Full title: High-fat diet-induced obesity increases susceptibility to endogenous and ethyl carbamate-induced somatic mutagenesis

Short title: High-fat diet and mutagenesis

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Abstract

Obesity is associated with increased risk for certain cancers. We conducted a set of experiments designed to investigate both the inherent mutagenicity of high-fat diet (HFD) and the effect of HFD on sensitivity to ethyl carbamate, a known mutagen, in a mouse model of HFD-induced obesity. Short term HFD exposure was found to induce both hepatic Cyp2e1 activity and ethenoadenosine, a marker of lipid peroxidation DNA damage. After one year, greater body mass in females was associated with higher frequency of Pig-a mutant erythrocytes as well as hepatic precancerous transformation, suggesting greater risk of tumor formation. HFD-induced obese female mice exhibited increased sensitivity to ethyl carbamate-induced mutagenesis compared to control mice. Together, these findings indicate that HFD exposure can induce both endogenously generated changes related to xenobiotic metabolism, mutagenesis, and pre-carcinogenesis, and sensitivity to exogenous mutagens, in a sex-specific manner.

Introduction

Obesity and overweight are highly prevalent in the United States and are associated with a number of conditions, including certain cancers. Of all new cancer cases diagnosed in 2015, 55% of those diagnosed in women and 24% of those diagnosed in men can be linked to obesity, including cancers of the breast, uterus, colon and liver[1]. In fact, incidence of obesity related cancers rose 7% between 2005 and 2014, while non-obesity related cancer declined by 13%, and the fastest growing obesity-related cancer type during that period was liver cancer, which rose by 29%[1,2]. Specifically among

46 women, rates of obesity-related cancers rose rapidly between 2001 and 2018, while
47 cancers due to other risk factors declined[3].

48 There are many factors underlying the progression from obesity to carcinogenesis,
49 including environmental exposures such as diet and mutagenic toxicants. In the context
50 of obesity and cancer risk, it is useful to consider diet as a type of environmental exposure
51 that leads to high internal levels of triglycerides and adipose tissue-secreted hormones
52 and proteins. Because obesity is often a lifelong condition, with childhood and adolescent
53 obesity predictably persisting into adulthood, this exposure can be chronic[4].

54 It has been known in the medical community that patients with obesity process
55 certain drugs at different rates than non-obese patients, due to altered levels of
56 cytochrome P4502E1, a promiscuous enzyme responsible for phase I metabolism of a
57 range of substrates, often to intermediate products with enhanced toxicity[5,6]. Despite
58 this, very few studies of diet-induced obesity in mouse models have measured enzymatic
59 activity of hepatic Cyp2e1, the majority favoring measurement of changes at the gene
60 expression level, [7–9]. CYP2E1/Cyp2e1 catalyzes both the genotoxic peroxidation of
61 endogenous lipids and synthesis of several toxic intermediates from environmental
62 mutagens, including ethyl carbamate[10,11]. Industrially, ethyl carbamate was used as
63 an intermediate in textile, pesticide and cosmetic manufacturing and is produced in food
64 and beverage fermentation[12]. While the risk to the general public is low, those that
65 regularly consume stone fruit brandy or fermented soy products may have a lifetime tumor
66 risk of 0.01%, 100-times the risk that is generally considered acceptable[13–15]. Ethyl
67 carbamate is largely desaturated by CYP2E1/Cyp2e1 to vinyl carbamate, and oxidized
68 into vinyl carbamate epoxide[10,16]. Vinyl carbamate epoxide is highly reactive with DNA,

69 forming stable, mutagenic etheno-DNA adducts[17]. These adducts are also formed in
70 the presence of excess lipids due to lipid peroxidation[18].

71 We have previously shown that high-fat diet (HFD) exposure in male C57BL/6J
72 mice leads to higher frequency of spontaneous somatic mutations using the Pig-a gene
73 mutation assay[19]. Here we present the results from three experiments including both
74 male and female mice. We hypothesize that the physiological state of HFD-induced
75 obesity, and resultant excess lipid accumulation provides activity-inducing substrate for
76 Cyp2e1, both raising endogenous levels of lipid peroxidation and metabolism of
77 exogenous compounds to genotoxic intermediate metabolites. First, we designed a short-
78 term HFD exposure experiment to determine if mice reared on HFD (60% calories from
79 fat) have higher hepatic Cyp2e1 activity and retain more ethenoadenosine adducts. We
80 then designed a long-term HFD exposure cohort to determine the effect of HFD over time
81 in both males and females on frequency of spontaneous somatic mutations using the Pig-
82 a gene mutation assay and markers of hepatic precancerous transformation. Given the
83 convergent mechanism of toxicity of HFD and ethyl carbamate we designed a third, two-
84 factorial experiment whereby mice with long-term HFD exposure were challenged with
85 ethyl carbamate and assessed for Pig-a gene mutation frequency. Together, this set of
86 experiments explores both the endogenous and exogenous factors underlying the
87 pathway to carcinogenicity in the context of diet-induced obesity.

88

89 # Methods

90 ## General animal care, sample processing, and analysis

91 Mice were reared at the Tulane University Downtown Vivarium on a 12 hour
92 light/dark cycle. Research staff supplied C57BL/6J mice with HFD containing 60%
93 calories from fat from Research Diets, Inc. (D12492) or with a low-fat diet (LFD) containing
94 10% calories from fat (D12450J), *ad libitum*. The LFD formulation serves as the most
95 appropriate control condition to the HFD formulation because it is source-matched and
96 contains equivalent sucrose content. Water and veterinary care were provided by
97 vivarium staff. Organs were harvested and flash frozen in liquid nitrogen, serum or plasma
98 was isolated from whole blood, and all samples were stored at -80°C until further analysis
99 unless otherwise indicated. Prism software (GraphPad; San Diego, CA, USA) was used
100 to conduct statistical analysis and to generate figures. Unless otherwise indicated, column
101 charts present arithmetic mean ±standard error, and regression charts present individual
102 data points and resulting regression curve with a truncated x-axis. Statistical significance
103 was determined using α=0.05.
104

105 # Short-term HFD experiment

106 ## Husbandry and sample collection

107 This experiment was conducted under Tulane IACUC protocol 4374. Mice were
108 maintained on HFD or LFD conditions starting at 8 weeks old, and the experiment was
109 terminated when mice reached 22 weeks old. Each dietary treatment group consisted of
110 three males and three females. Samples were collected following CO_2 overdose and
111 exsanguination.

112 ## Biomarker assays

113 Serum triglycerides and leptin content were analyzed via the Triglyceride
114 Quantification Assay Kit (Abcam, ab65336) and the Mouse/Rat Leptin Quantikine ELISA
115 Kit (R&D Systems, MOB00). To assess pre-mutagenic changes to DNA that might occur
116 in HFD mice, ethenoadenosine, a modification to the adenosine nucleoside that results
117 from lipid peroxidation, was measured in liver tissue using the OxiSelect Aldehyde-
118 Induced DNA Damage Kit (Ethenoadenosine Quantitation) (Cell BioLabs, STA-820).

119 Cyp2e1 activity assay

120 Cyp2e1 activity was measured in liver tissue by first isolating the rough
121 endoplasmic reticulum (RER) of liver cells and then incubating the isolated microsomal
122 fraction with *p*-nitrophenol, a Cyp2e1 substrate with colorimetric properties. Functional
123 RER isolation was achieved with a modified version of the Ca^{2+} precipitation protocol
124 outlined for Endoplasmic Reticulum Isolation Kit (Sigma-Aldrich, ER0100). The protocol
125 was followed as written, with the exception of the final resuspension, in which 50 mM
126 potassium phosphate with EDTA-free protease inhibitor cocktail tablets (Roche,
127 05892791001) replaced the supplied Isotonic Extraction Buffer. The conversion of *p*-
128 nitrophenol to *p*-nitrocatechol was quantified by colorimetric assessment at OD 535 nm
129 via spectrophotometry.

130 Statistical analysis

131 Differences in mean body mass, serum leptin, serum triglycerides, liver
132 ethenoadenosine, and liver Cyp2e1 activity were compared using the independent two-
133 way Student's t test within males and females separately. Body mass was also correlated
134 with liver ethenoadenosine and hepatic Cyp2e1 activity using simple linear regression
135 analysis and the slope of each curve was tested for significant deviation from zero using

136 an F test with the null hypothesis that the overall slope is zero. Correlation coefficient (R^2)
137 values were also reported to allow interpretation of the predictive value of body mass on
138 each outcome of interest. Sample size analyses were conducted using G*Power software
139 (Heinrich-Heine-Universität Düsseldorf; Dusseldorf, NRW, Germany)[19].
140

141 # Long-term HFD experiment

142 ## Husbandry and sample collection

143 This set of experiments was conducted under the approval of Tulane University
144 IACUC protocol 546. Mice were maintained on HFD or LFD conditions from 8 weeks old
145 until 52 weeks old. Each dietary group consisted of 12 males and 12 females. Blood was
146 taken at three time points, when mice were 17, 33 and 52 weeks of age. This was
147 accomplished through repeated submandibular venipuncture under isoflurane
148 anesthetization[21]. Mouse livers were excised from 52 week old mice following final
149 submandibular venipuncture. Under isoflurane anesthetization, mice were exsanguinated
150 via cardiac puncture and half of the left hepatic lobe was excised.

151 ## Erythrocyte Pig-a mutant frequency analysis

152 Each blood sample was immediately transferred to a K_2EDTA-coated microtainers (BD,
153 365974) and kept on ice. Samples were packed into Whirl-Pak® bags and shipped to
154 Litron Laboratories (Rochester, NY). Pig-a mutant frequency analysis was carried out by
155 Litron research staff following *In Vivo* MutaFlow® kit protocols[19,22,23].

156 ## Hepatocyte proliferation index and Ki67 content analysis

157 A MicroFlow^{PLUS} Rat Liver (Prototype) kit was supplied *gratis* by Litron Laboratories
158 to Tulane University. Following excision, left liver lobes were placed into a tube of ice cold

159 HBSS with EDTA and 1% DMSO. Samples were transferred to 4°C, and within 6 hours,
160 each preserved liver was transferred into fresh buffer with EDTA and DMSO and stored
161 overnight. The following day, one gram of each sample was rinsed twice in Liver Rinse
162 Solution and then in Erythrocyte Clearing Solution. The liver samples were then digested,
163 and individual cells were isolated in collagenase. The outer cell membranes were lysed,
164 leaving nuclei intact, and SYTO 13 nucleic acid dye and an anti-Ki67-eFluor660 antibody
165 were applied. A MACSQuant Analyzer10 flow cytometer was used to acquire cellular
166 phenotype data and analysis was conducted using FlowLogic™ software (Inivai
167 Technologies; Mentone, VIC, Australia). Hepatocyte proliferation index (HPI) was
168 calculated using the following formula:

169 $$HPI = \frac{j + 2k + 3l}{j + k + l}, \text{where } j = 2\text{n events}, k = 4\text{n events}, l = 8\text{n events}$$

170 **Statistical analysis**

171 Body mass and mutant frequency were analyzed using mixed effects analysis of
172 variance (ANOVA) for repeated measures with Bonferroni's post hoc multiple
173 comparisons test performed at each time point between LFD and HFD subjects. Simple
174 linear regression analysis of the relationship between body mass and Pig-a mutant
175 frequency was also conducted at each time point, and the slope p value and R^2 were
176 reported as described for the short-term HFD experiment. HPI and Ki67 content was
177 compared between LFD and HFD groups using the two-tailed independent Student's t
178 test. Males and females were analyzed separately.

179

180 **Ethyl carbamate challenge experiment**

181 ## Husbandry and sample collection

182 This experiment was conducted under approved Tulane University IACUC protocol
183 36-105. 48 males and 48 females were reared on HFD or LFD from 8 weeks old until they
184 reached 42 weeks old, when the experiment was terminated. Nine weeks prior to
185 termination, at 33 weeks, mice were divided into groups of 6, and exposed to 0, 100, 200,
186 or 400 mg/kg/day of ethyl carbamate (CAS # 51-79-6; Sigma, U2500, Lot # WXBC3505V)
187 in deionized water via oral gavage for 3 consecutive days following the procedure outlined
188 by Labash et al. [23]. At 42 weeks, to maximize yield and quality of blood samples, mice
189 were anesthetized with no more than 5% isoflurane (v/v) and 100% oxygen saturation at
190 a 1 L/min flow rate. The sternal cavity was opened for full visualization of the heart, and
191 blood was collected from the left ventricle with a heparin-coated 22G needle and 1 mL
192 syringe. The blood was then transferred to a microtainer containing EDTA (BD, 365974)
193 to prevent coagulation. Immediately after collection, 60 μL of each sample was transferred
194 to a tube containing an anticoagulant solution provided by Litron Laboratories and kept
195 on ice for further processing following the Mouse MutaFlow^{PLUS} Kit manual (Litron
196 Laboratories, v170925). Exsanguinated tissues were excised and flash frozen in liquid
197 nitrogen and stored at -80°C.

198 ## Pig-a mutant frequency analysis

199 Pig-a mutant frequency analysis was conducted at Tulane University using a
200 modified MutaFlow method. In summary, white blood cells and platelets were removed
201 with Lympholyte®-Mammal (CedarLane, CL5110) and remaining reticulocytes,
202 erythrocytes, and platelets were labeled with Anti-CD24-PE (Miltenyi, 130-110-688) and
203 Anti-CD61-PE (Miltenyi, 130-102-628) antibodies and PE-conjugated magnetic

204 microbeads (Miltenyi, 130-048-801). A small portion of each sample was retained for cell
205 density determination and the remainder was enriched for mutant cells via
206 immunomagnetic separation on LS columns (Miltenyi, 130-042-401) within a magnetic
207 field. SYTO 13 nucleic acid dye (Invitrogen, S7575) was added to discriminate mature
208 erythrocytes from immature reticulocytes and remaining lymphocytes. Total mature
209 erythrocyte and *Pig-a* mutant erythrocyte counts were enumerated via the Miltenyi
210 MACSQuant Analyzer 10 flow cytometer. *Pig-a* mutant erythrocytes were classified as
211 cells exhibiting absence of the PE fluorescence signal and intracellular SYTO 13,
212 indicating a lack of both CD24 surface markers and nucleic acids. Mutant frequency was
213 calculated as the number of mutant erythrocytes per 10^6 total mature erythrocytes.

214 Hepatic Cyp2e1 activity

215 Hepatic Cyp2e1 activity was quantified at described above for the short-term HFD
216 experiment.

217 Statistical analysis

218 Pig-a mutant frequency was analyzed using two-way ANOVA with diet and ethyl
219 carbamate dose as the two factors, with Bonferroni's post hoc comparison test between
220 LFD and HFD groups within each dose group. A dose-response curve was plotted to
221 compare the effect of ethyl carbamate dose on Pig-a mutant frequency between LFD and
222 HFD groups, with slope comparison and R^2 calculation as well as an F test to compare
223 dose-response curve slopes between HFD and LFD groups. Cyp2e1 activity was
224 analyzed using a two-tailed independent Student's t test and simple linear regression, as
225 described for the short-term experiment. Males and females were analyzed separately.
226

Results

Short-term HFD experiment

Males gain more weight than females and exhibit biomarkers of diet-induced obesity after short term HFD treatment

All mice survived the duration of the short-term HFD experiment. When mice reached 22 weeks of age, the experiment was terminated. HFD males were significantly heavier (t_4=3.85, p=0.018) than LFD males and HFD females were also heavier than LFD females, though this difference was not statistically significant (t_4=2.48, p=0.068) (Fig 1A). HFD males exhibited significantly elevated serum leptin (t_4=4.745, p=0.009) and elevated serum triglycerides, (t_4=2.756, p=0.051) (Fig 1B-C). Serum leptin and triglycerides were not significantly elevated in HFD females (t_4=2.23, p=0.09; t_4=1.453, p=0.22) (Fig 1B-C).

Fig 1. Effects of diet on body mass, serum leptin, and serum triglycerides. Body mass (A), total serum triglycerides (B), and serum leptin (C) of female (left) and male (right) mice at collection (22 weeks). * p<0.05, **p<0.01, two-tailed independent Student's t test conducted within males and females separately, between LFD and HFD groups (n=3).

Short term HFD exposure induces DNA damage linked to lipid peroxidation and increased activity of xenobiotic metabolizing enzyme Cyp2e1

248 13 weeks of HFD treatment led to elevated levels of the adducted nucleotide
249 ethenoadenosine, a marker of lipid peroxide-induced DNA damage. Female and males
250 exhibited similarly elevated levels of liver etheno adenosine, though this difference was
251 significant in females (t_4=3.897, p=0.0176) and not significant in males (t_4=1.609,
252 p=0.1829) (Fig 2A). To further evaluate the effect of HFD-induced weight gain on lipid
253 peroxidation, linear regression analysis was conducted to determine the correlation
254 between body mass and liver ethenoadenosine. Among females, regression analysis
255 suggested a correlation between body mass and liver ethenoadenosine though not
256 significantly (R^2=0.5557, p=0.0889; Fig 2B, Table 1). Among males, body mass was
257 significantly positively correlated with liver ethenoadenosine content with an R^2 value of
258 0.77 (p=0.0216; Fig 2C, Table 1).
259
260 **Fig 2. Effect of diet on liver ethenoadenosine.** (A) Mean liver ethenoadenosine in
261 females and males, LFD compared to HFD. (B) Correlation of body mass and liver
262 ethenoadenosine in female and (C) males by simple linear regression. *p<0.05 per two-
263 tailed independent Student's t test conducted within males and females separately,
264 between LFD and HFD groups (n=3).
265
266 **Table 1. Simple linear regression correlating body mass and liver ethenoadenosine**
267 **in female and male mice following short term HFD exposure.**

Sex	Equation	R^2	Deviation from zero p value
Female	y = 1.970x-20.68	0.5557	0.0889
Male	y = 2.998x-87.49	0.7700	**0.0216**

268 Bold type indicates the slope deviates significantly from zero (α=0.05).
269

270 To evaluate the potential for HFD-induced sensitivity to xenobiotic toxification,
271 enzymatic activity of Cyp2e1 was measured in liver. Hepatic Cyp2e1 activity was elevated
272 in males (t_4=3.076, p=0.0371), but not significantly elevated in females (t_4=1.851,
273 p=0.1378; Fig 3). Simple linear regression suggested a correlation between body mass
274 and hepatic Cyp2e1 activity, but the association was not statistically significant in males
275 (R^2=0.5805, p=0.0783) or females (R^2=0.6416, p=0.0555) (Table 2). This pilot study was
276 limited by a small sample size, but post hoc power analysis indicated a sample size of
277 n=11 would yield significant differences in male ethenoadenosine, given the observed
278 effect size of 1.3. A sample size of n=8 per group would yield significant elevation in
279 female Cyp2e1, given the observed effect size of 1. Together, these results suggest an
280 increase in Cyp2e1-mediated synthesis of genotoxic lipid metabolites due to short-term
281 HFD exposure alone.
282
283 **Fig 3. Effects of diet on liver Cyp2e1 activity.** (A) Mean hepatic Cyp2e1 activity,
284 reported as fold difference between LFD and HFD groups, within males and females
285 separately. (B) Correlation of body mass and Cyp2e1 activity in female and (C) males by
286 simple linear regression. *p<0.05 per two-tailed independent Student's t test conducted
287 within males and females separately, between LFD and HFD groups (n=3).
288
289 **Table 2. Simple linear regression correlating body mass and liver Cyp2e1 activity**
290 **in male and female mice following short term HFD exposure.**

Sex	Equation	R^2	Deviation from zero p value
Female	y=0.07249x-0.5783	0.6416	0.0555
Male	y=0.09217x-2.023	0.5805	0.0783

291

292 # Long-term HFD experiment

293 ## Higher rates of somatic mutations and precancerous markers develop
294 over time with long term HFD in females.

295 To assess the mutagenicity of long-term HFD exposure, the erythrocyte Pig-a
296 mutation assay was conducted with sequential blood sampling at 17, 33 and 52 weeks
297 old. By week 52, one LFD female was euthanized due to injury, and two HFD males died
298 due to undetermined causes. Both HFD males and HFD females were significantly
299 heavier than their LFD counterparts at each sampling timepoint. HFD females were 6.98
300 ($t_{17.11}$=6.334, p<0.0001), 19.99 ($t_{15.12}$=8.281, p<0.0001), and 32.10 ($t_{14.92}$=13.27,
301 p<0.0001) grams heavier than LFD females at 17, 33, and 52 weeks, respectively (Fig
302 4A). HFD male were 10.62 ($t_{14.24}$=8.151, p<0.0001), 14.83 ($t_{11.34}$=11.18, p<0.0001), and
303 15.90 ($t_{11.07}$=9.494, p<0.0001) grams heavier than LFD males at 17, 33, and 52 weeks,
304 respectively (Fig 4B).
305
306 **Fig 4. Effect of diet on body mass at each sampling timepoint.** Male (A) and female
307 (B) body mass in LFD and HFD groups at 17, 33 and 52 weeks of age.
308 **** p<0.0001 per mixed effects analysis (for repeated measures) with Bonferroni's
309 multiple comparisons test, between LFD and HFD groups at each timepoint, conducted
310 in males and females independently (n=10-12).
311
312 One extreme outlier LFD female was excluded from all Pig-a mutant frequency
313 analyses due to very high frequency of Pig-a mutant erythrocytes detected at 33 and 52
314 weeks (>4 standard deviations). Both raw and fold-difference erythrocyte mutant

315 frequency values were reported to normalize interexperiment variability. Despite a trend
316 of an approximately 1.5-2-fold elevation, there was no significant difference in mutant
317 frequency at any sampling timepoint between LFD and HFD females [24,25]. Because of
318 the variability in body mass observed in response to HFD, simple linear regression was
319 performed to assess the association between body mass and mutant frequency at each
320 sampling time point. Simple linear regression for the association between body mass and
321 erythrocyte mutant frequency per million total erythrocytes indicated the curve slope was
322 not significantly different from zero at 17 weeks (Fig 5C, Table 3). By week 33, there was
323 significant positive association between body mass and mutant frequency, with an R^2 of
324 0.2004 and significantly non-zero slope (p=0.0322). By week 52, there was a stronger
325 association, with an R^2 of 0.2518 and a significant slope (p=0.0286) (Fig 5D-E), indicating
326 higher body mass was associated with higher incidence of somatic mutations, and this
327 association grew stronger with age in females. Among males, while there was a similar
328 trend towards higher mutant frequency among the HFD group, there was no significant
329 differences between the LFD and HFD groups at any time point (Fig 6A-B), and there was
330 no significant correlation between body mass and mutant frequency at any sampling
331 timepoint (Fig 6C-E, Table 4).
332
333 **Figure 5. Effects of diet and body mass on spontaneous mutation frequency in**
334 **erythrocytes at 17, 33 and 52 weeks of age in females.** (A) Mutant frequency per
335 million erythrocytes, and (B) Mutant frequency fold-difference between LFD and HFD
336 groups at each time point, per mixed effects analysis (for repeated measures) with
337 Bonferroni's multiple comparisons test, between LFD and HFD group sat each timepoint
338 (n=10-12). (C) Correlation between body mass and erythrocyte mutant frequency per

339 million cells at 17 weeks. (D) Correlation between body mass and erythrocyte mutant
340 frequency per million cells at 33 weeks. (E) Correlation between body mass and
341 erythrocyte mutant frequency per million cells at 52 weeks.
342
343 **Table 3. Simple linear regression correlating body mass and Pig-a mutant**
344 **frequency in females after 17, 33 and 52 weeks of dietary exposure.**

Week	Equation	R^2	Deviation from zero p value
17	y=0.01042x+0.07179	0.01873	0.5763
33	y=0.008555x-0.01226	0.2004	**0.0322**
52	y=0.007024x+0.06099	0.2518	**0.0286**

345 Bold type indicates the slope deviates significantly from zero (α=0.05).
346
347 **Fig 6. Effects of diet and body mass on spontaneous mutation frequency in**
348 **erythrocytes at 17, 33 and 52 weeks of age in males.** (A) Mutant frequency per million
349 erythrocytes, and (B) Mutant frequency fold-difference between LFD and HFD groups at
350 each time point, per mixed effects analysis (for repeated measures) with Bonferroni's
351 multiple comparisons test, between LFD and HFD group sat each timepoint (n=10-12).
352 (C) Correlation between body mass and erythrocyte mutant frequency per million cells at
353 17 weeks in males. (D) Correlation between body mass and erythrocyte mutant frequency
354 per million cells at 33 weeks in males. (E) Correlation between body mass and erythrocyte
355 mutant frequency per million cells at 52 weeks in males.
356
357 **Table 4. Simple linear regression correlating body mass and Pig-a mutant**
358 **frequency in males after 17, 33 and 52 weeks of dietary exposure.**

Age (weeks)	Equation	R^2	Deviation from zero p value
17	y=0.01836x-0.4354	0.1339	0.0939
33	y=0.05517x-1.474	0.1504	0.1118
52	y=0.0112x-0.2016	0.02489	0.4946

To assess the impact of long term HFD exposure on development of precancerous hepatic biomarkers, hepatocyte nuclei were assessed for proliferative capacity by calculating the HPI and the nuclear Ki67 percent positivity. The HPI was significantly higher in HFD females compared to LFD females at 52 weeks old (t_6=8.171, p=0.0002), while there was no difference between HFD and LFD males (t_6=1.886, p=0.1082) (Fig 7A). Similarly, the percentage of hepatic nuclei that were Ki67+ was greater in HFD females than in LFD females (t_6=4.204, p=0.0057), with no difference seen between HFD and LFD males (t_6=0.1165, p=0.9110) (Fig 7B). These findings indicate HFD is a risk factor for cellular and molecular markers that are suggestive of precancerous transformation in females.

Fig 7. Effects of diet on precancerous hepatic markers at 52 weeks of age. (A) HPI in females (left) and males (right), (B) Percentage of hepatocyte nuclei that contained the Ki67 nuclear marker in females (left and males (right). ** p<0.01, *** p<0.001 per two-tailed independent Student's t test between LFD and HFD groups, conducted separately in males and females (n=3-5).

Ethyl carbamate challenge experiment

HFD increases sensitivity to ethyl carbamate-induced somatic mutagenesis in females.

To determine the effect of HFD on sensitivity to a known mutagen, mice were reared on HFD or LFD starting at 7 weeks of age, exposed to ethyl carbamate at 33 weeks, and the experiment was terminated following a 9 week mutation manifestation period. Dietary conditions were maintained for the entirety of the experiment, and 93 of 96 total mice survived until termination at 42 weeks old. One female HFD mouse was sacrificed at 17 weeks due to excessive pruritus with unhealed dermal lesions and lack of weight gain. One male HFD mouse was sacrificed at 22 weeks due to sudden rapid weight loss, several large obstructive growths were observed in the colon during necropsy. One female LFD mouse was cannibalized at 29 weeks.

Final body mass did not differ between ethyl carbamate dose groups, and all HFD groups were significantly heavier than their corresponding LFD controls (S1 Fig). Similarly to the long-term HFD only experiment, the proportional difference between HFD and LFD female body weight was greater than the body mass difference between LFD and HFD males. HFD exposure led to elevated liver triglycerides and elevated plasma leptin (S2 Fig) as well as AST and ALT in both males and females, indicating physiological obesity and liver damage due to HFD exposure (S3 Fig).

To determine the effect of longer term HFD exposure alone on the potential for generation of toxic lipid metabolites, hepatic Cyp2e1 activity was also assessed at 42 weeks in samples that had not been exposed to ethyl carbamate (vehicle controls). Hepatic activity of Cyp2e1, the enzyme that metabolizes ethyl carbamate to its mutagenic metabolite, vinyl carbamate, was elevated approximately 2.5-fold in females (t_9=2.872,

401 p=0.0184; Fig 8). There was no significant difference in hepatic Cyp2e1 activity in males
402 due to die (t_9=1.337, p=0.2141; Fig 8). Higher body mass was significantly associated
403 with higher hepatic Cyp2e1 activity in vehicle control females (R^2 =0.5083, p<0.05), but
404 not males (R^2=0.08934, p=0.4015) (Table 5). Because of the elevated endogenous
405 Cyp2e1 activity, females with long-term HFD exposure are predisposed to generation of
406 genotoxic lipid peroxides and vinyl carbamate epoxides.
407
408 **Fig 8. Effects of diet on hepatic Cyp2e1 activity in 0 mg/kg/day ethyl carbamate**
409 **dose group.** (A) Mean hepatic Cyp2e1 activity, reported as fold difference between LFD
410 and HFD groups, within males and females separately (n=5-6). (B) Correlation of body
411 mass and Cyp2e1 activity in female and (C) males by simple linear regression. * p<0.05
412 per Student's t test.
413
414 **Table 5. Simple linear regression correlating body mass and liver Cyp2e1 activity**
415 **in male and female mice following 52 weeks of dietary exposure.**

Sex	Equation	R^2	Deviation from zero p value
Female	y=0.07241x-0.9335	0.5083	**0.0138**
Male	y=0.01470x+0.4762	0.08934	0.4015

416 Bold type indicates the slope deviates significantly from zero (α=0.05).
417
418 HFD exposure in females led to increased susceptibility to somatic mutations
419 induced by exposure to 400 mg/kg/day ethyl carbamate (t_{38}=4.67, p=0.0001; Fig 9A). The
420 difference was on average 3.8-fold higher in the HFD group than the LFD group, within
421 those who received 400 mg/kg/day ethyl carbamate (t_{38}=4.67, p=0.0001; Fig 9B). To
422 determine if HFD females were more sensitive than LFD females to the same ethyl

423 carbamate dose regimen, dose response curves were plotted with regression analysis
424 between ethyl carbamate dose and Pig-a mutant frequency response. Simple linear
425 regression indicated the dose-response slope does not significantly differ from zero in the
426 LFD group (p=0.3872) and there was no correlation between ethyl carbamate dose and
427 mutant frequency response (R^2=0.0376; Fig 9C, Table 6). The HFD regression curve is
428 highly significantly different from zero (p<0.0001) and there is a strong correlation
429 between ethyl carbamate dose and mutant frequency (R^2 =0.5975; Fig 9C, Table 6). The
430 slope of the HFD curve is significantly higher than the LFD curve, indicating HFD
431 significantly increases the susceptibility of female mice to ethyl carbamate induced
432 somatic mutagenesis (p=0.0003; Table 6).
433
434 **Fig 9. Effects of diet and body mass on mutation frequency in erythrocytes of**
435 **female mice exposed to 0, 100, 200, and 400 mg/kg/day ethyl carbamate.** (A) Mutant
436 frequency per million erythrocytes in each ethyl carbamate dose group. (B) Mutant
437 frequency fold-difference between LFD and HFD groups in each ethyl carbamate dose
438 group. (C) Dose-response curves for ethyl carbamate dose and erythrocyte mutant
439 frequency with simple linear regression analysis, points represent mean ±standard error.
440 *** p<0.001 per two-way ANOVA with Tukey's multiple comparison test between LFD and
441 HFD groups (n=4-5).
442
443 **Table 6. Simple linear regression dose-response analysis correlating ethyl**
444 **carbamate dose and Pig-a mutant frequency response in females.**
445

Diet	Equation	R^2	Deviation from zero p value	Slope comparison p value
LFD	y=0.0006029x + 0.1626	0.4032	0.3872	**0.0003**
HFD	y=0.005166x - 0.02898	0.5924	**<0.0001**	

446 Bold type indicates the slope or slope difference deviates significantly from zero (α=0.05).

447

448 Among males there was no significant difference between the response of the HFD and
449 LFD groups to any level of ethyl carbamate treatment (Fig 10A). Though there was a
450 trend suggesting HFD males exhibit an approximately 2-fold increase in Pig-a mutant
451 frequency, regardless of ethyl carbamate treatment, these differences were not
452 statistically significant (Fig 10B). Linear regression analysis of the correlation between
453 ethyl carbamate dose and mutant frequency with slope comparison between the HFD
454 and LFD groups suggest a positive trend, although the slopes were not significant and
455 the R^2 values were weak (LFD: p=0.1032, R^2=0.1486; HFD: p=0.0951, R^2=0.1471; Fig
456 10C). There was ultimately no difference between the slopes for the LFD and HFD curves,
457 suggesting both dietary groups are equally sensitive to ethyl carbamate-induced
458 mutagenesis (p=0.5654, Table 7).

459

460 **Fig 10. Effects of diet and body mass on mutation frequency in erythrocytes of**
461 **male mice exposed to 0, 100, 200, and 400 mg/kg/day ethyl carbamate.** (A) Mutant
462 frequency per million erythrocytes in each ethyl carbamate dose group. (B) Mutant
463 frequency fold-difference between LFD and HFD groups in each ethyl carbamate dose
464 group. (C) Dose-response curves for ethyl carbamate and erythrocyte mutant frequency
465 with simple linear regression analysis, points represent mean ±standard error.

466

467 **Table 7: Simple linear regression dose response analysis between ethyl carbamate**

468 **dose and Pig-a mutant frequency response in males.**

Diet	Equation	R^2	Deviation from zero p value	Slope comparison p value
LFD	y=0.0002403x+0.0.1553	0.1486	0.1032	0.5654
HFD	y=0.003947x+0.5569	0.1471	0.0951	

469

470 # Discussion

471 The purpose of this set of experiments was to examine elements of the pathway
472 that link diet-induced obesity to a physiological state primed for carcinogenesis from three
473 angles. First we wanted to determine the early effects of short-term HFD exposure on two
474 key outcomes linked to lipid peroxidation, ethenoadenosine adducts in genomic DNA and
475 hepatic Cyp2e1 activity. Cyp2e1 catalyzes the reduction of lipids to reactive peroxides.
476 This phenomenon is well studied in the context of alcoholic liver disease, methionine-
477 choline deficient diet-induced NASH, and human NAFLD, but few studies have linked
478 early Cyp2e1 induction and ethenoadenosine production where short term HFD was the
479 only exposure in a controlled mouse model. Because of the small sample size used in
480 this experiment, and the variability of body weight seen as a result of HFD, linear
481 regression analysis was also performed to determine the relationship between body mass
482 and the lipid peroxidation endpoints of interest. At the early time point, males and females
483 responded somewhat differently to HFD. Males had significantly higher body mass, serum
484 triglycerides, and serum leptin after 13 weeks of HFD, and females followed similar trends
485 but were less responsive (Fig 1A-C). HFD females did have significantly higher liver
486 ethenoadenosine levels than LFD females (Fig 2A-B), indicating even small, non-

487 significant increases in body mass and Cyp2e1 activity (Fig 3A-B) are linked to
488 accumulation of mutagenic DNA damage. Males exhibited similar trends. While HFD
489 males did not have significantly heightened levels of ethenoadenosine, male body mass
490 correlated significantly with liver ethenoadenosine. (Fig 2A and 2C, Table 1). Similarly,
491 though only males had significantly elevated Cyp2e1 activity, both males and females
492 exhibited a nearly significant correlation between Cyp2e1 activity and body mass (Fig 3A
493 and 3C, Table 2) . Together, these experiments were instrumental in suggesting that even
494 short-term HFD exposure alone leads to a hepatic environment primed for mutagenesis,
495 prompting the next set of experiments.

496 A year-long cohort was used to determine the effects of long-term HFD exposure
497 on somatic mutagenesis and pre-cancerous hepatic transformation in both male and
498 female mice. This was the first time this type of experiment was conducted including both
499 sexes. Surprisingly, though the short-term experiment indicated females are initially less
500 responsive, females in this long-term experiment were more responsive to HFD as they
501 aged than males, as indicated by the fact that HFD females were twice as heavy as LFD
502 females, while HFD males were less than 50% heavier than LFD males (Fig 4). This was
503 largely due to the fact that even males on the LFD become obese over time while females
504 do not, a phenomenon well-documented in the C57BL/6J strain[26]. Because of this
505 tendency of males to gain weight over time, regardless of diet, we postulate that both LFD
506 and HFD males represent some degree of biological overweight/obesity.

507 In females, linear regression analysis demonstrates that the association between
508 body mass and Pig-a mutant frequency strengthens with age and continued HFD
509 exposure (Fig 5C-D, Table 3), suggesting that the length of the duration of obesity over

510 the lifespan is linked to degree of cancer risk. While somatic mutagenesis is a key
511 component of the pathway from obesity to carcinogenesis, increased cell proliferation rate
512 is also often used as a biomarker to predict the likelihood of future cancer development
513 and progression[27,28]. Here we demonstrated that hepatocytes isolated from 52 week
514 old HFD females were more likely to be in an active proliferation state than age-matched
515 LFD controls, indicated by HPI and the percentage of hepatocytes with Ki67+ nuclei (Fig
516 7A-B). Both LFD and HFD males exhibited proliferation biomarker levels similar to those
517 found in HFD females. This result is consistent with our other findings that LFD and HFD
518 males exhibit similar characteristics at baseline.

519 The final set of experiments was designed to test the hypothesis that HFD-induced
520 obesity increases sensitivity to ethyl carbamate, a known Cyp2e1 substrate and
521 hepatotoxicant. Hepatic Cyp2e1 activity analysis was first repeated in the vehicle treated
522 group of this cohort to determine the effect of longer-term (until 42 weeks old) HFD. In
523 contrast to the short-term HFD cohort, females exhibited approximately 2.3-fold higher
524 Cyp2e1 activity due to HFD, while males had relatively similar activity regardless of diet
525 (Fig 8). HFD females developed, on average 3.8-times more somatic mutations in
526 response to high-dose ethyl carbamate than LFD females (Fig 9B). In fact, obesity
527 appears to potentiate the mutagenicity of ethyl carbamate in females, as indicated by the
528 significance of the ethyl carbamate-mutant frequency dose-response slope among HFD,
529 but not LFD, females (Fig 9C, Table 6). Among HFD males, mutagenicity of ethyl
530 carbamate appears to saturate between 100 and 400 mg/kg/day, with a peak at 200
531 mg/kg/day (Fig 10A-C). HFD males tended to exhibit higher mutant frequencies than LFD
532 males in each ethyl carbamate dose group, however the dose-response curves for LFD

533 and HFD males shared a common slope (Fig 10C, Table 7). These results suggests that
534 males on both diets were similarly sensitive to ethyl carbamate, further strengthening our
535 postulation that C57BL/6J male on the HFD and LFD formulation used in these studies
536 do not differ sufficiently. We attribute the overall lack of association between diet and
537 mutant frequency with the underlying weight gain observed in LFD males, and this
538 supports our assertion that studies that only include male C57Bl6/J mice are not sufficient
539 to explore the interactive effects of high-fat diet-induced obesity and environmental
540 toxicants.

541 Interpretation of sex differences observed in these three sets of experiments is
542 complicated by the different trends in weight gain between males and females. That is,
543 males on the LFD gained weight consistently throughout the duration of the study, while
544 females on the LFD maintain proportionally lower body masses. Increased weight gain
545 by LFD males maintained for longer periods of time may mask differences that might be
546 evident a study that included in truly lean (non-overweight, non-obese) males as a control
547 group. It is likely that increased metabolism of ethyl carbamate to mutagenic vinyl
548 carbamate epoxide by Cyp2e1 is at least partially responsible for the increased *Pig-a*
549 mutant frequency observed in HFD females. This observation, in conjunction with the lack
550 of obesity-related Cyp2e1 activity elevation and response to ethyl carbamate due to HFD,
551 further emphasizes that C57Bl6/J males are not the most suitable model for long-term
552 studies of diet-induced obesity with toxicant exposure. Additional calorie restriction or
553 reduced dietary fat may be required to generate a male equivalent of the female non-
554 obese phenotype within this model system.

555

556 # Conclusions

557 The findings presented here emphasize the importance of studying obesity as a
558 mutagen and a carcinogen and the necessity of including both sexes in diet-induced
559 obesity research. The current paradigm regarding body weight and chemical safety is that
560 excess body weight is protective, due to the larger volume of distribution. This is a
561 dangerous assumption for chemicals that are metabolized by CYP2E1, if not other
562 CYP450s as well. If the purpose of chemical risk assessment is to protect the most
563 vulnerable groups, obesity must be factored in. In our female model, obesity appears to
564 act to both directly induce *de novo* mutations and hepatic proliferation, and to increase
565 sensitivity to xenobiotic-induced mutagenesis. We demonstrated that obesity is likely
566 involved in both the initiation (mutagenesis) and promotion (proliferation) stages of cancer
567 progression, however, further studies are needed to investigate the differences in male
568 and female response to HFD exposure. The limited scale of our experiment rendered
569 this research group unable to follow tumor formation, but it will be important to conduct
570 studies in the context of diet-induced HCC and other cancers as well.
571

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576

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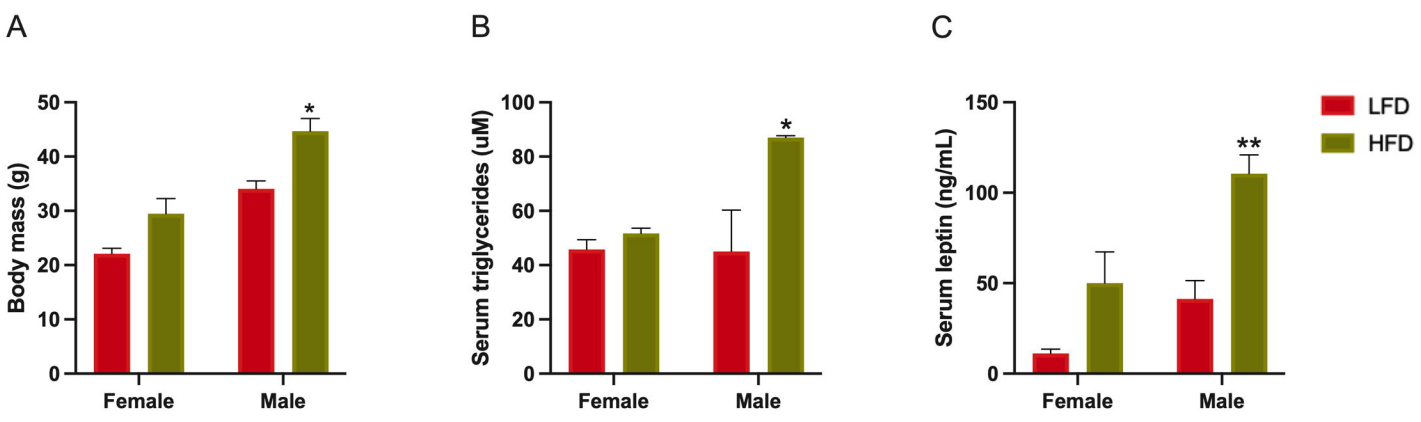
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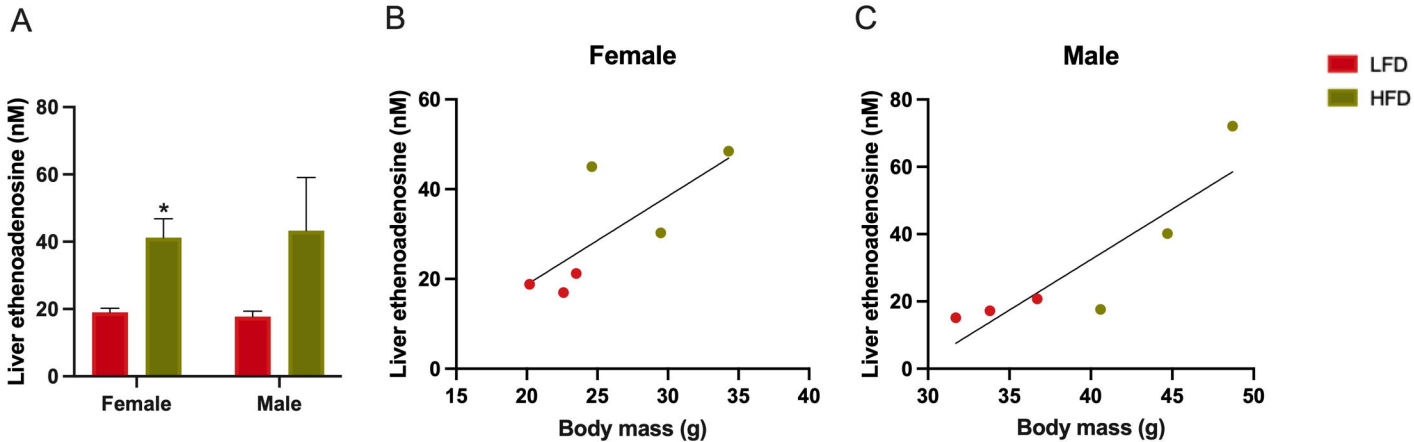
Body mass (g)
50
40
30
20
10
0
*
Female
Male
B
Serum triglycerides (uM)
100
80
60
40
20
0
*
Female
Male
C
Serum leptin (ng/mL)
150
100
50
0
**
Female
Male
LFD
HFD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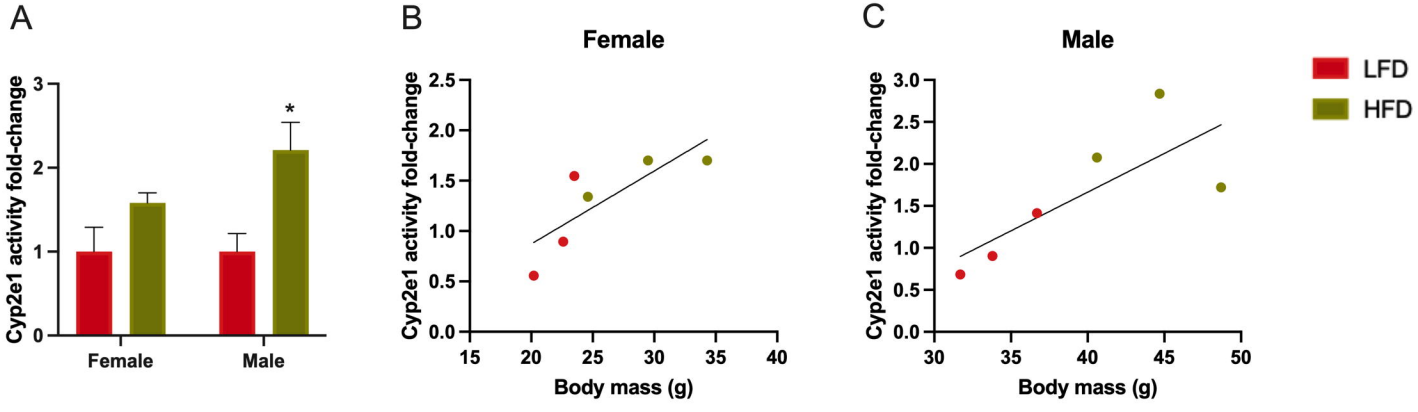
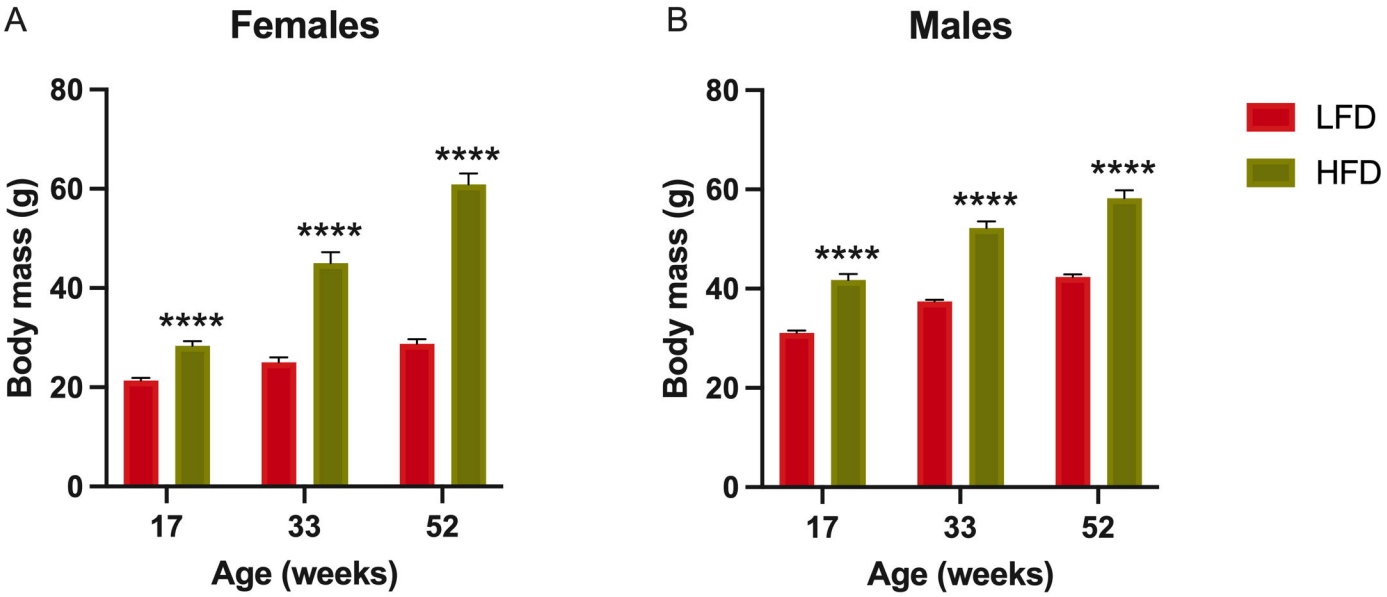
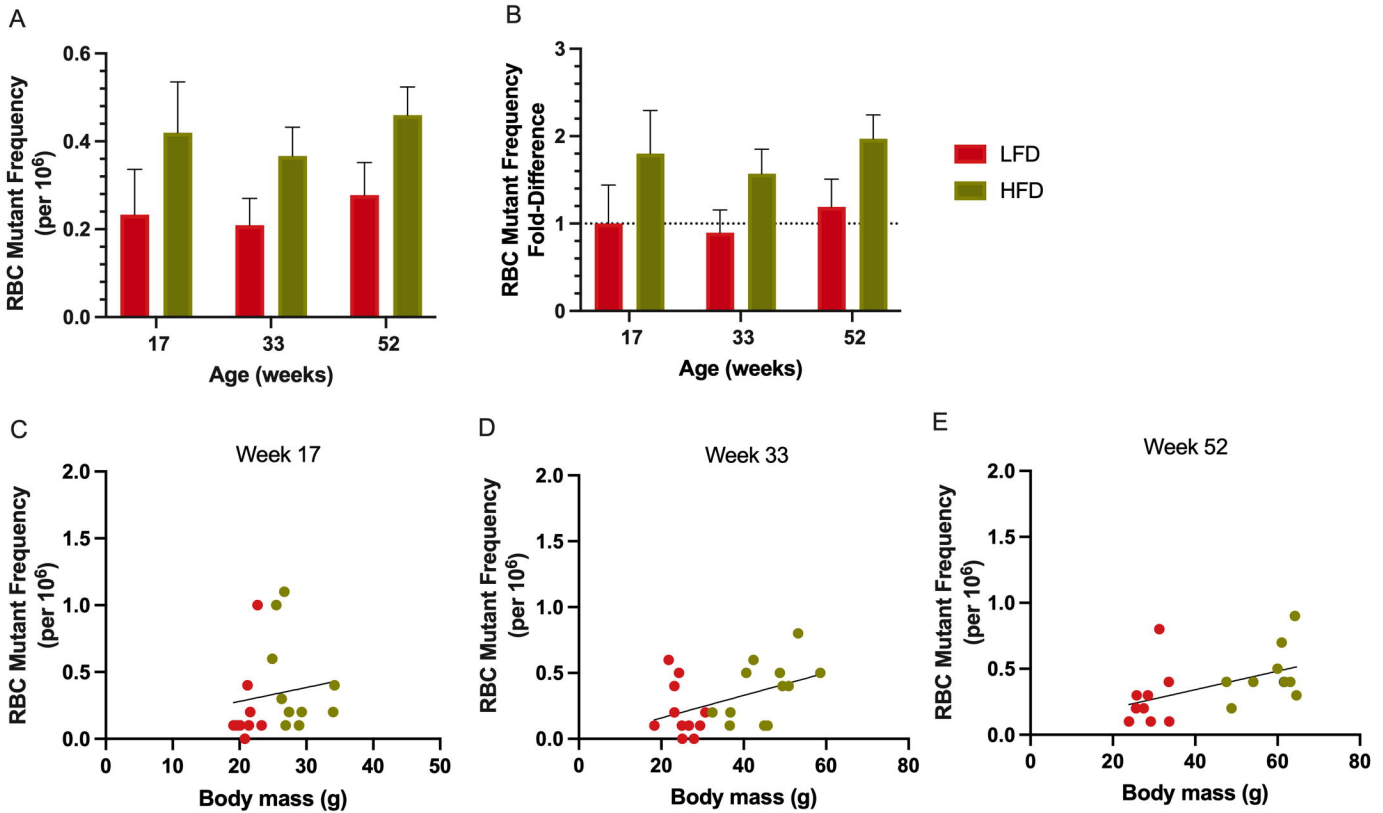
Liver ethenoadenosine (nM)
80
60
40
20
0
*
Female
Male
B
Female
Liver ethenoadenosine (nM)
60
40
20
0
15
20
25
30
35
40
Body mass (g)
C
Male
Liver ethenoadenosine (nM)
80
60
40
20
0
30
35
40
45
50
Body mass (g)
LFD
HFD


A
Cyp2e1 activity fold-change
3
2
1
0
*
Female
Male
B
Female
Cyp2e1 activity fold-change
2.5
2.0
1.5
1.0
0.5
0.0
15
20
25
30
35
40
Body mass (g)
C
Male
Cyp2e1 activity fold-change
3.0
2.5
2.0
1.5
1.0
0.5
0.0
30
35
40
45
50
Body mass (g)
LFD
HFD


A
Females
Body mass (g)
80
60
40
20
0
****
****
****
17
33
52
Age (weeks)
B
Males
Body mass (g)
80
60
40
20
0
****
****
****
17
33
52
Age (weeks)
LFD
HFD


A
RBC Mutant Frequency (per 10^6)
0.6
0.4
0.2
0.0
17
33
52
Age (weeks)
B
RBC Mutant Frequency Fold-Difference
3
2
1
0
17
33
52
Age (weeks)
LFD
HFD
C
Week 17
RBC Mutant Frequency (per 10^6)
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
50
Body mass (g)
D
Week 33
RBC Mutant Frequency (per 10^6)
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
Body mass (g)
E
Week 52
RBC Mutant Frequency (per 10^6)
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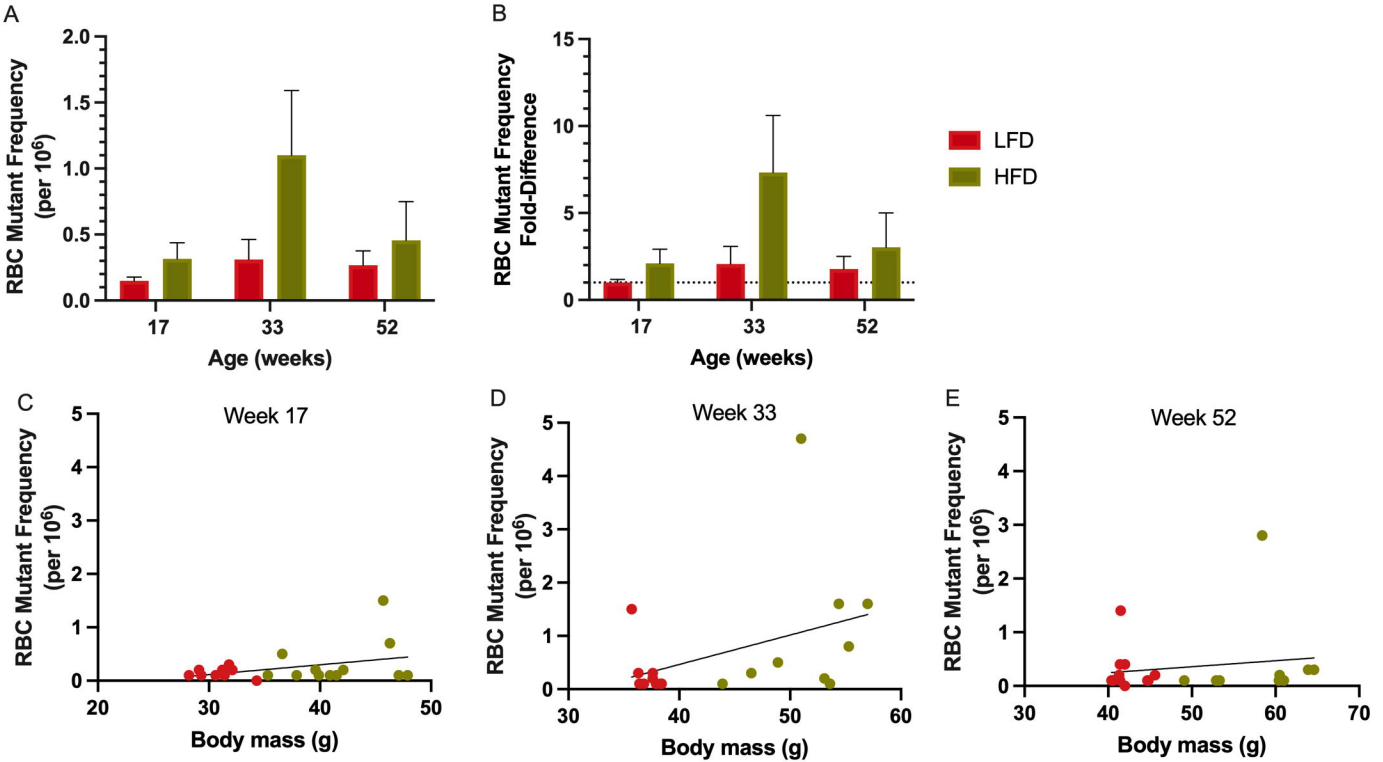
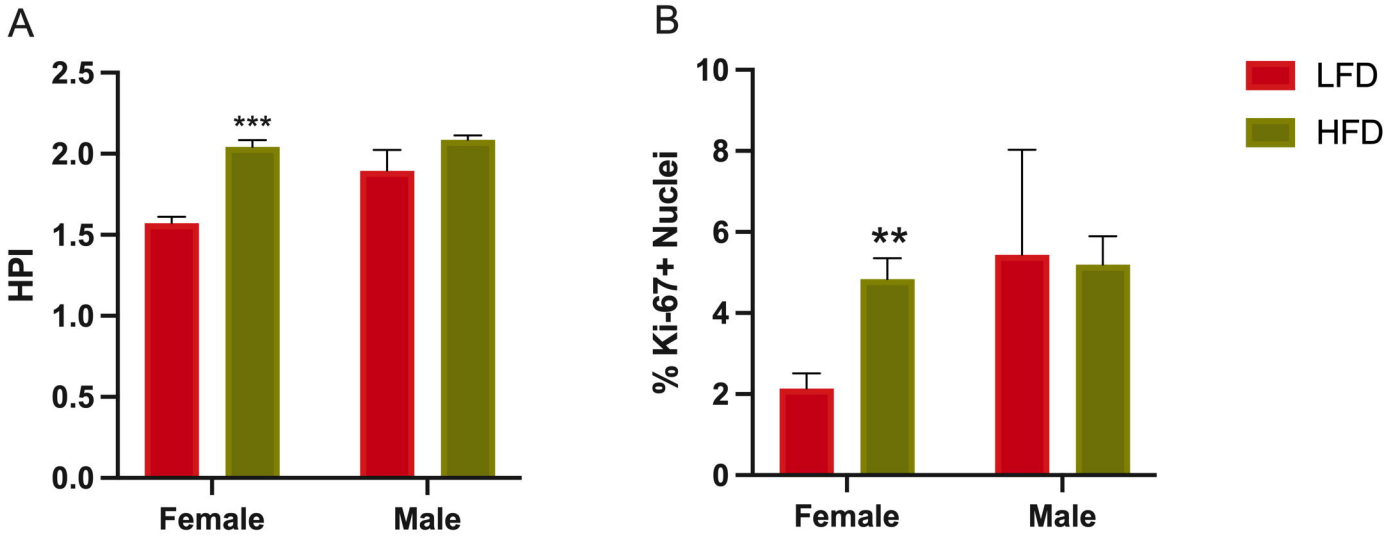
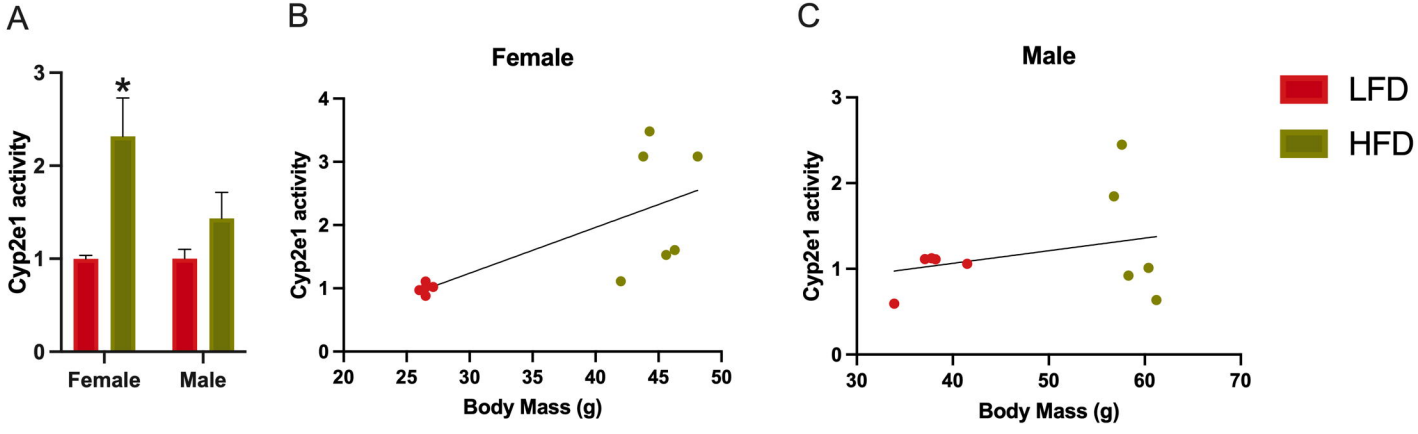
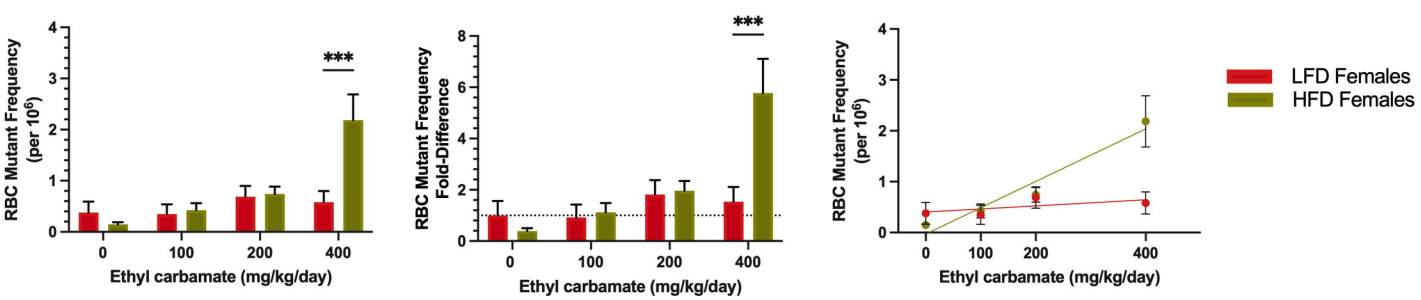
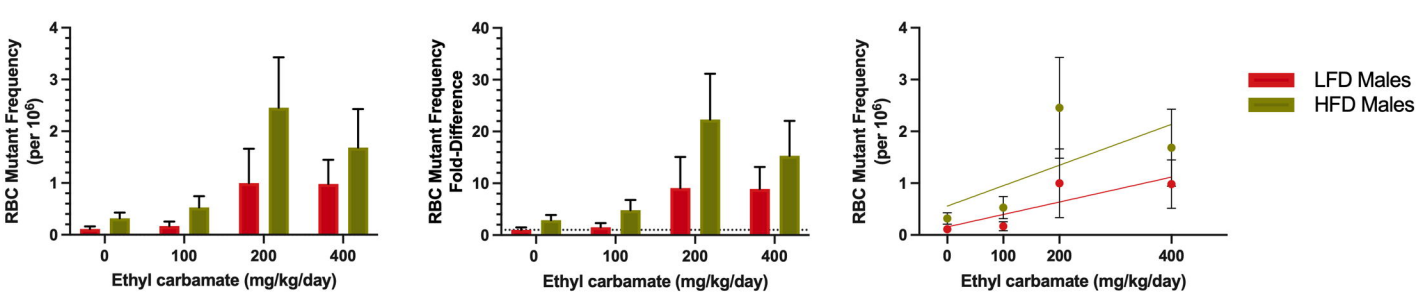
1.5
1.0
0.5
0.0
0
20
40
60
80
Body mass (g)


A
RBC Mutant Frequency (per 10^6)
2.0
1.5
1.0
0.5
0.0
17
33
52
Age (weeks)
B
RBC Mutant Frequency Fold-Difference
15
10
5
0
17
33
52
Age (weeks)
LFD
HFD
C
Week 17
RBC Mutant Frequency (per 10^6)
5
4
3
2
1
0
20
30
40
50
Body mass (g)
D
Week 33
RBC Mutant Frequency (per 10^6)
5
4
3
2
1
0
30
40
50
60
Body mass (g)
E
Week 52
RBC Mutant Frequency (per 10^6)
5
4
3
2
1
0
30
40
50
60
70
Body mass (g)


A
2.5
2.0
1.5
1.0
0.5
0.0
HPI
***
Female
Male
B
10
8
6
4
2
0
% Ki-67+ Nuclei
**
Female
Male
LFD
HFD


A
B
C
Cyp2e1 activity
Female
Male
*
Body Mass (g)
LFD
HFD


RBC Mutant Frequency (per 10^6)
***
0
1
2
3
4
0
100
200
400
Ethyl carbamate (mg/kg/day)
RBC Mutant Frequency Fold-Difference
***
0
2
4
6
8
0
100
200
400
Ethyl carbamate (mg/kg/day)
RBC Mutant Frequency (per 10^6)
0
1
2
3
4
0
100
200
400
Ethyl carbamate (mg/kg/day)
LFD Females
HFD Females


RBC Mutant Frequency (per 10^6)
0
1
2
3
4
0
100
200
400
Ethyl carbamate (mg/kg/day)
RBC Mutant Frequency Fold-Difference
0
10
20
30
40
0
100
200
400
Ethyl carbamate (mg/kg/day)
RBC Mutant Frequency (per 10^6)
0
1
2
3
4
0
100
200
400
Ethyl carbamate (mg/kg/day)
LFD Males
HFD Males