

1 **Metabolomics biomarkers of frailty: a longitudinal study of**
2 **aging female and male mice**

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36 **Abstract**

37 Frailty is an age-related geriatric syndrome, for which the mechanisms remain largely unknown.
38 We performed a longitudinal study of aging female (n = 40) and male (n = 47) C57BL/6NIA
39 mice, measured frailty index and derived metabolomics data from plasma samples. We identify
40 differentially abundant metabolites related to aging, determine frailty related metabolites via a
41 machine learning approach, and generate a union set of frailty features, both in the whole cohort
42 and in sex-stratified subgroups. Using the features, we perform an association study and build a
43 metabolomics-based frailty clock. We find that frailty related metabolites are enriched for amino
44 acid metabolism and metabolism of cofactors and vitamins, include ergothioneine, tryptophan,
45 and alpha-ketoglutarate, and present sex dimorphism. We identify B vitamin metabolism related
46 flavin adenine dinucleotide and pyridoxate as female-specific frailty biomarkers, and lipid
47 metabolism related sphingomyelins, glycerophosphoethanolamine and glycerophosphocholine
48 as male-specific frailty biomarkers. These associations are confirmed in a validation cohort, with
49 ergothioneine and perfluorooctanesulfonate identified as robust frailty biomarkers. In summary,
50 our results identify sex-specific metabolite biomarkers of frailty in aging, and shed light on
51 potential mechanisms involved in frailty.

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58 **Key words:** aging, frailty, sex difference, metabolites, frailty index, association, longitudinal
59 study, mouse models

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76 Introduction

77 With the success of medical innovations and public health interventions, people are living much
78 longer. However, aging is highly heterogeneous and there is extreme variability in health and
79 function amongst different individuals of the same age¹. Such variability in health can be
80 captured by the concept of 'frailty', a measurement of overall decline in health with age². Frailty
81 can be quantified using a frailty index (FI), which counts the proportion of age accumulated
82 health-related deficits present in an individual^{3,4}. Higher FI values indicate a greater degree of
83 frailty and are associated with an increased susceptibility to diseases and mortality^{4,5}. Frailty
84 indices have been adopted for use in other mammals, including mice⁶.

85
86 Whilst frailty assessments are commonly used in both the clinic and research, there are no
87 accepted frailty biomarkers⁷, and very little is known about the underlying molecular
88 mechanisms of frailty, distinct from aging. Identification of frailty biomarkers would be beneficial
89 in enabling earlier identification and tracking of frailty over time, development and testing of
90 treatments and interventions⁷ and contribute to our understanding of the biological pathways
91 underlying the development of frailty⁸. Metabolomics is an emerging field that enables
92 comprehensive and quantitative metabolite assessment in biological samples. Circulating
93 metabolites can provide a snapshot of the metabolic status of an individual, and as such have
94 the potential to be both biomarkers, and provide insight into biological pathways changed in
95 frailty and age.

96
97 There are known metabolic changes in aging, and in fact many of the 'hallmarks' of aging are
98 linked to unfavorable metabolic shifts⁹. Less is known about metabolic changes in frailty,
99 although studies have shown that glucose intolerance and insulin dynamics are closely linked to
100 physical frailty in both humans and mouse models^{10,11}. Metabolomics studies of aging in
101 humans are beginning to identify specific metabolite markers¹². Elevated high- and decreased
102 low-density lipoproteins are well established in older individuals, and associated with poor
103 clinical outcomes¹³. Changes in amino acids are observed in aging, including increased tyrosine
104 and decreased tryptophan^{14,15}. Both lipids and amino acids are extensively related to nutrient
105 sensing pathways, such as the mammalian target of rapamycin (mTOR)¹⁶ that acts as a central
106 regulator in aging¹⁷. Oxidative stress and inflammation- related metabolites are also associated
107 with aging, particularly acylcarnitines, sphingomyelins¹⁸, and cytochromes P450 metabolites¹⁹.
108 However, the majority of these metabolomics studies are cross-sectional in design, comparing
109 separate groups of young and old individuals, and there are few studies exploring how
110 metabolites change longitudinally within the same individuals as they age^{20,21}. Although early
111 metabolomics studies focused on associations with chronological age only, there is a growing
112 focus on metabolomics studies of frailty in humans, and these studies have revealed
113 associations with energy and nutrition metabolism²² and with amino acid metabolism^{23,24}. While
114 these studies hint at a strong link between frailty and metabolism, they are limited by small
115 sample sizes, and cross-sectional designs.

116
117 Additionally, sex dimorphism in aging is widely observed across many levels. Most notably, at
118 every age, women are more frail than men, despite having longer life expectancy²⁵. There are
119 also clear sex differences in the risk and prevalence of age-related diseases, including

120 metabolic diseases^{26,27}. Many studies have revealed clear sex differences in metabolic aging
121 across multiple tissues including blood²⁸, brain²⁹, and adipose tissue³⁰. Sex differences in
122 metabolites related to lipid metabolism, such as cholesterol³⁰ and sex steroid hormones³⁰,
123 amino acids and acylarnitines³¹ are widely observed, and such differences can be age-
124 dependent. The mechanisms, and especially metabolic mechanisms, underlying these sex
125 differences in aging and frailty are not well understood, despite some recent efforts^{25,32,33}.
126 Although studies have explored baseline sex differences in circulating metabolites, as far as we
127 are aware, there are currently no longitudinal metabolomics studies exploring sex differences in
128 frailty.

129
130 Here, we completed a longitudinal study of female and male mice and generated matched
131 metabolomics and frailty data across 5 time points. We use time-course and network analysis to
132 identify age related metabolites, apply machine learning algorithms to select frailty-related
133 metabolite features, perform an association study on frailty features, and build a metabolite
134 frailty clock. We reveal that age-related metabolites are enriched in lipid metabolism, and
135 suggest that amino acid metabolism and metabolism of cofactors and vitamins are enriched for
136 frailty related metabolites. In particular, we demonstrate strong sex differences in metabolite
137 features and their associations with frailty. We confirm these findings in a validation cohort,
138 specifically finding consistent associations for 9 candidate frailty biomarkers, and the metabolite
139 frailty clock achieves better prediction performance than age and sex alone, but only in male
140 samples. Our results provide candidate metabolomic biomarkers of frailty for future testing in
141 clinical studies, and provide insights into possible mechanisms underlying sex differences in
142 frailty and aging.

143 **Results**

144 ***Metabolomics data variation***

145 We performed a longitudinal study of female ($n = 40$) and male ($n = 47$) C57BL/6NIA mice at 5
146 time points, and derived metabolomics data for a total of 321 samples that have valid
147 metabolomics data (**Table1**). In order to investigate aging- and frailty- related metabolites and
148 mechanisms in naturally aging mice, we used non-NMN treated mice (female, $n = 20$; male, $n =$
149 24) as the discovery cohort for the ensuing analysis (**Fig.1**). To investigate the metabolomic
150 data variation, we performed a principal component analysis (PCA), including a set of 781
151 metabolites. The PCA plot indicated clear separation of samples across time points (by PC1)
152 and by sex (by PC2) (**Supplementary Fig.1a**). We then performed linear regression analyses
153 on PCs and observed clustering of factors of interest (e.g., sex, time point, mouse ID) in the
154 associations with PCs (**Supplementary Fig.1b**) based on p -values. We selected time points
155 and sex as representative variables in the ensuing analysis as they showed the smallest p -
156 values among the factors within the same cluster. Mouse ID also showed an association with
157 PC2 at a significant level and was included to account for repeated measurements on the same
158 mouse.

159

160 ***Metabolomic signatures of aging across sexes***

161 After the determination of covariates, we performed metabolite differential abundance analyses
162 to identify metabolites that were related to general aging. That is, metabolite abundances that
163 significantly changed for these mice across the sampled time points. We considered the pattern
164 of metabolite abundance globally over time, by fitting a time series smoothing spline, accounting
165 for mouse ID and sex. We found 527 (67.5% of total detected metabolites) differentially
166 abundant metabolites (DAMs) over the time-course within all mice (both females and males)
167 (**Supplementary Fig.2a**).

168

169 In order to select subsets of metabolites with similar abundance over the time course and, more
170 importantly, highly related to aging independent of sex, we performed co-abundance network
171 analysis on the 527 metabolites derived above. We derived two metabolite subsets, subset1 (n
172 = 200) and subset2 (n = 125) (**Supplementary Fig.2b**) of which the eigenvalues showed strong
173 associations ($p < 0.001$) with age, presenting a generally decreasing trend in abundance in
174 aging (**Fig.2a**). Significantly higher proportions of metabolites within the amino acids super-
175 pathway were observed in subset 1 (69 metabolites, 34.5% of subset1, χ^2 (df = 2, $N = 781$) =
176 25.3, $p < 0.001$) and those in the lipids super-pathway for subset 2 (100 metabolites, 80% of
177 subset2, χ^2 (df = 2, $N = 781$) = 141.1, $p < 0.001$), compared to the rest of metabolites.

178 Metabolite set enrichment analysis results on two subsets aligned with the above classification,
179 with amino acid metabolism (subset1) and lipid metabolism (subset2) pathways over-
180 represented (**Supplementary Fig.2c** and **d**). To further select metabolites that play important
181 roles in aging, we selected 86 hub metabolites, 46 metabolites from subset1 and 40 from
182 subset2, based on module membership in the network and significance (correlation coefficient
183 between eigenvalue and age) (**Supplementary Fig.2e**). These 86 metabolites were defined as
184 core age-related metabolites in the ensuing analyses, and include guanidinoacetate,
185 methylmalonate (MMA) and sphingomyelin species (**Supplementary Table1**). 54.7% of these
186 metabolites (47 total, 9 from subset1 and 38 from subset2) are from the lipid super-pathway,
187 also evidenced by enrichment analysis (**Fig.2b**). This result suggests lipid metabolism is among
188 the key mechanisms contributing to general aging.

189

190 ***Sex specific metabolomic signatures of aging***

191 To identify sex specific metabolomic signatures, we investigated DAMs within 1) females only
192 (significant change in abundance in the whole time frame), $n = 498$ DAMs (63.8%, 498/781), 2)
193 males only, $n = 253$ DAMs (32.4%, 253/781); and 3) sex differences (significantly differentially
194 abundant in females and males considering the whole time frame), $n = 331$ DAMs, (42.4%,
195 331/781). The results suggest significant sex differences in metabolite abundance in the aging
196 process.

197

198 It was interesting to observe a common set of 97 metabolites after merging the above three sets
199 of DAMs with the 527 DAMs derived from the mixture of both sexes (sex-independent) (**Fig.3a**;
200 **Supplementary Table2**). These metabolites not only were related to aging in both sexes, but
201 also presented sex differences in aging (**Supplementary Fig.3**). Notably, these included 8
202 acylcarnitines, for instance, oleoylcarnitine (C18:1) and palmitoleoylcarnitine (C16:1). Among

203 the 97 metabolites, 41 are in lipid and 22 in amino acid super pathways, representing 64.9% of
204 the 97 metabolites. Enrichment analysis revealed 11 KEGG pathways overrepresented
205 (**Fig.3b**), mostly within lipid metabolism and digestive system pathways.

206
207 Apart from the common set, among the 331 metabolites that are different between males and
208 females across the investigated timeframe, there are only 21 metabolites that present sex
209 differences (distinct abundance in females and males) and do not present significant abundance
210 differences over time (**Supplementary Table2**). This indicates that the majority of sex
211 differential metabolites also change with age. In terms of female-specific metabolites that are
212 changed with age, that is, metabolites detected in both female and sex difference DAM sets, the
213 187 in this category included amino acids and acylcarnitines and were enriched for amino acid
214 metabolism pathways (**Fig.3c; Supplementary Table2**). Many fewer male-specific age-related
215 metabolites were observed, with a total of only 23, including phosphocholine and spermine. One
216 metabolite, corticosterone, changed with age in both females and males separately
217 (**Supplementary Fig.4**), but was not detected to change with age when the entire cohort of
218 mice was considered. Altogether, we found metabolites involved in lipid metabolism and
219 digestive system pathways contribute to aging and present strong sex differences. Specifically,
220 amino acid metabolism-related metabolites are associated with aging in female mice.

221 222 ***Sex independent metabolite features of frailty***

223 Having identified age-related metabolites, we were interested to identify metabolites specifically
224 associated with frailty. Frailty is a complex geriatric syndrome. For each mouse at a certain time
225 point, FI is composed of the base FI (median FI of the corresponding sex and age group) and
226 devFI (the deviation of individual FI from the median FI at corresponding age- and sex-specific
227 group). By definition, base FI is highly related to age, but devFI is age independent
228 (**Supplementary Fig.5**).

229
230 In order to find metabolites related to frailty, we performed feature selection outlined in **Fig.4a**.
231 We investigated metabolites that were related to both FI and devFI, by performing feature
232 selection with elastic net regularization, via a 100 times repeated 5-fold cross validation
233 approach. Based on the rank of presence frequency, we selected 156 and 149 metabolites
234 predictive of FI and devFI, respectively (**Supplementary Fig.6a; Supplementary Table3**). 86 of
235 these metabolites were identified as both devFI and FI features (**Fig.4b**), suggesting both
236 overlapping and distinct metabolite signatures of FI and devFI. The majority of identified FI and
237 devFI metabolites were within the amino acid and lipids super pathways (**Supplementary**
238 **Fig.6b**). Three metabolites were simultaneously identified as FI-, devFI- and age-metabolites,
239 including ergothioneine that decreases with age and frailty in both females and males
240 (**Supplementary Fig.7**). When looking at the top enriched KEGG pathways for FI and devFI,
241 there were 11 common pathways (out of the top 15 by p-value) across both groups
242 (**Supplementary Fig.6c and d**), including 7 amino acid metabolism pathways, nicotinate and
243 nicotinamide metabolism, pantothenate and CoA biosynthesis, pyruvate metabolism and ABC
244 transporters. To further identify core-metabolites related to frailty, we derived 21 FI-age and 86
245 devFI features by merging age-related metabolites (86 hub metabolites) and devFI metabolites
246 with the FI metabolites respectively, resulting in a set of 104 union features (**Fig.4b**) which are

247 enriched for amino acid metabolism and metabolism of cofactors and vitamins pathways
248 (**Fig.4c**). These results suggest these metabolic pathways, notably, amino acids and B vitamin
249 metabolism are specifically important in the development of frailty.

250
251 Despite the common metabolites, we observed 61 metabolites that are unique to devFI
252 (**Fig.4b**), including hippurate, choline, hypotaurine, phenylacetyltaurine, and adenosine 5'-
253 diphosphoribose. Enrichment analysis based on these metabolites led to efferocytosis and ABC
254 transporters. The results suggest these metabolites and pathways are associated with frailty in
255 a completely age- and sex-independent way.

256

257 ***Association study of metabolite features with frailty outcomes***

258 To test the associations of the individual metabolite features with frailty outcomes (**Fig.5a**), we
259 applied linear mixed regression models and subjected the 104 union frailty features to a
260 longitudinal association study. First, we considered only metabolite abundance at the current
261 timepoint (Age_c). For the current FI (FI_c) and devFI ($devFI_c$), we found 47 and 16 metabolites,
262 respectively presented coefficients significantly different from 0 (**Supplementary Table4**).
263 Among the 47 FI_c metabolites, three metabolites (leucine, N-acetylthreonine, and X-25422),
264 presented a significant metabolite abundance by age interaction term (**Supplementary Table5**),
265 indicating that the association of these metabolites with FI_c is age-dependent. Despite this,
266 leucine showed a generally consistent positive correlation with FI_c at each age group, but this
267 was not the case for N-acetylthreonine and X-25422 (**Supplementary Fig.8a**). The remaining
268 45 metabolites (those without significant interaction terms, plus leucine) are associated with FI_c
269 independent of age. That is, individual mice with higher abundance of these 45 metabolites are
270 either more (19 metabolites, $\beta > 0$) or less (26 metabolites, $\beta < 0$) frail in a cohort. For $devFI_c$,
271 individual metabolites were also associated with both higher (9 metabolites, $\beta > 0$) and lower (7
272 metabolites, $\beta < 0$) frailty scores. Eight metabolites were identified as significantly associated
273 with both FI and devFI (**Fig.5b**). For each of these metabolites, the coefficient of association
274 was in the same direction, indicating the same trend of association with both FI and devFI.

275

276 Given the longitudinal nature of our dataset we were interested to observe whether metabolite
277 abundance at any specific timepoint was associated with frailty at a future timepoint (**Fig.5a**).
278 Unfortunately, we didn't observe any metabolites that showed an overall significant association
279 with future FI (FI_f) or future devFI ($devFI_f$). When focusing only on the abundance of metabolites
280 at the baseline time point (~400 days), we found a single metabolite, alpha-ketoglutarate, was
281 negatively associated with both FI_f and $devFI_f$ (**Supplementary Fig.8b**). Next, we considered
282 whether there were associations between current metabolite abundances, and a change in FI
283 from one timepoint to the next (ΔFI or $\Delta devFI$, **Fig.5a**). We saw no associations with $\Delta devFI$
284 but found 27 metabolites that showed significant associations with ΔFI (**Fig.5c** and
285 **Supplementary Table4**). No significant interaction terms were observed for these metabolites,
286 indicating that these associations were not age-dependent. 20 metabolites ($\beta > 0$, e.g.
287 creatinine) were associated with increased frailty and the remaining 7 ($\beta < 0$, e.g. phenyllactate)
288 were associated with decreased frailty (**Supplementary Fig.8c**). Finally, we considered whether
289 changing abundances of a metabolite over time (ΔMA), might be associated with frailty (**Fig.5b**)
290 but found no significant associations.

291

292 Combining the 3 sets of metabolites above (those significantly associated with FI_c , ΔFI , or
293 $devFI_c$) gives a total of 63 metabolites, of which 23 are present in 2 or more sets (**Fig.5d**).
294 These metabolites represent candidate biomarkers for frailty and include phenyllactate,
295 ergothioneine, nicotinamide riboside, creatinine, alpha-ketoglutarate, isoleucine and valine.

296

297 ***Sex specific metabolite features of frailty***

298 Sex differences are common in aging and frailty. We investigated the performance of the
299 generalized linear models that were trained to predict FI or $devFI$ in the whole cohort (**Fig.4a**), in
300 the females and males separately. We found significant differences (two-tailed t-test, $p < 0.001$)
301 between the R-squared values derived from female and male samples (**Supplementary**
302 **Fig.9a**), suggesting the associations of metabolites with FI and $devFI$ are sex specific and
303 stratification by sex is appropriate for this analysis. Interestingly, the model performance was
304 better in the females than males.

305

306 In order to select sex specific metabolites related to frailty, we stratified the whole cohort into
307 female and male subgroups and re-selected metabolite features as above. For females, we
308 derived 133 and 45 metabolites related to FI and $devFI$, and for males, we obtained 32 and 92,
309 respectively. Of these only 7 were associated with FI, and 8 with $devFI$, in both sexes. Despite
310 this, for both males and females, the majority of the identified metabolites were within amino
311 acids and lipids super pathways (**Supplementary Fig.9b**), and the enriched pathways were
312 similar between males and females. They predominantly included amino acid metabolism,
313 metabolism of cofactors and vitamins, mineral absorption, and protein digestion and absorption
314 related to the digestive system (**Supplementary Fig.9c and d**).

315

316 Following the union feature workflow (**Fig.4a**), we obtained 58 union features for females and 21
317 for males related to overall FI. Within the female union features, 50% of the metabolites were
318 related to amino acid and lipid pathways, whilst the male union features were enriched in lipid
319 super pathways ($\chi^2(df = 2, N = 781) = 4.11, p\text{-value} = 0.042$). Excluding the union features
320 associated with frailty in the whole cohort, we identified 25 and 9 metabolites that are unique
321 metabolite features identified only in females or males (**Fig.6a**). These sex specific features
322 include kynurenate and quinolinate for females and sphingomyelin and creatinine for males.
323 These results suggest sex specific biomarkers for frailty may be appropriate.

324

325 ***Association study of sex specific frailty features***

326 To investigate the association of individual metabolites with frailty in each sex, we performed
327 mixed linear model regressions using the FI union features for females and males separately,
328 as above. In females, we first considered solely the current metabolite abundance and found
329 that 38 and 16 metabolites, respectively, were significantly associated with FI_c and $devFI_c$
330 (**Supplementary Table6**). As with the whole cohort, metabolites were both positively (21 for FI_c
331 and 11 for $devFI_c$) and negatively (17 for FI_c and 5 for $devFI_c$) associated with frailty outcomes.
332 Notably, 6 metabolites were identified as both FI_c and $devFI_c$ related (**Fig.6b**). When considering
333 associations between current metabolite levels and either future frailty, or changing frailty levels
334 (**Fig.5a**), we found 26 metabolites were associated with $\Delta devFI$ (**Supplementary Table6**).

335 These associations were independent of age, suggesting a relationship between metabolite
336 levels and either increasing (10 metabolites, $\beta > 0$) or decreasing (16 metabolites, $\beta < 0$) rate of
337 development of frailty. (**Fig.6c**). Next, we considered the relationship between frailty outcomes
338 and changing metabolite abundances over time, and found one metabolite, ergothioneine,
339 which was significantly associated with devFI_c. We combined the four datasets from these
340 female-specific frailty association studies to identify a total of 52 metabolites, of which 3
341 metabolites were present across 3 lists, including FAD and ergothioneine, and 23 metabolites
342 were present across 2 lists (**Supplementary Fig.10a**). These 26 metabolites are potential
343 female specific frailty biomarkers.

344

345 In male samples, we followed the same analysis flow. Considering current metabolite
346 abundance, we found 19 and 12 metabolites that were associated with FI_c and devFI_c
347 (**Supplementary Table6**). Of these, 11 metabolites were excluded as they had significant
348 interaction terms, indicating that the association of the metabolite with FI_c depends on age
349 (**Supplementary Table7**). No significance was found for the remaining regressions for the male
350 samples. The results include three GPEs, one GPC, creatine, and phenyllactate, that may be
351 potential male specific frailty biomarkers (**Supplementary Fig.10b**).

352

353 ***Validation of the frailty associated metabolites***

354 In order to validate the metabolites associated with frailty in an external cohort, we used female
355 ($n = 20$) and male ($n = 23$) mice samples under long-term NMN treatment (**Table1**). To
356 investigate if the associations of our identified frailty features with frailty outcomes persist under
357 the intervention, we used union features identified from the whole cohort, and females and
358 males separately, and performed the same association analysis. For sex-independent features,
359 we found one metabolite, perfluorooctanesulfonate, that was significantly associated with
360 current frailty (FI_c) in the validation cohort. There were seven metabolites associated with FI
361 change over time (Δ FI, **Fig.5a**), including ergothioneine, guanidinoacetate, N-
362 glycolylneuraminic acid, X-12798, creatinine, dimethylarginine (ADMA + SDMA), and N-acetyl-
363 beta-alanine. In male samples only, 2-hydroxydecanoate maintained a significant association
364 with FI_c at one time point (**Supplementary Fig.11**). Although NMN treatment delays frailty³⁴,
365 the persistent association of these metabolites with frailty outcomes reveals evidence for their
366 robustness as possible frailty biomarkers.

367

368 ***Development of a metabolite-based frailty clock***

369 To build a model to accurately predict frailty using metabolomics features in aging mice, we fit a
370 random forest model in the discovery cohort, with FI as the dependent variable and the
371 combination of identified union features from the whole cohort, female- and male- specific
372 analysis (total $n = 139$ metabolites) as the independent variables. We further determined the top
373 63 metabolite features ranked by the presence frequency (**Supplementary Fig.6a**) gave the
374 best performance in predicting FI (**Supplementary Fig.12**). Among the 63 metabolite features
375 that can be found in the whole cohort derived features, 24 metabolites were also identified as
376 female-specific frailty related metabolites, and 4 as male-specific. Our final model, metabolite
377 frailty clock, included these 63 informativity-based metabolites, age and sex. We also fit a model
378 using all 781 metabolites detected in our study, as a comparison. Both random forest models

379 performed well in the discovery cohort ($R^2 = 0.95$, $RMSE = 0.022$ for RF with 781 metabolites;
380 $R^2 = 0.96$, $RMSE = 0.019$ for metabolite frailty clock), and outperformed a benchmark model of
381 merely age+sex ($R^2 = 0.51$, $RMSE = 0.053$) (**Fig.7a**). Importantly, we examined the performance
382 of the metabolic frailty clock in the validation cohort, and although it achieves similar
383 performance as the age+sex model trained in the validation cohort across the entire cohort
384 (**Fig.7b**), it outperforms age+sex in male samples (**Fig.7c**). Despite the fact that there is clearly
385 room for improvement, these results suggest that frailty can be accurately predicted in aging
386 mice using metabolite features.

387 Discussion

388 Using a longitudinal study of female and male mice, we identified both sex-independent and
389 sex-specific metabolomic signatures of aging and frailty. Overall, we found that age related
390 metabolites are enriched for lipid metabolism, while frailty related metabolites are enriched for
391 amino acid metabolism and metabolism of cofactors and vitamins. B vitamin metabolism-related
392 metabolites and lipid metabolism-related metabolites, respectively, are determined as candidate
393 female- and male-specific frailty biomarkers.

394

395 **Age-related metabolites**

396 Using a time course analysis in mice, we found a total of 527 metabolites significantly changed
397 with age, representing the majority of measured metabolites. This result suggests dramatic
398 change in the abundance of most metabolites in aging, and aligns with a previous longitudinal
399 human study²¹. Among these age-related metabolites, we identified 86 hub metabolites by
400 network analysis, and observed that these metabolites were enriched for lipid metabolism,
401 including biosynthesis of unsaturated fatty acids, primary bile acid biosynthesis, and fatty acid
402 elongation. Interestingly, several studies in mice have demonstrated manipulation of lipid
403 metabolism as a method to extend longevity^{35,36}. Our results using longitudinal data provide
404 further evidence of the importance of lipid metabolism in aging. In terms of the individual
405 metabolites changed in age, we identified 10 sphingomyelin species, which are of interest given
406 the established link between sphingomyelins and longevity in humans²⁰.

407

408 Additionally, we found that 42% of measured metabolites were significantly different between
409 males and females, and the majority of these were also changed with age. Metabolites involved
410 in aging and sex differences were enriched for a spectrum of pathways involved in lipid
411 metabolism and digestive system. While sex differences in lipid metabolism have been widely
412 recognised, our finding provides further evidence in the context of aging and aligns with the
413 result from humans that the lipidome exhibits significant age-dependent differences between
414 sexes³⁷. Moreover, liver is the primary tissue for bile acid metabolism³⁸, fatty acid metabolism³⁹
415 and taurine metabolism (conjugation with bile acid⁴⁰), all pathways identified in our study as
416 displaying sex differences in aging. This suggests that the liver is strongly influenced by
417 biological sex in aging, which aligns with transcriptomic results from our lab⁴¹. Furthermore, the
418 presence of the mineral absorption and ferroptosis pathways in our findings^{42,43} reveal that the
419 impact of aging on iron homeostasis⁴⁴ is sex specific.

420

421 Sex stratified analysis revealed female specific metabolite markers of aging include amino acids
422 and acylcarnitines. Previous work has extensively shown the role of amino acids and
423 acylcarnitines in the regulation of aging^{45,46}. Our results indicate significant changes of these
424 metabolites in females in aging, but not necessarily in males, which is also implied in other
425 studies⁴⁷. As for the male specific aging biomarkers, we found phosphocholine (adjusted for
426 sex) and spermine (only in males), both of which have been previously linked to overall
427 aging^{48,49}. Together, these findings give clues about how metabolic aging may occur differently
428 in males and females.

429

430 ***Frailty related metabolites***

431 Although age-related metabolic markers are of interest, markers that are associated with health
432 in aging may provide more clues about underlying mechanisms of the aging process, rather
433 than the passing of time. To this end, we sought to identify metabolic features of frailty, a
434 validated quantification of health in aging in both humans and mice. As frailty is strongly
435 correlated with age, it was important that we identify metabolic markers of frailty, independent of
436 age. We used a novel approach of calculating devFI, the deviation from the median frailty index
437 of the corresponding age and sex group. In this way we are able to identify metabolites
438 associated with individual variations in frailty at a given age and sex group, and distinguish
439 these from metabolic changes that arise from aging. We applied a machine learning approach
440 to select metabolites that are associated with both outcomes, FI and devFI. In the whole cohort
441 study, we identified 149 metabolites features for devFI, among which 61 were not also
442 associated with FI or age (**Fig.4b**). These include hippurate, a gut microbiome derived
443 metabolite that has been previously associated with aging⁵⁰. These metabolites are particularly
444 interesting for further analysis as underlying markers of health, independent of age.

445

446 Overall, frailty-related metabolites were heavily enriched in amino acid metabolism and
447 metabolism of cofactors and vitamins. The majority of the 20 proteinogenic amino acids
448 metabolism pathways were enriched, suggesting amino acids serve as the main driver of frailty
449 dynamics in mice. In humans, altered amino acid metabolism is also suggested to be
450 associated with frailty²⁴, in particular tryptophan metabolism⁵¹. Interestingly, nicotinate and
451 nicotinamide metabolism were also over-represented in these candidate frailty biomarkers.
452 Recent work has shown that boosting nicotinamide levels is associated with improved health in
453 aging, including improved frailty^{34,52,53}. These results suggest pivotal differences in the metabolic
454 mechanisms underlying aging and frailty.

455

456 Additionally, we applied linear mixed models to the metabolite features identified for frailty to
457 look at their specific univariate association with frailty outcomes (**Fig.5a**). Notably, we found 23
458 metabolites that were associated with more than one frailty outcome (i.e., current FI, current
459 devFI and/or change in FI over time) (**Fig.5d**), including ergothioneine, nicotinamide riboside
460 (NR), phenyllactate, and creatinine. Interestingly, ergothioneine is one of the most robust markers
461 in our study, which is identified as FI, devFI and age-associated across both males and females.
462 It has been previously identified as a frailty biomarker⁵⁴, and is thought to promote healthy
463 aging⁵⁵. NR is an NAD precursor, part of the nicotinamide metabolism pathway, and boosting
464 levels of NR are associated with improved health in aging^{53,57}. Phenyllactate, is a catabolite of

465 phenylalanine (phenylalanine metabolism is identified as enriched from frailty related
466 metabolites) derived from *Lactobacillus*⁵⁸, providing further evidence of the possible involvement
467 of the microbiome in the development of frailty. Creatinine, a muscle breakdown product, has
468 been associated with sarcopenia, functional limitation and frailty⁵⁹. Additionally, alpha-
469 ketoglutarate was the only metabolite for which the abundance in middle-aged mice was
470 predictive of future frailty, suggesting it could be an early-biomarker of frailty, and/or a viable
471 target for early intervention. In support of this, a recent study shows that alpha-ketoglutarate
472 supplementation in mice reduced frailty⁵⁶. Taken together, our results provide preclinical
473 evidence for several potential biomarkers for frailty.

474

475 ***Sex dimorphism in frailty***

476 In order to identify sex-specific metabolic markers of frailty, we completed sex-stratified
477 analysis. We identified vitamin B3/tryptophan metabolites, kynurenine and quinolinate, as being
478 specifically associated with frailty in females. The findings for these two metabolites are
479 consistent with previous studies^{60,61}, where the link to frailty is sex-specific. FAD (vitamin B2),
480 was also significantly associated with multiple frailty outcomes in females (**Supplementary**
481 **Fig.9a**). FAD is one of the active forms of vitamin B2, however, previous studies in both sexes
482 found that intake of vitamin B2 has no association with frailty^{62,63}. Given our novel findings, we
483 suggest further investigation into FAD as a female-specific marker of frailty. Another female-
484 specific frailty biomarker is pyridoxate (vitamin B6), which is reported to be related to frailty⁶⁴. In
485 male mice, we identified mainly lipid metabolism-related metabolites, including sphingomyelins,
486 three GPE species and one GPC species. These metabolites are lipid species that have been
487 previously associated with frailty^{65,66} in both sexes, so the male specificity needs further
488 investigation. Taken together, our results reveal evidence of sex specific biomarkers for frailty,
489 and imply that B vitamin metabolism is a key feature of frailty development in females and lipid-
490 related metabolism for males. We highly recommend applying the sex stratification approach in
491 the future study of frailty biomarkers and mechanisms.

492

493 Importantly, and often ignored in other frailty biomarkers studies, we confirmed whether the
494 same metabolites were associated with frailty outcomes in an independent validation cohort.
495 Although the association of not all metabolites held in this cohort, we did find 9 metabolites
496 showing persistent significance in the association with frailty outcomes, including ergothioneine
497 and creatinine. Our validation cohort included mice that had long-term treatment with the NAD
498 booster, NMN, suggesting that the association of these metabolites with frailty outcomes may
499 be universal even under interventions, so these biomarkers should be investigated further.

500

501 Many 'clocks' have been built to predict chronological age based on either epigenetic or
502 metabolomic features^{67,68}. There is a growing focus, however, on building models to predict
503 health- rather than age-related outcomes. Here, we build the first clock to directly predict frailty
504 in mice. Our model performs extremely well in our discovery cohort, and although the
505 performance of the frailty clock is similar to that of an age+sex only model (trained within the
506 validation cohort) in females for the validation cohort, our clock outperforms the simple model in
507 male samples. These results provide preliminary evidence that it is possible to predict frailty

508 using metabolites, but suggest further work should be done in large datasets to develop a more
509 universal metabolomics-based frailty clock.

510

511 There are some limitations to this study. Our validation dataset was relatively small, and mice
512 were treated with NMN that may alter metabolite abundance levels. We suggest future work
513 should validate these potential frailty biomarkers in larger cohorts, as well as in other mouse
514 strains and humans. Additionally, the sample size for metabolomics data is relatively small,
515 especially at the older ages, which might decrease the power of statistical analysis. Survival
516 bias is also an issue to consider, as mice died over the course of the study and only those that
517 were longest lived made it to timepoint 4 and 5. For future work, it will be ideal to conduct
518 studies in a broader age range with an increased number of mice.

519

520 In summary, we performed the first longitudinal study of naturally aging female and male mice
521 looking at metabolomics of frailty. We found aging related metabolites are mainly involved in
522 lipid metabolism while frailty related metabolites are predominantly parts of amino acid
523 metabolism and metabolism of cofactors and vitamins. Apart from whole cohort frailty
524 biomarkers, we demonstrated the sex dimorphism in the associations between metabolite and
525 frailty, and proposed sex specific frailty biomarkers.

526 **Material and methods**

527 ***Mice samples***

528 Mice used in this study are from a larger intervention study, so detailed methods can be found in
529 Kane et al (2024)³⁴. Briefly, C57BL/6NIA mice, female ($n = 40$) and male ($n = 47$) were obtained
530 from the National Institute on Aging (NIA) Aging Rodent Colony, among which, 20 female and
531 23 male mice were subjected to nicotinamide mononucleotide (NMN) treatment. Mice were
532 group housed (4-5 mice per cage, although over the period of the experiment mice died and
533 mice were left singly housed), at Harvard Medical School in ventilated microisolator cages, with
534 a 12-hour light cycle, at 71°F with 45-50% humidity. Mice were fed AIN-93G Purified Rodent
535 Diet (Dyets Inc, PA). All animal experiments were approved by the Institutional Animal Care and
536 Use Committee of the Harvard Medical Area. In order to investigate aging and frailty related
537 metabolites and mechanisms in naturally aging mice, we used non-NMN treated mice (female, n
538 = 20; male, $n = 24$) as the discovery cohort for principal component analysis, feature selection,
539 sex stratified analysis, association study, and metabolite frailty clock model building (**Fig.1**). We
540 then tested the selected metabolite features and model in the NMN treated mice (validation
541 cohort).

542

543 ***Mouse Frailty assessment***

544 Behavioral and clinical variables for clinical frailty index were measured in both the discovery
545 and validation cohorts, at each time point (**Table1**). We utilized the mouse clinical frailty index²
546 (FI) that contains 31 health-related items for this study. Briefly, mice were scored either 0, 0.5 or
547 1 for the degree of deficit they showed in each item with 0 representing no deficit, 0.5
548 representing a mild deficit and 1 representing a severe deficit⁶⁹. Apart from FI score itself, we

549 introduced devFI score, that is the deviation of individual FI from the median FI for the
550 corresponding sex, at the corresponding time point.

551

552 ***Blood collection and processing***

553 Mice were fasted for 5-6 hours, anesthetized with isoflurane (5%) and then blood was collected
554 from the submandibular vein with a lancet (maximum 10% of mouse body weight, approx. 200-
555 300 ul), into a tube containing 20ul of 0.5M EDTA. Blood was mixed and stored on ice. Whole
556 blood was centrifuged at 1500×g for 15 mins, plasma was removed and frozen at -80°C for
557 subsequent metabolomics.

558

559 ***Metabolites extraction, quantification and processing***

560 Global metabolomics analysis was completed by Metabolon. Samples were prepared using the
561 automated MicroLab STAR® system (Hamilton Company), and analyzed using Ultrahigh
562 Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS). We used
563 raw metabolite data (peak area). We performed batch effects mitigation by calculating the mean
564 metabolite value for the baseline time point across all mice, comparing it to the mean value of all
565 other time points and excluding metabolites that presented the mean of the baseline
566 significantly lower (0.05x, compared to the mean of all other time points) or significantly higher
567 (10x compared to the mean of all other time points). We normalized each sample by dividing the
568 metabolite value by the median of metabolite values for that sample to account for any
569 collection batch effects and then derived the (natural) log-transformed values as the metabolite
570 abundance. We derived 781 metabolites with greater than 5% unique abundance across
571 samples.

572

573 ***Metabolomics data variations***

574 The number of metabolomics data is summarised in **Table1**. Metabolite abundance data was
575 subjected to principal component (PC) analysis. We derived PC1 to PC10 and for each PC as
576 the dependent variable, we applied linear regression models and obtained *p*-values, where we
577 used mouse ID, time points, sex, cage (categorical variables), and age at assessment
578 (continuous variables), respectively as the independent variable. Independent variables tested
579 were then clustered according to Euclidean distance.

580

581 ***Differential abundance analysis of metabolites***

582 Log-transformed metabolite abundance data were subjected to differential abundance analysis
583 by using the 'limma' pipeline with a spline. Briefly, metabolite abundance data was subjected to
584 the limma time-course spline analysis, excluding time point 5 due to absence of female samples
585 at this time point. We generated a matrix for a natural cubic spline based on the remaining time
586 points, with degrees of freedom set at 3, and the matrix was used as the time factor. Design
587 matrices for global differential abundance analysis included sex and sex by time interaction
588 term, without assigning a reference level. The data along with the multi-factor design matrix
589 were then subjected to linear modeling with the intra-block correlation based block on mouse
590 ID, and empirical bayes smoothing of metabolite-wise standard deviations. We then determine
591 metabolite abundance differences by defining a contrast matrix for each of the following four

592 categories: 1) mixture of female and male samples, 2) female samples, 3) male samples, and 4)
593 sex differences.

594

595 **Co-abundance analysis**

596 We performed the co-abundance analysis of metabolites (excluding time point 5), with a soft
597 threshold set at 9 to select metabolite abundance modules. For a given module, we derived the
598 first principal component as the eigenvalue. To identify the association of metabolite modules
599 with age, we applied a linear mixed model using the module eigenvalue as independent variable
600 and age as dependent variable with adjustment for sex, allowing a random intercept for each
601 mouse. P-values were then adjusted by the Bonferroni correction method. For each identified
602 subset, metabolites that showed significance greater than 0.2 (correlation coefficient between
603 the metabolite abundance and the age) and module membership greater than 0.8 were selected
604 as the hub metabolites in the module.

605

606 **Pathway enrichment analysis**

607 Metabolite set enrichment analysis was performed by using the hypergeometric test from R
608 package *FELLA* (v. 1.20.0) to identify KEGG pathways that were overrepresented, with a cutoff
609 of p -value set at 0.05.

610

611 **Feature selection**

612 We applied a machine learning approach to identify FI/devFI related metabolites in the
613 discovery cohort (**Fig. 4a**). We performed feature selection by fitting generalized linear
614 regression models using the frailty assessment score (FI or devFI) as the dependent variable
615 and the 781 metabolites abundance data as the independent variables, through a 100 x 5-fold
616 cross validation approach. Briefly, we performed 100 runs of multivariate generalized
617 regression with elastic net regularization. Within each run, the hyperparameters for the least
618 Root mean square error (RMSE) were tuned using 5-fold cross-validation, and a list of
619 metabolite features assigned a non-zero coefficient was derived. These lists (from 100 runs)
620 were merged into a list of metabolite features, which were then ranked according to the
621 importance, i.e. the presence percentage of the metabolites. We selected metabolites that
622 made to the top 20% percentile as FI/devFI metabolites. FI is composed of the age-related base
623 FI and devFI. Hence, we derived FI-age features by combining age metabolites (hub
624 metabolites from co-abundance analysis) and FI metabolites, and devFI features by combining
625 devFI metabolites with FI metabolites. We then obtained union features from the union of FI-age
626 features and devFI features.

627

628 **Analysis of metabolite associated with frailty outcomes**

629 For the association study, we applied mixed linear models allowing variations in individual mice
630 as the random effect. We considered three outcomes for FI and devFI respectively (six in total),
631 1) the score at current age (age_c), FI_c and $devFI_c$; 2) a score at a future time point (age_f), FI_f and
632 $devFI_f$; and 3) score change to a future time point, ΔFI and $\Delta devFI$ (**Fig.5a**). We considered two
633 scenarios in the analysis, where abundance of metabolites from a previous time point (age_p)
634 are: a) absent, only the current abundance of metabolites (MA_c) is available. For each

635 metabolite, we used one of the six score outcomes as the dependent variable and MA_c as the
636 independent variable, adjusting for sex, age_c and age change from age_c to age_t (Δage_2 , only for
637 outcomes 2) and 3)) in the non-interaction models. For interaction models, we considered
638 abundance by age term and abundance by age change term; and b) present, the current and a
639 previous abundance of metabolites and the age interval are available. We focused on
640 metabolite abundance change (ΔMA) in this scenario. We used one of the six score outcomes
641 as the dependent variable and ΔMA as the independent variable, adjusting for MA_c , age change
642 from age_p to age_c (Δage_1), sex, age_c and age Δage_2 (for outcomes 2) and 3)) in the non-
643 interaction models. For interaction models, we included abundance change by age (and/or age
644 change) terms and current abundance by age (and/or age change) term. devFI score is the
645 deviation from the median at the age- and sex- specific group. Hence, current age was not
646 included in the analyses of devFI in both non- and interaction models. The age variable used
647 above was the actual days of assessment divided by 1,000, in order to be within the same
648 scale.

649

650 ***Metabolite frailty clock model building***

651 After obtaining three sets of union features for the whole cohort, females, and males, we
652 generated a single set of metabolite features from the above three sets. We ranked these
653 features by occurrence frequency from the feature selection process (100 times repeated cross
654 validation) within the whole cohort. Via a cross validation approach, we selected 'mtry' (the
655 number of randomly drawn candidate variables out of which each split is selected when growing
656 a tree) and the number of informativity-based top metabolite features that gave the least RMSE
657 in predicting FI. The final metabolite frailty clock model was fit in the discovery cohort,
658 constructed using a random forest regression with FI as the dependent variable and the top
659 metabolites features, age and sex as the independent variables. We also fit linear regression
660 models with age and sex as the independent variables in respective the discovery and
661 validation cohort, and a random forest model using all the 781 metabolites, age and sex in the
662 discovery cohort for comparative purposes.

663

664 ***Statistics***

665 All statistical analyses were performed using R (version 4.3.0). Differentially abundant
666 metabolites (DAMs) are selected by controlling for a 5% Benjamini-Hochberg false discovery
667 rate (adjusted p -values < 0.05). For univariate association study, the significance was
668 determined by controlling for a 5% Benjamini-Hochberg false discovery rate (adjusted p -values
669 < 0.05).

670 **Data availability**

671 Mice metadata, metabolite abundance data, and R markdown file for data analysis are available
672 at <https://github.com/Kane-Lab-LSB/longitudinal-metabolite-analysis-in-mice.git>.

673 **References**

- 674 1. Mitnitski, A., Howlett, S. E. & Rockwood, K. Heterogeneity of Human Aging and Its
675 Assessment. *J. Gerontol. A Biol. Sci. Med. Sci.* **72**, 877–884 (2017).
- 676 2. Whitehead, J. C. *et al.* A clinical frailty index in aging mice: comparisons with frailty index
677 data in humans. *J. Gerontol. A Biol. Sci. Med. Sci.* **69**, 621–632 (2014).
- 678 3. Graber, T. G., Ferguson-Stegall, L., Liu, H. & Thompson, L. V. Voluntary Aerobic Exercise
679 Reverses Frailty in Old Mice. *J. Gerontol. A Biol. Sci. Med. Sci.* **70**, 1045–1058 (2015).
- 680 4. Mitnitski, A. B., Mogilner, A. J. & Rockwood, K. Accumulation of deficits as a proxy measure
681 of aging. *ScientificWorldJournal* **1**, 323–336 (2001).
- 682 5. Heinze-Milne, S. D., Banga, S. & Howlett, S. E. Frailty Assessment in Animal Models.
683 *Gerontology* **65**, 610–619 (2019).
- 684 6. Bisset, E. S. & Howlett, S. E. The biology of frailty in humans and animals: Understanding
685 frailty and promoting translation. *Aging Med (Milton)* **2**, 27–34 (2019).
- 686 7. Kane, A. E. & Sinclair, D. A. Frailty biomarkers in humans and rodents: Current approaches
687 and future advances. *Mech. Ageing Dev.* **180**, 117–128 (2019).
- 688 8. Pan, Y., Ji, T., Li, Y. & Ma, L. Omics biomarkers for frailty in older adults. *Clin. Chim. Acta*
689 **510**, 363–372 (2020).
- 690 9. López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M. & Kroemer, G. Hallmarks of aging:
691 An expanding universe. *Cell* **186**, 243–278 (2023).
- 692 10. Kalyani, R. R., Varadhan, R., Weiss, C. O., Fried, L. P. & Cappola, A. R. Frailty status and
693 altered glucose-insulin dynamics. *J. Gerontol. A Biol. Sci. Med. Sci.* **67**, 1300–1306 (2012).
- 694 11. Sinclair, A. J. & Abdelhafiz, A. H. Metabolic Impact of Frailty Changes Diabetes Trajectory.
695 *Metabolites* **13**, (2023).
- 696 12. Panyard, D. J., Yu, B. & Snyder, M. P. The metabolomics of human aging: Advances,
697 challenges, and opportunities. *Sci Adv* **8**, eadd6155 (2022).

- 698 13. Mutlu, A. S., Duffy, J. & Wang, M. C. Lipid metabolism and lipid signals in aging and
699 longevity. *Dev. Cell* **56**, 1394–1407 (2021).
- 700 14. Dunn, W. B. *et al.* Molecular phenotyping of a UK population: defining the human serum
701 metabolome. *Metabolomics* **11**, 9–26 (2015).
- 702 15. Rist, M. J. *et al.* Metabolite patterns predicting sex and age in participants of the Karlsruhe
703 Metabolomics and Nutrition (KarMeN) study. *PLoS One* **12**, e0183228 (2017).
- 704 16. Dato, S. *et al.* Amino acids and amino acid sensing: implication for aging and diseases.
705 *Biogerontology* **20**, 17–31 (2019).
- 706 17. Weichhart, T. mTOR as Regulator of Lifespan, Aging, and Cellular Senescence: A Mini-
707 Review. *Gerontology* **64**, 127–134 (2018).
- 708 18. Yu, Z. *et al.* Human serum metabolic profiles are age dependent. *Aging Cell* **11**, 960–967
709 (2012).
- 710 19. Peters, K. *et al.* Metabolic drift in the aging nervous system is reflected in human
711 cerebrospinal fluid. *Sci. Rep.* **11**, 18822 (2021).
- 712 20. Mielke, M. M. *et al.* Factors affecting longitudinal trajectories of plasma sphingomyelins: the
713 Baltimore Longitudinal Study of Aging. *Aging Cell* **14**, 112–121 (2015).
- 714 21. Darst, B. F., Kosciuk, R. L., Hogan, K. J., Johnson, S. C. & Engelman, C. D. Longitudinal
715 plasma metabolomics of aging and sex. *Aging* **11**, 1262–1282 (2019).
- 716 22. Mishra, M., Wu, J., Kane, A. E. & Howlett, S. E. The intersection of frailty and metabolism.
717 *Cell Metab.* **36**, 893–911 (2024).
- 718 23. Cesari, M., Calvani, R. & Marzetti, E. Frailty in Older Persons. *Clin. Geriatr. Med.* **33**, 293–
719 303 (2017).
- 720 24. Calvani, R. *et al.* Amino Acid Profiles in Older Adults with Frailty: Secondary Analysis from
721 MetaboFrail and BIOSPHERE Studies. *Metabolites* **13**, (2023).
- 722 25. Gordon, E. H. *et al.* Sex differences in frailty: A systematic review and meta-analysis. *Exp.*
723 *Gerontol.* **89**, 30–40 (2017).

- 724 26. Yu, H., Armstrong, N., Pavela, G. & Kaiser, K. Sex and Race Differences in Obesity-
725 Related Genetic Susceptibility and Risk of Cardiometabolic Disease in Older US Adults.
726 *JAMA Netw Open* **6**, e2347171 (2023).
- 727 27. de Ritter, R. *et al.* Sex differences in the risk of vascular disease associated with diabetes.
728 *Biol. Sex Differ.* **11**, 1 (2020).
- 729 28. Bell, J. A. *et al.* Sex differences in systemic metabolites at four life stages: cohort study with
730 repeated metabolomics. *BMC Med.* **19**, 58 (2021).
- 731 29. Zhao, L., Mao, Z., Woody, S. K. & Brinton, R. D. Sex differences in metabolic aging of the
732 brain: insights into female susceptibility to Alzheimer's disease. *Neurobiol. Aging* **42**, 69–79
733 (2016).
- 734 30. Varghese, M., Song, J. & Singer, K. Age and Sex: Impact on adipose tissue metabolism
735 and inflammation. *Mech. Ageing Dev.* **199**, 111563 (2021).
- 736 31. Costanzo, M. *et al.* Sex differences in the human metabolome. *Biol. Sex Differ.* **13**, 30
737 (2022).
- 738 32. Hägg, S. & Jylhävä, J. Sex differences in biological aging with a focus on human studies.
739 *Elife* **10**, (2021).
- 740 33. Hägg, S., Jylhävä, J., Wang, Y., Czene, K. & Grassmann, F. Deciphering the genetic and
741 epidemiological landscape of mitochondrial DNA abundance. *Hum. Genet.* **140**, 849–861
742 (2021).
- 743 34. Kane, A. E. *et al.* Long-term NMN treatment increases lifespan and healthspan in mice in a
744 sex dependent manner. *bioRxiv* (2024) doi:10.1101/2024.06.21.599604.
- 745 35. Cnaan, A. *et al.* Extended lifespan and reduced adiposity in mice lacking the FAT10 gene.
746 *Proc. Natl. Acad. Sci. U. S. A.* **111**, 5313–5318 (2014).
- 747 36. Streeper, R. S. *et al.* Deficiency of the lipid synthesis enzyme, DGAT1, extends longevity in
748 mice. *Aging* **4**, 13–27 (2012).
- 749 37. Tabassum, R. *et al.* Lipidome- and Genome-Wide Study to Understand Sex Differences in

- 750 Circulatory Lipids. *J. Am. Heart Assoc.* **11**, e027103 (2022).
- 751 38. Phelps, T., Snyder, E., Rodriguez, E., Child, H. & Harvey, P. The influence of biological sex
752 and sex hormones on bile acid synthesis and cholesterol homeostasis. *Biol. Sex Differ.* **10**,
753 52 (2019).
- 754 39. Palmisano, B. T., Zhu, L., Eckel, R. H. & Stafford, J. M. Sex differences in lipid and
755 lipoprotein metabolism. *Mol Metab* **15**, 45–55 (2018).
- 756 40. Sjovall, J. Dietary glycine and taurine on bile acid conjugation in man; bile acids and
757 steroids 75. *Proc. Soc. Exp. Biol. Med.* **100**, 676–678 (1959).
- 758 41. Zhu, D. *et al.* Sex dimorphism and tissue specificity of gene expression changes in aging
759 mice. *Biol. Sex Differ.* **15**, 89 (2024).
- 760 42. Dixon, S. J. *et al.* Ferroptosis: an iron-dependent form of nonapoptotic cell death. *Cell* **149**,
761 1060–1072 (2012).
- 762 43. Lindeman, R. D. Mineral metabolism in the aging and the aged. *J. Am. Coll. Nutr.* **1**, 49–73
763 (1982).
- 764 44. Zeidan, R. S., Han, S. M., Leeuwenburgh, C. & Xiao, R. Iron homeostasis and organismal
765 aging. *Ageing Res. Rev.* **72**, 101510 (2021).
- 766 45. Austad, S. N., Smith, J. R. & Hoffman, J. M. Amino acid restriction, aging, and longevity: an
767 update. *Front Aging* **5**, 1393216 (2024).
- 768 46. Jarrell, Z. R. *et al.* Plasma acylcarnitine levels increase with healthy aging. *Aging* **12**,
769 13555–13570 (2020).
- 770 47. Sol, J. *et al.* Plasma acylcarnitines and gut-derived aromatic amino acids as sex-specific
771 hub metabolites of the human aging metabolome. *Aging Cell* **22**, e13821 (2023).
- 772 48. Jové, M. *et al.* Human Aging Is a Metabolome-related Matter of Gender. *J. Gerontol. A Biol.*
773 *Sci. Med. Sci.* **71**, 578–585 (2016).
- 774 49. Xu, T.-T. *et al.* Spermidine and spermine delay brain aging by inducing autophagy in
775 SAMP8 mice. *Aging* **12**, 6401–6414 (2020).

- 776 50. De Simone, G., Balducci, C., Forloni, G., Pastorelli, R. & Brunelli, L. Hippuric acid: Could
777 became a barometer for frailty and geriatric syndromes? *Ageing Res Rev* **72**, 101466
778 (2021).
- 779 51. Al Saedi, A., Chow, S., Vogrin, S., Guillemin, G. J. & Duque, G. Association Between
780 Tryptophan Metabolites, Physical Performance, and Frailty in Older Persons. *Int. J.*
781 *Tryptophan Res.* **15**, 11786469211069951 (2022).
- 782 52. Zhang, H. *et al.* NAD⁺ repletion improves mitochondrial and stem cell function and
783 enhances life span in mice. *Science* **352**, 1436–1443 (2016).
- 784 53. Mills, K. F. *et al.* Long-Term Administration of Nicotinamide Mononucleotide Mitigates Age-
785 Associated Physiological Decline in Mice. *Cell Metab.* **24**, 795–806 (2016).
- 786 54. Kameda, M., Teruya, T., Yanagida, M. & Kondoh, H. Frailty markers comprise blood
787 metabolites involved in antioxidation, cognition, and mobility. *Proc. Natl. Acad. Sci. U. S. A.*
788 **117**, 9483–9489 (2020).
- 789 55. Katsube, M. *et al.* Ergothioneine promotes longevity and healthy aging in male mice.
790 *Geroscience* (2024) doi:10.1007/s11357-024-01111-5.
- 791 56. Asadi Shahmirzadi, A. *et al.* Alpha-Ketoglutarate, an Endogenous Metabolite, Extends
792 Lifespan and Compresses Morbidity in Aging Mice. *Cell Metab.* **32**, 447–456.e6 (2020).
- 793 57. Imai, S.-I. & Guarente, L. NAD⁺ and sirtuins in aging and disease. *Trends Cell Biol* **24**,
794 464–471 (2014).
- 795 58. Preidis, G. A. *et al.* Microbial-Derived Metabolites Reflect an Altered Intestinal Microbiota
796 during Catch-Up Growth in Undernourished Neonatal Mice. *J. Nutr.* **146**, 940–948 (2016).
- 797 59. Shlipak, M. G. *et al.* The presence of frailty in elderly persons with chronic renal
798 insufficiency. *Am J Kidney Dis* **43**, 861–867 (2004).
- 799 60. Westbrook, R. *et al.* Kynurenines link chronic inflammation to functional decline and
800 physical frailty. *JCI Insight* **5**, (2020).
- 801 61. Chung, T. *et al.* Deletion of quinolinate phosphoribosyltransferase gene accelerates frailty

- 802 phenotypes and neuromuscular decline with aging in a sex-specific pattern. *Aging Cell* **22**,
803 e13849 (2023).
- 804 62. Cheng, X. *et al.* Association between B-vitamins intake and frailty among patients with
805 chronic obstructive pulmonary disease. *Aging Clin. Exp. Res.* **35**, 793–801 (2023).
- 806 63. Balboa-Castillo, T. *et al.* Low vitamin intake is associated with risk of frailty in older adults.
807 *Age Ageing* **47**, 872–879 (2018).
- 808 64. Kato, N. *et al.* Relationship of Low Vitamin B6 Status with Sarcopenia, Frailty, and Mortality:
809 A Narrative Review. *Nutrients* **16**, (2024).
- 810 65. Laurila, P.-P. *et al.* Sphingolipids accumulate in aged muscle, and their reduction
811 counteracts sarcopenia. *Nat Aging* **2**, 1159–1175 (2022).
- 812 66. Marron, M. M., Yao, S., Shah, R. V., Murthy, V. L. & Newman, A. B. Metabolomic
813 characterization of vigor to frailty among community-dwelling older Black and White men
814 and women. *Geroscience* **46**, 2371–2389 (2024).
- 815 67. Mutz, J., Iniesta, R. & Lewis, C. M. Metabolomic age (MileAge) predicts health and life
816 span: A comparison of multiple machine learning algorithms. *Sci Adv* **10**, eadp3743 (2024).
- 817 68. Mak, J. K. L. *et al.* Temporal Dynamics of Epigenetic Aging and Frailty From Midlife to Old
818 Age. *J Gerontol A Biol Sci Med Sci* **79**, (2024).
- 819 69. Kane, A. E. *et al.* A Comparison of Two Mouse Frailty Assessment Tools. *J. Gerontol. A*
820 *Biol. Sci. Med. Sci.* **72**, 904–909 (2017).

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825 **Author contributions**

826 A.E.K. and D.A.S. conceived and designed the study. A.E.K. performed the experiments. D.Z.
827 conducted the data analysis, with contribution from J.Z.W., P.G., and B.A.S. P.G. provided

828 critical feedback. D.Z., J.Z.W., and A.E.K. drafted and revised the manuscript with help from all
829 authors. All authors have read and agreed to the published version of the manuscript.

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831 Correspondence to Alice E. Kane.

832 Competing interests

833 D.A.S. is a founder, equity owner, advisor to, director of, board member of, consultant to,
834 investor in and/or inventor on patents licensed to Revere Biosensors, UpRNA, GlaxoSmithKline,
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841 (aka Longevity Sciences) and Bold Capital. D.A.S. is an inventor on a patent application filed by
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843 info on D.A.S. affiliations can be found at <https://sinclair.hms.harvard.edu/david-sinclairs->
844 affiliations. The other authors declare no competing interests.

845 Tables

846 **Table1. List of female and male samples.**

847

	Discovery cohort						Validation cohort					
	Female (n = 20)			Male (n = 24)			Female (n = 20)			Male (n = 23)		
Time points	Samples ^a	Age ^b	Frailty index ^b	Samples	Age	Frailty index	Samples	Age	Frailty index	Samples	Age	Frailty index
BL	19	393	0.14 [0.12, 0.16]	21	386	0.19 [0.18, 0.21]	16	393	0.15 [0.13, 0.16]	18	386	0.19 [0.18, 0.22]
T2	20	541	0.24 [0.22, 0.26]	22	539	0.23 [0.21, 0.25]	19	541	0.21 [0.20, 0.23]	23	539	0.22 [0.20, 0.24]
T3	17	624	0.29 [0.26, 0.31]	23	635	0.26 [0.24, 0.28]	19	624	0.24 [0.21, 0.26]	23	635	0.25 [0.23, 0.32]
T4	11	756	0.28 [0.26, 0.34]	19	773	0.27 [0.23, 0.28]	12	756	0.28 [0.23, 0.32]	20	773	0.27 [0.26, 0.31]
T5	NA	NA	NA	9	910	0.37 [0.35, 0.45]	4	899	0.33 [0.29, 0.36]	6	910	0.44 [0.40, 0.45]

848 a. Number of samples that have valid metabolomics data

849 b. Age (days) at assessment for frailty

850 c. Median [Lower quartile, Upper quartile]

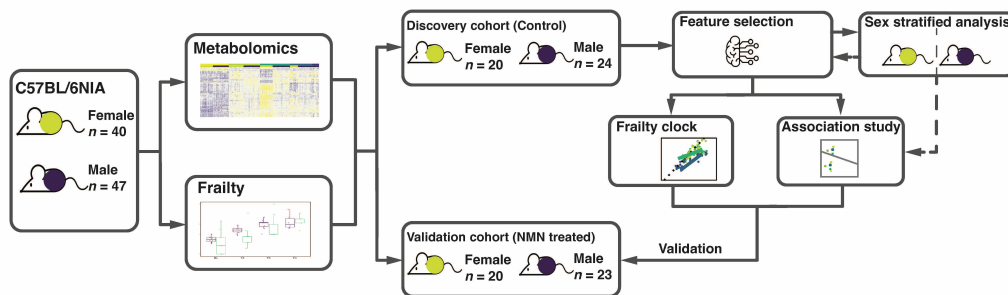
851 **Figures**

852 **Fig.1. Schematic diagram of the workflow.**

853 The longitudinal study starts with female ($n = 40$, yellow circles) and male ($n = 47$, blue circles)
854 C57BL/6NIA mice. Frailty was assessed and blood samples were collected at 5 time points from
855 BL to T5 (exact days of experiments are shown in Table1). Plasma samples were derived from
856 blood samples and were then subjected to metabolite quantification. In order to investigate
857 metabolites related with natural aging and frailty, feature selection, sex stratified analysis,
858 association study and the frailty clock were all performed in the control samples without
859 intervention as the discovery cohort. The metabolite biomarkers and a metabolite clock for frailty
860 were then tested in the validation cohort.

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Fig.1

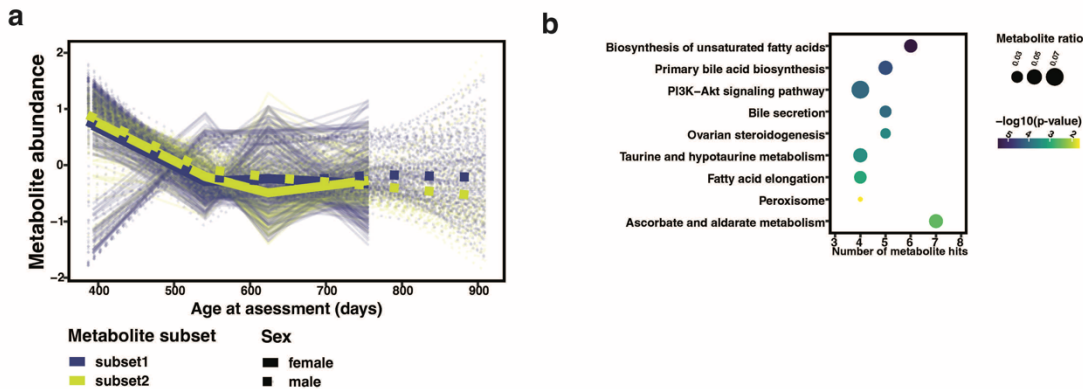


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870 **Fig.2. Sex independent age-related differentially abundant metabolites in**
871 **longitudinal study.**

872 Differential abundance analysis was performed using all samples (excluding time point T5) in
873 the study. Sex-independent age-related differentially abundant metabolites (DAMs) were
874 selected from comparisons of the mixture of female and male samples at different time points
875 and by controlling for a 5% Benjamini-Hochberg false discovery rate (adjusted p -values < 0.05).
876 These DAMs were then subjected to a co-abundance analysis, and subset1 and subset2 were
877 determined to be significantly associated with age by linear mixed models. **(a)** Dynamics of
878 metabolite abundance in each sex, derived from two subsets (subset1, $n = 200$ metabolites;
879 subset2, $n = 125$). After determining the hub metabolites based on metabolite correlation with
880 age and module membership, hub metabolites were subjected to metabolite set enrichment
881 analysis. **(b)** Over-represented pathways (y-axis) from the hub metabolites from the two
882 subsets. The number of hits (metabolite) from the hub metabolites set is shown by x-axis, ratio
883 of the hit number to total metabolites in the enriched pathway is represented by dot size and p -
884 value is colored by levels.

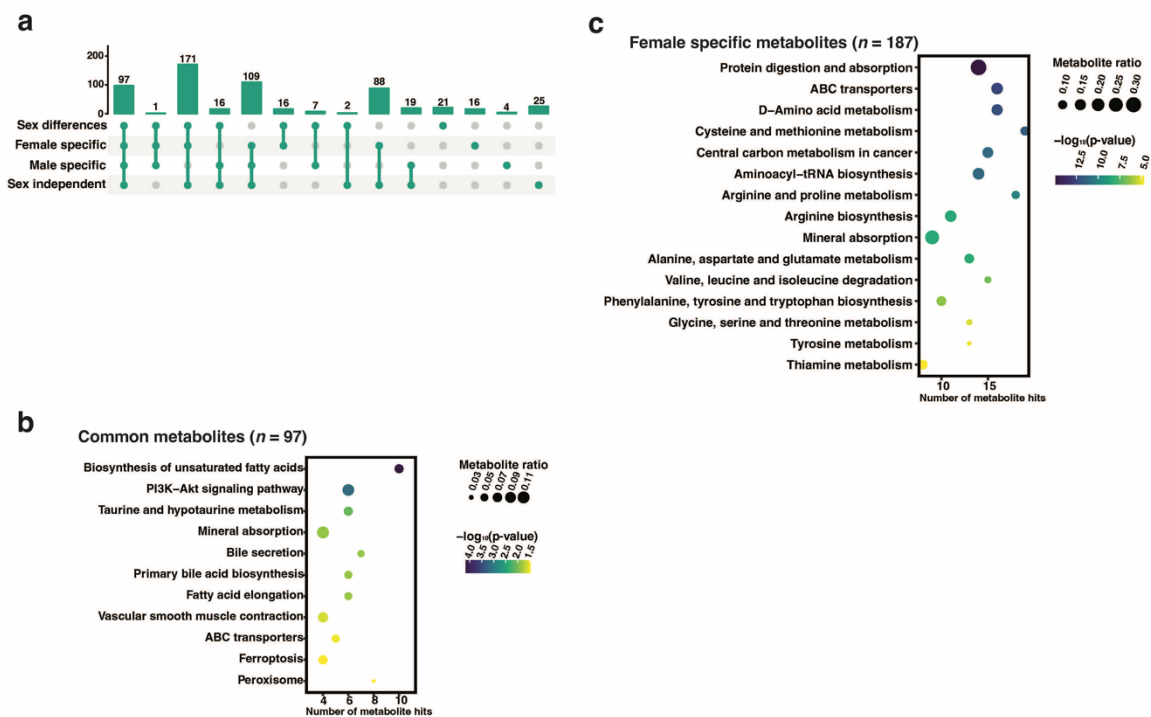
885
886 **Fig.2**



910 **Fig.3. Comparisons of differentially abundant metabolites determined in four**
 911 **groups.**

912 Differential abundance analysis was performed using all samples (excluding time point T5) in
 913 the study. Age-related differentially abundant metabolites (DAMs) were determined by
 914 comparisons within four groups: the mixture of females and males (sex independent), female
 915 specific, male specific, and sex differences, and by controlling for a 5% Benjamini-Hochberg
 916 false discovery rate (adjusted p -values < 0.05). **(a)** UpSet plot showing the common DAMs
 917 derived from the comparisons. **(b)** Over-represented pathways (y-axis) from the 97 common
 918 metabolites of four groups by metabolite set enrichment analysis. **(c)** Over-represented
 919 pathways (y-axis) from the 187 female specific metabolites markers that also present sex
 920 differences. The number of hits (metabolite) from the hub metabolites set is shown by x-axis,
 921 ratio of the hit number to total metabolites in the enriched pathway is represented by dot size
 922 and p -value is colored by levels.

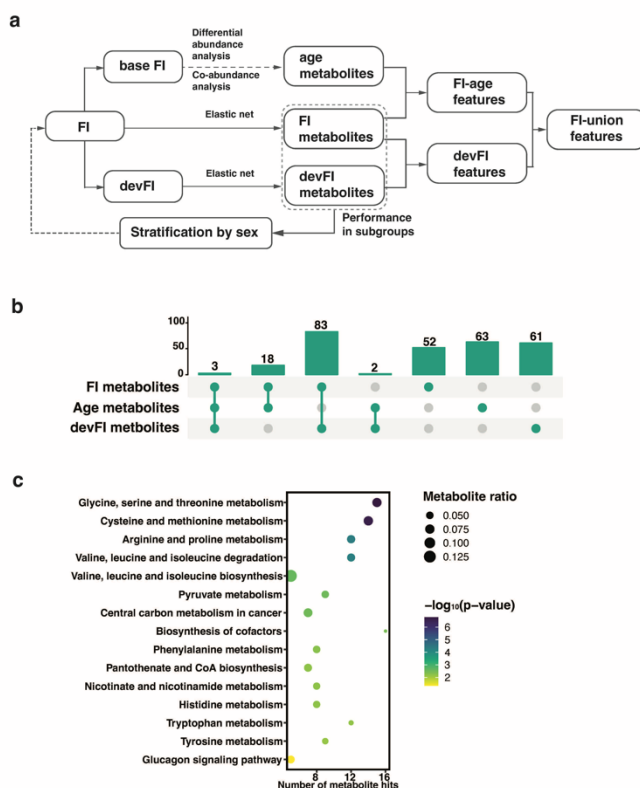
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 926 **Fig.3**
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952 **Fig.4. Selection of frailty related features**

953 **(a)** Schematic diagram for the workflow of the feature selection. Frailty index (FI) is composed of
 954 base FI and devFI (deviation from the age- and sex- group median FI). Base FI is age related,
 955 hence leads to age metabolites. FI and devFI metabolites are derived from elastic net
 956 regularization regression via a 100 times repeated 5-fold cross validation approach. FI
 957 metabolites are merged with age metabolites into FI-age features and with devFI metabolites
 958 into devFI features. The FI-union features are the union of FI-age and devFI features. The
 959 workflow is performed in the whole cohort, as well as females and males after the stratification
 960 by sex. **(b)** UpSet plot showing the overlapping metabolite features from the FI-, age- and
 961 devFI- metabolites. **(c)** Over-represented pathways (y-axis) from the 104 FI-union features from
 962 the whole cohort. The number of hits (metabolite) from the hub metabolites set is shown by x-
 963 axis, ratio of the hit number to total metabolites in the enriched pathway is represented by dot
 964 size and p-value is colored by levels.

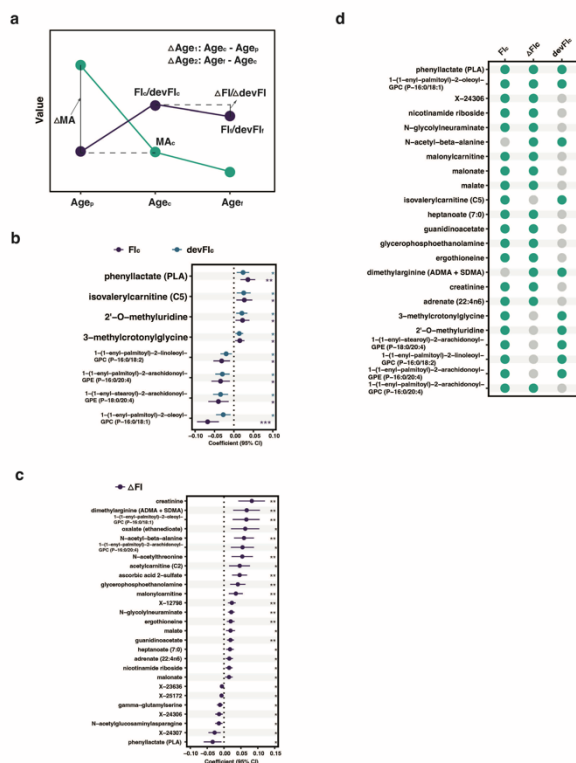
966 Fig.4



993 **Fig.5. Association study in the whole cohort**

994 (a) Schematic diagram showing the dependent and independent variables in the linear mixed
 995 models for the association study. Dependent variables include Frailty Index (FI_c) and devFI
 996 ($devFI_c$, deviation from median FI of the age- and sex- specific group) at the current age (age_c),
 997 FI and devFI ($FI_f/devFI_f$) at a future age (age_f), and FI/devFI change from age_c to age_f
 998 ($\Delta FI/\Delta devFI$). Independent variables include current abundance of metabolites (MA_c),
 999 abundance change from a previous age (age_p) to age_c , ΔAge_1 and ΔAge_2 . For each frailty
 1000 outcome, FI-union features identified were individually subjected to linear mixed models. (b)
 1001 Coefficients of eight metabolites of which MA_c presents significance in the association with both
 1002 FI_c and $devFI_c$. Metabolites are arranged by coefficients (represented by dots) for FI_c in
 1003 descending order. The line represents the 95% confidence interval of each coefficient. (c) 27
 1004 metabolites of which the current metabolite abundance presents significance in the association
 1005 with ΔFI . Metabolites are arranged by coefficients (represented by dots) in descending order.
 1006 Significance was determined by adjusted p -values via Benjamini-Hochberg false discovery rate
 1007 procedure at a cutoff of 0.05, with * for $p < 0.05$, ** for $p < 0.01$, and, *** for $p < 0.001$. (d) List of
 1008 23 metabolites that show occurrences greater than or equal to 2. That is, $MA/\Delta MA$ of metabolite
 1009 presents significance in the association with frailty outcomes of the column.
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Fig.5

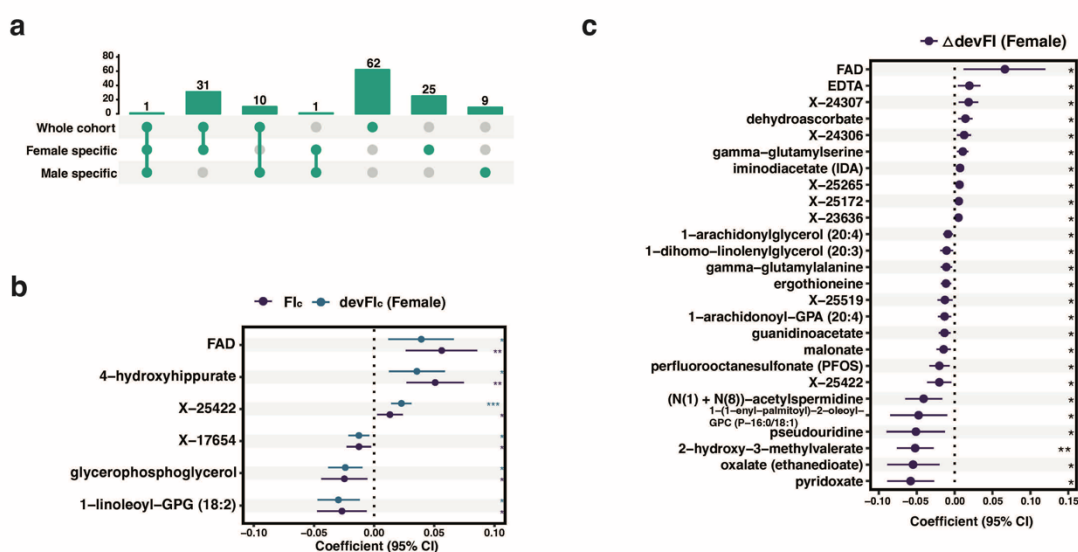


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1033 **Fig.6. Sex independent metabolite features and association**

1034 (a) UpSet plot showing the overlapping metabolites of frailty index union features of the whole
 1035 cohort, females and males. (b) Coefficients of six metabolites of which metabolite change
 1036 presents significance in the association with both FI_c and $devFI_c$ in females. Metabolites are
 1037 arranged by coefficients (represented by dots) for FI_c in descending order. (c) Coefficients of 26
 1038 metabolites of which metabolite change presents significance in the association with $devFI$
 1039 change in females. The significance was determined by adjusted p -values via Benjamini-
 1040 Hochberg false discovery rate procedure at a cutoff of 0.05, with * for $p < 0.05$, ** for $p < 0.01$,
 1041 and, *** for $p < 0.001$. Metabolites are arranged by coefficients (dots) in descending order. The
 1042 line represents the 95% confidence interval of each coefficient.

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1045 **Fig.6**
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1074 **Fig.7. Performance of metabolite frailty clock in the discovery and validation**
 1075 **cohorts.**

1076 Frailty models were built via machine learning approaches, with frailty index scores as the
 1077 dependent variable and three sets of variables as the independent variables: 1) The age+sex
 1078 model, linear regression models using age and sex, trained in the discovery and validation
 1079 cohorts respectively; 2) The RF (781 metabolites) model, a random forest model using all 781
 1080 metabolites detected in this study, age and sex; and 3) metabolite frailty clock model, a random
 1081 forest model using 63 informativity-based metabolites, age and sex. The performance of models
 1082 in the corresponding cohort/subcohort (Female, F and male, M) are presented using R^2 and
 1083 Root-mean-square deviation ($RMSE$).

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 1085
 1086 **Fig.7**

