Maternal dietary deficiencies in folic acid and choline change metabolites levels in offspring after ischemic stroke

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Keywords

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Abstract

Ischemic stroke is a debilitating disease, with nutrition being a modifiable risk factor. Changes in levels of metabolites can be used to measure the alterations in the gut, a significant marker for the etiology of diseases. This study utilized untargeted metabolomics to investigate changes in fecal samples of offspring in response to maternal dietary deficiencies and ischemic stroke. Female mice were placed on control (CD), folic acid- (FADD), or choline-deficient (ChDD) diets prior to, during pregnancy, and lactation. Offspring were weaned on to CD and at 2 months of age an ischemic stroke was induced. Fecal samples were collected prior to ischemic stroke, and at 1- and 4-weeks post-stroke for analysis. Sex and maternal dietary differences in metabolites were observed at both the 1- and 4-week post-stroke timepoints. At the 1-week post-stroke, female FADD offspring had more changes in metabolites than males. Comparatively, at the 4-week post-stroke timepoint, female offspring on either FADD or ChDD demonstrated metabolite changes. This study demonstrates a long-lasting impact of maternal dietary deficiencies on central nervous system and gut microbiome function after ischemic stroke.

Summary Statement

Our study investigated metabolite changes in female and male offspring fecal samples from mothers maintained on folic acid or choline deficient diet. We report that female offspring metabolite levels were impacted.

Introduction

Ischemic stroke is one of the leading causes of morbidity and mortality globally. The incidence of stroke in adults has been increasing due to factors such as high blood pressure, vascular disorders, diabetes, and obesity (Mozaffarian et al., 2015). By 2030, the number of

63 individuals affected by stroke is predicted to increase by 3.4 million (Ovbiagele et al., 2013). While
64 there has been a higher prevalence of ischemic stroke in older age individuals (> 65 years) (Feigin
65 et al., 2014; Mozaffarian et al., 2015), recent studies have shown that the incidence of stroke among
66 people under 50 years of age is alarmingly increasing (Feigin et al., 2014; Smajlović, 2015; Tong
67 et al., 2016). More importantly it is becoming more evident that nutrition is a well-established and
68 a modifiable risk factor for vascular dysfunction leading to ischemic stroke (Benjamin et al., 2018;
69 Hankey, 2012; Spence et al., 2017).

70 Although previous studies have established nutrition as a modifiable risk factor for vascular
71 dysfunction and ischemic stroke (Benjamin et al., 2018; Hankey, 2012; Spence et al., 2017),
72 logistical challenges in conducting longitudinal studies have limited research on the impact of
73 early-life nutrition on late-life neurological outcomes. Pregnancy and early life are critical periods
74 for neurodevelopment and lay the foundation for future brain function and health (Carolan-Olah
75 et al., 2015; Kolb et al., 2016; Pannia et al., 2016). In the US, health disparities lead many women
76 to receive inadequate vitamins and nutrients during pregnancy. For example, nearly one in three
77 African-American women do not get enough folic acid daily (Bailey et al., 2010). The same is true
78 for Spanish-speaking Mexican-American women; they often do not get enough folic acid (Hamner
79 et al., 2011). Furthermore, approximately one-fourth of women in the US do not consume enough
80 choline from their diet (Jensen et al., 2007; Zeisel, 2013), and women in low-income countries
81 often face nutritional deficiencies during pregnancy, making this a serious global health issue
82 (Gernand et al., 2016). Despite recent attention to the potential effects of maternal dietary
83 deficiency on early life and childhood development, the long-term impact of maternal dietary
84 deficiencies during pregnancy on the child's future vascular health is not yet well understood.
85 Evidence suggests that maternal nutrition plays an important role in disease onset and outcome

later in life (Di Meco & Praticò, 2019; Gaillard, 2015; Palinski, 2014; Yessoufou & Moutairou, 2011). For example, maternal obesity during pregnancy is implicated in the onset of metabolic diseases, such as diabetes in offspring(Gaillard, 2015; Yessoufou and Moutairou, 2011), but more life course studies are required (Gaillard, 2015). Furthermore, it has been shown that the maternal environment plays an important role in cardiovascular risk in offspring (Palinski, 2014). Using animal models, many groups have shown that maternal diet (e.g., high-fat diet, and caloric restriction, etc.) can modulate cerebrovascular structure and function, leading to endothelial dysfunction, reduced smooth muscle contractility, and decreased myogenic tone, all of which significantly influence peripheral and cerebral vascular health in mouse and rat adult offspring (Campbell et al., 2005; Durrant et al., 2014; Lin et al., 2018). Our group has demonstrated that maternal dietary deficiencies in folic acid or choline impact offspring ischemic stroke outcome (Clementson et al., 2023; Hurley et al., 2023; Pull et al., 2023). However, the mechanisms are not yet clear, our hypothesis is that maternal diet deficient in either folic acid or choline combined with ischemic stroke will result in changes to metabolites within the gut. The aim of the present study was to investigate changes in metabolite levels of fecal matter in male and female offspring exposed to FADD and ChDD during early neurodevelopment and an ischemic stroke during adulthood. Untargeted metabolite analysis has been shown to provide some insight to changes after stroke (Zhao et al., 2022), predict stroke outcome (Chi et al., 2021), as well as generate data for biomarkers (Li et al., 2018; M.-H. Wu et al., 2023).

Results

Maternal diet deficiencies in FADD or ChDD during pregnancy and lactation impacted metabolite levels at the 1- and 4-week post-stroke timepoints. The number of changes in

metabolites because of experimental manipulations (maternal diet or offspring sex) are summarized in Figure 1. The number of metabolites measured in offspring stools samples was extensive. We have listed the metabolites in Table 1 with the corresponding p-values for interactions between maternal diet and offspring sex, as well as main effects of maternal diet and sex.

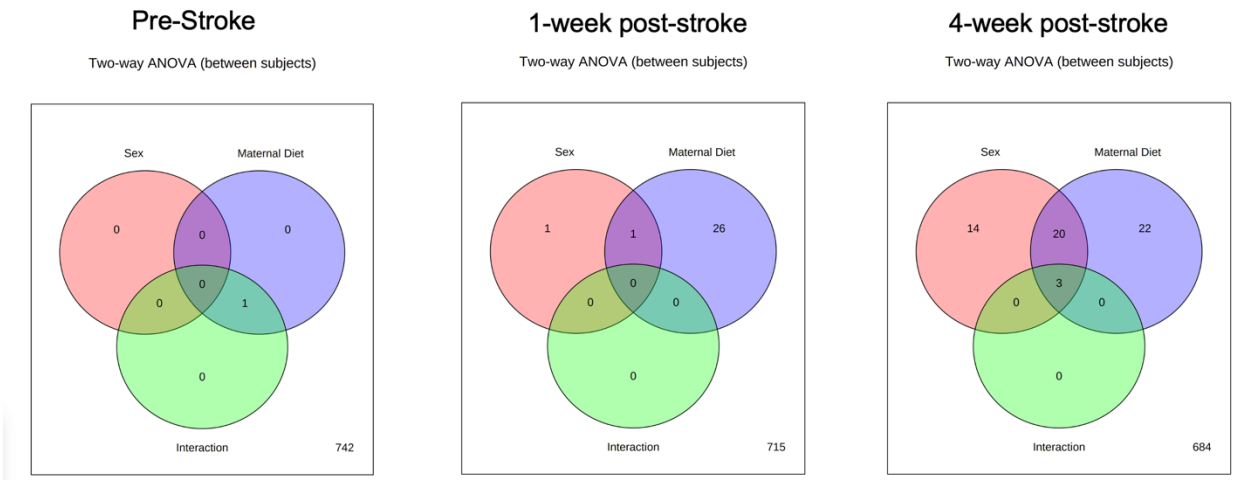


Figure 1. Summary of two-way ANOVA analysis demonstrating the metabolite changes based on sex, maternal diet, or interaction. (A) Demonstrate difference in one metabolite pre-stroke due to change in maternal diet. (B) Demonstrate metabolite changes based on sex, maternal diet, and combined effect of both maternal diet & sex in 1-week post-stroke with no interaction. (C) Significant and enhanced changes in metabolites seen at 4-weeks post stroke based on sex, maternal diet, or combined with 3 metabolites changes being affected differently by sex and maternal diet.

Pre-Stroke

At the pre-stroke time point, a significant difference in HEPES levels was observed between maternal diet groups ($p = 0.00052$), with female ChDD offspring having elevated levels of this metabolite (Table 1). There was also a difference between male and female offspring ($p = 0.0087$) and interaction between maternal diet and sex of animals ($p < 0.0001$).

One-Week Post-Stroke

126 ***Interactions between maternal diet and sex***

127 There were interactions between maternal diet and offspring sex in PALDA (Table 1; p =
128 0.213), 3-Oxopalmitic Acid (p = 0.001), 7-Dehydrocholesterol (p = 0.001),
129 Tetrahydrodeoxycorticosterone (p = 0.001), and Avenasterol (p = 0.011).

130

131 ***Impact of Maternal Diet***

132 After one-week post-stroke we observed maternal dietary differences in stool
133 measurements for the following metabolites, PALDA (Table 1; p = 0.0006), N6-(2-1Himidazol-
134 4-yl) ethyl)lysine (p = 0.001), 3-Oxopalmitic Acid (p = 0.017), 7-Dehydrocholesterol (p = 0.0001),
135 Yakkasterone (p = 0.0001), Tetrahydrodeoxycorticosterone (p = 0.013), Cromide BEM
136 (p=0.0063), certonardosterol (p = 0.0001), 4betamethylzymosterol-4-carbaldehyde (p = 0.001),
137 4a,10-Dihydroxy-2,2,6a,6b,9,9,12a-heptamethyl-2,3,4,4a,6,6a,6b (p = 0.04), 3-dehydro-6-
138 deoxoteasterone (p = 0.001), 2-Aminoethyl(2R)-3-[(1Z)-1-h(14Z)-14-Tricosen-10-one (p =
139 0.0001), 2-Aminoethyl(2R)-3-[(1Z)-1-h(14Z)-14-Tricosen-10-one (p = 0.0001), 1-(3,5-
140 Dihydroxyphenyl)-2-heptadecanone (p = 0.0001), (R)-palmiticmonoisopropanolamide (p =
141 0.0002), (R)-palmiticmonoisopropanolamide (p = 0.006), and (3S,5E,7E,24R)-(6,19,19-
142 ~2~H_3_)-9,10-Secocholesta-5,7,10-trie (p = 0.0001). Significant maternal diet differences in
143 metabolites were followed up with pairwise comparisons, details are listed in table 2.

144 ***Sex differences***

145 Sex differences were observed in in PALDA (p = 0.0307) and N6-(2-1Himidazol-4-yl)
146 ethyl) lysine (p = 0.0221). Significant sex differences were followed up with pairwise
147 comparisons, details are listed in Table 2.

148

149 ***Four-Week Post-Stroke***

150 ***Interactions between maternal diet and sex***

151 There was an interaction between maternal diet and offspring sex for the following
152 metabolites, HEPES (p = 0.032), 1-(4-Aminobutyl)urea (p = 0.0015), PRIMA-1 (p = 0.0005),
153 PRIMA-1 (p = 0.022), N-lauroylglycine (p = 0.0036), N~6~,N~6~-Dimethyllysine (p = 0.0088),
154 Misoprostol (p = 0.032), Gly-Leu (p = 0.003), Fenamole (p < 0.001), Chamazulene (p = 0.02),
155 Esmolol (p = 0.013), Dodecylethanolamide (p = 0.038), Creatine (p = 0.01), C2 Dihydroceramide
156 (p = 0.003), BAR501 (p = 0.006), APM (p = 0.024), Adenine (p = 0.012), Acetylcadaverine (p =
157 0.0038), Acetohydroxamic acid (p = 0.0084), 1-PP (p = 0.011), (4R)-5-Hydroxy-L-leucine (p =
158 0.003), and (+)-castanospermine (p = 0.028).

159 ***Impact of Maternal Diet***

160 At four weeks post-stroke there were differences in offspring metabolites, as a result of
161 maternal diet in the following: HEPES (p = 0.0072), 2,3-Dihydroxypropylstearate (p = 0.0004),
162 JWH 133 (p < 0.0001), Sphinganine (p = 0.053), 1-(4-Aminobutyl)urea (p = 0.025), Propoxur (p
163 = 0.003), phytosphingosine (p = 0.02), N-lauroylglycine (p = 0.021), nicaraven (p = 0.003),
164 N~6~,N~6~-Dimethyllysine (p = 0.049), Misoprostol (p = 0.036), lovastatin (p = 0.0081), Gly-
165 Leu (p=0.026), Chamazulene (p = 0.02), Esmolol (p = 0.0364), Dodecylethanolamide (p = 0.009),
166 BAR501(p = 0.042), APM (p = 0.038), 5-Hydroxytryptophol (p = 0.003), and (+)-
167 castanospermine (p = 0.026). Significant differences in metabolite levels as a result of maternal
168 diet was followed up with pairwise comparisons, details are listed in table 3.

169 ***Sex Differences***

170 There were sex differences in the following metabolites, n-Hexanamide (p=0.0019), N-
171 Acetyl-Lglutamic Acid (p = 0.051), Sphinganine (p = 0.02), Pyrrolidine (p = 0.04), Propoxur (p =

172 0.006), Phytosphingosine (p = 0.012), HMMNI (p = 0.0014), Chamazulene (p = 0.05), Esmolol (p
173 = 0.025), and 5-Hydroxytryptophol (p = 0.006). There were interactions between maternal diet and
174 sex in the following metabolites, n-Hexanamide (p = 0.0073), N-Acetyl-Lglutamic Acid (p =
175 0.0029), Homocitrulline (p = 0.0001), Sphinganine (p = 0.018), R-(+)-Etiracetam (p = 0.0005),
176 and Pyrrolidine (p = 0.01). Significant sex differences between females and males were followed
177 up with pair wise comparisons, details are listed in table 3.

178 **Discussion**

179 Ischemic stroke is a major health concern, with nutrition being a modifiable risk factor. We
180 have previously demonstrated offspring from female mice deficient in either folic acid or choline
181 have worse outcome after stroke (Clementson et al., 2022; Hurley et al., 2023; Pull et al., 2023) .
182 Metabolites are important marker to measure the changes in the gut microbiota, which can be
183 significant markers for different pathophysiological diseases in the body. We collected fecal
184 samples from female and male offspring whose mothers were maintained on either FADD, ChDD,
185 and CD. The fecal samples were collected at three timepoints including prior to, 1, and 4 weeks
186 post-ischemic stroke to measure metabolites using untargeted analyses. This untargeted
187 metabolomics analysis revealed a wide spectrum of detrimental effects caused by stroke and
188 maternal diet deficiencies in 3-month-old offspring. Our analysis revealed that maternal folic acid
189 dietary deficiency has significant impact on microbiota of offspring after ischemic stroke. The
190 results demonstrate that sex and maternal diet impact metabolites levels after ischemic stroke and
191 may impact outcomes.

192 At pre-stroke stage we observed changes in levels of HEPES because of maternal diet. The
193 role and involvement of these metabolites remain unclear. The differences in maternal diet and sex
194 impact on the stroke outcome become more prominent in 1-and 4-week post-stroke time points.

195 A total of 17 metabolites differences observed in different diet groups at 1 week post stroke.
196 FADD mice had lower level of metabolites compared to ChDD and CD, while ChDD mice had
197 similar metabolites level to CD mice. Male mice had lower levels of metabolites at 1 week post
198 stroke. Most of the observed metabolites observed in lower levels are involved in various survival
199 and developmental cellular mechanisms including cholesterol biogenesis, cell division,
200 macrophage polarization, and neuroprotection. Tertahydrodeoxycorticsterone, a neurosteroid,
201 have been observed to play a role in neuroprotection and neural analgesic affects (Kang et al.,
202 2021; Márk & Paragh, 2007; Mediratta et al., 2001; Patchev et al., 1997; Piette, 2020; Rupprecht,
203 1997; Wetzel et al., 1999; Womack et al., 2006). Another metabolite, 7-dehydrocholesterol
204 reductase (DHCR7) is an enzyme that catalyzes 7-dehydrocholesterol (7-DHC) to cholesterol, a
205 final step in the biogenesis of cholesterol (Huang et al., 2017; Waterham & Wanders, 2000; Xiao
206 et al., 2020). DHCR7 mutations in humans cause a clinical autosomal recessive genetic disease,
207 namely Smith-Lemli-Opitz syndrome (SLOS), which is characterized with multiple abnormalities
208 including growth deficiency, intellectual disability, and frequent infections(Honda et al., 1998).
209 JWH 133 activates cannabinoid 2 receptors in reduced ovarian ischemia-reperfusion injury due to
210 its antioxidant and anti-inflammatory effects (Aydogan Kirmizi et al., 2021; López-Dyck et al.,
211 2017; Onat et al., 2021; Wojcieszak et al., 2016). JWH-133 showed anti-obesity effects that
212 ameliorated pro-inflammatory M1 macrophage polarization through the Nrf2/HO-1 pathway (Q.
213 Wu et al., 2020). The lower levels of the involved metabolites seem to have effects on
214 neuroprotective mechanism during neurodevelopment in early age as well as have effects on the
215 immune systems and fatty acid mechanism. Dysfunction in such metabolites can also signal
216 towards growth deficiency and more prone to infections. However, the mechanism of the effects
217 remains unclear and require additional investigation.

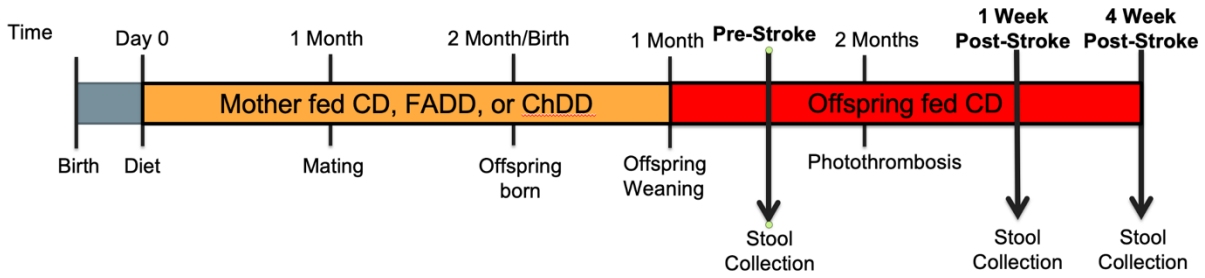
218 At 4-week post stroke, metabolites significantly lower compared to one-week timepoint
219 are observed to have come back to normal levels while new metabolites were found to be in
220 abnormal levels. Female mice were affected more adversely with lower levels of all the metabolites
221 found in 4-week post stroke compared to pre-stroke levels. Mice on either ChDD or FADD were
222 both at lower levels as compared mice of CD. Homocitrulline levels were lower in both genders
223 on FADD diet which plays role in cell viability and mitochondrial function in menadione-treated
224 astrocytes (Zanatta et al., 2016). N-Acetyl-glutamic acid, Noradrenaline, and Creatine which play
225 roles in liver ureagenesis, cognitive process, and cellular metabolism essential for cognitive
226 abilities respectively were found in lover levels (Caldovic et al., 2010; Harper et al., 2009;
227 Prokopová, 2010; Sumien et al., 2018). Other metabolites, Dehydrocostus which have antibacterial
228 activity by inhibiting LPS-induced production of proinflammatory mediators were in lower levels
229 (Nie et al., 2019). Metabolite which are indicators of central nervous system health and play
230 protective roles were detected at lower levels in female with both FADD and CHDD diet. Some
231 of the metabolites are (+)-Castanospermine involved in reduces inflammation in brain, 5-
232 Hydroxytryptophol responsible for elicits cerebro arterial contractions (Saul et al., 1983; Tharappel
233 et al., 2020), Acetylcadaverine lower levels is markedly found Alzheimer's Disease (Paik et al.,
234 2006), BAR501 responsible for reversing intestinal inflammation and shifts activation of intestinal
235 macrophages reducing expression of inflammatory genes (Biagioli et al., 2017). C2
236 Dihydroceramide associated with increased apoptosis in human leukemia HL-60 cells (Shikata et
237 al., 2003), and Gly-Leu, Glycine acts as a neurotransmitter in central nervous system; antioxidant,
238 anti-inflammatory, cryoprotective, and immunomodulatory in peripheral and nervous tissues and
239 leucine essential amino acid used in ATP generation, protein synthesis, tissue regeneration, and
240 metabolism (Pedroso et al., 2015; Razak et al., 2017).

241 Overall, after 4-week post stroke, it is strongly evident that female offspring that come
242 from mothers maintained on FADD or ChDD have more changes in their metabolites compared
243 to males. The changes are magnified at the four-week timepoint despite being on CD compared to
244 1 week post stroke. The abnormal metabolites levels suggest that there is significant inflammation
245 found both in brain and gut. It is indicated that the body's anti-inflammatory and defense system
246 is also highly affected. Interestingly, markers for Alzheimer's disease, cancers, gut, and brain
247 inflammation were found to be changed suggesting that these subjects might experience an early
248 physical deterioration and hindered development. In a previous study, there was evidence of
249 phenotypical behavioral issues in mice despite any changes in the ischemic size of the brain infarct.
250 This is suggestive of activation of multiple pathophysiological mechanisms that have long lasting
251 effects on a developing body. This is the first study to report changes in fecal sample metabolites
252 after changes in maternal diet and ischemic stroke. More work is required, as the mechanism and
253 molecular pathway are not clear. Possible next steps may include sequencing samples to determine
254 changes in DNA. The present study provides insights that maternal dietary deficiencies in folic
255 acid when combined with ischemic stroke impact offspring metabolites in a sex dependent manner.
256
257 **Materials and methods**

258 **Animals and experimental design**

259 All experiments were conducted in accordance with the guidelines of the Midwestern
260 University Institutional Animal Care Users Committee (IACUC 2983). C57/BL6J mice (both
261 female and male) were obtained from Jackson Laboratories. The breeding pairs produced male (n
262 = 17) and female (n = 15) offspring.

263 Experimental manipulations are summarized in Figure 2. Two-month-old female mice
264 were habituated for seven days before they were placed on either control (CD), folic acid (FADD,
265 folic acid: 0.3 mg/kg), or choline deficient diets (ChDD, choline bitrate: 300 mg/kg) (Clementson
266 et al., 2023; Dam et al., 2017; Hurley et al., 2023; Jadavji, Deng, et al., 2015; Jadavji, Farr, et al.,
267 2015) (Envigo, Indianapolis, IN). The control diet contains the minimum amount of folic acid (2
268 mg/kg) and choline bitrate (1150 mg/kg). The dams were maintained on the diets 4 weeks prior to
269 pregnancy, during pregnancy, and lactation. Once female and male offspring were weaned from
270 their mothers, they were maintained on a CD. At 2 months of age, the offspring were subjected to
271 ischemic stroke, using the photothrombosis model to the sensorimotor cortex. Stool samples were
272 collected from the same offspring at three timepoints: prior to ischemic stroke, 1 week after, and
273 4 weeks after the stroke.



274 **Figure 2.** Displays the timeline of the experiment. Pregnant mothers were fed either the CD,
275 FADD, or ChDD diet throughout the months of pregnancy and lactation until the offspring
276 were weaned. Once the offspring were weaned, they were maintained on the CD. At 2 months
of age, the offspring were subjected to ischemic stroke via the PT model. Tissue and fecal
matter was collected at 1-Week Post Stroke and 4-Week Post Stroke.

277

278 **Photothrombosis Model for Ischemic Stroke**

279 At 2 months of age all female and male offspring mice were subjected to photothrombosis
280 to induce a unilateral ischemic stroke in the sensorimotor cortex. Mice were anesthetized with

281 isoflurane (1.5%) in a 70:30 nitrous oxide: oxygen mixture. Core body temperature was monitored
282 with a rectal thermometer (Harvard Apparatus, Holliston, MA) and maintained at 37 ± 0.2ºC using
283 a heating blanket. 10mg/kg of the photosensitive Rose Bengal (Sigma, St. Louis, MA) dye was
284 injected intraperitoneally 5 minutes prior to irradiation. A 532 nm green laser was placed 3 cm
285 above the animal and directed to the sensorimotor cortex (mediolateral + 0.24mm)(Abato et al.,
286 2020; Clementson et al., 2023; Hurley et al., 2023; Jadavji et al., 2017, 2019, 2019; Lee et al.,
287 2004; Mbs et al., 2023; Poole et al., 2022) for 15 min.

288 **Fecal preparation**

289 Fecal samples were collected in a microfuge tube at three timepoints, prior to, 1 and 4
290 weeks after ischemic stroke. The samples were stored at -80°C until analysis.

291 Each fecal sample (~20 mg) was homogenized in 200 µL MeOH:PBS (4:1, v:v, containing
292 1,810.5 µM $^{13}C_3$-lactate and 142 µM $^{13}C_5$-glutamic Acid) in an Eppendorf tube using a Bullet
293 Blender homogenizer (Next Advance, Averill Park, NY). Then 800 µL MeOH:PBS (4:1, v:v,
294 containing 1,810.5 µM $^{13}C_3$-lactate and 142 µM $^{13}C_5$-glutamic Acid) was added, and after
295 vortexing for 10 s, the samples were stored at -20ºC for 30 min. The samples were then sonicated
296 in an ice bath for 30 min. The samples were centrifuged at 14,000 RPM for 10 min (4ºC), and 800
297 µL supernatant was transferred to a new Eppendorf tube. The samples were then dried under
298 vacuum using a CentriVap Concentrator (Labconco, Fort Scott, KS). Prior to MS analysis, the
299 obtained residue was reconstituted in 150 µL 40% PBS/60% ACN. A quality control (QC) sample
300 was pooled from all the study samples.

301

302 **Solutions for Experiments**

303 Solutions used to complete analysis included, acetonitrile (ACN), methanol (MeOH),
304 ammonium acetate, and acetic acid, all LC-MS grade, were purchased from Fisher Scientific
305 (Pittsburgh, PA). Ammonium hydroxide was bought from Sigma-Aldrich (Saint Louis, MO). DI
306 water was provided in-house by a Water Purification System from EMD Millipore (Billerica, MA).
307 PBS was bought from GE Healthcare Life Sciences (Logan, UT). The standard compounds
308 corresponding to the measured metabolites were purchased from Sigma-Aldrich (Saint Louis, MO)
309 and Fisher Scientific (Pittsburgh, PA).
310
311 **Untargeted LC-MS Metabolomics**
312 The untargeted LC-MS metabolomics method used has developed and used in a growing
313 number of studies (Garcia et al., 2024; Gu et al., 2015; D. Scieszka et al., 2023; D. P. Scieszka et
314 al., 2023; Wei et al., 2021). Briefly, all LC-MS experiments were performed using the Thermo
315 Vanquish UPLC-Exploris 240 Orbitrap MS instrument (Waltham, MA). Fecal samples were
316 injected twice, 10 µL for analysis using negative ionization mode and 4µL for analysis using
317 positive ionization mode. Both chromatographic separations were performed in hydrophilic
318 interaction chromatography (HILIC) mode on a Waters XBridge BEH Amide column (150 x 2.1
319 mm, 2.5 µm particle size, Waters Corporation, Milford, MA). The flow rate was 0.3 mL/min, auto-
320 sampler temperature was kept at 4℃, and the column compartment was set at 40℃. The mobile
321 phase was composed of Solvents A (10 mM ammonium acetate, 10 mM ammonium hydroxide in
322 95% H_2O/5% ACN) and B (10 mM ammonium acetate, 10 mM ammonium hydroxide in 95%
323 ACN/5% H_2O). After the initial 1 min isocratic elution of 90% B, the percentage of Solvent B
324 decreased to 40% at t = 11 min. The composition of Solvent B maintained at 40% for 4 min (t=15
325 min), and then the percentage of B gradually went back to 90%, to prepare for the next injection.

326 Using mass spectrometer equipped with an electrospray ionization (ESI) source, we will collect
327 untargeted data from 70 to 1050 m/z.

328 To identify peaks from the MS spectra, we made extensive use of the in-house chemical
329 standards (~600 aqueous metabolites), and in addition, we searched the resulting MS spectra
330 against the HMDB library, Lipidmap database, METLIN database, as well as commercial
331 databases including mzCloud, Metabolika, and ChemSpider. The absolute intensity threshold for
332 the MS data extraction was 1,000, and the mass accuracy limit was set to 5 ppm. Identifications
333 and annotations used available data for retention time (RT), exact mass (MS), MS/MS
334 fragmentation pattern, and isotopic pattern. Thermo Compound Discoverer 3.3 software was used
335 for aqueous metabolomics data processing. The untargeted data were processed by the software
336 for peak picking, alignment, and normalization. To improve rigor, only the signals/peaks with CV
337 < 20% across quality control (QC) pools, and the signals showing up in >80% of all the samples
338 were included for further analysis.

339

340 **Data Preprocessing**

341 Raw data files generated from the mass spectrometer were converted to a matrix of peak
342 intensities, with each row representing a sample and each column representing a metabolite
343 feature. Quality control samples were also included in the matrix for inter-batch normalization.
344 Raw data were normalized to the median of quality control samples.

345

346 **Multivariate Analysis**

347 Principal component analysis (PCA) and orthogonal partial least squares-discriminant
348 analysis (OPLS-DA) were performed to explore the separation of different groups and identify
349 important metabolites contributing to the separation.

350 To investigate metabolite differences between sex and maternal dietary groups, as well as
351 their interactions, two-way ANOVA was conducted using GraphPad Prism 10. Significant main
352 effects of two-way ANOVAs were followed by Tukey's post-hoc test to adjust for multiple
353 comparisons.

354 Statistical analysis was performed using R (version 4.1.1). For each metabolite, normality
355 was assessed using the Shapiro-Wilk test, which is suitable for small to medium sample sizes and
356 provides a good balance between sensitivity and specificity.

357 If the data for a metabolite followed a normal distribution ($p > 0.05$ in the Shapiro-Wilk
358 test), independent t-tests were used to compare the means between groups. If the data did not
359 follow a normal distribution ($p \leq 0.05$ in the Shapiro-Wilk test), non-parametric Wilcoxon rank-
360 sum tests were employed to compare the medians between groups. This test was chosen because
361 it does not assume normality and is robust against outliers. To adjust for multiple testing and
362 control the rate of type I errors, a false discovery rate (FDR) correction was performed using the
363 Benjamini-Hochberg procedure.

364 Linear regression analysis was conducted to assess the correlation between metabolites and
365 stroke outcomes. The regression models included sex, maternal diet, and their interaction terms to
366 determine the influence of these factors on metabolite levels.

367

368 **Pathway and Enrichment Analysis**

Pathway analysis was performed using MetaboAnalyst (version 5.0). The pathway analysis involved the use of the hypergeometric test and pathway topological analysis. The p-values were not Bonferroni corrected because the related hypotheses are not completely independent, but instead share underlying biological mechanisms or functional relationships. Enrichment analysis was performed to identify the over-representation of metabolites in specific pathways using Fisher's exact test.

Visualization

Heatmaps were generated to visualize the clustering of metabolites and samples. Pathway and enrichment analysis results were visualized using bubble plots and bar plots, respectively. The level of significance was set at $p < 0.05$ for all analyses.

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None

Competing Interests

No competing interest declared

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Data statement

All relevant data can be found within the article and supplementary material

Author contrition statement

Conceptualization: N.M.J.; Methodology: H.G., J.C.; Formal analysis: P.J., F.A., N.M.J; Writing-review & editing: F.A., M.T.M, P.J., H.G., N.M.J; Visualization: P.J., F.A., N.M.J; Project administration: N.M.J.; Funding acquisition: N.M.J.

Diversity and inclusion statement

All authors were given opportunities to be involved in the study and writing up of the study.

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687 **Table 1**. The impact of maternal dietary deficiencies in folic acid or choline on offspring fecal
688 metabolite levels prior to stroke, 1 and 4-week post-stroke, summary of two-way ANOVA results.

		Prior-Stroke			***p-values* Interaction Sex Maternal diet**	**1-week post-stroke**			***p-values* Interaction Sex Maternal diet**	**4-week post-stroke**			***p-values* Interaction Sex Maternal diet**
Metabolite		**CD**	**FADD**	**ChDD**		**CD**	**FADD**	**ChDD**		**CD**	**FADD**	**ChDD**	
HEPES	Females	5.56 ± 0.04	5.29 ± 0.02	6.41 ± 0.22	0.01	5.56 ± 0.09	5.06 ± 0.15	5.34 ± 0.02	n.s.	5.27 ± 0.03	5.41 ± 0.02	5.50 ± 0.06	0.032
	Males	5.68 ± 0.20	5.39 ± 0.04	5.41 ± 0.03	0.994	5.44 ± 0.09	5.12 ± 0.02	5.39 ± 0.04	n.s.	5.39 ± 0.03	5.44 ± 0.04	5.42 ± 0.03	n.s.
					0.00052				n.s.				0.0072
Fenamole	Female	6.52 + 0.15	6.44 + 0.10	6.12 + 0.15	n.s.	6.33 + 0.93	6.42 + 0.14	6.53 + 0.10	n.s.	5.89 + 0.14	6.52 + 0.067	6.12 + 0.085	0.0001
	Male	6.28 + 0.18	5.86 + 0.13	6.45 + 0.26	n.s.	6.21 + 0.14	6.51 + 0.07	6.31 + 0.17	n.s.	6.46 ± 0.12	5.92 ± 0.058	6.37 ± 0.042	n.s.
					n.s.				n.s.				n.s.
PALDA	Female	5.79 ± 0.19	4.37 + 0.05	5.41 ± 0.25	n.s.	5.80 ± 0.193	4.32 ± 0.07	5.41 ± 0.25	0.0213	4.93 ± 0.29	5.14 ± 0.14	5.32 ± 0.25	n.s.
	Male	5.04 ± 0.27	4.78 ± 0.19	4.68 ± 0.13	n.s.	5.04 ± 0.27	4.65 ± 0.19	4.69 ± 0.14	0.0307	5.18 ± 0.21	4.95 ± 0.18	5.20 ± 0.19	n.s.
					n.s.				0.0006				n.s.
N6-(2-1Himidazol-4-yl)ethyl)lysine	Females	5.05 ± 0.02	4.82 ± 0.03	5.04 ± 0.03	n.s.	5.05 ± 0.03	4.84 ± 0.03	5.04 ± 0.03	n.s.	4.90 + 0.05	4.99 ± 0.02	4.93 + 0.02	n.s.
	Males	4.96 ± 0.02	4.81 ± 0.02	4.97 ± 0.03	n.s.	4.97 ± 0.02	4.83 ± 0.02	4.97 ± 0.03	0.0221	5.00 ± 0.03	4.95 ± 0.03	4.98 + 0.03	n.s.
					n.s.				0.0001				n.s.

3-Oxopalmitic Acid	Female	4.87 ± 0.01	5.05 ± 0.07	5.00 ± 0.14	n.s.	4.87 ± 0.01	4.79 ± 0.08	4.86 ± 0.04	0.001	4.85 ± 0.12	5.36 ± 0.19	4.91 ± 0.08	n.s.
	Male	5.56 ± 0.13	4.80 ± 0.10	4.85 ± 0.03	n.s.	5.56 ± 0.13	5.15 ± 0.11	5.01 ± 0.14	n.s.	5.29 ± 0.25	4.89 ± 0.05	5.02 ± 0.12	n.s.
					n.s.				0.017				n.s.
7-Dehydrocholesterol	Female	6.26 ± 0.04	4.46 ± 0.01	6.08 ± 0.09	n.s.	6.27 ± 0.04	4.464 ± 0.01	6.087 ± 0.10	0.0001	5.66 ± 0.30	6.03 + 0.08	6.13 ± 0.16	n.s.
	Male	5.35 ± 0.18	4.98 ± 0.15	5.46 ± 0.13	n.s.	5.35 ± 0.19	5.352 ± 0.19	5.459 ± 0.13	n.n.	5.70 ± 0.11	5.66 ± 0.19	5.65 ± 0.03	n.s.
					n.s.				0.0001				n.s.
Yakkasterone	Female	6.49 ± 0.07	4.73 ± 0.05	6.58 ± 0.07	n.s.	6.05 ± 0.16	4.89 ± 0.26	6.097 ± 0.10	n.s.	6.11 ± 0.10	6.41 + 0.05	6.41 ± 0.08	n.s.
	Male	6.26 ± 0.37	5.78 ± 0.27	6.45 ± 0.16	n.s.	6.21 ± 0.35	5.01 ± 0.13	6.23 ± 0.10	n.s.	6.40 ± 0.02	6.50 ± 0.06	6.46 ± 0.02	n.s.
					n.s.				0.0001				n.s.
Tetrahydrodeoxycorticosterone	Female	6.05 ± 0.16	4.87 ± 0.03	6.09 ± 0.09	n.s.	6.49 ± 0.38	6.58 ± 0.33	4.68 ± 0.16	0.001	5.17 ± 0.16	5.81 ± 0.14	5.66 ± 0.14	n.s.
	Male	6.21 ± 0.35	5.13 ± 0.06	6.29 ± 0.09	n.s.	6.26 ± 0.08	5.53 ± 0.08	6.45 ± 0.06	n.s.	5.75 ± 0.21	5.59 ± 0.18	5.62 ± 0.16	n.s.
					n.s.				0.013				n.s.
Avenasterol	Female	6.18 ± 0.17	6.28 ± 0.14	6.20 ± 0.05	n.s.	6.36 ± 0.11	4.64 ± 0.04	6.08 ± 0.10	0.011	5.80 ± 0.21	6.24 ± 0.08	6.33 ± 0.11	n.s.
	Male	6.37 ± 0.04	6.23 ± 0.23	6.29 ± 0.11	n.s.	5.86 ± 0.28	5.50 ± 0.30	6.17 ± 0.09	n.s.	6.29 ± 0.08	6.32 ± 0.09	6.49 ± 0.05	n.s.

					n.s.				n.s.				n.s.
4betamethylzymosterol-4-carbaldehyde	Female	6.12 ± 0.16	6.05 ± 0.31	6.25 ± 0.08	n.s.	6.52 ± 0.06	5.01 ± 0.04	6.44 ± 0.12	n.s.	6.16 ± 0.04	6.26 ± 0.08	6.36 ± 0.13	n.s.
	Male	6.11 ± 0.05	6.26 ± 0.08	6.13 ± 0.14	n.s.	6.16 ± 0.36	5.69 ± 0.26	6.59 + 0.12	n.s.	6.42 ± 0.13	6.24 ± 0.17	6.36 ± 0.03	n.s.
					n.s.				0.001				n.s.
4a,10-Dihydroxy-2,2,6a,6b,9,9,12a-heptamethyl-2,3,4,4a,6,6a,6b	Female	5.97 ± 0.19	6.15 ± 0.42	6.18 ± 0.100	n.s.	6.71 ± 0.09	5.12 ± 0.20	6.713 ± 0.13	n.s.	6.27 ± 0.15	6.47 ± 0.14	6.54 ± 0.13	n.s.
	Male	6.20 ± 0.12	6.24 ± 0.26	6.19 ± 0.20	n.s.	6.38 ± 0.43	5.88 ± 0.40	6.907 ± 0.07	n.s.	6.74 ± 0.07	6.62 ± 0.04	6.60 ± 0.05	n.s.
					n.s.				0.004				n.s.
3-dehydro-6-deoxoteasterone	Female	6.11 ± 0.15	6.14 ± 0.13	6.23 ± 0.08	n.s.	6.14 ± 0.16	4.87 ± 0.09	5.83 ± 0.15	n.s.	5.74 ± 0.29	6.23 ± 0.13	6.18 ± 0.12	n.s.
	Male	6.25 ± 0.11	6.13 ± 0.13	6.20 ± 0.13	n.s.	5.96 ± 0.21	5.48 ± 0.26	6.01 ± 0.03	n.s.	6.37 ± 0.07	6.25 ± 0.12	6.25 ± 0.09	n.s.
					n.s.				0.0001				n.s.
2-Aminoethyl(2R)-3-[(1Z)-1-h(14Z)-14-Tricosen-10-one	Female	5.59 ± 0.10	5.97 ± 0.27	5.68 ± 0.18	n.s.	6.18 ± 0.19	4.89 ± 0.11	5.74 ± 0.30	n.s.	5.53 ± 0.34	6.17 ± 0.088	5.97 ± 0.18	n.s.
	Male	5.76 ± 0.21	5.27 ± 0.22	5.59 ± 0.16	n.s.	5.71 ± 0.21	5.16 ± 0.13	5.96 ± 0.081	n.s.	6.10 ± 0.34	6.02 ± 0.14	6.07 ± 0.078	n.s.
					n.s.				0.0001				n.s.
1-Piperidinocyclohexanecarbonitrile	Female	6.12 ± 0.11	6.53 ± 0.16	6.12 ± 0.11	n.s.	6.41 ± 0.18	5.75 ± 0.12	6.69 ± 0.14	n.s.	6.37 ± 0.21	6.54 ± 0.11	6.38 ± 0.19	n.s.
	Male	6.29 ± 0.05	6.12 ± 0.18	6.30 ± 0.10	n.s.	6.28 ± 0.15	5.70 ± 0.06	6.52 ± 0.11	n.s.	6.35 ±	6.29 ± 0.14	6.29 ± 0.13	n.s.

										0.13			
					n.s.				0.0001				n.s.
1-(3,5-Dihydroxyphenyl)-2-heptadecanone	Female	5.95 ± 0.08	6.02 ± 0.30	5.93 ± 0.10	n.s.	6.13 ± 0.07	4.66 ± 0.07	6.00 ± 0.13	n.s.	5.15 ± 0.12	5.76 ± 0.21	5.79 ± 0.18	n.s.
	Male	5.86 ± 0.10	5.92 ± 0.10	6.07 ± 0.13	n.s.	6.23 ± 0.40	4.82 ± 0.07	6.30 ± 0.14	n.s.	5.93 ± 0.13	5.59 ± 0.23	5.63 ± .019	n.s.
					n.s.				0.0001				n.s.
(R)-palmiticmonoisopropanolamide	Female	6.23572978	6.1420727	6.07534708	n.s.	6.46 ± 0.09	3.81 ± 0.03	6.10 ± 0.31	0.05	5.82 ± 0.46	6.11 ± 0.16	6.53 ± 0.13	n.s.
	Male	6.20827593	6.20034822	6.26524215	n.s.	5.78 ± 0.48	5.15 ± 0.61	6.49 ± 0.12	n.s.	6.36 ± 0.08	6.35 ± 0.08	6.48284968	n.s.
					n.s.				0.0002				n.s.
(22S)-22-hydroxycampest-4-en-3-one	Female	6.93 ± 0.17	6.91 ± 0.25	7.06 + 0.11	n.s.	6.81 ± 0.16	4.73 ± 0.02	6.53 ± 0.18	0.0099	6.69 ± 0.08	6.83 ± 0.05	6.98 ± 0.15	n.s.
	Male	7.06 ± 0.06	6.78 ± 0.27	6.75 ± 0.17	n.s.	6.23 ± 0.37	6.00 ± 0.41	6.40 ± 0.21	n.s.	6.86 ± 0.09	6.92 ± 0.10	7.02 ± 0.06	n.s.
					n.s.				0.006				n.s.
(14Z)-14-Tricosen-10-one	Female	6.93 ± 0.17	6.89 ± 0.27	7.06 ± 0.11	n.s.	6.81 ± 0.16	4.84 ± 0.04	6.44 ± 0.39	0.0004	6.69 ± 0.08	6.83 ± 0.05	6.98 ± 0.15	n.s.
	Male	7.06 ± 0.06	6.78 ± 0.27	6.75 ± 0.18	n.s.	6.24 ± 0.36	6.58 ± 0.12	6.02 ± 0.21	n.s.	6.86 ± 0.09	6.92 ± 0.10	7.02 ± 0.06	n.s.
					n.s.				0.0208				n.s.
(3S,5E,7E,24R)-(6,19,19-~2~H_3_)-9,10-Secocholesta-5,7,10-trie	Female	5.80 ± 0.21	5.93 ± 0.33	5.71 ± 0.14	n.s.	5.92 ± 0.09	4.35 ± 0.02	5.98 ± 0.09	n.s.	5.45 ± 0.11	5.74 ± 0.11	5.82 ± 0.09	n.s.
	Male	5.55 ± 0.26	5.94 ± 0.07	5.93 ± 0.07	n.s.	5.78 ±	5.01 ±	5.93 ±	n.s.	5.81	5.92 ±	5.86 ± 0.04	n.s.

						0.32	0.26	1.00		± 0.01	0.08		
					n.s.				0.0001				n.s.
2,3-Dihydroxypropylstearate	Female	6.06 ± 0.08	6.29 ± 0.17	5.98 ± 0.14	n.s.	6.27 ± 0.22	5.90 ± 0.21	6.65 ± 0.13	n.s.	6.43 ± 0.21	6.41 ± 0.12	6.32 ± 0.21	n.s.
	Male	6.17 ± 0.03	5.98 ± 0.13	6.04 ± 0.10	n.s.	6.27 ± 0.11	5.69 ± 0.03	6.30 ± 0.13	n.s.	6.24 ± 0.15	6.13 ± 0.07	6.27 ± 0.11	n.s.
					n.s.				n.s.				0.0004
n-Hexanamide	Female	6.49 ± 0.06	6.52 ± 0.09	6.24 ± 0.16	n.s.	6.49 ± 0.07	6.52 ± 0.09	6.24 ± 0.16	n.s.	5.89 ± 0.29	6.69 ± 0.04	6.67 ± 0.02	0.0073
	Male	6.62 ± 0.37	6.89 ± 0.41	7.12 ± 0.31	n.s.	6.62 ± 0.37	6.89 ± 0.41	7.12 ± 0.32	n.s.	7.25 ± 0.13	7.25 ± 0.35	6.57 ± 0.30	0.0019
					n.s.				n.s.				n.s.
N-Acetyl-Lglutamic Acid	Female	6.67 ± 0.42	6.79 ± 0.14	7.02 ± 0.07	n.s.	6.67 ± 0.42	6.79 ± 0.14	7.02 ± 0.07	n.s.	7.01 ± 0.15	7.27 ± 0.12	7.24 ± 0.10	0.0029
	Male	7.16 ± 0.07	7.11 ± 0.13	7.26 ± 0.25	n.s.	7.17 ± 0.07	7.11 ± 0.13	7.26 ± 0.25	n.s.	7.75 ± 0.11	7.18 ± 0.11	7.20 ± 0.13	0.051
					n.s.				n.s.				n.s.
JWH 133	Female	7.43 ± 0.05	5.73 ± 0.16	7.31 ± 0.09	n.s.	7.43 ± 0.05	5.479 ± 0.29	7.22 ± 0.11	n.s.	6.48 ± 0.29	6.68 ± 0.08	6.75 ± 0.07	n.s.
	Male	6.87 ± 0.44	6.51 ± 0.39	7.31 ± 0.06	n.s.	6.88 ± 0.44	6.19 ± 0.44	7.31 ± 0.06	n.s.	6.63 ± 0.06	6.68 ± 0.07	6.74 ± 0.05	n.s.
					n.s.				n.s.				<0.0001
Homocitrulline	Female	6.22 ± 0.22	5.90 ± 0.17	5.88 ± 0.04	n.s.	5.68 ± 0.03	6.28 ± 0.1	5.91 ± 0.07	n.s.	5.68 ± 0.03	6.27 ± 0.09	5.91 ± 0.07	0.0001

	Male	5.98 ± 0.08	5.48 ± 0.34	6.05 ± 0.10	n.s.	6.31 ± 0.08	5.72 ± 012	5.94 ± 0.16	n.s.	6.32 ± 0.08	5.72 ± 0.12	5.94 ± 0.16	n.s.
					n.s.				n.s.				n.s.
Sphinganine	Female	6.48 ± 0.03	6.54 ± 0.02	6.45 ± 0.04	n.s.	6.51 ± 0.02	6.57 ± 0.05	6.53 ± 0.05	n.s.	6.73 ± 0.17	7.14 ± 0.03	7.07 ± 0.03	0.018
	Male	6.48 ± 0.03	6.52 ± 0.04	6.53 ± 0.02	n.s.	6.54 ± 0.04	6.68 ± 0.12	6.56 ± 0.02	n.s.	7.16 ± 0.03	7.11 ± 0.03	7.07 ± 0.01	0.02
					n.s.				n.s.				0.053
R-(+)-Etiracetam	Female	5.47 ± 0.29	5.85 ± 0.17	5.63 ± 0.14	n.s.	5.61 ± 0.16	5.70 ± 0.16	5.57 ± 0.12	n.s.	5.21 ± 0.22	5.99 + 0.04	5.71 ± 0.05	0.0005
	Male	5.68 ± 0.16	5.56 ± 0.09	5.44 ± 0.16	n.s.	5.88 ± 0.16	5.07 + 0.29	5.50 ± 0.13	n.s.	5.81 ± 0.06	5.60 ± 0.10	5.57 ± 0.09	n.s.
					n.s.				n.s.				n.s.
Pyrrolidine	Female	5.81 ± 0.15	5.89 ± 0.18	5.78 ± 0.19	n.s.	5.93 + 0.14	5.82 ± 0.01	5.74 ± 0.09	n.s.	5.59 ± 0.10	6.08 ± 0.12	5.75 ± 0.12	0.01
	Male	5.84 ± 0.13	5.88 ± 0.09	5.84 ± 0.09	n.s.	5.98 ± 0.14	5.50 ± 0.09	5.78 ± 0.16	n.s.	6.14 ± 0.09	5.91 ± 0.12	5.93 ± 0.10	0.04
					n.s.				n.s.				n.s.
1-(4-Aminobutyl)urea	Female	5.19 ± 0.33	5.11 ± 0.17	5.21 ± 0.23	n.s.	5.07 ± 0.09	4.88 ± 0.23	5.56 ± 0.29	n.s.	4.61 ± 0.19	5.94 ± 0.08	5.44 ± 0.17	0.0015
	Male	5.52 ± 0.23	5.28 ± 0.28	4.71 ± 0.19	n.s.	5.18 ± 0.15	4.56 ± 0.45	5.08 ± 0.21	n.s.	5.40 ± 0.09	5.15 ± 0.34	5.30 ± 0.24	n.s.
					n.s.				n.s.				0.025
Propoxur	Female	5.90 ± 0.14	5.72 ± 0.12	5.92 ± 0.07	n.s.	5.70 ± 0.29	5.81 ± 0.16	5.97 ± 0.06	n.s.	5.31 ±	6.017 ±	5.93 ± 0.07	n.s.

										0.19	0.07		
	Male	5.63 ± 0.24	6.01 ± 0.08	5.67 ± 0.14	n.s.	5.63 ± 0.24	5.61 ± 0.19	5.44 ± 0.16	n.s.	5.94 ± 0.14	6.072 ± 0.09	6.18 ± 0.14	0.006
					n.s.				n.s.				0.003
PRIMA-1	Female	5.46 ± 0.29	5.85 ± 0.17	5.62 ± 0.14	n.s.	5.61 ± 0.16	5.70 ± 0.16	5.57 ± 0.12	n.s.	5.20 ± 0.22	6.00 ± 0.04	5.71 ± 0.05	0.0005
	Male	5.68 ± 0.16	5.56 ± 0.14	5.44 0.16	n.s.	5.88 ± 0.16	5.07 ± 0.29	5.50 ± 0.13	n.s.	5.81 ± 0.06	5.60 ± 0.10	5.57 ± 0.09	n.s.
					n.s.				n.s.				n.s.
Phytosphingosine	Female	7.17 ± 0.04	7.04 ± 0.17	7.16 ± 0.08	n.s.	7.29 ± 0.02	7.07 ± 0.09	7.28 ± 0.05	n.s.	7.56 ± 0.21	8.14 ± 0.19	8.02 ± 0.07	0.022
	Male	7.11 ± 0.07	7.01 ± 0.17	7.15 ± 0.06	n.s.	7.17 ± 0.09	7.41 ± 0.22	7.21 ± 0.04	n.s.	8.13 ± 0.07	8.13 ± 0.018	8.15 ± 0.02	0.012
					n.s.				n.s.				0.02
N-lauroylglycine	Female	6.28 ± 0.04	6.24 ± 0.07	6.37 ± 0.08	n.s.	5.94 ± 0.06	5.52 ± 0.18	5.98 ± 0.11	n.s.	5.96 ± 0.13	6.46 ± 0.04	6.50 ± 0.09	0.0036
	Male	6.35 ± 0.02	6.18 ± 0.05	6.25 ± 0.07	n.s.	5.98 ± 0.19	6.02 ± 0.09	6.24 ± 0.07	n.s.	6.34 ± 0.09	6.22 ± 0.11	6.32 ± 0.04	n.s.
					n.s.				n.s.				0.021
Nicaraven	Female	5.19 ± 0.09	5.08 ± 0.09	5.21 ± 0.07	n.s.	5.09 ± 0.12	5.38 ± 0.11	5.18 ± 0.05	n.s.	5.24 ± 0.09	4.88 ± 0.07	4.84 ± 0.05	n.s.
	Male	5.21 ± 0.08	5.33 ± 0.06	5.19 ± 0.09	n.s.	5.09 ± 0.10	5.28 ± 0.08	5.04 ± 0.06	n.s.	5.06 ± 0.07	5.02 ± 0.10	4.94 ± 0.03	n.s.
					n.s.				n.s.				0.003
N~6~,N~6~-Dimethyllysine	Female	5.45 ± 0.17	5.49 ± 0.12	5.45 ± 0.16	n.s.	5.52 ±	5.52 ±	5.36 ±	n.s.	5.05	5.78 ±	5.47 ± 0.13	0.0088

						0.19	0.09	0.06		± 0.14	0.07		
	Male	5.60 ± 0.08	5.59 ± 0.12	5.35 ± 0.14	n.s.	5.57 ± 0.13	5.18 ± 0.35	5.55 ± 0.17	n.s.	5.45 ± 0.12	5.31 ± 0.16	5.63 ± 0.18	n.s.
					n.s.				n.s.				0.049
Misoprostol	Female	6.37 ± 0.02	6.34 ± 0.05	6.27 ± 0.07	n.s.	6.37 ± 0.06	6.53 ± 0.01	6.42 ± 0.03	n.s.	6.18 ± 0.14	5.85 ± 0.08	5.69 ± 0.05	0.032
	Male	6.35 ± 0.04	6.39 ± 0.04	6.41 ± 0.04	n.s.	6.41 ± 0.05	6.17 ± 0.22	6.38 ± 0.04	n.s.	5.88 ± 0.05	5.82 ± 0.11	5.91 ± 0.10	n.s.
					n.s.				n.s.				0.036
Lovastatin	Female	7.15 ± 0.02	7.07 ± 0.06	7.05 ± 0.07	n.s.	6.98 ± 0.05	7.39 ± 0.04	7.09 ± 0.05	n.s.	6.86 ± 0.13	6.43 ± 0.10	6.52 ± 0.05	n.s.
	Male	7.09 ± 0.04	7.07 ± 0.07	7.08 ± 0.03	n.s.	7.10 ± 0.06	7.01 ± 0.021	7.11 ± 0.05	n.s.	6.61 ± 0.04	6.50 ± 0.05	6.53 ± 0.08	n.s.
					n.s.				n.s.				0.0081
HMMNI	Female	5.83 ± 0.15	5.81 ± 0.11	5.68 ± 0.05	n.s.	5.67 ± 0.03	5.37 ± 0.10	5.31 ± 0.39	n.s.	4.87 ± 0.19	5.48 ± 0.23	5.50 ± 0.05	n.s.
	Male	6.11 ± 0.09	6.04 ± 0.12	5.91 ± 0.10	n.s.	5.58 ± 0.32	5.96 ± 0.13	6.25 ± 0.18	n.s.	6.08 ± 0.19	6.03 ± 0.19	5.78 ± 0.44	0.0014
					n.s.				n.s.				n.s.
Gly-Leu	Female	5.74 ± 0.41	5.98 ± 0.16	5.72 ± 0.22	n.s.	5.95 ± 0.08	5.66 ± 0.29	5.82 ± 0.05	n.s.	5.38 ± 0.10	6.46 ± 0.13	5.96 ± 0.11	0.003
	Male	6.20 ± 0.23	6.00 ± 0.10	5.81 ± 0.14	n.s.	6.19 ± 0.21	5.20 ± 0.45	5.89 ± 0.11	n.s.	6.21 ± 0.15	6.07 ± 0.30	5.88 ± 0.20	n.s.
					n.s.				n.s.				0.026

Chamazulene	Female	6.30 ± 0.06	6.27 ± 0.07	6.29 ± 0.08	n.s.	6.46 ± 0.02	6.02 ± 0.08	6.39 ± 0.12	n.s.	5.78 ± 0.35	5.01 ± 0.01	4.929 ± 0.02	0.02
	Male	6.24 ± 0.06	6.23 ± 0.08	6.22 ± 0.05	n.s.	6.34 ± 0.08	5.94 ± 0.37	6.33 ± 0.06	n.s.	4.99 ± 0.03	4.97 ± 0.03	4.99 ± 0.02	0.05
					n.s.				n.s.				0.02
Esmolol	Female	5.49 ± 0.13	5.56 ± 0.10	5.59 ± 0.09	n.s.	5.74 ± 0.11	5.95 ± 0.03	5.99 ± 0.10	n.s.	6.00 ± 0.22	6.39 ± 0.07	6.43 ± 0.04	0.013
	Male	5.81 ± 0.11	5.65 ± 0.07	5.61 ± 0.14	n.s.	5.78 ± 0.19	5.90 ± 0.25	6.02 ± 0.06	n.s.	6.59 ± 0.03	6.51 ± 0.08	6.35 ± 0.06	0.025
					n.s.				n.s.				0.0364
Dodecylethanolamide	Female	6.39 ± 0.05	6.54 ± 0.07	6.43 ± 0.07	n.s.	6.51 ± 0.04	6.63 ± 0.05	6.63 ± 0.06	n.s.	6.38 ± 0.09	6.15 ± 0.03	6.16 ± 0.01	0.038
	Male	6.46 ± 0.04	6.42 ± 0.04	6.46 ± 0.04	n.s.	6.59 ± 0.05	6.53 ± 0.15	6.63 ± 0.05	n.s.	6.24 ± 0.02	6.23 ± 0.03	6.20 ± 0.02	n.s.
					n.s.				n.s.				0.009
Creatine	Female	7.15 ± 0.11	6.93 ± 0.15	7.18 ± 0.07	n.s.	7.39 ± 0.21	6.80 ± 0.08	7.69 ± 0.15	n.s.	7.21 ± 0.17	6.86 ± 0.20	7.06 ± 0.14	0.01
	Male	6.89 ± 0.16	6.89 ± 0.13	6.91 ± 0.11	n.s.	6.39 ± 0.29	7.12 ± 0.40	7.45 ± 0.37	n.s.	6.21 ± 0.22	6.98 ± 0.10	7.15 ± 0.33	n.s.
					n.s.				n.s.				n.s.
C2 Dihydroceramide	Female	5.44 ± 0.07	5.53 ± 0.06	5.46 ± 0.10	n.s.	5.55 ± 0.01	5.47 ± 0.01	5.68 ± 0.04	n.s.	5.55 ± 0.09	5.87 ± 0.06	5.88 ± 0.03	0.003
	Male	5.49 ± 0.13	5.45 ± 0.03	5.46 ± 0.05	n.s.	5.61 ± 0.07	5.53 ± 0.06	5.57 ± 0.03	n.s.	5.93 ± 0.04	5.73 ± 0.13	5.84 ± 0.05	n.s.
					n.s.				n.s.				n.s.

BAR501	Female	4.59 ± 0.04	4.71 ± 0.05	4.61 ± 0.04	n.s.	5.46 ± 0.22	4.52 ± 0.06	5.66 ± 0.41	n.s.	4.61 ± 0.06	5.15 ± 0.04	4.96 ± 0.09	0.006
	Male	4.64 ± 0.05	4.64 ± 0.07	4.72 ± 0.06	n.s.	5.29 ± 0.19	4.55 ± 0.16	5.52 ± 0.19	n.s.	5.06 ± 0.08	4.99 ± 0.11	4.97 ± 0.15	n.s.
					n.s.				n.s.				0.042
APM	Female	5.35 ± 0.29	6.06 ± 0.14	5.81 ± 0.27	n.s.	5.76 ± 0.24	5.87 ± 0.12	5.89 ± 0.24	n.s.	5.59 ± 0.26	6.42 ± 0.07	6.21 ± 0.10	0.024
	Male	6.01 ± 0.15	6.04 ± 0.23	5.79 ± 0.24	n.s.	5.77 ± 0.17	5.36 ± 0.39	6.12 ± 0.15	n.s.	6.16 ± 0.12	6.13 ± 0.14	6.14 ± 0.13	n.s.
					n.s.				n.s.				0.038
Adenine	Female	6.79 ± 0.15	7.13 ± 0.18	6.64 ± 0.29	n.s.	6.81 ± 0.17	6.48 ± 0.23	6.89 ± 0.26	n.s.	6.51 ± 0.14	7.04 ± 0.20	6.91 ± 0.21	0.012
	Male	7.04 ± 0.20	7.06 ± 0.14	6.71 ± 0.08	n.s.	6.96 ± 0.12	6.24 ± 0.24	6.97 ± 0.17	n.s.	7.39 ± 0.16	6.76 ± 0.19	7.00 ± 0.15	n.s.
					n.s.				n.s.				n.s.
Acetylcadaverine	Female	5.35447326	6.19094134	5.33010269	n.s.	5.37 ± 0.02	5.85 ± 0.47	5.32 ± 0.03	n.s.	5.31 ± 0.01	5.80 ± 0.16	5.42 ± 0.05	0.0038
	Male	5.6323512	5.60670829	5.51076697	n.s.	5.45 ± 0.04	5.32 ± 0.03	5.99 ± 0.50	n.s.	5.58 ± 0.07	5.44 ± 0.06	5.38 ± 0.03	n.s.
					n.s.				n.s.				0.041
Acetohydroxamic acid	Female	7.51 ± 0.06	7.63 ± 0.10	7.44 ± 0.11	n.s.	7.71 ± 0.18	7.46 ± 0.12	7.65 ± 0.07	n.s.	7.31 ± 0.08	7.75 ± 0.07	7.48 ± 0.09	0.0084
	Male	7.56 ± 0.13	7.64 ± 0.05	7.61 ± 0.08	n.s.	7.65 ± 0.12	7.18 ± 0.16	7.56 ± 0.06	n.s.	7.83 ± 0.12	7.53 ± 0.11	7.73 ± 0.17	n.s.
					n.s.				n.s.				n.s.

5-Hydroxytryptophol	Female	5.90 ± 0.14	5.72 ± 0.12	5.92 ± 0.07	n.s.	5.71 ± 0.28	5.81 ± 0.16	5.96 ± 0.07	n.s.	5.32 ± 0.14	6.02 ± 0.07	5.93 ± 0.07	n.s.
	Male	5.63 ± 0.24	6.01 ± 0.08	5.67 ± 0.14	n.s.	5.63 ± 0.24	5.61 ± 0.19	5.45 ± 0.16	n.s.	5.94 ± 0.19	6.07 ± 0.09	6.18 ± 0.14	0.006
					n.s.				n.s.				0.003
1-PP	Female	5.75 ± 0.09	5.93 ± 0.12	5.73 ± 0.12	n.s.	5.97 ± 0.14	5.78 ± 0.12	5.94 ± 0.08	n.s.	5.61 ± 0.08	6.07 ± 0.08	5.79 ± 0.10	0.011
	Male	5.85 ± 0.14	5.96 ± 0.05	5.91 ± 0.09	n.s.	5.94 ± 0.12	5.05 ± 0.26	5.79 ± 0.04	n.s.	6.14 ± 0.13	5.85 ± 0.12	6.01 ± 0.16	n.s.
					n.s.				n.s.				n.s.
(4R)-5-Hydroxy-L-leucine	Female	6.27 ± 0.23	6.28 ± 0.19	6.13 ± 0.21	n.s.	6.28 ± 0.04	5.34 ± 0.53	6.06 + 0.07	n.s.	5.91 ± 0.16	6.66 ± 0.10	6.20 ± 0.13	0.003
	Male	6.55 ± 0.17	6.37 ± 0.12	6.23 ± 0.12	n.s.	6.64 ± 0.10	5.62 ± 0.26	6.26 ± 0.15	n.s.	6.64 ± 0.13	6.25 ± 0.25	6.37 ± 0.08	n.s.
					n.s.				n.s.				n.s.
(+)-castanospermine	Female	5.41 ± 0.24	5.59 ± 0.15	5.51 ± 0.09	n.s.	5.61 ± 0.05	5.69 ± 0.22	5.71 ± 0.14	n.s.	5.05 ± 0.29	5.94 ± 0.08	5.59 ± 0.10	0.028
	Male	5.17 ± 0.19	5.54 ± 0.09	5.28 ± 0.17	n.s.	5.38 ± 0.19	5.07 ± 0.18	5.58 ± 0.20	n.s.	5.60 ± 0.08	5.59 ± 0.10	5.69 ± 0.14	n.s.
					n.s.				n.s.				0.026

Values are expressed as the mean ± SEM. Abbreviations: CD: Control diet; ChDD: choline-deficient diet; FADD: folic acid-deficient diet.

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702 **Table 2**. One-week post-stroke Tukey's pairwise comparison summary determining the impact of
703 maternal dietary deficiencies in folic acid or choline on offspring fecal metabolite levels.
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	Female				Male		
Metabolite	**CD vs. FADD**	**CD vs. ChDD**	**ChDD vs. FADD**		**CD vs. FADD**	**CD vs. ChDD**	**ChDD vs. FADD**
PALDA	0.002	n.s.	0.0045		n.s.	n.s.	n.s.
N6-(2-1Himidazol-4-yl)ethyl)lysine	0.0002	n.s.	0.0003		0.0044	n.s.	0.0002
3-Oxopalmitic Acid	n.s.	n.s.	n.s.		< 0.001	< 0.001	n.s.
7-Dehydrocholesterol	< 0.001	< 0.001	n.s.		n.s.	n.s.	n.s.
Yakkasterone	0.0013	n.s.	0.001		0.003	n.s.	0.0001
Tetrahydrodeoxycorticosterone	n.s.	0.0002	0.0001		n.s.	n.s.	0.0276
Avenasterol	0.001	n.s.	0.0003		n.s.	n.s.	n.s.
4betamethylzymosterol-4-carbaldehyde	0.0004	n.s.	0.0007		n.s.	n.s.	0.0004
4a,10-Dihydroxy-2,2,6a,6b,9,9,12a-heptamethyl-2,3,4,4a,6,6a,6b	0.0034	n.s.	0.003		n.s.	n.s.	0.0354
3-dehydro-6-deoxoteasterone	0.0003	n.s.	0.0043		n.s.	n.s.	n.s.
2-Aminoethyl(2R)-3-[(1Z)-1-h(14Z)-14-Tricosen-10-one	0.0008	n.s.	0.0016		n.s.	n.s.	0.0306
1-Piperidinocyclohexanecarbonitrile	0.0085	n.s.	0.0002		0.0003	n.s.	0
1-(3,5-Dihydroxyphenyl)-2-heptadecanone	0.003	n.s.	0.0001		0.0009	n.s.	n.s.
(R)-palmiticmonoisopropanolamide	0.004	n.s.	0.0004		n.s.	n.s.	0.0017
(22S)-22-hydroxycampest-4-en-3-one	0.0002	n.s.	0.0006		n.s.	n.s.	n.s.
(14Z)-14-Tricosen-10-one)	0.0002	n.s.	0.0018		n.s.	n.s.	n.s.

(3S,5E,7E,24R)-(6,19,19-~2~H_3_)-9,10-Secocholesta-5,7,10-trie	0.0255	n.s.	0.007		0.0001	n.s.	< 0.001

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706 Abbreviations: CD: Control diet; ChDD: choline-deficient diet; FADD: folic acid-deficient diet.
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714 **Table 3.** Four-week post-stroke Tukey's pairwise comparison summary determining the impact of
715 maternal dietary deficiencies in folic acid or choline on offspring fecal metabolite levels.
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	Female				**Male**		
Metabolite	**CD vs. FADD**	**CD vs. ChDD**	**ChDD vs. FADD**		**CD vs. FADD**	**CD vs. ChDD**	**ChDD vs. FADD**
2,3-Dihydroxypropylstearate	n.s.	n.s.	0.0062		0.0163	n.s.	0.0115
n-Hexanamide	0.0277	0.0307	n.s.		n.s.	n.s.	n.s.
N-Acetyl-Lglutamic Acid	n.s.	n.s.	n.s.		0.0096	0.0127	n.s.
JWH 133	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.
Homocitrulline	0.0004	n.s.	0.0265		0.0008	0.0349	
Sphinganine	0.0024	0.0111	n.s.		n.s.	n.s.	n.s.
R-(+)-Etiracetam	0.0001	0.0089	n.s.		n.s.	n.s.	n.s.
Pyrrolidine	0.0094	n.s.	n.s.		n.s.	n.s.	n.s.

1-(4-Aminobutyl)urea	0.0001	0.0114	n.s.		n.s.	n.s.	n.s.
Propoxur	0.0012	0.0042	n.s.		n.s.	n.s.	n.s.
PRIMA-1	0.0001	0.0088	n.s.		n.s.	n.s.	n.s.
Phytosphingosine	0.0012	0.0083	n.s.		n.s.	n.s.	n.s.
N-lauroylglycine	0.0014	0.0007	n.s.		n.s.	n.s.	n.s.
Nicaraven	0.0029	0.0011	n.s.		n.s.	n.s.	n.s.
N~6~,N~6~-Dimethyllysine	0.0014	n.s.	n.s.		n.s.	n.s.	n.s.
Misoprostol	0.0424	0.0019	n.s.		n.s.	n.s.	n.s.
Lovastatin	0.003	0.0203	n.s.		n.s.	n.s.	n.s.
HMMNI	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.
Gly-Leu	0.0003	0.0448	n.s.		n.s.	n.s.	n.s.
Chamazulene	0.0037	0.0015	n.s.		n.s.	n.s.	n.s.
Esmolol	0.0355	0.0204	n.s.		n.s.	n.s.	n.s.
Dodecylethanolamide	0.0018	0.0009	n.s.		n.s.	n.s.	n.s.
Creatine	n.s.	n.s.	n.s.		0.0355	0.0091	n.s.
C2 Dihydroceramide	0.009	0.0062	n.s.		n.s.	n.s.	n.s.
BAR501	0.0005	0.0225	n.s.		n.s.	n.s.	n.s.
APM	0.0017	0.018	n.s.		n.s.	n.s.	n.s.
Adenine	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.
Acetylcadaverine	0.0008	n.s.	0.007		n.s.	n.s.	n.s.

Acetohydroxamic acid	0.0176	n.s.	n.s.		n.s.	n.s.	n.s.
5-Hydroxytryptophol	0.0034	n.s.	n.s.		n.s.	n.s.	n.s.
1-PP	0.0181	n.s.	n.s.		n.s.	n.s.	n.s.
(4R)-5-Hydroxy-L-leucine	0.0034	n.s.	n.s.		n.s.	n.s.	n.s.
(+)-castanospermine	0.0011	0.0443	n.s.		n.s.	n.s.	n.s.
Fenamole	0.0003	n.s.	0.0174		0.0032	n.s.	0.0207

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718 Abbreviations: CD: Control diet; ChDD: choline-deficient diet; FADD: folic acid-deficient diet.
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