

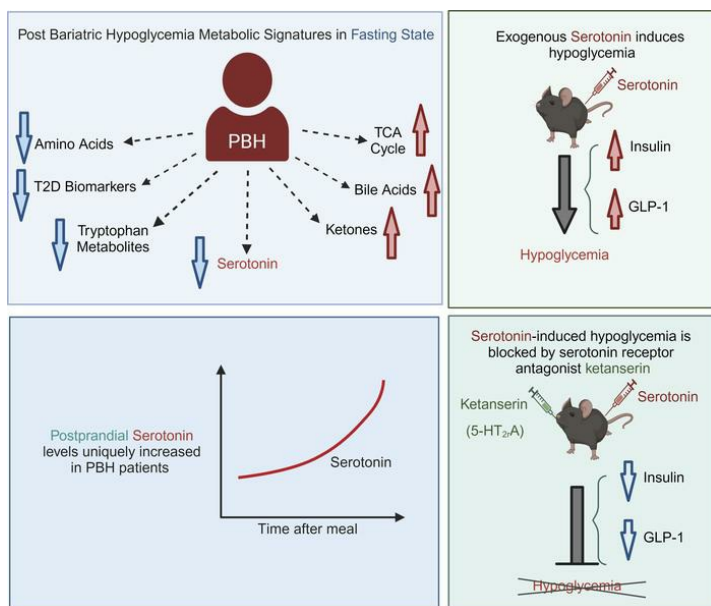
# Postprandial metabolomics analysis reveals disordered serotonin metabolism in post-bariatric hypoglycemia

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## Graphical abstract



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1 **Postprandial metabolomics analysis reveals disordered serotonin metabolism in post-bariatric**  
2 **hypoglycemia**

4 **Running Title: Serotonin and Post-Bariatric Hypoglycemia**

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27 **One Sentence Summary:** Metabolomics analysis reveal reduced fasting and elevated postprandial  
28 serotonin levels in individuals with hypoglycemia after bariatric surgery.

33 **Abstract**

34

35 **BACKGROUND.** Bariatric surgery is a potent therapeutic approach for obesity and type 2 diabetes  
36 but can be complicated by post-bariatric hypoglycemia (PBH). PBH typically occurs 1-3 hours after  
37 meals, in association with exaggerated postprandial levels of incretins and insulin.

38 **METHODS.** To identify mediators of disordered metabolism in PBH, we analyzed plasma  
39 metabolome in fasting state and 30 and 120 minutes after mixed meal in 3 groups: PBH (n=13),  
40 asymptomatic post-RYGB (n=10), and non-surgical controls (n=8).

41 **RESULTS.** In the fasting state, multiple tricarboxylic acid cycle intermediates and the ketone beta-  
42 hydroxybutyrate were increased by 30-80% in PBH vs. asymptomatic. Conversely, multiple amino  
43 acids (BCAA, tryptophan) and polyunsaturated lipids were reduced by 20-50% in PBH vs.  
44 asymptomatic. Tryptophan-related metabolites, including kynurenate, xanthurenate, and serotonin,  
45 were reduced by 2-10-fold in PBH in fasting state. Postprandially, plasma serotonin was uniquely  
46 increased by 1.9-fold in PBH vs. asymptomatic post-RYGB. In mice, serotonin administration lowered  
47 glucose and increased plasma insulin and GLP-1. Moreover, serotonin-induced hypoglycemia in mice  
48 was blocked by the nonspecific serotonin receptor antagonist cyproheptadine and the specific serotonin  
49 receptor 2 antagonist ketanserin.

50 **CONCLUSION.** Together these data suggest that increased postprandial serotonin may contribute to  
51 the pathophysiology of PBH and provide a potential therapeutic target.

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55

56 **Introduction**

57 Bariatric and upper gastrointestinal surgery exerts profound improvements in glucose metabolism and can  
58 even induce remission of type 2 diabetes (T2D) (1, 2), thus demonstrating the importance of the  
59 gastrointestinal tract as a key regulator of systemic glucose metabolism. While the mechanisms  
60 responsible for these potent effects remain incompletely understood, contributors include altered  
61 postprandial nutrient delivery to the intestine, increased secretion of glucagon-like peptide 1 (GLP-1) by  
62 intestinal L-cells, increased insulin secretion, intestinal mucosal adaptations, alterations in the gut-brain  
63 axis, and changes in bile acid content and composition (3, 4).

64 Despite the metabolic benefits of bariatric surgery, one increasingly recognized and often-severe  
65 complication of bariatric surgery is postprandial hypoglycemia, termed post-bariatric hypoglycemia  
66 (PBH). PBH is characterized by hypoglycemia with neuroglycopenia, typically developing 1-3 years or  
67 more postoperatively. Hypoglycemia is most commonly recognized 1 to 3 hours after meals (5),  
68 potentially related to the even greater increases in meal-stimulated GLP-1 and insulin secretion in patients  
69 with PBH as compared with unaffected post-surgical patients (5). Indeed, incretin hormone action can  
70 reduce postprandial insulin secretion and improve hypoglycemia (6). However, hypoglycemia may also  
71 occur with activity and during mid-nocturnal hours (7), suggesting additional mechanisms unique to those  
72 affected by PBH. These include increased insulin-independent glucose uptake (8) and increased levels of  
73 the intestinally-derived hormone FGF-19 (9). Given that this disorder represents a model of “extreme”  
74 modification of glucose metabolism in response to bariatric/metabolic surgery, understanding the  
75 metabolic pathways perturbed in PBH may inform our understanding of intestinal regulation of glucose  
76 homeostasis and uncover new therapeutic targets for PBH and, more broadly, for non-surgical approaches  
77 to improve systemic glucose metabolism.

78 We hypothesized that additional perturbations in the metabolome unique to PBH could modulate key  
79 substrates for gluconeogenesis and/or other pathways contributing to hypoglycemia. We thus performed  
80 untargeted semiquantitative metabolomic analysis of plasma samples collected in the fasting state and at  
81 30 and 120 minutes after administration of a liquid mixed meal (MM) in 3 groups of human volunteers:  
82 individuals with PBH, those without hypoglycemia after RYGB (asymptomatic post-RYGB), and  
83 overweight/obese individuals without history of gastrointestinal surgery. We report that metabolic  
84 signatures in PBH suggest an accelerated metabolic return to the fasting state, as evidenced by increases  
85 of multiple tricarboxylic acid cycle intermediates and the ketone  $\beta$ -hydroxybutyrate. In addition, PBH is  
86 characterized by reduction in fasting plasma levels of amino acids (AA), multiple lipid species, and  
87 tryptophan intermediates including kynurenate, xanthurenate, and serotonin. Moreover, plasma serotonin  
88 levels are uniquely increased in the postprandial state in patients with PBH. We demonstrate that serotonin  
89 administration in mice increases insulin and GLP-1 levels and reduces plasma glucose; these effects are  
90 blocked by the nonspecific serotonin antagonist cyproheptadine. Moreover, the serotonin receptor 2  
91 antagonist ketanserin abolished the impact of serotonin to induce hypoglycemia. Together these data  
92 suggest that increased postprandial serotonin may contribute to the pathophysiology and provide a new  
93 therapeutic target for PBH.

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## 100 **Results**

### 101 **Clinical parameters**

102 Demographic information was self-reported by participants and is provided in **Table S1**. Participants were  
103 a subset from a prior study (5) for whom pristine residual samples were available for analysis. These  
104 included 13 individuals with PBH (11F, 2 M; mean age  $48 \pm 11$ , BMI  $30.6 \pm 4.9$ ,  $3.1 \pm 1.6$  years post-  
105 RYGB), 10 individuals post-RYGB without symptomatic hypoglycemia (Asx) (8F, 2M; age  $48 \pm 8$ , BMI  
106  $29.9 \pm 4.4$ ,  $2.6 \pm 1.0$  years post-RYGB), and 8 overweight/obese individuals without T2D and without  
107 history of gastrointestinal surgery (Ow/Ob) (5F, 2M; age  $48 \pm 9$ , BMI  $41.8 \pm 10.8$ ). Hemoglobin A1C did  
108 not differ between groups. Fasting glucose, insulin, triglycerides, HOMA-IR, and Matsuda index were  
109 significantly lower, and HDL significantly higher, in both surgical groups as compared with nonsurgical  
110 Ow/Ob individuals, consistent with improved glucose metabolism and insulin sensitivity postoperatively,  
111 but did not differ between PBH and Asx.

112 During mixed meal testing, nonsurgical participants displayed the expected meal-related rise in glucose  
113 with return to baseline by 120 minutes. However, patterns were distinct in the asymptomatic post-surgical  
114 patients, with greater glycemic excursions vs. controls; participants with PBH had lower glucose levels at  
115 30 minutes vs. asymptomatic ( $128 \pm 32.3$  mg/dL vs.  $162 \pm 38.3$  mg/dL at 30 minutes,  $p=0.019$ , **Fig. S1A**).  
116 At 2 hours, PBH and Asx glucose levels were similar, but significantly lower than in non-surgical  
117 individuals (PBH  $68.7 \pm 11.3$  mg/dL; Asx  $70.3 \pm 8.6$  mg/dL; Ow/Ob  $98.3 \pm 11.4$  mg/dL,  $p<0.001$  for both  
118 PBH vs. Ow/Ob and Asx vs. Ow/Ob).

119 Fasting insulin and C-peptide were reduced in PBH and Asx groups compared to Ow/Ob, but did not  
120 differ between PBH and Asx. After meal ingestion, insulin and C-peptide levels increased markedly in  
121 PBH, returning to baseline by 2 hours (**Fig. S1B/C**). Plasma GLP-1 was significantly increased in both  
122 surgical groups, with even higher levels in PBH at 30 minutes ( $209.3$  vs  $125.8$  pmol/L for Asx,  $p<0.001$ )

123 (Fig. S1D). GIP levels did not differ between PBH and Asx, but GIP levels in Asx were higher than in  
124 Ow/Ob at 30 minutes ( $p<0.01$ ) (Fig. S1E). Glucagon levels did not differ in the fasting state but were  
125 higher post-meal in both surgical groups (Fig. S1F). Plasma cortisol did not differ between groups (Fig.  
126 S1G).

127

### 128 **Metabolite patterns differ in PBH**

129 Unsupervised hierarchical clustering of the 189 metabolites detected at all 3 time points and with  
130 significant  $FDR<0.05$  (F-test) is presented in the heat map in **Figure 1A**, with full list of cluster  
131 components provided in **Table S2** and full statistical analysis in **Table S3**. As expected, ingestion of the  
132 mixed meal in nonsurgical controls resulted in decreased abundance of multiple lipid species and  
133 increased abundance of amino acids at 30 minutes, with return to baseline at 120 minutes.

134 Exploratory analysis of these 189 metabolites revealed that 158 metabolites were altered in Asx  
135 individuals vs. nonsurgical controls, reflecting the impact of surgery ( $FDR<0.25$ ). 62 metabolites were  
136 altered in PBH vs. Asx post-surgical individuals, and 178 metabolites were differentially abundant in both  
137 comparisons. Interestingly, 19 of these 178 had opposite directionality between PBH and Asx, including  
138 adenine, oxalate, and hydroxyphenylpyruvate (Fig. S2A-C). These findings support the concept that PBH  
139 is not simply an “extreme” example of post-surgical metabolic perturbations.

140 Pathway analysis was performed using the Fry function of the Rotation Gene Set Test (Roast) in the limma  
141 R package. Multiple pathways were enriched ( $FDR<0.25$ ) in the comparison of PBH and Asx post-  
142 surgical groups (Figure 1B-D). In the fasting state, pathways downregulated in PBH include  
143 spermidine/spermine biosynthesis, tryptophan and methionine metabolism, phospholipid synthesis, and  
144 glycolysis (Figure 1B). Post-meal, both spermidine/spermine and methionine metabolism pathways

145 remained downregulated at 30 minutes (**Figure 1C**). By 2 hours, enriched pathways were upregulated in  
146 PBH, including starch/sucrose and pyruvate metabolism, gluconeogenesis and citric acid cycle pathways  
147 (**Figure 1D**). Additional comparisons are presented in **Fig. S3** (PBH vs. Ow/Ob) and **Fig. S4** (Asx vs.  
148 Ow/Ob).

149

### 150 **Correlation of metabolites with glucose**

151 As expected, both amino acids and lipids were highly correlated with glucose levels. The top metabolites  
152 that correlated with glucose in fasting state (FDR<0.05, **Table S4**) were amino acids (Phe, Tyr, Met, Leu,  
153 Trp, His, Ala and Asn), with similar patterns at 30 minutes. Additional metabolites correlating with  
154 glucose in the fasting state included NMMA (N-methylmalonic acid), carnitine, UDP (uridine-5'-  
155 diphosphate), and oxalate. At 30 minutes metabolites correlating with glucose included amino acids (Leu,  
156 Met, Ile, Phe, Tyr, Gln, Pro, Arg, Trp, Gly, Ser, His, Val, Lys, Thr), citrulline, choline, NMMA and  
157 ornithine, and the triglycerides C56:1, C48:0, and C50:0 (FDR<0.05). There were no metabolites  
158 significantly correlating (FDR<0.05) with glucose at 120 minutes (**Table S4**).

159 Given that hypoglycemia typically develops 2-3 hours after meals, we asked whether metabolites present  
160 in the fasting state correlated with post-meal glucose. While relationships were not as robust as for the  
161 matched-time analyses, fasting levels of 17 metabolites were positively correlated with glucose at 120  
162 minutes, including lactate, amino acids (Gln, Pro, Trp, Tyr, betaine), carnitine, and multiple lipids (LPC  
163 18:0, 18:2, 20:3, 22:6, LPE 18:1 and 22:6) (FDR<0.25, **Fig. S5**).

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167 **Plasma levels of TCA cycle intermediates are increased in PBH**

168 Hypoglycemia ultimately may reflect interaction between excessive insulin secretion, inadequate  
169 counterregulatory hormone levels and/or action, reduced glycogen stores, or inadequate substrate or  
170 activity within gluconeogenic pathways. As noted above, multiple AA were reduced in PBH, potentially  
171 contributing to reduced formation of intermediates such as pyruvate (alanine), acetyl-CoA (tryptophan,  
172 tyrosine, leucine, isoleucine and phenylalanine) and succinyl-CoA (methionine, valine and threonine).  
173 We interrogated metabolite patterns in central glucose metabolic pathways, projecting differences in PBH  
174 onto a pathway map at each time point (**Figure 2**).

175 In the fasting state (**Figure 2A**), PBH was characterized by decreased levels of not only glucose (20%  
176 lower,  $p=0.001$ ,  $FDR=0.04$ ) but also decreases in gluconeogenesis intermediates such as glucose-6-  
177 phosphate (40% reduction,  $p=0.02$ ,  $FDR=0.18$ ) and pyruvate (80% lower,  $p<0.0001$ ,  $FDR=0.003$ ).  
178 Conversely, TCA cycle intermediates such as aconitate, isocitrate, and succinate were increased by 30%  
179 in PBH vs. Asx in the fasting state ( $p<0.05$  for all,  $FDR<0.25$ ). Moreover, the ketone  $\beta$ -hydroxybutyrate  
180 was increased by 80%, as noted above ( $FC=1.8$ ,  $p=0.04$ ,  $FDR<0.25$ ).

181 At 30 minutes post-meal, only glucose and pyruvate remained lower (20 and 50% lower,  $p<0.01$ ,  
182  $FDR<0.1$ ), and isocitrate remained higher (30% higher,  $p=0.009$ ,  $FDR=0.14$ ) in PBH vs. Asx (**Figure**  
183 **2B**). At 2 hours, 3-phosphoglycerate was 2-fold higher ( $p=0.002$ ,  $FDR=0.002$ ), while several TCA  
184 intermediates were again higher in PBH, including 30-40% increases in aconitate, isocitrate, and malate  
185 (all  $p<0.01$ ,  $FDR<0.01$ ) (**Figure 2C**).

186

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188

189 **Lipid, ketone, and bile acid metabolism is perturbed in PBH.**

190 Multiple lipid species were decreased in both surgical groups at 30 minutes, consistent with postprandial  
191 rises in insulin secretion. Additional lipid species differentially abundant in PBH vs. Asx included LPE  
192 20:4 and C22:6, LPC C22:6, and TAG C50:5, C54:5 and C56:7, all of which were decreased by 30-50%  
193 in PBH vs. Asx ( $p < 0.05$ ,  $FDR < 0.25$ , **Fig. S6A-F**). By contrast, C56:1 TAG was markedly increased in  
194 PBH vs. Asx at 30 and 120 minutes (29 and 59-fold higher,  $p = 0.01$ ,  $FDR < 0.25$  for both, **Fig. S6G**). In  
195 agreement, abundance of C56:1 TAG was inversely correlated with glucose levels at both 30 ( $r = -0.67$ ,  
196  $p = 0.01$ ) and 120 minutes ( $r = -0.65$ ,  $p = 0.02$ ) in PBH, but not in the fasting state.

197 Ketones, produced in the liver from 2-carbon products of  $\beta$ -oxidation, can be utilized by some tissues as  
198 an alternative fuel when glucose availability is limited, as with prolonged fasting. Interestingly, the ketone  
199  $\beta$ -hydroxybutyrate was increased in the fasting state by 80% in PBH vs. Asx ( $p = 0.04$ ,  $FDR < 0.25$ ) and by  
200  $>3$ -fold in comparison to Ow/Ob ( $p < 0.001$ ,  $FDR = 0.01$ ). These differences remained at 120 minutes (**Fig.**  
201 **S6H**).

202 Bile acids have been proposed as mediators of glycemic improvement after RYGB (4), potentially via  
203 effects to increase both incretin hormones and FGF19 (4, 10). Fasting levels of the conjugated bile acid  
204 taurocholate were 2.5-fold higher in PBH vs. Asx ( $p = 0.03$ ,  $FDR = 0.22$ ) (**Fig. S6I**). However, there were  
205 no significant differences in fasting or postprandial levels of other measured bile acids in PBH vs.  
206 asymptomatic post-surgical participants (4, 9, 11).

207

208 **Biomarkers of insulin resistance and T2D are decreased in PBH.**

209 Previous studies have identified metabolite biomarkers of insulin resistance and risk for T2D (12, 13).  
210 These include amino acids (BCAA, Phe, and Tyr), and triglyceride species characterized by lower carbon

211 number and double bond content (14). Indeed, post-surgical individuals had reductions in these biomarker  
212 AA and lipids, consistent with improved metabolism after RYGB, with further reductions in PBH.  
213 Moreover,  $\alpha$ -hydroxybutyrate, an organic acid derived from  $\alpha$ -ketobutyrate (produced by amino acid  
214 catabolism) and associated with insulin resistance, was 30% lower in PBH at both fasting ( $p=0.03$ ,  
215  $FDR=0.21$ ) and 30 minutes post-meal ( $p=0.03$ ,  $FDR=0.22$ ), as compared to Asx (**Fig. S7A**). Likewise, 2-  
216 aminoadipate, a product of lysine degradation associated with insulin resistance (13, 15), was also 6-fold  
217 lower in PBH vs. Asx ( $p=0.001$ ,  $FDR=0.04$ ) in the fasting state (**Fig. S7B**).

218

#### 219 **Levels of B-complex vitamins are reduced in PBH.**

220 B-complex vitamins play important roles as cofactors for key reactions in cellular metabolism. Several  
221 water-soluble B vitamins, including niacinamide (B3) and pantothenate (B5) were decreased by 2-fold in  
222 the fasting state in PBH vs. Asx ( $p<0.05$  for all,  $FDR<0.25$ ). Pantothenate was also 2-fold lower at 30 and  
223 120 minutes ( $p<0.01$  for both,  $FDR<0.05$ ) (**Fig. S7C-D**). Moreover, fasting glucose was positively  
224 correlated with levels of both vitamins B3 (fasting  $r=0.48$ ,  $p=0.02$ ,  $FDR 0.11$ ) and B5 (fasting  $r=0.52$ ,  
225  $p=0.009$ ,  $FDR=0.07$ ; 30 minutes:  $r=0.49$ ,  $p=0.017$ ,  $FDR=0.14$ ).

226

#### 227 **Amino acid levels are reduced in PBH in the fasting state.**

228 In the fasted state, 10 amino acids (AA) were significantly reduced in PBH as compared with both  
229 asymptomatic and nonsurgical groups. Leucine, isoleucine, valine, alanine, tryptophan, phenylalanine,  
230 threonine, tyrosine, methionine and asparagine were reduced by 17-29% ( $p<0.05$ ,  $FDR<0.25$ ) in PBH vs.  
231 Asx and Ow/Ob groups (**Figure 3A-J**). After meal ingestion, amino acid levels increased markedly in  
232 both surgical groups, but only leucine, tryptophan and phenylalanine remained lower in PBH as compared

233 to Asx. AA levels for each participant are presented in **Fig. S8**. Notably, the reductions in AA in PBH  
234 included both essential and non-essential AA, and both gluconeogenic (e.g. Ala) and ketogenic (e.g. Leu)  
235 AA. Arginine abundance was increased in both PBH and Asx compared to Ow/Ob (**Figure 3K**), while  
236 glutamine did not differ between groups (**Figure 3L**). The sum of all amino acids was strongly positively  
237 correlated with glucose in the fasting state ( $r=0.78$ ,  $p=0.001$ ) and at 30 minutes ( $r=0.59$ ,  $p=0.033$ ); these  
238 correlations did not remain at 120 minutes ( $r=0.07$ ,  $p=0.82$ ) (**Figure 3M-O**). The top-ranking specific  
239 amino acids correlated with glucose levels in both the fasting state and at 30 minutes are provided in **Fig.**  
240 **S9**.

241

#### 242 **Serotonin levels are uniquely increased in the postprandial state in individuals with PBH**

243 Both pathway and individual metabolite analysis revealed differential abundance of tryptophan pathway  
244 intermediates in PBH, including tryptophan itself and its downstream metabolites kynurenic acid,  
245 xanthurenate, and serotonin (**Figure 4**). For example, tryptophan levels were 40% lower in the fasting  
246 state in PBH vs. Asx ( $p=0.003$ ,  $FDR=0.06$ ), and 20-30% lower at 30 and 120 minutes ( $p=0.028$  and  $0.019$ ,  
247  $FDR<0.25$  respectively) (**Figure 4A**). Similarly, kynurenic acid was reduced by 70% at all 3 time points  
248 ( $p<0.01$  for all,  $FDR<0.25$ ), while xanthurenate was 8-fold lower in the fasting state ( $p=0.003$ ,  $FDR=0.06$ )  
249 and 6-fold lower at 2 hours ( $p=0.008$ ,  $FDR<0.01$ ) (**Figure 4B/C**).

250 Serotonin levels were 10-fold lower in the fasting state in PBH, as compared to Asx ( $p=0.019$ ,  $FDR=0.16$ )  
251 (**Figure 4D**). Interestingly, serotonin patterns in response to meal ingestion were distinct in PBH,  
252 increasing by 3.2 and 4.7- fold at 30 and 120 minutes; by comparison, serotonin levels decreased after  
253 meal ingestion in Asx and nonsurgical groups.

254 To confirm alterations in serotonin dynamics in PBH, we analyzed serotonin using ELISA on samples  
255 collected in the fasting state and at 30 and 120 minutes after meal originating from a newly recruited  
256 cohort described in **Table S5**. Consistent with the metabolomics data, serotonin levels in individuals with  
257 PBH increased 1.9-fold after a meal ( $p < 0.001$ ), whereas Asx and Ow/Ob patients had a 1.4 to 1.6 fold  
258 ( $p < 0.001$  for both) decrease in serotonin levels over the same time course (**Figure 4E**). Serotonin levels  
259 for each participant are presented in (**Figure 4F-H**), demonstrating the distinct responses in all individuals  
260 with PBH.

261 Correlation analysis between metabolites and glucose levels during MMTT showed that tryptophan was  
262 correlated with glucose in the fasting state ( $r=0.67$ ;  $p < 0.001$ ), and at 30 ( $r=0.65$ ;  $p < 0.001$ ) and 120 minutes  
263 ( $r=0.47$ ;  $p=0.023$ ) (**Table S4**). While there was no correlation between serotonin (measured by  
264 metabolomics assay) and glucose (**Table S4**), plasma serotonin, measured by ELISA, was inversely  
265 correlated with glucose at 120 minutes after the meal in PBH ( $r=-0.78$ ;  $p=0.007$ ) (**Fig. S10**).

266 To exclude the possibility that high plasma insulin or low glucose levels could contribute to observed  
267 increases in serotonin in the postprandial state, we analyzed serotonin levels in response to  
268 hyperinsulinemic hypoglycemic clamps in PBH, Asx and Ow/Ob participants (**Fig. S16A**). Consistent  
269 with the design of the clamp, plasma glucose levels at 100 minutes were similarly reduced in all groups  
270 (PBH:  $51 \pm 7$  mg/dL; Asx:  $51 \pm 7$  mg/dL; Ow/Ob:  $53 \pm 5$  mg/dL; **Fig. S16B**). However, serotonin levels did  
271 not differ between groups, either in the fasting state (PBH:  $103 \pm 57$  ng/ml; Asx:  $152 \pm 73$  ng/mL; Ow/Ob:  
272  $174 \pm 81$  ng/mL) or during experimental hypoglycemia (PBH:  $142 \pm 72$  ng/mL; Asx:  $119 \pm 89$  ng/mL;  
273 Ow/Ob  $187 \pm 53$  ng/ml, **Fig. S16C**). Similarly, serotonin levels did not change after insulin injection in  
274 mice (2U/kg, intraperitoneally) (**Fig. S16D-E**) as compared with equal-volume saline at either 15 min  
275 (insulin:  $430 \pm 166$  ng/mL vs. saline:  $344 \pm 56$  ng/mL) or 30 minutes (insulin:  $344 \pm 87$  ng/mL vs. saline:  
276  $351 \pm 175$  ng/mL) (**Fig. S16F**). Together, these results in both humans and mice suggest that

277 hyperinsulinemia or hypoglycemia per se are not likely to mediate increases in postprandial serotonin in  
278 PBH.

279

## 280 **Serotonin induces hypoglycemia in mice**

281 Given that serotonin levels were uniquely increased in the postprandial state in individuals with PBH (4.7-  
282 fold at 120 min), and not influenced by experimental hypoglycemia or hyperinsulinemia, we sought to  
283 determine whether serotonin could modulate glucose levels in vivo in healthy C57Bl/6J mice. We injected  
284 wild type mice with serotonin (20 mg/kg, intraperitoneally) or equal volume of saline (**Figure 5A**). In  
285 serotonin-injected mice, serotonin levels increased rapidly to a peak of 539 ng/mL at 30 minutes (**Figure**  
286 **5B**). To determine the impact of serotonin on glucose metabolism, we measured blood glucose at baseline  
287 and at 15, 30 and 60 minutes. Glucose levels were significantly reduced in serotonin-injected male mice  
288 at 15 (-39%,  $p < 0.001$ ), 30 (-43%,  $p < 0.002$ ) and 60 min (-44%,  $p < 0.018$ ) (**Figure 5C**), with similar  
289 magnitude reductions in females (-29% at 15 min,  $p < 0.001$ , -48% at 30 min,  $p < 0.001$ , and -48% at 60  
290 min,  $p = 0.005$ ) (**Fig. S11**). We next assessed whether serotonin-induced reduction in glucose was  
291 mediated by an insulin-dependent mechanism; plasma insulin increased by 5 and 2.5-fold at 30 and 60  
292 min after serotonin injection vs. saline ( $p = 0.004$  and  $p = 0.002$ , **Figure 5D**). Interestingly, serotonin  
293 administration also significantly increased plasma levels of GLP-1 by 1.6 to 2.1-fold at 15, 30, and 60  
294 min after injection ( $p = 0.011$ ,  $p < 0.001$ , and  $p < 0.001$ , **Figure 5E**).

295 To mimic the postprandial glucose dynamics and exaggerated insulin secretion characteristic of PBH in  
296 mice, we developed an insulin-augmented mixed meal tolerance test. After a four-hour fast, mice were  
297 gavaged with a liquid mixed meal (200  $\mu$ l Ensure Compact, Abbott) and injected with insulin (2 U/kg  
298 intraperitoneal, Humulin R, Lilly), prior to injection with serotonin or saline (**Figure 6A**). Consistent with

299 prior experiments, serotonin (20 mg/kg) reduced also glucose by 38% vs. saline in this setting, achieving  
300 levels below fasting level (serotonin: 80 vs. saline: 128 mg/dL at 60 minutes,  $p=0.021$ , **Figure 6B**).

301 Given that serotonin increased GLP1 levels, we asked whether GLP1 receptor blockade would attenuate  
302 serotonin affects. We first confirmed that the GLP1 receptor antagonist avexitide (30 mmol/kg) blocked  
303 semaglutide-induced increases in insulin secretion (**Fig. S13B**, and in parallel, attenuated the  
304 hypoglycemic effects of semaglutide (**Fig. S13C**) and reduce GLP-1 levels (**Fig. S13D**). Next, we tested  
305 whether avexitide could block serotonin-induced hypoglycemia (**Fig. S14A**). Treatment of mice with  
306 avexitide rapidly increased glucose levels as compared to saline-injected mice ( $p=0.012$  at 15 minutes).  
307 However, avexitide was unable to block serotonin-induced hypoglycemia (**Fig. S14B**). Likewise,  
308 avexitide did not affect serotonin-induced increases in insulin and GLP-1 (**Fig. S14C/D**). Thus, these data  
309 suggest GLP-1 receptor-mediated signaling is not likely a primary mechanism mediating serotonin-  
310 induced hypoglycemia.

311

### 312 **Serotonin-induced hypoglycemia in mice is blocked by serotonin receptor antagonism**

313 First, we sought to investigate pathways by which serotonin induced hypoglycemia. Serotonin has  
314 pleiotropic effects on many aspects of metabolism, mediated via multiple receptor subtypes (16).

315 To test whether the effects of serotonin on glucose levels could be blocked by broad antagonism of  
316 serotonin receptor subtypes, the nonspecific antagonist cyproheptadine or equal volume of saline was  
317 injected intraperitoneally 30 minutes before the mixed meal (**Figure 6A**). Cyproheptadine completely  
318 blocked the hypoglycemia induced by serotonin administration, with glucose of 170 mg/dL at 60 minutes  
319 vs. 80 mg/dL with serotonin alone ( $p=0.005$ , **Figure 6B**). Interestingly cyproheptadine pretreatment also  
320 blunted the increase in plasma serotonin as compared with saline at 15 (75%,  $p<0.001$ ), 30 (72%,  $p=0.008$ )

321 and 60 minutes (74%,  $p=0.034$ , **Fig. S12A**). Likewise, serotonin-induced increase in GLP-1 (1.7 to 2.1-  
322 fold,  $p<0.001$ , **Fig. S12B**) was abolished by pretreatment with cyproheptadine (-1.9 to -2.1-fold,  $p<0.001$   
323 for all, **Fig. S12B**). These results suggest that increased plasma serotonin may contribute to regulation of  
324 postprandial glucose metabolism, potentially via effects to increase both GLP-1 and insulin secretion.

325 We next investigated whether inhibition of specific serotonin receptor subtypes could block serotonin-  
326 induced hypoglycemia, selecting inhibitors of 5HTR3 and 5HTR2, given their demonstrated role in  
327 metabolism and insulin secretion (17-19). The 5HTR3 antagonist ondansetron (3 mg/kg) was  
328 administered 30 minutes before injection of serotonin (20 mg/kg) or equal volume of saline (**Fig. S15A**).  
329 Ondansetron did not impact serotonin-induced reductions in glucose (**Fig. S15B**), nor was it able to block  
330 serotonin-stimulated increases in insulin (**Fig. S15C**) or GLP-1 (**Fig. S15D**).

331 We next tested the impact of the 5HTR2 antagonist ketanserin, injecting male mice with ketanserin (5  
332 mg/kg) 30 minutes prior to serotonin administration (20 mg/kg) (**Figure 6C**). Ketanserin abolished the  
333 impact of serotonin, increasing blood glucose by 86% ( $p<0.05$ , **Figure 6D**). Likewise, ketanserin reduced  
334 serotonin-stimulated insulin levels by 44% ( $p<0.05$ , **Figure 6E**) and reduced serotonin-stimulated GLP-  
335 1 levels by 33% ( $p<0.05$ , **Figure 6F**). These data suggest that 5HTR2 may mediate glycemic effects of  
336 serotonin and that inhibition could be tested as a potential strategy for PBH.

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343 **Discussion**

344 PBH can be observed in up to 20-30% of patients following bariatric surgery. When severe, it can be  
345 associated with neuroglycopenia, reduced awareness, and impaired safety. Accelerated gastric emptying  
346 with rapid increases in glucose and amino acid absorption, together with elevations in GLP-1 and other  
347 intestinally-derived hormones, contribute to increased postprandial insulin secretion and subsequent  
348 hypoglycemia (20). Increased insulin-independent glucose uptake (4) and reduced counterregulatory  
349 responses (21) may also contribute to hypoglycemia in patients with PBH. Given that maintenance of  
350 normal glucose homeostasis also requires adequate substrate availability and metabolism, we performed  
351 and now report an unbiased metabolomics analysis of the plasma metabolome in the fasting state and in  
352 response to a standard mixed meal stimulus in individuals with PBH as compared with unaffected post-  
353 bariatric participants.

354 Our data suggest that metabolism in PBH is not simply an extreme example of post-bariatric metabolism,  
355 as multiple metabolites exhibited unique patterns in PBH, with differential direction of change as  
356 compared with asymptomatic post-RYGB individuals. For example, we observed PBH-specific increases  
357 in abundance of adenine, oxalate and hydroxyphenylpyruvate. It is possible that unique differences in  
358 dietary patterns, intestinal or microbial metabolism could contribute to these PBH-specific metabolic  
359 responses. More detailed studies analyzing the intestinal microbiome in individuals with PBH will be  
360 required.

361 Amino acid and lipid metabolism ultimately yield carbons for key metabolic pathways, such as oxidative  
362 TCA cycle activity and gluconeogenesis. Interestingly, several glycolytic intermediates were reduced in  
363 PBH in the fasting state, alongside an increase in the TCA cycle intermediates aconitate, isocitrate and  
364 succinate. These patterns were largely normalized at the 30-minute time point but were observed again at  
365 2 hours – patterns reminiscent of both the accelerated onset of postprandial physiology after RYGB,

366 potentially linked to rapid emptying of nutrients from the pouch (22), as well as accelerated return to a  
367 fasting state. Levels of the ketone  $\beta$ -hydroxybutyrate were reduced in response to the meal in all 3 groups,  
368 as expected. However, levels were significantly increased in PBH in both the fasting state and after 120  
369 minutes, potentially reflecting increased fatty acid oxidation with fasting, an accelerated return to fasting  
370 physiology after meals (despite high postprandial insulin levels), and protective adaptation to provide  
371 alternative fuels in the setting of recurrent hypoglycemia (23).

372 Thus, we hypothesize that lipid metabolism is the dominant contributor to whole-body metabolism in both  
373 the fasting state and at 2 hours postprandially in PBH. In this setting, if gluconeogenic flux and/or  
374 glycogenolysis are also compromised due to inadequate substrates for gluconeogenesis (e.g. reduced  
375 amino acids), subnormal glycogen storage (low caloric intake), or reduced counterregulatory hormones,  
376 hypoglycemia at late postprandial time points could ensue. The increase in 3-phosphoglycerate at 120  
377 minutes suggests that reductions in gluconeogenesis at more distal steps could potentially contribute to  
378 hypoglycemia. While studies of flux will be required to dissect these interesting patterns, we hypothesize  
379 that PBH is characterized by enhanced lipid fuel utilization in the fasting state, but inadequate glucose  
380 fuel availability in the fed-fasted transition.

381 Individuals with PBH also had reductions in levels of B-complex vitamins such as niacinamide (B3) and  
382 pantothenate (B5) and levels of both B3 and B5 were correlated with fasting glucose. Reductions in these  
383 cofactors for critical enzymes could potentially impair the function of central metabolic pathways  
384 including the TCA cycle (24), and could suggest deficiencies in micronutrient intake or absorption in  
385 PBH.

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387 Bile acids, now recognized as metabolically active signaling molecules (25), are increased after RYGB  
388 (26). While the sum of measured BA was not increased in PBH vs. asymptomatic participants in this

389 cohort, the conjugated bile acid taurocholate was 2.5-fold higher in PBH in the fasting state, consistent  
390 with prior reports (9, 11) and several species tended to be higher in post-surgical participants. Additional  
391 targeted studies of bile acid synthesis, conjugation, and microbial metabolism in the fasting and  
392 postprandial state will be required to further define these patterns.

393

394 Abundance of multiple amino acids, including BCAA, was reduced in individuals with PBH as compared  
395 with both asymptomatic and non-surgical groups in the fasting state. Amino acids increased rapidly after  
396 mixed meal ingestion in both surgical groups, potentially reflecting rapid gastric emptying and absorption.  
397 However, lower levels persisted in PBH over time, and total AA levels correlated with glucose levels in  
398 both the fasting state and at 30 minutes. These data are of interest for several reasons. Firstly, amino acids  
399 have long been recognized as important substrates for gluconeogenesis and central metabolic pathways.  
400 For example, alanine, reduced in PBH, is a major substrate for hepatic gluconeogenesis (27). Multiple  
401 BCAA were also reduced in PBH, including the ketogenic Leu, forming acetyl-CoA, the glucogenic Val,  
402 yielding succinyl-CoA, and isoleucine, which can be metabolized into both acetyl-CoA and succinyl-CoA  
403 . Secondly, our data are concordant with prior reports showing reductions in AA, BCAA, and downstream  
404 products of BCAA catabolism following RYGB (22, 28, 29). Given that elevations in AA are associated  
405 with obesity and insulin resistance (30) and predict risk of T2D (31), the further reduction in AA in patients  
406 with PBH as compared with unaffected post-surgical patients may reflect the impact of weight loss, altered  
407 nutrition, and improved insulin sensitivity to yield an exaggerated “anti-diabetes” metabolic state  
408 contributing to reductions in gluconeogenesis. Alterations in AA in PBH could also contribute to the  
409 significant reduction in 2-aminoadipate and  $\alpha$ -hydroxybutyrate, biomarkers of diabetes risk and insulin  
410 resistance (12, 13, 32).

411 We do not fully understand the mechanisms underlying reductions in multiple AA in PBH. Given that  
412 differences were observed mainly for essential amino acids, it will be important to determine whether  
413 sources or quantity of dietary protein intake, absorption (33), or metabolism differs in post-surgical  
414 individuals with and without PBH. The rapid prandial excursions in AA after RYGB could also contribute  
415 to prandial rises in insulin and glucagon (34, 35). Additional studies of in vivo flux will be required to  
416 clarify these possibilities.

417 A key finding is that multiple metabolites within the tryptophan pathway are downregulated in the fasting  
418 state in PBH as compared with asymptomatic individuals, including tryptophan and its downstream  
419 metabolites kynurenic acid and xanthurenic acid. Tryptophan was also significantly correlated with  
420 glucose at all 3 time points. It is possible that altered intake and/or microbial metabolism of tryptophan  
421 could contribute to reduced absorption and reduced plasma levels of tryptophan and downstream  
422 metabolites after bariatric surgery (36, 37).

423 Even more striking were differences in levels of serotonin in individuals with PBH, which were validated  
424 by ELISA in samples from an independent validation cohort. Firstly, fasting serotonin levels were 10-  
425 fold lower in PBH, as compared with both unaffected post-surgical individuals and nonsurgical  
426 participants. Secondly, responses to the mixed meal were distinct and divergent in PBH. Consistent with  
427 prior reports in healthy individuals (38), serotonin levels decreased after meal ingestion in both Asx and  
428 nonsurgical groups. By contrast, patients with PBH had a 5-fold *increase* in meal-stimulated serotonin.

429 Several lines of evidence suggest that observed changes in serotonin in the postprandial state may  
430 contribute mechanistically to postprandial increases in GLP1 and insulin in PBH. Firstly, our studies in  
431 both humans and mice indicate that neither experimental hyperinsulinemia or hypoglycemia result in  
432 increased serotonin. Secondly, we demonstrate that experimental injection of serotonin can potently  
433 reduce blood glucose in mice, consistent with prior studies (39, 40), in parallel with >2-fold increases in

434 insulin and GLP1. We also extended these observations to the postprandial state; serotonin injection at  
435 the time of oral mixed meal gavage also induced hypoglycemia.

436 Unique patterns of alterations in serotonin levels in both the fasting and postprandial state are of great  
437 interest for the pathophysiology of PBH given its role in multiple key processes in both peripheral tissues  
438 and the central nervous system linked to maintenance of normal glucose homeostasis (41-45). Serotonin  
439 modulates insulin secretion (46, 47) and contributes to expansion of  $\beta$ -cell mass during pregnancy.  
440 Furthermore, production and release of serotonin by  $\beta$ -cells (48) inhibits glucagon secretion from  $\alpha$ -cells  
441 (46). Serotonin also impacts intestinal metabolism, including stimulation of GLP-1 secretion by intestinal  
442 L cells (49, 50) and modulation of the local enteric and central nervous system (51). Central serotonergic  
443 signaling also regulates food intake; loss of the 5HT<sub>2C</sub> receptor leads to obesity. Moreover, serotonergic  
444 neurons are required for glucagon secretion, a key element of the counterregulatory response to  
445 hypoglycemia (52).

446 Clinically, modulation of serotonin has been linked to hypoglycemia. Antidepressant selective serotonin  
447 reuptake inhibitors or serotonin norepinephrine reuptake inhibitors (SSRI/SNRI) and MAO inhibitors,  
448 which inhibit metabolism of serotonin, can cause hypoglycemia. Moreover, we recently reported that use  
449 of SSRI and SNRI was a significant risk factor for self-reported hypoglycemia symptoms in post-RYGB  
450 patients enrolled in the Longitudinal Assessment of Bariatric Surgery (LABS) cohort (53). Interestingly,  
451 symptoms experienced by PBH patients in the postprandial state show some similarities to those in  
452 individuals who have serotonin syndrome (SS), a condition induced by SSRIs and other serotonergic  
453 medications (54), and manifested by confusion, sweating, tremors and, in more severe cases, seizures (55).  
454 However, there were no differences in medication classes, including SSRI and SNRI in any of the cohorts  
455 utilized for the current studies (**Table S5**).

456 Taken together, our data support the hypothesis that dysregulation of serotonin metabolism, with reduction  
457 in fasting levels and increased meal-stimulated levels, may contribute to excessive prandial insulin  
458 secretion and hypoglycemia in patients with PBH. We do not yet understand the mechanisms responsible  
459 for the reduction in basal serotonin levels and the unique increases in postprandial serotonin levels in  
460 individuals with PBH. Potential mediators of increased postprandial serotonin levels in PBH could include  
461 increased numbers of serotonin-secreting enterochromaffin cells, or increased release of serotonin by these  
462 same cells, potentially linked to alterations in luminal contents (56). Conversely, altered expression or  
463 activity of serotonin synthesis enzymes or cofactors (e.g. vitamin B6) and serotonin-metabolizing  
464 enzymes (e.g. monoamine oxidase, MAO) could also result in increased postprandial levels. Indeed Ben-  
465 Zvi and colleagues (57) demonstrated that expression of MAO is reduced by 2-5-fold in jejunum and  
466 ileum after bariatric surgery in mice. In turn, increased serotonin could contribute to increases in meal-  
467 stimulated GLP1 and insulin secretion characteristic of PBH, as we now demonstrate in mice. It is  
468 possible that these interactions are potentiated by PBH-specific alterations in gut microbiome and/or bile  
469 acid signaling or turnover (58). This concept is supported by several studies demonstrating that the  
470 intestinal microbiome plays a critical role in serotonin metabolism, BA production and even intestinal  
471 motility (59-61). Whether differences in serotonin are pre-existing prior to surgery in those who develop  
472 PBH, or solely develop in response to the altered intestinal anatomy postoperatively, is an interesting  
473 question for future studies.

474 Our findings suggest opportunities for targeting serotonin metabolism as a potential approach to the  
475 management of PBH. Our pilot experiments using the nonspecific serotonin receptor antagonist  
476 cyproheptadine reduced serotonin-induced effects on glucose. While previous studies identified the  
477 serotonin 5-HT<sub>3</sub> receptor in  $\beta$ -cells as a mediator of serotonin-induced insulin secretion (47, 62), here we  
478 demonstrate that serotonin receptor antagonist 3 (ondansetron) was not able to block serotonin-induced

479 hypoglycemia. By contrast, administration of serotonin receptor 2 antagonist (ketanserin) abolished the  
480 impact of serotonin to induce hypoglycemia, in concert with decreased insulin and GLP-1 secretion.  
481 These data suggest that serotonin receptor subtype 2 might mediate serotonin effects on hypoglycemia,  
482 potentially via impact on pancreatic beta cells and intestinal L-cells (18, 19, 56). Future studies will be  
483 required to test whether serotonin receptor blockade could be an effective strategy for PBH therapy, and  
484 to ascertain whether additional serotonin receptor subtypes also contribute in diverse tissues and cell types  
485 relevant for PBH, such as L-cells, enterocytes, enterochromaffin cells, islets, liver, and brain (56, 63).

486 We acknowledge several limitations of our study. Firstly, the mass spectrometry methodology utilized to  
487 analyze metabolomics are semi-quantitative, permitting only relative between-group analysis. However,  
488 differences in serotonin between groups were confirmed by ELISA. Secondly, the sample size is relatively  
489 small, with a majority of women, which does not allow generalization to other populations. In mouse  
490 studies, we demonstrate that serotonin-mediated reduction in glucose are similar in both males and  
491 females. Thirdly, due to the observational nature of our study, it is not possible to determine whether  
492 observed changes in levels of metabolites reflect changes in dietary patterns, nutrient absorption,  
493 catabolism or flux. Due to the postoperative period being between 8-12 years, we do not have a record of  
494 current or previous nutritional intake of supplements such as B-complex vitamins. Fourthly, while the  
495 intestine is the dominant source of circulating serotonin, future studies will be required to determine  
496 whether intestinally-derived serotonin is the mediator of metabolic and hormonal changes characteristic  
497 of PBH and whether neural signaling is required. Finally, we acknowledge that the diet, the gut  
498 microbiome, and its interactions with the host may contribute to differences in metabolite profiles both in  
499 the fasting and postprandial states between patients with PBH and asymptomatic and nonsurgical controls  
500 (64).

501 In summary, we identify metabolic signatures of PBH including reduction in circulating AA and  
502 tryptophan intermediates, multiple lipid species and increased TCA cycle intermediates. These potentially  
503 contribute to alterations in glucose metabolic flux in both the fasting and postprandial state which  
504 collectively increase risk for hypoglycemia. We also identified PBH-specific patterns of serotonin  
505 dynamics in both the fasting and postprandial state. Using a mouse model, we demonstrate that exogenous  
506 serotonin administration in mice lowers plasma glucose, potentially via increases in both plasma insulin  
507 and GLP1 levels. Moreover, serotonin-induced hypoglycemia after a mixed meal was blocked by the  
508 serotonin antagonist cyproheptadine and more specifically by serotonin receptor 2 antagonist ketanserin.  
509 Thus, increases in postprandial serotonin levels may contribute to postprandial symptoms and reduction  
510 in glucose levels, and provide a potential therapeutic target for PBH.

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522 **Methods**

523 **Sex as a biological variable**

524 In both cohorts of human participants, both male and female individuals were included. Sex was not  
525 considered as a biological variable, given the low percentage of males in our study population, consistent  
526 with greater percentage of females among the post-surgical population, limiting power for sex-specific  
527 analysis. However, for animal studies, we evaluated both male and female mice; similar results suggests  
528 relevance for both sexes

529

530 **Participants**

531 The Joslin Diabetes Center Institutional Review Board approved the study. Written informed consent was  
532 obtained from all participants. A detailed description of the previous clinical study that generated residual  
533 samples for analysis of the present study is provided in (5). In brief, 3 study groups were analyzed (**Table**  
534 **S1**): (1) PBH: 13 participants with history of RYGB and neuroglycopenia recruited from the  
535 hypoglycemia clinic at Joslin Diabetes Center, (2) asymptomatic post-RYGB group (Asx): 10 participants  
536 who underwent uncomplicated RYGB 2–4 years previously with no history of hypoglycemia; and (3)  
537 overweight/obese (Ow/Ob): 8 individuals without history of gastrointestinal surgery (control group).  
538 Blood samples were obtained after an overnight fast, and at 30 and 120 minutes after consumption of a  
539 liquid mixed meal (MMTT) (Ensure, 9 g protein, 40 g carbohydrate, 6 g fat, 240 ml; Abbott Laboratories,  
540 Abbott Park, IL).

541 A new cohort (**Table S5**), was recruited from 2020-2023, and included 15 participants with PBH, 15 Asx  
542 and 10 Ow/Ob. Samples from this cohort were used to validate serotonin levels by ELISA during mixed  
543 meal tolerance test and during hyperinsulinemic hypoglycemic clamps.

544 **Metabolomics analysis**

545 Plasma collected at time 0, 30 and 120 minutes after mixed meal (5, 9) was utilized for untargeted  
546 semiquantitative metabolomic analysis using flow injection-tandem mass spectrometry (FI-MS/MS), with  
547 detection of 189 metabolites.

548 **Hyperinsulinemic hypoglycemic clamp**

549 A primed, continuous infusion of insulin (80 mU/m<sup>2</sup>/min) was used together with a 20% glucose infusion,  
550 titrated every 5 minutes to gradually lower the glucose from fasting levels to a target of 50 mg/dL at a rate  
551 of 1 mg/dl/min (65); the target glucose was maintained for 30 minutes to ensure a consistent stimulus for  
552 counterregulatory responses.

553 **Animal studies**

554 All animal studies were approved by the Institutional Animal Care and Use Committee. C57BL/6J male  
555 and female mice, age 6 weeks, were purchased from JAX. Mice were maintained on a 12 hr light-dark  
556 cycle with ad libitum access to water and chow diet.

557 The impact of serotonin was assessed after a 4 hour fast; mice were injected with 20 mg/kg of serotonin  
558 (serotonin hydrochloride, Sigma-Aldrich, St. Louis, MO, USA) or an equal volume of saline  
559 intraperitoneally. Blood was collected at baseline (pre-injection) and at 15, 30 and 60 min after injection  
560 from the tail vein or blood collected via cardiac puncture at time of euthanasia in EDTA-coated microtubes  
561 containing DPP-IV inhibitor (Millipore, St. Louis, MO, USA); blood glucose was measured using the  
562 Prodigy Autocode meter (Charlotte, NC, USA).

563 To mimic the postprandial glucose dynamics and high insulin secretion characteristic of PBH, the effect  
564 of serotonin on glucose metabolism in response to an insulin-augmented mixed meal tolerance test was  
565 assessed. After a four-hour fast, mice were gavaged with a liquid mixed meal (200 µl Ensure Compact,

566 Abbott, Columbus, OH, USA) and intraperitoneal injection of insulin (2 U/kg, Humulin R, Lilly,  
567 Indianapolis, IN, USA). Serotonin (20 mg/kg) or equal volume of saline was injected intraperitoneally,  
568 immediately after insulin injection. For some experiments, mice were treated with the nonspecific  
569 serotonin antagonist cyproheptadine (50 mg/kg, Millipore&Sigma, Burlington, MA, USA)  
570 intraperitoneally or equal volume of saline, 30 minutes before mixed meal. For all, blood glucose was  
571 measured in the fasting state, at the time of gavage (time 0) and at 15, 30 and 60 min thereafter.

572 In additional studies, mice were pretreated with the GLP-1 receptor antagonist avexitide (30mmol/kg,  
573 Eiger Biopharmaceuticals, Palo Alto, CA, USA), serotonin receptor 3 antagonist ondansetron (3mg/kg,  
574 Cayman Chemical, Ann Arbor, MI, USA) and serotonin receptor 2 antagonist ketanserin (5mg/kg, Sigma,  
575 Burlington, MA, USA).

576 To determine whether insulin could impact serotonin levels in mice, 4h fasted mice were injected IP with  
577 insulin (2U/kg, Humulin R, Lilly, Indianapolis, IN, USA) or saline. Blood was collected via tail vein at  
578 time 0 and 15 min and 30 minutes post-injection.

579

#### 580 **ELISA and metabolite assays**

581 Human samples: Plasma collected at baseline (fasting) and at 30 and 120 min following the mixed meal  
582 or plasma collected at baseline (fasting) and at 40 and 100 min after initiation of the hyperinsulinemic  
583 hypoglycemic clamp was assayed by serotonin ELISA (Enzo, Farmingdale, NY, USA, ADI-900-175)  
584 following the manufacturer's instructions.

585 Mouse samples: Plasma collected at fasting (time 0) and subsequently at 15, 30 and 60 min after injections  
586 was assayed by Ultra-Sensitive Mouse Insulin ELISA (Crystal Chem, Elk Grove Village, IL, USA,  
587 90082), serotonin ELISA (Enzo, Farmingdale, NY, USA, ADI-900-175), and mouse GLP-1 ELISA

588 (Crystal Chem, Elk Grove Village, IL, USA, 81508). All sampled blood was collected via tail vein or  
589 cardiac puncture in EDTA-coated microtubes containing DPP-IV inhibitor (Millipore, St. Louis, MO,  
590 USA). All assays were performed according to manufacturers' instructions.

591

## 592 **Statistical analysis**

593 All metabolite data were log-transformed, and those with >75% missing values were filtered out. The  
594 remaining missing values were imputed with half of the minimum values of the corresponding  
595 metabolites. Each sample's abundances were scaled so that all samples had the same median abundance.  
596 Differential abundance was assessed by using linear regression modeling with limma, which applies an  
597 empirical Bayesian approach to moderate each metabolite's variance (66). Two-group comparisons were  
598 done by using moderated t-tests, with moderated F-tests for multiple-group comparisons (both 2-tailed,  
599 unpaired). P-values were corrected using the Benjamini-Hochberg false discovery rate (FDR) (67).  
600 Metabolite clusters in a hierarchical dendrogram were detected using a variable cut height approach (68).  
601 The volcano plot was made using ggplot2 (69) and heatmap by pheatmap (70). Metabolite pathway sets  
602 were downloaded from the Small Molecule Pathway Database (SMPDB) (71) and metabolite set  
603 enrichment was tested using the limma Roast method (72). All bioinformatics analysis was done in the R  
604 software and graphs were generated using R and Graphpad Prism 9 (GraphPad Software, Inc).

605

606 For non-metabolic data, data were analyzed using either 1 or 2-way ANOVA, as specified in figure  
607 legends. Graphical abstract was created with BioRender.com.

608

## 609 **Study approval**

610 The study was approved by the Committee on Human Subjects, Joslin Diabetes Center, Boston, MA.  
611 Written informed consent was obtained from all participants. Animal procedures were performed using  
612 protocols approved by the Institutional Animal Care and Use Committee.

613

614 **Data availability**

615 All data points presented in the graphs are detailed in the Supporting Data Values file.

616 **Author contributions:** Study concept and design: MEP. Acquisition, analysis, or interpretation of data:  
617 RFB, BO, CC, VE, PCQ, LP, HW, VN, HS, CM, DS, HP, JD, DCS and MEP. Drafting of the manuscript:  
618 RFB, MEP. Critical revision of the manuscript for important intellectual content: All authors. Statistical  
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798 **Competing interests:** MEP serves on the data safety monitoring board for Fractyl, has received  
799 investigator-initiated research support from Dexcom, and is a consultant for Eiger, Hanmi, and MBX  
800 Pharmaceuticals. The other authors declare no competing interests.

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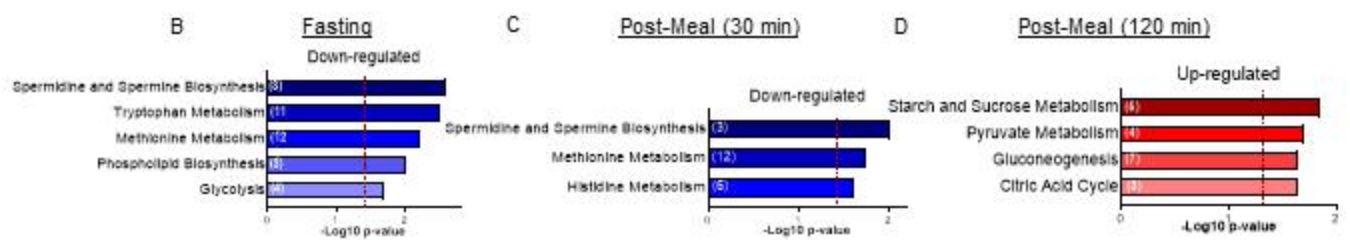
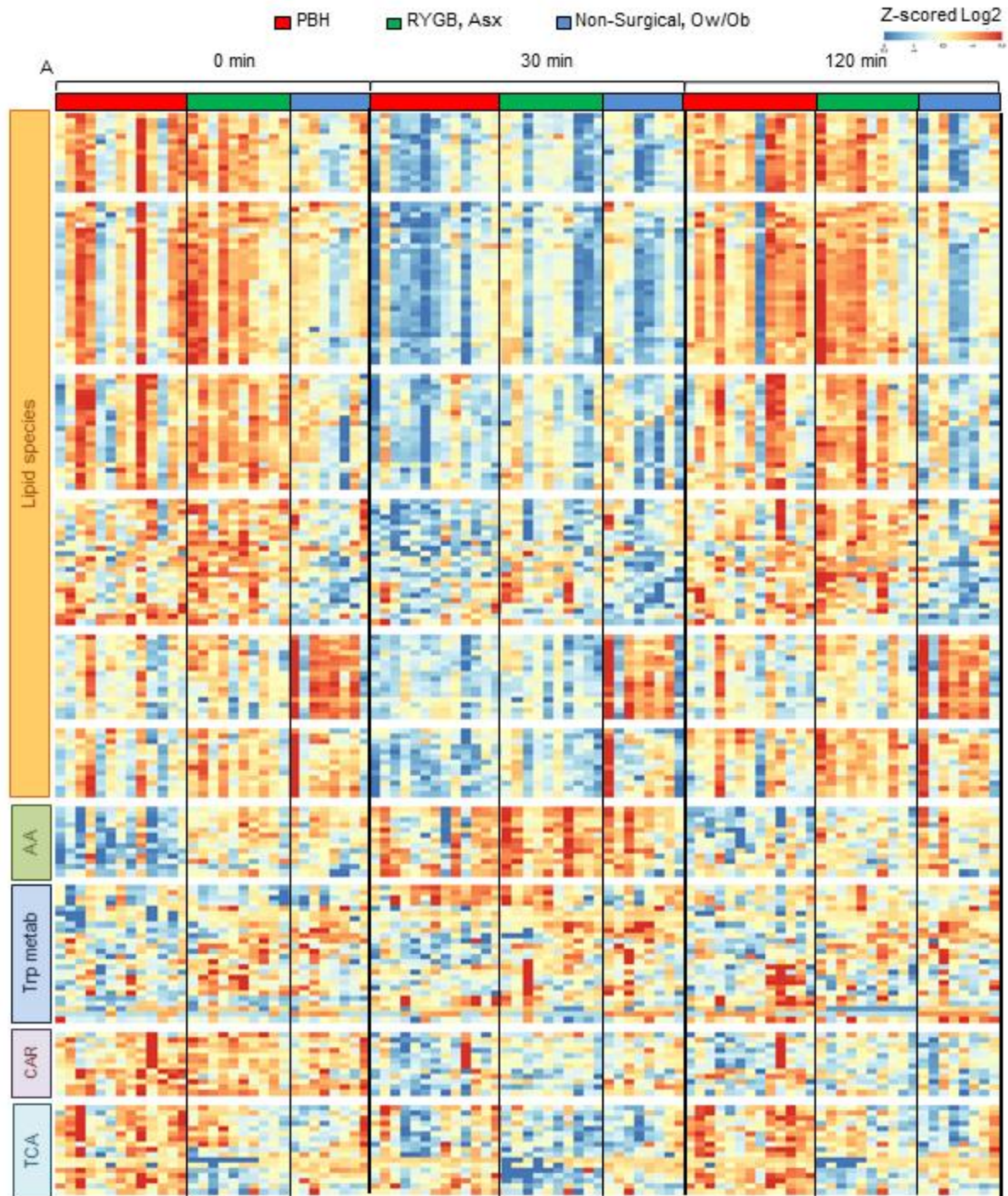
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811 **Figure. 1. Metabolite abundance in individuals with post-bariatric hypoglycemia (PBH),**  
812 **asymptomatic post-RYGB (Asx) and without history of gastrointestinal surgery (Ow/Ob) with**  
813 **fasting state and at 30 and 120 minutes after mixed meal ingestion. (A)** Heatmap of the Z-scored  
814 Log2 transformed metabolites abundance for the top 190 most variant metabolites between groups, with  
815 unsupervised hierarchical clustering of rows. Cluster annotations include lipid species (clusters 1-6),  
816 amino acids (AA, cluster 7), tryptophan metabolites (8), carnitines (CAR, 9), and tricarboxylic acid cycle  
817 (TCA) metabolites (10). **(B-D)** Pathway enrichment analysis for PBH vs. Asx individuals in fasting state,  
818 30 minutes and 120 minutes post meal ingestion, respectively. Down-regulated pathways are colored blue,  
819 while up-regulated pathways are colored in red. The  $-\log_{10}$  p value for enrichment is represented on the  
820 x axis; the vertical line indicates nominal  $p < 0.05$ .

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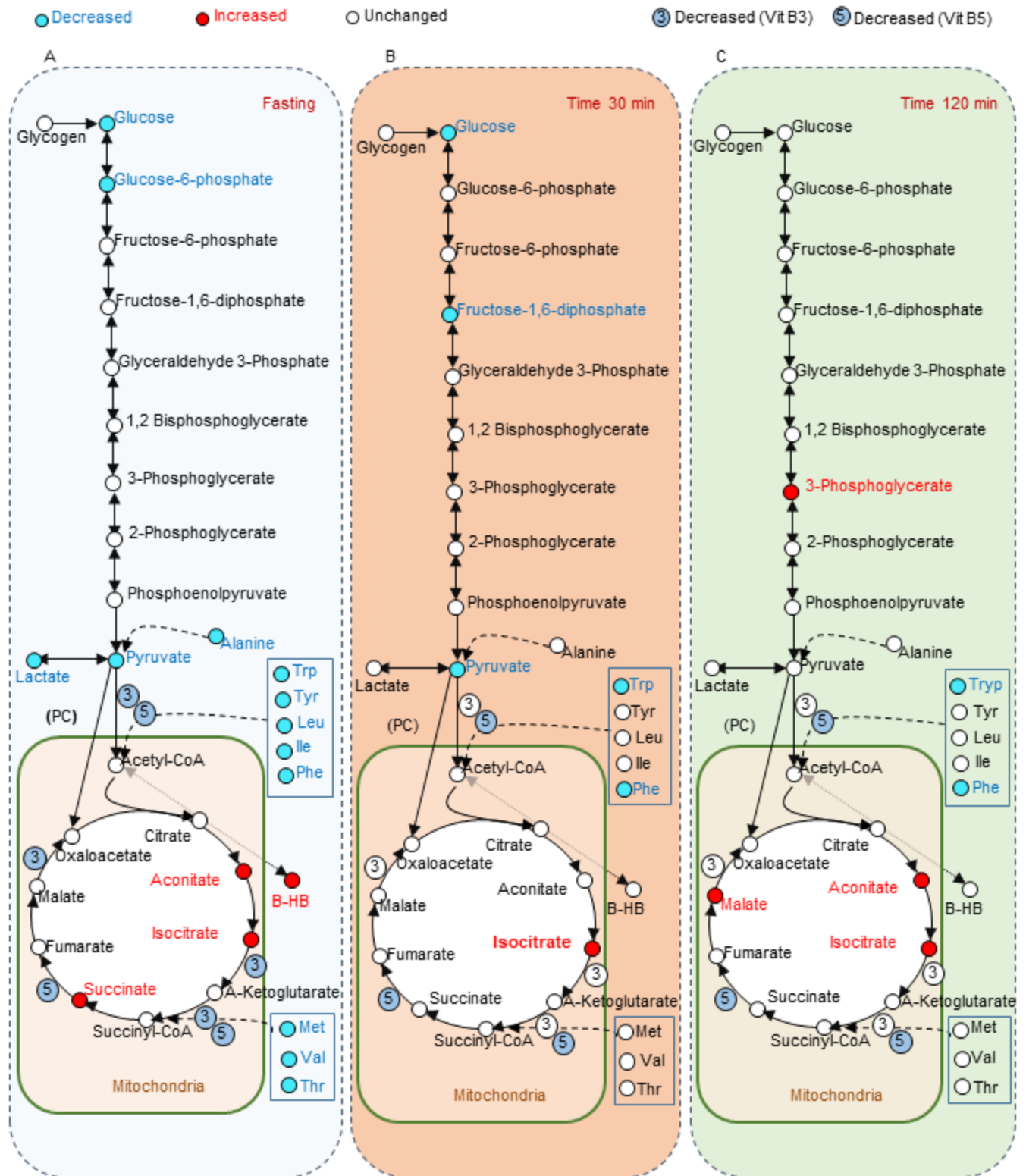
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833 **Figure. 2. Model demonstrating the metabolites of the glycolysis, gluconeogenesis and TCA cycle**  
834 **pathways during MMTT comparing individuals with PBH and Asx in (A) fasting state, (B) 30 minutes**  
835 **and (C) 120 minutes after mixed meal ingestion. Metabolites are colored blue if decreased (nominal**  
836 **p<0.05), red if increased (nominal p<0.05), and white if unchanged in PBH vs. Asx.**

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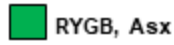
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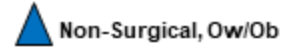
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PBH

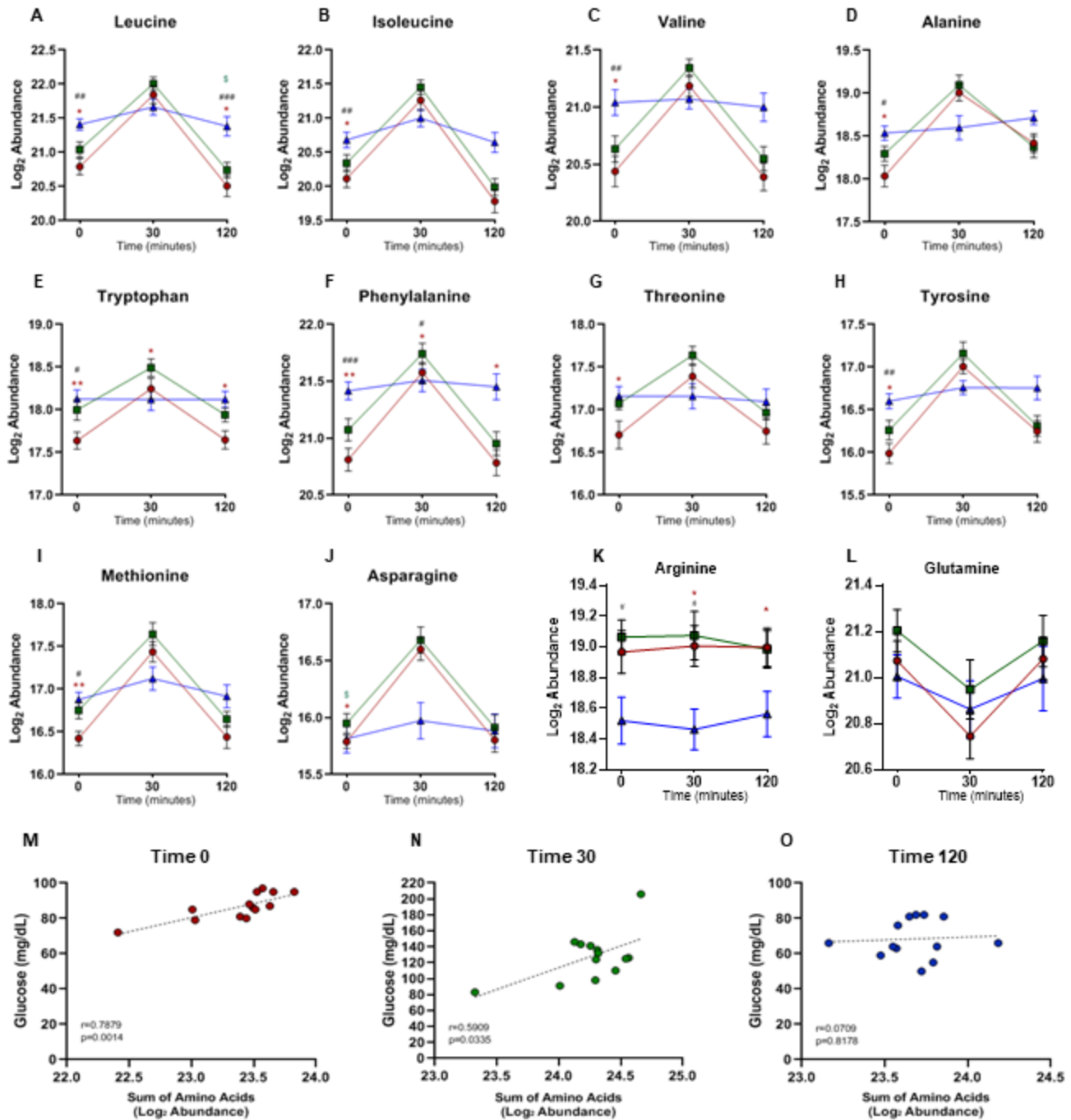


RYGB, Asx



Non-Surgical, Ow/Ob

BCAA



854 **Figure. 3. Abundance of 10 plasma amino acids acid in PBH, Asx and Ow/Ob individuals in the**  
855 **fasting state and at 30 and 120 minutes after mixed meal ingestion. (A) Abundance of Leucine, (B)**  
856 **Isoleucine, (C) Valine, (D) Alanine, (E) Tryptophan, (F) Phenylalanine, (G) Threonine, (H) Tyrosine, (I)**  
857 **Methionine, (J) Asparagine, (K) Arginine and (L) Glutamine. (M-O) Correlation between glucose levels**  
858 **and sum of amino acids abundance in fasting state (red), and at 30 minutes (green) and 120 minutes after**  
859 **meal (blue) respectively. Data are mean  $\pm$  SD. \*, #, \$,  $P < 0.05$ ; \*\*, ##, \$\$,  $P < 0.01$ ; \*\*\*, ###, \$\$\$,  $P <$**   
860 **0.001. PBH (post-bariatric hypoglycemia), Asx (asymptomatic post-RYGB) and Ow/Ob**  
861 **(Overweight/obese without history of gastrointestinal surgery). ANOVA 2-way with Tukey's multiple**  
862 **comparisons test was performed.**

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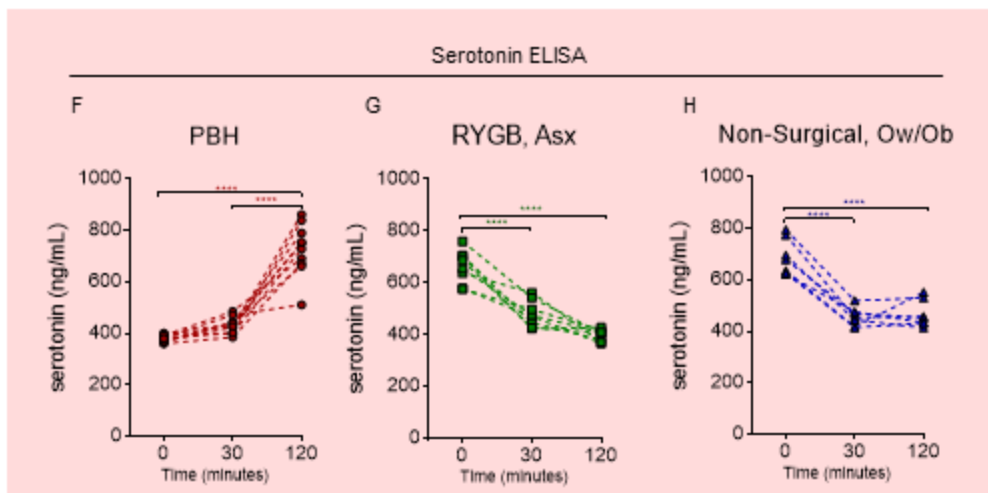
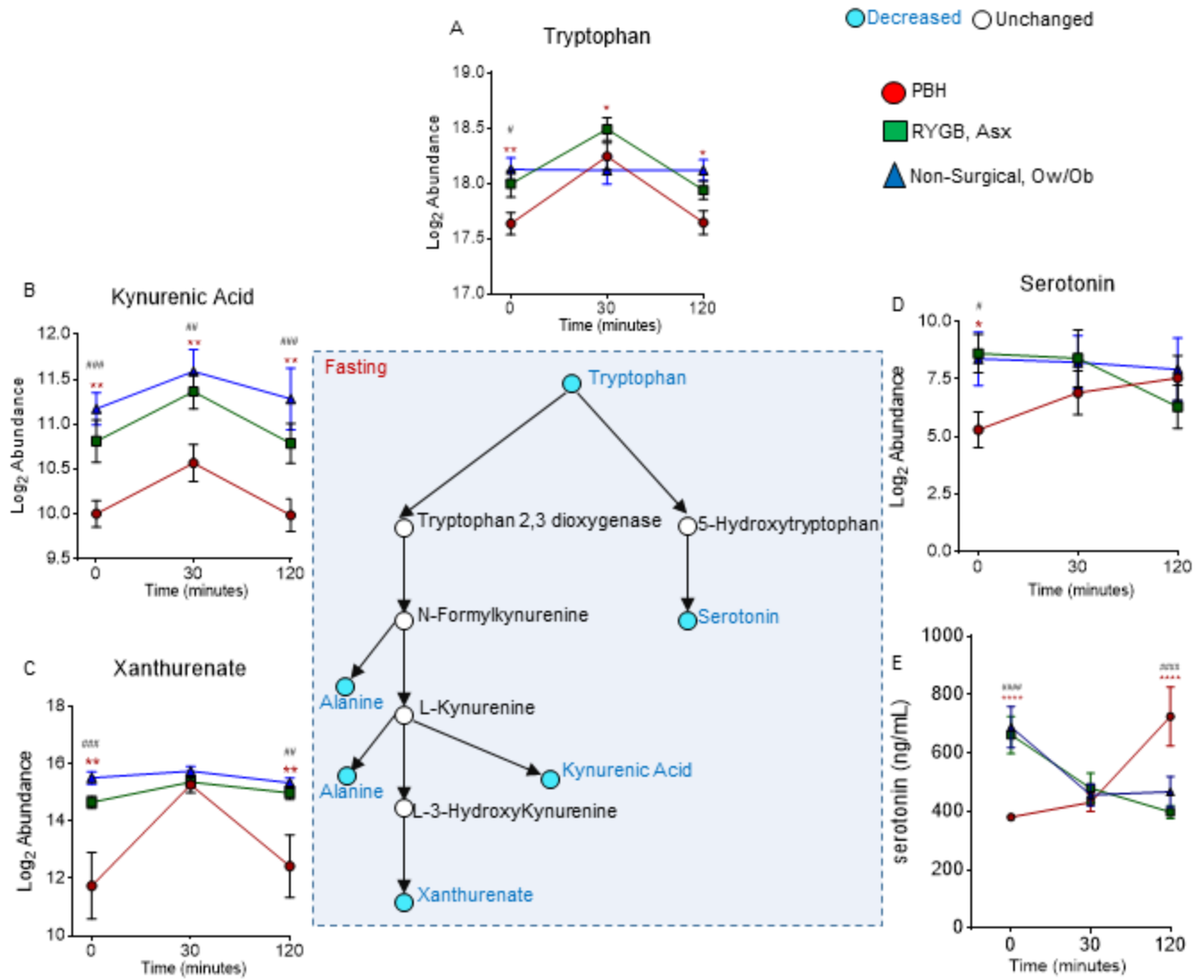
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876 **Figure. 4. Schematic of tryptophan metabolism and downstream metabolites.** (A) Tryptophan, (B)  
877 Kynurenic Acid, (C) Xanthurenate, (D) Serotonin, (E) Serotonin (by ELISA). (F-H) Serotonin ELISA of  
878 individual participants in PBH (red), Asx (green), Ow/Ob (blue) groups. Data are mean  $\pm$  SD. \*, #, \$,  $P$   
879  $< 0.05$ ; \*\*, ##, \$\$,  $P < 0.01$ ; \*\*\*, ###, \$\$\$,  $P < 0.001$ . PBH (post-bariatric hypoglycemia), Asx  
880 (asymptomatic post-RYGB) and Ow/Ob (Overweight/obese without history of gastrointestinal surgery).  
881 ANOVA 2-way with Tukey's multiple comparisons test was performed. Blue color of circles in the  
882 schematic indicate metabolites with decreased abundance, while white color indicates unchanged  
883 abundance.

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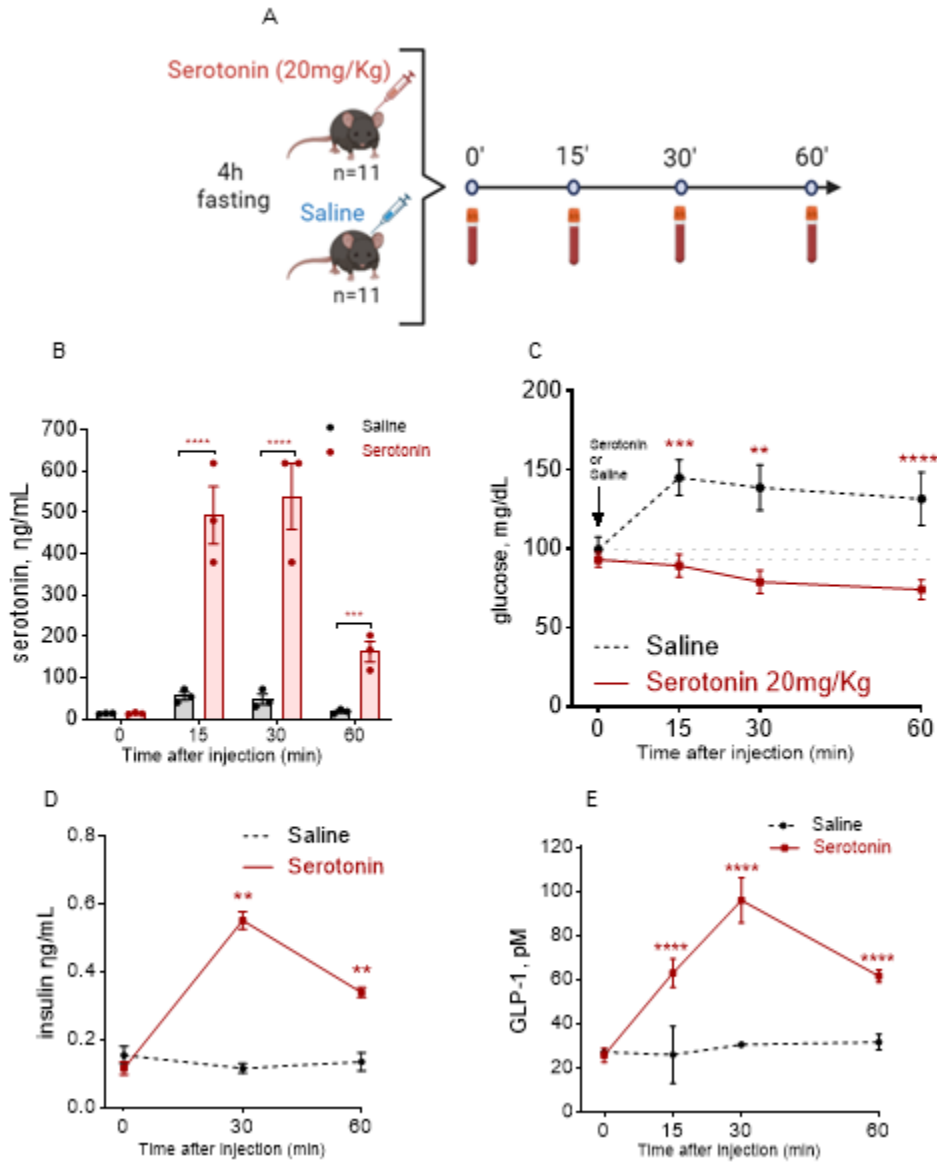
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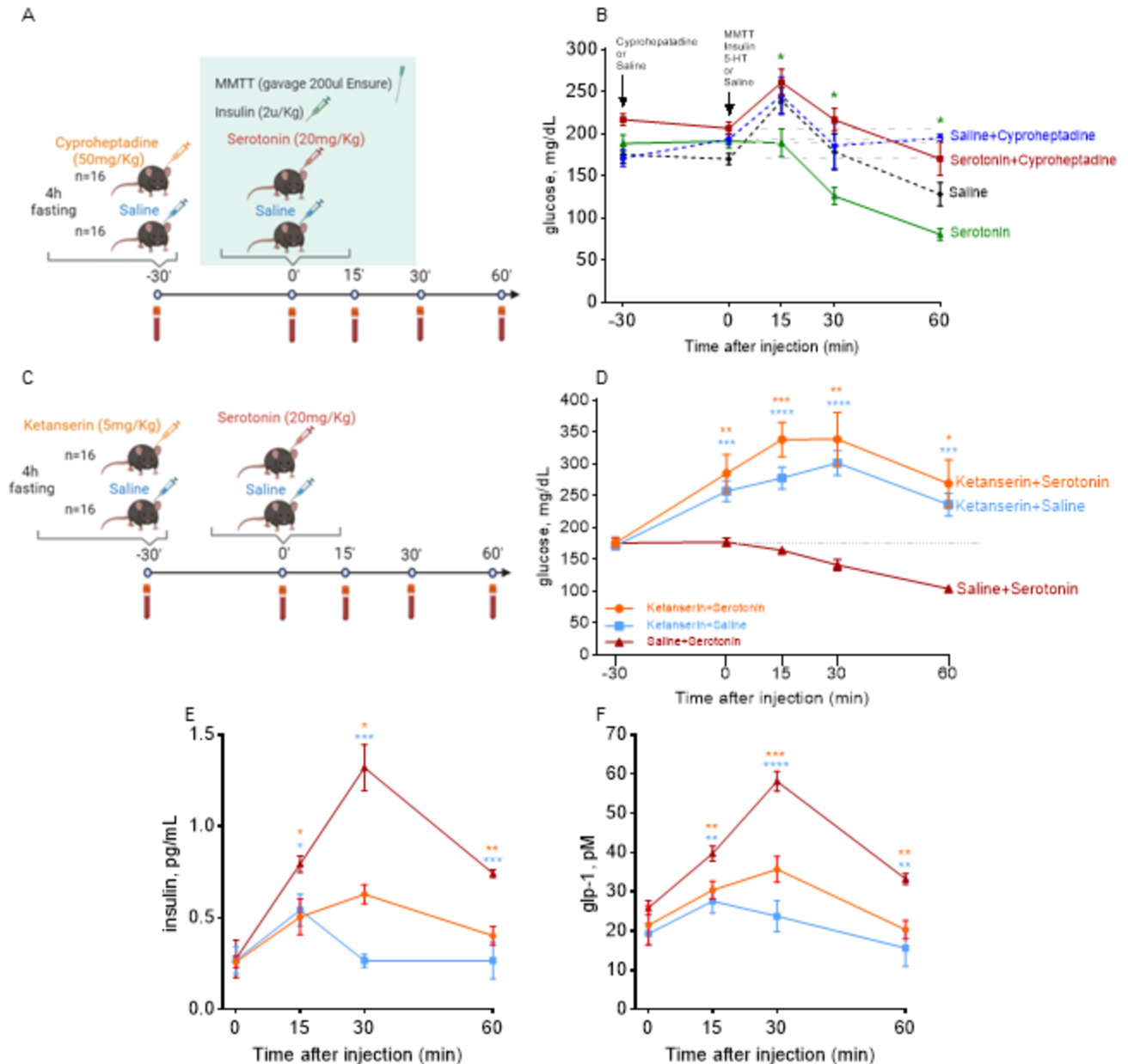
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898 **Figure 5. Serotonin reduces glucose and increases insulin and GLP-1 secretion.** (A) Experimental  
 899 protocol showing exogenous administration of serotonin (20 mg/kg) or saline in C57BL/6J mice. (B)  
 900 Serotonin levels achieved by serotonin injection. (C) Glucose levels after serotonin or saline injection.  
 901 (D) Insulin levels and (E) GLP-1 levels after serotonin or saline injection. In all panels, \*\*p<0.01,  
 902 \*\*\*p<0.001, \*\*\*\*p<0.0001 by ANOVA 2-way with Tukey's multiple comparisons test.

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905 **Figure 6. Serotonin induces hypoglycemia during meal tolerance test.** Reductions in glucose are  
 906 blocked by the serotonin antagonist cyproheptadine and the specific serotonin receptor 2 antagonist  
 907 ketanserin. (A) Scheme showing administration of cyproheptadine (50mg/Kg) or saline and after 30 min  
 908 oral gavage with 200ul of Ensure, insulin injection (2 U/Kg) and administration of serotonin (20mg/Kg)  
 909 or saline in C57BL/6J mice. (B) Glucose levels during experiment. (C) Scheme illustrating experiment

910 showing administration of ketanserin (5mg/Kg) and exogenous administration of serotonin (20mg/Kg) or  
911 saline in C57BL/6J mice. **(D)** Glucose levels after serotonin or saline injection. **(E)** Insulin levels, and **(F)**  
912 GLP-1 levels after serotonin or saline injection. In all panels, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001 by  
913 ANOVA 2-way with Tukey's multiple comparisons test.

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