Psychological processes in bipolar affective disorder: negative cognitive style and reward processing

Emma Van der Gucht, Richard Morriss, Gill Lancaster, Peter Kinderman and Richard P. Bentall

Background
Psychological processes in bipolar disorder are of both clinical and theoretical importance.

Aims
To examine depressogenic psychological processes and reward responsivity in relation to different mood episodes (mania, depression, remission) and bipolar symptomatology.

Method
One hundred and seven individuals with bipolar disorder (34 in a manic/hypomanic or mixed affective state; 30 in a depressed state and 43 who were euthymic) and 41 healthy controls were interviewed with Structured Clinical Interview for DSM–IV and completed a battery of self-rated and experimental measures assessing negative cognitive styles, coping response to negative affect, self-esteem stability and reward responsiveness.

Results
Individuals in all episodes differed from controls on most depression-related and reward responsivity measures. However, correlational analyses revealed clear relationships between negative cognitive styles and depressive symptoms, and reward responsivity and manic symptoms.

Conclusions
Separate psychological processes are implicated in depression and mania, but cognitive vulnerability to depression is evident even in patients who are euthymic.

Declaration of interest
None. Funding detailed in Acknowledgements.

Progress in understanding the role of psychological processes in bipolar disorder may facilitate the development of interventions for people with this highly disabling illness and also research on underlying biological mechanisms, for example by identifying candidate endophenotypes or by providing targets for neuroimaging studies. Some studies have identified depression-related psychological abnormalities in people with bipolar disorder, for example low1 or unstable self-esteem,2 rumination,3 dysfunctional attitudes to self-evaluation4 and a pessimistic explanatory style,5 which have sometimes been interpreted as evidence that mania arises from dysfunctional strategies for avoiding depression.6 Other studies have found that manic episodes are preceded by goal attainment life events,6 suggesting that excessive reward responsiveness7 may be important in the condition. It is not clear whether these mechanisms are trait vulnerability factors or related to symptoms and episodes. To answer this question we administered multiple measures of depressogenic cognitive biases and reward responsivity to people with bipolar disorder who had remitted, were currently depressed or were currently manic and healthy controls. We predicted that depressive symptoms would be specifically related to depressogenic cognitive styles and manic symptoms would be specifically related to reward responsivity.

Method
The design was a cross-sectional study with four groups broadly matched for age, gender and premorbid intelligence.

Participants
One hundred and seven people with bipolar disorder were recruited from across the North West of England. Potential in-patients were identified and approached by either their consultant psychiatrist or the senior nurse in charge on participating wards. Potential out-patients were identified and approached by their consultant psychiatrist, community mental health team keyworker or lithium clinic doctor to obtain verbal consent to be approached by the researcher. Adverts were also placed in out-patient waiting areas, day hospitals and Pendulum (the Manic Depression Fellowship quarterly magazine for service users).

The inclusion criteria for the patient sample were: a DSM–IV8 diagnosis of bipolar disorder; age 18 years or over; ability to read and write English; and willingness to give written informed consent to the study. Diagnoses were confirmed by Structured Clinical Interview for DSM–IV (SCID)9 and the intellectual comparability of the groups was assessed using the National Adult Reading Test.10 Individuals were excluded if they had a clear organic cause for their disorder or medical comorbidity that put the diagnosis of any bipolar episode in the past 24 months in doubt. Thirty-four participants were allocated to the mania group because they met DSM–IV criteria for manic or hypomanic episode (31) or mixed affective state (3); 30 to the depression group because they met DSM–IV criteria for major depressive episode; and 43 to the remitted group because they had not met DSM–IV criteria for a major depressive, hypomanic, mixed affective or manic episode in the past 2 months and also had a Hamilton Rating Scale for Depression (HRS11) score of eight or less and a score on the Mania Rating Scale (MRS)12 of three or less.

Forty-one healthy controls were recruited via adverts placed around the Royal Liverpool University Hospital, in local libraries, and on the University of Liverpool staff internet message board. Inclusion criteria were not meeting SCID criteria for any psychiatric disorder within the last 2 years; age 18 years or over; ability to read and write English; and willingness to give written informed consent to the study.
Procedures
The study was approved by a National Health Service Multi-centre Research Ethics Committee in accordance with the Helsinki Declaration of 1975. Once the suitability of participants had been confirmed by SCID, participants were administered the HRSD, the Bech–Rafaelsen Mania Rating Scale\(^\text{12}\) and the Cassidy Scale for Manic States.\(^\text{13}\) Assessments were then conducted within the following 7 days in meetings between 11:00 and 18:00 to control for diurnal mood variation in the following order: Personal Style Inventory (PSI);\(^\text{14}\) Behavioural Inhibition System/Behavioural Activation System Scales (BIS/BAS);\(^\text{15}\) Autobiographical Memory;\(^\text{16}\) Pragmatic Inference Task (PIT);\(^\text{17}\) Rosenberg Self-Esteem Scale (RSE);\(^\text{18}\) Modified Response Style Questionnaire (RSQ);\(^\text{19}\) Card Arranging Reward Responsivity Objective Test (CARROT);\(^\text{20}\) and the diary. Participants were debriefed and paid £20 plus travel expenses.

Psychological measures
The following psychological measures were used.
(a) The Personality Style Inventory (PSI)\(^\text{14}\) is a 48-item self-schema measure with sub-scales of autonomy and sociotropy.
(b) The Behavioural Inhibition System/Behavioural Activation System scale (BIS/BAS)\(^\text{15}\) has 20 items with sub-scales measuring behavioural inhibition and three scores for behavioural activation; drive, reward responsiveness and fun seeking.
(c) Autobiographical Memory was assessed using the method of Williams & Broadbent.\(^\text{16}\) Participants attempted to retrieve specific memories to six positive and six negative cue words. Memories were categorised as general extended (e.g. ‘The last time I was in hospital’); general categorical (e.g. ‘When I go to the park’); specific (a memory linked to a particular occasion) or delusional. Reliability of the classification was good (96% agreement between independent raters on the first 50 participants).
(d) The Pragmatic Inference Task (PIT)\(^\text{17}\) is an implicit measure of attributional (explanatory) style, giving internality scores for positive and negative events.
(e) The Rosenberg Self-Esteem Scale (RSE)\(^\text{18}\) is a widely used 10-item self-reported measure of self-esteem.
(f) Modified Response Style Questionnaire (RSQ)\(^\text{19}\) is a 38-item questionnaire with sub-scales measuring rumination, adaptive coping (distraction and problem solving), and risk-taking.
(g) The Card Arranging Reward Responsivity Objective Test (CARROT)\(^\text{20}\) is a three-trial card sorting task. On the third trial participants receive monetary reward for speed and increased speed in this trial is taken as a measure of reward responsivity.
(h) Participants completed a self-esteem diary\(^\text{2,21}\) twice daily for 4 consecutive days at approximately 10:00 and at 22:00. At each time, participants completed the RSE and the Positive and Negative Affect Scale,\(^\text{22}\) a brief measure of mood. Within-participant standard deviations (s.d.’s) were calculated as a measure of variability on these scales.

Statistical analysis
Analyses were carried out using SPSS 14 for Windows. Groups were compared with ANOVA followed by post hoc Tukey honestly significant difference (HSD) tests. Partial correlations were used to examine relationships between psychological measures and symptom scores, controlling for Bech–Rafaelsen scores when analysing relationships with depression, and controlling for HRSD scores when examining relationships with mania. Factor analysis (principal components with varimax rotation) on the psychological measures from the complete sample of 148 was then used to reduce the 17 psychological measures to a minimum number of dimensions, which were then compared between groups. The ratio of sample size to measures, approximately 9:1 exceeded the recommended minimum of 5:1.\(^\text{23}\)

Results
Demographic and clinical measures
Demographic and clinical data are shown in online Table DS1 and Table 1, which also give the results of univariate tests. No differences were observed between the groups for age, IQ, the proportion of males or educational achievement, but the people in the bipolar groups were less likely to be employed and more likely to be living alone compared with the controls. The mania group scored higher on the Bech–Rafaelsen Scale compared with all other groups (minimum \(P<0.0001\)). On the HRSD, the depression group scored higher than all other groups (\(P<0.001\)) but the mania group scored higher than both the euthymia group and controls (\(P<0.001\)). An identical pattern was observed on the Cassidy negative mood sub-scale. On the psychomotor agitation, psychosis and increased hedonic functioning Cassidy sub-scales the mania group scored higher than all other groups (\(P<0.001\)). Finally, on the Cassidy paranoia sub-scale, the mania and depression groups scored higher than the euthymia and control groups (\(P<0.05\)) but not differently from each other.

Diary mood scores are also shown in Table 1. People in the mania group reported more positive affect than those in the euthymia (\(P<0.01\)) and depression groups (\(P<0.001\)), who reported less positive affect than the euthymia (\(P<0.01\)) and control groups (\(P<0.001\)). All three bipolar disorder groups reported higher negative affect than the control group (\(P<0.001\) for all comparisons) and the depression group reported more negative affect than both the mania (\(P<0.001\)) and euthymia groups (\(P<0.001\)), who did not differ on this measure. No differences were observed in variability of positive affect (measured by the within-participant standard deviation of scores) but the depression (\(P<0.001\)) and mania groups (\(P<0.005\)) had greater variability in negative affect than the control group, and the depression group also showed greater variability in negative affect than the euthymia group (\(P<0.01\)).

Depression-related psychological measures
Scores for depressogenic cognitive style are shown in Table 2. Significant main effects were observed on all measures with the exception of the PIT and RSQ adaptive coping. All bipolar groups scored higher than the control group on sociotropy and autonomy (\(P<0.0001\) for each comparison) and the depression group scored higher than the euthymia group on sociotropy (\(P<0.001\)). The control group reported higher self-esteem on the RSE than all three patient groups (\(P<0.005\)) but the mania and euthymia groups scored higher than the depression group (\(P<0.001\) for each comparison). On the twice daily ratings of self-esteem, post hoc analysis revealed that the depression group had a lower mean score than all the other groups (\(P<0.005\) level) and the euthymia group had a lower mean score than the control group (\(P<0.05\)) but there was no significant difference between the controls and mania groups. The control group also showed less variability (as measured by the within-participant standard deviation of scores) in self-esteem compared with the
euthymia (P < 0.05) and depression groups (P < 0.05). No significant differences were observed on the PIT internality scores. On the autobiographical memory test, the mania group recalled fewer specific negative memories than the control group (P < 0.001) but no differences were observed for the recall of positive memories. On the RSQ, all three bipolar groups reported fewer specific negative memories than the control group (P < 0.01 for each comparison) and mania group (P < 0.05 for each comparison) scored lower than the euthymia and depression groups. On BAS drive, the mania group scored higher than those in the depression (P < 0.001) but not the controls. On BAS fun seeking the mania group scored higher than the euthymia group (P < 0.05) but no differences were observed for the reward responsiveness sub-scale. No significant differences were observed on the CARROT.

**Reward processes**

Table 3 shows scores on the BIS/BAS scale and the CARROT. On the BIS (a measurement of sensitivity to punishment-related stimuli) the controls (P < 0.001 for each comparison) and mania group (P < 0.05 for each comparison) scored lower than the euthymia and depression groups. On BAS drive, the mania group scored higher than those in the depression (P < 0.001) and euthymia groups (P < 0.05) but not the controls. On BAS fun seeking the mania group scored higher than the euthymia group (P < 0.05) but no differences were observed for the reward responsiveness sub-scale. No significant differences were observed for the reward responsiveness sub-scale. No significant differences were observed on the CARROT.

**Relationships between psychological measures and symptoms**

Bech–Rafaelsen and HRSD scores were significantly correlated (r = 0.24, P < 0.005, d.f. = 146). Therefore, partial correlations, shown in Table 4, were used to examine the relationships between psychological variables and mania and depression scores, in each case controlling for the effects of the other mood state. A clear pattern can be seen, with strong correlations between depression and measures of negative cognitive style (sociotropy, autonomy, self-esteem and rumination) and weaker correlations between measures of positive cognitive style (hedonic functioning, positive events, positive scores).
manic symptoms and reward-related measures (the BAS scale, with a trend towards significance with the CARROT). These observations were confirmed using factor analysis (principal components with varimax rotation, missing data replaced by mean values) on all questionnaire measures and the CARROT, which yielded five easily interpretable factors: negative cognitive style (high loadings for sociotropy, autonomy, BIS and rumination, 22.5% of the variance), excitement (high loadings for the three BAS scores and RSQ risk taking, 18%), PIT pessimism (interanal for negative events, 10%), PIT optimism (interanal for positive events, 9%) and CARROT scores (9%); total variance accounted for 69.80%. When these factor scores were partially correlated against the Bech–Rafaelsen and HRSD scores, the former robustly correlated with the excitement factor (r=0.36, P<0.001, d.f.=144) but not the negative cognitive style factor (r=-0.01, P=0.64, d.f.=144), whereas the latter robustly correlated with the negative cognitive style factor (r=0.43, P<0.001, d.f.=144) but not the excitement factor (r=-0.09, P=0.25, d.f.=144).

Significant differences between the groups were observed for the negative cognitive style (F(3,144)=22.32, P<0.001), excitement (F(3,144)=5.69, P<0.001) and pessimism factors (F(3,144)=3.87, P=0.01). Interestingly, ANCOVA with Bech–Rafaelsen and HRSD scores as covariates revealed that the differences observed for negative cognitive style remained even after symptom scores were controlled for (F(3,144)=10.08, P<0.001); planned contrasts revealed that both the euthymia (P<0.0001) and depression groups (P<0.0001) scored higher than the controls in this analysis. The group differences in excitement and pessimism did not remain after controlling for symptoms.

### Discussion

In this study we aimed to investigate depressogenic cognitive styles and reward responsivity in a well-characterised and large sample of people diagnosed with bipolar disorder. When individuals in different episodes were compared with healthy controls, the results largely replicated those obtained in previous studies, with people with bipolar disorder in all phases showing high levels of sociotropy and autonomy and low self-esteem, self-esteem instability, rumination and, less clearly, impairment in the ability to recall specific autobiographical memories. On all except the autobiographical memory measure, the euthymia group’s results were abnormal compared with the controls. Less robust evidence of abnormal reward responsivity was evident in the group comparisons, but the mania group scored higher than the controls on two of the BAS scores (drive and fun seeking). Only the results from the PIT failed to replicate previous studies, which had indicated a pessimistic attributional style in patients with remitted2,17 and currently symptomatic bipolar disorder.

### Table 3 Mean (95% CI) scores for controls, and mania, depression and euthymia bipolar disorder groups on the study instruments

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Control (n=157)</th>
<th>Euthymia (n=149)</th>
<th>Depression (n=145)</th>
<th>Mania/mixed (n=148)</th>
<th>F (3,141)</th>
<th>Tukey HSD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BIS/BAS scale</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drive</td>
<td>9.95 (9.05–10.81)</td>
<td>9.72 (8.91–10.53)</td>
<td>8.66 (7.55–9.76)</td>
<td>11.65 (10.19–13.10)</td>
<td>5.01**</td>
<td>M=D=E</td>
</tr>
<tr>
<td>Reward responsiveness</td>
<td>15.55 (14.74–16.38)</td>
<td>15.72 (14.91–16.53)</td>
<td>15.66 (14.50–16.81)</td>
<td>16.24 (15.03–17.44)</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td><strong>CARROT, reward responsivity</strong></td>
<td>3.59 (3.95–5.22)</td>
<td>2.49 (2.11–4.87)</td>
<td>5.03 (4.32–7.38)</td>
<td>4.35 (3.10–8.76)</td>
<td>0.73</td>
<td></td>
</tr>
</tbody>
</table>

**BIS/BAS, Behavioural Inhibition System/Behavioural Activation System; CARROT, Card Arranging Reward Responsivity Objective Test; HSD, honestly significant difference; D, depression group; M, mania/mixed group; E, euthymia group; C, control group.**

A crucial issue when interpreting these and previous findings is the relationships between the measures and mood state. For example, previous reports of depressogenic processes during mania, although plausibly reflecting defensive processes, might more simply reflect the coactivation of depressogenic and reward-related processes in mixed episodes. Our factor analysis, which attempted to address this problem, yielded an easily interpretable model that accounted for a very large amount of the variance in the data. The high loadings of poor self-esteem, sociotropy, autonomy, BIS and rumination on a single factor suggest that these are highly related processes and might be considered to form a negative cognitive syndrome. The fact that the PIT and CARROT appeared as separate factors suggests that they are poor indicators of negative cognitive style and reward processing respectively, which probably accounts for their failure to discriminate between the groups.

A clear and specific relationship was observed between negative cognitive style and depression. However, it is notable that...
the negative cognitive style was still evident in the euthymia group, even after symptoms were controlled for. One possible interpretation of this finding is that negative cognitive style is a vulnerability factor in people with bipolar disorder that, when activated, leads to spiraling negative thoughts about self, rumination and eventually severe depression. By contrast, the factor we have characterised as excitement, consisting of high responsivity to reward signals and excessive risk taking, seems to be clearly state-related and associated with current mania.

The finding that bipolar depression and bipolar mania are related to distinct psychological processes is consistent with recent findings that have suggested that mania is not simply the opposite of depression. It is also consistent with the finding that depression and mania are provoked by distinct kinds of life events.

Limitations
A limitation of this study is that we were unable to take into account the effects of treatment on the psychological processes we were measuring. However, any such effects would reduce the variance in our data and thereby reduce our opportunity to demonstrate significant associations between the measures and psychopathology. A more important limitation is that the study was cross-sectional, and hence could not address the evolution of bipolar symptoms (an inherently dynamic process) over time. It has recently been suggested that neither depressogenic cognitive styles nor reward sensitivity are sufficient to account for the complexity of bipolar phenomena and that higher level cognitive appraisals play an important role in the ascent into mania. More complex longitudinal designs will be required to test these accounts.

Implications
The major clinical implication of the present study is that attention must be given to the two types of psychological processes we have identified when designing interventions for people with bipolar disorder. Given that dopaminergic function is implicated in the anticipatory processing of reward stimuli, it is unsurprising that dopamine-blocking drugs are effective in treating manic states. It might be useful to consider the effects of other pharmacological interventions, for example mood stabilisers, on negative cognitive styles and reward processing. In humans there is evidence that lithium and carbamazepine normalise self-esteem thereby preventing relapse. In animals, there is evidence that lithium can attenuate some dopamine-induced behaviours thought to be related to reward responsivity.

With respect to psychological interventions, our recent trial of cognitive therapy yielded disappointing results and it might be argued that other trials have been more successful because they have more thoroughly incorporated techniques to prevent excessive responding to reward stimuli. Further advances in the psychological treatment of people with bipolar disorder are likely to be achieved following a more thorough understanding of the processes involved in the evolution of bipolar symptoms.

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References


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**Poems by doctors**

**Watching someone die**

Lenrie Peters

Watching someone die is a fraudulent experience
The deep significance is felt
the meaning escapes
like a child’s first punishment.
The dying ravish your strength
whether by throttle of convulsive gasp
or tideless fading away
like ancient familiar sounds in sea shells
the moment is the same
reinforced brutality to life
a rugged cliff bloodstained
with the agonising rhythm of many heads.
A cold demise; each
successive moment a banishment.
The terror is in leaving behind
the ache is in departing.
Humming fantasies crowd their stings
to seize and record the moment
the hands curl in spasm
to hold it back; this life, this infidel.
It is too late. Everything and nothing
has happened. A huge machine
the earth, grinds to a bolt-knocking halt.
It is the changing of the tide
at the boundary hour
Life like a handful of feathers
engulfed by cliff winds
one like yourself swept
Oh so swiftly into the anchorage of history
Tears and sighs; sighs and tears
stamping the leaden feet
the solid agony of years
they all abound.
One life or a million
contrived by nature or by man
greatly obscures the issue.
Face to face with dying
you are none-the-wiser
Yet it seems a most ignoble epitaph
“He was a man and had to die; after all.”

Lenrie Peters was born in Bathurst (Banjul), The Gambia, in 1932. He studied medicine at Trinity College Cambridge and later trained as a surgeon. He currently practises in The Gambia. He has been Chairman of the West African Examinations Council. He has published one novel, *The Second Round*, and four volumes of poetry – *Poems* (Mbari Press, 1964), *Satellites* (Heinemann, 1967), *Katchikali* (Heinemann, 1971) and *Selected Poetry* (Heinemann, 1981). He is the Officer of the Republic of the Gambia. This poem is taken from *Satellites* by kind permission of the author.

Selected by Femi Oyebode.
## Data supplement

### Table DS1  Demographic and clinical characteristics of participants

<table>
<thead>
<tr>
<th></th>
<th>Control (n=41)</th>
<th>Euthymia group (n=43)</th>
<th>Depression group (n=38)</th>
<th>Mania/mixed group (n=34)</th>
<th>Statistical comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, years: mean (95% CI)</strong></td>
<td>47.90 (43-31–52.50)</td>
<td>47.53 (43.61–51.56)</td>
<td>46.17 (42.62–49.71)</td>
<td>45.55 (41.03–50.09)</td>
<td>F=0.28</td>
</tr>
<tr>
<td><strong>Female, n (%)</strong></td>
<td>21 (51)</td>
<td>25 (58)</td>
<td>22 (73)</td>
<td>21 (62)</td>
<td>χ²=3.65</td>
</tr>
<tr>
<td><strong>Employed (full or part time), n (%)</strong></td>
<td>28 (68)</td>
<td>18 (42)</td>
<td>11 (37)</td>
<td>9 (27)</td>
<td>χ²=14.73**</td>
</tr>
<tr>
<td><strong>Educated over 16 years old, n (%)</strong></td>
<td>32 (78)</td>
<td>31 (72)</td>
<td>21 (70)</td>
<td>21 (62)</td>
<td>χ²=3.05</td>
</tr>
<tr>
<td><strong>NART IQ, mean (95% CI)</strong></td>
<td>120.63 (119.49–121.76)</td>
<td>119.74 (118.33–121.14)</td>
<td>121.29 (120.01–122.57)</td>
<td>118.58 (117.04–120.13)</td>
<td>F=2.55</td>
</tr>
<tr>
<td><strong>Living alone, n (%)</strong></td>
<td>7 (17)</td>
<td>12 (28)</td>
<td>8 (27)</td>
<td>18 (53)</td>
<td>χ²=11.93*</td>
</tr>
<tr>
<td><strong>Prescribed mood stabiliser, n (%)</strong></td>
<td>37 (88)</td>
<td>25 (83)</td>
<td>27 (79)</td>
<td>27 (79)</td>
<td>χ²=0.60</td>
</tr>
<tr>
<td><strong>Prescribed antidepressant, n (%)</strong></td>
<td>17 (40)</td>
<td>17 (57)</td>
<td>14 (41)</td>
<td>14 (41)</td>
<td>χ²=2.37</td>
</tr>
<tr>
<td><strong>Prescribed antipsychotic, n (%)</strong></td>
<td>7 (16)</td>
<td>8 (27)</td>
<td>19 (56)</td>
<td>19 (56)</td>
<td>χ²=7.50</td>
</tr>
<tr>
<td><strong>Currently in receipt of psychological therapy, n (%)</strong></td>
<td>2 (5)</td>
<td>1 (3)</td>
<td>1 (3)</td>
<td>1 (3)</td>
<td>χ²=0.17</td>
</tr>
<tr>
<td><strong>Current in-patient, n (%)</strong></td>
<td>0 (0)</td>
<td>9 (30)</td>
<td>26 (77)</td>
<td>26 (77)</td>
<td>χ²=50.58***</td>
</tr>
<tr>
<td><strong>Age at first episode, years: mean (95% CI)</strong></td>
<td>24.05 (21.63–26.47)</td>
<td>21.00 (17.02–24.98)</td>
<td>23.87 (20.97–26.77)</td>
<td>23.87 (20.97–26.77)</td>
<td>F=1.22</td>
</tr>
<tr>
<td><strong>Bipolar episodes (self-report), n: median (range)</strong></td>
<td>12 (4-30+)</td>
<td>22 (5-30+)</td>
<td>10 (2-30+)</td>
<td>10 (2-30+)</td>
<td>F=0.62</td>
</tr>
<tr>
<td><strong>Psychiatric admissions (self-report), n: median (range)</strong></td>
<td>4 (0-30+)</td>
<td>3 (0-22)</td>
<td>5 (1-19)</td>
<td>5 (1-19)</td>
<td>F=0.62</td>
</tr>
</tbody>
</table>

NART, National Adult Reading Test.  
*P<0.01; **P<0.005; ***P<0.001.
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