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# Metabolic features of recurrent major depressive disorder in remission, and the risk of future recurrence

Roel J. T. Mocking<sup>1</sup>, Jane C. Naviaux<sup>2,3</sup>, Kefeng Li<sup>2,4</sup>, Lin Wang<sup>2,4</sup>, Jonathan M. Monk<sup>2,4</sup>, A. Taylor Bright<sup>2,4,9</sup>, Caroline A. Figueroa<sup>1,10</sup>, Aart H. Schene<sup>5,6</sup>, Henricus G. Ruhé<sup>5,6</sup>, Johanna Assies<sup>1</sup> and Robert K. Naviaux<sup>1,2,4,7,8</sup>

## Abstract

Recurrent major depressive disorder (rMDD) is a relapsing-remitting disease with high morbidity and a 5-year risk of recurrence of up to 80%. This was a prospective pilot study to examine the potential diagnostic and prognostic value of targeted plasma metabolomics in the care of patients with rMDD in remission. We used an established LC-MS/MS platform to measure 399 metabolites in 68 subjects with rMDD ( $n = 45$  females and 23 males) in antidepressant-free remission and 59 age- and sex-matched controls ( $n = 40$  females and 19 males). Patients were then followed prospectively for 2.5 years. Metabolomics explained up to 43% of the phenotypic variance. The strongest biomarkers were gender specific. 80% of the metabolic predictors of recurrence in both males and females belonged to 6 pathways: (1) phospholipids, (2) sphingomyelins, (3) glycosphingolipids, (4) eicosanoids, (5) microbiome, and (6) purines. These changes traced to altered mitochondrial regulation of cellular redox, signaling, energy, and lipid metabolism. Metabolomics identified a chemical endophenotype that could be used to stratify rMDD patients at greatest risk for recurrence with an accuracy over 0.90 (95%CI = 0.69–1.0). Power calculations suggest that a validation study of at least 198 females and 198 males (99 cases and 99 controls each) will be needed to confirm these results. Although a small study, these results are the first to show the potential utility of metabolomics in assisting with the important clinical challenge of prospectively identifying the patients at greatest risk of recurrence of a depressive episode and those who are at lower risk.

## Introduction

Major depressive disorder (MDD) affects 16.1 million US adults and costs \$210 billion annually<sup>1</sup>. MDD's worldwide point prevalence is 6%<sup>2</sup>. Recurrence risk after a first MDD-episode is 3–6 times the background

population risk<sup>3</sup>, with most patients having a recurring-remitting course with five lifetime episodes on average. This lifelong recurrence risk accounts substantially to the overall burden of MDD<sup>4</sup> and to the risk of suicide<sup>5</sup>. If we could better understand molecular bases of recurrent major depressive disorder (rMDD), we might develop prospective risk markers and novel targets for prevention.

Standard clinical variables moderately predict recurrence risk<sup>4</sup> but more robust biomarkers are needed. Evidence for psychological theories of MDD-recurrence is limited and has not yet resulted in risk prediction tools that have been included in clinical guidelines<sup>6</sup>. Several biological pathways have been related to MDD in general, but only a limited number of studies specifically investigated rMDD. A recent meta-analysis

Correspondence: Roel J. T. Mocking (r.j.mocking@amc.uva.nl) or Johanna Assies (J.Assies@amsterdamumc.nl) or Robert K. Naviaux (rnaviaux@health.ucsd.edu)

<sup>1</sup>Department of Psychiatry, Amsterdam UMC, Academic Medical Center, University of Amsterdam, Meibergdreef 5, 1105 AZ Amsterdam, The Netherlands

<sup>2</sup>The Mitochondrial and Metabolic Disease Center, University of California, San Diego School of Medicine, 214 Dickinson St., Bldg CTF, Rm C107, San Diego, CA 92103-8467, USA

Full list of author information is available at the end of the article

These authors contributed equally: Roel J. T. Mocking, Jane C. Naviaux, Johanna Assies, Robert K. Naviaux

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comprehensively reviewed all evidence on biological factors predicting recurrence, including neuroimaging, immunological, and hormonal biomarkers<sup>7</sup>. It showed that only increased cortisol had a small predictive effect on recurrence, but even this effect disappeared when baseline clinical diagnoses, publication bias, or study quality were considered.

More recently, increasing focus has been on metabolic alterations in MDD<sup>8</sup>. Metabolomic and lipidomic studies showed more widespread alterations in MDD<sup>9–11</sup>, that have been hypothesized to constitute a trait associated with recurrence<sup>12</sup>. Recently, a targeted metabolomic study focusing on neurotransmitters and their metabolites in plasma, found a biochemical signature that could diagnose MDD-patients with up to 95% accuracy<sup>13,14</sup>. Moreover, metabolomics may predict MDD-recovery<sup>15</sup>, and response to therapy<sup>16,17</sup>. However, similar studies of biochemical signatures of remitted rMDD (rrMDD), with prospective follow-up to identify subjects at increased risk of future recurrence have not yet been conducted. The emerging recognition that the brain controls metabolism through neuroendocrine, autonomic, immune, and microbiome circuits<sup>18</sup>, implies that peripheral blood metabolomics can provide a uniquely accessible set of biomarkers that are diagnostic of real-time changes in brain-body function. This has now been shown in studies of myalgic encephalomyelitis/chronic fatigue syndrome<sup>19</sup>, schizophrenia<sup>20</sup>, Gulf War Illness<sup>21</sup>, response to treatment in autism spectrum disorder<sup>22</sup>, and treatment-refractory MDD with suicidal ideation<sup>23</sup>.

### Aims of the study

In this discovery phase pilot study, we tested the utility of metabolomic analysis for two purposes: (1) as a diagnostic tool to distinguish patients with rrMDD from controls, and (2) as a prognostic tool to assess the future risk of recurrence in patients with rMDD in drug-free remission at the time of sample collection.

## Patients and methods

### Study design

A cross-sectional patient-control design was used to compare medication-free rrMDD patients with age- and sex-matched, never-depressed controls to identify a metabolic profile of rrMDD patients<sup>4</sup>. In addition, rrMDD patients were followed prospectively every 4 months for 2.5 years by measuring depressive symptoms. Occurrence and time to recurrence of a new major depressive episode were documented.

### Approvals, inclusion and exclusion criteria

We included subjects who experienced  $\geq 2$  previous MDD-episodes according to the structured clinical

interview for Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) diagnoses (SCID), but were in stable remission ( $\geq 8$  weeks 17-item Hamilton Depression Rating Scale (HAM-D)  $\leq 7$  (the lowest qualifying score for remission<sup>24</sup>), and not currently in an MDD-episode (SCID). Participants were 35–65 years, to include a homogeneous age group and minimize the risk of later conversion to bipolar disorder. Second, we included never-depressed controls without personal psychiatric history by SCID-analysis, or first-degree familial psychiatric history, matched to the rrMDD subjects for age, sex, educational level, working class and ethnicity. Both groups were recruited using identical advertisements in freely available sources and from previous studies<sup>4</sup>. This study was approved by the accredited Academic Medical Centre (AMC) Medical Ethical Committee (METC), and conformed to the Declaration of Helsinki<sup>25</sup>. Eleven of 40 healthy control females and 7 out of 19 healthy control males were recruited at the University of California, San Diego (UCSD) under IRB-approved protocol #140072 with signed informed consent. We excluded subjects with current diagnoses of alcohol and/or drug dependence, psychotic or bipolar symptoms, predominant anxiety or severe personality disorder. Other exclusion criteria included standard MRI-exclusion criteria, history of severe head trauma or neurological disease, or severe general physical illness. All participants had to be without psychoactive medication for  $\geq 4$  weeks.

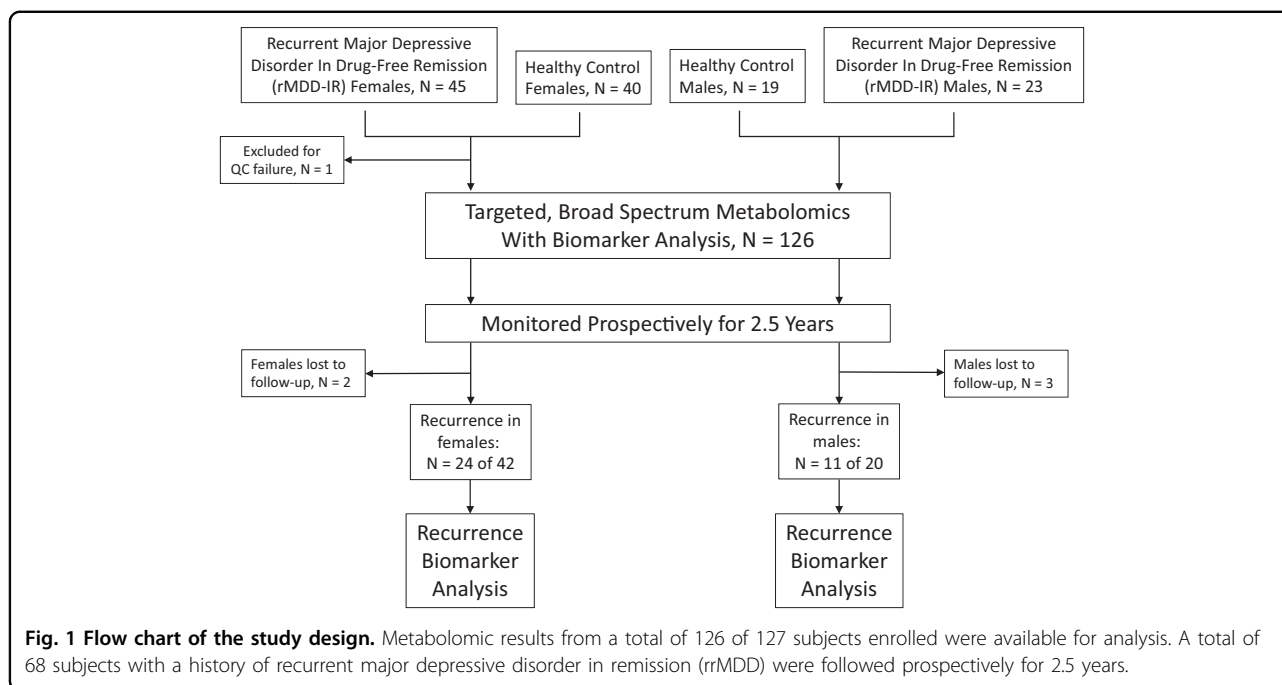
### Psychometrics

At baseline we administered the SCID and HAM-D to check inclusion criteria and residual depressive symptoms. Subsequently, we followed-up rrMDD subjects using the SCID for 2.5 years to prospectively assess time to recurrence with  $\geq 5$  depressive symptoms lasting at least 2 weeks according to the DSM-IV criteria. HAM-D evaluations were conducted upon enrollment of 41 of the 59 healthy controls. Healthy controls were not followed prospectively.

### Metabolomics

Targeted, broad-spectrum, metabolomic analysis of 612 intracellular and plasma metabolites was performed by LC-MS/MS as described<sup>26</sup> with minor modifications. A total of 399 of the 612 targeted metabolites were measurable in plasma of both males and females. This platform broadly interrogates 63 biochemical pathways and permits analysis of many of the metabolites known to be core features of the cell danger and integrated stress response (CDR and ISR)<sup>27–29</sup>.

See Supplementary Methods for additional methods



## Results

### Study cohort

Sixty-eight drug-free rrMDD patients were enrolled and followed for 2.5 years (Flow diagram: Fig. 1). Fifty-nine age- and sex-matched controls were also enrolled.

### Participant characteristics

The rrMDD patients had a long history of illness ( $25 \pm 2.4$  years for males, and  $27 \pm 1.9$  years for females), with a high total number of MDD-episodes (mean =  $9.7 \pm 2.5$  for males;  $8.1 \pm 1.9$  for females) (Table 1). Mean HAM-D score for the rrMDD subjects was 2 and 3 for males and females, respectively. A major epidemiologic difference between males and females with rrMDD was the median time to recurrence after enrollment (males: 5.5 months IQR = 1.9–7.6; females: 10.1 IQR = 4.9–17.2; Table 1). During follow-up three participants restarted antidepressants when experiencing a recurrence and two participants restarted antidepressants while in remission during follow-up. This occurred after blood sample collection for metabolomics at enrollment and did not affect the drug-free analysis.

### Metabolomics overview

Drug-free rrMDD subjects had a metabolic profile that could be distinguished from healthy controls. Multivariate analysis showed a clear separation between the 2 groups in both males and females (Fig. 2A, B). Top discriminating metabolites are shown in Fig. 2C, D. Relative metabolic impact and significance of these differences are shown in Fig. 2E, F. Tables S1 and S2 report the raw data

and Tables S3 and S4 list the rank order of biochemical pathways that were disturbed in rrMDD female and male subjects, respectively, when compared to healthy age- and sex-matched controls. Figure 2G summarizes shared and gender-specific metabolic differences. Principal components analysis (PCA) showed that metabolomics explained up to 39.1% of the phenotypic variance between patients with rrMDD and healthy controls in both males and females (Fig. S1AC). Metabolomics explained up to 43.7% of the phenotypic variance between females who experienced recurrence of depressive symptoms and those with non-recurrence over the 2.5 years of prospective observation (Fig. S1B), and up to 50.6% in males (Fig. S1D).

Overall, alterations in lipid metabolism dominated the rrMDD metabolic signature. Lipid abnormalities constituted 80% of the top 10 pathway alterations in females (Table S3 and Fig. S1). In males, lipid abnormalities constituted 70% of the top 10 pathways (Table S4 and Fig. S2). The 8 lipid pathways most affected were phospholipids, fatty acids and acyl-carnitines, cardiolipins, two classes of sphingolipids (ceramides and sphingomyelins), eicosanoids, bile acids, sterols, and non-gonadal steroids.

The top non-lipid pathway alteration was purine metabolism (Tables S3 and S4 and Fig. S2). Pyrimidines, microbiome metabolites, GABA-glutamate-pyrroline-5-carboxylate-proline, folate-1-carbon, inositol, and tryptophan-serotonin metabolism were also altered in rrMDD subjects (Fig. 2 and S2). Gender-specific differences are discussed in Supplementary Results.

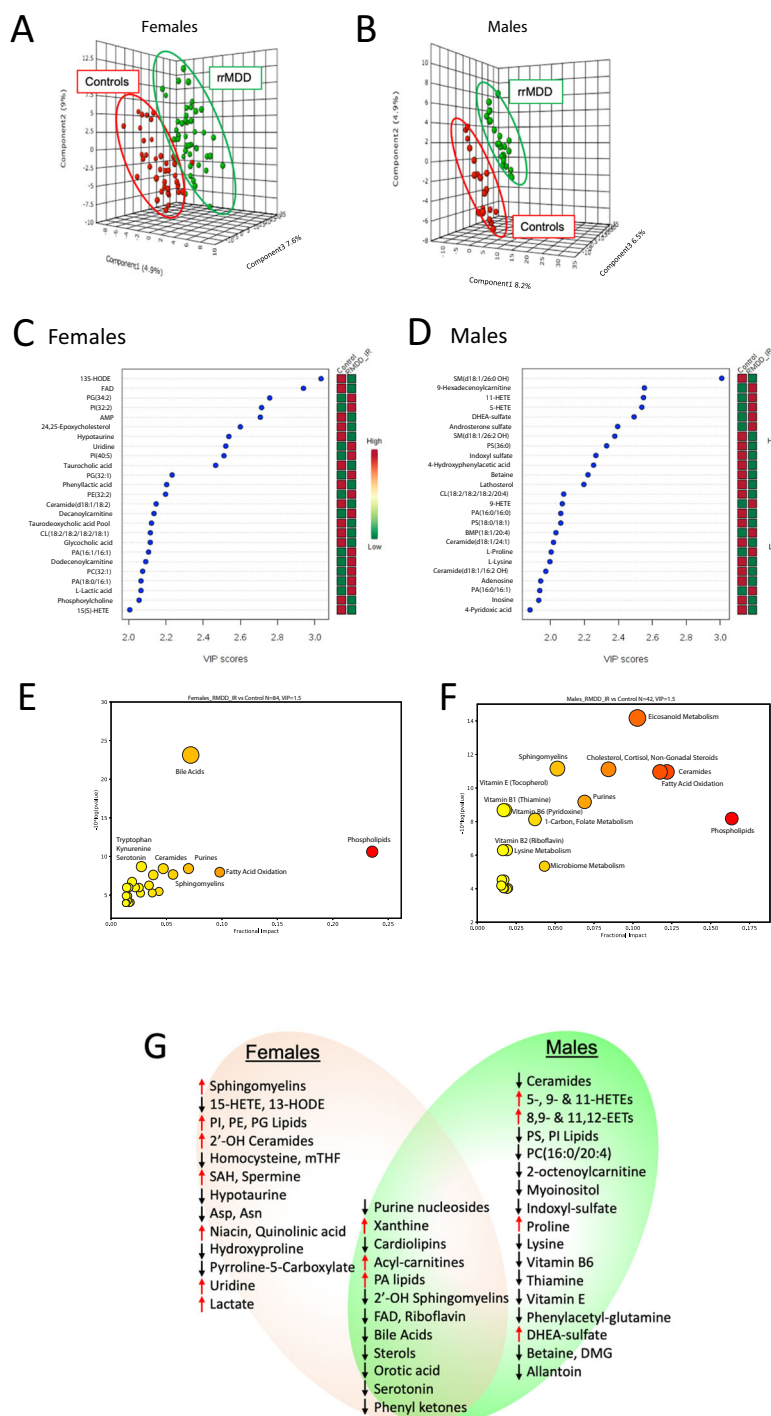
**Table 1 Participant characteristics.**

|   | Males                    |                             |         | Females                  |                             |         | rrMDD                     |  |
|---|--------------------------|-----------------------------|---------|--------------------------|-----------------------------|---------|---------------------------|--|
|   | rrMDD mean ± SEM (range) | CONTROLS mean ± SEM (range) | p value | rrMDD mean ± SEM (range) | CONTROLS mean ± SEM (range) | p value | Males vs. females p value |  |
| Subjects enrolled                                   | 23                       | 19                          |         | 45                       | 40                          | –       | –                         |  |
| Age   | 54 ± 1.4 (42–65)         | 56 ± 1.2 (46–64)            | 0.28    | 53 ± 1.2 (36–64)         | 51 ± 1.2 (36–65)            | 0.22    | 0.41                      |  |
| Metabolomic samples analyzed                        | 23                       | 19                          |         | 44                       | 40                          | –       | –                         |  |
| Subjects living alone                               | 9                        | 5                           | 0.51    | 19                       | 13                          | 0.38    | 0.99                      |  |
| Waist circumference (cm)                            | 101 ± 2.4 (86–127)       | 104 ± 3.8 (91–129)          | 0.56    | 92 ± 2.1 (67–124)        | 85 ± 2.6 (57–111)           | 0.05*   | 0.01*                     |  |
| HAM-D score   | 2.0 ± 0.4 (0–6)          | 0.7 ± 0.4 (0–4)             | 0.02*   | 3.0 ± 0.3 (0–9)          | 1.2 ± 0.3 (0–5)             | 0.0003* | 0.12                      |  |
| Age of 1st episode of MDD                           | 30 ± 2.1 (9–43)          | n/a                         |         | 26 ± 1.7 (9–49)          | n/a                         | –       | 0.15                      |  |
| Duration of rMDD (years)                            | 25 ± 2.4 (6–50)          | n/a                         |         | 27 ± 1.9 (3–55)          | n/a                         | –       | 0.39                      |  |
| Lifetime episodes of depression at entry            | 9.7 ± 2.5 (2–45)         | n/a                         |         | 8.1 ± 1.9 (2–60)         | n/a                         | –       | 0.63                      |  |
| Years since last depressive episode                 | 4.1 ± 0.8 (0.1–12)       | n/a                         |         | 5.1 ± 0.8 (0.1–21)       | n/a                         | –       | 0.49                      |  |
| Subjects completing 2.5 years of follow-up          | 20                       | 19                          | 0.23    | 42                       | 40                          | 0.50    | 0.33                      |  |
| Subjects suffering recurrence                       | 11                       | n/a                         |         | 24                       | n/a                         |         | 0.99                      |  |
| Median time to recurrence (QR, months) <sup>a</sup> | 5.5 (1.9–7.6)            | n/a                         |         | 10.1 (4.9–17.2)          | n/a                         |         | 0.02*                     |  |
| Subjects taking CNS medications <sup>b</sup>        | 1                        | 0                           | 0.99    | 3                        | 1                           | 0.62    | 0.99                      |  |
| Vitamins, supplements, and OTC medications          | 0.7 ± 0.19 (0–3)         | 0.6 ± 0.22 (0–2)            | 0.81    | 1.5 ± 0.22 (0–5)         | 0.9 ± 0.24 (0–5)            | 0.05*   | 0.03*                     |  |
| Non-CNS prescription medications                    | 1.4 ± 0.36 (0–6)         | 0.5 ± 0.23 (0–2)            | 0.12    | 1.1 ± 0.24 (0–7)         | 0.3 ± 0.1 (0–2)             | 0.03*   | 0.46                      |  |
| Years of education <sup>c</sup>                     | 16 ± 0.8 (9–20)          | 16 ± 1.2 (9–25)             | 0.90    | 14 ± 0.6 (9–20)          | 14 ± 0.6 (10–25)            | 0.22    | 0.04*                     |  |
| Ethnicity   |                          |                             |         |                          |                             |         |                           |  |
| Northern European                                   | 22                       | 19                          | 0.99    | 35                       | 34                          | 0.42    | 0.08                      |  |
| Non-N. European                                     | 1                        | 1                           | 0.99    | 10                       | 6                           | 0.42    | 0.08                      |  |

rMDD recurrent major depressive disorder in remission, HAM-D Hamilton depression rating scale, OTC over the counter, CNS central nervous system.

\*Significant p value ≤ 0.05.

<sup>a</sup>Data from N = 24 females and 11 males with recurrence in 2.5 years post-enrollment. Excludes data from N = 18 females and 9 males without recurrence. <sup>b</sup>No subjects were taking antidepressants (selective or non-selective serotonin or norepinephrine reuptake inhibitors, or tricyclics). The numbers indicate the 1–3 subjects taking low-dose benzodiazepines. These subjects stopped the medication for 1–2 days before the blood draw, depending on the half-life of the drug, then to restarted as indicated. <sup>c</sup>Years of education: US system, High School=12; AA = 14; Bachelor's=16; Master's=18; JD = 19; MD, DO, or ND = 20; PhD=21; MD-PhD=25; Dutch system by Verhage score: 1 = 5 years, 2 = 6 years, 3 = 8 years, 4 = 9 years, 5 = 10 years, 6 = 15 years, 7 = 20 years.



**Fig. 2 Metabolite and biochemical pathway abnormalities in recurrent major depressive disorder in remission.** Females: **A, C, E.** Males: **B, D, F.** **AB.** Multivariate metabolomic discrimination of remitted recurrent major depressive disorder (rrMDD) from controls by partial least squares discriminant analysis. **CD.** Rank order of top 25 discriminating metabolites by variable importance in projection (VIP) scores. **EF.** Bubble impact plot of pathway alterations. **G.** Venn diagram of shared and gender-specific metabolites diagnostic for rrMDD. Red arrows indicate an increased, and black arrows indicate a decreased concentration was associated with rrMDD risk. VIP scores  $\geq 1.5$  were significant. rrMDD subjects  $n = 44$  females, 23 males. Controls  $n = 40$  females, 19 males.

### Metabolic alterations shared by males and females

When analyzed at the pathway level, 16 pathways were shared by both males and females with rrMDD (Fig. 2E, F and Tables S3 and S4). All 8 lipid pathways found to be abnormal were shared by both males and females. The other 8 shared pathways involved diverse non-lipid pathways.

### Acyl-carnitines, cardiolipins, and vitamin B2

One of the most consistent lipid abnormalities found in both males and females was an increase in acyl-carnitines (Tables S3 and S4), which are a marker of decreased mitochondrial fatty acid oxidation<sup>30</sup>. In females, this was confined to increases in medium chain (C6–C10) acyl-carnitines, while in males, the long-chain (C12–C18), and very long-chain ( $\geq$ C20) acyl-carnitines were increased (Tables S5 and S6). Cardiolipins, markers for inner mitochondrial membrane complexity and biomass, were also decreased. Phosphatidic acids (PA-lipids), precursors for cardiolipin synthesis, were increased in both males and females (Fig. 2G). Vitamin B2-metabolism needed for mitochondrial fatty acid oxidation was decreased in both males and females. In males this was reflected in a decrease of plasma riboflavin. In females, this was associated with a decrease in plasma flavin adenine dinucleotide (FAD; Fig. 2G, Tables S5 and S6).

### Sphingolipids

Sphingolipids (ceramides, sphingomyelins, and glycosphingolipids) are major structural and signaling lipids that facilitate the exchange of materials between lysosomes and the plasma membrane to regulate cell growth and inflammation<sup>31,32</sup>, form membrane lipid rafts<sup>33</sup>, exosomes released from cells<sup>34</sup>, and are involved in synapses<sup>35–37</sup>. rrMDD males and females shared a specific abnormality in sphingolipid metabolism: a decrease in 2'-hydroxy sphingomyelin SM(d18:1/26:0 OH) (Fig. 2G). The 2'-hydroxylation (2'-OH) of the fatty acid precursor of the amide acyl chain of sphingolipids is catalyzed by the peroxisomal enzyme fatty acid 2'-hydroxylase (FA2H)<sup>38</sup>. 2'-hydroxy sphingomyelins are precursors for the 2'-OH glycosphingolipids needed for cell differentiation, neuronal connectivity, myelin stability, and have antitumor properties<sup>39</sup>.

### Eicosanoids and oxylipins

Both males and females had alterations in eicosanoid (20-carbon, polyunsaturated) lipids made from arachidonic acid<sup>40</sup>, but the specific metabolites and the direction of change differed. Females also showed a decrease in an 18-carbon oxylipin made from linoleic acid<sup>41</sup> called 13(S)-hydroxyoctadecadienoic acid (13-HODE), which was unchanged in males. 15(S)-hydroxyeicosatetraenoic acid (15(S)-HETE) was decreased in females, but not in males

(Fig. 2C and Tables S5 and S6). 13-HODE and 15-HETE are anti-inflammatory and pro-resolving oxylipins that also have antitumor effects<sup>42</sup>. In contrast, rrMDD-males had increases in 3 eicosanoids: 11-HETE, 9-HETE, and 5-HETE, which are pro-inflammatory mediators made by neutrophils, eosinophils, and mast cells<sup>43,44</sup>. Males also had an increase in the vasodilatory and anti-inflammatory epoxyeicosatrienoic acids 8,9-EET, and 11,12-EET (Fig. 2G). The large number of alterations in eicosanoid metabolism made this the most statistically significant pathway alteration in males (Fig. 2F).

### Sterols

Sterols are needed for the synthesis of cholesterol, glucocorticoid, and steroid hormones and bile acids. Sterol synthesis requires coordinated enzyme activity in the endoplasmic reticulum (ER) and mitochondria. Sterols were decreased in both male and female rrMDD subjects (Fig. 2G, Tables S3 and S4). In females, both 24,25-epoxycholesterol and cholesteryl-sulfate were decreased. In males, cholesterol precursors 24,25-dihydrolanosterol and lathosterol were decreased (Tables S5 and S6).

### Bile acids

Bile acid metabolism requires coordinated activities of enzymes located in mitochondria, peroxisomes, ER, and the gut microbiome<sup>45</sup>. Bile acids are signaling molecules that bind to several classes of nuclear receptors (FXR, PXR, and CAR), and permit real-time coordination between food intake, the microbiome, liver, and systemic detoxification systems<sup>46</sup>. Bile acids are made from cholesterol and represent the major disposal route for excess cholesterol. Both males and females had decreased plasma levels of bile acids. rrMDD-females had decreased levels of four glycine- and taurine-conjugated secondary bile acids including glycocholic and taurocholic acids (Tables S3–S6 and Fig. 2). Bile acid abnormalities were the most statistically significant single pathway alteration in females (Fig. 2E). rrMDD males had decreased levels of the secondary bile acid, deoxycholic acid, which is formed by dehydroxylation of cholic acid by normal gut bacteria.

### Purines

Purine nucleosides adenosine, guanosine, and inosine were decreased and xanthine was increased in both males and females with rrMDD (Fig. 2G and Tables S3 and S4). Plasma purine nucleosides are derived by dephosphorylation of purine nucleotides like ATP, ADP, AMP, GMP, and IMP. Xanthine is a purine nucleobase that has been shown to connect purine metabolism with the immune system, memory, and anxiety<sup>47</sup>. De novo synthesis, salvage, and metabolism of purine nucleotides depends on cooperative activities of extracellular, cell

membrane-associated, cytosolic, and mitochondrial enzymes.

### Metabolomics as a diagnostic tool

Area under the receiver operator curve (AUROC)-analysis was used to test the accuracy of metabolites to distinguish between rrMDD subjects and healthy controls (Fig. S3). The classifier for females used 12 metabolites, resulting in an AUC of 0.83 (95%CI = 0.68–0.96; sensitivity = 80%, 95%CI = 0.66–0.89; specificity = 87%, 95%CI = 0.74–0.95). For males, seven metabolites were used as classifier, resulting in an AUC of 0.83 (95%CI = 0.64–1.0; sensitivity = 74%, 95%CI = 0.53–0.87; specificity = 79%, 95%CI = 0.57–0.91).

### Metabolomics as a prognostic tool

#### *Predictors shared by males and females*

Median time to recurrence during the 2.5 years follow-up for all rrMDD subjects was 588 days for females and 291 days for males (Kaplan–Meier analyses; Fig. 3A, B). Multivariate analysis permitted the metabolomic signature of subjects who experienced recurrence to be distinguished from those who did not (Fig. 3C, D and Tables S7 and S8). The top discriminating metabolites are shown in Fig. 3E, F. Overall, most predictive metabolite classes for both genders were sphingomyelins and phospholipids (Fig. 3G, H). AUROC-analysis was used to test the prognostic accuracy of a female-specific classifier using 7 metabolites and a male-specific classifier that used 3 metabolites (Fig. 3I, J). The predictive accuracy for recurrence in females was 0.90 (95%CI = 0.69–1.0; sensitivity = 0.88; specificity = 0.89; Fig. 3I, K). Although different from female rrMDD subjects, the predictive accuracy for recurrence of depression in males was 0.99 (95% CI = 0.9–1.0; sensitivity = 0.91; specificity = 1.0; Fig. 3J, L).

Three metabolite classes predicted recurrence risk in both males and females by Cox proportional hazard analysis (Fig. 4 and Tables S7–S10). These were a decrease in 2'-hydroxy sphingomyelins (2'-OH SM), trihexosylceramides (THC), and phosphatidylcholine (PC) lipids. In both males and females, nearly 80% of the predictive metabolites identified had a negative correlation with recurrence risk (Tables S9 and S10). This means that when the blood level was low compared to the other rrMDD subjects, recurrence risk was higher, and conversely.

#### *Female-specific predictors of recurrence*

All of the strongest predictors of recurrence were gender specific. In females the strongest predictor was low methylcysteine (Fig. 3K, M and Table S9). Female rrMDD subjects in the bottom half of plasma methylcysteine were found to have a median time to recurrence of 1.1 years (403 ± 63 days, mean ± SEM). Women in the top 50th methylcysteine percentile were much slower to experience recurrence

(median = 2.3 years; 848 ± 58 days; Cox beta coefficient = -1.8;  $p < 0.00006$ ; Table S9 and Fig. 3M). Monohexosylceramide (MHC(d18:1/20:0)) was another example of a metabolite that protected against future recurrence (Fig. 3O and Table S9). Two metabolites that increased recurrence risk were lysophosphatidylcholine 16:0 (LysoPC(16:0)) and arachidonic acid (20:4). These two lipids can be produced from the same parental phosphatidylcholine lipid, PC(16:0/20:4) through the action of lipoprotein associated phospholipase A2 (LP-PLA<sub>2</sub>) and other PLA<sub>2</sub>-types under conditions of stress<sup>48</sup>. Metabolites found to increase and decrease recurrence risk are listed in Table S9. Of the 399 measured metabolites, 33 were significant predictors of recurrence risk in females, and in 82% of these metabolites (27/33) higher levels decreased recurrence risk (Table S7 and S9). Correcting for number of previous episodes and residual symptoms did not change hazard-ratios of these metabolites.

#### *Male-specific predictors of recurrence*

Of the 399 measured metabolites, 17 were significant predictors of recurrence risk in males (Table S8 and S10). The eicosanoid lipid, 15-hydroxyeicosatetraenoic acid (15-HETE) was the top predictor of recurrence in males. When males were stratified into top and bottom 50th percentiles, rrMDD subjects in the bottom half for this plasma 15-HETE were found to have a median time to recurrence of 0.7 years (245 ± 85 days). Males in the top 50th percentile for this anti-inflammatory eicosanoid were much slower to experience recurrence (median = 1.7 years; 614 ± 108 days; Fig. 3N; Cox beta coefficient = -1.5;  $p < 0.0006$ ; Table S10). Beta carotene was also protective. Males in the lowest 50th percentile for plasma beta-carotene had a median time to recurrence of 0.5 years (175 ± 103 days), while the upper 50th percentile had a median time to recurrence of 1.9 years (677 ± 104 days; Fig. 3N and P; Table S8 and S10). Increased alanine and allantoin were associated with an increased risk of recurrence in males. Increased alanine is produced as a transamination product of pyruvate and is a marker of mitochondrial dysfunction<sup>49</sup>. Allantoin is produced non-enzymatically from uric acid by exposure to reactive oxygen species (ROS) and is a marker of oxidative shielding<sup>50</sup> and stress<sup>51</sup>. Increased levels of 14 of 18 (78%) predictors in males decreased recurrence risk. Figures S4 and S5 illustrate the Kaplan–Meier style recurrence profiles of the top predictive metabolites determined by Cox proportional hazard analysis in females and males, respectively. Correcting for number of previous episodes and residual symptoms did not change the hazard-ratios of these metabolites, except for beta-carotene, SM(d18:1/20:2 OH), PS(18:0/18:1), PE(34:1), or SM(d18:1/20:1), where correction for especially previous lifetime episodes of depression increased the hazard-ratios.

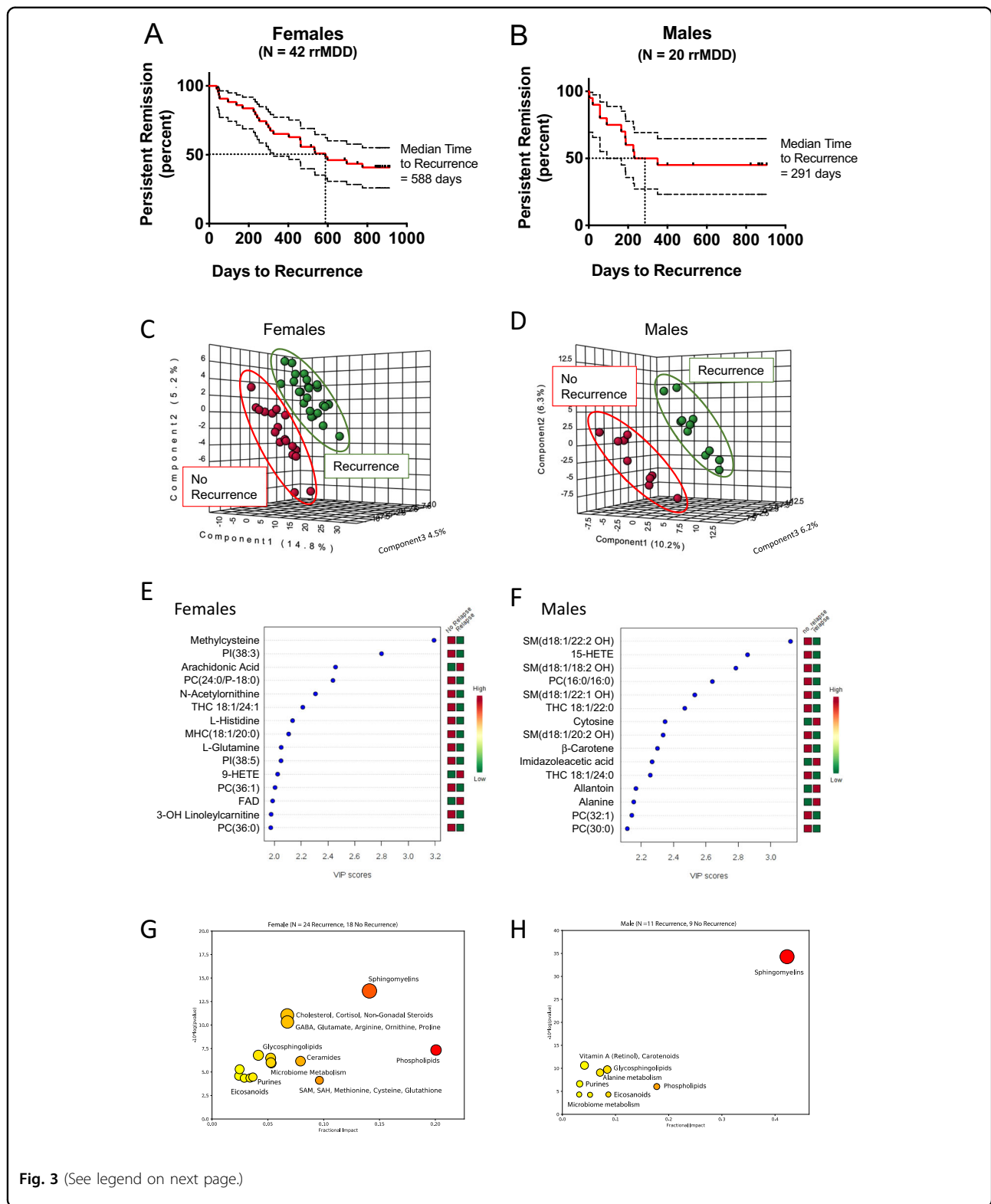


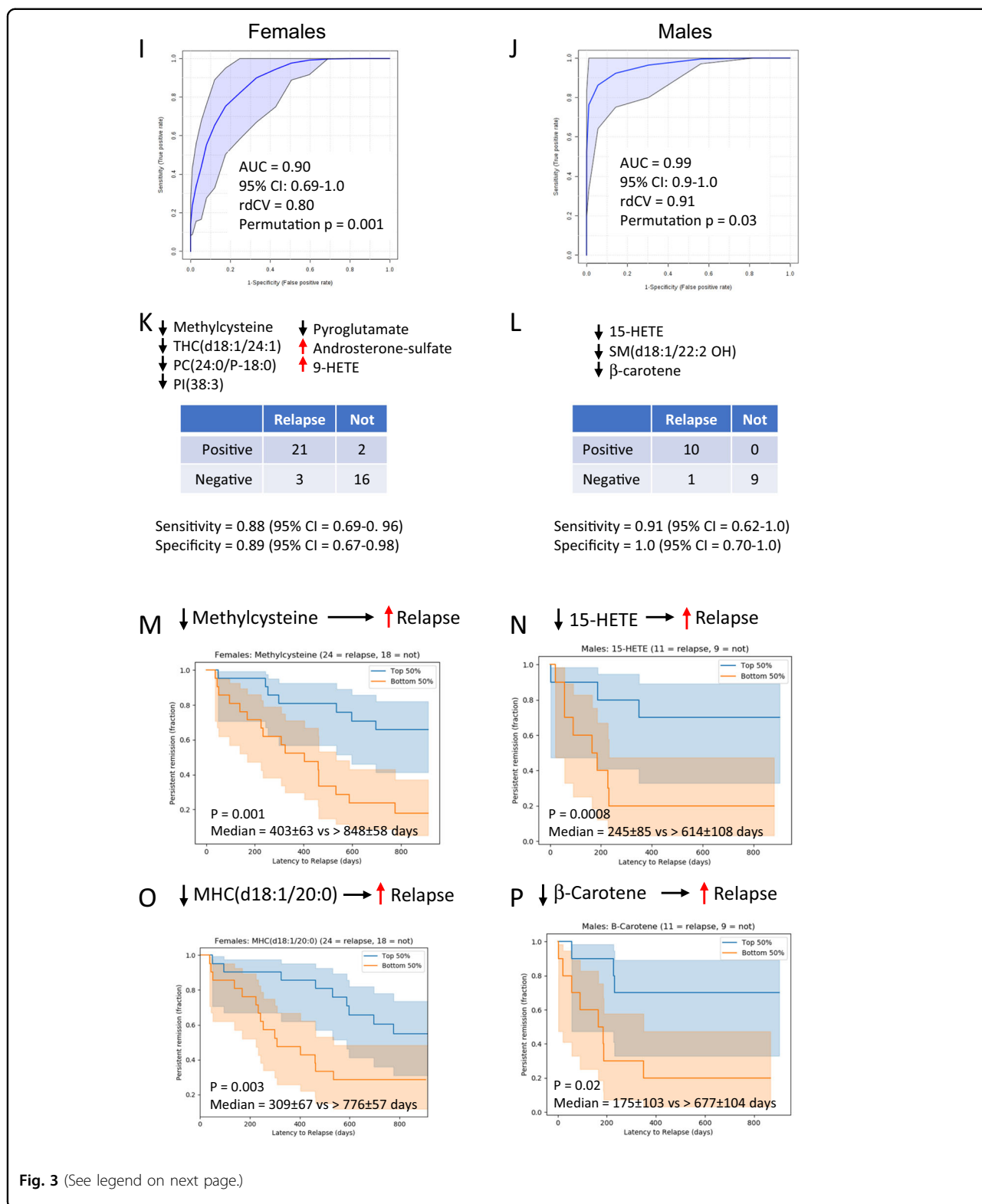
Fig. 3 (See legend on next page.)

**Sample size calculation for validation studies**

Three methods were used to estimate the sample size needed in future studies to validate the results of this

pilot study (see Supplemental Methods). The median Pearson  $r$  for the metabolites in females having VIP scores  $\geq 1.5$  was  $r = 0.2$ . The median Z-score difference



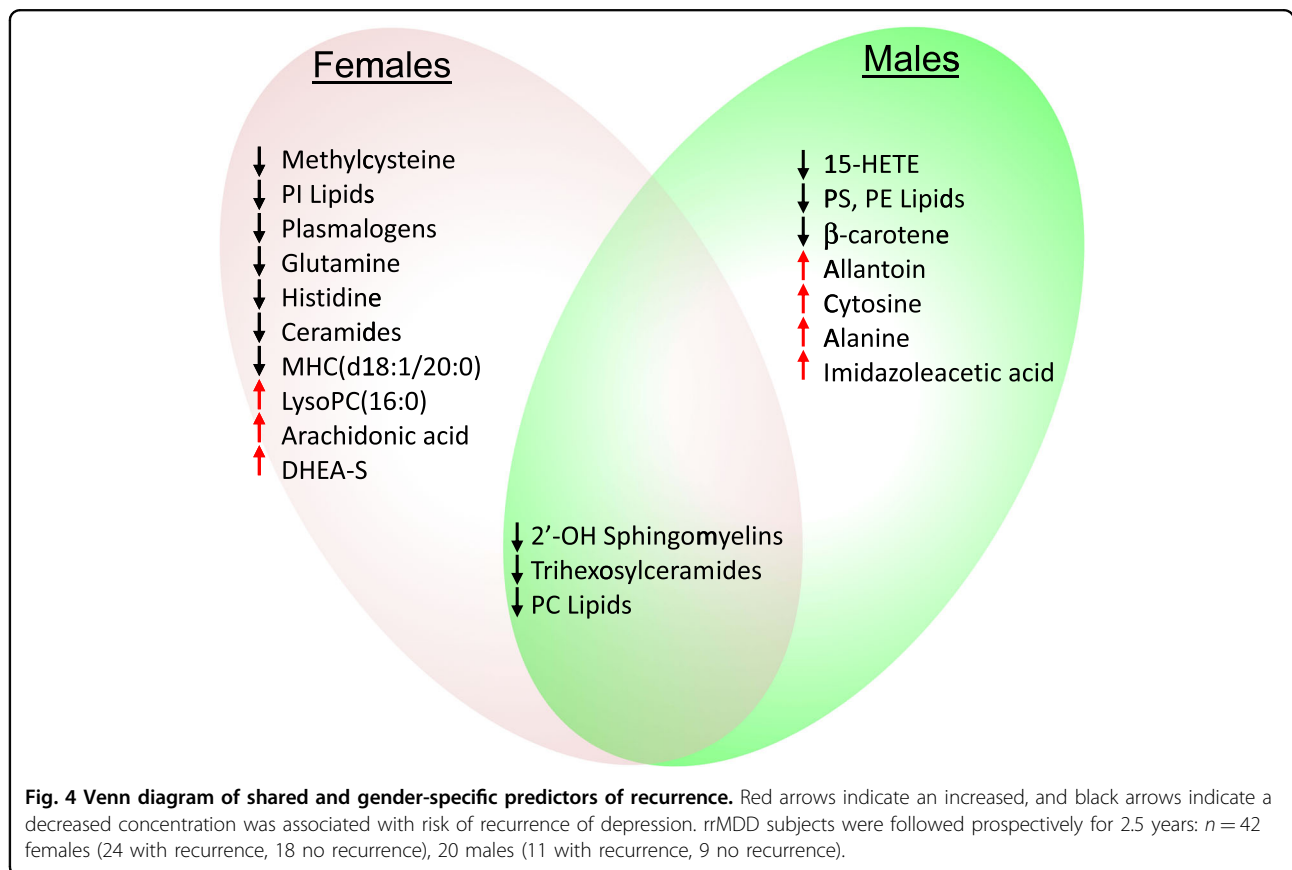


was 0.39 (Tables S5, S11–S14). Using correlation analysis and a requirement for a Pearson  $r \geq 0.2$ , the total number of subjects of a single sex (cases plus controls)

was 194. Using multiple regression analysis and a threshold of at least 35 significant metabolites and a Cohen’s  $f^2 = 0.15$ , the study size was 201. Using a

(see figure on previous page)

**Fig. 3 Metabolomic predictors of recurrence in remitted recurrent major depressive disorder (rrMDD).** **A** Kaplan–Meier analysis of latency to recurrence in subjects with remitted recurrent major depressive disorder (rrMDD), **Females**, **B** Males. Dotted boundaries indicate the 95% confidence intervals. Metabolic predictors of recurrence in recurrent major depressive disorder. **Females**: **C, E, G**. **Males**: **D, F, H**. **CD** Multivariate metabolomic discrimination of subjects with rMDD who experienced recurrence in the next 2.5 years, and those who did not, analyzed by partial least squares discriminant analysis. **EF** Rank order of top 15 discriminating metabolites by variable importance in projection (VIP) scores. **GH** Bubble impact plot of pathway alterations. Receiver operator characteristic (ROC) curve analysis of multianalyte diagnostic classifiers for rrMDD. **I**. The classifier for females used 7 metabolites. **J** The classifier for males used three metabolites. AUC area under the curve, rdCV repeated double cross validation accuracy. **KL** 2 × 2 contingency table analysis. Cox proportional hazard analysis of selected metabolites. **M** Decreased methylcysteine predicted a higher risk of recurrence in females. **N** Decreased 15-hydroxyeicosatetraenoic acid (15-HETE) predicted a higher risk of recurrence in males. **O** Decreased monohexosyl ceramide (MHC(d18:1/20:0)) predicted a higher risk of recurrence in females. **P** Decreased β-carotene predicted a higher risk of recurrence in males. rrMDD subjects were followed prospectively for 2.5 years: *n* = 42 females (24 with recurrence, 18 no recurrence), 20 males (11 with recurrence, 9 no recurrence).



Z-score threshold of  $\geq 0.4$  (significant metabolites in cases must differ from controls by at least 0.4 standard deviations), the study size was 198. The mean estimate was  $198 \pm 3.5$  (mean  $\pm$  sd). These results showed that a validation-scale study of at least 198 females and 198 males (99 cases and 99 controls each) will be needed to confirm the results.

See Supplementary Results for additional results.

## Discussion

Metabolomic analysis revealed an underlying biochemical signature in remitted recurrent major

depressive disorder (rrMDD) that distinguished patients from healthy controls. This difference was psychometrically inapparent as patients were studied during antidepressant-free remission. Patterns of metabolic abnormalities that we found reflected alterations in chemical communication across lipid membranes and between organelles, cells, organ systems, and the microbiome. Lipid abnormalities constituted 60–70% of the total metabolic impact. Even more striking was the finding that metabolomic analysis was able to unmask a latent signature of future risk of recurrence with 90–99% accuracy. If replicated, this finding could have a

significant impact on clinical practice by permitting patients with major depressive disorder to be stratified according to future risk, and new preventive treatments to be tested systematically in clinical trials. To the best of our knowledge, this is the first report to show that broad-spectrum targeted metabolomics can predict future recurrence risk.

Interestingly, the top metabolites that distinguished rrMDD cross-sectionally from controls were not the same biomarkers that predicted future recurrence risk. Reciprocally, the top predictors of future risk were not the same biomarkers that distinguished patients with recurrent MDD in drug-free remission cross-sectionally from healthy controls. A similar pattern of metabolites being used differently in health than in disease has been observed previously for other biological markers. For example, a study showed that cortisol<sup>52</sup> was increased in rrMDD subjects compared to controls, while decreased cortisol predicted recurrence in the same study. In this example, higher levels of cortisol actually protected against future recurrence in patients at risk. Altogether, these differences between diagnostic and prognostic biomarkers makes it unlikely that these observations merely represent epiphenomena or consequences of earlier MDD episodes.

### Measuring latent risk

These data suggest that once a new disease state such as MDD has been entered, asymptomatic remission comes with a latent (hidden) future risk that can be objectively measured, permitting patients to be stratified. In this new state of latent risk, biochemical and endocrine pathways are used differently to prevent disease progression and recurrence than in healthy control subjects. These results suggest that natural recovery from disease is not the simple reversal of the sequence of pathogenic events that led to disease. Metabolomic analysis unmasked new interaction patterns between metabolites that changed the future risk in patients in a disease state, but that had either no utility, or even an opposite effect, in predicting the future risk of developing that disease state in healthy controls<sup>53</sup>. Rephrasing this, the clinical context of the patient in health or disease determines the meaning of the data, and the pathophysiologic sequence of events that produced the original disease is different than the path that is used to minimize future complications, or to recover and heal from that disease<sup>18</sup>.

### Mechanistic implications

Membrane properties are determined by lipids and transporters that conduct material across those membranes. Changes in membrane-dependent cell-to-cell and interorganellar communication provide a rationale for why over half of all the prognostic metabolites for

recurrence risk in rrMDD were lipids, and why those that were not lipids, like purines, are potent regulators of membrane and transporter properties. We found that several nucleosides of purines such as inosine and guanosine were decreased while certain nucleobases like xanthine were increased. This has been reported in independent studies of MDD<sup>54</sup> and confirms an important role of purines and purinergic signaling in regulating affective and several other neuropsychiatric and neurodegenerative disorders<sup>55,56</sup>. Metabolic abnormalities found in rrMDD support the notion that interorganellar and intercellular exchange and the transformation of metabolites across membranes—the connectivity and communication between organelles, cells, and organ systems—is altered. Impaired communication across membranes, as measured by plasma metabolomics, permits the pathogenesis of major depressive disorder to be reframed as a neurometabolic disorder<sup>8,57,58</sup>. When reframed under this new paradigm as a neurometabolic disorder, novel approaches to treatment become apparent that may never have been considered under the old paradigm of MDD as an isolated disorder of brain function independent of whole-body changes in metabolism.

### The mitochondrial nexus

Mitochondria have been shown to coordinate many of the metabolic features of stress that can play a causal role in mental health disorders<sup>27,59,60</sup>. Mitochondria are the hub of the wheel of cellular metabolism. This nexus point is a crossroads for many neurodevelopmental and psychiatric disorders<sup>61</sup>. Mitochondria naturally respond to chemical and physical environmental changes, making their membrane structures of fundamental interest<sup>62</sup>. Although typically thought of as an energy factory, mitochondria catalyze over 700 biochemical reactions<sup>63</sup> needed to produce building blocks for cell growth, repair, and signaling. Mitochondria can also shift dynamically between three major modes of function, or functional types designated M1-, M0-, and M2-organelles, according to cellular needs<sup>18</sup>. Cells in which M1- and M0-mitochondria predominate are pro-inflammatory and dependent on glycolytic metabolism for energy. M1- and M0-cells release more lactic acid in order to maintain intracellular redox. Lactic acid elevation was found in females with a history of rMDD in this study (Fig. 2G), and is a known feature of symptomatic depression that decreases with treatment<sup>64,65</sup>. Moreover, both males and females had several other markers of decreased mitochondrial function and/or biomass. These included decreased purines like adenosine, inosine, and guanosine, and decreases in the pyrimidine precursor orotic acid, and decreased cardiolipins, which are markers of inner mitochondrial membrane surface area and biomass. Mitochondria specialized for oxidative phosphorylation are designated M2-organelles, needed for burning fatty acids

for energy to make ATP. When M2-mitochondrial function is impaired, several fatty acyl-carnitines increase in the blood<sup>30</sup>. Acyl-carnitines were increased in both males and females with antidepressant-free rrMDD, and the major vitamins needed for fatty acid oxidation (riboflavin and FAD) were decreased. Disturbances in mitochondrial function leading to acyl-carnitine abnormalities have been observed in several other independent studies of MDD<sup>66</sup>. These findings support the hypothesis that even in remitted rrMDD a shift in mitochondrial function exists and persists. This change is functionally dynamic, shifting mitochondria away from the M2-polarized oxidative phosphorylation phenotype, to the M1 and M0 phenotypes associated with inflammation and proliferation, respectively<sup>18,63</sup>.

### Study limitations

A limitation of this study was the relatively smaller number of rrMDD subjects enrolled. This was a greater problem for males than for females. The smaller sample size for males captured a smaller proportion of the natural phenotypic variation, making the results vulnerable to overfitting. The small sample size influenced the calculated false discovery rates (FDRs). FDRs for the significant metabolites in females ranged from 0.6 to 0.96. FDRs for the 54 discriminating metabolites in males ranged from 0.82 to 0.91. As expected for a small-scale pilot study, these FDRs were higher than would be desired for a validation study. To further test the statistical validity of these results we used repeated double cross validation (rdCV) analysis. rdCV analysis showed modest values of 0.74–0.76 for males and females in remission, while a similar analysis of the prognostic classifiers for high and low risk of future recurrence of MDD symptoms reached rdCV values of 0.91 and 0.80 for males and females, respectively. Despite these high values for the in-sample validation statistics, the generalizability of these findings is not currently known, and will require independent testing in future cohorts. Larger studies will be needed for validation, and verification of the sensitivity and specificity of our results. Moreover, we did not include first-episode MDD-patients. Although this allowed focus on recurrent patients with a high recurrence risk, it precluded comparative analyses with patients with a relatively low risk. Future studies applying comprehensive metabolomics in untreated first-episode MDD may further elucidate the role of different metabolites in the prognosis of MDD.

See Supplementary Discussion for additional discussion

### Conclusions

Targeted broad-spectrum LC-MS/MS-metabolomics of 399 plasma metabolites was used to study recurrent major depressive disorder in remission (rrMDD), and the time to recurrence, in 126 cases and controls. Lipid abnormalities dominated rrMDD's metabolic signature. The most powerful

statistical inferences came from the 2.5-year prospective study of subjects at risk for recurrence. Stratification of rrMDD subjects using metabolomics was able to predict recurrence risk with >90% accuracy in both males and females. Nearly 80% of the metabolites with greatest predictive accuracy for recurrence risk belonged to just six pathways: phospholipids, sphingomyelins, glycosphingolipids, eicosanoids, microbiome, and purines. Abnormalities in these pathways support the emerging conceptual framework that mitochondria act as a metabolic nexus<sup>61</sup>—as sensors and regulators of cell function that respond to environmental threat, stress, or injury<sup>18,27,62</sup>. After correcting for residual symptoms and lifetime MDD-episodes, metabolomic analysis was found to add new information that was not statistically correlated with any other neuropsychiatric parameter measured during remission and was highly significant in prospectively identifying the rrMDD patients at greatest risk of recurrence. New clinical trials designed to restore normal lipid metabolism<sup>67</sup>, mitochondrial health<sup>68</sup>, metabokine signaling<sup>18,22</sup>, and microbiome health<sup>69,70</sup> through dietary, supplement, psychological stress reduction, and medical interventions will be required to confirm these results.

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### Author details

<sup>1</sup>Department of Psychiatry, Amsterdam UMC, Academic Medical Center, University of Amsterdam, Meibergdreef 5, 1105 AZ Amsterdam, The Netherlands. <sup>2</sup>The Mitochondrial and Metabolic Disease Center, University of California, San Diego School of Medicine, 214 Dickinson St., Bldg CTF, Rm C107, San Diego, CA 92103-8467, USA. <sup>3</sup>Department of Neurosciences, University of California, San Diego School of Medicine, 214 Dickinson St., Bldg CTF, Rm C107, San Diego, CA 92103-8467, USA. <sup>4</sup>Department of Medicine, University of California, San Diego School of Medicine, 214 Dickinson St., Bldg CTF, Rm C107, San Diego, CA 92103-8467, USA. <sup>5</sup>Department of Psychiatry, Radboud University Medical Center, Nijmegen, the Netherlands. <sup>6</sup>Donders Institute for Brain, Cognition and Behavior, Radboud University Nijmegen, Nijmegen, the Netherlands. <sup>7</sup>Department of Pediatrics, University of California, San Diego School of Medicine, 214 Dickinson St., Bldg CTF, Rm C107, San Diego, CA 92103-8467, USA. <sup>8</sup>Department of Pathology, University of California, San Diego School of Medicine, 214 Dickinson St., Bldg CTF, Rm C107, San Diego, CA 92103-8467, USA. <sup>9</sup>Colt Neck Labs, 838 E High St 202., Lexington, KY 40503, USA. <sup>10</sup>Present address: School of Social Welfare, University of California, Berkeley, CA 94720, USA

### Author contributions

R.K.N., K.L., J.C.N., L.W., A.T.B., and J.M.M. developed the metabolomics methods, performed the metabolomic analysis, analyzed the data, and wrote and edited the manuscript. R.J.T.M. and C.A.F. enrolled the subjects and collected the samples. R.J.T.M., C.A.F., and H.G.R. coordinated the sample and clinical data collection and analyzed the neuropsychiatric data. J.A. directed the study,

designed the metabolic analyses, and analyzed the data. R.J.T.M., J.A., and R.K.N. wrote the initial version of the manuscript and edited the manuscript. H.G.R., R. J.T.M., and C.A.F. designed and wrote the human subjects protocol. H.G.R. and A.H.S. directed the study and edited the manuscript.

#### Data availability

Raw AUC data from the LC-MS/MS analysis and recurrence data are provided in Tables S1 and S2.

#### Conflict of interest

The authors declare that they have no conflict of interest.

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## Supplementary Information

*Translational Psychiatry*

10 December 2020

# Metabolic Features of Recurrent Major Depressive Disorder in Remission, and the Risk of Future Relapse

Roel J. T. Mocking; Jane C. Naviaux; Kefeng Li; Lin Wang; Jonathan Monk; A. Taylor Bright; Caroline A. Figueroa; Aart H. Schene; Henricus G. Ruhé; Johanna Assies; Robert K. Naviaux

## SUPPLEMENTARY METHODS

### Study Enrollment

Participants were enrolled between December 1, 2011 and November 20, 2014. Plasma samples were analyzed in September 2015.

### Sample Collection

Venous blood was collected at baseline between the hours of 8 am and 1 pm, after an overnight fast, into lithium-heparin vacutainer tubes. Plasma was separated by centrifugation at 1500g for 10 minutes at room temperature within one hour of collection, aliquoted into 0.5 ml samples and stored at -80°C in cryotubes until analysis.

### Statistical Analysis

Demographic data were analyzed by t-tests or non-parametric Mann-Whitney U tests. Categorical data was analyzed by Fisher's exact test. Peak area under the curve (AUC) data from metabolomics were log<sub>2</sub> transformed, scaled by control standard deviations, and the resulting z-scores (Tables S11-S14) analyzed by multivariate partial least squares discriminant analysis (PLSDA) in MetaboAnalyst<sup>1,2</sup>. Post hoc correction for multiple hypothesis testing was done by the false discovery rate (FDR)

method of Benjamini and Hochberg<sup>3</sup>. Bayesian false discovery rates were estimated using the Storey q value<sup>4</sup>. Metabolites with PLSDA variable importance in projection (VIP) scores  $\geq 1.5$  were considered significant. Significant metabolites were grouped into pathways and their VIP scores summed to determine the rank-ordered significance of each biochemical pathway. Random forest analysis<sup>5</sup> was used to rank metabolites for their ability to distinguish rrMDD cases and controls using mean decrease in accuracy (MDA) scores. *k*-nearest neighbor (*k*-NN) clustering was used to identify metabolite groups that contributed in different ways to the discrimination of rrMDD and controls. Time to recurrence was evaluated by Kaplan-Meier analysis. Models for future risk stratification were constructed using Cox proportional hazard methods<sup>6</sup>. Significant prognostic metabolites were identified by Cox analysis with *p* values  $< 0.05$ , were aggregated into biochemical pathways, and ranked according to the sum of the absolute value of the Cox  $\beta$  coefficients as a measure of predictive impact. Hazard ratios were corrected for residual symptoms using the HAM-D scores and the natural log of the lifetime episodes of depression as previously described<sup>7</sup>. Classifiers of 5-15 metabolites were selected and tested for diagnostic accuracy using area under the receiver operator characteristic (AUROC) curve and random forest analysis. Confidence intervals for the ROC curves were calculated by bootstrap resampling. Sample size calculations for future validation studies were performed using three methods: 1) Correlation analysis; two-sided  $\alpha = 0.05$ ,  $\beta = 0.2$  (power = 0.8), and a Pearson  $r \geq 0.2$  to calculate the number of cases and controls needed ( $n = 194$ ; <https://sample-size.net/all-calculators-on-this-site/>), 2) Multiple regression analysis using two-sided  $\alpha = 0.05$ , power = 0.8, and F-test Cohen's  $f^2 = 0.15$ , to detect at least 35 significant metabolites ( $n = 201$ ; <https://www.danielsoper.com/statcalc/calculator.aspx?id=1>), and 3) Z-score analysis; two-sided  $\alpha = 0.05$ ,  $\beta = 0.2$ , and a metabolite z-score difference of  $\geq 0.4$  ( $sd = 1$ ;  $n = 198$ ; <https://sample-size.net/sample-size-means/>). Classifiers were validated within sample using repeated double cross validation (rdCV)<sup>8</sup>, with bootstrapping 100 times to test random subsamples of 2/3 in and 1/3 out, and by permutation analysis<sup>9</sup>. Results were organized into biochemical pathways and visualized in Cytoscape version 3.4.0. Principal components analysis (PCA) and scree plots were used to quantify the fractional contribution of metabolomics to phenotypic variance. Statistical methods were



implemented in Stata (Stata/SE12.1, StataCorp, College Station, TX), Prism (Prism 6, GraphPad Software, La Jolla, CA), Python, or R.

## **SUPPLEMENTARY RESULTS**

### **Gender-Specific Differences**

Many of the strongest metabolic changes found in rrMDD were gender-specific (Figure 2G, S3, and S4). Gender-specific differences in the metabolic response in major depressive disorder have been documented in independent studies<sup>10</sup>. Ten of these are grouped by pathway and discussed below.

#### *Phospholipids*

Although 15 of the same biochemical pathways were disturbed in both males and females, the direction of change was sometimes different. All the major phospholipid classes found to be abnormal in females (phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylcholine (PC), and phosphatidylethanolamine (PE)) were increased, except for cardiolipin which was decreased. Myoinositol, a precursor for PI lipids was not significantly decreased in females. The only phospholipid intermediate that was decreased in females was phosphorylcholine (Table S3 and S5). In contrast, PC(16:0/20:4), PS, and PI lipids, and myoinositol were decreased in males (Table S4 and S6).

#### *Non-gonadal steroids*

The sulfated forms of the non-gonadal, androgenic steroids androsterone-sulfate and dehydroepiandrosterone-sulfate (DHEA-S) were both increased in males with rrMDD, but not in females (Table S3-S6).

#### *Eicosanoids and Oxylipins*

Eicosanoids are a major class of pro-inflammatory and anti-inflammatory lipids synthesized from 20-carbon, polyunsaturated fatty acids, including the omega-6 fatty acid, arachidonic acid (C20:4), and 20-carbon omega-3 and omega-9 fatty acids. 13(S)-hydroxyoctadecadienoic acid (13-HODE) was decreased in females, but not changed in males. Similarly, 15-hydroxyeicosatetraenoic acid (15-HETE) was decreased in females, but not in males (Figure 2C-G, Table S3, S4). 13-HODE and 15-HETE are anti-inflammatory and pro-resolving oxylipins that also have antitumor effects <sup>11</sup>. In contrast, males with rrMDD had increases in 3 eicosanoids: 11-HETE, 9-HETE, and 5-HETE, which are proinflammatory mediators made by neutrophils, eosinophils, and mast cells <sup>12,13</sup>. Males also had an increase in the vasodilatory and anti-inflammatory epoxyeicosatrienoic acids 8,9-EET, and 11,12-EET, but females did not (Figure 2CD and G). The large number of disturbances in eicosanoid metabolism made this the most statistically significant single pathway disturbance in males (Figure 2F).

#### *Acyl-carnitines and lactate*

Associated with the decreased mitochondrial medium chain (C4-C10) fatty acid oxidation capacity in females was an increase in medium chain acyl-carnitines and L-carnitine. There was also an increase in lactate, reflecting an increase in glycolytic ATP production. While males had a defect in mitochondrial long-chain (C12-C18) fatty acid oxidation and an associated increase in long-chain acyl-carnitines. Lactate was not significantly increased in males ( $p = 0.22$ ; Figure 2D, Table S4). A C8 medium chain, monounsaturated acyl-carnitine (2-octenoylcarnitine) known to be associated with obesity and risk of diabetes <sup>14</sup> was decreased in males but not in females with rrMDD (Figure 2G).

#### *Sphingolipids*

Sphingolipid metabolism, including ceramides and sphingomyelins, was disturbed in both males and females with a history of recurrent major depressive disorder. However, in females, the non-2'-hydroxylated sphingomyelins were increased, while the 2'-hydroxy sphingomyelins were decreased

(Table S3 and S5). In males, only the 2'-hydroxysphingomyelins were decreased and other sphingomyelins were unchanged (Figure 2G, Table S4 and S6).

#### *Folate, 1-Carbon, methylation, transsulfuration, and polyamine metabolism*

Although males and females with rrMDD shared disturbances in folate-1-carbon metabolism, the metabolites used were different. Females had decreases in 5-methyltetrahydrofolic acid (mTHF), homocysteine, and hypotaurine, with an increase in the methylation inhibitor S-adenosylhomocysteine (SAH). The polyamine spermine was also increased in females but not males (Figure 2G, Table S3-S6). Spermine synthesis depends on the decarboxylation of S-adenosylmethionine (SAM) to dcSAM and its use as a 3-carbon aminopropyl donor in polyamine synthesis.

#### *Tryptophan and indole metabolism*

In addition to a decrease in plasma serotonin, females with rrMDD also had an increase in the pro-inflammatory and neurotoxic metabolite of tryptophan, quinolinic acid and its downstream product, the vitamin B3 redox cofactor, niacinamide (Figure 2G, Tables S3-S6). Males did not. Indoxyl-sulfate, a metabolite regulated by both microbiome and liver metabolism, was decreased in males but not in females (Figure 2G).

#### *Asparagine and aspartate metabolism*

Both asparagine, and its biosynthetic precursor aspartate, were decreased in females with rrMDD, but were unchanged in males (Figure 2G, Tables S3-S6).

#### *Collagen, hydroxyproline, lysine, proline, and pyrroline-5-carboxylate*

Hydroxyproline is a marker of collagen turnover and vitamin C metabolism and was decreased in females with rrMDD, but unchanged in males (Figure 2G, Tables S3 and S4). In males, two other amino acids that are enriched in collagen, proline and lysine, were dysregulated in opposing directions. Lysine was decreased and proline was increased in males but were unchanged in females.

In females, the stress-associated oxidation product of proline, pyrroline-5-carboxylate (P5C), was decreased. P5C was not changed in males (Figure 2G, Tables S3-S6).

#### *Vitamin metabolism (B1, B6, E)*

Vitamins B1 (thiamine), a vitamin B6 metabolite (4-pyridoxic acid), and the trimethylated  $\alpha$ -tocopherol, a readily absorbed form of vitamin E, were decreased in males, but were unchanged in females with rrMDD (Figure 2G, Tables S3-S6).

### **Metabolic Alterations Shared by Males and Females**

#### *Pyrimidines*

Orotic acid was decreased in both males and females with rrMDD (Figure 2G). Orotic acid is the product of the 4<sup>th</sup> step of *de novo* pyrimidine synthesis catalyzed by the mitochondrial enzyme dihydroorotate dehydrogenase (DHOD).

#### *Serotonin and tryptophan metabolism*

Serotonin was decreased in both males and females, but its precursor tryptophan, was unchanged in this cohort of drug-free rrMDD subjects (Figure 2G).

#### *Phenylalanine and tyrosine metabolism*

Phenylketones is the name given to molecules made largely by gut bacteria from phenylalanine and tyrosine left over after, or diverted from, catecholamine neurotransmitter and protein synthesis.

Phenyllactic acid is a phenylketone that was decreased in females. Hydroxyphenylacetic acid is a phenylketone that was decreased in males with rrMDD (Figure 2G, Table S3, S4).

## **SUPPLEMENTARY DISCUSSION**

### **Study Limitations**

A small but significant difference was found in some participant characteristics among the female subjects (Table 1). These included a small increase in waist circumference and the number of over-the-counter (OTC) supplements, vitamins, and non-CNS prescription medications. We chose not to correct for these differences statistically for two reasons. First, in the case of waist circumference, we and others have found that this and other measures of obesity such as body mass index (BMI) are intrinsically linked to disturbances in mitochondrial function leading to oxidative stress<sup>15</sup>, and as such is not a confounder of the metabolomic signature, but a causal factor or mediator. Statistical efforts to correct for weight can inadvertently over-correct for a mediator of the metabolic phenotype and lead to an increase in type II error. Second, in the case of non-CNS prescription medications, no more than 1-4 patients (2%-10%) were taking any particular drug. As only a minority of subjects was found to take any particular medication, we felt the chances of introducing an error by inadvertent over-correction or over-simplification by grouping different types of drugs by category, were greater than the potential benefit of correcting for a factor that was present in only a minority of the subjects. Larger studies will be required to sort out the effects of non-CNS medications on the metabolomic features of recurrent major depressive disorder.

### **Mechanistic Implications**

Lipids dominated both the diagnostic and prognostic metabolic markers found in rrMDD. Purine abnormalities were also a consistent feature in both diagnostic and prognostic markers. The importance of lipids and purines in major depressive disorder<sup>16</sup>, anxiety<sup>17</sup>, autism spectrum disorder<sup>18</sup>, and other mental health disorders<sup>19,20</sup> has been underscored by several recent studies.

The prognostic metabolites that were found to regulate the risk of recurrence were united in serving dual functions in the cell: 1. as matter, these molecules function as building blocks and intermediates in metabolism; 2. as information, they can act to modify macromolecular targets and change their function by phosphorylation, methylation, acetylation, myristoylation, farnesylation, etc. In addition, these metabolites act as ligands for transmembrane G-protein coupled or nuclear receptors that

change gene expression, or act as allosteric regulators that change the conductance of solute carriers (SLCs) and ATP-binding cassette (ABC) transporters that conduct metabolites as organic anions and cations, and ion channels that conduct inorganic cations like  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$ , and  $\text{Ca}^{2+}$ , and anions like  $\text{Cl}^-$ . In the case of purines, a third function is also well known: the role of molecules like ATP as energy. Metabolites that have both metabolic and signaling functions have been called “metabokines”. All neurotransmitters currently targeted by drug therapies for major depressive disorder are metabokines<sup>21</sup>, and purines are purinergic metabokines that act as ligands for ionotropic and metabotropic receptors<sup>22,23</sup>. A large majority of metabokines have been found to be coordinately regulated in response to cellular injury or threat. The coordinated regulation of metabokines has been placed in evolutionary context as the biological process that underlies healing and aging and called the cell danger response (CDR)<sup>21,24,25</sup>. The molecular aspects of the CDR include the integrated stress response (ISR)<sup>26</sup>. The long-distance coordination of the CDR is effected by metabokines used for remote sensing and signaling (RSS)<sup>27</sup>. The interconversion of these 3 functions of metabolites as matter, information, and energy requires the movement of molecules up and down gradients between membrane-bounded compartments—between organelles, between cells, the extracellular space, and between organ systems—such that information is stored in the form of dynamically-interacting structures acting as sub-systems in the larger whole-body system. The subsystems of the body are connected and coordinated by the flow of materials they exchange<sup>28-30</sup>, the energy they produce<sup>31,32</sup>, and the information they store as structural order<sup>33,34</sup> and epigenetic modifications<sup>35,36</sup>.

### **Potential Clinical Implications for Prognosis, Management and Treatment**

Low methylcysteine was the single most predictive risk factor for recurrence found in females with recurrent major depression in remission. Low methylcysteine has been found to be a risk factor for a number of other mental health disorders including schizophrenia<sup>37,38</sup>. Dietary sources of methylcysteine include onions, garlic, and cruciferous vegetables like cabbage, broccoli, and kale and many legumes like peas, soy, and kidney beans<sup>39</sup>. Recent clinical trials<sup>40,41</sup> have found that dietary interventions can increase plasma methylcysteine levels, in addition to several sphingolipids and

phospholipids. However, methylcysteine alone accounted for only 6% of the risk of recurrence in females and was not a predictor of recurrence in males. The broader benefits of tailored healthy dietary interventions should be considered as a potential intervention for patients with recurrent major depressive disorder. Low phospholipid and sphingolipids, which can also be increased by including a healthy diet, together added another 47% of the recurrence risk in females and 68% in males. The importance of sphingolipids in depression has been underscored by the recent demonstration that a common mechanism of action for many antidepressant drugs is to inhibit the stress-related conversion of sphingomyelin to ceramide by inhibiting acid sphingomyelinase (ASM), thereby restoring more normal sphingomyelin levels, autophagy, and organellar quality control<sup>42</sup>. Abnormalities in metabolites traceable to the microbiome also accounted for 6% of the recurrence risk in both males and females. Although low methylcysteine was the top predictor of recurrence in females, a decrease in 10 sphingolipids and 5 phospholipids was responsible for a larger proportion of the overall metabolic risk. Low 15-HETE,  $\beta$ -carotene, and 7 sphingolipids were the top predictors in males.

If methylcysteine, phospholipids, sphingolipids (ceramides, sphingomyelins, glycosphingolipids),  $\beta$ -carotene, and the microbiome are addressed together, more than 80% of the metabolic risk of recurrence might be amenable to intervention. However, vitamin supplementation alone is not a solution. Accumulating evidence suggests that over-supplementation with purified vitamins can distort their natural stoichiometric proportions *in vivo*. Vitamin ratio distortions can produce relative deficiencies in unsupplemented vitamins because balanced proportions in vitamin cofactors are required to maintain balanced metabolic fluxes. Improvements in weekly activity and exercise can be used to facilitate the whole body integration of metabolism, and improve brain-body and interorgan communication, mitochondrial quality control by mitophagy<sup>43</sup>, lipid, endocrine, inflammation, and microbiome health<sup>44-46</sup>. Clinical trials of a whole food diet rich in plant-based foods containing methylcysteine, carotenoids, phospholipids, sphingolipids, and fiber for a healthier microbiome, with

and without judicious supplementation in selected subjects, and exercise, could be a natural next step in the application of the results of this metabolomic study.



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## Single PDF

### Supplementary Methods, Results, and Discussion.

**Figure S1.** Principal component analysis of metabolomics in rrMDD. **A.** Diagnostic differences, rrMDD vs healthy controls ( $n = 84$  females). **B.** Prognostic differences, prospective analysis of rrMDD subjects with recurrence and non-recurrence in 2.5 years ( $n = 42$  females). **C.** Diagnostic differences, rrMDD vs healthy controls ( $n = 42$  males). **D.** Prognostic differences, prospective analysis of rrMDD subjects with recurrence and non-recurrence in 2.5 years ( $n = 20$  males). The green lines indicate the cumulative proportion of phenotypic variation explained by up to 5 principal components. The blue lines indicate the fraction of variance explained by each component.

**Figure S2.** Cytoscape map of metabolic pathways altered in rrMDD compared to healthy controls, **A.** Females, **B.** Males. rrMDD subjects  $n = 44$  females, 23 males. Controls  $n = 40$  females, 19 males.

**Figure S3.** Shared and gender-specific metabolic differences that distinguish subjects with recurrent major depressive disorder in remission from controls.

**Figure S4.** Kaplan-Meier style recurrence curves for the top metabolic predictors of recurrence risk in females with a history of recurrent major depressive disorder in drug-free remission identified by Cox proportional hazard analysis. Subjects were stratified by risk observed in the top vs the bottom 50<sup>th</sup> percentile for each metabolite.

**Figure S5.** Kaplan-Meier style recurrence curves for the top metabolic predictors of recurrence risk in males with a history of recurrent major depressive disorder in drug-free remission identified by Cox proportional hazard analysis. Subjects were stratified by risk observed in the top vs the bottom 50<sup>th</sup> percentile for each metabolite.

## Single Excel File

**Table S1.** Raw AUC and recurrence data. Females

**Table S2.** Raw AUC and recurrence data. Males

**Table S3.** Biochemical pathways disturbed with recurrent major depressive disorder in remission: Females.

**Table S4.** Biochemical pathways disturbed with recurrent major depressive disorder in remission: Males.

**Table S5.** Statistical analysis. Females

**Table S6.** Statistical analysis. Males

**Table S7.** Prognostic pathways. Females

**Table S8.** Prognostic pathways. Males

**Table S9.** Representative diagnostic and prognostic metabolites for recurrent major depressive disorder in remission and the risk of recurrence. Females

**Table S10.** Representative diagnostic and prognostic metabolites for recurrent major depressive disorder in remission and the risk of recurrence. Males

**Table S11.** Log transformations. Females

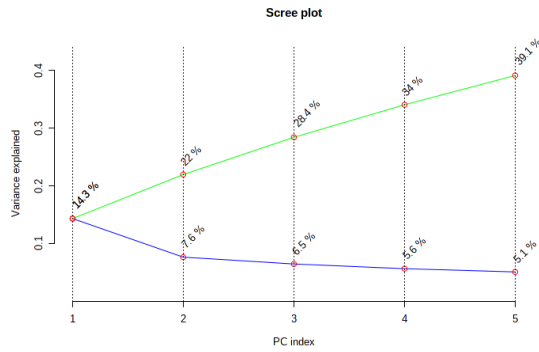
**Table S12.** Z-score transformations. Females

**Table S13.** Log transformations. Males

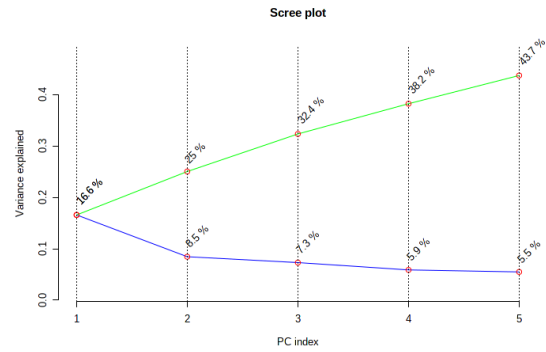
**Table S14.** Z-score transformations. Males

## Supplementary Figures and Legends

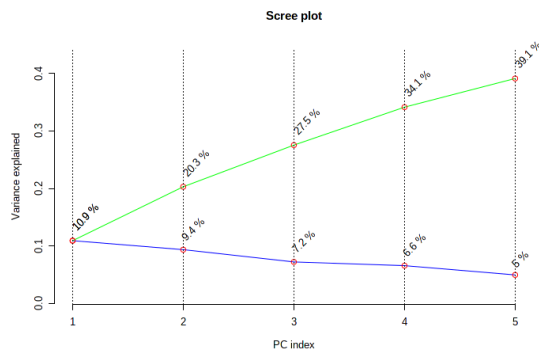
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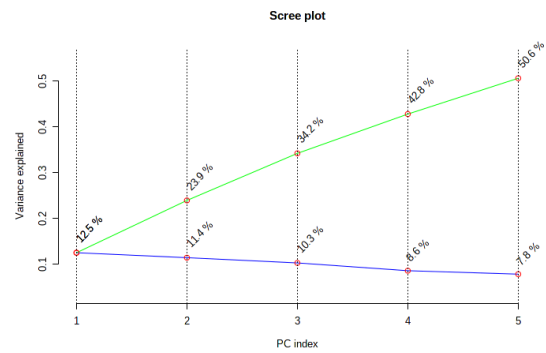
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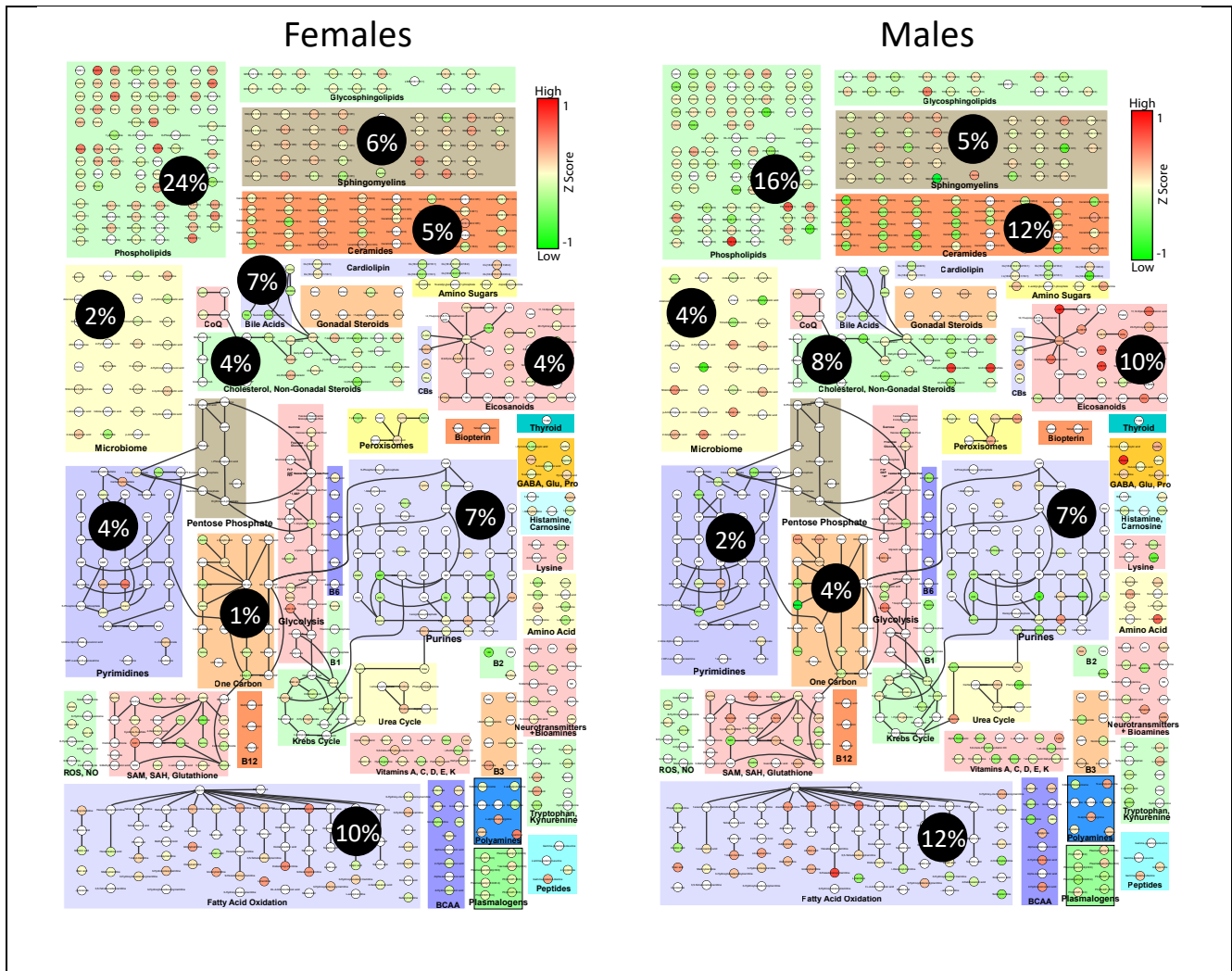
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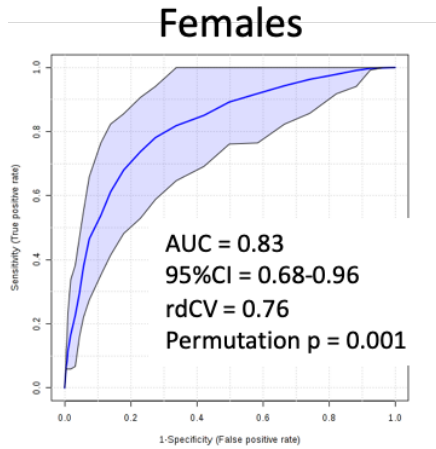
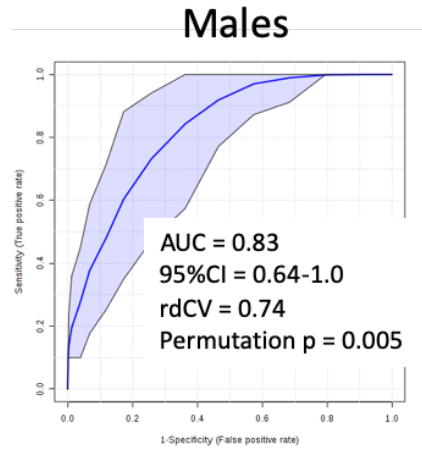
**D**



**Figure S1.** Principal component analysis of metabolomics in rrMDD. **A.** Diagnostic differences, rrMDD vs healthy controls (n = 84 females). **B.** Prognostic differences, prospective analysis of rrMDD subjects with recurrence and non-recurrence in 2.5 years (n = 42 females). **C.** Diagnostic differences, rrMDD vs healthy controls (n = 42 males). **D.** Prognostic differences, prospective analysis of rrMDD subjects with recurrence and non-recurrence in 2.5 years (n = 20 males). The green lines indicate the cumulative proportion of phenotypic variation explained by up to 5 principal components. The blue lines indicate the fraction of variance explained by each component.



**Figure S2.** Cytoscape map of metabolic pathways altered in rrMDD compared to healthy controls, **A.** Females, **B.** Males. rrMDD subjects  $n = 44$  females, 23 males. Controls  $n = 40$  females, 19 males.

**A****B****C**

- |                   |                           |
|-------------------|---------------------------|
| ↓ 13(S)-HODE      | ↓ 24,25-Epoxycholesterol  |
| ↓ FAD             | ↓ CL(18:2/18:2/18:2/18:1) |
| ↓ AMP             | ↓ Phenyllactic acid       |
| ↓ Hypotaurine     | ↑ Octanoylcarnitine       |
| ↑ Quinolinic acid | ↑ Decanoylcarnitine       |
| ↑ PG(34:2)        | ↑ Proline                 |

|          | RMDD-IR | Control |
|----------|---------|---------|
| Positive | 35      | 6       |
| Negative | 9       | 34      |

Sensitivity = 0.80 (95% CI = 0.66-0.89)  
Specificity = 0.87 (95% CI = 0.74-0.95)

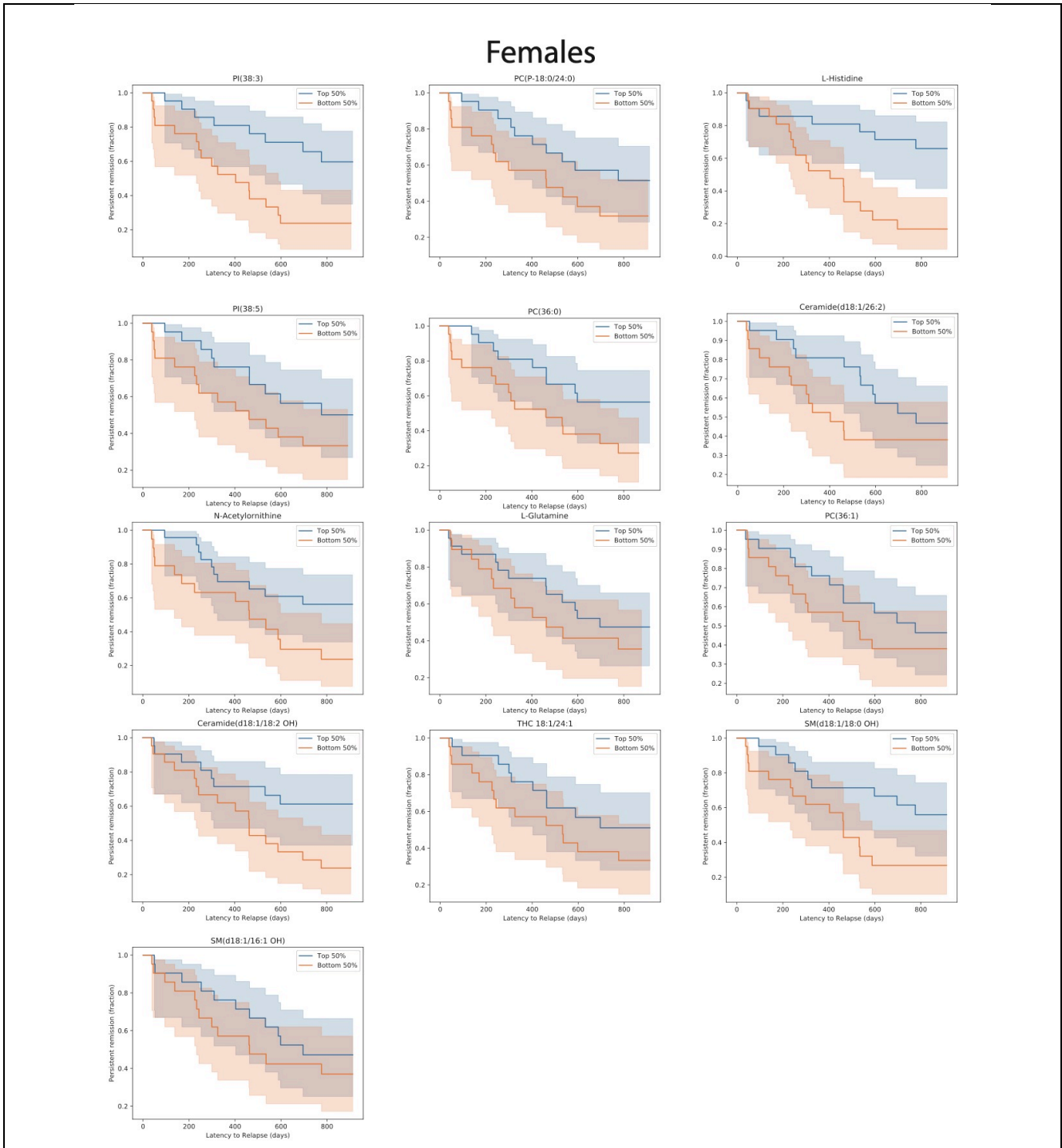
**D**

- |                           |
|---------------------------|
| ↓ SM(d18:1/26:0 OH)       |
| ↑ DHEA-S                  |
| ↑ 11-HETE                 |
| ↓ CL(18:2/18:2/18:2/20:4) |
| ↓ Lathosterol             |
| ↓ Betaine                 |
| ↓ SM(d18:1/26:2 OH)       |

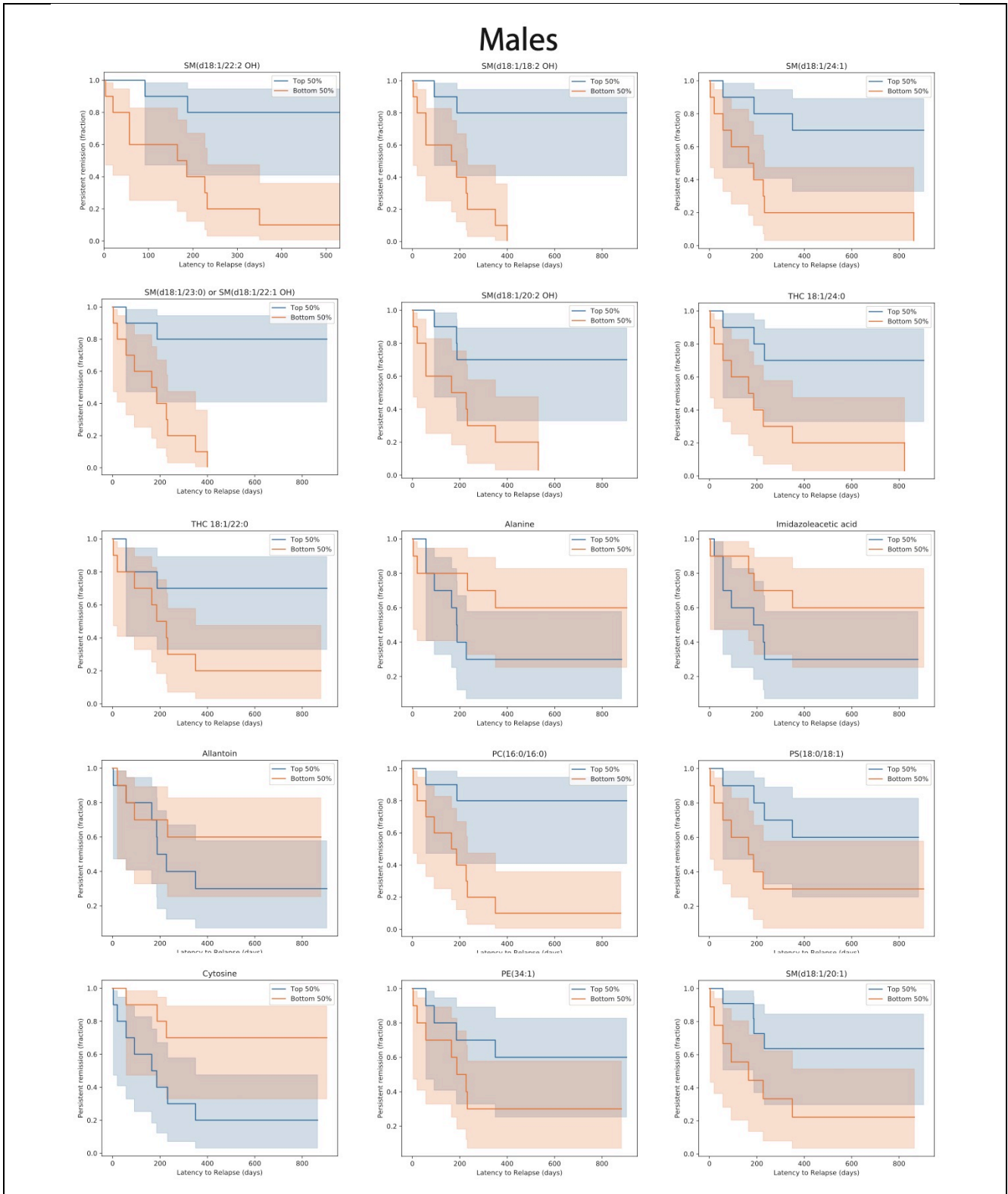
|          | RMDD-IR | Control |
|----------|---------|---------|
| Positive | 17      | 4       |
| Negative | 6       | 15      |

Sensitivity = 0.74 (95% CI = 0.53-0.87)  
Specificity = 0.79 (95% CI = 0.57-0.91)

**Figure S3.** Shared and gender-specific metabolic differences that distinguish subjects with recurrent major depressive disorder in remission from controls. Females: **A** and **C**. Males: **B** and **D**. **AB** Receiver operator characteristic (ROC) curve analysis of multianalyte diagnostic classifiers for rrMDD. The classifier for females used 12 metabolites. The classifier for males used 7 metabolites. AUC: area under the curve; rdCV: repeated double cross validation accuracy. **CD**. 2 x 2 contingency table analysis. rrMDD subjects  $n = 44$  females, 23 males. Controls  $n = 40$  females, 19 males.



**Figure S4.** Kaplan-Meier style recurrence curves for the top metabolic predictors of recurrence risk in females with a history of recurrent major depressive disorder in drug-free remission identified by Cox proportional hazard analysis. rrMDD subjects were followed prospectively for 2.5 years:  $n = 42$  females (24 with recurrence, 18 no recurrence).



**Figure S5.** Kaplan-Meier style recurrence curves for the top metabolic predictors of recurrence risk in males with a history of recurrent major depressive disorder in drug-free remission identified by Cox proportional hazard analysis. rrMDD subjects were followed prospectively for 2.5 years:  $n = 20$  males (11 with recurrence, 9 no recurrence).