 per 1000 births to 2.19 per 1000 births (RR 0.09, 95% CI 0.042 - 0.21) that was statistically significant (P-value<0.01). Decline in NTD prevalence rate in this study was also consistent with the observed increase in serum folate levels and folic acid intake in the women of reproductive age after flour fortification with folic acid. This findings confirm that, mandatory flour fortification in Iran, would be effective in reducing NTD risk in women who regularly consume fortified bread.

We examined whether folic acid fortification would mask vitamin B12 deficiency. The reason is that vitamin B12 and folic acid have the same metabolic pathway and additional folic acid could correct the hematological signs of vitamin B12 deficiency and postpone clinical diagnosis, which could lead to neurological damage. We did not find the evidence of masking vitamin B12 deficiency after folic fortification. The proportion of women without macrocytosis was higher after flour fortification (82.9%) than before flour fortification (80%), and the difference was not significant (p = 0.370). Our results provide important reassurance that folic acid exposure due to flour fortification has not resulted in an increase in the proportion of women with a low serum vitamin B12 concentration but normal MCV values.
Our findings, therefore, support the view expressed by many that mandatory fortification with folic acid is the only means of ensuring that all women of reproductive age can benefit in terms of reduced risk of NTDs.

We found a high prevalence of low serum B12 among the women of child bearing age both in the pre–fortification and post-fortification period. On the other hand, vitamin B12 intake in almost 50% of the women was lower than the RDA (2.4 μg/day) in the periods of before and after flour fortification, respectively. Our findings suggest that vitamin B12 status of general population should be monitored, during folic acid fortification in developing countries, where the probability of vitamin B12 deficiency could be high. Vitamin B12 is a risk factor, independent of foate status, in NTDs occurrence especially in the era after folic acid flour fortification, Consideration should be given to confirm our finding, and possibly, to the adding of B12 to folic acid fortified flour.
7. Zusammenfassung

Folsäure ist ein kritischer Nährstoff für Frauen im gebärfähigen Alter. Eine adäquate Aufnahme in der Zeit um die Empfängnis beugt der Entstehung einer Reihe angeborener Missbildungen wie vor allem Neuralrohrdefekten (NRD), die zu den häufigsten Folgen eines Folatmangels in der Schwangerschaft zählen.


Infolge der Anreicherung verringerte sich die Prävalenz niedriger Serumfolatwerte um 84%, der mittlere Gehalt stieg signifikant um 25% (von 13,6 nmol/l auf 18,1 nmol/l, p=0,000). Das Vorkommen von Hyperhomocysteinämie nahm um 88% ab (von 38,8% auf 7,3 %, p=0,000). Diese Effekte blieben auch nach Adjustierung hinsichtlich soziodemographischer Merkmale der untersuchten Frauen statistisch signifikant. Die Messung der Serumfolatkonzentration zeigte, dass mit zunehmender Aufnahme des Vitamins aus angereichertem Brot ein signifikant höherer Anteil an Frauen einen optimalen Folsäurestatus erreichte.

Hinsichtlich der Häufigkeit von NRD wurde nach der Anreicherung des Mehls mit Folsäure eine Verringerung um 31% beobachtet von 3,16 auf 2,19 Fällen pro 1000 Geburten (RR 0,09, 95% KI 0,042-0,21). Dieser Effekt war statistisch signifikant (p<0,01). Die Abnahme der NRD-Häufigkeit entsprach dem beobachteten Anstieg der Serumfolatspiegel und der Folataufnahme bei Frauen in gebärfähigem Alter. Diese Ergebnisse bestätigen, dass eine gesetzlich vorgeschriebene Anreicherung von Mehl mit Folsäure im Iran eine wirksame Maßnahme zur Verringerung von NRD bei Kindern von Frauen, die regelmäßig angereichertes Brot verzehren, darstellt.

Es wurde auch untersucht, ob die Gefahr einer Verschleierung eines vorhandenen Mangels an Vitamin B12 besteht. In der Tat besteht eine Verbindung des Stoffwechsels der Folsäure mit jenem von Vitamin B12, da Letzteres für die Regenerierung der Folsäure benötigt wird und eine Unterversorgung sekundär zu einem Mangel an Folsäure führen kann. Eine Supplementierung mit Folsäure kann die hämatologischen Symptome eines Vitamin B12 Mangels überdecken und die Diagnose erschweren, während die neurologischen Schäden bestehen bleiben.
In der vorliegenden Stichprobe ergab sich jedoch keine Maskierung eines B12 Mangels infolge Anreicherung.

Der Anteil der Frauen, bei denen keine Makrozytose vorlag, war nach der Anreicherung höher als vorher (82,9% gegenüber 80%). Dieser Unterschied war allerdings nicht statistisch signifikant (p=0,370). Insgesamt dürfte die Gefahr einer Zunahme des Anteils an Frauen mit niedrigen B12 Serumspiegeln und gleichzeitig normalem mittlerem korpuskulärem Volumen (MCV) infolge Aufnahme von angereichertem Mehl gering sein.

Zusammenfassend zeigen diese Ergebnisse, dass eine gesetzlich geregelte Mehlanreicherung mit Folsäure eine geeignetes Mittel ist, die Versorgung von Frauen in gebärfähigem Alter mit diesem Vitamin zu verbessern und so dem Auftreten von NRD vorzubeugen, mit dem der überwiegende Teil dieser Zielgruppe erreicht werden kann.

Sowohl vor als auch nach der Intervention traten niedrige Serumspiegel an Vitamin B12 häufig auf. Gleichzeitig lag die tägliche Zufuhr in beiden Phasen bei fast 50% der untersuchten Frauen unterhalb der empfohlenen Menge von 2,4 µg/Tag. Vitamin B12 Mangel ist ebenfalls ein Risikofaktor für NRD, unabhängig vom Folatstatus, besonders nach Anreicherungsmaßnahmen mit Folsäure. Insofern sollte im Zuge einer Lebensmittelanreicherung mit Folsäure in Entwicklungsländern der Vitamin B12 Status in der gesamten Bevölkerung überwacht werden, wenn die Gefahr einer mangelhaften Versorgung mit diesem Nährstoff gegeben ist. Weitere Studien sind nötig, um diese Ergebnisse zu bestätigen und es ist zu erwägen, die Anreicherung des Mehls auf Vitamin B12 auszudehnen.
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11. Curriculum Vitae

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Education

Bachelor degree: Public Health, Shahid Beheshti University of Medical Sciences, Tehran, Iran, 1981
Master degree: Public Health Nutrition, Tehran University of Medical Sciences, Tehran, Iran, 1991
Diploma on management of food and nutrition programs, International Agriculture Center (IAC), Netherlands, 1995

Experiences:

1- National program manager on prevention and control micronutrients deficiency in MOH&ME, from 1991.
4- Member of the national committee of developing the national nutrition program for the 3th development national plan in MOH&ME, 1999.
5- Member of the scientific committee of research in the National Food and Nutrition Council, 2001.
6- Member of the scientific committee of food and nutrition security in MOH&ME, 2001.
7- Member of the national scientific and planning committee of flour fortification program in MOH&ME from 2002.
8- Member of the national committee of the national plan of action for the project on “Nutrition Improvement in Children under 2, Pregnant and Lactating Women” in MOH&ME, sponsored by the World Bank, 2003-2006.
9- Member of the national committee of the Iron Deficiency Anemia prevention program in MOH&ME from 2003.
10- Member of the national committee of developing the national nutrition program for the 4th national development plan, in MOH&ME, 2004.

Research papers

3- Consumption of unworthy junk food by children under 3 in urban and rural areas nationwide, published in the special issue of the 5th Iranian Nutrition Congress, 2000.
4- Iron Deficiency Anemia and evaluation of iron supplementation program for pregnant women in Iran. *Articles collection of International congress on prevention of micronutrient deficiencies, Vietnam, 2000.*

5- Prevalence of different kinds of malnutrition in children under 5 in urban and rural areas nationwide, *Pazhoohandeh seasonal scientific journal, Shahid Beheshti University of Medical Sciences and Health Services, Tehran, Iran; 2002.*

6- The food consumption pattern in children of 12 – 23 months of age in urban and rural areas nationwide, *published in the special issue of the 7th National Nutrition Congress – Gilan University of Medical Sciences, Iran; 2002.*


8- Prevalence of iron deficiency, anemia and iron deficiency anemia among child-bearing age women (15-49 years old) in urban and rural areas nationwide. *Iranian Journal of Teb-VA-Tazkieh 2002; 7:37- 44.*


14- Iron deficiency anemia among adolescent boys and girls in Iran, 2nd national epidemiology congress, published in the special issue of the scientific magazine of Zahedan University of Medical Science, Iran; 2004.
15- Iron deficiency anemia among Iranian children 6 years old, 2\textsuperscript{nd} national epidemiology congress, published in the \textit{special issue of the scientific magazine of Zahedan University of Medical Science, Iran}; 2004.

16- Zinc deficiency among pregnant women in Iran, 2\textsuperscript{nd} national epidemiology congress, published in the \textit{special issue of the scientific magazine of Zahedan University of Medical Science, Iran}; 2004.

17- Nutrition intervention for reducing PEM among children under 3 years in Islamic Republic of Iran, published in the \textit{special issue of the 9\textsuperscript{th} international clinical nutrition congress, Westminster university, London}, 2002.


19- Prevalence of overweight and obesity among Iranian adult males and females, published in the \textit{special issue of the 8\textsuperscript{th} Iranian Nutrition Congress, Tehran, 2004}.


22- Prevalence of iron deficiency anemia in Iranian pregnant women, published in the \textit{special issue of the 9\textsuperscript{th} Iranian Nutrition Congress, Tabriz, 2006}.

23- Prevalence of iron deficiency among Iranian adults, published in the \textit{special issue of the 9\textsuperscript{th} Iranian Nutrition Congress, Tabriz, 2006}.

24- Interpretation of the trend of malnutrition among Iranian children under 6, published in the \textit{special issue of the 9\textsuperscript{th} Iranian Nutrition Congress, Tabriz, 2006}.

25- Micronutrient status of Iranian children 15-23 months, published in the \textit{special issue of the 9\textsuperscript{th} Iranian Nutrition Congress, Tabriz, 2006}.

26- Prevalence of Zinc deficiency among Iranian pregnant women, published in the \textit{special issue of the 9\textsuperscript{th} Iranian Nutrition Congress, Tabriz, 2006}.

28- Prevalence of iron deficiency anemia among adolescent’s boys and girls in Iran, published in the special issue of the scientific magazine of International Micronutrient Forum, Turkey, 2007.

29- Zinc and iron deficiency among Iranian children under 6 years, published in the special issue of the scientific magazine of International Micronutrient Forum, Turkey, 2007.


Studies and Researches

1- Epidemiological survey on endemic goiter in urban and rural areas of the central city of each province nationwide, baseline data gathering, 1995. (co investigator)

2- A Knowledge, Attitude and Practice (KAP) survey on iodized salt in urban and rural households, 1994 – 1997. (co investigator)

3- Epidemiological goiter survey, measurement of urinary iodine and serum thyroid hormones levels in 25 provinces of the country, 1996. (co investigator)

4- Multidisciplinary interventional model for decreasing malnutrition among children under 3 years (pilot study), ranking the best in Razi Research festival, 1996. (main investigator)

5- National survey on growth and nutrition indicators in children (defined in urban and rural areas, and either of the sexes), ranking first in Razi Research festival, 1998. (co investigator)
6- National survey on the mother’s knowledge about the growth-monitoring chart, 1998. (co investigator)
7- National food and Nutrition Security survey, 1999. (co investigator)

8- Consumption of unworthy junk food in children under 5 nationwide, 1999. (co investigator)
9- Effectiveness of weekly iron supplementation among adolescent’s girls, 1999. (main investigator)
10- Prevalence of Iron deficiency anemia among women in childbearing age in Boushehr province, 2000. (co investigator)
11- Micronutrients national survey (iron, zinc, vitamin a, vitamin D) in different age groups of Iranian population, 2001. (co investigator)
12- National survey on prevalence of goiter, usage of iodized salt and measuring urinary iodine among schoolchildren 8-10 years old ,2001. (co investigator)
13- National survey on the prevalence of Low Birth Weight, 2002. (co investigator)
14- Effectiveness of fortification of milk with vitamin D (pilot study), 2003. (co investigator)
15- Effectiveness of flour fortification with iron and folic acid in Boushehr province (Mid Term Evaluation, 2003. (co investigator)
16- Effectiveness of flour fortification with multi micronutrients in one district of Boushehr province, 2004. (co investigator)
17- KAP survey on Nutrition and micronutrients in 3 provinces of Iran, 2004. (co investigator)
18- Iron status of women of childbearing age in Golestan province, 2006. (main investigator)
19- Final evaluation of flour fortification program in Boushehr province, 2006. (co investigator)
National Program planning

1- Food and nutrition security program in the 3rd development national plan, 1994.
3- Prevention and control malnutrition among children under 5 through Primary Health Care (PHC) system.
4- Iron supplementation in pregnant women through PHC system, 1993.
7- Food and nutrition security program in the 4th development national plan , 2004.
9- National program for flour fortification with micronutrients, 2006.

Publications (Persian language)

2. Training manual on nutrition counseling” for health workers in PHC system, 1996.
16. Training booklet on the healthy foods and heart for nutrition officers in PHC system, 2005.
17. Training manual on prevention and control of micronutrient deficiencies with focus on food fortification for physicians and health workers, 2005.