

## ORIGINAL ARTICLE

## Therapeutic efficacy of atypical antipsychotic drugs by targeting multiple stress-related metabolic pathways

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Schizophrenia (SZ) is considered to be a multifactorial brain disorder with defects involving many biochemical pathways. Patients with SZ show variable responses to current pharmacological treatments of SZ because of the heterogeneity of this disorder. Stress has a significant role in the pathophysiological pathways and therapeutic responses of SZ. Atypical antipsychotic drugs (AAPDs) can modulate the stress response of the hypothalamic–pituitary–adrenal (HPA) axis and exert therapeutic effects on stress by targeting the prefrontal cortex (PFC) and hippocampus. To evaluate the effects of AAPDs (such as clozapine, risperidone and aripiprazole) on stress, we compared neurochemical profile variations in the PFC and hippocampus between rat models of chronic unpredictable mild stress (CUMS) for HPA axis activation and of long-term dexamethasone exposure (LTDE) for HPA axis inhibition, using an ultraperformance liquid chromatography–mass spectrometry (UPLC–MS/MS)-based metabolomic approach and a multicriteria assessment. We identified a number of stress-induced biomarkers comprising creatine, choline, inosine, hypoxanthine, uric acid, allantoinic acid, lysophosphatidylcholines (LysoPCs), phosphatidylethanolamines (PEs), corticosterone and progesterone. Specifically, pathway enrichment and correlation analyses suggested that stress induces oxidative damage by disturbing the creatine–phosphocreatine circuit and purine pathway, leading to excessive membrane breakdown. Moreover, our data suggested that the AAPDs tested partially restore stress-induced deficits by increasing the levels of creatine, progesterone and PEs. Thus, the present findings provide a theoretical basis for the hypothesis that a combined therapy using adenosine triphosphate fuel, antioxidants and omega-3 fatty acids as supplements may have synergistic effects on the therapeutic outcome following AAPD treatment.

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## INTRODUCTION

Stress has a powerful effect on the brain and body, and a significant role in the development and course of mental illness.<sup>1</sup> Specifically, stress has been related to the development of schizophrenia (SZ) and its potential therapeutic targets.<sup>2–5</sup> The hypothalamic–pituitary–adrenal (HPA) axis is central to the stress response. Both hyper- and hypofunction of the HPA axis have been linked to presentations of SZ, such as first-episode psychosis, acute or clinically stable chronic psychosis, and so on.<sup>6</sup>

Despite great interest in this area of research, the mechanisms of the effects of antipsychotic medication, especially atypical or second-generation drugs, on stress and the relation to their pharmacological effects are not fully understood.<sup>6</sup> To date, the data from available animal models cannot fully explain the manifestations of SZ.<sup>7</sup> Nevertheless, endocrine and neuroimaging markers are often used to evaluate HPA axis activity in humans.<sup>6,8</sup> A previous study suggested that atypical antipsychotic drugs (AAPDs) can mediate a nonspecific inhibition of stress-induced activation of the HPA axis after achieving symptom relief in acute psychosis or a direct pharmacological effect on cortisol secretion.<sup>6</sup> By contrast, such an effect is minimal in healthy subjects.<sup>9</sup> Other

biochemical and neuroimaging studies have also suggested that AAPDs increase the activity of the HPA axis following long-term treatment.<sup>10–12</sup> In short, whether AAPDs are able to influence the function of the stress axis requires further investigation, especially because SZ is a heterogeneous brain disease involving abnormalities from multiple biochemical pathways, and none of the current treatments is fully beneficial to all SZ patients.

In the brain, the prefrontal cortex (PFC) and hippocampus have been shown to modulate the HPA axis to produce negative feedback regulating glucocorticoid release during the stress response.<sup>13</sup> The PFC and hippocampus are also the two most-vulnerable brain regions in response to stress.<sup>13</sup> Several stress-induced increases in adrenal glucocorticoid hormones have been shown to contribute to the pathophysiology of SZ.<sup>14,15</sup> Although the HPA axis is not a direct target of AAPDs, both the PFC and hippocampus regions are intimately involved in the action of AAPDs<sup>16</sup> and are linked to HPA axis functioning.<sup>17,18</sup>

Biomarkers are increasingly needed to predict therapeutic outcomes in the treatment of SZ and other psychotic disorders.<sup>19</sup> Understanding the systemic metabolic effects of AAPDs on stress and identifying specific biomarkers will enhance

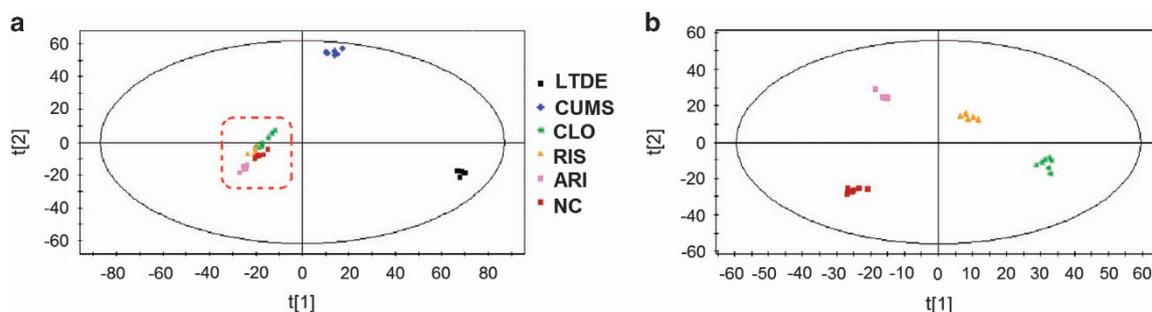
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**Figure 1.** Partial least square-discriminant analysis (PLS-DA) modeling of ultraperformance liquid chromatography–mass spectrometry (UPLC–MS/MS) spectral data derived from aqueous extracts of prefrontal cortex samples in the positive ion mode. **(a)** All seven groups; **(b)** atypical antipsychotic drug (AAPD) groups versus normal control (NC). ARI, aripiprazole; CLO, clozapine; CUMS, chronic unpredictable mild stress; LTDE, long-term dexamethasone exposure; RIS, risperidone.

our current knowledge of their pharmacological actions in SZ. We hypothesized that there are multiple targets for therapeutic efficacy in the stress-related metabolic pathways following AAPD treatment. Compared with that offered by biofluids, such as plasma and urine, which can reveal the overall metabolic state of a given organism, target tissue samples can offer a unique perspective on localized metabolic information, which will yield knowledge that is most relevant to the activity of the stress axis.<sup>20,21</sup> In the present study, using a metabolomic approach, AAPD effects on the stress-related regions of interest were investigated in the PFC and hippocampus from rats subchronically treated with the AAPDs clozapine (CLO), risperidone (RIS) and aripiprazole (ARI). In addition, the chronic unpredictable mild stress (CUMS)<sup>22</sup> and long-term dexamethasone exposure (LTDE) rat models<sup>23</sup> served as metabotypes of stress axis activation and inhibition, respectively. Using multivariate and univariate statistics to extract the shared and unique features of the metabolic signatures from rats exposed to AAPDs, CUMS and LTDE, we were able to identify potential biomarkers implicated in stress-related metabolic pathways.

## MATERIALS AND METHODS

### Animals

The study was approved by the Ethics Committee of the Central South University, and all of the experimental procedures conformed to the Declaration of Helsinki and local guidelines. A total of 42 male Sprague–Dawley rats (weighing 150–200 g) were used in this study and randomized into six groups: normal control (NC), CUMS, LTDE, CLO, RIS and ARI. The procedure for carrying out the animal experiments was in accordance with our previously published work<sup>24,25</sup> and is described in detail in the Supplementary Methods.

### Sample preparation, UPLC–MS/MS assay, data acquisition and pretreatment

Both the left and right sides of the PFC and hippocampus in each rat were homogenized. The tissue samples were harvested and handled according to a previously published protocol<sup>21</sup> and as indicated in Supplementary Figure S1 of the Supplementary Methods. The method used for metabolomic profiling was conducted as previously described,<sup>26</sup> but with minor adjustments (details in the Supplementary UPLC–MS/MS assay).

### Multivariate and univariate statistics

The three-dimensional data matrix compiled following pretreatment was subsequently analyzed using supervised multivariate statistics to extract useful information. A partial least square-discriminant analysis (PLS-DA) was performed using SIMCA-P v12.01 software (Umetrics, Umea, Sweden).

The variable selection procedure was based on a modified multicriteria assessment (MCA) strategy.<sup>20</sup> Herein, the MCA was applied to narrow down and explore those variables that were most sensitive to the interventions, using a combination of the variable importance in the projection (VIP)

statistic, the correlation coefficient ( $p(\text{corr})$ ) of the S-plot and the jack-knife-based confidence interval ( $\text{CIJ}_{jk}$ ). Finally, we selected those variables that satisfied the threefold criteria (that is,  $\text{VIP} > 5.0$ ,  $|p(\text{corr})| > 0.6$ , and the span of  $\text{CIJ}_{jk}$  excluding zero) as the most significant and reliable variables that could serve as candidate biomarkers.

A nonparametric Kruskal–Wallis one-way analysis of variance followed by pairwise multiple comparisons were performed to estimate the difference in biomarker levels among the groups. The significance level was set at  $P < 0.05$ . Given that the aim of this work was to find stress-induced biomarkers, only those with a distinct response were considered. That is, compared with that observed in the NC group, the stress-induced biomarkers should exhibit tendencies to change in an opposite direction in the LTDE versus the CUMS group. When the metabolites were compared across all groups, those that presented the same trend (that is, were either decreased or increased in all groups analyzed) or showed a nonsignificant trend after univariate analyses were further excluded. The final selected biomarkers were identified using previously established procedures.<sup>26</sup>

Two-tailed Spearman rank correlation analyses were performed to examine the relation between different categories of biomarkers with a significance level of  $P < 0.05$ . Moreover, the total list of metabolites was also analyzed using the bioinformatic tool Metabolites Biological Role (MBRole; <http://csbg.cnbc.csic.es/mbrole/>) to identify over-represented or enriched biological pathways that could be putatively active ( $P < 0.05$  was considered significant).<sup>27</sup>

All statistical analyses were performed blindly without knowledge of the origin of the samples. The schematic flowchart of the metabolic profiling and biomarker selection is illustrated in Supplementary Figure S3 of the Supplementary UPLC–MS/MS assay.

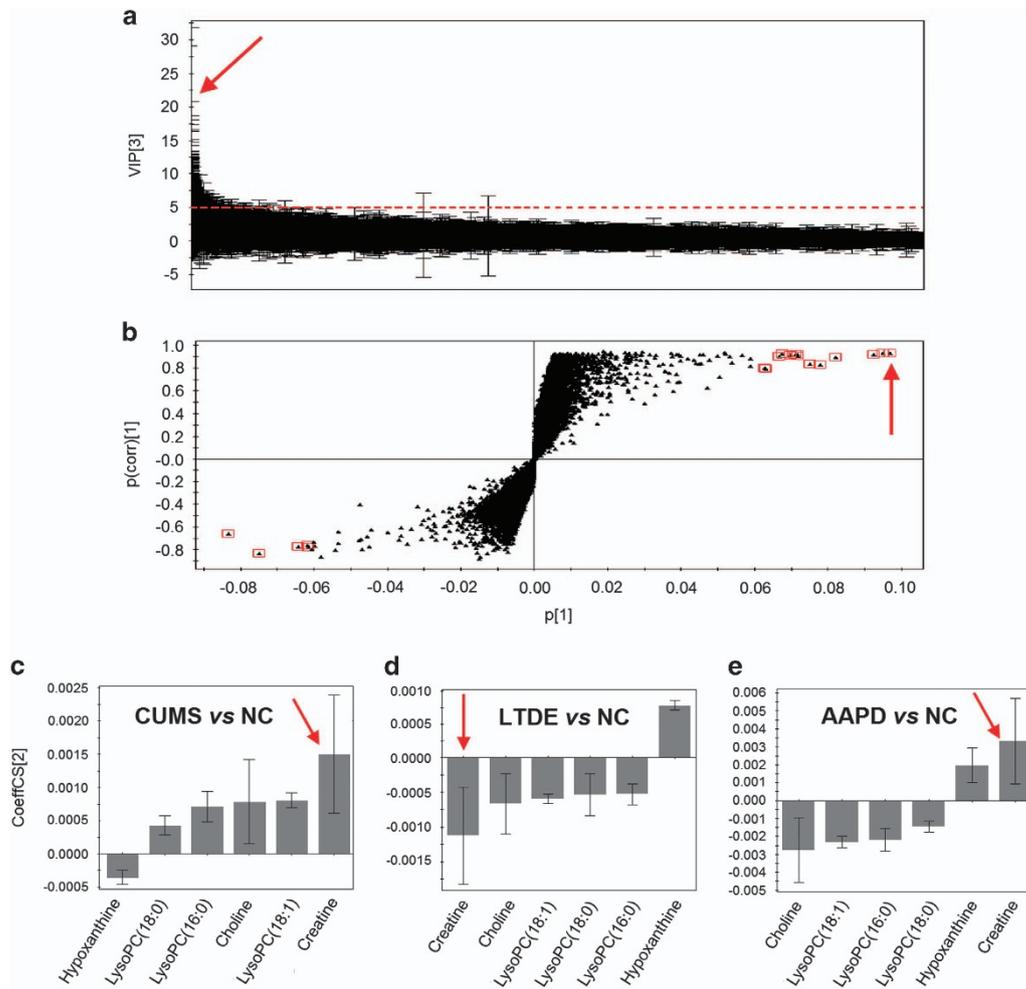
## RESULTS

### Multivariate analysis of UPLC–MS/MS data

Metabolites from aqueous or organic extracts were identified by both positive and negative ion modes of mass spectrometry. For illustration, the score plots of related PLS-DA models projecting all six groups were initially obtained for the metabolic profile from the positive ion mode ultraperformance liquid chromatography–mass spectrometry (UPLC–MS/MS) data set derived from the aqueous extracts of the PFC tissue samples. As depicted by the PLS-DA model (Figure 1a), CUMS and LTDE metabolic profiles showed distinct separation from each other, whereas the AAPD and NC groups clustered in the lower left area. However, after amplifying the dotted region in Figure 1a, the inherent metabolic differences among the CLO, RIS, ARI and NC groups could be readily visualized (Figure 1b). The amplified score plot showed that the AAPD groups were clearly separate from the NC group, and CLO was the group that was the most remote (Figure 1b).

### Data mining for biomarker discovery

To identify which variables were accountable for the significant separation described above, an MCA strategy was initially used to preselect stress-induced biomarkers (Figure 2). According to the

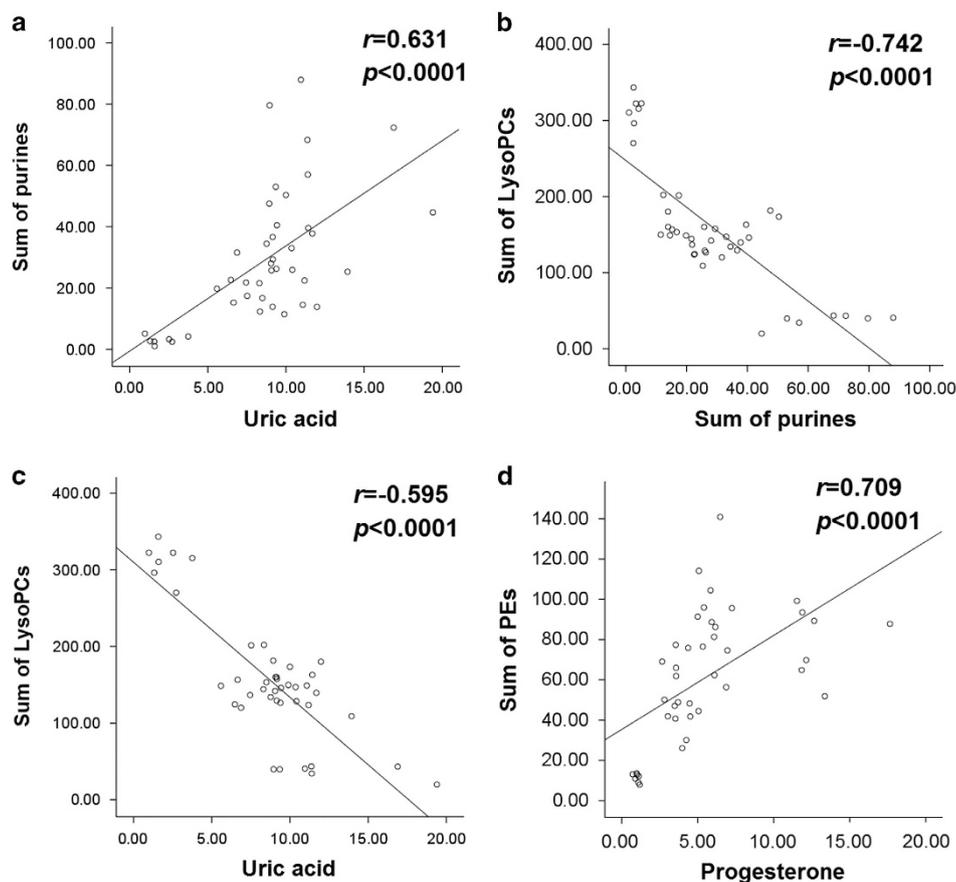


**Figure 2.** Multicriteria strategy for the selection of stress-induced biomarkers. (a) Value of importance (VIP) plot, (b) S-plot and (c–e) loading plots with the jack-knife confidence interval ( $CIJ_{jk}$ ). Specifically, creatine is labeled with a red arrow in these plots. For further interpretation, please see the Results section. AAPD, atypical antipsychotic drugs; CUMS, chronic unpredictable mild stress; LTDE, long-term dexamethasone exposure.

**Table 1.** The stress-induced biomarkers identified by UPLC–MS/MS in prefrontal cortex among different groups and their change trends

Retention (min)		m/z		Metabolites <sup>a</sup>	P-value <sup>b</sup>				
Aqueous	Organic	Positive	Negative		CUMS versus NC	LTDE versus NC	CLO versus NC	RIS versus NC	ARI versus NC
0.4	—	103.8	—	Choline	0.003↑	0.0001↓	ns	ns	ns
0.5	—	131.8	—	<b>Creatine</b>	0.033↑	0.020↓	0.001↑	0.001↑	0.003↑
0.7	—	136.9	—	Hypoxanthine	0.045↓	0.001↑	ns	ns	ns
0.6	—	—	167.1	Uric acid	0.004↓	0.045↑	ns	ns	ns
0.7	—	—	267.1	<b>Inosine</b>	0.023↓	0.010↑	0.006↑	ns	ns
0.8	—	—	174.9	Allantoic acid	0.002↑	0.043↓	ns	ns	ns
9.7	—	496.5	540.5	LysoPC(16:0)	0.033↑	0.001↓	ns	ns	ns
10.1	—	522.5	566.5	LysoPC(18:1)	0.045↑	0.001↓	ns	ns	ns
10.6	—	524.5	568.5	LysoPC(18:0)	0.031↑	0.001↓	ns	ns	ns
—	0.6	347.5	—	<b>Corticosterone</b>	0.025↑	0.001↓	0.023↓	ns	ns
—	0.7	315.4	—	<b>Progesterone</b>	0.027↓	0.0001↑	0.023↑	ns	ns
—	8.6	—	762.9	<b>PE(16:0/22:6)</b>	0.012↓	0.029↑	0.010↑	0.026↑	ns
—	10.9	—	790.7	<b>PE(18:0/22:6)</b>	0.001↓	0.014↑	0.005↑	ns	ns

Abbreviations: ↓, decreased; ↑, increased; ARI, aripiprazole; CLO, clozapine; CUMS, chronic unpredictable mild stress; LTDE, long-term dexamethasone exposure; LysoPC, lysophosphatidylcholine; NC, normal control; ns, not significant; PE, phosphatidylethanolamine; RIS, risperidone; UPLC–MS/MS, ultraperformance liquid chromatography–tandem mass spectrometry. <sup>a</sup>Metabolites responding to atypical antipsychotics are bolded. <sup>b</sup>Nonparametric Kruskal–Wallis one-way analysis of variance followed by pairwise multiple comparisons.



**Figure 3.** Spearman rank correlations between different categories of biomarkers in the prefrontal cortex (PFC). (a) The sum of purines (inosine and hypoxanthine) was positively correlated with uric acid in the PFC. (b) The sum of purines was negatively associated with the sum of lysophosphatidylcholines (LysoPCs 16:0, 18:0 and 18:1). (c) There was a negative correlation between uric acid and the sum of LysoPCs. (d) There was a positive association between progesterone and the sum of phosphatidylethanolamines (PEs).

criterion for the VIP statistics ( $VIP > 5.0$ ), a total of 194 retention and  $m/z$  pairs were obtained for their contribution to discriminate the metabolic profiles among the CUMS, LTDE, AAPD and NC groups (Figure 2a). Subsequently, using the MCA strategy in which the variables met all three criteria, including  $VIP > 5.0$ ,  $|p(\text{corr})| > 0.6$  (Figure 2b), and the span of  $CIJF_{jk}$  excluding zero, we were able to reduce the 194 variables down to six representing individual metabolites (that is, creatine, choline, hypoxanthine, lysophosphatidylcholine (LysoPC; 16:0), LysoPC (18:1) and LysoPC (18:0)). These six metabolites could be considered as potential biomarkers. Specifically, creatine labeled with a red arrow in the VIP plot, S-plot and loading plot with  $CIJF_{jk}$  (Figure 2c–e) exhibited the top VIP value of 20.7, the highest positive correlation coefficient ( $p(\text{corr})=0.93$ ), and a small confidence interval that did not cross zero. Thus, the biological significance of creatine deserves further investigation.

Metabolic profiling further revealed the 13 most significant stress-induced biomarkers in the PFC after applying MCA strategies and univariate analyses (Table 1). Among them, creatine, progesterone and phosphatidylethanolamines (PE (16:0/22:6) and PE (18:0/22:6)) were also identified in the hippocampus and showed the same pattern of changes as that observed in the PFC (Supplementary Table S2 in Supplementary Data Analysis). The relative intensities of these biomarkers after normalization are provided in Supplementary Table S3 in the Supplementary Data Analysis.

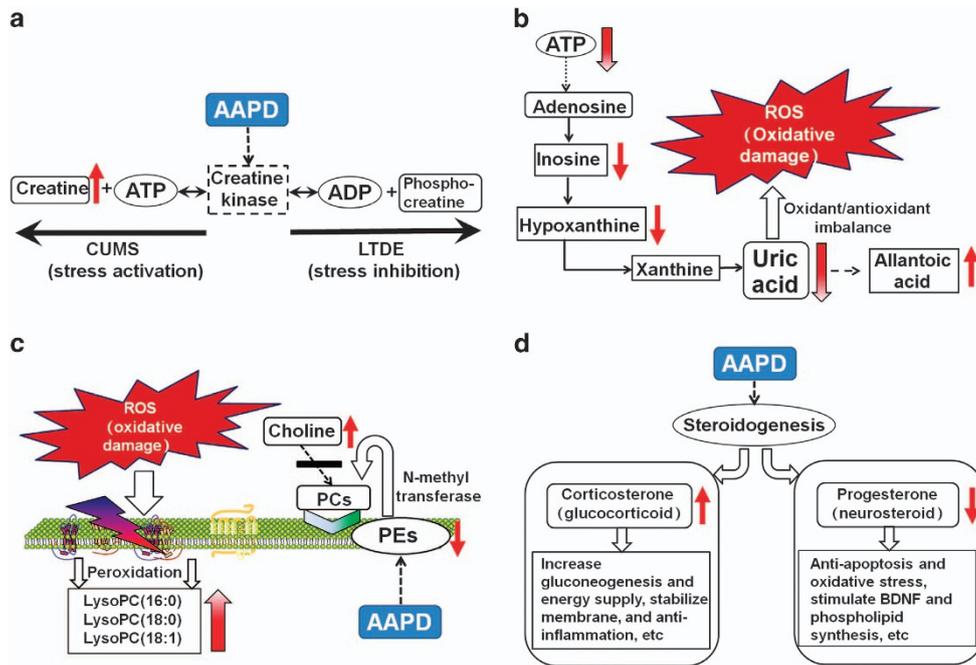
Of these above-mentioned stress-induced biomarkers, six metabolites responded to AAPDs, especially to CLO (in bold in

Table 1). In response to treatment with CLO, RIS or ARI, there was a common feature of increased levels of creatine in the PFC. Concomitantly, progesterone and PEs were also increased after treatment with CLO or RIS, but not with ARI. Similar effects of these AAPDs on stress-induced biomarkers were also found in the hippocampus (Supplementary Table S2 in the Supplementary Data Analysis).

#### Inter-relation of stress-induced biomarkers

To assess whether there were links among the different metabolites from purine signaling, membrane phospholipids and neurosteroid catabolism, we performed correlation analyses among specific candidate biomarkers in the PFC (Figure 3). First, uric acid was positively correlated with the sum of inosine and hypoxanthine (Figure 3a) and negatively associated with the sum of LysoPCs (Figure 3c). Second, the sum of purine metabolites (inosine and hypoxanthine) was inversely correlated with the sum of LysoPCs (16:0, 18:0 and 18:1; Figure 3b). Third, progesterone was positively correlated with the sum of PEs (16:0/22:6 and 18:0/22:6; Figure 3d). The positive correlation between progesterone and the sum of PEs was also present in the hippocampus (Supplementary Figure S5 in the Supplementary Data Analysis).

Cellular pathway enrichment analysis was performed with MBRole. We summarized the disturbed stress-related metabolic pathways revealed by the Kyoto Encyclopedia of Genes and Genomes (KEGG; <http://www.genome.jp/kegg/>) and the Small Molecule Pathway Database (SMPDB; <http://smpdb.ca/>). As listed



**Figure 4.** The perturbed stress-related metabolic pathways and responses to AAPD treatment. **(a)** An imbalanced creatine–phosphocreatine circuit results in an inability to satisfy the demand for ATP; **(b)** insufficient ATP leads to abnormal purine metabolism and oxidative stress; **(c)** peroxidation and biosynthetic dysfunction of membrane phospholipids; and **(d)** altered steroidogenesis by stress and subsequent changes in multiple arrays of physiological function. AAPD, atypical antipsychotic drug; ADP, adenosine diphosphate; ATP, adenosine triphosphate; BDNF, brain-derived neurotrophic factor; CUMS, chronic unpredictable mild stress; LTDE, long-term dexamethasone exposure; LysoPC, lysophosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; ROS, reactive oxidative species.

in Supplementary Table S4 of the Supplementary Data Analysis, the following six metabolites were associated with glycerophospholipid metabolism ( $P=0.000352$ ): choline, LysoPC (16:0), LysoPC (18:1), LysoPC (18:0), PE (16:0/22:6) and PE (18:0/22:6). The following four metabolites were associated with purine metabolism ( $P=0.000138$ ): inosine, hypoxanthine, uric acid and allantoin acid. Metabolites associated with steroid hormone biosynthesis (progesterone and corticosterone;  $P=0.0392$ ) and glycine, serine and threonine metabolism (creatine and choline;  $P=0.0104$ ) were also found. Purine metabolism ( $P=0.0103$ ), phospholipid metabolism ( $P=0.0166$ ) and steroidogenesis ( $P=0.0448$ ) were also identified with the SMPDB.

## DISCUSSION

Previous studies have reported the roles of mitochondrial dysfunction,<sup>28</sup> purine catabolism disturbances,<sup>29</sup> membrane phospholipid abnormalities<sup>30</sup> and neurosteroid biosynthesis dysregulation<sup>31</sup> in the pathophysiology of SZ. Herein, by metabolomic mapping of stress-induced biomarkers, we provide multiple novel targets for a combined therapy for the treatment of SZ.

**Initial energy deficiency: an imbalanced creatine–phosphocreatine circuit**

The primary physiological function of creatine is to buffer the energy supply in tissues with significant and fluctuating energy demands, especially muscles and the brain. It has become increasingly evident that endogenous creatine has a pivotal role in a range of cognitive functions, including learning, memory, attention, speech and language, and possibly emotion.<sup>32</sup> Recently, evidence of alterations in brain total creatine (creatine plus phosphocreatine) in psychiatric disorders has been provided by studies in various brain regions *in vivo*.<sup>33</sup> However, previous studies in the brain have shown no consistent pattern of

abnormalities in total creatine in SZ.<sup>34</sup> Therefore, the dynamic transformation between creatine and phosphocreatine, rather than their total amount, might be more indicative of the pathogenesis of creatine abnormalities.

In the current study, we found significantly increased creatine levels in the PFC and hippocampus in response to CUMS, which suggests a compensatory mechanism. This mechanism implies that stress in SZ consumes more energy than usual in these brain regions and leads to the conversion of phosphocreatine to creatine to generate additional adenosine triphosphate (ATP; Figure 4a). Most ATP synthesis occurs during aerobic cellular respiration, which starts with glycolysis.<sup>32</sup> Unfortunately, these complex, multistep metabolic pathways require time and energy. To this end, the creatine–phosphocreatine circuit could be considered to be a bioenergetics thermostat that quickly replenishes ATP in tissue to maintain stable levels when there are sudden and significant energy demands.<sup>32</sup> Under long-term stress, mitochondrial dysfunction might arise when reserved phosphocreatine is repeatedly depleted and, thus, is no longer available to complement ATP. Instead, the cell has to shift back to the less-efficient glycolysis pathway to satisfy its energy needs, which could set the stage for a cascade of events causing related pathology (that is, energy shortage and altered phospholipid metabolism) in the brain.<sup>35</sup>

**Insufficient ATP leading to a homeostatic imbalance of purine catabolism and oxidative stress**

Impaired brain energy metabolism and mitochondrial dysfunction are among the plausible hypotheses for the pathogenesis of SZ.<sup>28</sup> Given that psychosocial stress and abnormalities in stress axis function occur at different clinical stages of SZ, they are frequently considered to be the precipitating factors.<sup>2</sup> Similarly, the results of the current study also indicated that stress can deprive the energy supply of ATP by compromising creatine–phosphocreatine

shuttling, which could provide a link between stress and SZ pathology. Interestingly, another set of aqueous metabolites affected by stress in the two brain regions studied of the CUMS and LTDE rats included, but were not limited to, inosine, hypoxanthine, uric acid and allantoinic acid. This is the first report, to our knowledge, showing disturbed purine metabolism in brains affected by stress. These biomarkers are involved in the purine pathway, indicating that stress-induced ATP deficiency and the disturbance of purine metabolism are tightly integrated.

The precursors inosine and hypoxanthine are converted to uric acid, whereas allantoinic acid is the end product of uric acid degradation (Figure 4b). In the present study, we found that significant reductions in inosine and hypoxanthine, together with an increase in allantoinic acid, resulted in a decrease in levels of uric acid in the PFC. However, the enzyme (that is, uricase) required for the conversion of uric acid to allantoin and subsequently to allantoinic acid is not present in humans.<sup>36</sup> Therefore, uric acid is the end product of purine catabolism in humans.

Nevertheless, uric acid is a powerful antioxidant as well as a scavenger of singlet oxygen and radicals.<sup>37</sup> It is about as effective an antioxidant as ascorbate but with considerably higher levels in plasma, making it one of the most abundant antioxidants in humans.<sup>37</sup> In fact, plasma levels of uric acid have been shown to be significantly lower in clinically stable patients with chronic SZ<sup>38</sup> and in first-episode antipsychotic-naïve schizophrenia patients (FEAN-SZ)<sup>26,29,39</sup> than in healthy control subjects. Moreover, plasma uric acid levels were inversely correlated with psychosis.<sup>38</sup> A homeostatic imbalance of purine catabolism is likely to impair the antioxidant defense system, leading to oxidative damage in the PFC. This stress-induced deficit in the antioxidant defense system is also consistent with the notion of free radical-mediated neurotoxicity in SZ pathology.<sup>3-5</sup>

#### Degradation of membrane phospholipids and peroxidation of polyunsaturated fatty acids

Excess free radicals can cause cellular dysfunction, loss of membrane integrity and even cell death. The brain, which is rich in polyunsaturated fatty acids (PUFAs), is particularly susceptible to free radical-mediated damage. Thus, membrane pathology, which is secondary to a free radical-mediated insult, can contribute to specific aspects of SZ symptomatology and complications of its treatment.<sup>4</sup> One of the best-described effects of free radicals on the cell is the oxidative modification of fatty acids within the membrane phospholipids.<sup>40</sup> This lipid peroxidation predominantly occurs at the sn-2 position of phospholipids via the action of phospholipase A<sub>2</sub>, yielding oxidized fatty acid and LysoPCs.<sup>41</sup> Our data showed that CUMS increased the release of LysoPCs (16:0), (18:0) and (18:1), which suggested increased membrane breakdown (Figure 4c). Furthermore, increased LysoPCs were also associated with decreased purine metabolites (Figure 3b and c).

In addition to PUFAs, choline is another product from the degradation of membrane phospholipids and is considered to be a marker of membrane turnover.<sup>42</sup> Levels of choline were found to be significantly higher in the PFC of the CUMS group than in that of the NC group. The accumulation of choline suggests blocked biosynthesis of choline-containing phospholipids, especially phosphatidylcholine (PC), possibly because of ATP depletion under stressful conditions.<sup>43</sup> Even if PC synthesis via the choline pathway is blocked in chronic stress, methylation of PE via phosphatidylethanolamine N-methyltransferase might be available to consume the contents of PE to compensate for the PCs that are degraded in the membrane.<sup>44</sup> This could be one reason why we found that PEs were decreased concurrently with increased choline after stress stimulation (Table 1 and Figure 4c). Moreover, the decrease in PEs could indicate the early loss of myelin because these lipids are abundant in myelin.<sup>45</sup>

#### Steroidogenesis for coping with stress

As stated above, stress-induced energy depletion (Figure 4a), oxidative stress (Figure 4b) and membrane degradation (Figure 4c) are all metabolic signals that impair the structural and functional integrity of the brain. It appears that the human body also generates several feedback mechanisms for coping with stress. As expected, corticosterone, the major glucocorticoid in rodents, was also elevated in the CUMS group. Its increase is a classic endocrine response to stress (Figure 4d). The actions of glucocorticoid as a result of stress include a series of physiological consequences, such as an increase in gluconeogenesis and energy supply, stabilization of the membrane and anti-inflammation.<sup>46</sup>

Interestingly, our data revealed that chronic stress also decreased progesterone levels in the PFC and hippocampus. This could be related to a localized reduction in biosynthesis because circulating progesterone levels are not affected by stress.<sup>47</sup> By contrast, stress axis inhibition by dexamethasone increased progesterone levels in these regions, which is consistent with previous findings.<sup>48</sup> A range of actions of progesterone underlying its neuroprotective effects has been demonstrated, including a reduction in inflammation and oxidant capacity, preservation of mitochondrial functions, restoration of brain-derived neurotrophic factor and promotion of the survival of newborn neurons.<sup>49</sup> Therefore, the decline in progesterone will inevitably contribute to the deterioration of the PFC and hippocampus after long-term stress axis hyperactivity.

#### Pathways modulated by AAPDs in the stress response

Both preclinical and clinical evidence suggest that atypical antipsychotics modulate the stress response and antagonize stress-induced deficits.<sup>50</sup> Using the metabolomic approach, we investigated the emerging stress-modulatory profile of AAPDs to identify specific targets in the stress-related metabolic pathways for the therapeutic efficacy of these AAPDs.

**Cerebral energy metabolism.** One of the shared features among the AAPD-induced metabolic signatures is their regulatory effect on creatine levels, which is particularly strong in the PFC and hippocampus. CLO, RIS and ARI treatments have this feature in common. Creatine kinase (CK) catalyzes the reversible conversion of creatine and ATP to form phosphocreatine and adenosine diphosphate, respectively.<sup>51</sup> As mentioned above, the CUMS-induced elevation of creatine could result from increased CK activity to satisfy the increased demand for ATP. The association between increased CK activity and behavior changes has also been identified in a SZ animal model.<sup>52</sup> In fact, AAPDs, in contrast to typical antipsychotic drugs, have been shown to be able to regulate CK activity in the brain.<sup>53</sup> Interestingly, our data suggested that AAPDs increased creatine levels in the PFC, but decreased them in the hippocampus. This differential regulatory function is associated with the efficacy of AAPDs in the pathology of altered CK activity in the schizophrenic brain.<sup>54,55</sup>

**Steroidogenesis and phospholipid metabolism.** We have previously demonstrated the presence of a metabolic signature of increased progesterone after AAPD treatment and its correlation with symptomatology improvement in FEAN-SZ.<sup>26</sup> Consistent with our previous findings, the present findings also identified increased progesterone levels in the two brain regions studied in response to AAPDs. Given the psychotropic-like properties of progesterone,<sup>56</sup> its increment could account for the anxiolytic and neuroprotective effects of AAPDs through binding with intracellular and membrane progesterone receptors, as well as  $\gamma$ -aminobutyric acid type A receptors.<sup>49</sup> Meanwhile, AAPD-induced upregulation of membrane PE in these brain regions could provide a novel mechanism for membrane regeneration.<sup>57</sup>

Recently, we demonstrated that progesterone could regulate lipid biosynthesis via a specific membrane-binding site, named progesterone receptor membrane component 1.<sup>25</sup> Interestingly, the present data also showed that progesterone levels were positively associated with the sum of the PE concentrations in the CUMS, LTDE and AAPD treatment conditions. Given that PEs are rich in myelin, this association suggests a pivotal role for AAPD-induced progesterone elevation in myelination.<sup>58</sup> However, the exact biological mechanism of this correlation is still unclear.

*Potential synergistic effects with combined ATP fuel, antioxidant and PUFA supplementation.* Pharmaceutical treatment for the many patients worldwide with SZ is limited to a handful of antipsychotics. Despite the proven efficacy of these drugs, the overall outcome for SZ remains suboptimal.<sup>59</sup> Thus, alternative or adjunctive treatment options are urgently needed. As stated above, AAPDs could temporarily alleviate ATP shortage by regulating CK activity until the depletion of phosphocreatine. Thus, supplements of ATP fuel could help AAPDs to sustain mitochondrial function.<sup>60</sup> In the present study, levels of purines, choline and LysoPCs were not significantly influenced by AAPD treatment, suggesting that oxidative stress is not the primary target of AAPDs. This also offers a plausible adjunctive therapeutic approach using AAPDs in the form of antioxidants.<sup>61</sup> As illustrated in Figure 4, which depicts the altered stress-related metabolic pathways, ATP deficiency and membrane phospholipid breakdown are markedly linked. Moreover, Table 1 indicates that the stress-induced PUFA decreases mainly involved C22:6 (n-3; docosahexaenoic acid). This result implies that supplements of these essential PUFAs would facilitate PE synthesis under AAPD treatment in conditions of stress.<sup>62</sup> Taken together, the addition of combined oral supplements of ATP fuel, antioxidants and essential PUFAs could provide synergistic effects with AAPD treatment.

## CONCLUSION

To the best of our knowledge, this is the first metabolomic study that: (1) evaluates the metabolic profiles of different brain regions that are vulnerable to stress among CUMS, LTDE, AAPD and NC rats; (2) establishes a novel strategy for stress-induced biomarker screening; (3) identifies metabolic pathways specifically affected by stress that are responsive to AAPD treatment; and (4) validates stress-induced biomarkers as potential therapeutic targets for AAPD treatment. Taken together, these results show that stress can induce oxidative damage by disturbing the creatine-phosphocreatine circuit and purine pathway, leading to increased membrane lipid peroxidation. Moreover, the preliminary data suggest that AAPDs partially restore the stress-induced deficits by increasing the content of creatine, progesterone and PEs. These results provide a theoretical basis from which the development of novel therapeutic strategies, in combination with ATP fuel, antioxidant and omega-3 fatty acid supplementation, could occur.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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