Maternal dietary choline deficiencies during pregnancy and lactation reduce cerebral blood flow in 3-month-old female mice offspring following ischemic stroke to the sensorimotor cortex

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Abstract

A maternal diet that provides adequate nutrition during pregnancy and lactation is vital to the neurodevelopment of offspring. One-carbon metabolism plays an important role in the closure of the neural tube of the developing embryo; however, the impact of maternal one-carbon dietary deficiencies on offspring neurological function later in life remains relatively unknown. Stroke is one of the leading causes of death globally, and its prevalence is expected to increase in younger age groups as the incidence of various risk factors for stroke increases. The aim of our study was to determine the impact of maternal nutritional deficiencies on cerebral blood flow and peripheral hemodynamics after ischemic stroke in adult offspring. In this study, adult female C57BL/6J mice were placed on either control (CD), choline (ChDD) or folic acid (FADD) deficient diets for four weeks to deplete stores prior to mating and maintained on the assigned diet during pregnancy and lactation. Female offspring were weaned and transitioned to a CD for the duration of the study. Ischemic stroke was induced in the sensorimotor cortex of 2- and 10-month-old female offspring using the photothrombosis model. Six weeks after induction of stroke, cerebral and peripheral blood flow was measured using the Vevo2100 Pulse Wave Doppler tracing modality. Our data showed that 3.5-month-old female offspring from a ChDD mothers had reduced blood flow in the posterior cerebral artery compared to CD mice; this effect disappeared in older offspring. In 11.5-month-old females we observed changes in peripheral hemodynamics, but not in young animals. Our findings suggest that a maternal dietary deficiency in choline results in reduced cerebral blood flow in adult female offspring after ischemic stroke, but the long-term effects are not present. This result points to the key role of the maternal diet in early life neuro-programming, while emphasizing its effects on both fetal development and long-term cerebrovascular health.

1 Introduction

Maternal nutrition during pregnancy and lactation is recognized as a critical factor determining the health of offspring (1–5). Early life nutritional cofactors are critical for typical fetal development, but also in determining offspring disease outcome (6,7). The Developmental Origins of Health and Disease (DOHaD) theory suggests that prospective chronic diseases are programmed *in utero* (8–11). When already compensating for fetal nutrient accumulation and increased maternal metabolic demands (12), altered or insufficient maternal nutrition impacts both early development and future offspring health. In offspring, maternal dietary deficiencies have been associated with ventricular septal defects (13) and impaired glucose tolerance (14), as well as modified neural tube closure (15,16) and neurocognitive development (17–21). Beyond this evidence of suboptimal structural development in offspring, maternal nutritional deficiencies have been linked to programming of offspring metabolic (22) and epigenetic (23–28) adaptations, thereby predisposing that individual to life-long cardiovascular, metabolic, and neuroendocrine dysfunction.

Epidemiological studies have demonstrated the effect of maternal diet on lifelong cardiovascular and neurological function (29–31). Most of this population-level work reveals an effect of poor maternal health on birthweight and incidence of disease and cardiovascular risk factors in adulthood, such as hypertension and hyperlipidemia (4,10). Such relationships have been shown in numerous global populations and are apparent from birth through early childhood (32). Folate and choline are important players in healthy fetal neurodevelopment due to their involvement in the closure of the neural tube and are components of one-carbon metabolism (33,34). Folate and its chemically synthesized form folic acid are important for fetal neurodevelopment (35), as folate requirements during pregnancy are increased by 5- to 10-fold compared to non-pregnant women (36). Maternal folate and choline levels during pregnancy have also been shown to be important in the development

of the cerebellum and hippocampus (37), as well as affecting postnatal myelination trajectories (38), short-term memory (39), hyperactivity/attention (40), neurocognitive development (41), and risk of autism spectrum disorder (ASD) (42).

Recent work in rodent models has improved mechanistic understanding of how maternal levels of folate and choline impact neurodevelopmental processes (43). Akin to human epidemiological studies (29–31), murine maternal folate deficiencies have been implicated in adverse reproductive performance, implantation, and fetal growth (44). During pregnancy and lactation, maternal dietary folic acid availability has been shown to influence progenitor cell mitosis, and apoptosis in the fetal mouse forebrain (45) and hippocampus (39). Investigations of maternal perinatal folate deficiencies have revealed reduced hippocampal proliferation, impaired vesicular transport and synaptic plasticity, as well as poor neurite outgrowth (46), modified cellular neocortex composition, and diminished complexity and arborization of projection neurons (47) in offspring. In addition to these structural observations, both genetic and epigenetic modifications have been observed; maternal folate deficiencies reduced expressions levels of brain derived neurotropic factor (BDNF) and H3K9me2 in the fetal hippocampus, and folic acid deficiency for two generations, significantly enhancing *de novo* mutations accumulation during meiosis (48). Choline, another one-carbon cofactor implicated in a number of diverse biological processes (49), has yielded variable results in animal models of neurodevelopment. Effects of maternal choline deficiencies, such as defective layering of the cortex, reduced cortical size and brain weight (50), and modified hippocampal electrophysiology (51) and neurogenesis (52), have been observed in offspring. Beyond these physiological, and histological findings, choline and folate deficiencies have been shown to elicit similar adverse effects, such as impaired homocysteine remethylation, oxidative stress, and endothelial dysfunction in murine cerebrovasculature (59,60); effectively demonstrating the link between maternal one-carbon cofactors and typical fetal neurodevelopment.

The link between maternal nutrition and fetal development is abundantly clear, but the long-term effects of maternal nutritional deficiencies on adult offspring are less well-studied. Investigating the links between cerebral and peripheral blood flow and the maternal environment will improve our understanding of dietary requirements during pregnancy and provide information on the role of maternal nutrition in early life programming of adult neurovascular diseases, such as ischemic stroke. Stroke is among the leading causes of death globally and its prevalence as a major health concern is predicted to increase, as the global population ages and demographics of populations change (53,54). One of the many reasons these problems exist is that the majority of preclinical studies are targeted only towards male subjects (55). Over 90% of preclinical studies use strictly male mice whereas all clinical studies use equal part male and female participants (55,56). This makes clinical pharmaceutical findings favor better outcomes in males (57,58). The aim of this study was to determine the impact of perinatal maternal nutritional deficiencies in folic acid or choline on cerebral and peripheral (cardiac and aortic) hemodynamics flow after ischemic stroke in adult and mid-age female offspring.

2 Materials and Methods

2.1 Experimental Design

All animal experimentation was performed following approval by the Midwestern University Institutional Animal Care and Use Committee in accordance with animal welfare guidelines.

Experimental manipulations are summarized in Figure 1. Briefly, for the maternal cohort (n = 30) female and (n = 30) male C57BL/6J mice were purchased from Jackson Laboratories and acclimatized for one week to controlled housing conditions (22 ± 1°C, 12h-light/12h-dark cycle) with *ad libitum*

access to food and water (RRID: IMSR_JAX:000664, Jackson Laboratories). At two months of age (Day 0), females were randomized to control (CD, TD.190790), and commercially (Envigo) prepared folic acid (FADD, TD.01546) or choline deficient (ChDD, TD.06119) diets and maintained on these diets for four weeks prior to mating, and later throughout pregnancy and lactation (Figure 1). Levels of folic acid and choline bitartrate in experimental diets are listed in in Table 1 (39,59–61).

After weaning, female offspring were maintained on the CD *ad libitum*. Offspring were randomized to one of two cohorts undergoing photothrombotic (PT) stroke at either 2- or 10-months of age, followed by ultrasound measurements at 1.5 months post-stroke.

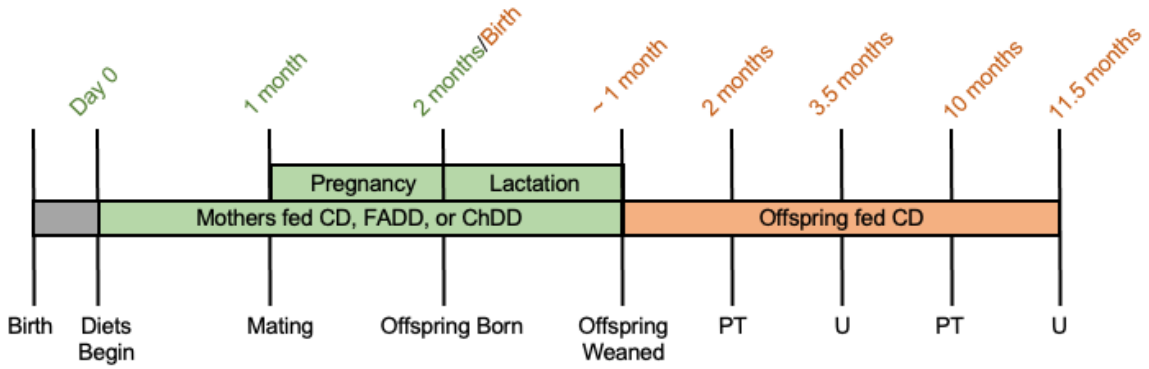

Figure 1. Experimental timeline. Beginning at 2-months-of age female mice were fed either control (CD), folic acid (FADD) or choline (ChDD) deficient diets. The female mice were maintained on these diets throughout the pregnancy and lactation until the offspring were weaned. Once the offspring were weaned, they were fed the CD. Separate cohorts of female offspring at 2 or 10 months of age had ischemic stroke induced via the photothrombosis (PT) model. At 3.5 (CD, n = 6 ; FADD, n = 7 ; ChDD, n = 6) and 11.5 (CD, n = 6 ; FADD, n = 6; ChDD, n = 6) months of age all female mouse offspring underwent ultrasound imaging (U).

Table 1. Experimental Diets. Concentration (in milligrams/kilogram) of folic acid and choline bitartrate in control (CD), folic acid-deficient (FADD), and choline-deficient (ChDD) diets fed to mothers throughout pregnancy and lactation.

	Diet		
	Control	**FADD**	**ChDD**
Folic Acid (mg/kg)	2	0.3	2
Choline Bitartrate (mg/kg)	1150	1150	300

2.2 Photothrombosis

When female offspring reached 2 or 10 months of age, ischemia was induced using the photothrombosis model. They were anesthetized with isoflurane (1.5%) in a 70:30 nitrous oxide:oxygen mixture. Core body temperature was monitored with a rectal thermometer (Harvard Apparatus) and maintained at 37 ± 0.2 ºC using a heating blanket. 10 mg/kg of the photosensitive Rose Bengal dye was injected intraperitoneally 5 minutes prior to irradiation. A 532 nm green laser was

placed 3 cm above the animal and directed to the sensorimotor cortex (mediolateral + 0.24mm) for 15 minutes (59,62–64).

2.3 Ultrasound imaging

Approximately 1.5 months after ischemic stroke, Vevo® 2100 ultrasound imaging system (FUJIFILM, Visual Sonics) was used to assess offspring *in vivo* cerebral (Figure 2A) and peripheral vascular function as previously reported (65,66). Measurements were completed in random order by investigators blinded to treatment groups. The high-frequency, high-resolution ultrasound system is equipped with a 40 MHz transducer (MS550S) with a focal length of 7.0 mm, frame rate of 557 fps (single zone, 5.08 mm width, B-mode), and a maximum two-dimensional field of view of 14.1×15.0 mm with a spatial resolution of 90 μm lateral by 40 μm axial.

Mice were anesthetized in an induction chamber with 3% isoflurane and 1 L/min flow of 100% oxygen for 1–2 mins, then placed supine on a heated platform and maintained with 1.5–2% isoflurane. Heart rate, electrocardiogram (ECG), and respiratory rate were measured by the four ECG electrodes embedded in the platform. Using a heat lamp and heated platform, body temperature was maintained at 36–38°C and monitored by a rectal probe throughout.

The left ventricular (LV) structural and functional parameters, including stroke volume, ejection fraction, fractional shortening, and cardiac output, were calculated from the LV parasternal short-axis M-mode view and recorded at the level of two papillary muscles. An M-mode cursor was positioned perpendicular to the anterior and posterior walls in the middle of the LV for measuring wall thickness. Interventricular septal wall (IVS) thickness during diastole (IVSd) and systole (IVSs) were also obtained from LV parasternal long-axis M-mode view.

Aortic diameters at the annulus, sinuses of Valsalva, and sinotubular junctions were measured from the B-mode aortic arch view. Ascending and descending aortic, and posterior cerebral artery (PCA) peak velocities were measured from the pulse wave (PW) Doppler-mode. Pulse wave velocity (PWV) was obtained from the B-mode and Doppler-mode aortic arch view, calculated as PWV (mm·s−1) = aortic arch distance (d2-d1)/transit time (T1-T2). The PW Doppler mode sample volume was placed in the ascending aorta to verify the time from the onset of the QRS complex to the onset of the ascending aortic Doppler waveform (T1). Using the same image plane, the time from the onset of the QRS complex to the onset of the descending aortic Doppler waveform (T2) was also measured, and the average values for T1 and T2 over 10 cardiac cycles were calculated. Furthermore, the aortic arch distance was measured between the two sample volume positions along the central axis of aortic arch on the B-mode image.

Transcranial Doppler sonography is a non-invasive, non-ionizing, inexpensive, portable, and safe technique that uses a pulsed Doppler transducer for assessment of intracerebral blood flow in the clinical practice (67,68) and has become an important translational tool to evaluate the intracerebral blood flow in animal models.

The posterior cerebral artery (PCA) peak blood flow was measured using the Vevo 2100 high-resolution ultrasound system and the 24MHz (MS250) transducer. The trans occipital window was used to visualize the posterior cerebral arteries and pulsed wave (PW), Doppler-mode was used to measure the PCA peak blood flow velocity.

2.4 Statistics

Ultrasound data was analyzed by two individuals that were blinded to experimental treatment groups using Vevo Lab ultrasound analysis software (VisualSonics, Toronto, Canada). Using GraphPad Prism 9.0., One-way ANOVA analysis was performed to analyze maternal dietary effects, and two-way ANOVA analysis was performed to assess aging effects. 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). Statistical tests were performed using a significance level (*P*) of 0.05.

3 Results

Cerebral blood flow in offspring after ischemic stroke

In 3.5-month-old offspring, there was a statistically significant difference in blood flow velocity within the posterior cerebral artery between maternal dietary groups (Figure 2B; F [2, 15] = 4.07, p = 0.04). Female offspring of ChDD mothers had significantly impaired blood flow velocity in the PCA compared to offspring from the CD group (p = 0.04). A maternal FADD reduced blood flow velocity in female offspring, but this did not reach significance (p = 0.14). In 11.5-month-old offspring, no statistically significant differences in cerebral blood flow velocity were observed between maternal diet groups (Figure 2C; F [2, 13] = 4.07, p = 0.08).

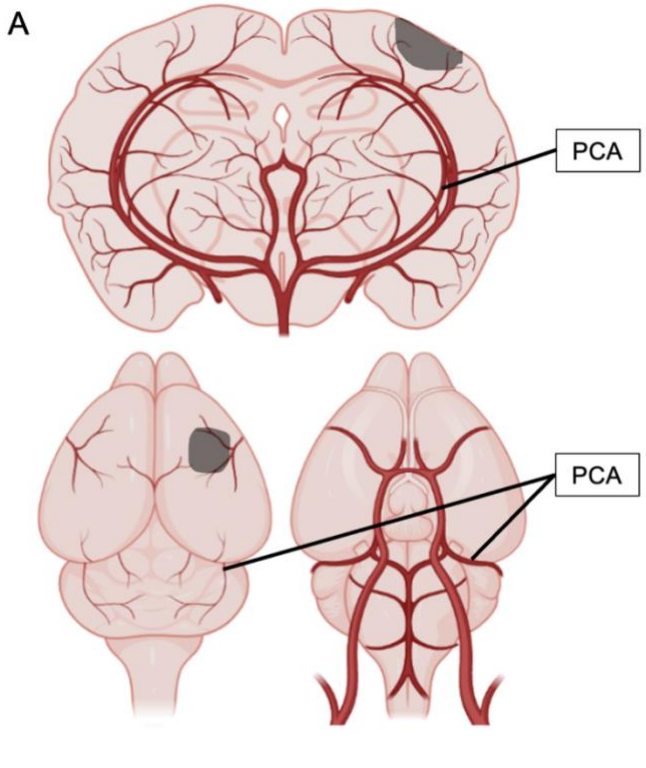

Figure 2. (A) Visual representation of mouse cerebral vasculature, posterior cerebral artery (PCA) and location of ischemic stroke. Blood flow velocity in the PCA after ischemic stroke in 3.5- **(B)** and 11.5- **(C)** month-old female offspring from control (CD), folic acid (FADD) and choline (ChDD) deficient diet mothers. Scatter plot with mean ± SEM of 5 to 7 mice per group. * $p < 0.05$, Tukey's pairwise comparison.

Cardiac/Aortic (Peripheral) hemodynamics in offspring after ischemic stroke

In 3.5-month-old offspring, no differences in cardiac/aortic (peripheral) hemodynamics were observed between maternal diet groups (Table 2). In 11.5-month-old offspring, there was a statistically significant difference in the coronary artery velocity ratio between maternal diet groups (Table 3; F (2, 13) = 4.07, p = 0.08). Female offspring of FADD mothers had a significantly increased systolic/diastolic ratio in the coronary artery compared to controls.

Table 2. Descriptive statistics (Mean ± SEM) of peripheral hemodynamics in 3.5-month-old female mouse offspring by maternal diet. Maternal diets included a control diet (CD), a folic acid deficient diet (FADD), and a choline deficient diet (ChDD). Mean ± SEM of 5 to 7 mice per group.

	Maternal Diet			
Measurement	**CD**	**FADD**	**ChDD**	***p-value***
Average Heart Rate (BPM)	494.2 ± 13.23	504.5 ± 12.68	513.0 ± 9.55	0.57
Average Stroke Volume (uL)	24.04 ± 2.54	28.67 ± 2.17	25.26 ± 2.06	0.33
Average Ejection Fraction (%)	86.57 ± 4.24	84.68 ± 3.09	86.46 ± 2.3	0.90
Average Fractional Shortening (%)	57.94 ± 6.28	53.89 ± 3.67	54.89 ± 3.13	0.81
Average Cardiac Output (mL/min)	11.77 ± 1.04	14.43 ± 1.08	12.94 ± 1.03	0.22
Coronary Artery Velocity Ratio (S/D)	0.34 ± 0.05	0.29 ± 0.02	0.36 ± 0.14	0.40
Aortic Pulse Wave Velocity ($mm \cdot s^{-1}$)	1.23 ± 0.24	3.52 ± 1.95	4.24 ± 3.28	0.57
Left Internal Diameter in Systolic (IVSs)	173.7 ± 28.62	163.7 ± 18.58	125.4 ± 19.83	0.42
Left Internal Diameter in Diastolic (IVSd)	531.2 ± 83.17	574.5 ± 45.33	361.9 ± 53.26	0.11

Table 3. Descriptive statistics (Mean ± SEM) of peripheral hemodynamics in 11.5-month-old female mouse offspring by maternal diet. Maternal diets included a control diet (CD), a folic acid deficient diet (FADD), and a choline deficient diet (ChDD). Mean ± SEM of 5 to 7 mice per group.

	Maternal Diet			
Measurement	**CD**	**FADD**	**ChDD**	***p-value***
Average Heart Rate (BPM)	524.8 ± 8.82	531.4 ± 4.02	531.3 ± 6.02	0.64
Average Stroke Volume (uL)	23.55 ± 4.55	37.47 ± 5.56	38.24 ± 2.08	0.05
Average Ejection Fraction (%)	84.88 ± 3.55	85.05 ± 2.85	74.93 ± 4.24	0.10
Average Fractional Shortening (%)	53.65 ± 4.18	53.92 ± 3.88	43.70 ± 4.04	0.11
Average Cardiac Output (mL/min)	20.32 ± 0.78	19.91 ± 2.97	20.28 ± 0.96	0.90
Coronary Artery Velocity Ratio (S/D)	0.27 ± 0.02	0.39 ± 0.04	0.28 ± 0.02	0.04*
Aortic Pulse Wave Velocity ($mm \cdot s^{-1}$)	3.28 ± 0.46	5.52 ± 2.83	6.11 ± 2.28	0.52
Left Internal Diameter in Systolic (IVSs)	90.72 ± 29.49	159.3 ± 27.05	167.5 ± 26.45	0.18
Left Internal Diameter in Diastolic (IVSd)	663.0 ± 72.03	427.8 ± 62.76	545.6 ± 58.14	0.10

The impact of aging on cerebral blood flow and cardiac/aortic hemodyamics

We compared the 3.5 and 11.5-month female cerebral and cardiac/aortic (peripheral) hemodynamics measurements (Table 4). For fractional shortening and end systole septal diameter, no differences were observed between experimental groups. However, further analysis revealed significant effects on other peripheral hemodynamic measures. Main effects of diet ($p = 0.03$) and offspring age ($p = 0.001$) were observed for average heart rate. While exclusively offspring age effects were observed for ejection fraction ($p = 0.01$), cardiac output ($p < 0.0001$), and pulse wave velocity ($p < 0.0001$). Finally, significant interaction effects were observed for stroke volume ($p = 0.03$), coronary artery velocity ratio ($p = 0.02$), and end diastole septal diameter ($p = 0.04$).

Table 4. Descriptive statistics (Mean ± SEM) of central and peripheral blood flow in 3.5 and 11.5-month-old female mouse offspring. Maternal diets included a control diet (CD), a folic acid deficient diet (FADD), and a choline deficient diet (ChDD). Mean ± SEM of 5 to 7 mice per group.

		Offspring Age		*p-values*		
Measurement	**Maternal Diet**	**3.5 months**	**11.5 months**	**Diet**	**Age**	**Diet x Age**
Posterior Cerebral Artery (mm/s)	CD	147.74 +10.24	122.37 _ 9.24	0.009	0.37	0.30
	FADD	136.43 + 8.78	146.27 + 21.01			
	ChDD	109.74 + 4.38	99.62 + 8.4			
Average Heart Rate (BPM)	CD	474.53 ± 15.19	524.78 ± 8.82	0.03*	0.001*	0.1374
	FADD	515.96 ± 5.84	531.42 ± 4.02			
	ChDD	511.16 ± 11.30	531.30 ± 6.02			
Average Stroke Volume (uL)	CD	24.04 ± 2.54	23.55 ± 4.55	0.2744	0.2319	0.03*
	FADD	28.67 ± 2.17	37.47 ± 5.56			
	ChDD	25.26 ± 2.06	38.24 ± 2.08			
Average Ejection Fraction (%)	CD	86.58 ± 4.24	84.88 ± 3.55	0.1638	0.009*	0.2929
	FADD	84.68 ± 3.09	73.10 ± 4.61			
	ChDD	86.46 ± 2.30	74.05 ± 2.49			
Average Fractional Shortening (%)	CD	57.94 ± 6.28	53.65 ± 4.18	0.3924	0.1811	0.4998
	FADD	53.89 ± 3.67	53.92 ± 3.88			
	ChDD	54.89 ± 3.13	43.70 ± 4.04			
Average Cardiac Output (mL/min)	CD	11.77 ± 1.04	20.32 ± 0.78	0.7197	<0.0001*	0.5400
	FADD	14.43 ± 1.08	19.91 ± 2.94			
	ChDD	12.94 ± 1.03	20.28 ± 0.96			
Coronary Artery Velocity Ratio (S/D)	CD	0.32 ± 0.04	0.27 ± 0.02	0.3813	0.9454	0.02*
	FADD	0.29 ± 0.02	0.39 ± 0.04			
	ChDD	0.36 ± 0.07	0.28 ± 0.02			
Pulse Wave Velocity ($mm \cdot s^{-1}$)	CD	1.23 ± 0.24	3.29 ± 0.46	0.8061	<0.0001*	0.1498
	FADD	1.58 ± 0.18	2.71 ± 0.36			
	ChDD	0.97 ± 0.18	3.92 ± 0.74			
Left Internal Diameter in Systolic (IVSs)	CD	173.72 ± 28.62	180.53 ± 24.50	0.4980	0.4745	0.6540
	FADD	163.72 ± 18.58	159.27 ± 27.05			
	ChDD	125.35 ± 19.83	167.50 ± 26.45			
Left Internal Diameter in Diastolic (IVSd)	CD	531.18 ± 83.17	662.98 ± 72.03	0.1063	0.3048	0.03*
	FADD	574.46 ± 45.33	427.78 ± 62.76			
	ChDD	361.92 ± 53.26	545.59 ± 58.14			

4. Discussion

The Developmental Origins of Health and Disease (DOHaD) theory suggests that prospective chronic diseases are programmed in utero- giving rise to programming of offspring cardiovascular, metabolic, and neuroendocrine dysfunction (8–11). Despite impressive evidence of the importance of the maternal environment for fetal growth and development, there have been few investigations surrounding the effects of maternal nutrition on cerebrovascular function in fully developed or adult offspring. Using an experimental model of ischemic stroke, our study aimed to determine the impact of perinatal maternal nutritional deficiencies in 1C metabolites on measures of cerebral and peripheral blood flow and cardiac function in offspring, following ischemic injury. Our results demonstrate a significant impairment in cerebral blood flow velocity following stroke in 3.5-month-old, but not 11.5-month-old offspring from choline-deficient mothers. However, 11.5-month-old offspring from folic acid-deficient mothers did display a significant increase in peripheral hemodynamic measures, including the coronary artery velocity ratio. Effects of both diet and offspring age, as well as interactions between these variables were observed for numerous peripheral indices.

The neurovascular unit (NVU) is comprised of a number of unique neuronal, glial, and endothelial cell types, and recent findings indicate unique cross-talk between neurons and the cerebral vasculature (69–72), emphasizing the complex, pivotal role the NVU plays during development and in the progression of neurovascular pathologies like ischemic stroke and neurodegenerative disorders (73–78). Further, the NVU is responsible for the maintenance of a highly selective blood–brain barrier (BBB) and cerebral homeostasis, as well as the control of cerebral blood flow (CBF) (79). The impact of maternal diet on the NVU, modulating integrity of cerebral blood vessels and closure of the neural tube, has been established (15,16,80–82). Our study adds to these investigations by assessing the hemodynamic response of blood flow within the posterior cerebral artery (PCA) in both young and aged offspring. The contralesional PCA was selected as an index of cerebral blood flow due to its spatial and functional independence from the sensorimotor cortex targeted during photothrombotic stroke (83), and evident correlation to measures of the Middle Cerebral Artery (MCA) (84).

In line with studies detailing the impact of maternal choline on neurovascular development (16), our results suggest that maternal choline levels during pregnancy and lactation impair cerebral blood flow in young mice following ischemic stroke. In rodent models, the importance of choline for optimal neurodevelopment is well-established (85,86). Recent work has examined the role of choline in neurovascular interactions as well, modulating levels of anti-angiogenic factors during gestation (87), fetal hippocampal angiogenesis (37), and promoting the proliferation of rat endothelial cells following hypoxic injury in cerebral vessels (88). In this way, choline has been shown to influence neurovascular health across the lifespan and may be implicated in both neurovascular structure and functional response to injury. The cardiovascular system has also demonstrated effects of choline deficiency, including heart defects (89,90), while higher intake of choline was associated with reduced risk of adult cardiovascular disease (91) and amelioration of impaired vagal activity and inflammation in hypertensive rodents (92). Our study revealed a diet effect of both maternal choline and folic acid, where deficiencies in either nutrient significantly increased offspring heart rate, regardless of offspring age. While heart rate is a well-known risk factor for cardiovascular disease, our results align well with recent findings associating low heart rate with better functional and cognitive outcomes following ischemic stroke (93) and high heart rate with impaired endothelial function and increased ischemic lesion size following stroke (94), as well as death due to vascular diseases (93). Overall, maternal diet has an established developmental influence on basic measures of cardiovascular and neurovascular health, and may impact offspring programming of the NVU, thereby influencing stroke protection via endothelial homeostasis via endothelial NO synthase (eNOS) (95,96).

We did not observe an effect of maternal diet on cerebral blood flow in 11.5-month-old offspring. This could be due to the well-investigated aging-associated changes in the structural and functional integrity of the vasculature (97–101). Therefore, we propose that the difference in effect between young (3.5m.o.) and old (11.5m.o.) offspring is a result of the aging of the control mice. In addition, the presumed damage or endothelial dysfunction induced by the deficient diets is long-lasting and may contribute to premature aging, generating a mathematically significant difference when compared to young, healthy controls, but only a minor difference when compared to senescent offspring displaying similar levels of vascular dysfunction. This result is supported by the interaction effect of diet and offspring age and requires further investigation. In addition, unique mechanisms drive vascular senescence in males and females (102), so our results may be obscured by our study of exclusively female mice. Another interesting result from our study is the significance of the coronary artery velocity (S/D) ratio in 11.5-month-old offspring. In this assessment, folic acid significantly increased the ratio, as occurs with moderate coronary atherosclerosis (103). This result may indicate cardiovascular impairment related to a maternal diet deficient in choline. However, because the incidence of coronary artery disease (CAD), valvular disease, rhythm disorders, and heart failure increases with age (104), it appears that folic acid may play a role in programming resistance to this age-related dysfunction.

Outside of heart rate, which is discussed above, age effects were observed for offspring ejection fraction, cardiac output, and pulse wave velocity. Ejection fraction, an index of the left ventricular output, has recently been used as a measure of cardiac mortality risk (105), with lower percentages indicating cardiac dysfunction. In our study, older mice displayed a significantly reduced ejection fraction, indicating cardiovascular impairment. In a similar manner, our cardiac output data indicate the expected increased cardiac dysfunction as a product of aging (106). Aortic pulse wave velocity (PWV) was also found to be significantly increased on the older cohort, in line with clinical findings (107). Finally, interactions between maternal folic acid deficiency and offspring age were found for the coronary artery velocity S/D ratio, an indicator of coronary atherosclerosis, and interventricular septal end diastole (IVSd), an indicator of ventricular hypertrophy.

Overall, our data points to the need for rodent models spanning a variety of ages for research in age-related diseases such as stroke and vascular dysfunction. We recognize that the exclusion of male subjects in this study may limit our ability to draw conclusions with respect to the impact of sex hormone in observed phenomenon. In future studies, we plan to include male animals, and design experiments that would allow us to investigate the role of paternal dietary effects. Additionally, we plan to further age animals to ~20mo after ischemic stroke as well as investigate the role of over supplementation on blood flow after stroke. A detailed analysis of angiogenesis after ischemic stroke might also be prudent. In conclusion, 1C metabolism metabolites have potentially compensatory, but unique roles. Maternal nutrition during pregnancy and lactation has effects, even after infancy and childhood. Our work demonstrated an age effect in animal models encourages further comprehensive longitudinal time-point studies that includes older age animals.

3 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

4 Author Contributions

Kasey Pull: data analysis, writing – original draft, writing – review and editing. **Robert Folk**: investigation and data analysis. **Jeemin Kang**: data analysis. Shaley Jackson: data analysis. **Brikena Gusek**: investigation and data analysis. **Mitra Esfandiareri:** Conceptualization, writing – review and editing, funding acquisition. **Nafisa M. Jadavji:** conceptualization, investigation, resources, data curation, writing – original draft, writing – review and editing, visualization, supervision, project administration, and funding acquisition

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