

## **A dietary vitamin B12 deficiency impairs motor function and changes neuronal survival and choline metabolism after ischemic stroke to the sensorimotor cortex in middle aged male and female mice**

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## Abstract

The majority of the world's population is growing older, in 2000, 10% of the total population of the world was over 60 years old and the proportion is projected to increase to 21% by 2050. Currently, ischemic stroke predominately affects the elderly. Nutrition is a modifiable risk factor for stroke, as people age their ability to absorb some nutrients decreases. A primary example is vitamin B12, most older adults are deficient in vitamin B12 because of changes in breakdown and absorption of the vitamin that take place during the aging process. Using a mouse model system, we investigated the role of vitamin B12 deficiency in ischemic stroke outcome and investigate mechanistic changes in ischemic versus non-ischemic brain tissue. At 10-weeks of age male and female C57Bl/6J mice were put on control or vitamin B12 deficient diets for 4-weeks prior to ischemic damage. At 14 weeks of age, we induced ischemic stroke in the sensorimotor cortex using the photothrombosis model. Animals were continued on diets for 4 weeks after damage. At 18 weeks of age, we assessed stroke outcome using the accelerating rotarod and forepaw placement tasks. After the collection of behavioral data, we euthanized animals and collected brain, blood, and liver tissues to assess histological and biochemical measurements. All animals maintained on the vitamin B12 deficient diet had increased levels of total homocysteine in plasma and liver tissue. Male and female mice maintained on a vitamin B12 deficient diet had impairments in balance and coordination on the accelerating rotarod compared to control diet animals after ischemic stroke. In ischemic brain tissue no difference between groups in lesion volume was observed. More neuronal survival was present in ischemic brain tissue of the vitamin B12 deficient group compared to controls. There were changes in choline metabolites in ischemic brain tissue as a result of diet and sex. In conclusion, the data presented in this study confirms that a vitamin B12 deficiency impacts

motor function in older adult male and female mice after ischemic stroke. The mechanisms driving this change may be a result of neuronal survival and compensation in choline metabolism within the damaged brain tissue.

### **Keywords**

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

### **Abbreviations**

Total homocysteine; tHcy, neuronal nuclei; NeuN, 4',6-diamidino-2-phenylindole; DAPI

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## Introduction

A large complication in public health today is overall health and diet. It is known that eating healthy is important for good health and can reduce the risk of diseases such as stroke (Spence, 2019). It is becoming increasingly popular to partake in a vegetarian or vegan diet, for many reasons. These kinds of diets eliminate meat, which is one of the main sources of our daily required B vitamins and are extremely important for maintaining good health and everyday functioning. Vitamin B12 is also referred to as cobalamin and is a water-soluble vitamin found naturally in foods such as red meat, poultry, eggs, dairy products, seafood, and fortified cereals (Langan and Goodbred, 2017). Vitamin B12 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 (Spence, 2016). It can affect all age ranges, however it has a much higher prevalence within the elderly population (Andrès et al., 2004; Spence, 2019).

In elderly patients, a vitamin B12 deficiency is commonly caused by malabsorption, decreased acid secretion, and reduced intrinsic factor production (Andrès et al., 2004). Having a deficiency in vitamin B12 results in a multitude of problems and can exacerbate outstanding conditions or developing conditions. The recommended daily amount of vitamin B12 is 2.4  $\mu\text{g}$  and the average daily diet contains a range of 3-30  $\mu\text{g}$  (Moridani and Ben-Poorat, 2006). Interestingly, only 50% of patients who are vitamin B12 deficient present with low levels of serum vitamin B12, resulting in a substantial number of undiagnosed patients (Langan and Goodbred, 2017). Clinical manifestations can be set back due to the high amounts of hepatic storage of vitamin B12 (Moridani and Ben-Poorat, 2006).

Adequate levels of vitamin B12 are needed for successful aging (Vogiatzoglou et al., 2008). 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 (Spence, 2007; Spence and Stampfer, 2011). Clinical data strongly suggest that low levels of vitamin B12 are a risk factor for ischemic stroke and also impacts stroke outcome (Ahmed et al., 2019; Yahn et al., 2021). There is a gap in the understanding of the mechanisms in which a vitamin deficiency leads to increased risk of stroke. This study aims to assess the effects of a vitamin B12 deficiency on stroke outcome and pathology.

## **Materials and methods**

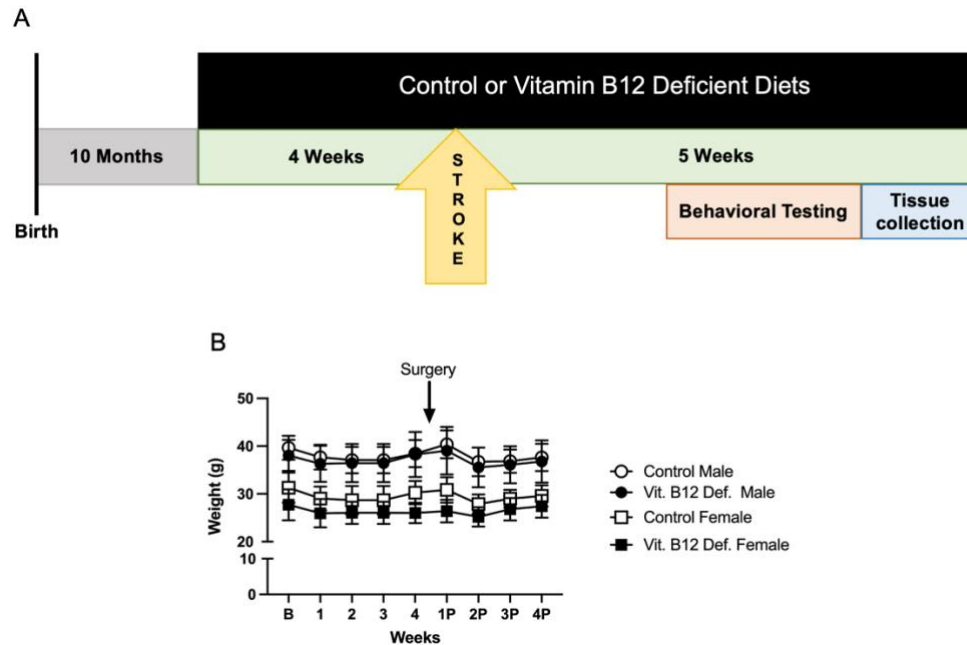
### **Animals**

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 1. After mice were habituated to the Midwestern University animal facility, 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 (Abato et al., 2020; Jadavji et al., 2018, 2017; Labat-gest and Tomasi, 2013; Lee et al., 2004) this corresponds to middle aged in humans (Flurkey et al., 2007). The ischemic stroke damaged the sensorimotor cortex, which allows 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.



**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 animals maintained on control and vitamin B12 deficient diets (B).

## 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. The control diet contains the recommended dietary amount of nutrients for mice (Reeves, 1997). The vitamin B12 deficient diet was formulated based on a previous study in mice that has shown it to be safe and have no negative side effects (Bernard et al., 2018). The mice had *ad libitum* access to food and water throughout the experiment. Body weights were recorded weekly.

### Photothrombosis Model

Using the photothrombosis model of ischemic stroke damage, mice were anesthetized with 4-5% isoflurane in oxygen. After anesthetization the mice had the top of their heads shaved and disinfected. Tear gel was used to prevent their eyes from drying out during while anesthetized. 0.03 mg/kg of Buprenorphine and one mL of saline were administered subcutaneously. 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). While keeping moist with saline, 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 (Balkaya et al., 2013; Jadavji et al., 2017, 2015). Thirty centimetres above the ground, mice were placed on a rotating rod 3 cm in diameter and 6 cm wide 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.

### *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 (Jadavji et al., 2017; Theoret et al., 2016). During a rear, the first forepaw placement on the wall was recorded as impaired, non-impaired, or both.



## Total Homocysteine Levels in Plasma and Liver

At the time of euthanization, blood was collected by cardiac puncture in EDTA coated tubes, centrifuged at 7000g for 7 minutes at 4°C degrees to obtain plasma. Liver tissue was also removed at the same time 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 (Ducros et al., 2006).

## Brain Tissue Sectioning

Brain tissue was sectioned using a cryostat at 30 µm and slide mounted in serial order. There were six slides full of brain tissue sections of the damaged area per mouse and each animal had a minimum of four sections that were used for quantification. ImageJ (NIH) was used to quantify ischemic damage volume by measuring the area of damaged tissue (Jensen, 2013).

## Immunofluorescence Experiments

Brain tissue was used in immunofluorescence analysis to assess molecular mechanisms and brain tissue staining was performed to investigate potential 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 stains were analyzed using a microscope (Zeiss) and all images were collected at the magnification of 40X.

In brain tissue within the ischemic region, co-localization of active caspase-3 or phospho-AKT with NeuN labelled neurons were counted and averaged per animal. 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. 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.

#### *Choline metabolites measurements*

Frozen brain tissue from ischemic and non-ischemic cortex was measured for acetylcholine, betaine, choline, glycerophosphocholine, phosphocholine, phosphatidylcholine, and sphingomyelin levels using the LC-MS method as previously reported (Koc et al., 2002).

#### *Statistics*

All data were analyzed by two individuals that were blinded to experimental treatment groups. GraphPad Prism 6.0 was used to analyze all 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  $\pm$  standard error of the mean (SEM). Statistical tests were performed using a significance level of 0.05.

## **Results**

### **Body weight**

To assess health animals were weighed weekly. There was difference between male and females in body weight (Figure 1B ( $F_{(1,38)} = 4.63$ ,  $p = 0.001$ ). There was no change in body weight between groups prior to or after photothrombosis damage (Figure 1B,  $F_{(1,33)} = 45.5$ ,  $p = 0.27$ ).

## Behavioral Testing

### *Accelerating Rotarod*

Four weeks after ischemic damage to the sensorimotor cortex, balance and coordination were measured via the accelerating rotarod task (Balkaya et al., 2013). We measured the latency to fall, there was no difference between diet groups (**Figure 2A**;  $F_{(1,32)} = 3.06$ ,  $p = 0.09$ ). Female mice stayed on the rotarod longer compared to male mice (**Figure 2A**;  $F_{(1,32)} = 5.52$ ,  $p = 0.025$ ).

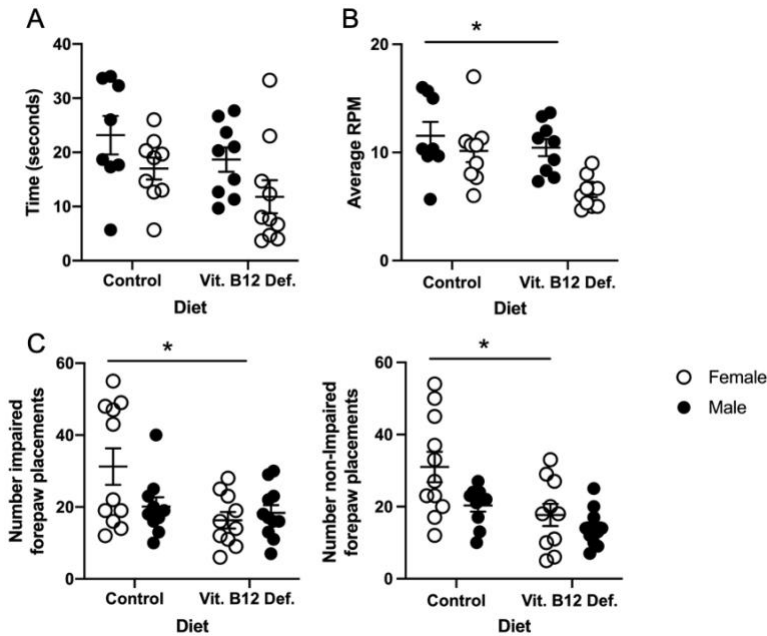
The revolutions per minute reached by each animal were also measured during the accelerating rotarod task. Control diet mice achieved higher levels of speed compared to vitamin B12 deficient mice (Figure 2B;  $F_{(1,32)} = 7.44$ ,  $p = 0.010$ ). Male control mice were able to achieve a higher RPM compared to vitamin B12 deficient animals (Tukey's pairwise,  $p = 0.021$ ). Female mice achieved higher speeds compared to male mice (Figure 2B;  $F_{(1,32)} = 9.33$ ,  $p = 0.0045$ ).

### *Forepaw Placement*

The forepaw placement task evaluated forepaw usage through natural rearing behaviors of rodents (Theoret et al., 2016). Four weeks after ischemic damage, there were differences in impaired forepaw usage scores between control and vitamin B12 deficient mice (Figure 2C;  $F_{(1,38)} = 6.27$ ,  $p = 0.017$ ). Female control mice used their impaired forepaw more than vitamin B12 deficient animals (Tukey's pairwise,  $p = 0.015$ ). There was no sex difference ( $F_{(1,38)} = 1.86$ ,  $p = 0.18$ )

There was a diet difference in non-impaired forepaw usage scores between control and vitamin B12 deficient mice (Figure 2D;  $F_{(1,38)} = 11.41$ ,  $p = 0.002$ , diet effect) and sex difference

( $F_{(1,38)} = 6.136$ ,  $p = 0.018$ ). Female control mice used their non-impaired forepaw more than vitamin B12 deficient animals (Tukey's pairwise comparison,  $p = 0.012$ ).



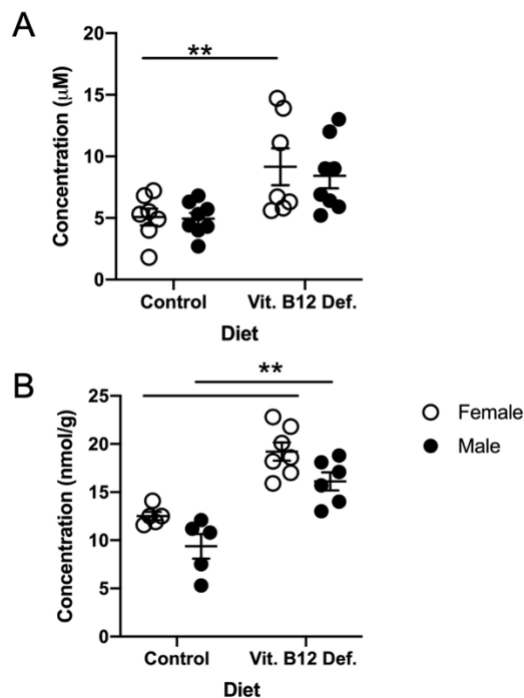
**Figure 2.** Impact of dietary vitamin B12 deficiency and ischemic stroke on motor function. Coordination and balance were assessed using the accelerating rotarod behavioral task. The latency to fall (A) and revolutions per minute (B) were measured. The forepaw placement task was used to measure forepaw usage. The number of impaired (A) and non-impaired (B) forepaw placements were recorded. Depicted are means of  $\pm$  SEM of 10 to 11 mice per group. Tukey's pairwise comparison \*  $p < 0.01$  between indicated groups.

### Total Homocysteine Levels

To confirm vitamin B12 deficiency, total homocysteine (tHcy) levels in the plasma and liver were measured. In plasma, vitamin B12 deficient mice, plasma homocysteine levels were elevated compared to control diet mice (Figure 3A;  $F_{(1,26)} = 15.05$ ,  $p = 0.0006$ ). Female vitamin B12 deficient mice had elevated levels of homocysteine compared to control animals (Tukey's

pairwise comparison,  $p = 0.048$ ). There was no difference between male and female mice (Figure 3A;  $F_{(1,26)} = 0.20$ ,  $p = 0.66$ ).

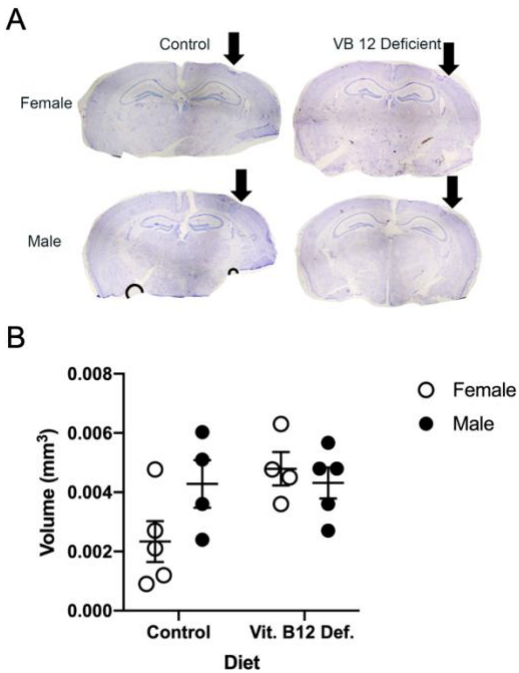
In vitamin B12 deficient mice, liver tHcy levels were elevated compared to control diet mice (Figure 3B;  $F_{(1,19)} = 47.86$ ,  $p < 0.0001$ ) and there was also a sex effect (Figure 3B;  $F_{(1,19)} = 10.30$ ,  $p = 0.005$ ). Female vitamin B12 deficient mice had elevated levels of tHcy compared to control animals (Tukey's pairwise comparison,  $p = 0.001$ ).



**Figure 3.** Impact of dietary vitamin B12 deficiency and ischemic stroke on total homocysteine levels. Plasma (A) and liver (B) homocysteine levels (B). Depicted are means of  $\pm$  SEM of 7 to 8 mice per group. \*\*\*  $p < 0.01$  between indicated groups.

### Ischemic Damage Volume Quantification

Ischemic brain damage to the sensorimotor cortex was measured. Representative images of brain tissue are shown in **Figure 4A**. There was no diet effect (Figure 4B;  $F_{(1,14)} = 3.65$ ,  $p = 0.077$ ) or sex effect ( $F_{(1,14)} = 1.27$ ,  $p = 0.28$ ).



**Figure 4.** Impact of dietary vitamin B12 deficiency and ischemic stroke on damage volume. Representative cresyl violet image (A) and ischemic damage volume quantification (B). Depicted are means of  $\pm$  SEM of 4 to 5 mice per group.

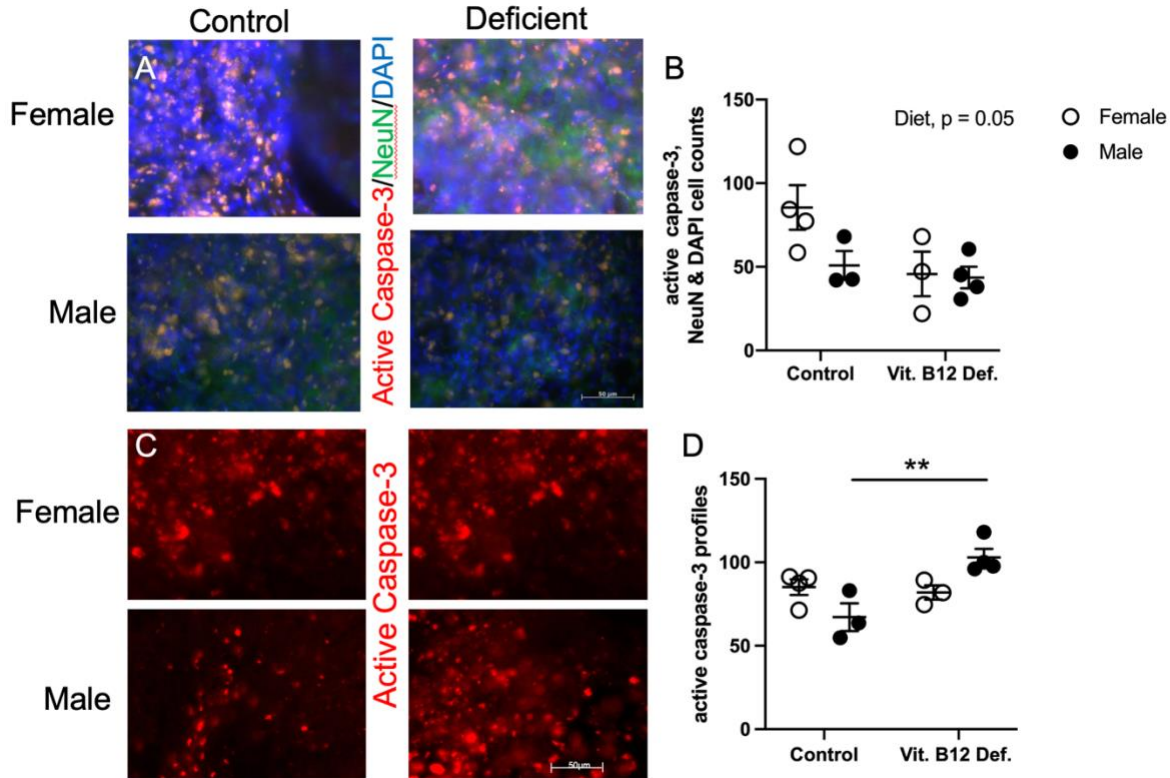
## Brain Tissue Immunofluorescence Staining

### *Neuronal Apoptosis*

Neuronal apoptosis was assessed within the damage area using active caspase-3 and neuronal nuclei (NeuN), a marker of neuronal nuclei immunofluorescence experiments. Representative images are shown in Figure 5A. Quantification of neuronal apoptosis revealed that there was no diet (Figure 5B;  $F_{(1,10)} = 4.60$ ,  $p = 0.056$ ) or sex ( $F_{(1,10)} = 2.81$ ,  $p = 0.13$ ) effects.

Evaluation of total active caspase-3 within the damage area was compiled by counting the number of antibody active caspase 3 positive profiles. Representative images are shown in Figure 5C. There was a main effect of diet (Figure 5D;  $F_{(1,10)} = 8.28$ ,  $p = 0.017$ ), but no effect of sex ( $F$

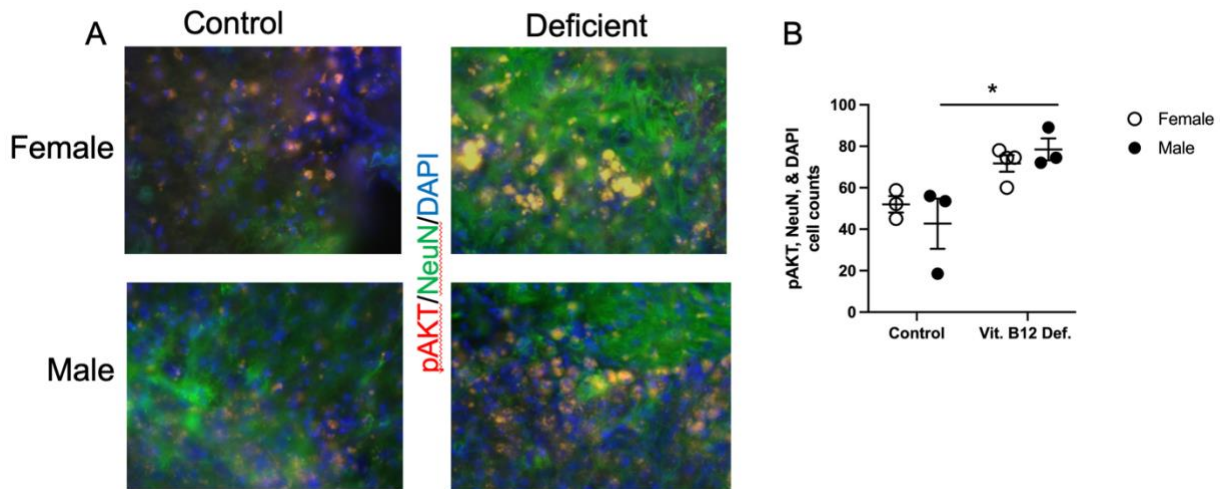
(1, 10) = 0.070,  $p = 0.80$ ). Male vitamin B12 deficient animals had more total active caspase-3 cells within the damage area (Tukey's pairwise comparison,  $p = 0.0055$ ).



**Figure 5.** Impact of dietary vitamin B12 deficiency and ischemic stroke on neuronal active caspase-3 cell counts. 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 caspase-3, NeuN, and DAPI cell counts (B). Depicted are means of  $\pm$  SEM of 3 to 4 mice per group. The scale bar = 50  $\mu$ m.

### Neuronal Cell Survival and Proliferation

Neuronal survival and proliferation were measured using colocalization of pAKT and NeuN. Representative images are shown in Figure 6A. Vitamin B12 deficient animals had more positive neuronal pAKT (Figure 6B,  $F(1,9) = 16.6$ ,  $p = 0.0028$ ), but no difference between male and females ( $F(1,9) = 0.03$ ,  $p = 0.856$ ).



**Figure 6.** Impact of dietary vitamin B12 deficiency and ischemic stroke on phospho-AKT (pAKT) cell counts. 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  $\pm$  SEM of 3 to 4 mice per group. The scale bar = 50  $\mu$ m.

### Choline Metabolite Measurements

A dietary vitamin B12 deficiency has been linked to changes in choline metabolism (Compher et al., 2002; Hegsted et al., 1967). Offspring choline metabolites were measured in brain tissue with ischemic (Table 1) and non-ischemic (Table 2) brain tissue.

**Table 1.** Choline metabolite concentration in cortical damaged hemisphere of male and female mice maintained on control and vitamin B12 diets.

Metabolites (nmol/g)	Control diet		Vitamin B12 Deficient diet		Effect	p-value
	Female	Male	Female	Male		
Methionine	62.91 $\pm$ 13.12	85.66 $\pm$ 5.83	97.00 $\pm$ 2.03	103.31 $\pm$ 11.79	Diet	0.0232
Acetylcholine	3.90 $\pm$ 0.92	4.92 $\pm$ 5.08	5.64 $\pm$ 1.59	3.95 $\pm$ 0.34		
Betaine	20.62 $\pm$ 1.10	13.15 $\pm$ 0.94	15.35 $\pm$ 1.63	11.61 $\pm$ 1.63	Sex and diet	0.0366; 0.0033
Choline	67.47 $\pm$ 4.11	51.44 $\pm$ 2.29	55.67 $\pm$ 6.89	64.05 $\pm$ 7.02		
GPC	679.61 $\pm$ 18.52	756.71 $\pm$ 60.35	676.34 $\pm$ 24.74	756.71 $\pm$ 60.35		
Phosphocholine	381.49 $\pm$ 33.30	333.18 $\pm$ 2.58	338.49 $\pm$ 20.54	329.26 $\pm$ 13.17		
Phosphatidylcholine	34162.62 $\pm$ 566.20	35640.89 $\pm$ 566.20	34706.88 $\pm$ 651.01	35626.43 $\pm$ 227.88		
Sphingomyelin	4269.26 $\pm$ 186.93	4075.13 $\pm$ 202.93	4268.67 $\pm$ 68.54	3999.57 $\pm$ 23.03		



**Table 2.** Choline metabolite concentration in cortical non-damaged hemisphere of male and female mice maintained on control and vitamin B12 diets.

Metabolites (nmol/g)	Control diet		Vitamin B12 Deficient diet		Effect	p-value
	Female	Male	Female	Male		
Methionine	77.51 ± 12.43	79.19 ± 9.36	90.77 ± 5.94	107.07 ± 9.84	Diet	0.029
Acetylcholine	3.64 ± 0.36	4.17 ± 0.012	5.03 ± 0.59	4.79 ± 0.33		
Betaine	25.53 ± 2.91	46.47 ± 1.45	15.14 ± 0.83	15.24 ± 4.93		
Choline	67.92 ± 6.39	46.47 ± 6.90	67.92 ± 6.39	82.09 ± 4.95		
GPC	635.66 ± 5.68	611.81 ± 40.95	649.40 ± 10.85	692.71 ± 2.11	Sex	0.0151
Phosphocholine	353.28 ± 9.50	385.74 ± 16.73	353.28 ± 9.50	309.66 ± 14.10		
Phosphatidylcholine	34546.56 ± 1271.33	32335.41 ± 1178.83	35283.09 ± 497.43	34727.34 ± 262.79		
Sphingomyelin	4342.97 ± 148.72	3653.92 ± 41.67	4518.94 ± 39.26	3766.14 ± 116.91	Sex	0.0001

Measurements within the ischemic cortex showed that methionine levels were increased in vitamin B12 tissue compared to controls (Table 1; diet,  $F_{(1,8)} = 7.84$ ,  $p = 0.023$ ). Betaine levels were reduced in male and female animals on a vitamin B12 deficient diet compared to control diet animals (Table 1; sex,  $F_{(1,8)} = 17.0$ ,  $p = 0.0033$ ; diet,  $F_{(1,8)} = 6.28$ ,  $p = 0.036$ ).

In the non-ischemic cortex acetylcholine levels were increased between vitamin B12 and control diet animals (Table 2, diet,  $F_{(1,8)} = 7.84$ ,  $p = 0.023$ ). Phosphocholine levels were decreased in vitamin B12 deficient animals compared to control animals (Table 2; diet,  $F_{(1,8)} = 0.95$ ,  $p = 0.0151$ ). Sphingomyelin levels were also increased in vitamin B12 deficient animals compared to controls (Table 2; diet,  $F_{(1,8)} = 9.50$ ,  $p = 0.015$ ).

## Discussion

Ischemic stroke is the third leading cause of death and long-term disability in the United States. Nutrition is a modifiable risk factor for stroke (Spence, 2017). The elderly population is at higher risk of stroke as well as a vitamin B12 deficiency (Andrès et al., 2004). The purpose of this study was to assess the impact of a vitamin B12 deficiency on ischemic stroke outcome using a mouse model. Our findings showed that vitamin B12 deficient mice had more impairments in coordination and balance compared to control diet mice after ischemic damage. Forepaw usage was decreased in female mice maintained on the vitamin B12 diet. Male mice on a vitamin B12

deficient diet showed impaired coordination and balance. In both plasma and liver there was an increase in plasma and liver tHcy because of dietary vitamin B12 deficiency. 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 the ischemic cortex. We also report increased neuronal survival in vitamin B12 deficient male and female mice, along with changes in choline metabolism in brain tissue.

Lacunar stroke patients with vitamin B12 deficiency reported significantly worse stroke outcome, including fatigue and depressive-like symptoms (Huijts et al., 2012). 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 (Li, 2017). We measured forepaw usage and saw that female mice had significantly reduced impaired and non-impaired forepaw usage. 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 (Yahn et al., 2021).

In the present study, we did not observe any differences in damage volume between dietary groups; however, this is a gross measurement. In our immunofluorescence experiments we report a difference in in apoptosis between dietary groups. 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 (Cirillo et al., 2020). 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 (Sairanen et al., 2006). Furthermore, in post-ischemic stroke there is elevated levels of neuronal death due to apoptosis in the damaged area of the brain (Uzdensky, 2019). A potential reason there is not an increase in apoptosis in female mice is the neuroprotective effects of estrogen and progesterone (Kim and Vemuganti, 2015). 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 (Yahn et al., 2021).

One-carbon metabolism incorporates several vitamins and nutrients. Other studies have shown that vitamin B12 status affects choline status in animals (Compher et al., 2002; Hegsted et al., 1967). We measured levels of choline in brain tissue in ischemic and non-ischemic cortical tissue. Our results show that ischemic brain tissue has higher methionine levels in vitamin B12 deficient mice. The increase in methionine levels might be in compensation to the dietary vitamin B12 deficiency, since the brain is sensitive to methionine (Selhub et al., 2000). We also report lower levels of betaine in ischemic brain tissue of vitamin B12 deficient mice, which might reflect what's happening in liver and plasma, and availability for uptake by brain.

Part of 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 (Persky et al., 2010; Singh et al., 2021). In our study, male and female mice maintained on vitamin B12 deficiency had impaired stroke outcome, although they had impairments of different motor tasks. 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 (Margalit et al., 2018). The

results of our study emphasizes the importance of studying of sex differences in preclinical stroke experiments and the possibility of individualized medicine (Bushnell et al., 2018).

## **Conclusion**

The data presented in this study provides evidence that a vitamin B12 deficiency impacts motor function in older adult male and female mice after ischemic stroke. 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.

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