ADDIS ABABA UNIVERISITY

COLLEGE OF NATURAL AND COMPUTATIONAL SCIENCE

CENTER FOR FOOD SCIENCE AND NUTRITION

THE RELATIONSHIP BETWEEN DIETARY FOLATE INTAKES AND SERUM FOLATE STATUS OF REPRODUCTIVE AGE WOMEN IN WestGOJJAM, ETHIOPIA

BY

HAWI BEKELE

July, 2019

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HAWI BEKELE

A thesis submitted to the Center for Food Science and Nutrition in partial fulfillment of the requirements for the Master Science in Food Science and Nutrition.

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ADDIS ABABA UNIVERSITY

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SCHOOL OF GRADUATE STUDIES

CENTER FOR FOOD SCIENCE AND NUTRITION

This is to declare that the thesis entitled "**THE RELATIONSHIP BETWEEN DIETARY FOLATE INTAKES AND SERUM FOLATE STATUS OF REPRODUCTIVE AGE WOMEN IN West GOJJAM, ETHIOPIA**" submitted in partial fulfillment of the requirements of MSc. degree in food science and Nutrition, to the school of Graduate, studies food science and nutrition program, Food Science and nutrition center is a record of original research carried out by HawiBekeleunder my supervision and no part of the thesis has been submitted for any other degree or diploma. The assistance and help received during the course of this study have been properly acknowledged. Therefore, I recommend that it be accepted as fulfilling the thesis requirements.

The Thesis has been approved for submission by:

Dr. KaleabBaye (PhD) **July, 2019**

_____ _____ _____

Name of major advisor **Signature** **Date**

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FINAL THESIS APPROVAL FORM

As participants of the Board of Examiners of the final MSc. open defense, we declare that we have read and evaluated the thesis prepared by Hawi Bekele under the title allowed "**THE RELATIONSHIP BETWEEN DIETARY FOLATE INTAKES AND SERUM FOLATE STATUS OF REPRODUCTIVE AGE WOMEN IN West GOJJAM, ETHIOPIA**" and recommend for the degree of Master of science in Food science and Nutrition.

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ABBREVIATIONS

AI	Adequate Intakes
AMD	Age related macular degeneration
CoA	Coenzyme A
DNA	Deoxyribonucleic acid
DRIs	Dietary reference intakes
DV	Daily Value
EARs	Estimated Average Requirements
EPBA	Enzyme Protein Binding assay
FNB	Food and Nutritional Board
GGH	Gamma – gultamyi hydrolase
Hcy	Homocysteine
HDI	Human Development Index
HPLC	High – performance liquid chromatography
IOM	Institute of Medicine
LC	Liquid chromatography
LDL	Low – density liporotien
MA	Microbiological assy
MCV	Mean Cell Volume
MMA	Methylmalonic acid
MTHFR	Methteneterahy drofolate reducase
5-Methyl – THF	5-mehtyenetetrahydrofolate

5-MTHFGlu	5-methyeneterahydrofolate-monglutamate
MS	Mass spectrometry
NTD	Neural tube defect
RBCs	Red blood cells
RNA	Ribonucleic acid
SAM	S- adenosyl methionine
THF-Glu1	Terahydrofolate – monogultamate
UL	Tolerable Upper intake level
WHO	World health organization

ABSTRACT

Folate is an essential nutrient, involved in several metabolic activity of the human body and it contributes to the prevention of neural tube defect. However, little is known about folate content of commonly consumed foods in Ethiopia. In addition research determining folate status among human subjects in Ethiopia is scanty.

In WsetGojjam zone of the Amhara region, Ethiopia serum folate was determined in women of reproductive age (n=179). In addition, folate content of commonly consumed foods among participating households was determined. Furthermore, effect of traditional processing on the retention of folate content was investigated.

Participating women had serum folate concentration of (26.8±5.8). Folate deficiency was found in 1.9% of the subjects. Staple crops from the households had folate concentration in the range of 4.4µg/100g (Maize) to 40.21µg/100g (Wheat). From legumes 9.7µg/100g (Pea) to 65.2µg/100g (Bean). The mean folate concentration of raw and cooked/baked food was 61.00 ± 13.7 Vs13.78 ± 3.9 (p=0.052)

Most cereals and legumes are better source of folate. The result varies form 4-40µg/100g in cereals and 10-65µg/100g in legumes. Though, vegetables and fruits are folate-contributing foods, they are not frequently consumed in this area.Though, legumes and cereals are good source of folate in their raw form, their retention affected by different factors. There is a significant difference between raw food items and cooked ones in *Shirowot*. Whereas there is no significance difference between raw food items and cooked ones in *Injera*. This could be result of Tefbehavior which is Teff is a relatively good source of folate and fermenting it to *Injera* could increase its folate content. This could be a condition that favors folate production during fermentation.

Almost all (95.4%) have optimal folate (>6.6ng/mL) in their blood, this shows that folate deficiency is not a public health problem. Also the result shows that there is no correlation between dietary folate intake and serum folate status. This could be due to some reasons like production of folate in the human colon, the method used to identify most frequent foods or because of serum usage for folate analysis.

As recommendation, to have adequate folate intake these women should have diversified fooditems. And consume foods that are rich in folate like green leafy vegetables. Even diet alone cannot make the RDA to be met, so if possible fortified foods and supplements should be given.

Key words: Folate, NTD, folate deficiency, reproductive age women, *Injera*, *Shirowot*,

1. Introduction

1.1 Background

Folate is a generic descriptive for a family of structurally related compounds that share a common pteroyglutamic acid core and function in the acceptance, redox processing, and transfer of one-carbon unit [1]. The name folate is derived from Latin word ‘folium’, which means leaf to indicate green leafy vegetables as the main dietary source. Folate is found from natural foods and folic is the synthetic form which is found in fortified foods and in the form of sup of 2-amino-4-hydroxy-6-methylpterin (petridine ring) linked through a methylene bridge to para-amionbenzoate which is conjugated with one or several L-glutamic acid residues with Û-peptide linkage [2].

Humans by nature cannot synthesize folate and thus are more dependent on dietary sources. Green leafy vegetables, liver, kidney and citrus fruits are folate-rich foods. Bread, potatoes, and dairy products are middle-grade sources but contribute significantly to the total folate intake as they are consumed in large quantities [3].

Folates in foods are transported via an ion-exchange mechanismthat is carried out against the pH gradient along the brush membrane of enterocytes. Folate is anionic at intraluminal pH and it is exchanged with a hydroxyl anion. Folate is mainly absorbed in the proximal part of small intestine, duodenum, and to a much lesser extent in the jejunum. Polyglutamyl folates are hydrolyzed into monoglutamyl folates by gamma-glutamyl hydrolase (GGH) and then all monoglutamyl folates are converted into 5-methyltetrahydrofolate-monoglutamate (5-MTHFGlu 1) in enterocytes. 5-MTHF-Glu1 is the plasma form of this vitamin and it is transported to the peripheral tissues, where it is converted into tetrahydrofolate-monoglutamat (THF-Glu1) via a reaction catalyzed by methionine synthase that uses vitamin B12 as a cofactor [4].

There is metabolism difference between folate and folic acid, whenfolate has higher metabolic activity and are better retained by cells. Whereas folic acid pass through cell walls more rapidly as folate require deconjugation to change into monoglutamates within the erythrocyte, comparing their bioavailability folate is bioavailable ~50% whereas folic acid (monoglutamates) ~85% bioavailable [5]. In addition antifolate components present in vegetables as well as exposure to heat and light during storage, all to lower folate availability. Therefore to meet daily requirement of folate the synthetic form (folic acid) need to be used in fortification and in supplement form.

Most women are affected by folate deficiency[6-8].A study done by Jemal Haider estimates, that the casual effect of folate deficiency in Ethiopia is inadequate dietary intake [9]. Folate is suboptimal in the diets of many women of childbearing age. This requires the need for sustainable folate intake through dietary diversification and alsoappropriate public health interventions through supplementation.

The intake of folate is limited by cooking losses and poor bioavailability, [10] estimated to be from 50% to 82%[11,12]. Folate deficiency can also be caused as a consequence of medical conditions that increase the need for folate or result in increased excretion of folate, including pregnancy, alcoholism, malabsorption, kidney dialysis, liver disease, certain anemias and medication that interfere with folate metabolism [13-16]. Fortification of grains with folic acid has increased folate intake in several developed countries [17] since, fortified foods are generally not available in Ethiopia, there increase the risk of women of child bearing age to have an off spring with neural tube defects (NTDs).

NTD is a defect which results from failure of the neural tube to close properly, approximately at 28 days where the women could realize as she conceived [18,19]. The two most common NTDs are spinal bifida and anencephalya. Spinal bifida results from failure of fusion of the posterior (caudal) neural tube, whereas anencephalya results from failure of fusion of the anterior (cranial) neural tube.

1.2 Significance of the study

- To evaluate the relationship between dietary folate intake and serum folate.
- To contribute on the prevention of neural tube defect and other degenerative disease caused by folate deficiency.
- To suggest if fortification is needed.
- The study may serve as baseline for further studies that can be done on folate analysis from serum.

1.3 Objectives

1.3.1 General objective

The main objective of this study is to investigate the contribution of the most frequently consumed foods to dietary intake and serum folate status of women of reproductive age in West Gojjam.

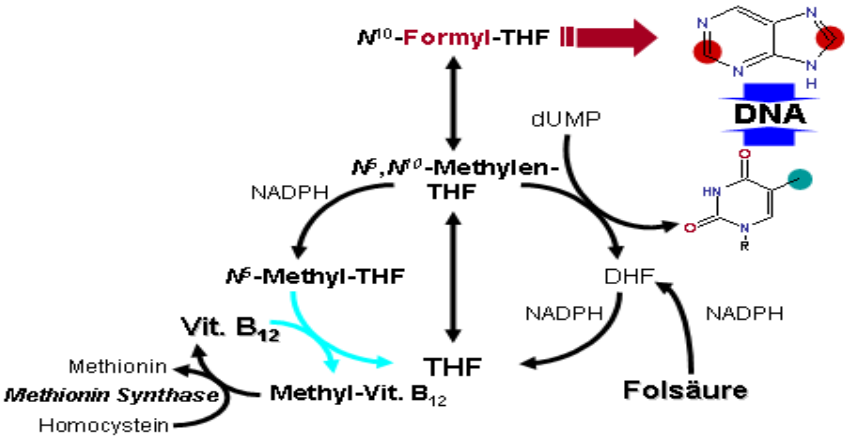
1.3.2 Specific objectives

- To determine the folate content of most commonly consumed foods among women of reproductive age in West Gojjam.
- Investigate the effect of cooking on folate retention.
- Estimate the correlation of the widely consumed foods with folate intake.

2. Literature review

2.1 Folate metabolism

There are two cycles in folate metabolism, which are DNA biosynthesis and methylation. Natural folates are hydrolysed to the monoglutamate form in the gut lumen. Folate and folic acid are metabolized to 5-methylenetetrahydrofolate (5-methyl-THF), during the passage through the intestine. 5,10-methylenetetrahydrofolate reductase (MTHFR) enzyme catalyzed the methyl group, which also links the DNA biosynthesis cycle with methylation cycle. Using the vitamin B-12 dependent enzyme methionine synthase 5-methyl-THF remethylated to the biologically active form of folate tetrahydrofolate (THF).THF is a coenzyme, which has its primary roles in metabolic transfer (DNA biosynthesis cycle) and donation of carbon-1-units (methylation cycle). The methylation cycle has two roles. It guarantees that the cell always has an adequate supply of S-adenosyl methionine (SAM), which is an activated form of the essential amino acid methionine. SAM acts as a methyl donor to a many methyltransferases, the enzymes which methylate a wide range of products (e.g. lipids, hormones, proteins, DNA). Another role of the methylation cycle is to provide the function of degrading methionine in the liver. Methionine is degraded to homocysteine (Hcy), which can leave the system with the help of the B6-dependent enzyme cystathionine synthase and be catabolised to sulphate and further to pyruvate. Homocysteine can also be remethylated to methionine with the help of the enzyme methionine synthase[20,21,22]. The most important folate cofactors in the methylation and DNA cycles are shown in the following Figure.



Source: Rady PL, Szucs S, Grady J, et al. Geneti polymorphisms of methylenetetrahydrofolate reductase (MTHFR) and methionine synthase reductase (MTRR) in ethnic populations in Texas; a report of a novel MTHFR polymorphic site, G1793A. Am J Med Genet. Jan 15 2002;107(2):162-168.

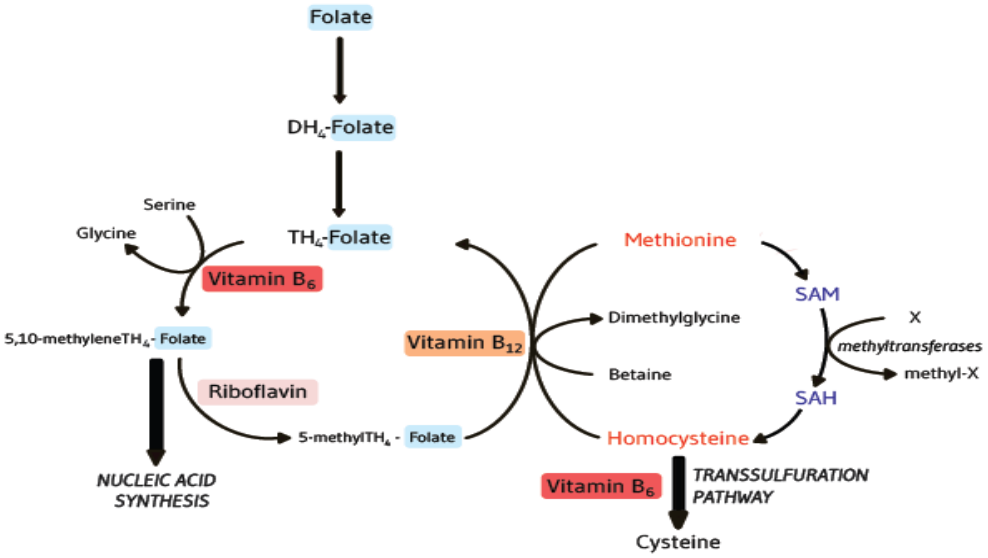
Figure 1.**Folate metabolism**

Genetics of folate metabolism Several variations have been identified in genes involved in folate absorption and metabolism. These polymorphisms may affect the beneficial effect of folates and other B vitamins that a play role in metabolism. The MTHFR gene has three commonly known polymorphisms, 677C>T, 1298A>C and 1793G>A that are involved in folate metabolism [23]. The MTHFR 677C>T polymorphism seems to be the most influential genetic variation affecting folate metabolism. The homozygous TT genotype of the 677C>T polymorphism is associated with higher plasma homocysteine and lower serum folate levels than the heterozygous (CT) and wild-type (CC) genotypes [24,25]. In contrast, the gene variation 1298A>C has not been shown to alter homocysteine or folate levels in the blood [26,27] and the MTHFR polymorphism 1793G>A has been shown to have a decreasing effect on plasma homocysteine[28].

2.2 Biological function

2.2.1 One-carbon metabolism

Folate coenzymes function in the body appears to be in mediating the transfer of one-carbon units[29]. It act as acceptors and donors of one-carbon units in a variety of reactions critical to the metabolism of nucleic acids and amino acids[30].



Source: Food and Nutrition Board, Institute of Medicine. Folate Dietary Reference Intakes: Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12. Panthenic Aci, Biotin, and choline. Washington D.C. National Academy Press; 1998:196-305.

Figure 2 **One-carbon metabolisms**

2.2.2 Nucleic acid metabolism

In DNA metabolism, folate coenzymes play a significant role through two different pathways [29]. The synthesis of DNA from its precursors (thymidine and purines) is dependent on folate coenzymes [31]. A folate coenzyme is required for the synthesis of methionine from homocysteine, and methionine is used for the synthesis of S-adenosylemethionine (SAM). SAM is a methyl group (one-carbon unit) donor used in most biological methylation reactions, including the methylation of a number of sites within DNA, RNA, protiens, and phospholipids. The methylation of DNA plays a role in controlling gene expression and is critical during cell differentiation. Aberrations in DNA methylation have been linked to the development of cancer. Folate deficiency hinders DNA synthesis and cell division affecting hematopoietic cells and neoplasms the most because of their greater frequency of cell division. RNA transcription, and subsequent protein synthesis, are less affected by folate deficiency, as the mRNA can be recycled and used again (as opposed to DNA synthesis, where a new genomic copy must be created). Since folate deficiency limits cell division, erythropoiesis, production of red cells, is hindered and leads to megaloblastic anemia, which is characterized by large immature red blood cell.

2.2.3 Amino acid metabolism

Metabolism Folate coenzymes also required for the metabolism of several important amino acids, such as cysteine, serine, glycine, and histidine. The synthesis of methionine from homocysteine is catalyzed by methionine synthase, an enzyme that requires not only folate (as 5-methyltetrahydrofolate) but also vitamin B12. Thus, folate (and/or vitamin B12) deficiency can result in decreased synthesis of methionine and an accumulation of homocysteine. Elevated blood concentrations of homocysteine have been considered for many years to be a risk factor for some chronic diseases, including cardiovascular disease and dementia.

2.3 Health benefits

2.3.1 Neural tube defects prevention

Neural tube defects (NTDs) a collective name given for all birth defects in which an opening in the spinal cord and brain will occur from early in human development. NTD develops in the 3rd week of pregnancy which is called gastrulation, when specialized cells on the de neural dorsal side of the embryo begin to change shape and from the neural tube does not close completely. The occurrence of NTDs varies from 0.5 to 4.0 per 1,000 births in North America [32]. The two most common types of NTDs which are spina bifida and anencephaly, are higher among Hispanic women and lowest among African American and Asia women [33].

Due to its use in the synthesis of DNA and other critical cell components, folate is essential during phase of rapid cell growth. [34] Clear clinical trial evidence shows that when women take folic acid periconceptionally, NTDs is prevented [32, 33, 35, 36, 37]. As estimated by scientists that periconceptionally folic acid use could reduce NTDs by 50% to 60%

Folic acid fortification was made mandatory in 1998 in the United States, since then NTD rates have declined by 25% to 30% [35].Though significant racial and ethnical disparities exit. Spina bifida and anencephaly rates have declined significantly among Hispanic and non-Hispanic white births in the United States, but not among no-Hispanic black births [38]. Difference in dietary habits and supplement-taking practice could be a factor in the disparities. [38]. In addition, factors other than folate status such as maternal diabetes, obesity, and intake of other nutrients such as vitamins B_{12} are believe to affect the risk of NTDs [39,40,37,41,42].

There are two forms of NTD: open, which are more common, and closed. Open NTDs occur when the brain and/or spinal cord are exposed at birth through a defect in the skull or vertebrae. Example of open NTDs are anencephaly, encephaloceles, hydranencephaly, iniencephaly, schizencephaly and spinal bifida. Closed NTDs are rare types occur when the spinal defects are covered by skin. Example of closed NTDs are lipomyelomeningocele, lipomeningocele, and tethered cord.

Types of NTDs include:

Anencephaly

Is a neural tube defect that occurs when the head end of the neural tube fails to close, usually during the 23^{rd} and 26^{th} days of pregnancy, resulting in an absence of a major portion of the brain and skull. Infants born with this condition are born without the main part of the forebrain-the largest part of the cerebrum-and are usually blind, deaf and unconscious. The lack of a functioning cerebrum will ensure that the infant will never gain consciousness. Infants are either stillborn or usually die within a few hours or days after birth.

Enecephaloceles

Neural tube defect which is characterized by protrusions of the brain through the skull that are sac-like and covered with membrane. They can be a groove down the middle of the upper part of the skull, between the forehead and nose, or the back of the skull. Encephaloceles are often obvious and diagnosed immediately. Sometimes small encephaloceles in the nasal and forehead are undetected.

Hydranencephaly

A condition in which the cerebral hemispheres are missing and instead filled with sacs of cerebrospinal fluid.

Iniencephaly

Is rare neural tube defect that results in extreme bending of the head to the spine. The diagnosis can usually be made on antenatal ultrasound scanning, but if not will undoubtedly be made immediately after because the head is bent backwards and the face looks upwards. Usually the neck is absent. The skin of

the face connects directly to the chest and the scalp connects to the upper back. The infant will usually not survive more than a few hours.

Spina bifida

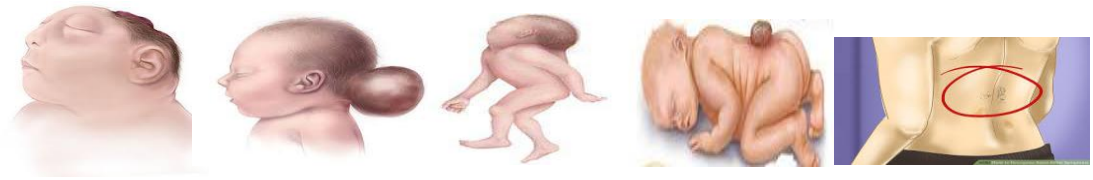
Spina bifida has two classifications: spina bifida cystica and spina bifida occulta.

Spina bifida cystica

This includes meningocele and myelomeningocele. Meningocele is less severe and is characterized by herniation of the meninges, but not the spinal cord, through the opening in the spinal canal. Myelomeningocele involves herniation of the meninges as well as the spinal cord through the opening [43].

Spina bifida occulta

In this type of neural tube defect, the meninges do not herniate through the opening in the spinal canal[43]. By definition, spina bifida occulta means hidden split spine [44]. The most frequently seen form of spina bifida occulta is when parts of the bones of the spine, called the spinous process, and the neural arch appear abnormal on a radiogram, without involvement of the spinal cord and spinal nerves [45]. The risk of recurrence in those who have a first degree relative (a parent or sibling) is 5-10 times greater compared to the general population.



(a) ***(b)*** ***(c)*** ***(d)*** ***(e)***

Source: Pittman, T (2008). "Spina bifida occulta". Journal of Neurosurgery.Pediatrics. **1**(2): 113

Figure 3.**Types of neural tube defects (a) Anencephaly, (b) Enecephaloceles, (c) Iniencephaly, (d) Spina bifida cystica, (e) Spina bifida occulta**

2.3.2 Pregnancy

Folate needs increase during the period of pregnancy because of the significant acceleration in single-carbon transfer reactions, including those required for synthesis of nucleotide and thus division of cells. Beside excretion of folate catabolite during late pregnancy has been documented as being higher than in non- pregnant women. Comparing to the mid pregnancy, urinary folate catabolites were not found to be increased. Folate deficiency is linked to placental abruption (pre mature detachment of placental) due to a weak but positive association between hyperhomocysteinerima and risk for placental abruption [46]. It has been found that maternal use of folic acid supplements 3 months before becoming aware of pregnancy and/or during the following 3 months was associated with improved gross motor development at 3 years of age in Africa-American children [47].

2.3.3 Fertility

Folate has a role in fertility in both men and women. It contributes to spermatogenesis. For this reason, it is necessary to receive sufficient amounts of folate through diet to avoid subfertility [48]. Polymorphisms in genes of enzymes which involved in folate metabolism could be one reason for fertility complications in women with unexplained infertility [49]. Also in men lower folate intake has been shown to have sperm with incorrect chromosomal structure [50]. A study made in 2012 reported that previously infertility patients who took a nutritional supplement, which include folic acid, experienced significant improvement in sperm motility and successfully achieved pregnancy with their partners [51].

2.3.4 Cancer prevention

Folate's cancer-preventive property is linked to its function in the de novo synthesis of thymidylate and purines-nucleotides which are needed for DNA replication and repair. In addition, folate is essential for the production of S-adenosylmethionine (SAM), which is a universal donor of methyl groups for several methylation reactions, including DNA methylation [52], which is central to gene slicing and probably to the suppression of repetitive DNA of viral origin [53]. Folate role in carcinogenesis is more complex than was initially thought [54, 55].

2.3.5 Other health benefits

Heart disease

Folic acid reduces the risk of cardiovascular disease by 4% [56]. Folate helps synthesis of homosysteine level increase, which is linked to atherosclerosis and cardiovascular problems [57]. Folate contributes the

breaking down of homocysteine. Consuming folic acid supplements during pregnancy may reduce the risk of heart defects in infants [58].

Stroke

Due to the role of folate in regulating homocysteine concentration, long term supplementation with folic acid reduces the risk of stork by 10% [59]. Asian population had greater protection against stroke with folate supplementation that did European or North American subjects [60], Observed stroke reduction is consistent with the reduction in pulse pressure produced by folate supplementation of 5mg per day, since hypertension is a key risk factor for stroke. Folic supplements are pensive and inexpensive and relatively safe to use, which is why stroke or Hyperhomocysteinemia patients are encouraged to consume daily B vitamins including folic acid [61],

Age-related hearing loss

One study suggest that folic acid supplements help show the progression of age-related hearing loss in elderly people with high homocysteine levels and low folate in their diet [62].

Age-related macular degeneration (AMD)

A Harvard Medical School trial of women with a risk of age-related macular degeneration (AMD) explored the impact of B vitamin therapy which includes folic acid, vitamin B_6, and vitamin B_{12}. The study found that women who took 2500mcg of folic acid along with 500mg of vitamin B6 and 100mcg of cyanocobalamin daily reduced the risk of developing AMD, an eye disease that can cause vision loss [63]. The controlled had a higher incidence of AMD than the group taking the B vitamin therapy. For this reason researchers conclude that daily supplementation might help the fight in reducing the risk of AMD [64].

Depression

Studies show that folate and depression have some links [65,66]. In an ethnically diverse population study of 2948 people aged 1 to 39 years in the United States, serum and erythrocyte folate concentrations were significantly lower in individuals with major depression than in those who had never been depressed [67]. Results from a study of 52 men and women with major depressive disorder showed that only 1 of 14 subjects with low serum folate levels responded to antidepressant treatment compared with 17 of 38 subjects with normal folate levels [68]. Although supplemental folic acid has not been proposed as a replacement for traditional antidepressant therapy, it might be helpful as an adjuvant treatment [69,70]. In a trial conducted in the United Kingdom, 127 patients with major depression were randomly assigned to receive either 500mcg folic acid or placebo in addition to 20mg of fluoxetine (an antidepressant medication) daily for 10weeks [71]. Although the effects in men were not statistically significance,

women who received fluoxetine plus folic acid had a significantly greater improvement in depressive symptoms than those who received fluoxetine plus folic acid had a significantly greater improvement in depressive symptoms that those who received fluoxetine plus placebo. Addition research is needed to fully understand the association between folate status and depression and whether folic acid supplementation might be helpful adjuvant treatment.

Overall cardiovascular support

Adequate dietary folate intake help keep blood levels of homocysteine in check. Homocysteine (Hcy) is a well-documented marker cardiovascular disease that when excessive, represents clearly increased risk of a variety of cardiovascular problems. By helping to keep Hcy levels in check, healthy intake of folate can help lower risk of cardiovascular disease. During the past 10 years, research on the role of folate in nervous system support has greatly overlapped with folate research as it relates to support of the cardiovascular system. In fact, it might be hard generated more excitement that this overlapping area of folate-related events critical for health of our cardiovascular and nervous systems.

Encourages normal Cholesterol Levels

A Polish study found folic acid supplementation encourages normal cholesterol levels. In the study of 124 individuals, researchers observed significant reduction in LDL cholesterol levels in subjects who'd supplemented with 4 mg of folic acid daily for 12 weeks. The result is believed that have been derived from reduced homocysteine levels [72].

2.4 Folate source

Folate is available in a wide variety of foods but in relatively low concentrations. Folate in foods is in the form of polyglutamates [73], which can be found especially in legumes, while green leafy vegetables are outstanding sources. And also foods like fruit juices, pulses, bans, whole grains, milk and liver are good source of folate. Naturally occurring folate are chemically unstable, as they lose their activity during cooking, processing and storage of food [73, 74]. Folic acid (pteroylmonoglutamic acid, PGA) is the synthetic form of folate used in supplements. It is not found naturally in foods. This form has a more stable chemical structure than the natural (dietary) forms, which makes it resistant to heat and light [69]. It also appears to have better bioavailability than naturally occurring folates [75, 76]. However, existing data on the bioavailability of food folates is still limited and varies a lot between different studies [77]. Fortified grain products also contribute to folate intake. The bioavailability of natural folate is affected by the removal of the polyglutamate chain by the intestinal conjugase. This process is apparently not complete, thereby reducing the bioavailability of natural folate by as much as 25-30 percent. In contrast, synthetic folic acid appears to have a bio-availability of close to 100 percent. The low bioavailability and, more importantly, the poor chemical stability of the natural folate have a profound influence on the

development of nutrient recommendations. Food fortification can add significant amounts of folic acid to the diet.

Table 1.Food folate content [78]

Food	µg Per serving	DEF Percent DV*
Beef liver, braised,3 ounce	215	54
Spinach, boiled,1/2 cup	131	33
Black-eyed peas (cowpeas),boiled,1/2 cup	105	26
Breakfast cereals, fortified with 25% of the DV	100	25
Rice, white, medium-grain, cooked,1/2 cup	90	23
Asparagus, boiled 4 spears	89	22
Spaghetti, cooked, enriched, ½ cup	83	21
Brussels sprouts, frozen, boiled, ½ cup	78	20
Lettuce, romaine, shredded, 1 cup	64	16
Avocado, raw, sliced, ½ cup	59	15
Spinach, raw, 1 cup	58	15
Broccoli, chopped, frozen, cooked, ½ cup	52	13
Mustard greens, chooped, frozen, boiled, ½ cup	52	13
Green peas, frozen, boiled, ½ cup	47	12
Kidney beans, canned, ½ cup	46	12
Bread, white, 1 slice	43	11
Peanuts, dry roasted, 1 ounce	41	10
Wheat germ, 2 tablespoons	40	10
Tomato juice, canned, ¾ cup	36	9
Crab, Dungeness, 3 ounces	36	9
Orange juice, ¾ cup	35	9
Turnip greens, frozen, boiled, ½ cup	32	8
Orange, fresh, 1 small	29	7
Papaya, raw, cubed, ½ cup	27	7
Banana, 1 medium	24	6
Yeast, baker's, ¼ teaspoon	23	6
Egg, whole, hard-boiled, 1 large	22	6
Vegetarian baked beans, canned, ½ cup	15	4
Cantaloupe, raw, 1 wedge	14	4
Fish, halibut, cooked, 3 ounce	12	3
Milk, 1% fat, 1 cup	12	3
Ground beef,85% lean, cooked, 3 ounce	7	2
Chicken breast, roasted, ½ breast	3	1

2.5 The Dietary Reference Intake (DRIs)

Intake recommendations for folate and other nutrients are provided in the Dietary Reference Intake (DRIs). Developed by the Food and Nutritional Board (FNB) at the Institute of Medicine (IOM) of the national Academies [79]. DRIs incorporate for reference values: Estimated Average Requirements (EARs)'

- Recommended Dietary Allowance (RDAs): average daily level of intake sufficient to meet the nutrient requirements of nearly all (97%-98%) healthy individuals.
- Adequate Intakes (AI): established when evidence is sufficient to develop an RDA and is set at a level assumed to ensure nutritional adequacy.
- Estimated Average Requirement (EAR): average daily level of intake estimated to meet the requirements of 50% of healthy individuals. It is usually used to assess the adequacy of nutrient intakes in populations but not individuals.
- Tolerable Upper Intake Level (UL): maximum daily intake unlikely to cause adverse health effects.

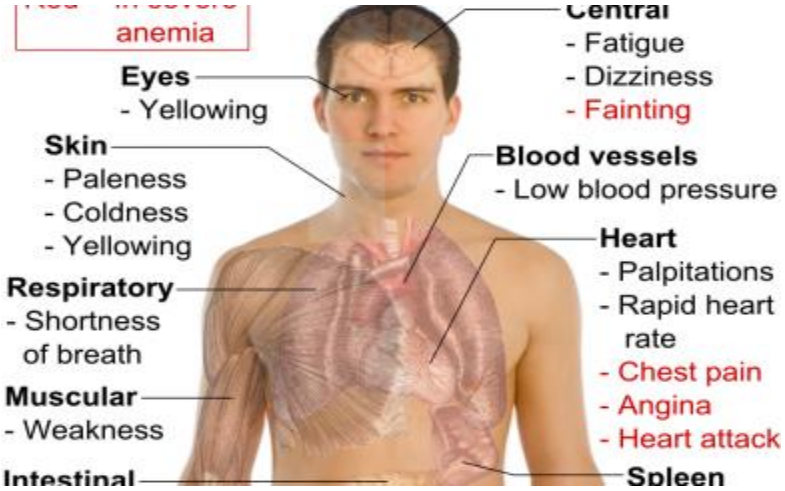
For U.S food and dietary supplement labeling purposes the amount in a serving is expressed as a present of Daily Value (%DV). For folic acid labeling purpose 100% of the Daily Value was 400µg. As of May 2016 update it was kept unchanged at 400µg.

Table 2.National institutes of health (US) nutritional requirements of folate (µg/day) [80].

Age	Infants (RDA)	Infants (UL)	Adults (RDA)	Adults (UL)	Pregnant women (RDA)	Pregnant Women (UL)	Lactating women (RDA)	Lactating women (UL)
0-6 Months	65	None set	-	-	-	-	-	-
7-12 Monts	80	None set	-	-	-	-	-	-
1-3 Years	-	-	150	300	-	-	-	-
4-8 Years	-	-	200	400	-	-	-	-
9-13 Years	-	-	300	600	-	-	-	-
14-18 Years	-	-	400	800	600	800	500	800
+19	-	-	400	1000	600	1000	500	1000

2.6 Folate deficiency

Not having enough folate in a diet can lead to a deficiency in just few weeks. Deficiency may also occur because of disease or genetic mutation that prevents the body from absorbing or converting folate to its unstable form.Isolated folate deficiency is uncommon it usually coexists with other nutrient deficiencies because of its strong association with poor diet, alcoholism, and sometimes, malabsorpitive disorders. Decreased if folate will cause decreased DNA synthesis this will lead to impaired maturation of the nuclei of erythropoietic precursors, resulting in larger than normal red blood cells (RBCs), i.e., macrocytes, and therefore a gradual increase in the mean cell volume (MCV). The hypercellular bone marrow with large erythroblasts with abnormally open, uncondensed chromatin can suggest a misdiagnosis of acute leukemia in severe megaloblastic anemia. Folate deficiency can also lead to elevated blood concentration of homocysteine.Women with insufficient folate intake are at increased risk of giving birth to infants with neural tube defects(NTDs) although the mechanism responsible for this effect is unknown. Inadequate maternal folate status has also been associated with low infant birth weight, preterm delivery, and fetal growth retardation [81].



Source: htpp//www.GoldBamboo.com

Figure 4. **Effects of folate deficiency on the body**

2.6.1 Groups at risk of folate inadequacy

People with alcohol dependence

People with alcohol dependence frequently have poor-quality diets that contain insufficient amounts of folate. Moreover, alcohols interfere with folate absorption and metabolism and accelerate its breakdown [80, 81]. An evaluation of the nutritional status of people with chronic alcoholism in Portugal, where the food supply is not fortified with folic acid, found low folatestatus in more than 60% of those studied [83]. Even moderate alcohol consumption of 240 ml (8 fluid ounces) red wine per day or 80 ml (2-.7 fluid ounces) vodka per day for two weeks can significantly decrease serum folate concentration in healthy men, although not below the cutoff level for folate adequacy of 3ng/ml [84].

Women child bearing age

All women capable of becoming pregnant should obtain adequate amounts of folate to reduce the risk of NTDs and other birth defects. Unfortunately, some women of childbearing age obtain insufficient folate even when intakes from both food and dietary supplements are included [85]. Women of childbearing age should obtain 400mcg/day of folic acid from dietary supplements and /or fortified foods in addition to the folate present in a varied diet [86].

Pregnant women

During pregnancy, demands for folate increase due to its role in nucleic acid synthesis [87]. To accelerate this need, the FNB increased the folate RDA from 400mcg/day for nonpregnant women to 600mcg/day during pregnancy [86]. This level of intake might be difficult for many women to achieve through diet alone. The American College of Obstetricians and Gynecologist recommends a prenatal vitamin

supplement for most pregnant women to ensure that they obtain adequate amount of folic acid and other nutrients [88].

People with malabsorpitive disorders

Several medical conditions increase the risk of folate deficiency. People with malabsorpitive disorders including tropical sprue, celiac disease, and inflammatory bowel disease& mdash might have lower folate absorption than people without these disorders [89]. Diminished gastric acid secretin associated with atrophic gastritis, gastric surgery, and other conditions can also reduce folate absorption [89].

2.6.2 Treatment of folate deficiency

The overarching and long-term strategy recommended for the control of folate deficiency is the consumption of a diet that meets the recommended intakes of the vitamin. However, in populations where it is unlikely that diet will provide recommended intakes of these nutrients, strategies such as supplementation and fortification should be considered. For safety reasons, when deciding the best way is delivering additional folic- either by supplementation or by fortification- the main criterion to be taken in to consideration is the need to target the population group to be reached.

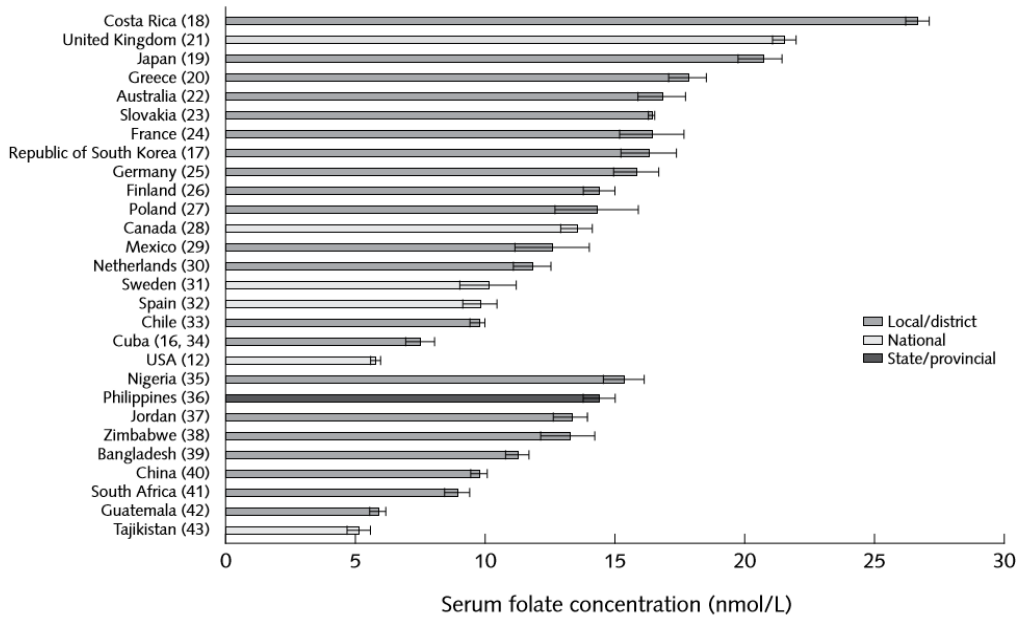
2.7 Prevalence of folate deficiency

Some studies show that there is an indication of the magnitude of folate deficiency around the world. Folate deficiency appears to be public health problem in most of the countries where national surveys are available. Six out of eight countries are deficient in folate. This deficiency differ depend upon different age and biological group. For folate, the main groups affected by deficiency are preschool children in Venezuela (33.8%), pregnant women in Costa Rica (48.8%) and United Kingdom (15.0%). In the United States, prior to folic acid fortification, folate deficiency was present in school-age children (2.3%), adults (24.5%), and the elderly (10.8%), whereas it is now under control.

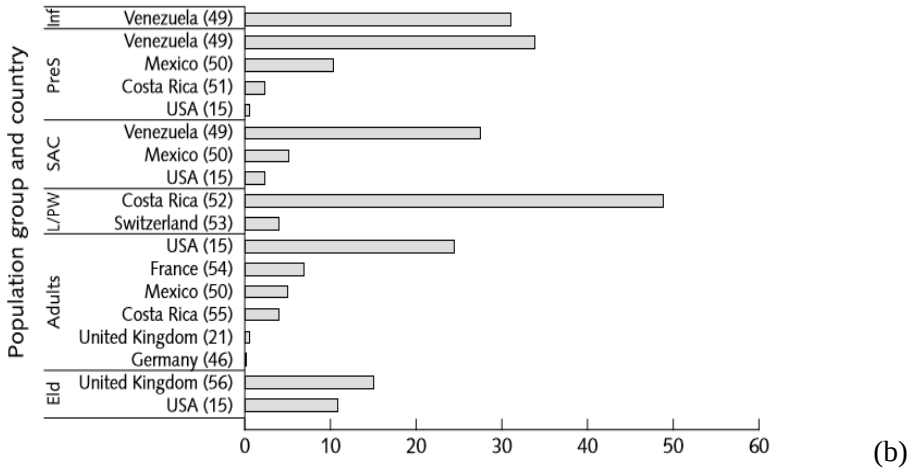
Table 3.Number of countries covered by folate and vitamin B12 status assessment surveys according to WHO Region [9]

WHO Region	No. of countries	Folate		
		National data (%)	Subnational data (%)	Total (%)
Africa	46	0	8(17.4)	8 (17.4)
America	35	4(11.4)	6 (17.4)	10 (28.6)
South-East Asia	11	0	6 (54.5)	6 (54.5)
Europe	52	5 (9.6)	13 (25.0)	18 (34.6)
Eastern Mediterran	21	0	4 (19.0)	4 (19.0)
Western Pacific	27	0	5 (18.5)	5 (18.5)
Total	192	9 (4.7)	42 (21.9)	51 (26.6)

There is some indication that folate deficiency may be a public health problem in some countries, but there is no evidence that the prevalence is associated with the level of development or the geographical location. There also no evidence that some specific groups are more affected by deficiency than others. However, surveys on preschool children and pregnant women, who are the most at risk for deficiencies, are underrepresented compared with the surveys on the adults or elderly. It is necessary to be cautious with these conclusions, because the data are few and there are concerns about the indicators used and the cutoffs applied to define deficiency [91].



(a)



(b)

Source: Review of the magnitude of folate and vitamin B12 deficiencies worldwide, Erin McLean, Bruno de Benoist, and Lindsay H.Allen

Figure 5.(a) Folate concentrations for surveys from countries according to Human Development Index (HDI) group and vitamin concentration, (b) % of population with folate deficiency

2.7.1 Prevalence of folate deficiency in Ethiopia

A study suggests that folate deficiency is clearly a concern throughout Ethiopia. The prevalence of folate deficiency was higher than in most of the countries that have national data. [91] Findings confirm that the Addis, Amhara, and southeren Ethiopia regions have among the lowest prevalence of folate deficient within the country [91].

Table 4.Folate status in women of reproductive age by region from nine regions of Ethiopia, 2005 [9]

Region	Number	Folate status		
		Severe deficiency[a] n(%)	Marginal deficiency[b] n (%)	Optimal status[c] n(%)
Afar	58	34(58.6)	11(19.0)	13(22.4)
Tigray	125	68 (54.4)	19(15.2)	38 (30.4)
Amhara	88	29 (33.0)	22(25.0)	37 (42.0)
Addis Ababa	126	28(22.2)	25(19.8)	73 (57.9)
Oromia	99	50(50.5)	5(5.1)	44(44.4)
SNNP	119	40(33.6)	48 (40.3)	31(26.1)
Benishangul-Gumuz	118	35(29.7)	44 (37.3)	39(33.1)
Harari	135	109(80.7)	17(12.6)	9(6.7)
Dire-Dewa	102	54 (52.9)	15(14.7)	33(32.4)
Overall	970	447(46.1)	206(21.2)	317(32.7)

Folate deficiency in Ethiopia is related to diet as study suggest. The variation in food patterns in the country might help to explain the disparities found in the different regions of the country. In Addis, the capital of the country and a more affluent area, food diversification is likely. Maize and fermented enset products are the major staple foods in the southern part of Ethiopia and animal products are consumed rarely. While it is expected that areas that rely on maize might have more folate deficiency, fermented foods may contribute some additional folate to the diet [92, 93]. The main dietary sources of folate are plant food [94, 95]; therefore, it is unsurprising that those women who eat plant foods less than once daily have higher levels of folate deficiency. In our sample, rates of folate deficiency varied with intake of animal products. Meats, eggs and milk are relatively poor sources of folate and increased frequency of these foods would not protect against moderate folate deficiency. Liver is a better source of folate than most meats, but in Ethiopia liver is usually consumed together with other meat, thus its folate content is weakened. As decreased frequency of consumption of animal products was more commonly associated with severe folate deficiency, it could be that those women had diets limited in several micronutrients, including vitamin B12. The diet consumed by most of the women in Ethiopia is generally poor and is likely deficient in several nutrients that interface with folate metabolism [96, 97].

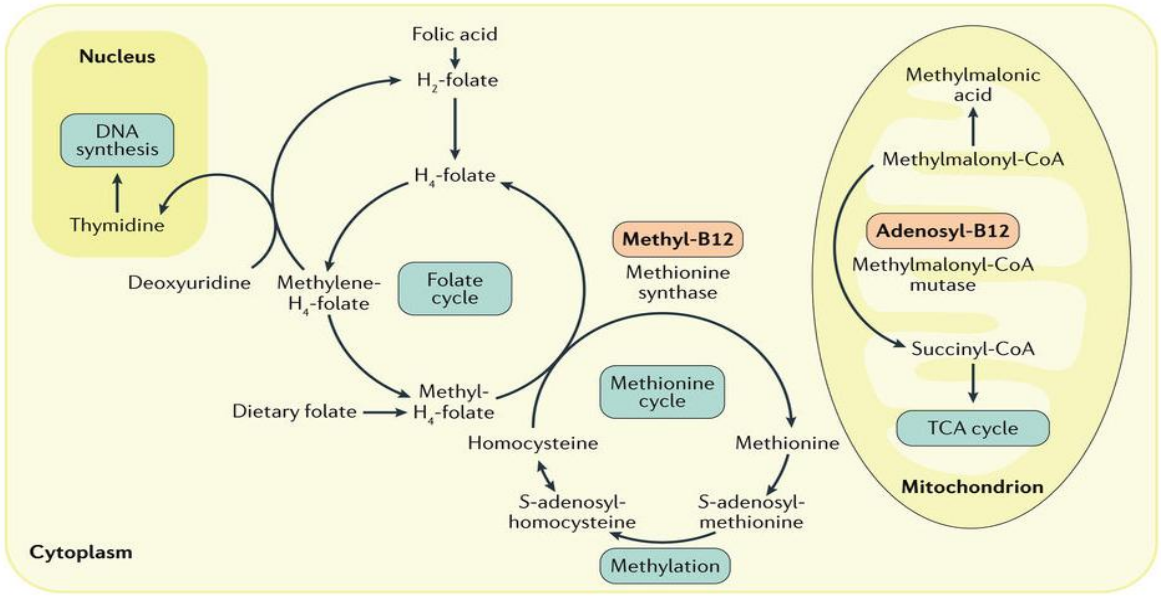
2.8 Folate and women of child bearing age

Folate is involved in one-carbon transfer reactions, which are necessary for DNA and RNA synthesis, amino acid metabolism, and form oxidation[98]. Data released by the World Health Organization (WHO) show that woman of reproductive age, along with infants younger than 2 years of age, are at their nutritionally most vulnerable stage in life [99]. In contrast to trends in developed countries over the past 70 years, high rates of maternal morbidity and mortality persist in developing regions, particularly among low-income women. Maternal mortality figures differ greatly between wealthy and poor nations, with the later accounting for 90% of such deaths [100]. Around 160 million women become pregnant every year, approximately 15% of whom develop serious preventable complications, and millions of newborns do not survive the first week of life due to the absence or lack of proper prenatal health care [101]. One of the indirect complications contributing to maternal health (and consequently to infant health) is maternal anemia. Anemia is especially common among women of reproductive age secondary to menstural bleeding and to physiological treatment services. From a nutritional stand point, deficiency in iron, vitamins B12, A and folate cause hematologic changes which if untreated can evolve to an anemic state [102] .Folic acid has been focus of research for the past 30 years given its role in the prevention of another condition: neural tube closure defects. The role of folic acid in biochemical reactions, such as those involved in metabolism of amino acids and in the synthesis of DNA, renders it a critical nutrient in embryogenesis. During embryogenesis closure of the neural tube, the structure from which the brain and spinal column are derived, take place [103]. Neural tube closure defects can lead to death of the infant or to serious life-long complications. Programs to increase the consumption of folate among women of reproductive age to 400µg/day may benefit countries where the NTD rates is higher than 0.6/1,000 live birth. To reduce the risk of NTD for women capable of becoming pregnant, the recommendation is to take 400µg of folic acid daily from fortified foods, supplements, or both, in addition to consuming food folate from a varied diet beginning at least 1 month prior to conception. The decision to choose between supplementation and fortification to deliver additional folic acid should be driven by the need to target women at risk for becoming pregnant. With that in mind, the main criteria to meet when considering a supplementation program are a high rate (80%) of planned pregnancies, easy to the health care system, a strong public health infrastructure capable of sustained promotional campaigns, and availability of social marketing interventions known to be effective in the targeted area. Where the conditions to implement folic acid supplementations are not met, targeted fortification should be considered.

2.9 Folate interaction with vitamin B12

The vitamins folic acid and B-12 serve as coenzymes in one-carbon metabolism. Specifically, a carbon unit form serine or glycine reacts with tetrahydrofolate (THF) to form methylene-THF. This may be used for the synthesis of thymidylate, a DNA nucleotide, or for purine synthesis. Folate deficiency related macrocytic anemia is due to failure of precursor blood cells to divide because of lack of DNA. The adverse effect of vitamin B-12 deficiency on DNA synthesis is explained by the “methylfolate trap

hypothesis" [104]. Vitamin B12 acts as a cofactor for methionine. The methyl group is donated by methyl-THF to methyl-THF by methyl-THF reductase. If MS is inactivated by a lack of vitamin B-12, the result is a functional folate deficiency (i.e., a lack of the nonmethylated folates needed for serine-glycine interconversion and the synthesis of purines and pyrimidine's) as folate becomes increasingly "trapped" as methyl-THF. Hyperhomocysteinemia is another consequence of deficiencies of either folate or vitamin B_{12} [105]. This effect is due, in part, to the requirement by MS for both folate and vitamin B-12. Furthermore, a to 5-adenosylemethionine, which regulates the one-carbon pathway by inhibiting methylene-THF reductase and activating the homocysteine-disposing enzyme, cystathionine-β synthase. 5-adenosylmethonine also serves as the sole methyl donor for the central nervous system, which may explain associations between folate deficiency and vitamin B_{12} deficiency and cognitive impairment and mental illness [106,107]. One of the most devasting consequences of vitamin B_{12} deficiency is a classic neuropathy called combined degeneration of the spinal cord [108]. The mechanism by which vitamin B_{12} deficiency lead to this fatal demyelinating illness is unknown, but its specific link to vitamin B_{12} deficiency, but not folate deficiency, may provide a clue to the causal pathway. Another unique consequence of vitamin B_{12} deficiency relates to its role in the isomerization of L-methylmalonyl-coenzymeA (coA) to succinyl-coA-a reaction that relates to its role in the isomerization of homocysteine, occurs in the mitochondria and does not involve folate. Thus, vitamin B_{12} deficiency specifically results in the increased methylmalonic acid (MMA) concentrations in both plasma and urine.



Source: Green, R et al. (2017) vitamin B12 deficiency

Figure 6.Folate interactions with vitamin B_{12} for the production DNA

2.10 Folic acid fortification

To address the dietary insufficiency in folate the fortification of flour and grain products (including ready-to-eat cereals and pasta) at a rate of 150 µg of folic acid/100 g was made mandatory. The objective of

folic acid fortification is to reduce the birth prevalence of NTDs by increasing total maternal folate, best estimated by a determination of red blood cell (RBC) folate. Food fortification aimed to increase the daily folic acid intake by about 100 μg on average, and one American study demonstrated a rise in RBC folate concentrations from 527 nmol/L to 741 nmol/L in 38,000 women of child-bearing age following the introduction of folic acid fortification. However, even this policy still leaves only 23% to 33% (depending on ethnicity) of North American women of child-bearing age meeting the daily recommended intake [108]. As a result, RBC folate levels in some women remain below the optimal estimated protective level of 900 nmol/L [110]. Moreover, a recent large study in China with pharmacokinetic modelling showed that even higher levels of RBC folate (up to 1500 nmol/L) might be additionally helpful [111]. Despite the fact that for many women daily folate intakes remain lower than the 400 μg Health Canada recommends, fortifying food with folic acid has been highly effective in reducing the birth prevalence of neural tube defects. Following folic acid fortification, the birth prevalence of spina bifida in Canada fell by over 50% and that of other NTDs by approximately one-third [112]. Moreover, the East-West gradient in the rates of NTDs flattened significantly following the introduction of folic acid fortification [113]. There may, however, be some under-ascertainment of cases because terminations at <20 weeks gestation/500 g are not uniformly recorded across the country. Similar 50% to 70% reductions in infants born with an NTD have been reported elsewhere following increased maternal folic acid intakeRegulations for mandatory fortification of wheat flour with folic acid are currently in place in 53 countries although in many cases these regulations have not been implemented [114].

2.11 Folic acid supplements

Despite overwhelming evidence that folic acid fortification is effective in reducing NTDS, a significant proportion of women remain folate-deficient in early pregnancy. Health Canada and the Public Health Agency of Canada recommend that women of child-bearing years take a daily supplement of 0.4 mg of folic acid to reduce the risk of NTDs. This recommendation is supported by detailed guidelines from the Society of Obstetricians and Gynecologists of Canada [115-117]. They recommend that women in good health eat a diet of folate-rich foods, along with daily supplementation with a multivitamin which includes folic acid (0.4 mg to 1.0 mg) for at least two to three months before conception and throughout the pregnancy and postpartum periods (for a minimum of four to six weeks and as long as breastfeeding continues). Factors known to increase the risk of NTDs in subsequent pregnancies include birth of a previous child with a NTD, a family history of NTDs, maternal obesity and maternal Hispanic origin, and the use of some anti-convulsants[118,119]. Pregestational or gestational diabetes is of low predictive value,(48) perhaps because the risk may vary with the level of glycemic control [120]. Since the etiology appears to be multifactorial, each risk factor is of similarly low predictive value, with the highest risk being for women with a previous affected child (at 2% to 5% before fortification [121]). Therefore, for women with a family history of NTD or other health complications, the SOGC recommends increasing

dietary intake of folate-rich foods and daily supplementation with multivitamins (including 5 mg of folic acid) at least three months before conception and continuing 10 to 12 weeks postconception [122].

2.12 Floate analysis

Method of analysis for folic acid and its analogous are grouped into biological, microbiological, bio-specific procedures (radioassay), chromatographic, and chemical methods. Although biological method using chick and rat assays were used before, they are no more used for folate except in studying nutritional aspects of the vitamin. Chemical methods are rarely used for routine folate analysis in food. A summary of strengths and shortcomings of microbiological, chromatographic (HPLC), and bio-spcific procedures is given in table. The method to be used for a particular biological material depends on the nature and purpose of the assay. Moreover, the sample preparation and extraction methods largely influence the amount of folate present in the extract, which is then assayed by a method of choice.

2.12.1 Serum folate analysis

Microbiological assay has been used for many years to estimate the concentration of folate in blood and other tissues. In the 1990s O' Brion et al. and Molloy et al. have introduced robust and reliable procedures that use microtiter plates for higher throughput and a cryopreserved antibiotic resistant microorganism to avoid having work under aseptic conditions. The herein described procedure is an adaptation of the O' Broin et al. method and is used to quantitatively measures serum and RBC folate in human specimens. The method is relatively easy to perform, reliable, and considerably less costly than chromatographic or commercial kit assays. Diluted serum or whole blood hemolysate is added to an assay medium containing Lactobacillus rhamnosus (formerly known as L. casei) and the entire nutrient necessary for the growth of L. rhamnosus except for folate. The inoculated medium is incubated for 45 hours at 37^{o}C. Since the growth of L. rhamnosus is with 5-mproportional to the amount of total folate present in serum or whole blood samples, the total folate level can be assessed by measuring the turbidity of the inoculated medium at 590nm ina microplate reader. The assay is calibrated with 5-methtyltetrahydrofolic acid (5MeTHF). If seven plates are processed in a run, 132 patient specimens can be analyzed.

2.12.2 Food folate analysis

Folate and its derivatives occur as polyglutamets in nature. The multiplicity of forms and the generally low levels in foods makes quantitative analysis of folate a difficult task. The assay of folates from foods generally involves three steps: liberation of folates from the cellular matrix; deconjugation from the polyglutamate to the mono and di-glutamate forms; and the detection of the biological activity or chemical concentration of the resulting folates. The detection methods used are the microbiological assay relying on the turbidimetric bacterial growth of Lactobacillus rhamnosus which is by far the most commonly used method; the HPLC and LC/MS techniques and bio-specific procedures. For this experiment microbiological assay is used.

Microbiological assay (MA)

The amount of growth of the folate dependent microorganism is proportional to the amount of folate in the medium. Growth measured by change in solution turbidity. Extraction of folate from matrix, deconjugation, growth of micro-organism, measurement of turbidity. Low equipment set up costs versatile, similar response to most folate isomers, can measure mono- to polyglutamates (>3), very sensitive, measure up to sup-nanogram levels, 'gold standard' for folate analysis Test organism may be stimulated or inhibited by non-folate substances, tedious and time consuming, require microbiological expertise, microorganism finicky and needs proper transfer and maintenance. This are the steps need to be followed to do the analysis:

Sample preparation and purification

Extraction

The sample preparation step has received the least attention, although it can have a significant effect on availability of folate for deconjugation and the subsequent detection. Most often, sample preparation involves grinding of sample and homogenization of ground food in a suitable buffer system followed by heating and centrifugation. Heating during the extraction procedure causes thermal denaturation of folate-binding proteins and enzymes that may catalyse the folate degradation or interconversion and at the same time also precipitates structural proteins. Various temperatures have been used to heat the homogenate: 70^{o}C boiling water bath or autoclaving at 100 or 121^{o}C. In all folate assays, a preservative is added to the extraction buffer to prevent the oxidative loss of labile reduced folates. Ascorbic acid and 2-mercaptoethanol are the two most common anti-oxidants used in the folate extraction buffer. 0.2% ascorbic acid in phosphate buffer (Ph 6.1) affords complete protection to 5-methyltetrahydrofolic acid for a routine autoclave cycle.

Tri-enzyme extraction

In the 1980s, a number of researchers reported that treatment with folate conjugase alone is usually not effective to liberate food-bound folate. The use of additional enzymes, prolytic or amylolytic, was shown to liberate folate from the foods, there by maximizing the folate activity in certain foods. Later reported a method of folate extraction where, in addition to the traditional treatment with folate conjugase, protease (EC3.4.24.31) and α-amylase (EC3.2.1.1) were also used. The extraction method

was named as the 'tri-enzyme treatment'. It was intended to accomplish a more complete extraction of folates that may be trapped in or bound to the matrices of protein and polysaccharides, by using protease and α-amylase in addition to the heat treatment and the conjugase. There was a remarkable increase observed in folate values when results from tri-enzyme extracts were compared with a single enzyme.

Microbiological assay

The realization of the fact that certain microorganisms require specific nutritional factors that they are unable to synthesize themselves led to the application of these microorganisms to the quantitative determination of vitamins in the early 1940s. The microbiological assay of folate started with the findings of Stokstad (1943) that growth of lactic acid bacteria such as Lactobacillus rhamnosus and streptococcus lactis is influenced by liver and yeast extracts which are rich in folate. Despite the advent of several alternative methods for determining folates in foods and biological samples, the microbiological assay using Lactobacillus rhamnosus (ATCC 7469) in casein based media remains the standard method for most applications. L.rhamnosus is the most commonly used assay organism for folate analysis because it responds to the widest variety of folate derivatives, including 5-methyltetrahydrofolate, the predominant folate form in plasma, red blood cell and liver, and the formyl derivatives. Some organisms used less widely for folate assay are Streptococcus faecalis, Pediococcus cerevisiae, Tetrahymena pyriformis (geleii) (ATCC 30008), and Bacillus coagulans. S. faecalis was formerly the most common assay organism but its use declined after it was learned that it does not respond to methyl folates.

Platting

In 1986, Newman and Tsai introduced a procedure for microbiological assay of folate in 96-well microtiter plates read with an automated plate reader. This assay has some advantages over the conventional microbiological assay, such as decreased reagent cost and shorter time spent in pipetting and manually reading the results for samples in the spectrophotometer. Use of cryoprotected L. rhamnosus shortened the assay time from 36-48h to 18h and simplified the 96-well microtiter plates technique. The detectability limit of this method is about 10 f mol as compared about 100 f mol in the standard microbiological assay. It is reported that reasonably good reproducibility of results with this procedure. Semi-automated microbiological assay for folates with a capacity of 600 tubes per day, using a micro-computer to control sample dilution, medium addition, turbidity determination, and data acquisition was developed. This method reportedly reduced human error and gave higher volume output and greater reproducibility when tested on folate content determinations.

Although the microbiological assay is regarded as the premium method of folate assay, there are some associated problems such as the assumption that the test organism has an absolute requirement for the compound being assayed, and therefore stimulation or inhibition of L. rhamnosus growth by any non-folate substances in the extracts would invalidate the assay. The assay itself is labor-intensive and time-consuming. Other common causes of difficulty with the microbiological assay include improper maintenance of assay organisms; improper dilution of assay media; folate contamination of reagents and glassware; inappropriate medium, inappropriate preparation and storage of folate standards; inappropriate dilutions of samples because of over-or underestimation of folate concentration; variation in incubation time and temperature; sterilization procedure; inoculation volume, conjugase treatment; and pH of the assay medium.

3. Materials and Methods

3.1 Study area

The study was conducted at Amhara region, in WestGojjam.

3.2 Study Design

Community-based cross sectional survey conducted from November 2017 to April 2018.

3.3 Study population

All women of reproductive age which arein theaverage age range from 26 to 33 year in seven clusters were considered as the study population. Household in the study area were allowed in the study if they meet the following criteria: women with age range between18-35, and who were in healthy condition and also have given consent to participate in the study. Women who have bad health condition were excluded. The health centers' data was completed through house-to-house visits.

3.4 Sampling techniques

From two woreda 7 clusters (kebele) were selected by simple random techniques. 3 clusters from Dangela woreda and 4 clusters from Bahir Dar zuria. In the selected cluster, all households who had eligiblewomen were listed and 29 household/study participants were selected from each cluster by systematic random sampling techniques

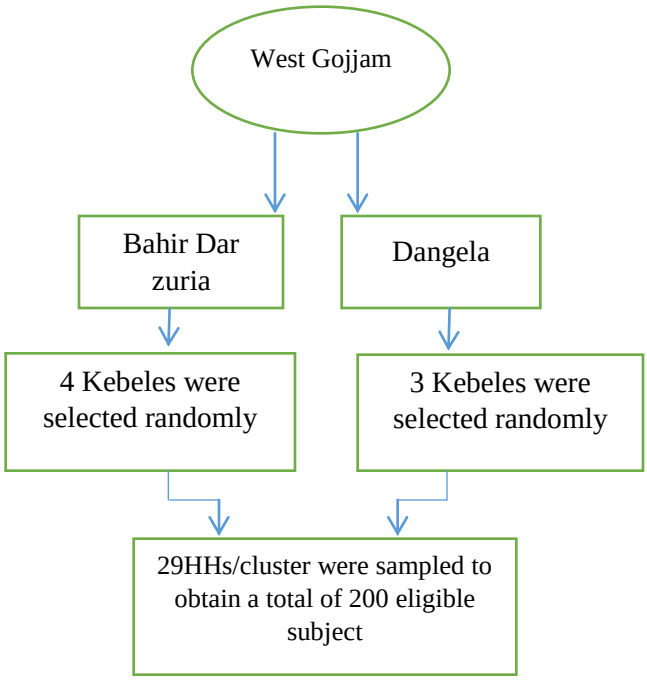


Figure 7.Schematic presentation of sampling procedure

3.5 Study variables

3.5.1 Dependent variables

- Serum folate status

3.5.2 Independent variables

- Dietary factors
- Socio-demographic factor

3.6. Dietary data collection

Based on the previously collected 24hr recall questionnaire done by Dr. Kaleab Baye, most frequently consumed foods by the women were identified. The data for target women were collected in three rounds. A digital kitchen scale was used to measure the amount of food consumed and ingredients used in food preparation. All food and drinks consumed by the women were measured and recorded in the household, starting from early morning until the next day morning. The dietary data obtained by a standard 24-hour recall. The 24-hour recall had three steps; the first was used to set a quick-list of food and list all the food that the women consumed during the 24 hours, second the ingredients/cooking methods used was asked, and finally portion size was 25 estimated by direct weighting of foods or using household measures. Dietary data were entered in duplicate to limit data entry errors and individual food intake was transformed in nutrients using (Nutriservay for windows, 2007) software.

3. 6.1. Assessment of energy and nutrient

The nutrient and energy content of foods consumed was calculated using the Ethiopian food composition table. The median daily intakes of energy and nutrients from diets, recipes or commercial food products were compared with the equivalent estimated needs for energy and selected nutrients based on FAO/WHO. The adequacy of the energy intakes was calculated by comparison with the total energy intakes as a percentage of estimated energy needs from foods.

3.6.2. Blood collection and measurement

Blood was drawn by trained phlebotomists. An appointment was made with the women and blood was drawn in the nearby health facilities (health post/health center). Approximately 2-6 ml blood was drawn into vacationers via venipuncture. Before puncturing, the subject's skin was wiped with 70% alcohol. Serum was separated by centrifugation at 3000 rpm for 10 minutes within 1hour of collection and was frozen at -18°C in a portable refrigerator (WAECO-CF35 portable compressor freezer). Before and after serum separation, the samples were kept away from the light, by putting them in black/opaque ziplock bags. The samples were transported to the nearest district laboratory for temporary storage at -35°C refrigerator. After completion of fieldwork, all samples were shipped to EPHI laboratory and were stored at -80°C until further analyses.

3.6.3. Dietary food collection and measurement

Based on the 24hr recall questionnaire, most frequently consumed foods were identified. This food items were bought from five different local markets to make composite. Five samples of each food were taken from different market, for each type of them. They were individually labeled and stored at room temperature until it's transported to the laboratory for analysis. Using this composite of the samples folate analysis was done. The effect of cooking on folate retention was determined. To this end, two foods (*Injera* and *Shiro-wot*) were selected, the samples of which were taken from five different households. The samples were taken both in their raw and cooked form. These cooked foods were stored at 4^{o}C until it was transported to the laboratory.

Duplicate of each food sample were separately analyzed for moisture content. Moisture was estimated for the raw samples.

3.7Folate analysis

3.7.1 Food folate analysis

Analytical procedures were carried out under yellow light. Samples and calibration solution were covered with aluminum foil. After extraction sample extracts were kept under nitrogen atmosphere whenever feasible.

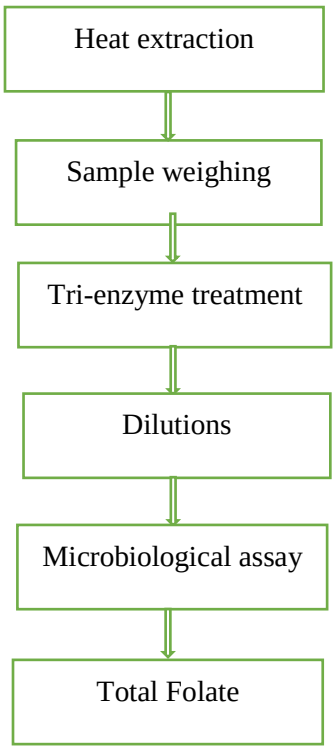


Figure 8.**Extraction and tri-enzyme treatment**

Extraction

The first step in the folate analysis was extraction followed by tri-enzyme treatments (conjugase, α-amylase and protease) for analyzing total folate with microbiological assay. The sample amount was 1 to 1.5 g, depending on the estimated folate content. Samples (usually in triplicates) were weighed to plastic tubes, and 15 mL of extraction buffer (50 mM Ches, 50 mM Hepes, containing 2 % sodium ascorbate and 10 mM 2-mercaptoethanol, pH 7.85) was added. Samples were flushed with nitrogen, placed in a boiling water bath for 10 min, cooled on ice and homogenized, whenever necessary. Samples were cooled and the pH was adjusted to 4.9 with HCl. Hog kidney conjugase (HK) was prepared from fresh pork kidneys according to Gregory et al. (1984). Its activity was tested in every batch [123]. Sample extracts were first incubated under a nitrogen atmosphere with α –amylase (EC 3.2.1.1, St.Louis, MO) and HK for 3 h at 37 ºC in a water bath. After that, the pH was adjusted to 7.0 with KOH and protease (EC 3.4.24.31; Sigma, St. Louis, MO) was added. Extracts were incubated under a nitrogen atmosphere for 1 h at 37 ºC, after which they were boiled for 5 min in boiling water bath to inactivate the enzymes, and cooled on ice. Samples were filled to an exact volume of 25 mL with 0.5 % (w/v) sodium ascorbate, pH 6.1 and then analyzed directly with the microbiological assay. A blank sample was analyzed in each set of samples and the results were corrected accordingly.

Microbiological assay

A chloramphenicol-resistant strain, Lactobacillus rhamnosus which is folate dependent microorganismobtained from Dr. Aynadis Tamenewas used as growth indicator. Eight levels of the calibrant, corresponding to 0 to 80pg of PGA, were pipetted into 96-well plates, four wells for each level. Sodium ascorbate (0.5%) was added to the calibrant-containing wells so that the final volume in each well before adding the inoculated medium was 100 µl. Different dilutions, typically varying from 1:330 to 1:50, were prepared from each sample to 0.5% (w/v) sodium ascorbate, pH 6.1, and 100 µl of each dilution was pipetted into four wells. Inoculated medium was then added into each well (200 µl). After 42 h incubation at 37 ºC the optical densities of the wells were measured with a microplate reader at 595 nm.

3.8 Ethical considerations

Ethical clearance was obtained from Addis Ababa University college of Natural Science, Institutional Review Board and Amhara national Health Institute. At the time of data collection, a written consent obtained from the participants. Confidentiality of responses was also ensured throughout the research process. The formal letter also summited and the study design was also explained to officials of the region, zone, and woreda Health Department for their permission and support. Data obtained from each study participant was kept confidential. All subjects who participated in the study were acknowledged, but did not receive any compensation.

3.9 Operational definitions

- *Shiro Wot:-*is a homogenous stew whose primary ingredient is powdered chickpeas or broad bean meal.
- *Injera:-* a flat spongy bread made of fermented teff flour.
- Bioavailability: - is defined as the proportion of a nutrient in food that is absorbed and utilized for normal metabolic and physiological functions or storage.
- Dietary intake: - The amount of energy, nutrients or anti-nutrients available in the food consumed by a preschool children.
- Dietary adequacy: - The amount of food taken daily by preschool children relates to the standard recommendations
- Estimated average requirement (EAR): - is the daily intake estimated to meet the requirements, as defined by a specified function or biochemical measurement of 50% of the individuals in a particular life-stage and sex.
- Prevalence: - is a measure of the number of persons with inadequate intakes of a nutrient or with malnutrition or disease at a given time.

4. Results

4.1 Socio demographic characteristic of women of reproductive age in West Gojjam

A total of 200 women of reproductive aged women were recruited from Bahir Dar zuriya and Dangela woreda and about 179 women participated in this study. The response rate was 89.5%. Their age ranged from 26 to 33 years. Most of them (68.7%) do not have formal education, and a majority of them were married (92%). Furthermore, about 50% of households were comprised of 4-6 tenants. Agriculture was the main source of income of the household(97.8%). About half of the women (49.7%) earn 1000-2000 Ethiopian birr (ETB) per month.

Table 5.Demographic characteristics of reproductive aged women in W.Gojjam.

Back ground	Categories	Frequency	Percent
Age of women	18-25 years	25	14.0
	26-33 years	72	40.2
	34-41 years	58	32.4
	42-49 years	24	13.4
Educational level of women	Illiterate	123	68.7
	Primary level	45	25.1
	Secondary level	10	5.6
	Semi college	1	0.6
Marital status	Married	164	91.6
	Divorced	9	5.0
	Window	5	2.8
	Other	1	0.6
Women's occupation	House wife	1	0.6
	Farmer	175	97.8
	Trader	1	0.6
	Government job	2	1.1
Monthly income	No formal income	2	1.1
	Below 1000	29	16.2
	1000-2000	89	49.7
	Above 3000	59	33.0
Number of children	None	1	0.6
	1-3	71	39.7
	4-6	66	36.9
	Above 6	41	22.9

4.2 Food consumption pattern of the participants

Food consumption pattern was determined using food frequency questionnaire, which is conducted in three rounds. This helped us to identify the most consumed food items in the area. The most consumed foods were *Injera* and *Shiro wot*, in addition Bread and most of the time they used to drink tella. To make these food items different kinds of cereals and legumes were used. To identify the common one, randomly from thirty households *Injera* flour and *Shiro* flour were taken (Table 7). Fromcereals which used to make *Injera* and bread are wheat, teff, sorghum, millet and barley are the most common one. From legumes which used to make *shiro wot* are beans, chick pea, grass pea, and peas are the commononones (Table 7).

Table 6.The most common cereals and legumes used to make *Injera* and *Shiro wot*

No	Cereal to make *Injera*	n	Legumes to make *Shiro wot*	N
1	75%Teff,25%Maize	3	Grass pea, Chick pea	2
2	75%Mize,25%Teff	4	Grass pea	20
3	50%Maize,50%Teff	5	Chick pea	1
4	75%Millet,25Maize	5	Grass pea, Chick pea, Cow pea	2
5	50%Millet,50%,Maize	2	Cow pea, Chick pea	2
6	2/3Millet,4/3Maize,4/3Teff	2	Grass pea, Cow pea	2
7	4/3Millet,4/3Maize,4/3Teff	2	Bean	
8	2/3Maize,4/3Millet,1/3Rice	1		
9	100%Maize	4		
10	100%Teff	1		
11	100%Millet	1		
	Total	30	Total	30

The folate content of these food items was determined. As indicated in Table 8 the major cereals with high folate content were wheat (40.2 µg/100g), teff (39 µg/100g) and sorghum (31 µg/100g), whereas, maize (4.4 µg/100g) has the lowest folate content. Beans (65 µg/100g), grass pea (48µg/100g) and chickpea (42 µg/100g) has the highest folate content. Whereas, peas (10µg/100g) has the lowest.

Table 7.Folate content of most frequently consumed cereals and legumes

Cereals	**Folate content in µg/100g**
Wheat	40.21
Teff	38.95
Sorghum	30.95
Millet	20.07
Barley	19.44
Maize	4.42
Legumes	
Bean	65.27
Grass pea	47.84
Chick pea	42.13
Pea	9.78

4.3 Effect of cooking on folate retention

As Table 9 shows there is a significant difference between the raw and cooked food items. On *Injera*it shows that there is no significant difference, whereas on *shiro wot* it has significant difference.

Table 8. Effect of cooking on folate retention

Sample	Folate content (µg/100 g) (Mean ± SD)	P-value
Teff flour	61.00 ± 13.7	0.052
Injera	13.78 ± 3.9	
Shiro (uncooked)	66.98 ± 14.9	0.000
Shiro wot	20.58 ± 9.2	

4.4 Serum folate status in women of reproductive age in West Gojjam

Out of 179 serum samples, almost all of all of the samples (95.4%) have an optimal folate status (> 6.6 ng/mL) and 2.6% have marginal folate deficiency (> 4–6.6 ng/mL) while few (1.9%) have severe folate deficiency (≤ 4 ng/mL).

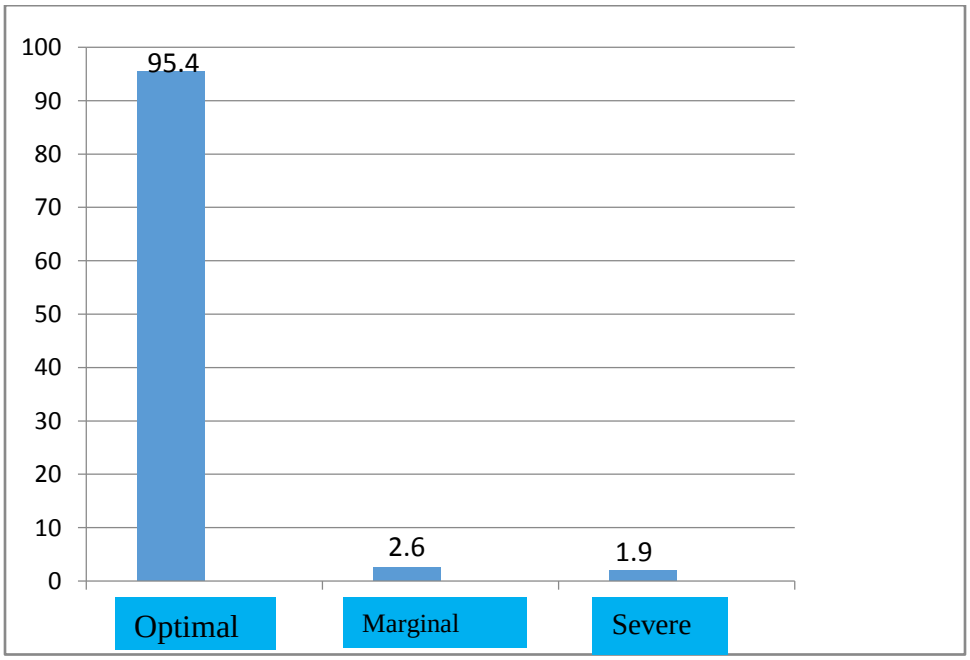


Figure 9.Serum folate status

4.5 Correlation between dietary folate intake and serum folate status

Correlation between food intake and serum status was done on a given sample. As the chart below shows that there is no correlation between them.

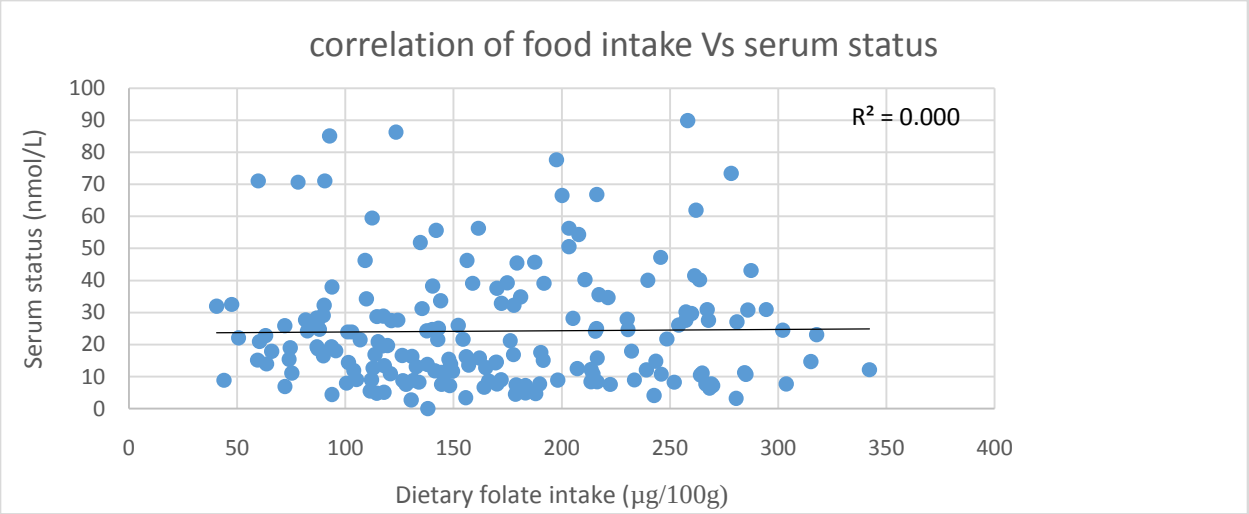


Figure 10.Correlation between dietary folate intake and serum folate status

5. Discussion

Folate has a great role in the health of reproductive age women, and inadequate folate status is associated with the risk of birth defects and other health problems. For this reason, conducting this study on current intake and status of folate is essential for implementation of an effective public health intervention.

A total of 200 women of reproductive aged women were recruited from Bahir Dar zuriya and Dangela woreda and about 179 women participated in this study. The response rate was 89.5%. Their age ranged from 26 to 33 years. Most of them (68.7%) do not have formal education, and a majority of them were married (92%). Furthermore, about 50% of households were comprised of 4-6 tenants. Agriculture was the main source of income of the household (97.8%). About half of the women (49.7%) earn 1000-2000 Ethiopian birr (ETB) per month.

Food consumption pattern was determined using food frequency questionnaire, which is conducted in three rounds. This helped us to identify the most consumed food items in the area. The most consumed foods were *Injera* and *Shiro wot*, in addition Bread and most of the time they used to drink tella. To make these food items different kinds of cereals and legumes were used. To identify the common one, randomly from thirty households *Injera* flour and *Shiro* flour were taken. From cereals which used to make *Injera* and bread are wheat, teff, sorghum, millet and barley are the most common one. From legumes which used to make *shiro wot* are beans, chick pea, grass pea, and peas are the common ones.

The mean dietary intake of women in this study was 171.1µg DFE/day. Compared to the intakes of other populations, the folate intake was found to be lower than those of American women (490 µg DFE/day)[124], British women (228 µg DFE/day)[125] and Chinese women (291.4 µg DFE/day)[126].

Folate which occurs naturally in the diet is more concentrated in selected foods, including green leafy vegetables, fruits, beans, and eggs. In addition to food folate, folic acid is fortified in ready-to-eat breakfast cereals, infant formulas, meal replacements and nutritional bars, which are not available in Ethiopia. By taking the most frequently consumed food items from West Gojjam, the contribution of these foods to folate were identified. As the data in table 4.3 shows, most cereals and legumes are better source of folate. The result varies form 4-40µg/100g in cereals and 10-65µg/100g in legumes. From cereals Wheat (40.2) and Teff (38.9) is the most contributor to folate intake. From legumes Bean (65.2) and Grass pea (47.8) have highest folate content. Though, vegetables and fruits are folate-contributing foods, they are not frequently consumed in this area. In Western countries such as the UK and US, cereals and cereals products are the largest contributors of folate, and large amounts of folate intake come from ready-to-eat breakfast cereals[127].

Though, legumes and cereals are good source of folate in their raw form, their retention affected by different factors. As a result of folate sensitiveness to heat, UV-light and oxidation. Retention of folate in foods after cooking is variable and highly dependent on type and food preparation method [126]. Folate losses during cooking and preparation of foods are the result of a combination of thermal degradation and leaching of vitamin into the cooking water [128], and it has been reported that food folate is reduced by 50% to 80% with food processing and preparation[129].On the result in table 9 shown, there is a significant difference between raw food items and cooked ones in *Shiro wot*. Whereas there is no significance difference between raw food items and cooked ones in *Injera*. This could be result of Tef behavior which is Teff is a relatively good source of folate and fermenting it to *Injera* could increase its folate content [130]. This could be a condition that favors folate production during fermentation.

The mean serum folate status of reproductive age women were 26.80nmol/L. the prevalence of folate deficiency (≤4 nmlo/L) in this study was 2.6%. More than half (95.4%) of the sample have optimal folate status (>6.6nmlo/L), this shows that folate deficiency is not a public health problem. Likewise, the prevalence of folate deficiency was higher than in most of the countries that have national data.1 Nonetheless, findings done by Jemal et.al confirm that the Addis, Amhara and southern Ethiopia regions have among the lowest prevalence of folate deficiency within the country. As stated above, folate intakeof reproductive age in West Gojjam is not sufficient, though their serum status shows that they are optimal. On the correlation that has been done between folate intake and serum folate status there is no correlation.

Several limitations may restrict the generalizability of the result in this study. The first one, method used for identifying most frequent foods is 24hr recall questionnaire. This method have limitations like not suitable for measuring distant meal and irregularly consumed foods, unsuitable for participant with memory issue, therefore intake of some nutrient could be underestimated. The second one, plasma folate was used to assess folate status. For population survey, plasma folate measurement is suitable to assess general folate status[131]. However, plasma (serum) folate level is subject to diurnal changes caused by recent food intake and chronic deficiency status [132]. Total homocysteine level in plasma is used as a functional test of folate deficiency. Future studies should confirm findings based on red blood cell folate or total homocysteine. The other one, the limited numbers and random selection of subjects in this study might limit the representatability of reproductive age women in West Gojjam. The other reason human colon is known to produce water soluble vitamins including folate [129].

6. Conclusion and recommendation

Most women of reproductive age found in West Gojjam consume mainly cereal and legume based foods. Measurement of the total folate content of these foods shows that legumes and cereals are good source of folate and also when we calculate the total energy using the software (Nutrition survey), it shows that it is lower than the RDA. Even though the folate is low in their dietary intake, the serum folate status shows that there is optimum folate in their blood. When the study was started correlation was expected between the dietary folate intake and serum folate status. But the result shows that there is no correlation. This could be due to some reasons like production of folate in the human colon, the method used to identify most frequent foods or because of serum usage for folate analysis. As recommendation, to have adequate folate intake these women should have diversified food items. And consume foods that are rich in folate like green leafy vegetables. Even diet alone cannot make the RDA to be met, so if possible fortified foods and supplements should be given. As discussed above there is limitation to this study so further investigation is needed.

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