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Serum S-100B as a Potential Biomarker for Meningitis in Febrile Infants: An Interim Analysis

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SERUM S-100B AS A POTENTIAL BIOMARKER FOR MENINGITIS IN FEBRILE INFANTS

AN INTERIM ANALYSIS

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SERUM S-100B AS A POTENTIAL BIOMARKER FOR MENINGITIS IN FEBRILE INFANTS – AN INTERIM ANALYSIS. Kelvin C. Lau, Melissa L. Langhan, and Kirsten A. Bechtel. Section of Emergency Medicine, Department of Pediatrics, Yale University School of Medicine, New Haven, CT.

The purpose of this study is to identify the utility of serum S-100B levels as a marker for meningitis in febrile infants younger than 3 months of age.

All infants younger than 3 months of age who presented to the Pediatric Emergency Department (PED) of Yale-New Haven Children's Hospital and required both a lumbar puncture and venipuncture due to either a confirmed rectal temperature ≥38.0ºC or an overall presentation that concerned the responsible physician for possible meningitis and required a lumbar puncture as part of their PED evaluation, were prospectively enrolled. A total of 111 patients participated after a 1.5-year recruitment period or about 40% of the 260 subjects calculated a priori to be required over 3 years in order to achieve 80% power. After informed written consent, approximately 1 mL of blood was obtained for the analysis of the serum level of S-100B, in addition to the volume normally drawn for standard laboratory analysis. Patients with confirmed meningitis as defined by a positive viral or bacterial culture, a positive polymerase chain reaction (PCR) for enterovirus or herpes simplex virus, and/or cerebrospinal fluid (CSF) pleocytosis, were compared with those subjects without meningitis. S-100B levels for 101 subjects were available for interim analysis, of which 27 (26.7%)met the criteria for meningitis and 74 (73.3%) did not.

The median S-100B level in infants with meningitis was 247.0 ng/L (95% CI: 103.5, 804), as compared to 199.1 ng/L (142.5, 384.0) in those without meningitis ($p>0.05$). Receiver operating characteristic analysis revealed an area under the curve of 0.4917 (0.3940, 0.5863). Ad hoc power calculations demonstrated a 57% probability of detecting a difference of 390 ng/L between the two groups when using this sample size.

At this time, this interim analysis of this ongoing study suggests that a larger sample size will still be required to determine if serum S-100B is a useful marker for meningitis in febrile young infants. Because CSF fluid analysis and the associated risks of lumbar puncture remain the only means by which to identify infants with meningitis, the search for a simple serum test for to determine the likelihood of meningitis will continue to be worthwhile.

Introduction

Fever among infants younger than 3 months of age is a common and distressing event for caregivers, which often leads to medical evaluation in either primary care physician offices or emergency departments. In this group of young febrile infants, the main concern for medical practitioners is the identification of a viral or bacterial source for the hyperpyrexia. Most worrisome of the potential serious infections in this age group is meningitis, especially bacterial meningitis.

Fortunately, self-limited viral infections have been cited as the most common cause of fever in young infants.[1] Recent evidence further suggest that particular viral agents, such as respiratory syncytial virus (RSV) or influenza A, tend to be the exclusive etiology of fever in young infants and subsequently reduce the risk of concomitant serious bacterial infection (SBI).[2, 3] However, the incidence of viral meningitis, most often due to enterovirus, in the group of young febrile infants may still be as high as 13.5 percent.[1]

SBI, defined as bacteremia, bacterial gastroenteritis, pneumonia, urinary tract infection, and meningitis, among febrile infants less than 90 days old has an average cumulative incidence of approximately 9 percent in a review of 18 studies from 1977-1993.[4] In turn, cumulative incidence of bacterial meningitis in the same age group was around 1.6 percent during this period of study. Since then, the incidence rate of the leading cause of bacterial meningitis in children less than 5 years old, *Haemophilus influenzae* type B, has continued to decline to near elimination due to the inclusion of the *H. influenza* type B conjugate vaccine into the vaccination schedule starting at age 2 months.[5] In spite of the low incidence of SBI in young infants today, a delayed or

missed diagnosis of bacterial meningitis may result in dire yet preventable consequences, such as subdural empyema, hydrocephalus, cerebral infarction, seizures, and death.[6, 7] Cognitive and executive functioning are also affected for at least a decade after the diagnosis, with a more pronounced deficit in children afflicted before 12-months of age.[8] Such persistent sequelae occurs in up to 50 percent of survivors, in spite of advancements in treatment.[9, 10] Early identification of patients at risk of meningitis, due to either bacteria or viruses, is thus imperative.

The assessment of young febrile infants has kindled passionate debate among physicians regarding the management of these patients.[11] What alarms physicians is not necessarily the ill-appearance of an infant with an obvious source of infection, as a quick physical examination in these patients likely would direct diagnostic approach and rapidly result in initiation of treatment. Rather, controversy centers on those infants who seemingly appear healthy, but in whom fever raises the possibility of an underlying serious illness. Various prospective studies of infants younger than 90 days old have shown that as many as 7% of well-appearing febrile infants may in fact have meningitis.[12-15] Similarly, fever may be the only dependable clinical manifestation of infants with meningitis.[6, 7, 16, 17] This is troubling, as the limited, if not virtual absence, of social interaction skills necessarily elevates the importance of visual inspection in the early assessment of febrile infants younger than 3 months. Measures developed to facilitate the evaluation of these patients, including one well-investigated clinical observation scale,[18] unfortunately have been undependable when specifically applied to either children under 2,[13, 19] and especially those 3 months of age.[1, 14] Physical examination of this young group of infants thus may still be inadequate in the

identification of an etiology for fever and may not confidently exclude meningitis and other serious illnesses. Research has thus since focused upon specific clinical and laboratory factors, or a combination thereof, that would identify healthy-appearing infants at particularly high risk for meningitis or SBI.

Several protocols for the management of fever in infants less than 3 months of age have been published.[1, 14, 15] Each of these protocols have excellent negative predictive values (94.6-99.7%) and are able to guide practitioners as to which febrile infants are at lower risk for SBI. However, because of their poor positive predictive values (12-14%), many infants, may undergo hospitalization and treatment with antibiotics due to erroneous classification as being high-risk for SBI. Although the positive predictive value for the Boston criteria was not provided, the authors did provide data that their strategy is more cost-effective because their empiric use of antibiotics in both high- and low-risk patients led to a lower overall hospital admission rate.[20]

Assessment for meningitis and other serious illnesses in young infants is further complicated by the fact that the risk of infection appears to be inversely proportional to age. In other words, the younger the infant, the higher the risk of meningitis and SBI.[21] Indeed, prospective application of the Philadelphia criteria to infants 3-28 days old resulted in an unacceptable 15.2 percent being misclassified as low-risk for serious infections compared to 1.5 percent in the originally studied older (29-56 days) age group.[19] The Boston protocol is similarly unreliable when the age range is expanded to include infants younger than 28 days, as a retrospective application in 225 febrile patients less than 1 months old mistakenly identified 25.8 percent with SBI as low-risk.[11] Only the Rochester protocol is inherently designed for febrile infants under 1 month of age.

While only 2 of 227 patients identified as low risk by the Rochester criteria indeed had SBI, this analysis was a combination of three separate studies. Because this study is not entirely composed of consecutively eligible infants, many have questioned the validity and usefulness of this data.[11, 15]

In addition, the choice of laboratory parameters used to determine risk for meningitis and SBI also differs among these three protocols. Of particular interest is the inconsistent use of cerebrospinal fluid (CSF) data, the current gold standard, for the diagnosis of meningitis. The threshold for an abnormal CSF white blood cell count (WBC) varies between the Boston and Philadelphia criteria (10 WBC/mm^3 versus 8 WBC/mm^3, respectively), while the latter also included a negative CSF Gram stain amongst its laboratory parameters.[1, 14] Most notably, a lumbar puncture is not included in the Rochester criteria. In a proposal of an alternative predictive model for SBI in young febrile infants, Bachur *et al.* retrospectively applied the Philadelphia and Rochester criteria to 5279 infants less than 90 days old, of whom 373 (7%) were diagnosed with SBI and 17 specifically identified as bacterial meningitis.[12] Because not all measures of either criterion were available for every patient, a complete and direct comparison could not be made in hindsight. The results are nonetheless suggestive. The Rochester criteria likely would have misclassified 6 patients with bacterial meningitis and 77 patients overall as low-risk for any SBI, while the Philadelphia criteria would have missed 2 patients with meningitis and 69 patients overall. The latter is in spite of the Philadelphia criteria's use of a CSF WBC parameter in addition to peripheral WBC count and urinalysis (UA). What is more, the novel decision-making algorithm tested by Bachur *et al.*, which consisted of UA, peripheral WBC, temperature, and age, fared only slightly

better. Sixty-six infants were misclassified as low risk for SBI, of whom 11 well- and ill-appearing patients were bacteremic and grew blood-borne organisms capable of causing meningitis. An additional infant with bacterial meningitis was also missed. Thus regardless of the laboratory parameters used, it is quite clear that no existing clinical criteria can confidently identify young febrile infants at low-risk for bacterial meningitis or, for that matter, any serious bacterial disease.

Evidence to this notion of a disappointing inability of current guidelines to facilitate the diagnosis of bacteremia, meningitis, or any other serious condition for febrile infants was provided by a recent large prospective study. Pantell *et al.* demonstrated that pediatricians in the United States more often than not utilized individual clinical judgment in the management of fever in this age group.[21] Of 1746 infants analyzed, these physicians correctly identified and treated bacteremia and bacterial meningitis as often as those who followed a "blended" version of current guidelines. These practitioners also utilized laboratory tests (UA and peripheral WBC) somewhat more efficiently and hospitalized fewer patients. Clear specificity values could not be presented by the authors for either approaches, but was concluded to have similarly suffered at the expense of greater sensitivity. This translates into a high rate of false positive diagnoses. Consequently, many infants with self-limited illnesses were subjected to excessive testing and treatment regardless of whether the responsible clinician followed current guidelines or evaluated febrile infants on an individual basis. That the particular tests utilized by physicians and the various clinical protocols lack simultaneously good sensitivity and specificity was no doubt in part to blame.

Of the currently available tools for the identification for bacteremia, meningitis, or other SBI, none has been found to be an adequately sensitive or specific indicator by itself. One such test most often obtained by physicians is the peripheral WBC. Recent research have clearly demonstrated that at no cutoff value does a peripheral WBC simultaneously have good sensitivity *and* specificity in infants younger than 90 days old.[22] This corroborates well with other studies involving a larger age range of children.[23-25] Fortunately, the value of the peripheral WBC increases once combined with other laboratory tests. For example, neither peripheral WBC nor UA in the Bachur *et al.* algorithm is a particular good predictor of SBI alone.[22] Combination of the two measures along with temperature and age improved sensitivity and specificity significantly. A similar result was obtained by Pantell *et al.*[21]

The above discussion have shown, that in spite of this incorporation of different laboratory parameters into various clinical criteria, these screening protocols still result in imprecise identification of serious infection febrile infants. Continued search for a more accurate indicator of SBI in young febrile infants to accompany or replace existing tests is therefore all the more justified in order to guide diagnostic assessment better. This is especially true in the diagnosis of meningitis, where lumbar puncture is the sole means by which it can be diagnosed.

Recent advances in molecular and imaging techniques have not replaced lumbar puncture as the procedure of choice to diagnose meningitis in young children.[26, 27] While CSF protein levels and pleocytosis can be highly suggestive of meningitis by themselves, Gram stain and culture of organisms in CSF is understandably the most reliable method to confirm bacterial or viral meningitis in young febrile infants. In addition, the

sensitivity of CSF Gram staining for bacteria ranges greatly from 25% to 97%, depending upon the number of colony-forming units.[7] Culture of CSF is also a time-consuming endeavor and is of little utility in the emergency setting. Lumbar puncture, however, is notoriously difficult to perform in young infants and has a failure rate of 5%.[28, 29] More important, concerns over the potential hazards of performing lumbar puncture have rightly or wrongly limited its use by physicians.[29]

A major contraindication to lumbar puncture is any sign of increased intracranial pressure (ICP), [26, 28] such as mental status change, focal neurological deficits, and papilledema. Profound elevations in ICP may result in cerebral herniation, although this is a rare complication.[30, 31] Moreover, there is question in the current literature as to whether or not lumbar puncture can directly result in herniation in this setting.[31, 32] Unfortunately, computed tomography cannot reliably detect an elevation of ICP or cerebral herniation in children or adults.[32, 33] Although there is little doubt regarding the value of lumbar puncture in the diagnosis of uncomplicated meningitis, clinical scenarios often exist in which this procedure is contraindicated. All this raises a critical question for physicians and researchers alike: Is there a blood test that can serve as a reasonable alternative to lumbar puncture to indicate the presence of meningitis?

Besides the peripheral WBC, at least two serum markers have been studied for their usefulness as indicators for SBI in febrile infants.[34] The first marker is C-reactive protein (CRP), an acute phase reactant known to be produced by hepatocytes within hours of tissue injury or inflammation. Studies by Pulliam *et al.*[23] and Galetto-Lacour *et al.*[25] both demonstrated CRP fared better than peripheral WBC as an indicator of SBI and may have promise as a marker. Neither study, however, specifically recruited the young

febrile infants younger than 3 months. More important, it is not possible to extrapolate the value of CRP as an indicator of meningitis given that no cases occurred within their patient population. The second such marker is procalcitonin (PCT). PCT rises more rapidly in response to infection than either WBC or CRP, although the function of this protein is not entirely clear and may include a role in sustaining inflammatory reactions. Interest in PCT stems from its previously shown dramatic increase in children afflicted with SBI compared to those with viral infections.[35] Current literature suggest that PCT overall has somewhat superior sensitivity and specificity when compared to both peripheral WBC and CRP as an indicator in cases of SBI, including several patients with bacterial meningitis.[24, 25, 36] Thus, these investigations has thus far suggested a potential role for CRP and PCT as serum markers for serious infections in young infants. To date, the use of CRP and PCT has not been specifically investigated for usefulness as a marker for meningitis in young infants.

Nonetheless, as meningitis is a disease of the central nervous system (CNS), the ideal indicator of this condition would logically be an exclusive product or component of the CNS that becomes elevated in the bloodstream only on occasions of infections, such as meningitis. Venipuncture would then become akin to an indirect sampling of the CNS, thus sparing young infants and their parents of the stress and pain of lumbar puncture. Because both are synthesized by hepatocytes, it is clear that neither CRP nor PCT would fit this description.

One potential protein that may serve as a surrogate marker of CNS inflammation is S-100B. First described in 1965 by a group searching for brain specific proteins,[37] S-100B is a 21-kDa calcium-binding dimeric protein found to be stable in serum for a

minimum of 7 months and also at -80°C. Its name derives from its solubility in 100% ammonium sulphate. S-100B is predominantly produced in high concentrations by nerve tissue, including astrocytes, oligodendrocytes, and Schwann cells, with at least 80-90 percent of the total S-100B pool found in the brain.[38, 39] Its gene is located on chromosome 21q22.3, and thus has been found to be excessively expressed in Down syndrome.[40] S-100B functions both extracellularly and intracellularly. Inside the cell, it is believed to play a role in signal transduction via the inhibition of protein phosphorylation and the maintenance of calcium homeostasis through cytosolic buffering.[39] But it is its effect once secreted that is of particular interest. Nanomolar concentration of extracellular S-100B has been found to be neurotrophic, favoring neuronal survival, whereas at micromolar concentrations, S-100B stimulates neuronal death via regulation of genes responsible for apoptosis and through an increase in calcium influx.[38, 41] In addition, secreted S-100B appears to stimulate the expression of inducible nitric oxide synthase by astrocytes and microglia; subsequent nitric oxide diffusion has led to co-cultured neuronal cell death.[42, 43] Expression of interleukin-6 (IL-6), a proinflammatory cytokine, by neurons is also elevated due to S-100B activity.[44] These functions of S-100B have led to its implication in the pathogenesis of neurological diseases in which inflammation may play a role, such as Alzheimer's,[45] or disorders characterized by the improper maturation of neurons, such as cerebral palsy and delayed development.[46]

S-100B has been postulated to be an important component of the infectious diseases of the CNS. Cerebrospinal fluid levels of S-100B have previously been found to be elevated in young infants (median age = 9 days) with bacterial meningitis.[47] In this

study, CSF S-100B levels also correlated well with CSF WBC and protein levels and inversely correlated with glucose concentrations in infants with meningitis. Another study of CSF S-100B involving older children with either aseptic or bacterial meningitis had significantly elevated CSF S-100B concentrations whereas those with alternative diagnoses did not. However, a statistical difference in CSF levels of S100B was not found between those children with aseptic and bacterial meningitis.[48] Thus, these studies suggest CSF S-100B may be a useful marker for meningitis of any etiology in the pediatric population, including very young infants.

A natural extension of these investigations is whether or not *serum* S-100B concentrations can serve as a marker of CNS infection, especially meningitis. Normally, serum S-100B concentrations do not differ with gender and are simply inversely correlated with age. Levels are greatest in healthy neonates less than 48 hours of age and progressively decrease until stabilizing after age 20 years.[49, 50] This age disparity may reflect neurodevelopmental processes. Of note, Lins *et al.* of Germany recently examined the potential of serum S-100B as a marker for meningitis.[51] Their investigation of 32 consecutively-admitted adults with laboratory confirmed infection of the CNS (bacterial or viral meningitis/ meningoencephalitis or neuroborreliosis) demonstrated that both CSF *and* serum S-100B levels drawn on admission were significantly elevated above controls with non-infectious CNS conditions, such as psychosomatic disorders or headaches. Moreover, initial S-100B concentrations in both serum and CSF could be used to distinguish bacterial CNS infection from viral meningitis/ meningoencephalitis and neuroborreliosis. A distinct correlation was also found between S-100B and standard CSF parameters for CNS infection, including pleocytosis.

Hypothesis

Based on this information, we hypothesize that serum S-100B levels in young febrile infants with meningitis will be significantly greater than those febrile infants without CNS infection.

Purpose

The aim of this study is to assess the potential of serum S-100B as an indicator of infantile meningitis of any etiology and ultimately to facilitate the diagnosis of this difficult-to-recognize disease and possibly reduce the need for lumbar puncture in this age group.

Methods

Study design. This was a prospective, cohort study conducted at the pediatric emergency department at the Yale-New Haven Children's Hospital, New Haven, Connecticut between April 2006 and September 2007. Our emergency department sees approximately 31,000 visits annually, of which about 200 are evaluations for sepsis in young infants that require lumbar puncture and venipuncture. The study protocol was reviewed and approved by the Yale University School of Medicine Human Investigation Committee prior to patient recruitment.

Study Population. The study attempted to enroll all eligible children between the ages of 1 day and 90 days old who presented to the emergency department and was deemed by the responsible physician to require venipuncture, lumbar puncture, and bladder

catheterization as part of their diagnostic work-up due to the presence of a fever or a history or clinical appearance that was worrisome for meningitis. Fever was defined as either a confirmed rectal temperature ≥38.0ºC in the emergency department or a history of a rectal temperature ≥38.0ºC. Informed, written consent was acquired by a physician from all parents or legal guardians of the eligible infants before inclusion of the subject into the study. Exclusion criteria for study subjects was set as the following: 1) no requirement for lumbar puncture; 2) no requirement for blood tests; 3) inability to obtain blood sample; 4) inability to obtain CSF sample; 5) history of recent seizure; 6) history of recent head trauma; 7) history of neurodegenerative disease; 8) history of gestational age less than 36 weeks; and 9) history of intraventricular hemorrhage.

Study Definitions. The definition of meningitis in this study was necessarily broad in recognition of the difficulties associated with lumbar punctures that often results in an inadequate volume of CSF for all analyses and/or the presence of a traumatic lumbar puncture that complicates the interpretation of the actual WBC count in the CSF. A subject is thus deemed to have meningitis if any of the following criteria are met: 1) positive CSF bacterial or viral culture or positive PCR for either enterovirus or herpes simplex virus ; 2) CSF pleocytosis in the absence of hemorrhage; or 3) a CSF white-blood-cell to red-blood-cell ratio (WBC:RBC) is >1/100.[52] CSF pleocytosis is defined as >15 cells/μL based on previous reports that WBC counts greater than this would have a high sensitivity for identifying children with bacterial meningitis.[1, 22-25] A traumatic lumbar puncture is defined as the presence of >500 red blood cells/ μL.[52, 53]

Study groups. Febrile infants were distributed into two groups according to presence or absence of meningitis. Patients with meningitis were defined as per above. Febrile infants without laboratory documentation of infection in their CSF made up the control group. Laboratory technicians performing the subsequent serum analysis for S-100B were blinded to the patient's final diagnosis based on CSF laboratory studies.

Sample procurement. Urine, CSF, and blood samples were obtained from all study patients as part of their evaluation for bacterial sepsis. Bladder catheterization via suprapubic or urethral techniques was used to yield urine samples for culture and urinalysis. Lumbar puncture was performed as per usual protocol, and the subsequent CSF was analyzed for cell count, protein, and glucose concentrations and cultures for bacteria and viruses. PCR for enterovirus and herpes virus was used to replace the viral culture in the event of a hemorrhagic tap.

Blood samples were obtained by standard venipuncture technique for the routine testing (complete blood count, culture) ordered by the treating physician. An additional 1.0 mL of blood for S-100B testing was also obtained at the initial venipuncture, but only after all necessary blood samples needed for treatment had been acquired. No additional venipuncture attempts were made to obtain a blood sample for the sole purpose of this study if a blood sample could not be gathered with the necessary laboratory tests for the evaluation of the patient’s fever. The 1.0 mL samples of blood were then centrifuged within 8 hours of venipuncture to yield serum. All serum samples were then subsequently frozen and stored until processing at the end of patient enrollment period.

Serum S-100B levels for each subject were determined by the Can Ag S-100B assay, an enzyme linked immunoassay (ELISA). Samples were thawed to room temperature. Wash buffer and standard and control reagents were reconstituted with deionized water. Fifty µL of each standard and control reagent were combined with 50 µL of serum sample. A volume of tracer equal to that of the latter mixture was added prior to an incubation period of 2 hours. After washing the unbound tracer and residual antibodies off with wash buffer, samples were then incubated once more for 15 minutes on a plate shaker at 800 RPM with 100 µL of TMB substrate. One hundred µL of TMB stop solution was utilized to halt the reaction, and the absorbance of each sample was read over a 15 minute period at 450 nm using a microplate reader. To minimize laboratory drift, control and case samples were analyzed in batches.

Statistical analysis. A previous study observed that the median serum S-100B levels in 19 healthy neonates is 1790 ng/L (interquartile range = 1570-2440) and 365 ng/L (267-410) in children between the ages of 4 and 9 years.[49] This yielded an estimated standard deviation of 960 and 140 ng/L in neonates and children, respectively. As the variability and central tendency of serum S-100B is not yet available, we utilized the most conservative (neonatal) estimate of standard deviation as a point of reference and postulated that a sample size of 130 subjects with meningitis and 130 without meningitis would provide a power of 80% to detect differences of 390 ng/L and a two-sided significance level of 0.016.

For this interim analysis, power calculations were repeated based on the sample recruited and a two-sided significance level of 0.05. Serum S-100B levels were reported

as medians and interquartile ranges. Comparison of serum S-100B levels between groups were performed using the Wilcoxon rank sum test. A non-parametric receiver operating characteristic (ROC) curve was generated for serum S-100B to evaluate its potential as a diagnostic test for meningitis. We examined for any potential relationship between discrete variables, such as gender and race, and the diagnosis of meningitis using the Pearson X^2 test or the Fisher exact test as necessary for situations with smaller sample sizes. A two-sided alpha level of 0.05 is considered to be significant. All statistical analyses were conducted with Stata/IC 10 (College Station, TX).

Results

A total of 111 eligible infants had been recruited by the end point date for this interim analysis, which was 18 months into the projected 3-year enrollment period. Several eligible patients were not included in this study predominantly due to guardian refusal or inadequate blood sampling. Prevalence of meningitis between participants and non-participants did not differ. Insufficient serum volume for analysis is also the reason for the unavailability of serum S-100B levels for 10 (9%) of the 111 participants. Meningitis occurred in 27 (26.7%) of the 101 infants with S-100B levels available compared to 2 (20%) of the 10 who did not ($X^2_{df=1}$=0.21, p=0.64). Three of the 27 cases of meningitis were apparently bacterial in etiology, 2 of which were identified as coagulase-negatvie *Staphylococcus*. No organisms were initially seen in any of these 3 suspected bacterial cases on Gram stain of the CSF. One of the 3 cases was simultaneously found to be positive by PCR for enteroviral infection of the CNS. In the non-meningitis group, the vast majority (57%) did not have a confirmed etiology for their

fever. Septicemia was, however, identified in 10 (13.5%) infants, urinary tract infections in 12 (16.2%), and respiratory tract infections, including pneumonia, in 13 (17.5%). Some of these patients had multiple possible etiologies as the source for fever.

Ad hoc power calculations based on our effective sample size of 101 and the approximately 1-to-3 ratio of case versus control suggested that this interim analysis only had a 57% probability of detecting a difference of 390 ng/L between the two groups.

Table A shows the demographics of those with and without meningitis. Overlapping 95 percent confidence intervals suggest that the mean age of the two groups did not differ. Neither male gender ($X^2_{df=1}$=1.49, p=0.22) nor Caucasian race ($X^2_{df=1}$=0.27, p=0.60) was associated with the diagnosis of meningitis. In an attempt to characterize the clinical presentation of the infants, information regarding the number of hours the child had already been afflicted with fever by the time of presentation to the emergency department was also collected. We realize, however, that the subjective nature of this data limits its reliability and therefore is not presented here. Similarly, an analysis of the various laboratory values, such as WBC count, between the two diagnostic groups is omitted due to their inadequate utility as measures of clinical severity in this age population.[34]

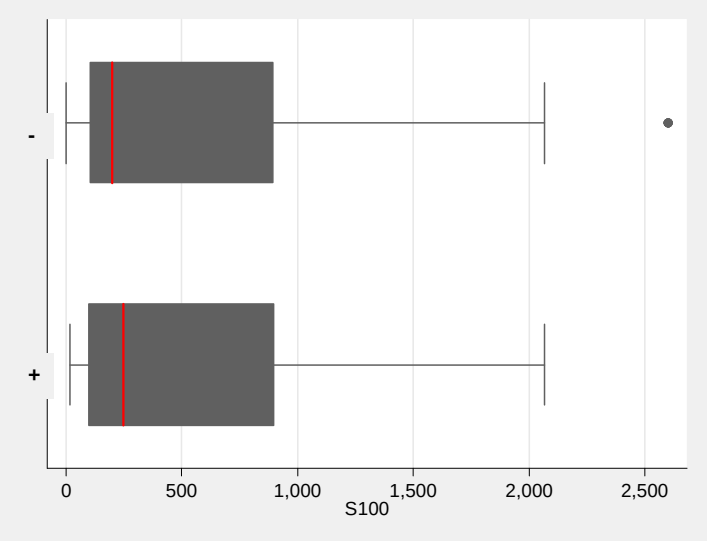
Table A. Demographics of subjects with meningitis (n=27) and without meningitis (n=74).

	+ Meningitis **# (95% CI) # (%)**	***- Meningitis*** **# (95% CI) # (%)**
Age (weeks)	5.45 (4.67, 6.23)	4.84 (4.28, 5.40)
Male	19 (65.5%)	43 (52.4%)
Caucasian	14 (48.3%)	35 (42.7%)

No differences exist between the age at presentation to the emergency department and the diagnosis of meningitis. No relationship exists between male gender or Caucasian race and the diagnosis of meningitis.

Figure 1 provides a box plot representation of the distribution of serum S-100B levels in the infants with (+) and without (-) meningitis. The 25th- and 75th- quartiles, as marked by the leftmost and rightmost boundaries of the boxes, appear similar between the groups. The median S-100B level (red line) for the meningitis group is 247.0 ng/L (103.5, 804), while that for the non-meningitis group is 199.1 ng/L (142.5, 384.0). No significant difference exists between these two values ($p>0.05$).

Figure 1. Box plot of serum S-100B (ng/L) distribution in infants 0-3 months of age with meningitis (+) and without meningitis (-).

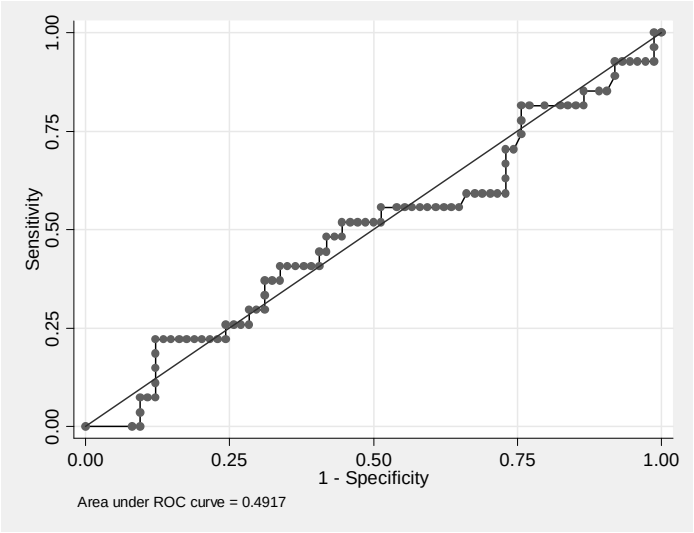


The serum S-100B distribution between the two groups is nearly identical. The boxes mark the interquartile ranges with the median indicated by the red line. The lower and upper bars represent the 5th and the 95th percentiles. An outlying value is shown by the diamond.

In addition, we utilized ROC curve analysis to evaluate the value of serum S-100B as a diagnostic test for meningitis. As figure 2 demonstrates, the curve nearly followed the 45-degree reference line of no significance. The area-under-the-curve was

0.4917 (0.3940, 0.5863). Thus, no value of serum S-100B had an acceptable combination of sensitivity and specificity.

Figure 2. Receiver operating characteristic (ROC) curve of serum S-100B as diagnostic test for meningitis in infants 0-3 months of age.



The sensitivity of serum S-100B for meningitis is plotted along the y-axis against its false positive rate (1-specificity) along the x-axis. The ROC curve follows the 45-degree reference line and the area under the ROC curve approximates 0.5, therefore indicating no significance.

Discussion

In this interim analysis, we found no difference in the serum S-100B levels of infants with meningitis and without meningitis. This is hardly surprising, as the sample size is less than half of the amount deemed a priori to be necessary to have sufficient power. This is confirmed by the ad hoc power calculation, which found the ability for this study to detect a meaningful difference in S-100B levels between the two groups to be only 57 percent. As a result, at this time, little can be said about the utility serum S-100B has as a biomarker for meningitis in infants. Any conclusions based on an

insufficiently powered analysis would be premature; Hennekens and Buring went as far to remark that to do so would have the potential to cause "great scientific harm."[54] In this case, a hasty conclusion of a null result may discourage continuation of this study, which given a full enrollment may reliably reject the null hypothesis instead. Because meningitis will remain a sought-after cause of fever in young infants, an alternative to the current gold standard of CSF analysis for the diagnosis of meningitis will continue to be necessary. The inconclusiveness of this interim analysis still leaves serum S-100B as a logical option for investigation.

Our review of current relevant literature suggests two general mechanisms by which S-100B could become serum marker for meningitis in very young children. The first relates to its presence in the CNS of healthy individuals, especially the relatively high levels of serum S-100B in term neonates.[49] S-100B is found predominantly within the cytosol of astrocytes within the CNS, where the concentration can be as high as 10 μL.[39, 55, 56] In the setting of the cell damage that is known to occur in acute meningitis, it is possible that the intracellular pool of S-100B is thereby first released into the CSF and finally through the blood-brain barrier to the peripheral bloodstreams.[57] The blood brain barrier has been found to become more permeable with the abundant release of cytokines, such as tumor necrosis factor α (TNF- α), which no doubt occurs during an acute infection.[58] The may be reflected by prior reports of elevated CSF and serum S-100 in not only meningitis itself,[47, 51] but also after traumatic brain injuries[59, 60] and hypoxic[60-62] strokes, where direct damage to astrocytes likely have occurred. Gazzolo *et al.* also found rising levels of S-100B in the urine of preterm infants with intraventricular

hemorrhage, although it should be noted that a nervous versus renal origin of this S-100B must be considered given the possibility of metabolization prior to renal filtration.[63, 64]

A rise in S-100B in the peripheral bloodstream secondary due to direct glial cell damage, however, is likely an over-simplification because it limits S-100B to a bystander role, in which it is an effect, rather than a part, of the disease process. In light of studies that have associated S-100B with several CNS-related conditions in which inflammation is also a significant part of their pathogenesis, a more active role for S-100B must be considered. In multiple sclerosis for instance, Massaro *et al.* and Missler *et al.* has respectively observed an increase in CSF (n=10) and plasma (n=7) S-100B during the acute phase of disease exacerbation.[65, 66] Similarly, Mokuno *et al.* noted greater levels and a longer duration of elevation of CSF S-100B in 16 of 24 Guillain-Barré patients who had an extended recovery course compared to the remaining 8 patients with lower CSF S-100B levels and a shorter recovery.[67] These studies, combined with current molecular data, suggest secreted S-100B has the potential to initiate or intensify the cytokine cascade of the inflammatory response seen in these diseases and meningitis.

Van Eldik and Zimmer first described the secretion of S-100B in an animal model.[68] While the means by which this is accomplished remains unclear, one important early proinflammatory cytokine in bacterial meningitis, interleukin-1(β) (IL-1 or IL-1β), has been reported to be stimulate astrocytes to release S-100B.[39] The beginning of our hypothetical pathway may thus consist of the activation of monocytes, macrophages, granulocytes, endothelial cells, microglial cells, and astrocytes within the CNS by various cell wall components or toxins of pathogens to release IL-1.[57] In turn, an overwhelming stimulation of IL-1 would then induce, among many chemical mediators in the

inflammatory cascade, the secretion of S-100B at non-physiological (micromolar) amounts by astrocytes. Excessive extracellular S-100B exerts its paracrine effect likely at least partly via the chronic engagement of the multiligand, transmembrane receptor for advanced glycation end products (RAGE) on neurons and microglia. This results in nuclear translocation and the activation of transcription factors, Sp1 and NFκB, in microglia and, Sp1, in cortical neurons, which ultimately leads to the formation of even more IL-1β.[69-71] Previous data that show a correlation between CSF IL-1β levels and the severity of disease in patients with acute bacterial meningitis would be consistent with this proposed mechanism.[72]

S-100B also exerts an autocrine effect on astrocytes via RAGE that further adds to cytokine cascade. More specifically, Ponath *et al.* demonstrated that S-100B upregulated interleukin-6 (IL-6) and TNF- α in a RAGE-dependent manner. Both cytokines are known to be markedly elevated in the acute bacterial meningitis.[57] As mentioned above, the permeability of the blood-brain barrier increases significantly because of the action of cytokines. S-100B would thus be able contribute to its own presence in the periphery during acute meningitis.

Of course, this interim analysis could not with any confidence comment on the plausibility of either of the aforementioned mechanisms by which serum S-100B could become a biomarker for meningitis. Nonetheless, even if a difference in the levels of serum S-100B were to be detected at the conclusion of patient recruitment, interpretation must still proceed with caution. Limitations exist regardless of our attempts to minimize the number via the study's design. Selection bias may occur due to the broad inclusion criteria as guardians of infants who are seemingly less ill may be less inclined to

participate. Thus far, however, the frequency of aseptic and bacterial meningitis is nearly the same between participants and the few non-participants. Another source of selection bias lies in the decision to perform a full diagnostic work-up on non-febrile infants whose clinical manifestation may or may not be worrisome to the particular physician responsible. Interrater agreement is therefore also of concern for those few patients enrolled through this route. Observation bias in the form of recall bias or loss of subjects to follow-up is fortunately unlikely in this study, as the outcome of interest is objective in nature and patient involvement is limited to the initial venipuncture, respectively. In contrast, the realities of clinical medicine do make misclassification possible. The sheer difficulty of performing an atraumatic or a volume-sufficient lumbar puncture has, to date, limited the number of subjects with CSF that is uncontaminated by hemorrhage and confirmed for infection via positive viral cultures. We, therefore, must rely upon the less-than-ideal combinationof viral and bacterial cultures, viral PCR, the presence of pleocytosis, and/ or CSF WBC to CSF RBC ratio to establish the diagnosis of meningitis. Finally, there always exist the possibility that an excessively traumatic venipuncture may elicit sufficient extracerebral S-100B to contaminate the serum sample.[73, 74] Thus far though, most of our venipunctures with our subjects have been uncomplicated. This may help keep the chance of this complication arising low.

In summary, serum S-100B does not appear to be a useful biomarker for meningitis at this time. If the result of this interim analysis were to continue to hold true, there are at least two explanations to account for this. First and foremost, the low cumulative incidence of bacterial meningitis in the vaccination era limits the cases to those secondary to viruses. This low incidence is reflected by our sample, in which only

3 cases of bacterial meningitis were found. These were, however, dubious, as 2 were apparently due to coagulase-negative Staphylococcus and none had visible organisms on Gram staining. The relatively mild nature of viral meningitis is epitomized by its more-often-than-not spontaneous resolution and limited long-term sequelae. When compared to bacterial cases, the extent of the inflammatory response and damage to CNS would be expected to be curtailed in viral cases. The release of S-100B into the periphery would thereby be minimized. While we are not aware of any reports in the literature of this, another possibility is that S-100B from corporeal sources may be elevated in the serum due to the hyperpyrexia itself through an unknown mechanism. This may be significant enough to obscure any additional serum S-100B derived from the CNS. Other potential reasons include those relating to the timing of venipuncture, our laboratory technique, and the power to detect a truly meaningful difference in serum S-100B levels.

The latter problem currently hinders our ability to draw a conclusion based on this interim analysis. Unfortunately, even our power calculations a priori relied upon estimates from a limited number of human subjects not derived from our region. Moreover, the calculated sample size for adequate power assumes a one-to-one ratio of cases and controls in order to limit the number of participants required. This no doubt is somewhat unrealistic with increasing immunizations against *H. influenza* type B and *Streptococcus pneumoniae*. Patient enrollment period therefore will likely increase beyond the stated three year time frame, and questions may be raised as to the comparability of patients at different points of recruitment. The importance of discovering a diagnostic test that can distinguish infants at risk for meningitis, however, continue to remain indisputable.

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