A dietary vitamin B12 deficiency impairs motor function and changes neuronal survival and choline metabolism after ischemic stroke in middle aged male and female mice

Gyllian B. Yahn BA[1], Brandi Wasek[2], Teodoro Bottiglieri PhD[2], Olga Malysheva MSc[3], Marie A. Caudill PhD[3], Nafisa M. Jadavji PhD[1,4]

Affiliations

[1] Department of Biomedical Sciences, Midwestern University, Glendale, USA
[2] Center of Metabolomics, Institute of Metabolic Disease, Baylor Scott & White Research Institute, Dallas, TX, USA
[3] Division of Nutritional Sciences, Cornell University, Ithaca, USA
[4] Department of Neuroscience, Carleton University, Ottawa, Canada

Sources of support: Midwestern University Start-up Funds
Conflict of Interest and Funding Disclosure: None
Corresponding Author:
Nafisa M. Jadavji, PhD
Department of Biomedical Sciences
College of Graduate Studies
Midwestern University
19555 N 59th Ave
Glendale, AZ 85308, USA
Phone: 623-572-3665
Fax : 623-572-3647
E-mail: njadav@midwestern.edu

Word Count: 4,505
Number of Figures: 6
Number of Tables: 3
Supplementary data submitted: One table
Running Title: Vitamin B12 deficiency and stroke
Abbreviations: Total homocysteine; tHcy, neuronal nuclei; NeuN, 4′,6-diamidino-2-phenylindole; DAPI

Abstract

Background

Nutrition is a modifiable risk factor for ischemic stroke. As people age their ability to absorb some nutrients decreases, an example is vitamin B12. Older individuals with a vitamin B12 deficiency are at a higher risk for stroke and have worse outcome. However, the mechanisms through which these occur remain unknown.

Objective

The aim of the study was to investigate the role of vitamin B12 deficiency in ischemic stroke outcome in a mouse model.

Methods

At 10-weeks of age male and female C57Bl/6J mice were put on vitamin B12 deficient (0 mg/kg) or control (0.025 mg/kg vitamin B12) diets for 4-weeks (n = 10/group). Using the photothrombosis model the sensorimotor cortex was damaged and at 18 weeks of age, motor outcome was measured. At the end of experiments, brain, blood, and liver tissue were collected to measure homocysteine levels, ischemic damage volume, apoptosis, or one-carbon metabolite levels.

Results

All animals maintained on the vitamin B12 deficient diet had increased levels of total homocysteine in plasma and liver tissue. In ischemic brain tissue no difference between groups in lesion volume were detected. There were changes in levels of one-carbon metabolites in ischemic and non-ischemic brain tissue because of a vitamin B12 deficiency or sex. There was an increase in total apoptosis in ischemic brain tissue of male vitamin B12 deficient animals. There was more neuronal survival within ischemic brain tissue of the vitamin B12 deficient animals compared to

controls. After ischemic stroke, vitamin B12 deficient male and female mice had impaired motor function compared to control animals.

Conclusions

The data presented in this study confirm that a dietary vitamin B12 deficiency impacts motor function in older adult male mice after ischemic stroke. The mechanisms driving this change may be a result of neuronal survival and compensation in one-carbon metabolism within the damaged brain tissue.

Keywords
Vitamin B12; stroke, sex differences; motor function; apoptosis; choline metabolism

Introduction

It is known that eating healthy is important for good health and can reduce the risk of diseases such as stroke (1). Vitamin B12 is also referred to as cobalamin, it is water-soluble found naturally in foods such as red meat, poultry, eggs, dairy products, seafood, and fortified cereals (2). It has very important roles in the human body as it is necessary for proper red blood cell formation, neurological function, myelin synthesis and repair, and DNA synthesis. A vitamin B12 deficiency is a serious and common complication that is defined as low plasma and tissue levels of vitamin B12 (3). It can affect all age ranges, however it has a much higher prevalence within the elderly population (1,4) commonly caused by malabsorption, decreased acid secretion, and reduced intrinsic factor production (4). Vitamin B12 is an important co-factor in the conversion of homocysteine to methionine. Elevated levels of homocysteine are well associated with increased risk for cardiovascular disease such as ischemic stroke (5).

The presence of atrial fibrillation, vitamin B12 deficiency, and resultant elevated levels of plasma total homocysteine (tHcy) increases with age and are a risk factor for stroke (6,7). Clinical data strongly suggest that low levels of vitamin B12 are a risk factor for ischemic stroke and also impacts stroke outcome in patients (8,9). There is a gap in the understanding of the mechanisms in which a vitamin deficiency leads to increased risk of stroke and worse stroke outcome. This study aims to assess the effects of a vitamin B12 deficiency on stroke outcome and related mechanisms.

Methods

Animals

All experiments in animals were approved by the Midwestern University IACUC committee. Female and male C57/BL6J mice were obtained for Jackson laboratories for this study.

A total of 44 mice were obtained, 22 males and 22 females. The mice 10-months-old upon arrival and were habituated for 1 week prior to the start of experiments.

Experimental design

An overview of all experimental manipulations is outlined in Figure 1A. Briefly, after mice were habituated to the Midwestern University animal facility for a period of one week, mice were randomly assigned to control or vitamin B12 deficient groups and placed on diets for 4 weeks. At 11 months of age, ischemic stroke was induced in animals using the photothrombosis model (10–14). The age of the mice corresponds to middle aged in humans (15). The ischemic stroke damaged the sensorimotor cortex in all animals, which allowed for motor function assessment. Four weeks after damage motor function was measured in animals using the forepaw placement and accelerating rotarod tasks. After the completion of behavioral testing animals were euthanized and tissue was collected, including brain, liver, and blood tissue to study mechanisms. Brain tissue for cryosectioning was collected from animals, after they were perfused with phosphate buffered saline and 4% paraformaldehyde tissue. Cryosectioned brain tissue was used to quantify damage volume quantification, and immunofluorescence experiments. For one-carbon metabolite analysis in brain tissue, cortical ischemic damage and healthy tissue was micro dissected and snap frozen using dry ice.

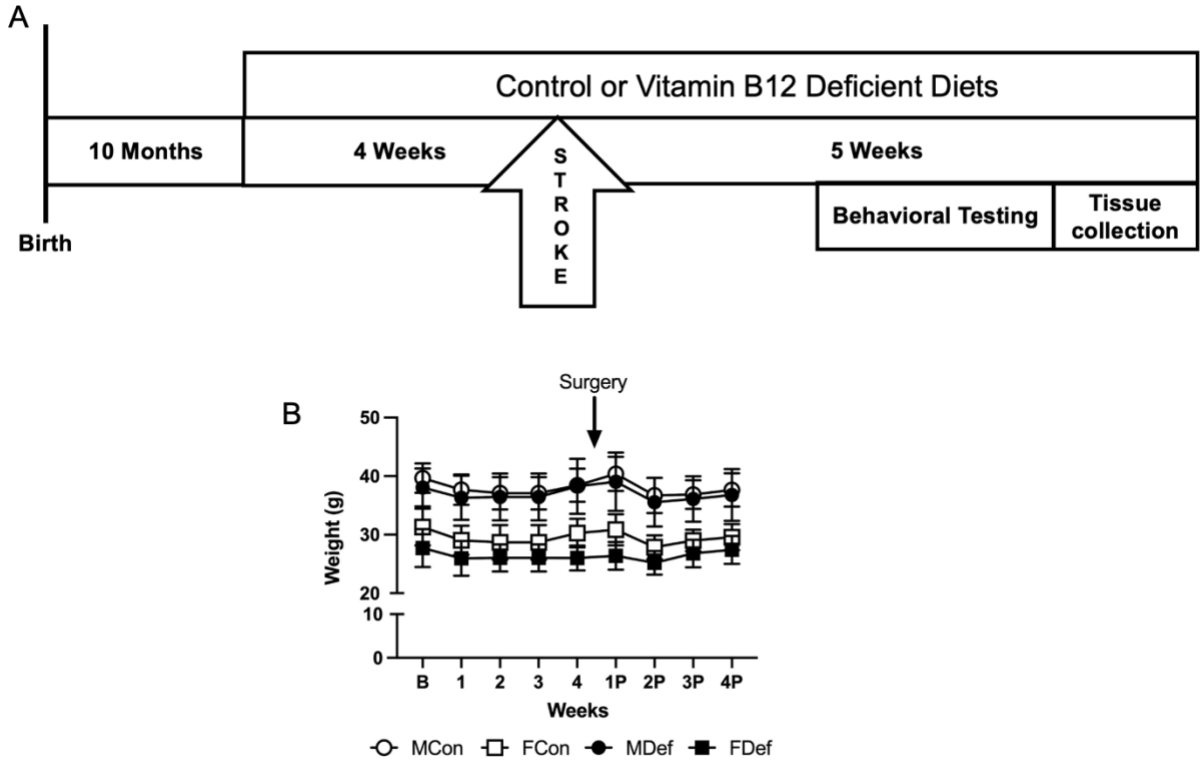

Figure 1. Timeline of experimental manipulation (A). Male and female C57Bl/6J mice arrived from Jackson Laboratories 10-months-old, and after one week of acclimation, animals were placed on a control or vitamin B12 deficient diet for four weeks. Following the four weeks, ischemic stroke was induced using the Photothrombosis model in the sensorimotor cortex. After stroke animals were maintained on respective diets for 4 additional weeks, after which motor function of the mice was measured using the accelerating rotarod and forepaw placement tasks. At the completion of *in vivo* experiments animals were euthanized, and brain and liver tissue, as well as plasma was collected for further analysis. Weekly body weights of male and female mice maintained on control and vitamin B12 deficient diets (B). Depicted are means of $\pm$ SEM of 10 to 11 mice per group. Abbreviations MCon, male control; FCon, female control; MDef, male deficient, FDec, female deficient.

Diet

The mice were placed on a vitamin B12 deficient (0 mg/kg) or a control (0.025 mg/kg vitamin B12) diet four weeks prior to photothrombosis damage and four weeks post photothrombosis damage. The diets were formulated by and purchased from Envigo (Table 1). The control diet (TD. 190790) contains the recommended dietary amount of nutrients for mice (16). The vitamin B12 deficient diet (TD. 190791) was formulated based on a previous study in

mice that has shown it to be safe and have no negative side effects (17). The mice had *ad libitum* access to food and water throughout the experiment. Body weights of each animal were recorded weekly. There were four experimental groups control male (MCon) and female (FCon); vitamin B12 deficient male (MDef) and female (FDef).

Table 1. Composition of diets

Category	Concentration	Category	Concentration
Amino acids	**(g/kg)**	**Lipids**	**(g/kg)**
Lysine	18	Total fat	60.0
Methionine	3.3	Saturated fat	9.0
Cystine	3.5	Monounsaturated fat	13.8
Arginine	12.1	Polyunsaturated fat	36.6
Phenylalanine	7.5	4:0 Butyric acid	0.0
Tyrosine	5.0	6:0 Caproic Acid	0.0
Histidine	4.5	8:0 Caprylic Acid	0.0
Isoleucine	8.2	10:0 Capric acid	0.0
Leucine	11.1	12:0 Lauric acid	0.0
Threonine	8.2	14:0 Myristic Acid	0.0
Tryptophan	1.8	16:0 Palmitic acid	6.6
Valine	8.2	16:1 Palmitoleic acid	0.0
Aspartic Acid	3.5	18:0 Stearic acid	2.4
Glutamic acid	40.0	18:1 Oleic acid	13.8
Alanine	3.5	18:2 Linoleic acid	31.8
Glycine	23.3	18:3 Linolenic acid	4.8
Proline	3.5	Cholesterol	8.8 mg/kg
Serine	3.5		
Fat-soluble Vitamins	**(IU/kg)**	**Minerals**	**(mg/kg)**
Vitamin A	4000	Calcium	5100
Vitamin D	1000	Phosphorus	3000

Vitamin E	75	Potassium	3600
Vitamin K	0.8	Sodium	1000
Water-soluble Vitamins	**(mg/kg)**	Chlorine	1600
Biotin	0.20	Magnesium	515
Choline	2.5	Copper	6.0
Folic acid	2.0	Iron	36.5
Niacin	30.0	Zinc	35.6
Pantothenate	16.0	Manganese	10.5
Riboflavin	6.0	Iodine	0.21
Thiamin	6.0	Selenium	0.15
Vitamin B_6	5.8	Molybdenum	0.15
Vitamin B_{12}	0.03	Chromium	1.00
Vitamin C	0.0		

List of micro- and macro-nutrient contents in Envigo control (TD.190790) and vitamin B12 deficient diet (TD.190791). The Envigo vitamin B12 deficient diet (TD.190791) was identical to the control diet (TD.190790) except it contained 0 mg/kg vitamin B12.

Photothrombosis model

Using the photothrombosis model of ischemic stroke damage as previously described (12,18). Briefly, mice were anesthetized with 4-5% isoflurane in oxygen. After anesthetization the mice had the top of their heads shaved and disinfected. Mice were then transferred to a stereotaxic apparatus (Harvard Apparatus) and maintained at 2-2.5% isoflurane. The mice were placed on a heating pad and a probe was rectally inserted to maintain a body temperature of 37°C. Prior to laser exposure, mice were intraperitoneally injected with 10 mg/kg of photoactive Rose Bengal (Sigma) followed by a 5- minute delay to allow the dye to enter circulation. Skin at the top of the head was surgically cut to expose the skull and then the sensorimotor cortex was targeted using stereotaxic coordinates (3 cm above, mediolateral + 0.24 mm from Bregma). The skull of the mice was exposed to a laser (Beta Electronics, wavelength: 532 nm) for 15 minutes. For recovery of post-operative pain, Buprenorphine was administered to all animals prior to ischemic damage.

Behavioral Testing

Accelerating Rotarod

A standard accelerating rotarod apparatus (Harvard Apparatus) was used to measure walking movements and balance previously described (12,19,20). Thirty centimeters above the ground, mice were placed on a rotating rod 3 cm in diameter and 6 cm in length in which the speed gradually increases from 4 to 60 revolutions per minute over 8 minutes. When mice fall off the rotarod, a digital sensor recorded the latency, in seconds, to fall off the accelerating rod. An average of three trials per mouse was taken with an inter trial interval of five minutes. The speed (rotations per minute) at which the animals fell off the accelerating rotarod was also measured and an average was calculated.

Forepaw placement task

To measure spontaneous forepaw usage, mice were placed in a 19 cm high, 14 cm diameter cylinder, and the placement of their forepaws on the cylinder wall during natural exploratory rearing behaviors was recorded using a digital camera for frame-by-frame analysis (12,21). During a rear, the forepaw placements were recorded as impaired, non-impaired, or both forepaws.

Total homocysteine levels

At the time of euthanization, blood was collected by cardiac puncture in EDTA coated tubes, centrifuged at 7000g for 7 minutes at 4°C to obtain plasma. Liver tissue was also removed flash frozen and samples were stored at -80°C, until time of analysis. Total homocysteine (tHcy) in plasma and liver were measured by liquid chromatography tandem mass spectrometry (LC-MS/MS) as previously described (22).

Brain tissue processing and ischemic damage volume quantification

Brain tissue was sectioned using a cryostat at 30 μm and slide mounted in serial order over three microscope slides. For ischemic damage volume brain tissue was stained with 0.1 % cresyl violet. There were six slides full of brain tissue sections of the damaged area per mouse that were used for ischemic damage quantification. ImageJ (NIH) was used to quantify ischemic damage volume by measuring the area of damaged tissue (23).

Immunofluorescence experiments

Brain tissue was used in immunofluorescence analysis to assess molecular mechanisms. Primary antibodies included, active caspase-3 (1:100, Cell Signaling Technologies) to measure apoptosis and phospho-AKT (protein kinase B) (1:100, Cell Signaling Technologies). All brain sections were stained with a marker for neuronal nuclei, NeuN (1:200, AbCam). Primary

antibodies were diluted in 0.5% Triton X and incubated with brain tissue overnight at 4°C. The next day, brain sections were incubated in Alexa Fluor 488 or 555 (Cell Signaling Technologies) and secondary antibodies were then incubated at room temperature for 2 hours and stained with 4', 6-diamidino-2-phenylindole (DAPI) (1:1000, Thermo Fisher Scientific). The staining was visualized using a microscope (Zeiss) and all images were collected at the magnification of 400X.

In brain tissue within the ischemic core region, co-localization of active caspase-3 or phospho-AKT with NeuN labelled neurons were counted and averaged per animal. Images were merged, and a positive cell was indicated by co-localization of the antibodies of interest located within a defined cell. Cells were distinguished from debris by identifying a clear cell shape and intact nuclei (indicated by DAPI and NeuN) under the microscope. All cell counts were conducted by two individuals blinded to treatment groups. Using ImageJ, the number of positive cells were counted in three brain sections per animal. For each section, three fields were analyzed. The number of positive cells were averaged for each animal.

One-carbon metabolite measurements

Snap frozen brain tissue from ischemic and non-ischemic cortex brain tissue was measured for acetylcholine, betaine, choline, glycerophosphocholine, methionine, phosphocholine, phosphatidylcholine, and sphingomyelin levels using the LC-MS method as previously reported (24).

Data analysis and statistics

All data were analyzed by two individuals that were blinded to experimental treatment groups. SPSS 27 was used to assess weight data using a repeated measures two-way ANOVA. GraphPad Prism 6.0 was used to analyze all remaining data from the study. Two-way ANOVA analysis was performed when comparing the mean measurement of both sex and dietary group for

behavioral testing, plasma tHcy measurements, lesion volume, immunofluorescence staining, and choline measurements. Significant main effects of two-way ANOVAs were followed up with Tukey's post-hoc test to adjust for multiple comparisons. All data are presented as mean ± standard error of the mean (SEM) (supplementary data, table 1). Statistical tests were performed using a significance level of 0.05.

Results

Body weight

There was no interaction between diet and sex for body weights (Figure 1B, $p = 0.18$). There was a diet main effect ($p = 0.001$), FDef animals weighed less compared to controls. There was a sex main effect ($p = 0.04$), male mice weight more compared to females.

Total homocysteine levels

In plasma, there was no interaction between diet and sex for homocysteine concentrations (p = 0.77). There was a diet main effect (Figure 3A; p = 0.0006). FDef mice had elevated levels of homocysteine compared to FCon (p = 0.05). There was no difference between male and female mice (Figure 2A; p = 0.66).

In liver tissue, there was no interaction between diet and sex in homocysteine levels ($p = 0.98$). In vitamin B12 deficient mice, liver tHcy levels were elevated compared to control diet mice

(Figure 2B, $p < 0.0001$) and there was also a sex effect (Figure 2B, $p = 0.005$). FDef mice had elevated levels of tHcy compared to FCon ($p = 0.001$).

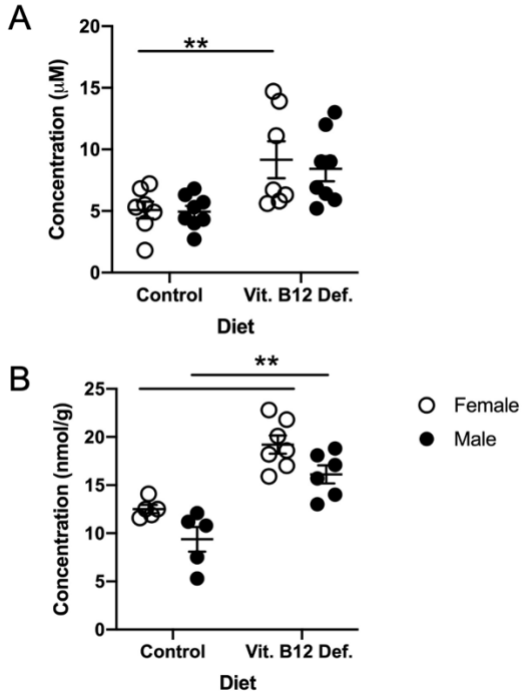

Figure 2. Impact of dietary vitamin B12 deficiency and ischemic stroke on total homocysteine levels in male and female mice. Plasma (A) and liver (B) homocysteine levels (B). Depicted are means of ± SEM of 7 to 8 mice per group. *** $p < 0.01$ between FCon and FDef or MCon and MDef.

One-Carbon Metabolite Measurements

In ischemic damaged brain tissue, there was no interactions in any brain tissue between diet and sex for any of the metabolites that were measured (Table 2). Methionine levels were decreased in vitamin B12 deficient brain tissue compared to controls (diet effect, $p = 0.02$). Betaine levels were reduced in MDef and MCon diet animals compared to females (sex main effect, $p = 0.003$; diet main effect, $p = 0.04$).

Table 2. One-carbon metabolites concentration in cortical ischemic brain tissue of male and female mice maintained on control or vitamin B12 deficient diets.[1]

Metabolites (µmol/g)	**FCon**	**MCon**	**FDef**	**MDef**	**Diet x sex**	**Diet**	**Sex**
Methionine	0.06 ± 0.13	0.09 ± 0.06	0.01 ± 0.02	0.01 ± 0.12	$p = 0.40$	$p = 0.02$ *	$p = 0.15$
Acetylcholine	0.004 ± 0.009	0.005 ± 0.0005	0.006 ± 0.00002	0.004 ± 0.003	$p = 0.18$	$p = 0.70$	$p = 0.73$
Betaine	0.02 ± 0.001	0.01 ± 0.0009	0.02 ± 0.002	0.01 ± 0.002	$p = 0.21$	$p = 0.04$ *	$p = 0.003$ **
Choline	0.07 ± 0.004	0.05 ± 0.002	0.06 ± 0.007	0.04 ± 0.007	$p = 0.06$	$p = 0.94$	$p = 0.50$
GPC	0.63 ± 0.019	0.76 ± 0.060	0.68 ± 0.03	0.77 ± 0.06	$p = 0.90$	$p = 0.93$	$p = 0.08$
Phosphocholine	0.38± 0.03	0.33 ± 0.03	0.34 ± 0.02	0.33 ± 0.01	$p = 0.37$	$p = 0.28$	$p = 0.20$
Phosphatidylcholine	34.2 ± 0.57	35.6 ± 0.57	34.7 ± 0.65	35.6 ± 0.23	$p = 0.57$	$p = 0.59$	p = 0.034
Sphingomyelin	4.27 ± 0.19	4.08 ± 0.20	4.27 ± 0.07	4.00 ± 0.02	$p = 0.80$	$p = 0.80$	$p = 0.14$

[1]Values are means ± SEMs. Abbreviations: FCon, female control; FDef, female deficient; GPC, glycerophosphocholine; MCon, male control, MDef, male deficient. * $p < 0.05$, ** $p < 0.01$, two-way ANOVA, main effects.

In the non-ischemic cortical brain tissue, there was no interactions in any brain tissue between diet and sex for any of the metabolites that were measured (Table 3). Acetylcholine levels were increased between vitamin B12 compared control diet animals (diet main effect, $p = 0.03$). Phosphocholine (sex main effect, $p = 0.01$). and sphingomyelin (sex main effect, $p = 0.001$) levels were decreased in males compared to female animals.

Table 3. One-carbon metabolites concentration in cortical non-ischemic brain tissue of male and female mice maintained on control and vitamin B12 diets.[1]

Metabolites (nmol/g)	**FCon**	**MCon**	**FDef**	**MDef**	**Diet x Sex**	**Diet**	**Sex**
Methionine	0.08 ± 0.01	0.08 ± 0.09	0.09 ± 0.06	0.11 ± 0.10	$p = 0.56$	$p = 0.08$	$p = 0.46$
Acetylcholine	0.004 ± 0.0004	0.004 ± 0.0001	0.005 ± 0.0006	0.005 ± 0.0003	$p = 0.34$	$p = 0.03$ *	$p = 0.72$
Betaine	0.026 ± 0.003	0.046 ± 0.001	0.015 ± 0.0008	0.015 ± 0.005	$p = 0.07$	$p = 0.42$	$p = 0.08$
Choline	0.07 ± 0.006	0.05 ± 0.007	0.06 ± 0.007	0.08 ± 0.005	$p = 0.06$	$p = 0.94$	$p = 0.50$
GPC	0.64 ± 0.006	0.61 ± 0.04	0.65 ± 0.01	0.69 ± 0.002	$p = 0.06$	$p = 0.16$	$p = 0.66$
Phosphocholine	0.39 ± 0.02	0.33 ± 0.02	0.35 ± 0.09	0.31 ± 0.01	$p = 0.80$	$p = 0.11$	$p = 0.01$ *
Phosphatidylcholine	34.5 ± 1.27	32.3 ± 1.18	35.2 ± 0.50	34.7 ± 0.26	$p = 0.39$	$p = 0.12$	$p = 0.17$
Sphingomyelin	4.34 ± 0.01	3.65 ± 0.04	4.52 ± 0.04	3.77 ± 0.12	$p = 0.76$	$p = 0.18$	$p = 0.001$**

[1]Values are means ± SEMs. Abbreviations: FCon, female control; FDef, female deficient; GPC, glycerophosphocholine; MCon, male control, MDef, male deficient. * $p < 0.05$, ** $p < 0.01$ two-way ANOVA.

Ischemic Damage Volume Quantification

Representative images of damage to the sensorimotor cortex are shown in Figure 3A. There was no interaction between sex and diet (Figure 3B, $p = 0.08$), diet ($p = 0.08$) or sex ($p = 0.28$) effects.

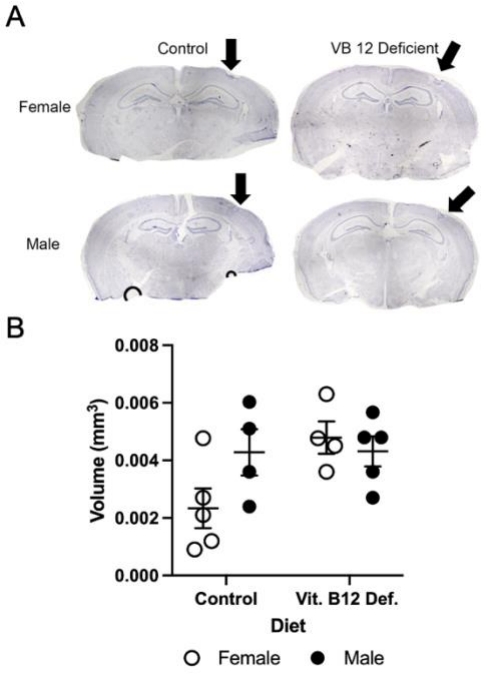

Figure 3. Impact of dietary vitamin B12 deficiency and ischemic stroke on ischemic damage volume in male and female mice. Representative cresyl violet image (A) and ischemic damage volume quantification (B). Depicted are means of ± SEM of 4 to 5 mice per group.

Brain Tissue Immunofluorescence Staining

Apoptosis

Representative images of neuronal apoptosis are shown in Figure 4A. Quantification of neuronal apoptosis revealed that there was no interaction (Figure 4B, $p = 0.17$), diet ($p = 0.05$) or sex ($p = 0.13$) effects.

Representative images of total active caspase-3 within the damage area are shown in Figure 4C. There was no interaction ($p = 0.17$) and sex main effect ($p = 0.80$). There was a main effect

of diet (Figure 5D, p = 0.02). MDef animals had more total active caspase-3 cells within the damage area (p = 0.006) compared to MCon mice.

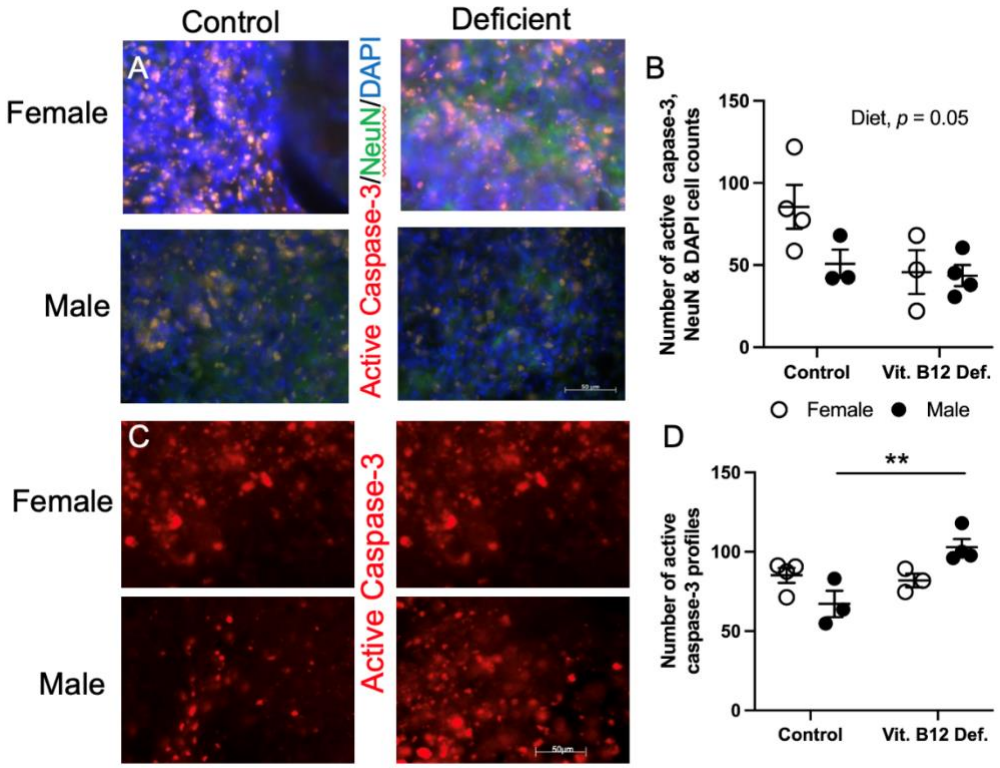

Figure 4. Impact of dietary vitamin B12 deficiency and ischemic stroke on neuronal active caspase-3 cell counts on male and female mice. Representative images of immunofluorescence staining with positive semi quantitative spatial co-localization of active caspase-3 with neuronal nuclei (NeuN) and 4′,6-diamidino-2-phenylindole (DAPI) (A). Quantification of active capsapse-3, NeuN, and DAPI cell counts (B). Depicted are means of ± SEM of 3 to 4 mice per group. The scale bar = 50 μm. *** p < 0.01 between MCon and MDef.

Neuronal Cell Survial and Proliferation

Representative images of neuronal survival and proliferation are shown in Figure 5A. There was no interaction (Figure 5B, p = 0.26) or sex effect](p = 0.86). There was a diet effect (p = 0.003), MDef animals had more positive neuronal pAKT (p = 0.05).

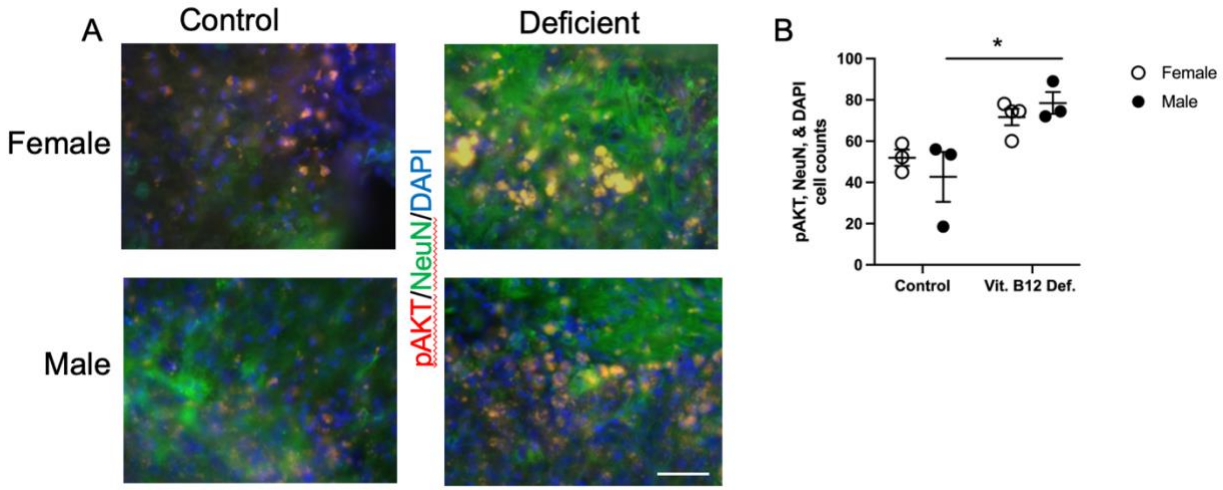

Figure 5. Impact of dietary vitamin B12 deficiency and ischemic stroke on phospho-AKT (pAKT) cell counts in male and female mouse brain tissue. Representative images of immunofluorescence staining with positive semi quantitative spatial co-localization of active caspase-3 with neuronal nuclei (NeuN) and 4′,6-diamidino-2-phenylindole (DAPI) (A). Quantification of pAKT, NeuN, and DAPI cell counts (B). Depicted are means of ± SEM of 3 to 4 mice per group. The scale bar = 50 μm. * $p < 0.05$ between MCon and MDef.

Behavioral Testing

Accelerating Rotarod

We measured the time it took for animals to fall of the accelerating rotarod (latency to fall), there was no interaction between diet and sex ($p = 0.90$) and diet groups ($p = 0.09$). There was a sex main effect (Figure 6A, $p = 0.02$). Male mice were not able to stay on the ladder beam as long as female mice (Figure 6A, $p = 0.03$).

In terms of speed (revolutions per minute; RPM) there were no interactions between diet and sex (Figure 6B, $p = 0.14$). There were differences in diet (p = 0.01) and sex ($p = 0.005$). Control diet mice achieved higher levels of RPM compared to vitamin B12 deficient mice (Figure 6B, $p = 0.01$). MCon mice achieved a higher RPM compared to MDef animals ($p = 0.021$). FCon

and MCon, as well as FDef mice achieved higher speeds compared to MDef mice (Figure 6B, p = 0.005).

Forepaw Placement

Four weeks after ischemic damage to the sensorimotor cortex, there was no interaction between diet and sex (p = 0.05), but there was a difference in impaired forepaw usage scores between diet groups (Figure 6C, p = 0.02). FCon mice used their impaired forepaw more than vitamin B12 deficient animals (p = 0.02). There was no difference between male and female animals (p = 0.18).

There was no interaction between diet and sex (p = 0.22) in non-impaired forepaw usage. There were diet (Figure 6D, p = 0.002) and a sex (p = 0.02) differences. FCon used their non-impaired forepaw more than FDef (p = 0.012) and MDef (p = 0.008).

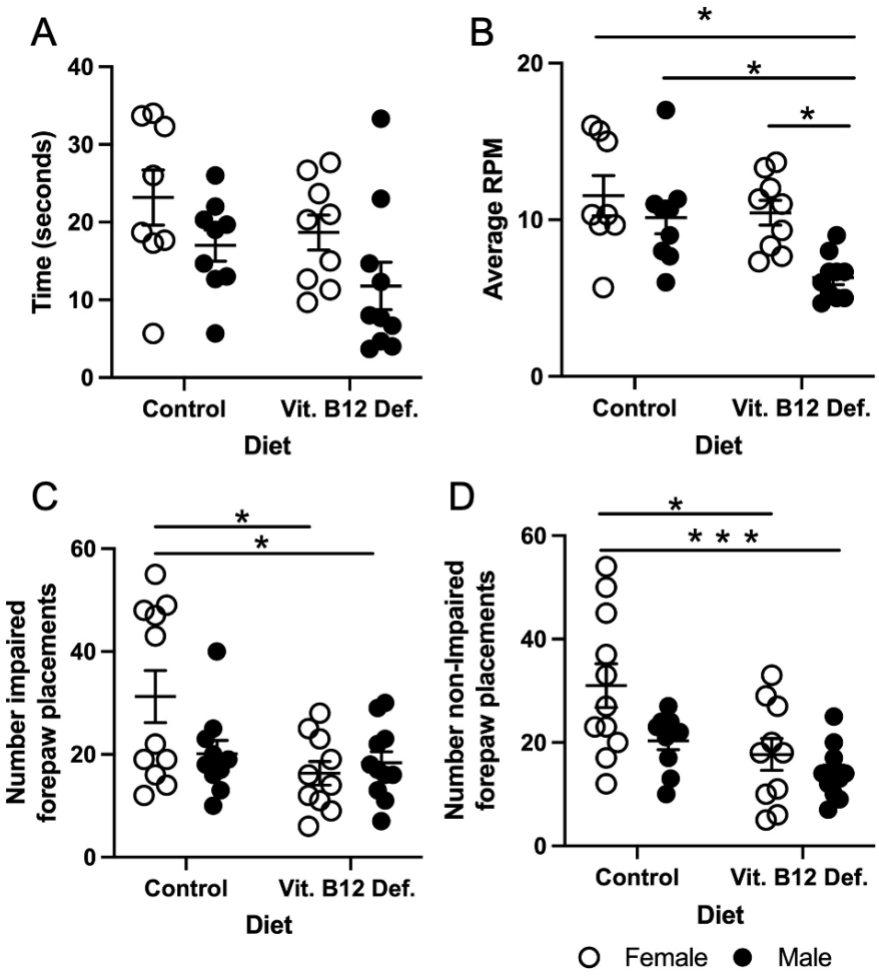

Figure 6. Impact of dietary vitamin B12 deficiency and ischemic stroke on motor function in male and female mice. Accelerating rotarod, the latency to fall (A) and revolutions per minute (B) were measured. The forepaw placement task number of impaired (A) and non-impaired (B) forepaw placements were recorded. Depicted are means of ± SEM of 10 to 11 mice per group. * $p < 0.05$ between FCon and FDef.

Discussion

Nutrition is a modifiable risk factor for stroke (25). The elderly population is at higher risk of stoke as well as a vitamin B12 deficiency (4). Furthermore, worse stroke outcome has been reported in patients with a vitamin B12 deficiency (8,9). The aim of the study was to investigate the role of vitamin B12 deficiency in ischemic stroke outcome and mechanistic changes in a mouse model. In both plasma and liver there was an increase in plasma and liver tHcy because of dietary

vitamin B12 deficiency. We measured one carbon metabolites in both healthy and ischemic brain tissue. In ischemic brain tissue methionine levels were decreased in vitamin B12 deficient animals and betaine levels were reduced in male control and vitamin B12 deficient diets. In healthy tissue acetylcholine was reduced in vitamin B12 deficient males and females. Whereas phosphocholine and sphingomyelin levels were reduced in male brain tissue. There was no difference in the damage size and neuronal apoptosis induced by the ischemic stroke between groups. There was an increase in overall apoptosis within ischemic cortical tissue. We also report increased neuronal survival in vitamin B12 deficient male and female mice. In terms of functional outcome after ischemic stroke, male mice on a vitamin B12 deficient diet showed impaired coordination and balance, as well as reduced usage of their impaired and non-impaired forepaws.

Lacunar stroke patients with vitamin B12 deficiency reported significantly worse stroke outcome, including fatigue and depressive-like symptoms (26). A common stroke outcome is impaired motor function, and the two most common motor deficits from stroke are spasticity and paresis, resulting in decreased usage of that limb. We measured forepaw usage and saw that vitamin B12 deficient male mice had significantly reduced impaired and non-impaired forepaw usage. Additionally, male vitamin B12 deficient mice showed impairments in coordination and balance using the rotarod task. Our preclinical study results are consistent with clinical observations in terms of worse outcome after ischemic stroke when patients are vitamin B12 deficient (9). It is curious that we observe reduced stroke outcome in male mice, not females.

In the present study, we did not observe any differences in damage volume between dietary groups; however, this is a gross measurement, and our analysis might have not picked up on a subtle difference in volumes. Interestingly, our immunofluorescence experiments we report a difference in in apoptosis between control and vitamin B12 deficient diet animals. In our neuronal

apoptosis data, there was a decrease in neuronal death in vitamin B12 deficient mice. This may be due to increased neuronal plasticity in the damaged area (27). However, total levels of apoptosis showed an increase in overall positive active caspase-3 levels in male vitamin B12 deficient mice. Increased levels of apoptosis have been reported in clinical studies, for example, in an autopsy cohort consisting of 13 cases of fatal ischemic stroke, TUNEL-labelled cells with apoptotic morphology were disproportionately more frequent in the ischemic core, compared percentage of the cells in the non-damaged hemisphere (28). Furthermore, in post-ischemic stroke there is elevated levels of neuronal death due to apoptosis in the damaged area of the brain (29). A potential reason there is not an increase in apoptosis in female mice is the neuroprotective effects of estrogen and progesterone (30). This finding could also reflect an increased vulnerability of glial cells, the other type of cell in the brain. Overall, these findings support clinical data being reported (9).

One-carbon metabolism incorporates several vitamins and nutrients. Other studies have shown that vitamin B12 status affects choline status in animals, these studies have reported increases in in choline levels when vitamin B12 is reduced, suggesting some sort of interaction (31,32). In the present study, we assessed levels of choline, as well as other one-carbon metabolites in ischemic and non-ischemic brain tissue from control and vitamin B12 deficient animals. We studied the impact of the stroke, diet, and sex on one-carbon metabolism. We report lower levels of betaine in ischemic brain tissue of male mice, these levels in brain tissue might be a reflection on what is occurring in liver and plasma as a result of dietary vitamin B12 deficiency. There might be a reduced availability for uptake by brain, because of low levels of vitamin B12. Additionally, our results show that within ischemic brain tissue has higher methionine levels in control diet. The increase in methionine levels might be in compensation to the dietary vitamin B12 deficiency

because of the role vitamin B12 plays in generating methionine. Brain health is dependent on methionine levels (33), there might be compensatory mechanisms in place to maintain adequate levels. In healthy brain tissue report dietary changes in acetylcholine, there are higher levels in vitamin B12 deficient animals, suggesting that there is a close interaction between choline and vitamin B12 metabolism (31,32). We have previously shown that folate, another B-vitamin and choline metabolism are tightly linked in healthy brain tissue (20,34,35). Additionally, it is important to note that there is a sex difference in phosphocholine and sphingomyelin levels in healthy brain tissue. Sex differences in one carbon metabolism have been reported by our group (36), as well as others (37–39).

This study investigated sex differences in response to ischemic stroke between dietary groups. This is an important component of preclinical studies as there have been a low number of studies using female mice (40,41). In our study, male and female mice maintained on vitamin B12 deficiency had impaired stroke outcome. Males and females had impairments on different motor tasks, males consistently showed worse outcome. In tissue, male mice showed more total apoptosis, however in our present study our data shows both males and females had increased neuronal survival within damage region in brain tissue. This is an interesting finding considering research has shown that men are more susceptible to a vitamin B12 deficiency (42) . The results of our study emphasizes the importance of studying of sex differences in preclinical stroke experiments and the possibility of individualized medicine (43).

Conclusion

The data presented in this study provides evidence that a vitamin B12 deficiency impacts stroke outcome differently in older adult male and female mice. The mechanisms driving this

change may be a result of increased apoptosis, changes in neuronal survival, and compensation in choline metabolism within the damaged brain tissue.

Acknowledgments

The authors would like to acknowledge the Chelsea Adamson for her assistance in photothrombosis surgeries.

Statement of authors' contributions to manuscript

Conceptualization, NMJ; Data curation, GBY, BW, TB, OM, MAC, NMJ; Formal analysis, GBY, TB, MAC, NMJ; Funding acquisition, NMJ; Investigation; Methodology, GBY, BW, TB, OM, MAC, NMJ; Supervision, NMJ; Visualization; Roles/Writing - original draft, GBY and NMJ; Writing - review & editing, GBY, TB, MAC, NMJ. N.M.J. had primary responsibility for final content. All authors read and approved the final manuscript.

References

1. Spence JD. Nutrition and risk of stroke. Nutrients 2019;11.

2. Langan RC, Goodbred AJ. Vitamin B12 Deficiency: Recognition and Management. Am Fam Physician 2017;96:384–9.

3. Spence JD. Metabolic B12 deficiency: A missed opportunity to prevent dementia and stroke. Nutrition Research Elsevier Inc.; 2016;36:109–16.

4. Andrès E, Loukili NH, Noel E, Kaltenbach G, Abdelgheni MB, Perrin AE, Noblet-Dick M, Maloisel F, Schlienger J-L, Blicklé J-F. Vitamin B12 (cobalamin) deficiency in elderly patients. CMAJ 2004;171:251–9.

5. Castro R, Rivera I, Blom HJ, Jakobs C, de Almeida IT. Homocysteine metabolism, hyperhomocysteinaemia and vascular disease: An overview. J Inherit Metab Dis 2006;29:3–20.

6. Spence JD. Homocysteine-lowering therapy: a role in stroke prevention? Lancet neurology 2007;6:830–8.

7. Spence JD, Stampfer MJ. Understanding the complexity of homocysteine lowering with vitamins: The potential role of subgroup analyses. JAMA - Journal of the American Medical Association 2011;306:2610–1.

8. Ahmed S, Bogiatzi C, Hackam DG, Rutledge AC, Sposato LA, Khaw A, Mandzia J, Azarpazhoo MR, Hachinski V, Spence JD. Vitamin B 12 deficiency and hyperhomocysteinaemia in outpatients with stroke or transient ischaemic attack: A cohort study at an academic medical centre. BMJ Open 2019;9:1–8.

9. Yahn G, Abato J, Jadavji N. Role of vitamin B12 deficiency in ischemic stroke risk and outcome. Neural Regeneration Research 2021;16.

10. Abato JE, Moftah M, Cron GO, Smith PD, Jadavji NM. Methylenetetrahydrofolate reductase deficiency alters cellular response after ischemic stroke in male mice. Nutritional Neuroscience [Internet] Taylor and Francis Ltd.; 2020; Available from: https://pubmed.ncbi.nlm.nih.gov/32448097/

11. Jadavji N, Emmerson JT, Shanmugalingam U, Willmore WG, Macfarlane AJ, Smith PD. A genetic deficiency in folic acid metabolism impairs recovery after ischemic stroke. Experimental neurology 2018;309:14–22.

12. Jadavji NM, Emmerson J, Willmore WG, MacFarlane AJ, Smith P. B-vitamin and choline supplementation increases neuroplasticity and recovery after stroke. Neurobiology of Disease 2017;103:89–100.

13. Labat-gest V, Tomasi S. Photothrombotic ischemia: a minimally invasive and reproducible photochemical cortical lesion model for mouse stroke studies. Journal of visualized experiments : JoVE 2013;e50370–e50370.

14. Lee J-K, Kim J-E, Sivula M, Strittmatter SM. Nogo receptor antagonism promotes stroke recovery by enhancing axonal plasticity. The Journal of neuroscience : the official journal of the Society for Neuroscience 2004;24:6209–17.

15. Flurkey K, Currer JM, Harrison DE. Mouse Models in Aging Research. In: Fox J, Barthold S, Davisson M, Newcommer C, Quimby F, Smith A, editors. The-Mouse-in-Biomedical-Research, Normative Biology, Husbandry, and Models. [Internet] 2nd ed. Elsevier.; 2007. p. 637–72. Available from: https://mouseion.jax.org/stfb2000_2009/1685

16. Reeves PG. Components of the AIN-93 Diets as Improvements in the AIN-76A Diet. The Journal of Nutrition 1997;127:838S-841S.

17. Bernard DJ, Pangilinan FJ, Cheng J, Molloy AM, Brody LC. Mice lacking the transcobalamin-vitamin B12 receptor, CD320, suffer from anemia and reproductive deficits when fed vitamin B12-deficient diet. Human Molecular Genetics 2018;27:3627–40.

18. Jadavji NM, Mosnier H, Kelly E, Lawrence K, Cruickshank S, Stacey S, McCall A, Dhatt S, Arning E, Bottiglieri T, et al. One-carbon metabolism supplementation improves outcome after stroke in aged male MTHFR-deficient mice. Neurobiology of Disease 2019;132.

19. Balkaya M, Kröber J, Gertz K, Peruzzaro S, Endres M. Characterization of long-term functional outcome in a murine model of mild brain ischemia. Journal of neuroscience methods 2013;213:179–87.

20. Jadavji NM, Farr T, Khalil A, Boehm-Sturm P, Foddis M, Harms C, Füchtemeier M, Dirnagl U. Elevated levels of plasma homocysteine, deficiencies in dietary folic acid and uracil-DNA glycosylase impair learning in a mouse model of vascular cognitive impairment. Behavioural brain research 2015;283:215–26.

21. Theoret JK, Jadavji NM, Zhang M, Smith PD. Granulocyte macrophage colony-stimulating factor treatment results in recovery of motor function after white matter damage in mice. European Journal of Neuroscience 2016;43.

22. Ducros V, Belva-Besnet H, Casetta B, Favier A. A robust liquid chromatography tandem mass spectrometry method for total plasma homocysteine determination in clinical practice. Clinical Chemistry and Laboratory Medicine (CCLM) 2006;44:987–90.

23. Jensen EC. Quantitative Analysis of Histological Staining and Fluorescence Using ImageJ. The Anatomical Record John Wiley & Sons, Ltd; 2013;296:378–81.

24. Koc H, Mar MH, Ranasinghe A, Swenberg JA, Zeisel SH. Quantitation of choline and its metabolites in tissues and foods by liquid chromatography/electrospray ionization-isotope dilution mass spectrometry. Analytical Chemistry 2002;74:4734–40.

25. Spence JD. Stroke Nutrition in Stroke Prevention. 2017;45–51.

26. Huijts M, Duits A, Staals J, van Oostenbrugge RJ. Association of vitamin B12 deficiency with fatigue and depression after lacunar stroke. PLoS One 2012;7:e30519.

27. Cirillo C, Brihmat N, Castel-Lacanal E, Le Friec A, Barbieux-Guillot M, Raposo N, Pariente J, Viguier A, Simonetta-Moreau M, Albucher J-F, et al. Post-stroke remodeling processes in animal models and humans. J Cereb Blood Flow Metab 2020;40:3–22.

28. Sairanen T, Karjalainen-Lindsberg M-L, Paetau A, Ijäs P, Lindsberg PJ. Apoptosis dominant in the periinfarct area of human ischaemic stroke--a possible target of antiapoptotic treatments. Brain 2006;129:189–99.

29. Uzdensky AB. Photothrombotic Stroke as a Model of Ischemic Stroke. Translational Stroke Research Springer US; 2018;9:437–51.

30. Kim T-H, Vemuganti R. Effect of sex and age interactions on functional outcome after stroke. CNS Neurosci Ther 2015;21:327–36.

31. Compher C, Kinosian B, Stoner N, Lentine D, Buzby G. Choline and vitamin B12 deficiencies are interrelated in folate-replete long-term total parenteral nutrition patients. JPEN J Parenter Enteral Nutr 2002;26:57–62.

32. Hegsted DM, Roach AM, McCombs HL. Effect of Vitamin B12 in Choline Deficiency in the Rat. The Journal of Nutrition 1967;92:403–11.

33. Selhub J, Bagley LC, Miller J, Rosenberg IH. B vitamins, homocysteine, and neurocognitive function in the elderly. The American journal of clinical nutrition 2000;71:614S-620S.

34. Jadavji NM, Deng L, Leclerc D, Malysheva O, Bedell BJ, Caudill MA, Rozen R. Severe methylenetetrahydrofolate reductase deficiency in mice results in behavioral anomalies with morphological and biochemical changes in hippocampus. Molecular genetics and metabolism 2012;106:149–59.

35. Dam K, Füchtemeier M, Farr TD, Boehm-Sturm P, Foddis M, Dirnagl U, Malysheva O, Caudill MA, Jadavji NM. Increased homocysteine levels impair reference memory and reduce cortical levels of acetylcholine in a mouse model of vascular cognitive impairment. Behavioural Brain Research 2017;321.

36. Bennett C, Green J, Ciancio M, Goral J, Pitstick L, Pytynia M, Meyer A, Kwatra N, Jadavji NM. Dietary folic acid deficiency impacts hippocampal morphology and cortical acetylcholine metabolism in adult male and female mice. Nutritional Neuroscience 2021;1–9.

37. Levav-Rabkin T, Blumkin E, Galron D, Golan HM. Sex-dependent behavioral effects of Mthfr deficiency and neonatal GABA potentiation in mice. Behavioural Brain Research Elsevier B.V.; 2011;216:505–13.

38. Sadre-Marandi F, Dahdoul T, Reed MC, Nijhout HF. Sex differences in hepatic one-carbon metabolism. BMC Systems Biology [Internet] BioMed Central Ltd.; 2018;12. Available from: https://pubmed.ncbi.nlm.nih.gov/30355281/

39. Blumkin E, Levav-Rabkin T, Melamed O, Galron D, Golan HM. Gender-Specific Effect of Mthfr Genotype and Neonatal Vigabatrin Interaction on Synaptic Proteins in Mouse Cortex. Neuropsychopharmacol 2011;36:1714–28.

40. Persky RW, Turtzo LC, McCullough LD. Stroke in women: Disparities and outcomes. Current Cardiology Reports 2010;12:6–13.

41. Singh D, Wasan H, Reeta KH. Preclinical Stroke Research and Translational Failure: A Bird's Eye View on Preventable Variables. Cell Mol Neurobiol 2021;

42. Margalit I, Cohen E, Goldberg E, Krause I. Vitamin B12 Deficiency and the Role of Gender: A Cross-Sectional Study of a Large Cohort. Ann Nutr Metab 2018;72:265–71.

43. Bushnell CD, Chaturvedi S, Gage KR, Herson PS, Hurn PD, Jiménez MC, Kittner SJ, Madsen TE, McCullough LD, McDermott M, et al. Sex differences in stroke: Challenges and opportunities. J Cereb Blood Flow Metab 2018;38:2179–91.

Supplementary file

Table 1. Mean and standard error of homocysteine levels, ischemic damage volume, apoptosis, pAKT, and behavioral data[1].

Measurements	FCon	MCon	FDef	MDef
Plasma homocysteine (uM)	5.07 ± 0.68	4.94 ± 0.48	9.16 ± 1.51	8.43 ± 1.01
Liver homocysteine (nmol/g)	12.5 ± 0.43	9.38 ± 1.28	19.2 ± 0.95	16.1 ± 0.94
Ischemic damage volume (mm^3)	0.002 ± 0.0007	0.004 ± 0.0008	0.005 ± 0.0006	0.004 ± 0.0005
Number of active-caspase-3, NeuN, and DAPI cell counts	85.5 ±13.3	50.9 ± 8.58	45.7 ± 13.3	43.6 ± 6.39
Number of active-caspase-3 profiles	85.2 ± 4.70	67.2 ± 8.32	81.9 ± 4.23	103 ± 5.10
Accelerating Rotarod Latency to Fall (seconds)	23.2 ± 3.56	17.0 ± 2.03	18.9 ± 2.24	11.8 ± 3.04
Accelerating Rotarod Rotations per Minute	11.5 ± 1.30	10.1 ± 1.05	10.4 ± 0.79	6.3 ± 0.44
Forepaw Placement Number of impaired forepaw placements	31.3 ± 5.01	20.1 ± 2.61	16.3 ± 2.3	18.4 ± 2.20
Forepaw Placement Number of non-impaired	31.0 ± 4.21	17.70 ± 3.10	20.3 ±1.69	14.1 ±1.54

[1]Values are means ± SEMs. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole, FCon, female control; FDef, female deficient; MCon, male control, MDef, male deficient; NeuN, neuronal nuclei.