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Original Article

Elevated levels of urinary markers of oxidatively generated DNA and RNA damage in bipolar disorder

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Objectives: The pathophysiological mechanisms underlying bipolar disorder and its multi-system nature are unclear. Oxidatively generated damage to nucleosides has been demonstrated in metabolic disorders; however, the extent to which this occurs in bipolar disorder *in vivo* is unknown. We investigated oxidatively generated damage to DNA and RNA in patients with bipolar disorder and its relationship with the affective phase compared with healthy control subjects.

Methods: Urinary excretion of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) and 8-oxo-7,8-dihydroguanosine (8-oxoGuo), markers of oxidatively generated DNA and RNA damage, respectively, was measured in 37 rapid cycling patients with bipolar disorder and in 40 age- and gender-matched healthy control subjects. Employing a longitudinal design, repeated measurements of both markers were evaluated in various affective phases in patients with bipolar disorder during a six- to 12-month period and compared with repeated measurements in healthy control subjects.

Results: In linear mixed models, adjusting for demographical, metabolic, and lifestyle factors, the excretion of 8-oxodG and 8-oxoGuo was significantly elevated in euthymic patients with bipolar disorder compared with healthy control subjects, with increases of 40% ($p < 0.0005$) and 43% ($p < 0.0005$), respectively. The increased oxidatively generated nucleoside damage was present through all affective phases of the illness, with no significant difference between affective states.

Conclusions: Our results indicate that bipolar disorder is associated with increased oxidatively generated damage to nucleosides. The findings could suggest a role for oxidatively generated damage to DNA and RNA as a molecular mechanism contributing to the increased risk of medical disorders, shortened life expectancy, and the progressive course of illness observed in bipolar disorder.

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Bipolar disorder is associated with an increased medical burden (1), shortened life expectancy (2), and increased mortality that, beyond increased suicide rates (3), appears largely driven by natural causes such as diabetes and cardiovascular and cerebrovascular diseases (4). Risk factors related to these diseases, such as obesity and metabolic syndrome, are highly prevalent in bipolar disorder (5, 6), and recent evidence indicates that while lifestyle and diet play important roles in mediating these risk factors, pharmacological side effects may only be partly responsible (7). Bipolar disorder is increasingly recognized as a multi-system disorder (8); however, the possible pathophysiological

mechanisms underlying the interaction between the illness, its progressive course (9), and the metabolic disturbances and comorbid medical disorders are not completely understood.

Evidence derived from gene expression studies (10), investigation of postmortem brain tissue (11), and clinical studies (12) indicate a role for oxidative stress in bipolar disorder and depression (13). Inflammatory and oxidative and nitrosative (IO & NS) pathways may generate damage to DNA, membrane lipids, and proteins, in turn causing cellular dysfunction through damage to mitochondria, the cell wall, DNA, and functional proteins, ultimately leading to apoptosis and cell death (14). These inter-related aberrations in oxidative and nitrosative stress (O & NS) and immune–inflammatory pathways are suggested to be key contributing pathophysiological mechanisms in both depression (15) and bipolar disorder (16). Oxidatively generated damage to nucleic acids has been demonstrated in several medical disorders with increased prevalence in bipolar disorder, among which are diabetes (17), atherosclerosis (18), and Alzheimer's disease (19). Recently, oxidative damage to RNA was proposed as a novel mechanism contributing to medical diseases (20).

In bipolar disorder, damage to DNA has been demonstrated in both postmortem brain tissue (21) and in peripheral blood (22), but investigation of nucleoside oxidation has been limited to one study of DNA oxidation *in vivo* (23) and one study of RNA oxidation in postmortem brain tissue (24). The extent of *in vivo* RNA oxidation in bipolar disorder and of nucleic acid oxidation in euthymic patients with bipolar disorder is unknown.

8-hydroxylation of guanine constitutes the prototypical measureable oxidation lesion of DNA, resulting in the formation of the 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) nucleoside. 8-oxodG is excreted in the urine along with its RNA counterpart, the 8-oxo-7,8-dihydroguanosine (8-oxoGuo) and their excretion, reliably measured by ultraperformance liquid chromatography with tandem mass spectrometry (25, 26), provides a quantitative estimate of the global DNA and RNA oxidation *in vivo* (27).

The aim of the present study was to compare levels of urinary markers of DNA and RNA oxidation between rapid cycling patients with bipolar disorder and healthy control subjects and to evaluate the extent of intra-individual natural variation in DNA and RNA oxidation in healthy individuals. Further, we wished to evaluate repeated measures of urinary markers of DNA and RNA oxidation in rapid cycling patients with bipolar disorder during various affective states and to assess their relationship with alterations of affective state. A naturalistic, longitudinal design aimed at assessing patients during multiple affective phases of varying polarity was employed. To our knowledge, this is the first study to implement this research strategy in identifying phase-specific intra-individual alterations in biomarker levels in bipolar disorder (10, 28).

Methods and materials

Participants

Patients with bipolar disorder. Patients with a potential diagnosis of rapid cycling bipolar disorder were recruited through referral by psychiatrists at hospitals or outpatient facilities throughout the region of Zealand, Denmark, or by contacting study researchers personally, with study recruitment taking place between June 2010 and May 2012. Inclusion criteria were: adults aged 18–70 years and a Diagnostic and Statistical Manual of Mental Disorders (DSM)-IV diagnosis of rapid cycling bipolar disorder, defined by the occurrence of at least four mood episodes (mania, hypomania, depression, or mixed) during the preceding year in the context of bipolar disorder. Exclusion criteria were: significant physical illness [i.e., autoimmune disorders, chronic pulmonary disease, inflammatory disease, hemochromatosis, chronic infectious disease, neurodegenerative or neuroinflammatory disease, and other conditions possibly associated with increased oxidative stress (15)], current drug abuse, inadequate Danish language skills, and pregnancy. A total of 37 patients with bipolar disorder were included, two of whom declined further examination after one- and three-months follow-up, respectively; the remaining patients were followed for a minimum of six months, with a mean [standard deviation (SD)] follow-up period of 11.9 (3.0) months. Upon signs of new affective episodes, patients were evaluated with clinical assessments of mood and the collection of urine samples and standard blood tests, which, when possible, were repeated after a return to a subsequent euthymic state or change to an affective episode of opposite polarity. When there were clinical signs of acute infection or other acute medical conditions, assessment and biochemical analysis were postponed. (A flow chart showing the study recruitment process is provided in *Supplementary Fig. 1* and additional information on follow-up procedures is available in the *Supplementary Methods*.)

Healthy control subjects. Forty healthy control subjects were recruited among blood donors

affiliated with the blood bank at Rigshospitalet, Copenhagen. Inclusion criteria were: (i) adults aged 18–70 years and (ii) no history of psychiatric disorder in the subjects or their first-degree relatives. Exclusion criteria were: (i) substance abuse, (ii) significant physical illness, (iii) pregnancy, and (iv) inadequate Danish language skills. Control subjects were evaluated with clinical assessments and the collection of urine samples and standard blood tests on two separate occasions, approximately three months apart. Mean (SD) follow-up time for the healthy control subjects was 2.9 (0.9) months.

All participants provided written informed consent and were reimbursed for their travel expenses. The study protocol was approved by the Committee on Health Research Ethics of the Capital Region of Denmark (Protocol no. H-4-2010-006) and the Danish Data Protection Agency (Approval no. 2010-41-4267). The study complied with the Declaration of Helsinki.

Clinical assessments

All participants, patients with bipolar disorder and healthy control subjects alike, were assessed by a specialist in psychiatry (KM) using standardized semi-structured interviews. The Schedules for Clinical Assessment in Neuropsychiatry (SCAN) interview (29) was used for diagnostic purposes and was based on available case material, referral reports, an interview with the participant, and the Hypomania Checklist (HCL-32) (30), which was completed by each participant. Data on illness duration, number of previous episodes, and hospitalizations were extracted from the SCAN interview. A DSM-IV diagnosis of rapid cycling bipolar disorder was established for the patients and absence of lifetime psychiatric morbidity was confirmed for healthy control subjects. In cases of uncertainty regarding the diagnosis or fulfillment of eligibility criteria, this was discussed among the study group (KM, LVK, MV) and a consensus decision was made.

A clinical diagnosis according to DSM-IV, without applying duration criteria, was established at each study visit concurrently with the collection of samples for laboratory analysis. The severity of depressive symptoms was assessed using the 17-item Hamilton Depression Rating Scale (HAMD-17) (31), employing a structured interview guide (32) translated into Danish; manic symptoms were assessed using the Young Mania Rating Scale (YMRS) (33), with a period of three days applied. Medication, physical activity, alcohol intake, and smoking habits during the two weeks prior to assessment were recorded.

Categories of affective states were based on clinical evaluation according to the SCAN interview combined with the scores from the HAMD-17 and YMRS, without applying duration criteria: euthymic (HAMD-17 and YMRS < 8), depressive (HAMD-17 > 7 and YMRS < 8), manic (YMRS > 7 and HAMD-17 < 8), and mixed state (HAMD-17 and YMRS > 7).

Laboratory methods

Blood and urine samples were obtained in the fasting state between 8:30 a.m. and 10:30 a.m., after a minimum period of 15 min of rest.

Blood collection. Blood samples were analyzed for standard clinical chemistry parameters at the hospital clinical chemistry department (see *Supplementary Methods* for additional details).

Urine collection and preparation. A freshly voided spot urine sample was obtained using a standard sampling kit without any additives (In Vitro as, Fredensborg, Denmark). The sample was kept on ice and centrifuged at 4°C and 1,590 *g* for 15 min, after which aliquots of 1.5 mL were transferred to Eppendorf tubes and stored at −80°C until analysis.

Urinary 8-oxodG and 8-oxoGuo. The frozen samples were thawed, mixed, and heated to 37°C for 5 min and then centrifuged at 10,000 *g* for 5 min. The supernatant was used for the analysis. Samples from the same participant were grouped together on the same plate in a randomly assigned sequence, with an even distribution of samples from patients and healthy control subjects across the assay. All samples were analyzed in two sessions, on two adjacent days. The urinary content of the oxidized nucleosides 8-oxodG and 8-oxoGuo were quantified using a modified ultraperformance liquid chromatography and mass spectrometry (UPLC-MS/MS) assay, described in detail elsewhere (26). Briefly, the chromatographic separation was performed on an Acquity UPLC system (Waters Corp., Milford, CT, USA) using an Acquity UPLC BEH Shield RP18 column (1.7 μm, 2.1 × 100 mm; Waters Corp.) and a VanGuard precolumn (1.7 μm, 2.1 × 5 mm; Waters Corp.) with a column temperature of 4°C. The mass spectrometry detection was performed on a Xevo-TSQ triple quadrupole mass spectrometer (Waters Corp.), using electrospray ionization in the positive mode for 8-oxodG and negative ionization mode for 8-oxoGuo. The 8-oxodG and 8-oxoGuo

urinary excretion was normalized to the urinary creatinine concentration, quantified by Jaffe's reaction. Laboratory personnel performing the analysis were blinded to the category of participants and the clinical state of patients with bipolar disorder. The average *within-day* and *between-day* variation (relative standard deviation (RSD), %) estimated from the method validation was 2.3% and 9.0% for 8-oxoGuo, respectively, and 3.8% and 7.4% for 8-oxodG.

Statistics

Independent *t*-tests were used to test differences in age between healthy control subjects and patients with bipolar disorder, and the chi-squared test was used to examine differences in categorical demographic and clinical variables.

For our main analysis, we employed a two-level linear mixed-effects model. Level one represented repeated measures of 8-oxodG and 8-oxoGuo and level two represented between individual variation. We conducted two sets of analyses, one on comparisons between patients with bipolar disorder and healthy control subjects (*set A*) and one on comparisons between affective states among patients with bipolar disorder (*set B*). In both sets, unadjusted mixed-model analyses, with levels of 8-oxodG and 8-oxoGuo as the dependent variables, were firstly conducted. Further, we specified several *a priori* models within each set of analyses. For the comparison of 8-oxodG and 8-oxoGuo levels between euthymic patients with bipolar disorder and healthy control subjects, we specified a main model (*model A-1*) where group (euthymic patient with bipolar disorder or healthy control subject) was entered as a covariate, along with demographic and lifestyle variables possibly associated with oxidative nucleoside damage [gender, age, body mass index (BMI), smoking, alcohol consumption, exercise, and oral contraceptive use]. Statistically significant covariates were subsequently kept in the model. For a second model (*model A-2*), blood level values related to metabolic disturbances possibly associated with oxidative stress (fasting blood glucose, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and triglyceride) were added as covariates in addition to group and significant covariates from model A-1. A possible effect of duration of euthymic episodes was analyzed by dividing the samples obtained in a euthymic state into subgroups by duration (< 2 weeks, 2–8 weeks, and > 8 weeks) (*model A-3*). Finally, the levels of oxidized nucleosides were compared between healthy control subjects and all samples from patients with bipolar disorder to assess differences between healthy control subjects and patients in a current affective episode (*model A-4*). Because smoking may be of particular importance in processes related to oxidative stress, we additionally carried out a supplemental set of analyses among non-smoking euthymic patients with bipolar disorder and healthy control subjects only, eliminating a possible confounding effect of smoking (*models A1S and A2S*). In *set B* analyses, differences in 8-oxodG and 8-oxoGuo levels between affective states were analyzed using the covariates affective state (euthymic, depressive, manic, or mixed) along with gender, smoking, exercise, age, and BMI (*model B-1*). In a further model (*model B-2*), we entered clinical covariates possibly related to oxidative nucleoside damage (previous number of episodes and illness duration) and current use of medication [lithium, anticonvulsant, antipsychotic agent, or antidepressant as categorical covariates (yes/no)] in addition to affective state and significant covariates from model B-1. Lastly, we analyzed a possible effect of symptom severity (*model B-3*), entering as covariates HAMD-17 scores and YMRS scores for samples obtained during depression and mania, respectively, in addition to age, BMI, smoking, and exercise. All models included a random intercept to accommodate correlations in the outcome variables over time within each participant. All other covariates were specified as fixed effects.

Differences between levels of 8-oxodG and 8-oxoGuo at the two time points in healthy control individuals were analyzed using a paired-samples *t*-test.

To evaluate the correlation between levels of 8-oxodG and 8-oxoGuo, a Pearson's correlation analysis was performed, using residual values produced by our mixed model.

For all parametric tests, levels of 8-oxodG and 8-oxoGuo were transformed by the natural logarithm. (Additional information on statistical methods is available in the *Supplementary Statistics*.) The statistical analysis was conducted using SPSS, version 20.0 (IBM Corporation, New York, NY, USA).

Results

Demographic and clinical characteristics

Demographic and clinical characteristics of the study participants are described in Table 1 (for a detailed view of medication intake at time of inclusion, see *Supplementary Table* 1).

All of the study participants were Caucasian. Overall, patients with bipolar disorder were on a

Table 1. Demographic and clinical characteristics of the study participants at inclusion

	Bipolar disorder (n = 37)	Healthy controls (n = 40)	Statistic	p-value
Age, years, mean ± SD [range]	40.9 ± 12.3 [23–66]	36.3 ± 12.5	$t = 1.828$	0.108
Gender, female/male	25/12	23/17	$\chi^2 = 0.830$	0.362
Education, years, mean ± SD [range]	16.1 ± 3.0 [9–22]	16.4 ± 2.3 [10–21]	$t = 0.608$	0.545
No. of smokers, n (%)	21 (76)	1 (0.03)	$\chi^2 = 18.547$	< 0.0001
Alcohol consumption, units/week, mean ± SD [range]	1.7 ± 2.4 [0–10]	5.1 ± 4.4 [0–15]	$t = 4.120$	< 0.0001
Exercise, hours/week, mean ± SD [range]	4.9 ± 3.0 [1–10]	7.1 ± 3.4 [2–20]	$t = 3.020$	0.003
Body mass index, mean ± SD [range]	24.6 ± 3.6 [19.4–32.4]	24.9 ± 3.9 [20.0–42.2]	$t = 0.353$	0.725
Oral contraceptive use (women), n (%)	3 (12)	11 (48)	$\chi^2 = 7.442$	0.006
Standard blood tests, mmol/L, mean ± SD [range]				
Fasting blood glucose	5.1 ± 0.5 [4.2–6.4]	4.9 ± 0.4 [4.0–5.8]	$t = 2.376$	0.020
Cholesterol	5.2 ± 0.8 [3.9–7.1]	4.7 ± 0.7 [3.2–6.3]	$t = 2.712$	0.008
HDL cholesterol	1.7 ± 0.6 [0.9–3.0]	1.7 ± 0.4 [1.0–2.6]	$t = -0.902$	0.370
LDL cholesterol	3.1 ± 0.7 [1.9–4.7]	2.7 ± 0.6 [1.1–4.0]	$t = 2.312$	0.024
Triglyceride	1.1 ± 0.4 [0.5–2.3]	0.9 ± 0.5 [0.4–3.5]	$t = 1.163$	0.249
Duration of illness, years, mean ± SD [range]	21.2 ± 13.0 [2–56]			
Bipolar I disorder, n (%)	22 (59.5)			
Bipolar II disorder, n (%)	15 (40.5)			
No. of depressive episodes	16.2 ± 15.4 [2–60]			
No. of hypomanic episodes	16.5 ± 19.1 [2–92]			
No. of manic episodes	3.2 ± 7.1 [0–35]			
No. of hospitalizations	10.2 ± 19.5 [0–75]			
Medications, n (%)				
Lithium	15 (40.5)			
Anticonvulsants	27 (73.0)			
Antipsychotic agents	27 (73.0)			
SSRI	8 (21.6)			
Newer antidepressant	2 (5.4)			
Older antidepressant	2 (5.4)			

The highest value of each participant's fasting blood glucose was used for the analysis. Cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, and triglyceride: mean values of repeated measures were used for each participant. SD = standard deviation; SSRI = selective serotonin reuptake inhibitors.

stable medication regimen during the course of the study, with few participants changing medication. During the study period, four patients stopped taking their medication, one patient started treatment with a selective serotonin reuptake inhibitor, two patients started lithium treatment, two started anticonvulsant treatment, and one started antipsychotic treatment.

The majority of the patients received specialized treatment at the Affective Disorders Clinic, Rigshospitalet, and all of the patients were outpatients at the time of inclusion.

Symptom severity and 8-oxodG and 8-oxoGuo levels of participants at sampling time are presented in Table 2.

Blood samples and urine samples were collected from patients with bipolar disorder, on average, 3.4 ± 1.67 (1–10) times during the study and on two occasions for all of the healthy control subjects. Samples were obtained during the euthymic phase in all but three patients [mean 2.0 ± 1.3 (0–6)], during major depression in 26 patients [mean 1.7 ± 1.7 (0–5)], during a manic phase in 11 patients [mean 0.7 ± 1.2 (0–5)], and during a mixed state in a total of six patients [mean 0.2 ± 0.4 (0–1)].

Oxidatively generated nucleoside damage in euthymic patients with bipolar disorder compared with healthy control subjects

In unadjusted mixed-model analysis, there was a significant difference between euthymic patients with bipolar disorder and healthy control subjects in both 8-oxodG [$F(1,73.062) = 10.300$, $p = 0.002$] and 8-oxoGuo [$F(1,68.594) = 52.035$, $p < 0.0005$]. These differences remained significant when adjusting for gender, age, BMI, smoking, alcohol consumption, exercise, and oral contraception use (model A-1) for both 8-oxodG [$F(1,75.438) =$

Table 2. Symptom severity and 8-oxodG and 8-oxoGuo levels of participants at time of collection of urine samples

	Healthy controls (n = 80)	Bipolar disorder (n = 168)			
		Euthymic (n = 75)	Depressive (n = 63)	Manic (n = 24)	Mixed state (n = 6)
HAMD-17	0.6 ± 0.9 [0–3]	3.7 ± 1.9 [0–7]	15.5 ± 5.1 [8–27]	3.4 ± 2.6 [0–7]	10.2 ± 1.8 [8–12]
YMRS	0.4 ± 0.8 [0–3]	1.0 ± 1.7 [0–7]	0.9 ± 1.4 [0–6]	15.3 ± 4.3 [9–24]	11.2 ± 2.8 [8–16]
8-oxodG	0.99 ± 0.45	1.22 ± 0.43	1.31 ± 0.56	1.05 ± 0.35	1.07 ± 0.20
8-oxoGuo	1.29 ± 0.37	1.81 ± 0.48	1.85 ± 0.94	1.87 ± 0.37	1.80 ± 0.36

Data are expressed as mean ± standard deviation [range]. Values are presented as raw values, unadjusted for repeated measures. 8-oxodG and 8-oxoGuo levels are expressed as nM/mM creatinine.
8-oxodG = 8-oxo-7,8-dihydro-2′-deoxyguanosine; 8-oxoGuo = 8-oxo-7,8-dihydroguanosine; HAMD-17 = 17-item Hamilton Depression Rating Scale; YMRS = Young Mania Rating Scale.

17.785, $p < 0.0005$] and 8-oxoGuo [$F(1,68.594) = 52.035$, $p < 0.0005$]. The differences were substantial, with 40% higher levels of 8oxodG and 43% higher levels of 8oxoGuo, compared with healthy control subjects (Fig. 1). Differences in 8-oxodG and 8-oxoGuo levels remained significant after additional adjustment for possible effects of fasting blood glucose and blood lipids (model A-2), with little impact on model parameters (Tables 3 and 4). In a supplementary analysis among non-smoking participants only (models A-1S and A-2S), differences between euthymic patients with bipolar disorder and healthy control subjects remained, with significantly elevated levels of both 8-oxodG and 8-oxoGuo in patients with bipolar disorder in both unadjusted analysis and after adjustment for possible confounding

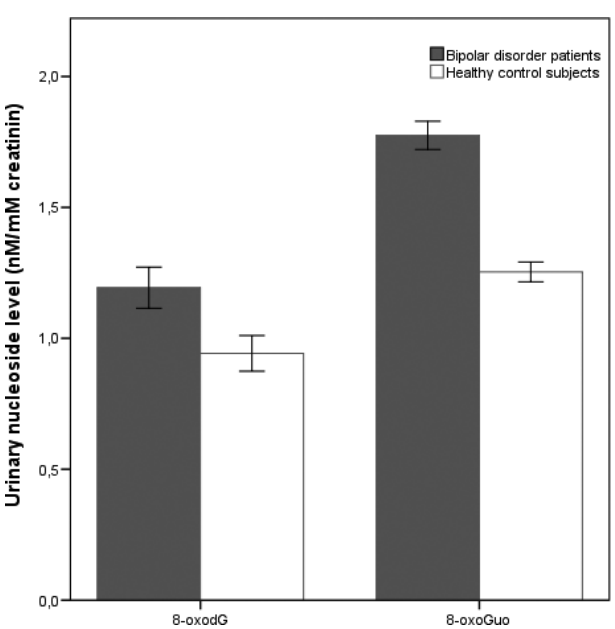

Fig. 1. Urinary levels of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) and 8-oxo-7,8-dihydroguanosine (8-oxoGuo) in euthymic patients with bipolar disorder (n = 37) compared with healthy control subjects (n = 40). Data are expressed as mean ± standard deviation. Linear mixed-model analysis of comparison between groups: $p < 0.0005$ for 8-oxodG and $p < 0.0005$ for 8-oxoGuo.

Table 3. Summary of mixed-model analysis of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) levels in euthymic patients with bipolar disorder versus healthy control subjects

	Loaded model		Final model	
Variable	*b* (SE)	p-value	*b* (SE)	p-value
Unadjusted				
Group (BD/HC)	1.29 (1.08)	0.002		
Model A-1				
Group (BD/HC)	1.47 (1.10)	< 0.0005	1.40 (1.08)	< 0.0005
Gender (male/female)	0.90 (1.09)	0.196		
Age	1.00 (1.00)	0.138		
BMI	1.02 (1.01)	0.135		
Smoking	1.00 (1.00)	0.579		
Alcohol	1.15 (1.01)	0.191		
Exercise	1.03 (1.01)	0.023	1.02 (1.01)	0.041
Oral contraceptive use (yes/no)	1.25 (1.10)	0.026	1.31 (1.09)	0.003
Model A-2				
Group (BD/HC)	1.39 (1.08)	< 0.0005		
Exercise	1.02 (1.01)	0.078		
Oral contraceptive use (yes/no)	1.35 (1.10)	0.002		
Fasting glucose	0.98 (1.64)	0.731		
Total cholesterol	0.98 (1.13)	0.847		
HDL cholesterol	0.83 (1.15)	0.168		
LDL cholesterol	0.98 (1.13)	0.861		
Triglyceride	1.00 (2.10)	0.994		

b = parameter estimate, expressing ratios of 8-oxodG levels based on back-transformed estimates from mixed-model analysis.
BD = bipolar disorder; BMI = body mass index; HC = healthy controls; HDL = high-density lipoprotein; LDL = low-density lipoprotein; SE = standard error.

Table 4. Summary of mixed-model analysis of 8-oxo-7,8-dihydroguanosine (8-oxoGuo) levels in euthymic patients with bipolar disorder versus healthy control subjects

	Loaded model		Final model	
Variable	*b* (SE)	p-value	*b* (SE)	p-value
Unadjusted				
Group (BD/HC)	1.43 (1.05)	< 0.0005		
Model A-1				
Group (BD/HC)	1.37 (1.06)	< 0.0005	1.43 (1.05)	< 0.0005
Gender (male/female)	0.91 (1.06)	0.111		
Age	1.00 (1.00)	0.126		
BMI	1.01 (1.01)	0.171		
Smoking	1.00 (1.00)	0.240		
Alcohol	0.98 (1.01)	0.721		
Exercise	0.99 (1.01)	0.783		
Oral contraceptive use (yes/no)	1.10 (2.03)	0.166		
Model A-2				
Group (BD/HC)	1.40 (1.06)	<0.0005		
Fasting glucose	1.01 (1.04)	0.714		
Total cholesterol	1.12 (1.11)	0.275		
HDL cholesterol	0.88 (1.12)	0.268		
LDL cholesterol	1.16 (1.12)	0.179		
Triglyceride	1.01 (1.06)	0.867		

b = parameter estimate, expressing ratios of 8-oxoGuo levels based on back-transformed estimates from mixed-model analysis.
BD = bipolar disorder; BMI = body mass index; HC = healthy controls; HDL = high-density lipoprotein; LDL = low-density lipoprotein; SE = standard error.

covariates (*Supplementary Table* 2).To investigate a possible effect of duration of the euthymic state on our analysis, euthymic patients with bipolar disorder were divided into three groups based on duration (model A-3). In this analysis, all three duration groups of patients with bipolar disorder continued to differ significantly compared with healthy control subjects in both 8-oxodG levels [$F(3,91.190) = 5.947$, $p = 0.001$] and 8-oxoGuo levels [$F(3,95.710) = 17.583$, $p < 0.0005$] (*Supplementary Table* 3).

We finally compared levels of DNA and RNA nucleoside oxidation between healthy control subjects and all samples from patients with bipolar disorder divided into groups of affective state (model A-4). This analysis showed that all affective states (euthymia, depression, mania, and mixed) differed from healthy control subjects for levels of both 8-oxodG [$F(4,151.479) = 5.415$, $p < 0.0005$] and 8-oxoGuo [$F(4,203.319) = 13.870$, $p < 0.0005$].

State-specific alterations of oxidatively generated nucleoside damage in patients with bipolar disorder and relationship with demographic and clinical variables

Levels did not differ significantly between affective states in patients with bipolar disorder in unadjusted analysis for either the DNA marker 8-oxodG [$F(3,135.937) = 0.813$, $p = 0.489$] or the RNA marker 8-oxoGuo [$F(3,148.668) = 1.199$, $p = 0.312$] (Fig. 2). Adjusting for demographic and lifestyle variables (model B-1) did not result in statistically significant differences for either 8-oxodG [$F(3,133.549) = 0.386$, $p = 0.477$] or 8-oxoGuo [$F(3,150.573) = 1.415$, $p = 0.241$]. In a secondary analysis (model B-2), there was no significant main effect of illness duration on either 8-oxodG levels [$F(1,32.968) = 0.130$, $p = 0.720$] or 8-oxoGuo levels [$F(1,25.741) = 2.601$, $p = 0.119$], or of total number of episodes on either 8-oxodG levels [$F(1,31.277) = 3.208$, $p = 0.83$] or 8-oxoGuo levels [$F(1,26.084) = 1.594$, $p = 0.218$]. There was no effect of medication on 8-oxoGuo levels, and for 8-oxodG levels there was a main effect of only anticonvulsant treatment [$F(1,146.814) = 6.494$, $p = 0.012$] and antidepressant treatment [$F(1,126.813) = 5.688$, $p = 0.019$]. In both cases, this effect was negative, with patients treated with anticonvulsants having 16% lower levels and patients treated with antidepressants having 15% lower

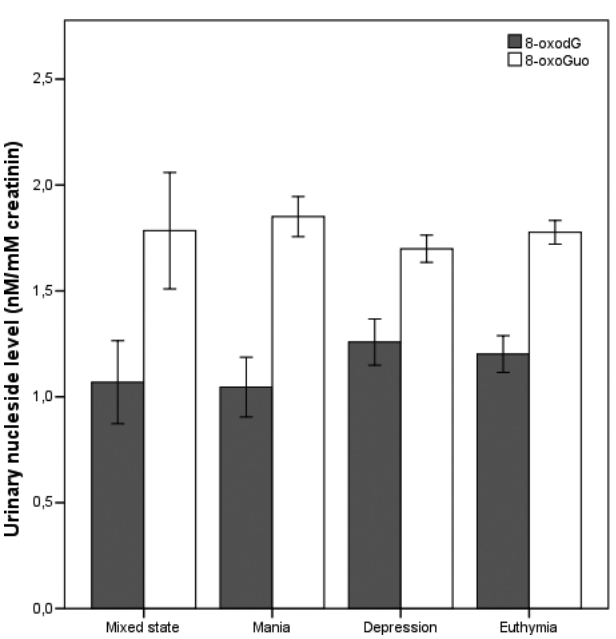

Fig. 2. Urinary levels of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) and 8-oxo-7,8-dihydroguanosine (8-oxoGuo) in patients with bipolar disorder in different affective states (n = 37). Data are expressed as mean ± standard deviation. Linear mixed-model analysis of comparison between affective states: p = 0.8 for 8-oxodG and p = 0.3 for 8-oxoGuo.

levels of 8-oxodG (*Supplementary Table* 4). In a tertiary analysis (model B-3) of possible effects of symptom severity on oxidatively generated nucleoside damage, there was no main effect of HAMD-17 scores on samples from depressive patients on 8-oxodG [$F(1,54.811) = 3.471$, $p = 0.068$] or 8-oxoGuo [$F(1,50.923) = 0.038$, $p = 0.847$], or of YMRS scores on samples from manic patients on 8-oxodG levels [$F(1,21.221) = 3.554$, $p = 0.073$] or 8-oxoGuo levels [$F(1,15.834) = 1.541$, $p = 0.233$].

Changes in oxidatively generated nucleoside damage over time in healthy control individuals

We evaluated the natural variation in both markers by measuring urinary excretion of 8-oxodG and 8-oxoGuo at two time points in healthy control subjects and used a paired-samples *t*-test to elucidate whether there was a statistically significant mean change between the repeated measures, expressed as a ratio between the first and the second measurement. There was no significant change for either 8-oxodG [mean = 1.07, 95% confidence interval (CI): 0.97–1.18, $t(38) = 1.472$, $p = 0.149$] or 8-oxoGuo [mean = 1.01, 95% CI: 0.92–1.11, $t(38) = 0.214$, $p = 0.831$] between the two measurements.

Overall, levels of 8-oxodG and 8-oxoGuo were moderately correlated [$r(246) = 0.46$, $p < 0.0005$].

Discussion

This was the first longitudinal study of patients with bipolar disorder to investigate oxidatively generated damage to nucleosides through different affective phases compared with repeated measurements in healthy control subjects. We demonstrated significantly elevated levels of both the urinary DNA oxidation marker 8-oxodG and the RNA oxidation marker 8-oxoGuo in euthymic patients compared with healthy control subjects, with substantial increases of 40% and 43%, respectively. The increased levels of oxidatively generated nucleoside damage were present through all affective phases of the illness, with no significant difference between affective states. Our results were independent of possible confounders of oxidative stress, indicating that the increased nucleic acid oxidation was not driven by differences in lifestyle or risk factors related to metabolic syndrome between participants.

To our knowledge, this study was the first to demonstrate increased systemic oxidatively generated damage to RNA in patients with bipolar disorder, and the first study specifically to demonstrate increased systemic DNA and RNA damage in *euthymic* patients with bipolar disorder. Our findings of elevated levels in symptomatic patients with bipolar disorder are in accordance with the findings of Soeiro-de-Souza et al. (23), demonstrating elevated levels of 8-oxodG in whole blood in depressed and manic patients with bipolar disorder compared with healthy control subjects, the only other study to investigate *in vivo* oxidation of DNA in bipolar disorder. However, their study employed the enzyme-linked immunosorbent assay (ELISA) for measurement of DNA oxidation, a method that may be less specific compared with UPLC-MS/MS in urinary samples (25, 34), and assessed levels in peripheral blood. Blood levels of 8-oxodG and 8-oxoGuo are mainly determined by kidney function and, when comparing different individuals, are thus unlikely to provide specific information about oxidative stress (35). While urinary 8-oxodG and 8-oxoGuo originate from tissue DNA and RNA, respectively, urinary excretion of oxidized nucleosides is suggested to be independent of changes in DNA repair and RNA breakdown changes and consequently is a more valid measure of 'oxidative stress' to DNA and RNA than blood levels (26, 35).

The present study informs on state versus trait characteristics of oxidatively generated DNA and RNA damage in bipolar disorder, with high levels of both markers across all affective states, indicating that alterations may not primarily be related to the affective state, and thus, more likely, are related to the illness as such. Along these lines, we found no association with illness duration or prior number of episodes and no association between levels of oxidative DNA and RNA damage and symptom severity in symptomatic patients. However, we obtained relatively few samples during manic and mixed states, resulting in low power to detect differences involving these states, possibly leading to type II error. Further, while not significant, our evaluation of repeated measures in healthy control subjects indicated a possible natural variation over time of up to 21% and 19% for the DNA and the RNA marker, respectively, which could result in obscuring smaller intra-individual alterations related to affective state in patients with bipolar disorder, so we cannot entirely rule out the possibility of an effect of affective state on oxidation markers. Conversely, we employed a longitudinal study design that allowed for high sensitivity towards assessing alterations between affective states. Surprisingly, for a disorder characterized by recurrent shifts in affective state, evaluation of intra-individual biological alterations, especially between a depressive and a manic state, is rarely done in bipolar disorder [see

the review on gene expression by Munkholm et al. (10) and the meta-analysis on cytokines by Munkholm et al. (28)]. Similarly, evaluation of the natural variation in biological markers among healthy individuals is usually omitted and not discussed. One other study, investigating variation between two measurements of 8-oxodG one week apart in healthy individuals, found an intra-individual variability of 20% (25), comparable with our findings. There are no similar previous data on intra-individual variability of 8-oxoGuo.

Another important consideration is that of the temporal relationship between changes in systemic conditions, disease status, and oxidative stress. If there is a significant delay in the excretion of oxidative stress markers, the levels may not necessarily reflect the true phenotypical presentation at the time of assessment. Excretion of 8-oxodG has been demonstrated to change within a matter of days (36) or weeks (37), perhaps shorter, after an intervention. This information is not known for 8-oxoGuo; however, the rapid turnover of RNA compared with DNA would suggest more immediate changes in 8-oxoGuo excretion in response to interventions or as a result of other alterations. In the present study, the naturalistic design resulted in samples being obtained at time points with different duration of the current episode. However, we did not find any significant effect of episode duration at sampling time, and the high levels of oxidatively generated damage observed in patients with bipolar disorder were equally present in early remission as well as late remission, compared with healthy controls, indicating either no effect of affective state or a delay of more than eight weeks in normalizing oxidatively modified nucleoside levels after acute affective episodes. However, one cannot entirely rule out the possibility that euthymic periods in this sample of rapid cycling patients with bipolar disorder are too short to ensure full resolution of any changes in oxidatively generated damage to nucleosides related to alterations of affective state, which ultimately could result in the high levels of 8-oxodG and 8-oxoGuo we observed.

Our finding of even higher levels of 8-oxoGuo in patients with bipolar disorder compared with healthy control subjects relative to 8-oxodG is noteworthy and suggests that oxidative damage to RNA could play a greater role than oxidative damage to DNA in patients with bipolar disorder. Oxidative modification of messenger RNA, and, significantly, also of non-coding RNAs (38), appears to affect the translational process in several ways, involving impairment of ribosome function and induction of translational errors, in turn resulting in reduced protein production or production of defective proteins (39). As such, oxidative modification of RNA could potentially constitute an epigenetic mechanism leading to severe and progressive disturbances of cellular function and the organism's ability to maintain cellular integrity.

The magnitude of RNA oxidation in the present study was considerably larger than that recently demonstrated in studies of schizophrenic patients (40) and of patients with depression (41), employing similar laboratory methods. One explanation could be that the episodic nature of bipolar disorder may be especially detrimental to maintaining systemic homeostasis, with a repeated need for adaptation and re-setting of parameters (42), leading to high levels of oxidative stress and increased allostatic load (43). An unstable, unremitting course of illness, as seen in rapid cycling bipolar disorder (44), could, along these lines, be associated with even higher cumulative allostatic load (45), perhaps paralleling findings from diabetes 2, where glucose fluctuations have been demonstrated to have a greater triggering effect than sustained hyperglycemia on oxidative stress (46).

These mechanisms may thus play a role in mediating the progressive nature of bipolar disorder, in which new affective episodes lead to a shortening of inter-episode intervals (47), and late-stage patients experience a poorer treatment outcome (48), factors that are suggested to contribute to a process of neuroprogression (49).

Our findings of increased levels of oxidatively generated damage to DNA and RNA are in line with current pathophysiological hypotheses, supporting a key role for activated IO & NS pathways in bipolar disorder (16). Importantly, oxidatively generated damage to not only DNA, but also RNA may be important an mechanism through which activated IO & NS pathways could potentially contribute to (neuro)degenerative processes in bipolar disorder, similar to that suggested in depression (50).

There are several limitations to the present study. First, the number of samples obtained from patients with bipolar disorder in a manic or a mixed episode was relatively low, possibly leading to a type II error in the comparison between affective states in patients with bipolar disorder. Our cohort consisted both of patients with bipolar I and with bipolar II disorder primarily treated in a specialized mood disorders clinic. This could have contributed to the relatively low number of manic episodes and the relatively low severity of affective episodes during the study period. It may be that affective episodes of increased severity would have resulted in higher levels of oxidative damage in

acute episodes compared with euthymia. Second, given the extent of natural variation in both oxidation markers, the study could have been underpowered to detect subtle differences between affective states. Third, given our limited understanding of the temporal aspects of changes in oxidatively generated nucleoside damage, a causal relationship between bipolar disorder and RNA and DNA oxidation cannot be inferred by our study alone. Fourth, it is possible that euthymic periods are too short to ensure full resolution of any changes in oxidatively generated damage to nucleosides related to alterations of affective state. While our analysis indicated that levels of 8-oxodG and 8-oxoGuo were similar in early and late remission, one cannot entirely rule out the possibility of alterations in oxidized nucleoside levels related to affective state. Finally, we cannot entirely rule out a confounding effect of medication, given the nature of comparison between healthy control subjects and medicated patients with bipolar disorder. Along the same lines, conducting prospective follow-up studies in patients with bipolar disorder without medication would not be realistic, for ethical reasons. However, we found no effect of any type of medication on 8-oxodG and 8-oxoGuo levels in patients with bipolar disorder; if anything, anticonvulsant and antidepressant treatment had a slightly inhibitory effect. Still sparsely studied, inhibition of oxidative processes may be among the mechanisms of action of the mood stabilizers lithium (51) and valproate (52) as well as antipsychotic agents (53) and antidepressants (54). This would be expected to lead to lower levels of oxidized nucleosides in patients treated with these compounds, and not the higher levels demonstrated in the present study, and could suggest that our results do not reflect an effect of medication.

In summary, the present study demonstrated substantially higher levels of oxidatively generated damage to DNA and RNA in euthymic patients with bipolar disorder compared with healthy control subjects, with increases present through all affective states. We suggest a role for oxidatively generated damage to DNA and RNA as a molecular mechanism contributing to the increased risk of medical disorders, shortened life expectancy, and the progressive course of illness observed in bipolar disorder.

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Disclosures

Within the last three years, LVK has been a consultant for Lundbeck and AstraZeneca. MV has been a consultant for Eli Lilly & Co., Servier, AstraZeneca, and Lundbeck. KM and HEP have no biomedical financial interests or potential conflicts of interest to report.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Supplemental material.