Revision

Utility of measuring (1,3) β-D-glucan in cerebrospinal fluid for the diagnosis of fungal central nervous system infection

(Running head: CSF and serum BDG for CNS fungal infections)

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Abstract

(1-3)-β-D-glucan (BDG) from cerebrospinal fluid (CSF) is a promising marker for diagnostic and prognostic aid of central nervous system (CNS) fungal infection, but its relationship to serum values has not been studied. Herein we detected BDG from CSF at levels 2-fold lower than serum in patients without evidence of fungal disease but 25-fold higher than in serum in non-cryptococcal CNS fungal infections. CSF BDG may be a useful biomarker in the evaluation of fungal CNS disease.
(1,3)-β-D-glucan (BDG) is a fungal cell wall polysaccharide (Cryptococcus and mucoralean fungi generally excepted). Circulating BDG is sometimes detectable at low levels (<60 pg/mL) in blood of asymptomatic individuals and is elevated in patients with invasive fungal infections (1). The United States (U.S.) Food and Drug Administration (FDA) has approved testing of BDG in serum to aid in diagnosis of invasive fungal infections. Clinical studies have demonstrated that the test marketed in the U.S. has a sensitivity of 75-77% and a specificity of 60-80% in the diagnosis of candidiasis in intensive care unit patients and aspergillosis in lung transplant patients with a cutoff value of ≤60 pg/mL as negative and ≥80 pg/mL as positive. (2-4).

Cerebrospinal fluid (CSF) BDG measurement has not been validated. Its utility was first described in a non-neutropenic rabbit model of experimental hematogenous Candida meningoencephalitis (5). Lyons, et al (6) and Litvintseva, et al (7) showed its diagnostic and prognostic utility during the 2012 U.S. fungal central nervous system (CNS) infection outbreak. Our study further explores the utility of CSF BDG and the relationship between serum and CSF BDG in CNS fungal and non-fungal disease.

We selected CSF and serum samples collected for routine care between 2007 and 2013 and frozen as part of a research protocol approved by Johns Hopkins Institutional Review Board. We selected subjects whose CSF and serum were collected within 24 hours of one another. There was only one serum and one CSF specimen available per patient. Diagnoses were confirmed by chart review and were simultaneous with the timing of sample acquisition.

Demographic information including gender, age, race, clinical diagnosis, and indication for lumbar puncture were documented. Pertinent clinical information gathered included history of fungal infection or other CNS infection, HIV status, and history of organ transplantation or other evidence of...
immunosuppression. Culture, serology, histopathology and neuroimaging data were collected for diagnostic purposes. Fungal infections were defined as proven, probable, or possible based on EORTC/MSG criteria (8) or CDC definitions for outbreak-associated meningitis (9).

Sterilely collected CSF and serum specimens had been frozen to -80°C and were shipped on dry ice to the Beacon Diagnostics Laboratory for testing via the Fungitell® assay (Associates of Cape Cod, Inc., East Falmouth, MA). All assays were performed in a biosafety cabinet that had not been used to manipulate fungal cultures. Glucan-free dilution tubes and pipette tips were used. CSF and serum samples were equilibrated to room temperature, vortexed, and tested in duplicate or triplicate using kit manufacturer protocol. The final result was the mean of the replicate or triplicate readings. Spectrophotometric analysis was performed using a BioTek ELx 808 iu Microplate Reader (BioTek Instruments Inc., Winooski, VT). Results were compared to a standard curve derived from serially diluted standard provided with limit of detection to <31 pg/mL. Samples with titers above 500 pg/mL were diluted and retested. Samples with titers below 31 pg/mL were rerun with extended low range standard curves (3.9 pg/mL to 125 pg/mL). Individuals performing the assay were blinded to all diagnostic and clinical information.

Patient demographic and clinical characteristics were summarized using appropriate descriptive statistics, e.g., counts and percents, median and interquartile range (IQR). Non-parametric analysis methods were used due to non-Gaussian distribution of values. Patients with no evidence of CNS fungal infection were compared to those with probable and proven CNS fungal disease with Wilcoxon rank-sum tests. Wilcoxon signed-rank tests were used to determine the statistical differences between levels of BDG in paired CSF and serum. Spearman correlation coefficients were used to determine the relationship...
between BDG levels and CSF pleocytosis. Logistic regression analysis and receiver operating characteristic (ROC) curves were plotted to determine the best cutoff value for CSF BDG to diagnose CNS fungal infection. Sensitivity, specificity and exact binomial 95% confidence intervals (CIs) for each were calculated. Analyses were performed using SAS version 9.3 (SAS Institute, Inc., Cary, NC). All tests were two-sided and considered significant at p<0.05.

Paired serum and CSF samples were available on 92 patients. Median (range) age was 47 (18–97) years; 44 (52%) were female, 39 (46%) African American, and 39 (46%) Caucasian. 27 (32%) patients were HIV infected with median (range) CD4 count of 98 (1–320) cells/uL and HIV plasma RNA 102,000 (<20–406,000) copies/mL.

Clinical characteristics of the patients are presented in Table 1. A total of 66 patients were categorized as having non-fungal infection, 10 had proven, probable, or possible fungal infection with no evidence of CNS involvement, 3 had proven or probable non-cryptococcal CNS fungal infection, and 6 had definitive cryptococcal meningitis (CM). Seven patients were excluded from analysis because their BDG testing results were inconclusive due to the presence of interfering substances.

Ten of 66 (15%) without fungal infection had undetectable CSF BDG levels (<4pg/mL), and 56 had detectable levels, ranging from 4 to 109 pg/mL. Median CSF BDG was lower than paired serum (p<0.001), with serum:CSF BDG ratio of 2.24 (range 0.3-45.3) (Table 1).

All 10 non-CNS proven, probable, or possible fungal infection patients had measurable CSF BDG, but the median was lower than that in serum (p=0.03), with median ratio of serum:CSF BDG of 14.9 (range 0.02-97.9) (Table 1).
In the six patients with CM infection, 5 (83%) had measurable CSF BDG levels that were comparable to serum values. Median ratio of serum:CSF BDG was 1.17 (range 0.43-7.5).

In the three patients with proven and probable CNS fungal infection, all had detectable CSF BDG, ranging from 110 to 1524 pg/mL, which were much higher than serum BDG (serum:CSF BDG=0.04 (0.02-0.13)).

ROC analysis for CSF BDG values demonstrated a cut point of 110 pg/mL. At this value, sensitivity was 100% (95% CI=29-100%) and specificity 96% (95% CI=89-98%), with an area under the curve of 0.982.

Patients with probable or definitive CNS fungal infection had significantly higher CSF BDG (p=0.01) and lower serum:CSF ratios (p=0.01) than patients with non-fungal CNS disease. Serum BDG was not significantly different between the groups (p=0.94).

There were no CSF BDG differences when analyzed by presence (N=33) or absence (N=52) of CSF pleocytosis (p=0.68). Among HIV patients (N=27), there was no difference between those with (N=14) and without (N=13) CSF pleocytosis (p=0.62).

We detected low CSF BDG levels in patients without evidence of fungal infection. CSF BDG levels remained low in non-CNS fungal infection but were significantly higher in three cases of CNS fungal infection. CSF BDG was lower than in paired serum for those without evidence of fungal infection, implying that the positive cutoff value for CSF may be lower than serum. However, the level of BDG was significantly higher in serum than in CSF in patients with non-CNS proven/probable/possible fungal infection versus those with CNS fungal infections. This suggests the integrity of the blood-brain barrier in walling off CNS fungal infections, preventing spillover. Conversely, we found BDG level was significantly higher in CSF than in serum in three cases of proven/probable CNS
fungal infection. This could suggest isolated CNS infection without significant
spillover into venous blood or that BDG may be more rapidly cleared in blood
than in CSF, as indicated in the fungal meningitis rabbit model (5) and suggests
that serum values may not be helpful in determining CNS invasion of a fungal
pathogen.

It is not clear why BDG levels were higher in CSF than serum in case
numbers 4 and 5 with non-CNS fungal infections (Table 1). In case number 4
with possible fungal sinusitis, by sheer proximity of the sinuses to the dura and
arachnoid and due to the chronicity of infections in the patient, it is plausible that
the process had extended to the subarachnoid space. This notion has been
documented in bacterial meningitis resulting from extension of sinusitis and in
rhinocerebral mucormycosis, which frequently arises from extension of a sinus
infection. In case number 5 with probable coccidioidomycosis, possible
involvement of CNS cannot be excluded.

Similar to that found by Litvintseva (7), our cutoff of 110 pg/mL
demonstrates both high sensitivity and specificity of CSF BDG, although given
the low number of true positives, the former is difficult to interpret. Our study
adds comparison of CSF BDG to serum BDG, and our findings suggest that
testing from CSF may be more useful than from serum in evaluating CNS
disease.

In the *Exserohilum* outbreak cases, CSF BDG levels anecdotally did not
correlate with CSF pleocytosis (6, 7). We found the same here. Likewise, in HIV
patients with pleocytosis, CSF BDG was not elevated, suggesting that the degree
of inflammation and fungal BDG shedding may be disparate.

Despite that *Cryptococcus* produces little to no BDG, studies have
reported detectable serum BDG in cryptococcosis (4, 10, 11). CSF BDG in CM
had not been assessed until a recent, proven case with CSF BDG of 331 pg/mL
and paired serum BDG of <7 pg/mL (12). In our six CM patients (cryptococcal
antigen titer ranging from <1:5 to 1:160; three patients were HIV positive [Table
1]), BDG was detectable but at low and comparable levels in serum and CSF,
suggesting limited utility in this disease, although additional factors, such as
fungal disease burden, may account for this discrepancy. In fact, rapid and
accurate diagnosis of the disease can be reliably achieved by detection of
cryptococcal antigen in CSF.

There are several limitations to this study, including its retrospective
design; therefore, circumstances that might give rise to false positive results were
not controlled for, especially since the role of the blood-brain barrier in preventing
BDG transport across has not been delineated. These circumstances include but
are not limited to history of hemodialysis, blood transfusion, intravenous
immunoglobulin use, and certain antibiotic use. Additionally, although we found
no differences in BDG levels from serum versus CSF between those with and
without HIV, it is difficult to know the effect HIV may have. Finally, the small
number of CNS fungal infection cases precludes generalizability of our findings
and can only be used to call for further study.

Our findings suggest elevated CSF BDG could be useful for diagnosing or
excluding fungal CNS infections. Additionally, it may be an important determinant
of fungal disease well before organism growth in culture. Testing of larger
cohorts is necessary to determine whether CSF BDG can be used to aid in
diagnosis, and predictive capabilities as well as evaluation for false positives
need to be determined.
Acknowledgement

Jennifer Lyons, Kiran Thakur, Rick Lee, Tonya Watkins, Carlos Pardo, Kathryn Carson, Karen Roos, and Sean Zhang declare no competing interests. Barbara Markley and Malcolm Finkelman are both employees of Associates of Cape Cod, the manufacturer of the Fungitell® assay. Kieren Marr has worked as a consultant, on advisory boards, and has received grants from Astrellas, Merck, and Pfizer. She has also licensed diagnostic technology to MycoMed Technologies. The statistical analysis was supported by the National Center for Research Resources and the National Center for Advancing Translational Sciences (NCATS) of the National Institutes of Health [grant number 1UL1TR001079] to Kathryn A. Carson.
References


Table 1. Level of BDG in paired serum and CSF samples of patients with or without CNS fungal infection

<table>
<thead>
<tr>
<th>Disease Category</th>
<th>Patient(s)</th>
<th>Paired Serum and CSF BDG levels (pg/mL)</th>
<th>No. patient w pleocytosis</th>
<th>No. patient w HIV</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Serum*</td>
<td>CSF*</td>
<td>p value*</td>
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<tr>
<td>Non fungal infections</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>- Multiple sclerosis</td>
<td>9</td>
<td>20 (10 – 35)</td>
<td>9 (4 – 28)</td>
<td>0.02</td>
</tr>
<tr>
<td>- Primary headaches</td>
<td>10</td>
<td>27.5 (12 – 64)</td>
<td>15.5 (&lt;4 – 27)</td>
<td>&lt;0.02</td>
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<td>- Pseudotumor cerebri</td>
<td>5</td>
<td>28 (18 – 58)</td>
<td>17 (13 – 43)</td>
<td>0.31</td>
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<tr>
<td>- Other CNS abnormalities</td>
<td>24</td>
<td>28 (11 – 1334)</td>
<td>14 (&lt;4 – 109)</td>
<td>0.10</td>
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<td>- Bacterial/Viral CNS infection</td>
<td>18</td>
<td>25.5 (7 – 397)</td>
<td>13 (&lt;4 – 71)</td>
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<td>Subtotal</td>
<td>66</td>
<td>26.5 (7 – 1344)</td>
<td>13.5 (&lt;4 – 109)</td>
<td>&lt;0.001</td>
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<td>Non CNS fungal infections</td>
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<td>- Proven invasive candidiasis</td>
<td>No.1</td>
<td>1051</td>
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<td>- Probable invasive aspergillosis</td>
<td>No.2</td>
<td>894</td>
<td>40</td>
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<td>- Proven fungal sinusitis/mastoiditis^1</td>
<td>No.3</td>
<td>201</td>
<td>57</td>
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<td>- Possible fungal sinusitis^1</td>
<td>No.4</td>
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<td>685</td>
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<td>- Probable disseminated coccidioidomycosis</td>
<td>No.5</td>
<td>14</td>
<td>139</td>
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<td>No.6</td>
<td>1179</td>
<td>158</td>
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<td>- Probable fungal pneumonia</td>
<td>No.7</td>
<td>2,100</td>
<td>34</td>
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* Data presented as median (range).
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<th></th>
<th>No.</th>
<th>260</th>
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<td>Subtotal</td>
<td>10</td>
<td>472.5 (11-2100)</td>
<td>48.5 (5-685)</td>
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<td>14.9 (0.02-97.9)</td>
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<td>-Cryptococcus meningitis</td>
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<td>1</td>
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<td>No.2</td>
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<td>-Probable cerebral</td>
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<td>110</td>
<td>-</td>
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<td>-Proven <em>Exserohilum</em></td>
<td>No.8</td>
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<td>-Probable <em>Exserohilum</em></td>
<td>No.9</td>
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<td>1524</td>
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<td>27</td>
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* Median (range) for group data or value for individual data.
# \( p \) value was determined by Wilcoxon signed-rank test
† CSF cryptococcal antigen titer determined by a lateral flow assay (IMMY, OK, USA). All six patients had positive serum cryptococcal antigen results by the lateral flow assay.
^ This fungal sinusitis case was proven based on histology examination. The right mastoid excision tissue from the patient showed fungal hyphae forms. However, culture was negative.
^ This patient had chronic sinusitis refractory to multiple courses of antibiotics empirically and was diagnosed clinically as having possible fungal sinusitis.
♪ This patient had a positive CSF Histoplasma antigen.