

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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14

15 **Abstract**

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

37 **1 Introduction**

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

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

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

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

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

97 **2 Materials and Methods**

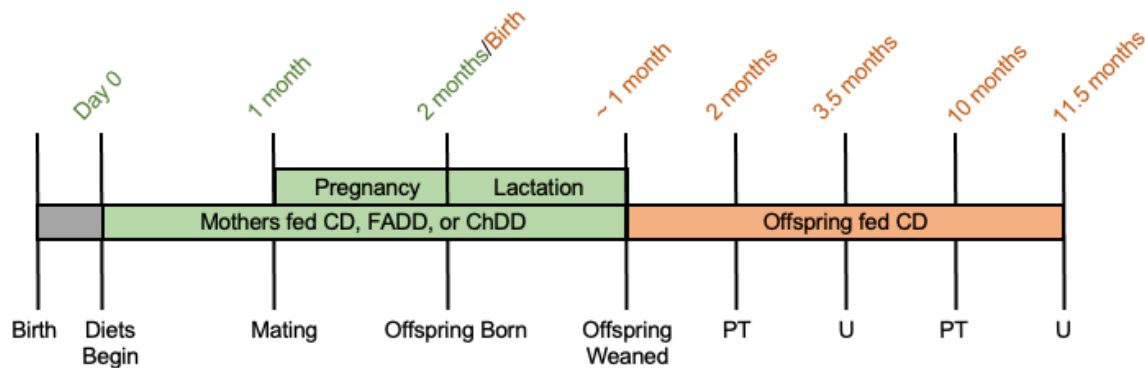
98 **2.1 Experimental Design**

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

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

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

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



112

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

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

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

123 2.2 Photothrombosis

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

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

131

132 **2.3 Ultrasound imaging**

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

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

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

151 Aortic diameters at the annulus, sinuses of Valsalva, and sinotubular junctions were measured from
152 the B-mode aortic arch view. Ascending and descending aortic, and posterior cerebral artery (PCA)
153 peak velocities were measured from the pulse wave (PW) Doppler-mode. Pulse wave velocity (PWV)
154 was obtained from the B-mode and Doppler-mode aortic arch view, calculated as $PWV \text{ (mm}\cdot\text{s}^{-1}\text{)} =$
155 $\text{aortic arch distance (d}_2\text{-d}_1\text{)}/\text{transit time (T}_1\text{-T}_2\text{)}$. The PW Doppler mode sample volume was placed
156 in the ascending aorta to verify the time from the onset of the QRS complex to the onset of the
157 ascending aortic Doppler waveform (T1). Using the same image plane, the time from the onset of the
158 QRS complex to the onset of the descending aortic Doppler waveform (T2) was also measured, and
159 the average values for T1 and T2 over 10 cardiac cycles were calculated. Furthermore, the aortic arch
160 distance was measured between the two sample volume positions along the central axis of aortic arch
161 on the B-mode image.

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

165
166 The posterior cerebral artery (PCA) peak blood flow was measured using the Vevo 2100 high-resolution
167 ultrasound system and the 24MHz (MS250) transducer. The trans occipital window was used to visualize the
168 posterior cerebral arteries and pulsed wave (PW), Doppler-mode was used to measure the PCA peak blood flow
169 velocity.

170
171

172

173 2.4 Statistics

174 Ultrasound data was analyzed by two individuals that were blinded to experimental treatment groups
175 using Vevo Lab ultrasound analysis software (VisualSonics, Toronto, Canada). Using GraphPad Prism
176 9.0., One-way ANOVA analysis was performed to analyze maternal dietary effects, and two-way
177 ANOVA analysis was performed to assess aging effects. Significant main effects of two-way ANOVAs
178 were followed up with Tukey's post-hoc test to adjust for multiple comparisons. All data are presented
179 as mean \pm standard error of the mean (SEM). Statistical tests were performed using a significance level
180 (P) of 0.05.

181 3 Results

182 *Cerebral blood flow in offspring after ischemic stroke*

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

190

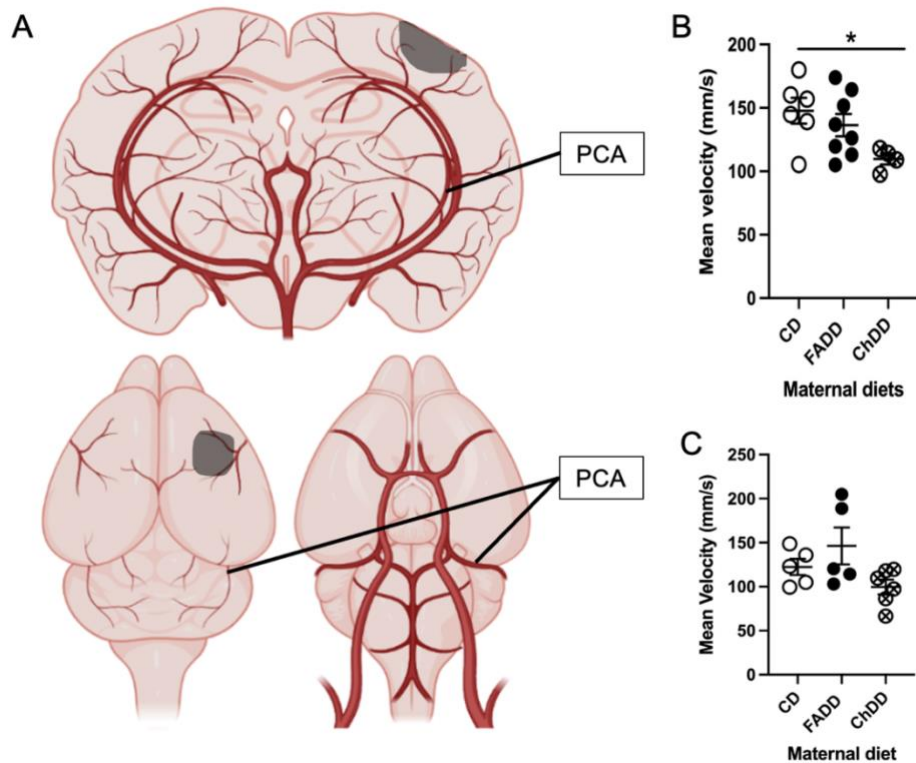


Figure 2. (A) Visual representation of mouse cerebral vasculature, posterior cerebral artery (PCA) and location of ischemic stroke. Blood flow velocity in the PCA after ischemic stroke in 3.5- (B) and 11.5- (C) month-old female offspring from control (CD), folic acid (FADD) and choline (ChDD) deficient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

212

213

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

215 In 3.5-month-old offspring, no differences in cardiac/aortic (peripheral) hemodynamics were observed
 216 between maternal diet groups (Table 2). In 11.5-month-old offspring, there was a statistically
 217 significant difference in the coronary artery velocity ratio between maternal diet groups (Table 3; $F(2,$
 218 $13) = 4.07, p = 0.08$). Female offspring of FADD mothers had a significantly increased
 219 systolic/diastolic ratio in the coronary artery compared to controls.

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

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

223

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

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

227

228 ***The impact of aging on cerebral blood flow and cardiac/aortic hemodynamics***

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

237

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

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

242 4. Discussion

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

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

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

288 We did not observe an effect of maternal diet on cerebral blood flow in 11.5-month-old offspring. This
289 could be due to the well-investigated aging-associated changes in the structural and functional integrity
290 of the vasculature (97–101). Therefore, we propose that the difference in effect between young
291 (3.5m.o.) and old (11.5m.o.) offspring is a result of the aging of the control mice. In addition, the
292 presumed damage or endothelial dysfunction induced by the deficient diets is long-lasting and may
293 contribute to premature aging, generating a mathematically significant difference when compared to
294 young, healthy controls, but only a minor difference when compared to senescent offspring displaying
295 similar levels of vascular dysfunction. This result is supported by the interaction effect of diet and
296 offspring age and requires further investigation. In addition, unique mechanisms drive vascular
297 senescence in males and females (102), so our results may be obscured by our study of exclusively
298 female mice. Another interesting result from our study is the significance of the coronary artery
299 velocity (S/D) ratio in 11.5-month-old offspring. In this assessment, folic acid significantly increased
300 the ratio, as occurs with moderate coronary atherosclerosis (103). This result may indicate
301 cardiovascular impairment related to a maternal diet deficient in choline. However, because the
302 incidence of coronary artery disease (CAD), valvular disease, rhythm disorders, and heart failure
303 increases with age (104), it appears that folic acid may play a role in programming resistance to this
304 age-related dysfunction.

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

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

326

327 **3 Conflict of Interest**

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

330 **4 Author Contributions**

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

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342 **7 References**

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631 The following formatting styles are meant as a guide, as long as the full citation is complete and
632 clear, Frontiers referencing style will be applied during typesetting.

633 **7.1 Harvard Referencing Style (Author-Date)**

634 Many Frontiers journals use the Harvard referencing system (Author-date), to find the style and
635 resources for the journal you are submitting to please go [here](#). For Humanities and Social Sciences
636 articles please include page numbers in the in-text citations

637 For some examples please click [here](#).

638 For more examples of citing other documents and general questions regarding Harvard reference
639 style, please refer to the [Chicago Manual of Style](#).

640 **7.2 Vancouver Referencing Style (Numbered)**

641 Many Frontiers journals use the numbered referencing system, to find the style and resources for the
642 journal you are submitting to please go [here](#). In-text citations should be numbered consecutively in
643 order of appearance in the text – identified by Arabic numerals in the parenthesis [square parenthesis
644 for Physics and Mathematics].

645 For some examples please click [here](#).

646 For more examples of citing other documents and general questions regarding Vancouver reference
647 style, please refer to [Citing Medicine](#).

648 **8 Supplementary Material**

649 Supplementary Material should be uploaded separately on submission, if there are Supplementary
650 Figures, please include the caption in the same file as the figure. Supplementary Material templates
651 can be found in the Frontiers Word Templates file.

652 Please see the [Supplementary Material section of the Author guidelines](#) for details on the different
653 file types accepted.

654 **9 Data Availability Statement**

655 The datasets [GENERATED/ANALYZED] for this study can be found in the [NAME OF
656 REPOSITORY] [LINK]. Please see the [Data Availability section of the Author guidelines](#) for more
657 details.