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The clinical usefulness of adenosine deaminase measurement in cerebrospinal fluid specimens

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2013-01-24
http://hdl.handle.net/10539/12291

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1.0 INTRODUCTION, ENCOMPASSING REVIEW OF THE LITERATURE

1.1 Physiology

Adenosine deaminase (ADA) physiologically acts as a catalyst in the deamination of adenosine or deoxyadenosine to inosine or deoxyinosine and ammonia – the ribonucleoside catabolic pathway (Sullivan, *et al.*, 1977; Barton, *et al*., 1979; Schutte, *et al*., 2001). It is present in most tissues, especially in the lymphatic organs (Adams & Harkness, 1976). In lymphocytes it is found in greater amounts in T lymphocytes compared to B lymphocytes (MacDermott, *et al*., 1980). Levels decrease as these cells differentiate and mature (Sullivan, *et al*., 1977; Barton & Goldschneider, 1979).

1.1.1 Isoforms

With polyacrylamide gel electrophoresis 3 isoforms can be visualized: ADA-1, ADA-1+CP and ADA-2 (Ungerer, *et al*., 1992). ADA-1 is a ubiquitous isoform essential for an efficient immune response. ADA-1+CP is a ubiquitous isoform consisting of two ADA-1 molecules connected by a combining protein. The function of this isoform is unclear. ADA-2 is mainly found in monocytes and macrophages and is released if any infectious agents have been phagocytosed (Chawla, *et al.*, 1991; Gakis, 1996). The molecular mass for each of these isoforms is (1) ADA-1 = 33 000 – 44 000 g/mol, (2) ADA-1+CP = +/- 280 000 g/mol, and (3) ADA-2 = 100 000 g/mol (Bota, *et al*., 2001). The isoforms have different affinities for adenosine and deoxyadenosine (Chawla, *et al.*, 1991).

1.2 Clinical significance

In 1975 congenital deficiency of mainly ADA-1 was described in patients with severe combined immunodeficiency (Parkman, *et al*., 1975). On the opposite side of the spectrum, characteristic elevated levels were noted in infectious diseases such as typhoid fever as early as the 1960s (Giusti, 1974). Measurement of ADA for these conditions has generally fallen out of current routine practice.

In 1973 Gakis and colleagues were the first to report elevations of ADA in the cerebrospinal fluid (CSF) of patients with meningitis due to *Mycobacterium tuberculosis* (TBM) (Gakis, *et al*., 1991). There is debate in the literature as to whether elevations of ADA are due to increased blood brain barrier permeability as proposed by Donald, *et al*., (1987) or local production in cells present in the CSF as documented by Gakis (1995).

Gakis (1995) reported that it is in fact ADA-2 that is predominantly elevated in the CSF of TBM patients. Eintracht, *et al*., (2000) found that an ADA-2 percentage (ADA-2 expressed in terms of total ADA) of greater than 80%, had a sensitivity of 100% and a specificity of 86.4% for the diagnosis of TBM. Schutte, *et al*., (2001) also found ADA-2 to be the major isoform noted in CSF in 90% of cases with TBM as well as 51% of cases with bacterial meningitis. No studies could be cited in the literature after 2001 that specifically measure ADA-2.

ADA can also be measured in other sample types, such as pleural fluid, in the context of suspected tuberculosis (TB) (Song, *et al*., 2010). Its utility in the diagnosis of TB pleural effusion, TB pericarditis and abdominal TB will not be discussed further in this report.

1.3 Measurement

The original measurement procedure for ADA is based on the method by Giusti described in 1974. In the presence of ADA and water, adenosine is broken down to inosine and ammonia. Hypochlorite and phenol are added to react with ammonia and result in the development of an intense blue indophenol (the Berthelot reaction). This intensity is measured colorimetrically at 630nm and is directly proportional to the amount of ADA present. This measurement procedure was described using serum or plasma samples (Giusti, 1974). Non-Giusti methods for the measurement of ADA are based on coupled enzymatic reactions. Numerous variations on the Berthelot reaction and coupled enzyme reactions have been described (Oosthuizen, *et al*., 1993). Differences in accuracy between these methods have been documented (Segura, *et al*., 1989). The method utilised in this study is based on coupled enzymatic reactions and will be described later.

1.4 Major health care concerns in South Africa

TB is a significant public health challenge in South Africa. The incidence has been documented to be 940 per 100 000 individuals in 2006 (Department: Health. Republic of South Africa. "National tuberculosis management guidelines 2009"). The numbers of people affected by TB increased greater than three-fold between 1996 and 2006: 109 000 in 1996 and 341 165 in 2006 (Department: Health. Republic of South Africa. "Tuberculosis strategic plan for South Africa, 2007-2011"). Several reasons may explain this increase, including poor management of the National TB Programme and the resistance patterns of *Mycobacterium tuberculosis* (Department: Health. Republic of South Africa. "National tuberculosis management guidelines 2009").

A further concern for health care in South Africa is the Human Immunodeficiency virus and Acquired Immunodeficiency syndrome (HIV/AIDS). The Joint United Nations Programme on HIV/ AIDS (UNAIDS) estimated that in 2007, 5 700 000 South African people (approximately 12% of the population) were affected by HIV/AIDS (UNAIDS, 2008).

The incidence of TB has increased in parallel to the increase in the estimated prevalence of HIV. HIV modifies the clinical presentation of TB. In HIV infected individuals TB may present more subtly and less characteristically than usual, with the diagnosis being made late or not at all. These patients may remain infectious for longer. Atypical presentations may be seen in patients with lower CD4 cell counts. Furthermore, 50% of cases of pulmonary TB are sputum negative for acid-fast bacilli (AFB), chest X-rays (CXRs) are often atypical and extra-pulmonary TB is common (Sepkowitz, *et al*., 1995).

1.5 Extra-pulmonary TB

The numbers of extra-pulmonary TB accounts for up to 25% of cases (Department: Health. Republic of South Africa. "National tuberculosis management guidelines 2009"). An important extra-pulmonary manifestation of TB is TBM. In some developing countries TBM comprises more than 20% of community-acquired meningitis (Tuon, *et al*., 2010). TBM occurs in 7 – 12% of patients with pulmonary TB. A high morbidity and mortality is found, with 20 – 30% of survivors suffering from permanent neurological sequelae and case-fatality ratios ranging between 20 – 50%. Despite chemotherapy, 30% of cases of TBM will die (Youssef, *et al*., 2006).

According to the South African National Department of Health there are no significant differences regarding clinical features and outcome between HIV-positive and HIV-negative individuals (Department: Health. Republic of South Africa. "National tuberculosis management guidelines 2009"). However, some studies have been cited that are not in agreement with this. Garg (2010) state that HIV-positive cases had a higher incidence of active TB on CXR, but less often on a computerized tomography (CT) scan noted with hydrocephalus and basal meningeal enhancement, as opposed to HIV-negative cases.

1.6 Diagnosis of TBM

The definitive microbiological diagnosis of TBM is based on culture of CSF, although the yield is not optimal (Sepkowitz, *et al*., 1995; Kashyap, *et al*., 2007; Thwaites, *et al*., 2009). Culture positivity has been documented to be in the range of 55 – 80% (Mvusi & Bloomberg, 2004). Other authors have reported a lower culture yield (Thwaites, *et al*., 2004; Bhigjee, *et al*., 2007). Culture takes long, with detection of growth usually occurring only after 2 weeks. Liquid culture as opposed to solid media may have better recovery (Thwaites, *et al*., 2009).

The presence of AFB in CSF may be seen in up to 80% of adult cases and 20% of paediatric cases, although this is heavily dependent on the volume and frequency of specimen submitted. It is recommended to perform microscopy for at least 30 minutes to improve diagnostic yield. Sensitivity of culture and smear falls rapidly during chemotherapy (Thwaites, *et al*., 2004; Thwaites, *et al*., 2009).

Molecular techniques such as nucleic acid amplification techniques (NAAT) have been documented to have a sensitivity of 56% and a specificity of 98%. NAAT can help with confirmation of TBM, but cannot rule it out. Repeat testing may improve yield. Deoxyribonucleic acid is detected in CSF for up to 1 month after the commencement of chemotherapy (Thwaites, *et al*., 2009).

Other evidence in support of the diagnosis of TBM may include clinical evidence (especially the presence of miliary TB) and radiological evidence (especially the presence of basal meningeal enhancement, tuberculoma or both). These radiological features have been documented to have a sensitivity of 89% and a specificity of 100% (Thwaites & Hien, 2005). In addition, biochemical evidence (such as CSF protein > 1 g/L and low CSF glucose) and cytological evidence (such as the presence of lymphocyte predominance in the CSF) may be useful (Department: Health. Republic of South Africa. "National tuberculosis management guidelines 2009").

1.7 Use of ADA in the context of TBM

Against this background, several additional investigations have been utilised to improve the diagnosis of TBM, one of which is the measurement of ADA in CSF (Tuon, *et al*., 2010). ADA assays are simple, rapid and affordable (Tuon, *et al*., 2010) and can be automated (Feres, *et al*., 2008). Xu, *et al*., (2010) in their systematic review with meta-analysis determined the diagnostic odds ratio for CSF ADA in the diagnosis of TBM to be 26.93, concluding CSF ADA to be a critical criterion for the diagnosis of TBM. They acknowledge that publication bias may have confounded their results. Donald, *et al*., (1986) measured ADA in plasma in

addition to CSF using the Giusti method and reported a CSF cut-off of 6 U/L and a CSF to plasma ratio of greater than 1.5 as supportive evidence for the diagnosis of TBM. This ratio was infrequently utilised in later studies. It was not until the research by Eintracht, *et al*., (2000) that found no benefit to measuring serum ADA in addition to determining the CSF ADA-2 percentage in the work-up of suspected TBM patients. Some authors have suggested the use of CSF ADA in monitoring treatment response (Ribera, *et al*., 1987; Mishra, *et al*., 1996). In a study of 26 children Jakka, *et al*., (2005) showed that an elevated ADA result was associated with poor neurological outcome.

1.8 Critical assessment and interpretation of the literature on the use of CSF ADA in the diagnosis of TBM

Key points in the history and usefulness of CSF ADA measurement are noted in sections 1.2 and 1.7 above. Otherwise for the most part the bulk of the studies cited in this research report attempt to validate a CSF ADA cut-off in different settings. In this section key issues are discussed relevant to interpretation of these studies. Most studies were performed in India.

In the investigator's experience there seems to be an overreliance by clinicians on the use of CSF ADA for the diagnosis of TBM. Its value can never be equal or superior to CSF culture. This overreliance may be due to improper assessment and interpretation of the literature.

It is important to assess CSF ADA results in context of the pre-test probability or prevalence of TBM in the particular population of interest (Tuon, *et al.*, 2010). Furthermore, drawing sound conclusions from the literature are hampered by differences in study design including:

number of participants/specimens used, age of participants, definition of TBM and control groups, HIV status, cut-offs for CSF ADA and ADA assay types utilised. It is important to adequately define the population in which CSF ADA performance is assessed and to know exactly how the measurement of ADA is made.

1.8.1 Total number of participants/specimens

The total number of study participants/specimens used in the studies cited in this research report is variable, ranging from as few as 26 (Schutte, *et al.*, 2001) to as many as 417 (Corral, *et al*., 2004). This may affect the statistical power of the study and the relevance of the study findings.

1.8.2 Age of participants

Some studies note the age of the patients assessed, whether it be children (Mishra, *et al*., 1996) or adults (Petterson, *et al*., 1991). Other authors pool data accounting for both children and adults (Blake & Berman, 1982; Donald, *et al*., 1986). Sometimes the ages of participants are not specified (Ribera, *et al*., 1987). Donald, *et al*., (1987) note 2 case series reflecting differences between CSF ADA in adults and in children: one cases series represented by 3 adults (mean CSF ADA of 26.2 U/L) and 27 children (mean CSF ADA of 14.5 U/L) and another cases series represented by 6 adults (mean CSF ADA of 20.8 U/L) and 41 children (mean CSF ADA of 10.4 U/L). Differentiating between adults and children with TBM may provide further evidence if indeed ADA levels differ between these 2 groups and whether different cut-offs need to be applied for each category.

1.8.3 Definition of TBM and control groups

When reviewing the literature it is important to note that participants in the TBM group in a study were not always placed there solely based on culture. Sometimes both clinical as well as culture evidence were used to group participants as TBM (Mishra, *et al*., 1996). This was probably due to the poor performance of culture as mentioned previously. The concern would be the introduction of bias into the results. Schutte, *et al*., (2001) used clinical criteria, typical CSF findings and response to anti-tuberculous treatment to place participants in the TBM group. Culture evidence from other sites has also been used to place participants in the TBM group (Ribera, *et al*., 1987).

Furthermore, the actual number of culture-positive cases considered has been cited in some studies as being less than 10 (Petterson, *et al*., 1991; Kaur, *et al*., 1992). Ideally one should compare ADA performance to the gold standard for the diagnosis of TBM, i.e. culture of CSF. Probably the only full-proof surrogate for culture may be if response to treatment has been documented, although the potential presence of resistance in the bacillus may prove this to be difficult.

Control groups of studies cited in this research report vary with regard to their inclusion of infectious and non-infectious pathologies involving the CSF. Schutte, *et al*., (2001) included only a bacterial meningitis control group, whereas Choi, *et al*., (2002) included control groups for bacterial meningitis, viral meningitis and fungal meningitis. Investigators such as Blake & Berman (1982) included infectious and non-infectious pathologies involving the CSF.

From a clinical point of view, it is important to realise that both infectious and non-infectious pathologies involving the CSF may require a lumbar puncture at admission and then ask the question as to how well ADA can distinguish between these pathologies. Blake & Berman (1982) measured ADA in a 134 CSF specimens and found levels to be significantly higher in TBM cases compared to non-TB neurological conditions. Later studies found considerable overlap between ADA levels in TBM cases and cases with bacterial meningitis (Malan, *et al*., 1984; Chaturvedi, *et al*., 2000). Others found elevated levels in non-infectious neuropathology, such as lymphoma (Corral, *et al*., 2004). These investigators report an overlap in the interquartile ranges for TBM (5 U/L – 11.15 U/L) and lymphomatous meningitis (3.3 U/L – 15.75 U/L).

1.8.4 HIV status

Upon reviewing the literature, it was not always clear if HIV-positive participants were included in studies (Choi, *et al.*, 2002). Lópes-Cortés, *et al*., (1995) explicitly mentions inclusion of HIV-positive participants. They found no difference between HIV-positive and HIV-negative participants. The CSF ADA was determined using a Non-Giusti method and the cut-off used was 10 U/L. Berenguer, *et al.*, (1992) found the sensitivity of CSF ADA for the diagnosis of TBM in HIV positive cases (n=37) to be 63% and HIV negative patients (n=19) to be 60%. The CSF ADA was determined using a Non-Giusti method and the cut-off used was 9 U/L. The body of evidence regarding utility of ADA in HIV-positive patients when assessing for the presence of TBM is limited.

1.8.5 Cut-offs for CSF ADA

The cut-offs for ADA utilised in the diagnostic work-up of suspected TBM patients cited in the literature varies considerably. Mishra, *et al*., (1996) reported a cut-off of 5 U/L with a sensitivity of 89% and specificity of 92%. Rana, *et al*., (2004) reported a cut-off of 10 U/L with a sensitivity of 66.6% and specificity of 90%. These 2 studies were performed in India and used the Giusti method for the determination of CSF ADA. Petterson, *et al*., (1991) reported a cut-off of 20 U/L with a sensitivity of 100% and specificity of 99%. This study was performed in Scandinavia and used the Giusti method with minor modifications for the determination of CSF ADA. One of the reasons for the difference between the performance of ADA in India and Scandinavia is the difference in prevalence of TB between these 2 countries. Despite this, the performance of ADA has been quite variable even between studies from India. For example, Bandyopadhyay, *et al*., (2008) used the Giusti method for the determination of CSF ADA, but expressed the intensity of the blue indophenol produced per minute. At a pre-determined CSF ADA cut-off of 10 U/L/min they reported a sensitivity of 47.7% and the specificity of 69.4%.

1.8.6 ADA assay types

Differences in ADA assay types utilised have been previously mentioned in this research report. Some investigators base their measurement on the Giusti method (Choi, *et al*., 2002; Kashyap, *et al*., 2007) and others on Non-Giusti methods (Blake & Berman, 1982; Lópes-Cortés, *et al*., 1995). Many of the methods used are developed in-house (Blake & Berman, 1982; Oosthuizen, *et al*., 1993). Furthermore, the ADA assay methodology utilised is not always elaborated upon (Karsen, *et al*., 2011) – it is not clear whether a Giusti or Non-Giusti

method was used. It is more meaningful to compare ADA results between different studies when the assay methodology utilised is the same.

1.9 Prediction rules

Some authors have designed prediction rules to further improve the diagnosis of TBM. The presence of 3 of more of the following findings had a sensitivity of 93% and a specificity of 77% in one study: (1) length of stay in hospital > 5 days, (2) headache, (3) total CSF white cell count < 1000 / mm^3, (4) clear appearance of CSF, (5) lymphocyte > 30% of the total CSF white cell count, or (6) protein content > 1 g/L (Youssef, *et al*., 2006). Another study found that the presence of 3 of more of the following findings had a sensitivity of 55% and a specificity of 98%: (1) symptoms longer than 6 days, (2) optic atrophy, (3) focal neurological deficit, (4) abnormal movements, or (5) polymorphonuclear cells comprising less than half of the total CSF white cell count (Thwaites & Hien, 2005). No prediction rule could be found that includes CSF ADA and therefore its value in a prediction rule has not yet been determined.

1.10 Aims of study

The aim of this study was to determine the clinical usefulness of measuring ADA levels in CSF samples in the local setting.

(1) *Primary objective*: Determine an appropriate ADA cut-off level to assist in the diagnosis of TBM.

(2) *Secondary objective*: Determine the usefulness of ADA together with clinical, radiological and other laboratory features in formulating a prediction rule to improve the clinical diagnosis of TBM, especially in the absence of definitive evidence for TBM. The combination of CSF ADA with the established prediction rules mentioned above as well as with other clinical, radiological and laboratory findings will be assessed.

2.0 MATERIALS AND METHODS

2.1 Introduction

This study was performed retrospectively at Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) in Johannesburg South Africa. Data mining of admission, near-admission and historical clinical and laboratory data (using the patients' files and the laboratory information system) from the years 2009 and 2010 was performed. All patients were already discharged or down-referred in the Gauteng healthcare system.

Clinical, radiological and laboratory data were collected, as used by clinicians, to categorise patients according to: (1) pathologies involving the CSF – 'TBM' ('Confirmed' and 'Suspected') and 'Non-TBM pathologies' ('Infective' and 'Non-infective'); pathologies not involving the CSF – 'Other'; and 'No diagnosis (dx) made'; and (2) the clinical research case definition as published by Marais, *et al*., (2010) – 'Definite tuberculous meningitis', 'Probable tuberculous meningitis', 'Possible tuberculous meningitis' and 'Not tuberculous meningitis'. See Appendix A for a detailed outline of this clinical research case definition.

'TBM' and infective 'Non-TBM pathologies' were generally categorised according to definitive evidence. In addition suspected diagnoses of infective origin were captured separately according to the evidence as used by the clinicians documented in patient files. ADA performance was assessed in both these confirmed and suspected categories. For all other categories clinical evidence as extracted from patient files took precedence.

Furthermore, ADA performance was assessed in the 4 categories of the clinical research case definition as proposed by Marais, *et al*., (2010). The CSF ADA results for the 'Suspected TBM' category, as defined by clinicians, were compared to the Marais, *et al.*, (2010) categories of 'Probable tuberculous meningitis', 'Possible tuberculous meningitis', and 'Not tuberculous meningitis'.

All criteria required by this clinical research case definition (Appendix A) as well as the general haematology and chemical pathology test results collected was utilised towards formulating the prediction rule. This clinical research case definition does not require the presence of optic atrophy or abnormal movements other than those associated with epilepsy. These were absent in all culture-confirmed TBM cases. The bulk of the general haematology and chemical pathology test results were selected from a time period of 24 hours on either side of the time when CSF sampling occurred. Sourcing of patients occurred by tracing them from a list of all CSF ADA requests for 2009 and 2010. Laboratory data was collected between January and May 2011 and clinical data was collected between June and September 2011.

Table 2.1 summarises how the study population was generated. This is discussed in more detail in the following 2 sections (section 2.2 and 2.3).

Table 2.1: Summary of how the study population was generated.

Number of patients at start of study	3495
Number of patients with more than one admission	117
Number of additional admissions captured as individual admissions	136
Number of patients deleted from the Excel spreadsheet	83
Number of patients comprising final population who had an ADA requested on CSF	**3548**
Of these 3548 patients, the number of patients who had both a culture and ADA requested on CSF	**1490**

2.2 Data collection procedure

2.2.1 Laboratory data collection

Initially a list of CSF samples (from CMJAH patients) submitted for mycobacterial culture for the diagnosis of TBM was generated for 2009 and 2010 to get an impression of the culture positivity rate for these 2 years (to ensure that there would be adequate numbers of culture positive TBM patients). This was followed by generating a list of ADA requests for 2009 and 2010 from patients emanating from CMJAH. This list of ADA requests was submitted to the Corporate Data Warehouse (CDW) of the National Health Laboratory Service (NHLS). An Excel spreadsheet of laboratory data pertaining to these ADA requests was generated by the CDW over a period of 3 months (March to May 2011). The data captured in this Excel spreadsheet covered the time period from 5 days before to 30 days after the date the CSF sampling occurred (up to 35 rows of data existed per patient). Please see Table 2.2 for the laboratory information collected: patient identifiers; patient demographics; CSF-related information; other microbiological, mycobacteriological and virological information; histology, including bone marrow aspirate and trephine biopsy results; and general haematology and chemical pathology test results. The dates of CSF sampling captured on the laboratory information system, were also noted.

Table 2.2: Laboratory information collected.

Patient identifiers:	Hospital number and laboratory report numbers for all relevant samples.
Patient demographics:	Age, sex, HIV-related diagnostic information (rapid tests, enzyme-linked immunosorbent assay results (ELISA - initial and confirmatory results), polymerase chain reaction (PCR) results, viral load), CD4 absolute cell count and CD4 percentage (the latter specifically in patients aged 5 years or younger) – the CD4 result closest to the time of CSF sampling in the current admission time period was captured.
CSF – related information:	ADA result (including the sample integrity comment made by the Chemical Pathology department); cerebrospinal fluid appearance (comment made by the Microbiology department); cell count; chemistry (protein and glucose); culture result for the particular sample (bacterial, fungal, mycobacterial, viral); India ink staining result; serology (cryptococcal latex antigen test (CLAT), syphilis serology – Venereal Disease Reference Laboratory (VDRL) and *Treponema pallidum* haemagglutination (TPHA) test results, cysticercosis serology); molecular results (PCR and viral load results); and cytology and flow cytometry results.
Other microbiological, mycobacteriological and virological information:	All samples submitted for culture; any other serology and molecular test results performed on non-cerebrospinal fluid samples (including syphilis serology – rapid plasma reagin (RPR) and TPHA test results, serology for *Toxoplasma gondii* and serology for cysticercosis; viral serology and molecular test results (including PCR and viral load results); and blood microscopy and rapid test results for malaria.
Histology, including bone marrow aspirate and trephine biopsy related results:	Specifically the diagnosis made on these samples was captured.
General Haematology and Chemical pathology test results:	*In the time period 24 hours on either side of the CSF sampling* – full blood count (specifically the white cell count, haemoglobin and platelet counts), differential counts (specifically polymorphonuclear cell, lymphocyte, monocyte, eosinophil and basophil counts) and blood smear result comments (presence of toxic granulation, toxic vacuolation, Döhle bodies, left shift and dysplastic features); serum urea, serum creatinine and serum electrolyte results (serum sodium, serum potassium and serum chloride); liver function tests (including serum total and conjugated bilirubin, serum total protein and serum albumin, serum enzymes – alkaline phosphatise, gamma-glutamyl transferase, alanine transferase and aspirate transferase); and infective markers – erythrocyte sedimentation rate, procalcitonin and C-reactive protein.

Table 2.2 continued

	Any result during the particular admission closest to the time of cerebrospinal fluid sampling – random and fasting plasma glucose.

2.2.2 Clinical data collection

Clinical data were obtained in the CMJAH archive over a period of 4 months (June to September 2011). Files for all patients are converted to microfilm upon their discharge from the hospital. The investigator extracted clinical and radiological evidence from 3495 patient files, at no more than 50 patient files per day.

A short summary of the clinical data was captured on the Excel spreadsheet next to each patient's laboratory data. Patients' discharge summaries, doctor notes, clinical notes and radiological reports for ultrasound, CT scans and magnetic resonance imaging served as the main source of information. The HIV status as per the patient file was captured as well as any other information that may have assisted in categorising the patient.

2.2.3 Checking procedures

The data extracted and captured in the Excel spreadsheet was not checked at a later date during or after the 4 months spent by the investigator in the archives or checked by a second person. Hence due to time limitations the data extraction occurred only once per patient file.

Each day after the clinical data collection, the laboratory data on the Excel spreadsheet was checked against the laboratory information system of the CMJAH NHLS laboratory. At the end of the 4 month data collection period, the list of cerebrospinal fluid cultures submitted for

the diagnosis of TBM was generated again to check the Excel spreadsheet against (to ensure that all TBM cases were considered).

2.3 Inclusion and exclusion criteria

All CMJAH adult and paediatric, male and female, as well as HIV-positive and HIV-negative patients for whom a CSF ADA was requested were included in this study. If a patient had more than 1 specimen taken during a particular admission period, only the CSF ADA result from the first specimen was considered for this study. If more than 1 admission occurred during the time period 2009 to 2010, each admission was considered as a separate patient. The number of patients with more than 1 admission totalled 117 and the number of additional admissions captured as individual patients totalled 136. All data previously captured on the laboratory information up to and including the admission data from which the ADA test request emanates, was considered.

Any subsequent admissions and associated data where ADA tests were not requested, was excluded for data collection. All results emanating from other hospitals were excluded. In total 83 of these 2 scenarios were deleted from the Excel spreadsheet.

At the end of clinical data collection 174 patient files were not yet found. An attempt was made to find these remaining patient files, but only 36 could be found with sufficient data available for categorisation. In 65 cases laboratory data could be used for categorisation. In 73 cases absolutely no diagnosis could be assigned based on the absence of the clinical data or any definitive laboratory. These were categorised under ‘No diagnosis made’. Reasons why

patient files could not be found include: misfiling, files being utilised outside the archive at the time of this study, and that some files never made their way to the archive for filing purposes.

2.4 Total population considered in this study

As reflected in Table 2.1 the total number of patients for whom a CSF ADA was requested total 3548. Of this population 1490 patients had both a CSF culture and CSF ADA requested.

2.5 Patient categorisation

2.5.1 Demographics

Patients considered for this study were categorised according to 8 demographic groups listed in Table 2.3 below.

Table 2.3: Main demographic categories.

Age	Adult patients: ages > 12 years
	Paediatric patients: ages ≤ 12 years
Sex	Male patients
	Female patients
HIV status	HIV positive
	HIV negative
CD4 absolute cell count	≤ 350 x 10^6/L
	> 350 x 10^6/L

The cut-off of 12 years was used to separate adult and paediatric patients and thus including adolescents in the adult patient category.

For the purpose of this study patients were considered HIV positive based on the following criteria: (1) presence of a positive rapid test as well as a positive ELISA, (2) presence of a positive initial and confirmatory ELISA, and (3) presence of a positive PCR for HIV (Maldarelli, 2005). In addition (1) the presence of an elevated HIV viral load, and (2) positivity for HIV as stated in the patient file (where tests were done at a different laboratory) were used to assign HIV positivity.

In patients aged 18 months or younger, only the HIV PCR result was considered to assign positivity to a patient in this age group (Maldarelli, 2005).

A CD4 absolute cell count of 350 x 10^6/L was used to further divide patients based on the current World Health Organisation (WHO) guidelines regarding treatment for HIV (WHO, 2010). In patients 5 years or younger both the CD4 absolute count and CD4 percentage was captured to assess patients' immunodeficiency status as per the Department of Health treatment guidelines for HIV (Department: Health. Republic of South Africa. "The South African antiretroviral treatment guidelines 2010"). The CD4 absolute count and CD4 percentage closest to the time of CSF sampling in the current admission time period was captured.

2.5.2 Categorisation of pathologies

Figure 2.1 summarises the categorisation of pathologies identified for this study.

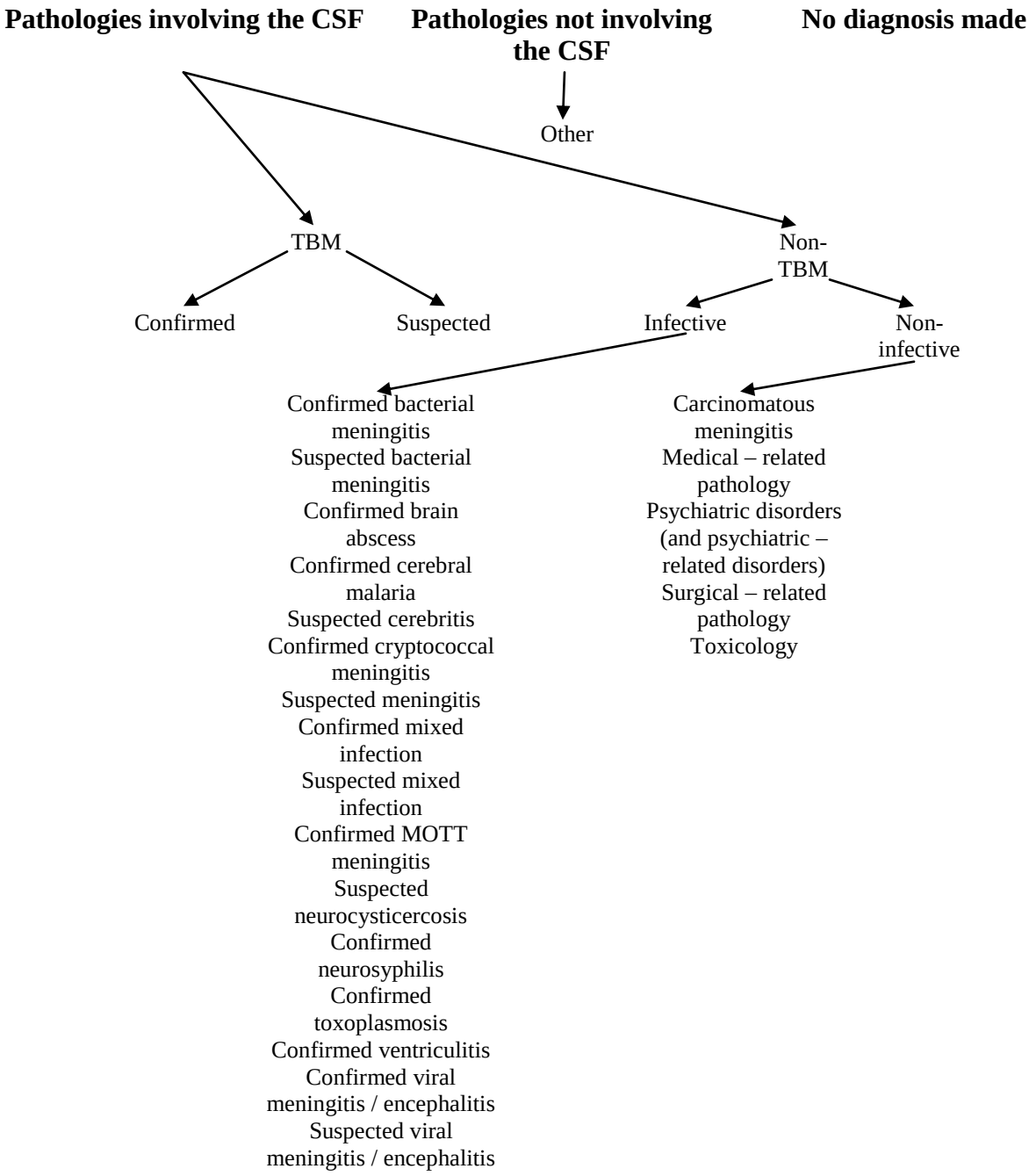


Figure 2.1: Categorisation of pathologies identified for this study. <u>Key to this figure</u>: MOTT – *Mycobacterium* other than tuberculosis.

2.5.2.1 Pathologies involving the CSF

2.5.2.1.1 TBM

2.5.2.1.1.1 Confirmed TBM

Patients that fell in this category either were culture-confirmed cases or those for whom only AFB were noted on microscopy (with no culture confirmation). In the mycobacteriology reference laboratory where analysis takes place, preliminary microscopy for the presence of AFB is performed, followed by incubation of the sample, microscopy of any growth, and finally identification of growth with the Hain® line-probe assay (personal communication, Dr Natalie Beylis, NHLS). Cases where, in addition to *Mycobacterium tuberculosis*, other organisms were also identified were captured as part of the 'Confirmed TBM' category and excluded from the other categories.

2.5.2.1.1.2 Suspected TBM

Patients were categorised as 'Suspected TBM' by clinicians based on clinical-radiological suspicion in the absence of CSF culture positivity. No standardized approach regarding diagnosis was noted in all patients.

2.5.2.1.2 Non-TBM pathologies

Categorisation in the following sub-categories was based on the absence of culture confirmation for TB or absence of AFB on microscopy and hence includes all other pathologies involving the cerebrospinal fluid or intracranial anatomy.

2.5.2.1.2.1 Infective

2.5.2.1.2.1.1 Confirmed bacterial meningitis

Culture and / or microscopy findings on cerebrospinal fluid (Gram stain results) were used to confirm bacterial meningitis.

2.5.2.1.2.1.2 Suspected bacterial meningitis

Patients were categorised as 'Suspected bacterial meningitis' by clinicians based on one or more of the following (in the absence of culture): (1) clinical-radiological suspicion, (2) predominant elevation in polymorphonuclear cells with high protein and low glucose on CSF, (3) usual pathogens causative of meningitis cultured on other specimen types: blood and / or sputum, (4) culture evidence noted in file (doctors' notes), but not found on the CMJAH NHLS laboratory information system, and (5) patients who were treated for bacterial meningitis.

2.5.2.1.2.1.3 Confirmed brain abscess

Patients were categorised as having a brain abscess based on clinical-radiological suspicion as well as the culture result.

2.5.2.1.2.1.4 Confirmed cerebral malaria

Based on clinical presentation together with positive blood smear microscopy and rapid tests on blood, patients were categorised as "Confirmed cerebral malaria".

2.5.2.1.2.1.5 Suspected cerebritis

Categorisation of patients as ‘Suspected cerebritis’, was based on the radiological picture. ‘Cerebritis’ was the primary pathology considered, although the exact aetiology was unclear.

2.5.2.1.2.1.6 Confirmed cryptococcal meningitis (*Cryptococcus neoformans*)

Culture positivity for *Cryptococcus neoformans* was used for categorisation of patients in this category. In some patients managed as ‘Confirmed cryptococcal meningitis’, the diagnosis was based on the CLAT result and / or the India ink result.

2.5.2.1.2.1.7 Suspected meningitis

Patients categorised here were generally seen in the admissions ward for the clinical suspicion of meningitis, but no culture evidence was found (hence the aetiology was not further defined). Furthermore cell counts were often difficult to interpret, especially due to the presence of erythrocyte contamination. Patients were very often empirically treated and discharged.

2.5.2.1.2.1.8 Confirmed mixed infection

Mixed infections involving culture-confirmed TBM or MOTT meningitis were classified under those categories, otherwise patients were categorised under the “Confirmed mixed infection” category (referring to other mixed infections) based on culture, serology or molecular evidence.

2.5.2.1.2.1.9 Suspected mixed infection

Patients were categorised as ‘Suspected mixed infection’ based on limited clinical-radiological and laboratory evidence (as documented in patients’ files). More than 1 diagnosis was suspected, but not confirmed with culture.

2.5.2.1.2.1.10 Confirmed *Mycobacterium* other than tuberculosis (MOTT) meningitis

Categorisation of patients in this category was based on culture.

2.5.2.1.2.1.11 Suspected neurocysticercosis

Categorisation here was based on clinical-radiological suspicion as well as sero-positivity on the serum sample and sero-negativity on the CSF sample.

2.5.2.1.2.1.12 Confirmed neurosyphilis

Patients were categorised as ‘Confirmed neurosyphilis’ based on clinical suspicion together with laboratory evidence. In most cases CSF VDRL and TPHA assays were positive.

2.5.2.1.2.1.13 Confirmed toxoplasmosis

Histology of suspected lesions, serum serology as well as radiological suspicion was used to categorised patients as ‘Confirmed toxoplasmosis’.

2.5.2.1.2.1.14 Confirmed ventriculitis

Culture confirmation as well as radiological evidence was used to categorise patients as ‘Confirmed ventriculitis’.

2.5.2.1.2.1.15 Confirmed viral meningitis / encephalitis

Clinical suspicion together with CSF chemistry and cell count, molecular, and / or serological evidence was used to categorise patients as ‘Confirmed viral meningitis / encephalitis’.

2.5.2.1.2.1.16 Suspected viral meningitis / encephalitis

Clinical suspicion together with an absence of culture, molecular or serological evidence, the presence of a lymphocytosis and an elevated CSF protein and normal CSF glucose (as documented in patient files) was used to categorise patients as ‘Suspected viral meningitis / encephalitis’.

2.5.2.1.2.2 Non-infective

2.5.2.1.2.2.1 Carcinomatous meningitis

Inclusion in this category was based on cytology or histology.

2.5.2.1.2.2.2 Medical – related pathology

Categorisation of patients as ‘Medical – related pathology’ was based on clinical and radiological data as well as other para-clinical data.

2.5.2.1.2.2.3 Psychiatric disorders (and psychiatric – related disorders)

The diagnoses included in this category were considered by clinicians / psychiatrists according to criteria as stipulated in the Diagnostic and Statistical Manual of Mental Disorders.

2.5.2.1.2.2.4 Surgical – related pathology

Categorisation of patients was based on clinical and radiological data as well as other para-clinical data.

2.5.2.1.2.2.5 Toxicology

Patients with drug overdose and drug toxicity, organophosphate poisoning, and traditional medicine-related intoxication were included in this category.

2.5.2.2 Pathologies not involving the CSF

2.5.2.2.1 Other

All pathologies not involving CSF or intracranial anatomy were included in this category.

2.5.2.3 No diagnosis made

No clinical, radiological or laboratory evidence was found to assign these patients to any of the above mentioned categories.

2.6 CSF cell counts and chemistry

The maximum reportable cell count value for CMJAH NHLS branch is 10 000 / mm^3. In some cases values > 10 000 / mm^3 were captured as the actual value on the laboratory information system (example: a polymorphonucleur cell count of 28 000 / mm^3) and reflected in the laboratory report as such. In other cases a value of 10 001 / mm^3 was inserted in the laboratory information system and reflected as > 10 000 / mm^3 in the laboratory report. All

values in this study > 10 000 / mm^3 was captured as 10 001 / mm^3. Although this may not be accurate, it was done as statistical analysis could only be performed on absolute values.

The Advia analyser (Siemens, United States of America) was used to determine the CSF protein (by means of a pyrogallol red-based method) and CSF glucose (by means of a glucose hexokinase-based method).

For CSF protein measurement the linear range for the Advia assay (Siemens, United States of America) is 0.01 – 5.00 g/L. Any value greater than 5.00 g/L is reported as > 5.00 g/L. The absolute maximum value accepted by the laboratory information system is 17.00 g/L. The actual value that was entered was captured for this study; 92 of the total 3548 CSF protein results were above 5 g/L. Although this may not be accurate, again this was done as statistical analysis could only be performed on absolute values.

Regarding CSF glucose: the linear range for the Advia assay (Siemens, United States of America) is 0 – 38.9 mmol/L. Only 1 sample had to be diluted into the linear and was reported as 43.3 mmol/L. The absolute maximum value accepted by the laboratory information system is 2000 mmol/L. Any value above a 1000 mmol/L is reported as > 1000 mmol/L (please note that these limits were arbitrarily set on the laboratory information system).

2.7 ADA assay utilized in this study

The Diazyme ADA assay (Diazyme, United States of America) is utilized at CMJAH NHLS Chemical Pathology Laboratory for measurement of ADA. In the presence of ADA and water,

adenosine is broken down to inosine and ammonia. Generation of inosine is subjected to a cascade of enzymatic reactions that culminate in the production of a quinine dye that is measured spectrophotometrically at 556nm. One unit of ADA is defined as the amount of ADA that generates 1 μmol of inosine from adenosine per minute at 37^{o}C. This assay measures total ADA levels. This assay was utilised by the laboratory in 2009 and 2010. See Appendix B for the Diazyme ADA kit package insert Catalog No. DZ117A-K (Diazyme, United States of America). The current clinical decision limit for TBM used by the CMJAH NHLS Chemical Pathology laboratory is 6 U/L. The origin of this cut-off is unclear.

The assay is performed on a Pentra analyser (Horiba Medical Diagnostics, Japan) with colorimetric detection. The linearity for the assay in the package insert is stated as 0 – 200 U/L. See Appendix B for the Diazyme ADA kit package insert Catalog No. DZ117A-K (Diazyme, United States of America). The reportable range on the laboratory information system is 1 – 400 U/L. Serum bovine albumin based calibration and quality control material is supplied by Diazyme. The Diazyme calibration material assigned value is 50 U/l. The supplied Diazyme control material (Diazyme, United States of America) assigned values are: 10.7 +/- 1.9 U/L for level 0, 29.8 +/- 5.1 U/L for level 1 and 137.6 +/- 23.4 U/L for level 2. The assigned values represented here are as per the current material used and have not changed significantly over the last 3 years. See Appendix B for the Diazyme ADA calibrator package insert Catalog No. DZ117A-CAL and Diazyme ADA control package inserts Catalog No. DZ117A (Diazyme, United States of America).

The assay is currently used for the measurement of ADA in peritoneal, pericardial and pleural fluid as well as CSF. For non-CSF specimens, the clinical decision limit used by the CMJAH NHLS Chemical Pathology laboratory is 30 U/L. All CSF ADA values captured for this study were taken as the exact value entered on the laboratory information system.

2.8 Ethics

An ethics clearance certificate (number M110212) for this study was obtained for this study from the University of the Witwatersrand Human Research Ethics Committee (Appendix C).

2.9 Statistical analysis of data collected

For the cell counts and chemistry as well as the CSF ADA results for each of the categories, the minimum, median, and maximum values as well as the interquartile ranges (IQRs) were determined. Because the data followed a non-parametric/non-Gaussian distribution, the IQR provides a measure of the spread of results.

All CSF ADA test results for (1) adults and paediatric patients, (2) male and female patients, (3) HIV-positive and HIV-negative patients, and (4) patients with a CD4 cell count > 350 x 10^6/L and those with a CD4 cell count ≤ 350 x 10^6/L, were compared in each of these groups using the Mann-Whitney and Kruskal-Wallis tests for non-parametric data (Dawson-Saunders & Trapp, 1994).

Using the Mann-Whitney test CSF ADA results were compared between the ‘Confirmed viral meningitis / encephalitis’, ‘Confirmed ventriculitis’, ‘Confirmed bacterial meningitis’ and

'Confirmed TBM' groups. Similarly 'Confirmed TBM' CSF ADA results were compared to all CSF ADA results for patients where TB culture on CSF was absent and or negative.

Receiver operating characteristic (ROC) curves and the Youden index were generated to establish the optimal cut-off point for ADA interpretation in patients for whom a CSF sample was submitted for both mycobacterial culture and ADA determination (Akobeng, 2007). The Youden index is defined as the maximum value determined by the equation: {sensitivity + specificity – 1}. It represents the maximum distance between the ROC curve generated and the line of 'No discrimination'. This methodology determines a cut-off point that maximises correct classification of individuals with disease and further represents a point on the curve farthest from chance. It is considered "optimal" when equal weighting is given to sensitivity and specificity (Perkins & Schisterman, 2006). The sensitivity and specificity, positive and negative likelihood ratios and the positive and negative predictive values was determined for this cut-off point. In addition to these values and ratios, the diagnostic odds ratio was determined (Glas, *et al*., 2003). A p-value of < 0.05 was considered statistically significant. For each of the individual groups (adult, paediatric, male, female, HIV-positive and HIV-negative patients as well as those with a CD4 cell count $> 350 \times 10^6$/L and those with a CD4 cell count $\leq 350 \times 10^6$/L) who had a CSF sample submitted for mycobacterial culture and ADA determination, ROC curve analysis was utilised to determine differences in these groups' cut-off point for ADA. The area under the curve (AUC) was used to compare the individual ROC curves (Dawson-Saunders & Trapp, 1994).

The investigator attempted to formulate a prediction rule for the diagnosis of TBM utilising the CSF ADA test result together with clinical, radiological and other laboratory data collected in this study using stepwise logistic regression analysis.

CSF ADA results for each of the categories in the clinical research case definition were compared by determining the minimum, median, and maximum values as well as the IQRs.

CSF sample integrity as defined by its appearance (clear, turbid, bloodstained and xanthochromic) was used to re-categorise CSF ADA results and these categories were compared by determining the minimum, median, and maximum values as well as the IQRs.

Statistical analyses were performed on Analyse-it (Analyse-it Software, Ltd., United Kingdom).

3.0 RESULTS

3.1 Demographics and Patient categorisation

CSF ADA and associated results for 3548 patients were captured as described in the 'Materials and Methods' section. Table 3.1 summarises details on the 8 demographic groups for this study. The bulk of the patients considered were adults (89.3%), of which 2009 individuals were between the ages of 20 years and 40 years. Male and female patients considered were roughly equal in number. Most patients were HIV-positive (55.5%). In the total population 41.0% of patients had CD4 cell counts available measuring $\leq 350 \times 10^6$/L. Of all the results available for CD4 cell count, 37 results accounted for patients 5 years or younger, of which 25 were $> 350 \times 10^6$/L and 12 were $\leq 350 \times 10^6$/L. These were excluded from analysis when comparing the 2 CD4 cell count-based groups.

Table 3.1: Demographic categories for the total population of 3548 patients.

Age	Adult patients: n=3170 (89.3%)
	Paediatric patients: n=365 (10.3%)
	No information regarding age available: n=13 (0.4%)
Sex	Male patients: n=1721 (48.5%)
	Female patients: n=1824 (51.4%)
	No information regarding sex available: n=3 (0.1%)
HIV status	HIV-positive patients: n=1970 (55.5%)
	HIV-negative patients: n=693 (19.5%)
	No information regarding HIV status available: n=885 (25%)
CD4 cell count	Patients with CD4 cell count $> 350 \times 10^6$/L: n=235 (6.6%)
	Patients with CD4 cell count $\leq 350 \times 10^6$/L: n=1456 (41.0%)
	No information regarding CD4 cell count available: n=1857 (52.3%)

Table 3.2 summarises the total number of patients considered for each of the categories (according to pathologies involving the CSF, pathologies not involving the CSF, and 'No diagnosis made').

Table 3.2: Number of patients per category in the total population (n=3548).

Category	Number of patients
Suspected bacterial meningitis	48
Suspected meningitis	299
Suspected mixed infection	62
Suspected neurocysticercosis	35
Suspected TBM	264
Suspected viral meningitis / encephalitis	34
Confirmed bacterial meningitis	134
Confirmed brain abscess	8
Carcinomatous meningitis	13
Confirmed cerebral malaria	3
Suspected cerebritis	9
Confirmed cryptococcal meningitis	302
Medical – related pathology	525
Confirmed mixed infection	3
Confirmed MOTT meningitis	4
Confirmed neurosyphilis	10
No diagnosis made	168
Other	1082
Psychiatric disorders (and psychiatric – related disorders)	298
Surgical – related pathology	93
Confirmed TBM	92
Toxicology	28
Confirmed ventriculitis	16
Confirmed viral meningitis / encephalitis	14

Culture results for 2 patients were cryptococcal species other than *Cryptococcus neoformans*: i.e. *Cryptococcus albidi* and *Cryptococcus laurentii*. These as well as the only 2 cases of 'Confirmed toxoplasmosis' were excluded from data analysis for cell counts and chemistry as

well from the determination of the minimum, median, maximum and IQR for CSF ADA results in each of the categories as the result number were insufficient for meaningful analysis.

In the 'Confirmed TBM' category 88 patients were culture-confirmed, of which 83 were admitted to hospital for the first time and showing culture positivity. The remaining 5 were admissions of known patients, with culture positivity determined in a previous admission and hence the patient still falling in the 9 – 12 months treatment period (the CSF sample for the current admission in these five patients was negative for TB culture). Four patients were positive for AFB on microscopy only. For the study period 2009 – 2010, 38 culture positive patients were not included as CSF ADA was not requested in those patients' samples.

TBM was suspected in other clinical scenarios in addition to the 264 patients categorised as 'Suspected TBM' (see Table 3.3). At least 126 of the 394 patients were treated with anti-tuberculous chemotherapy.

Table 3.3: Clinical scenarios where suspected TBM was considered by clinicians.

Suspected TBM	**Number**
Considered as primary diagnosis	264
Considered as part of a mixed infection	62
Considered in the presence of alternative culture confirmed diagnosis	68

Table 3.4 lists the organisms cultured and the number of patients with these organisms that made up the 'Confirmed bacterial meningitis' category. *Streptococcus pneumoniae* was the most commonly isolated organism.

Table 3.4: Culture results for the 'Confirmed bacterial meningitis' category.

Organisms	Number of patients
Enterococcus faecium	1
Escherichia coli	3
Haemophilus parainfluenzae	1
Klebsiella pneumoniae **(in 1 case** ***Enterobacter cloacae*** **was cultured together with the** ***Klebsiella pneumoniae*****)**	4
Listeria monocytogenes	4
Methicillin-resistant ***Staphylococcus aureus***	2
Neisseria meningitidis	28
Streptococcus agalactiae	2
Streptococcus Group G	1
Streptococcus mitis	1
Streptococcus pneumoniae	84
Pseudomonas aeruginosa **(in 1 case** ***Acinetobacter baumannii*** **was cultured together with the** ***Pseudomonas aeruginosa*****)**	3

In the 'Suspected bacterial meningitis' category 2 cases were included where the significance of the organism cultured was queried. One sample with significant erythrocyte contamination cultured *Salmonella* group D and one sample with no cellular response and no changes in CSF chemistry cultured *Pseudomonas aeruginosa*.

In the 'Confirmed brain abscess' category 2 cases were included where pus was noted at surgery, but not sent for culture. Culture results were available for 4 cases. One case of *Streptococcus milleri* was cultured on a pus sample. One case of Methicillin-resistant

Staphylococcus aureus was cultured from an ear swab; this was found together with prominent radiological findings. One case of *Acinetobacter baumannii* and *Proteus mirabilis* was cultured on a pus sample and a tissue sample. One case of *Proteus mirabilis* was cultured on a pus sample, a fluid sample and a tissue sample. In the remaining 2 cases, where the diagnosis was made in the absence of intra-operative evidence or culture, addition to this category made no difference to the data analysis.

In all patients making up the 'Confirmed cerebral malaria' category, *Plasmodium falciparum* was diagnosed on microscopy as well on a rapid test.

In amongst other categories 27 patients were found with radiological evidence of cerebritis This was not the primary pathology considered and not included in the 'Suspected cerebritis' category.

At least 19 patients in 'Confirmed cryptococcal meningitis' category was treated for TBM as well. Common reasons for commencing treatment included: elevated ADA, elevated CSF protein and elevated CSF lymphocyte count. TBM was not culture confirmed. At least 10 patients in this category were treated for bacterial meningitis as well. A common reason for commencing treatment was an elevated polymorphonuclear cell count. Bacterial meningitis was not culture confirmed. In at least 6 cases which form part of these treated patients, chemotherapy was commenced for both TBM and bacterial meningitis.

In the 'Confirmed mixed infection' 2 cases of culture-confirmed *Cryptococcus neoformans* was found together with PCR positivity for John Cunningham (JC) virus on CSF. One case of

‘Confirmed viral meningitis / encephalitis’ with a positive Epstein-Barr virus (EBV) viral load on CSF was found together with radiological evidence for neurocysticercosis and a positive serum serology for cysticercosis. In this patient the attending clinician was also concerned about the presence of bacterial meningitis; although no organism was cultured, erythrocyte contamination obscured the CSF cell count and CSF chemistry making interpretation difficult.

In the ‘Suspected mixed infection’ category, more than 1 diagnosis was suspected, but not confirmed with culture. These included 1 or more of the following: bacterial meningitis, cryptococcal meningitis, neurocysticercosis, TBM, and/or toxoplasmosis. In some cases patients were treated for more than 1 of these pathologies.

The category ‘Confirmed MOTT meningitis’ included 1 patient with *Mycobacterium intracellulare*, 1 patient with *Mycobacterium avium complex*, and 1 patient where both *Mycobacterium interjectum* and *Mycobacterium marinum* was cultured. One report only indentified the growth as a non-tuberculous mycobacterium.

In the ‘Confirmed neurosyphilis’ category for 2 patients only the TPHA assay on CSF was positive (1 of these patients had a positive RPR assay on serum). In 3 cases no syphilis serology was available for CSF (1 of these patients had a positive RPR assay on serum). Addition of these 3 cases to this category made no difference to the data analysis.

One patient was managed as toxoplasmosis based on radiological suspicion together with serum serology IgG positivity. The other patient in this category had histological confirmation of toxoplasmosis.

Fifteen patients with congenital hydrocephalus and 1 adult that was being worked up for dementia were classified as 'Confirmed ventriculitis'. In 7 patients culture for bacteria was positive: *Citrobacteri koserii* (1), coagulase-negative staphylococcus (1), *Klebsiella pneumoniae* (1), methicillin-sensitive *Staphylococcus aureus* (1), methicillin-resistant *Staphylococcus aureus* (2), and *Staphylococcus epidermidis* (1). In 1 case only microscopy was positive for Gram-positive cocci in clusters with no growth on culture. In 8 cases there was no culture nor microscopy evidence; only the cell count on the CSF was highly suggestive of pathology. Addition of these 8 cases to this category made no difference to data analyses.

Table 3.5 summarises the cases included in the 'Confirmed viral meningitis / encephalitis' category. The most common virus identified was the JC virus.

Table 3.5: Summary of cases categorised as 'Confirmed viral meningitis / encephalitis'.

Scenarios were virus were identified with identification method	Number of cases
JC virus (PCR on a CSF sample; in one case CSF cytology had features worrying for a large B-cell lymphoma; in one case the presence of oligoclonal banding was noted on CSF together with suspicious radiology)	6
Enterovirus (PCR on a CSF sample)	1
Coxsackie B virus (viral neutralization on a blood sample) and **Herpes simplex virus** (serology on a blood sample)	1
Cytomegalovirus (CMV) (viral load on a CSF sample)	1
CMV (viral load on a CSF sample) and **EBV** (viral load on a CSF sample)	1
EBV (viral load on a CSF sample)	1
EBV (viral load on a CSF sample) and **JC virus** (PCR on a CSF sample)	1

Table 3.5 continued

Measles (serology on a blood)	**1**
Mumps (serology on a CSF sample)	1

Non-Hodgkin's lymphoma accounted for the aetiology in the bulk of 'Carcinomatous meningitis' category: 9 patients were identified (in 1 of these 9 patients, although no malignant cells were noted, the comment was made that the cytological picture was suspicious for Non-Hodgkin's lymphoma). In 1 patient the radiology was suspicious for carcinomatous meningitis. In 2 patients the cytology was positive for involvement by breast carcinoma and 1 patient the cytology was positive for involvement by gastric carcinoma.

In the 'Medical – related pathology' category often more than 1 diagnosis existed for each patient. Thus the number of patients noted for this category in Table 3.2 includes any patient with 1 or more medical diagnosis. These diagnoses included any of the following (for each of these, the frequency of diagnoses follows in brackets): acute demyelinating encephalomyelitis (8), apnoea (7), benign intracranial hypertension (3), cerebral palsy (4), cerebral pontine myelinolysis (1), demyelinating disease (7), diabetic ketoacidosis (16), eclampsia (2), electrolyte abnormalities (7), epilepsy (138), headache (44), hepatic encephalopathy (5), hyperglycaemia (11), hyperosmolar non-ketotic coma (7), hypertensive encephalopathy (3), hypoglycaemia (33), hypoxic brain injury (6), intracranial venous sinus thrombosis (6), lactic acidosis (15), multiple sclerosis (6), neonatal jaundice (13), neurosarcoidosis (1), Pancerebellar syndrome (6), Posterior Reversible Encephalopathy syndrome (2), Progressive multifocal leucoencephalopathy (3), stroke (132), seizures (97), and transient ischaemic attack (1).

The diagnoses included in the 'Psychiatric disorders (and psychiatric – related disorders)' category were: primary mood disorders, primary psychosis-related disorders, mood- and psychosis-related disorders secondary to underlying medical disorders and drug abuse (especially cannabis and alcohol), adjustment disorder, and conversion disorder. Also included in this category are patients presenting with dementia and delirium in various stages of being worked up for underlying causes. As the various diagnoses considered here were in different stages of the diagnostic process (varying from suspected to confirmed) as well as the fact that this diagnostic process required long-term follow-up, it was difficult to assign actual numbers of patients to each of the diagnoses mentioned above.

In the 'Surgical – related pathology' category often more than 1 diagnosis existed for each patient. Thus the number of patients noted for this category in Table 3.2 includes any patient with 1 or more surgical diagnosis. These diagnoses included any of the following (for each of these, the frequency of diagnoses follows in brackets): aneurysm (6), arterio-venous malformation (3), hydrocephalus (these had no ventriculitis or meningitis and were noted in 55 cases), intracranial haemorrhage (28), and trauma (18).

In the 'Toxicology' category sometimes more than diagnosis existed for each patient. Thus the number of patients noted for this category in Table 3.2 includes any patient with 1 or more diagnosis. These diagnoses included any of the following (for each of these, the frequency of diagnoses follows in brackets): patients with drug overdose and drug toxicity (18), organophosphate poisoning (4), and traditional medicine-related intoxication (8) were included in this category. Alcohol and cannabis users usually presented with psychosis and were classified under the 'Psychiatric disorders (and psychiatric – related disorders)' category.

In the ‘Other’ category Haematology / Oncology – related diagnoses (225 patients), patients for work-up of disseminated TB (with no culture evidence for TBM accounting for 204 patients) and patients for work-up of sepsis (186 patients) accounted for the most prominent diagnoses. Regarding drug- induced hepatitis 4 patients of the 394 patients where TBM was suspected and treatment was commenced had this complication; the other 22 patients with drug-induced hepatitis were seen in patients with pulmonary TB and disseminated TB (these 22 patients were categorised as ‘Other’).

3.2 CSF cell counts and CSF chemistry for categories

A summary of the CSF cell counts (Table D1) and CSF chemistry (Table D2) for the categories mentioned above can be found in Appendix D. The minimum, median, and maximum values are noted in each case as well as the IQRs. CSF cell count and CSF chemistry patterns for ‘Confirmed bacterial meningitis’, ‘Confirmed viral meningitis / encephalitis’ and ‘Confirmed TBM’ followed textbook descriptions (Fitzgerald & Haas, 2005; Tunkel & Scheld, 2005). Similar patterns were noted in the ‘Suspected bacterial meningitis’, ‘Suspected viral meningitis / encephalitis’ and ‘Suspected TBM’ categories. In the investigator’s experience the CSF white cell count and CSF protein measurement in cryptococcal meningitis can vary from depressed, normal to elevated.

Very importantly Table D1 and Table D2 reflect the presence of erythrocyte contamination in many samples. Overall 2129 samples cell counts reflected the presence of erythrocytes. In 593 of these samples erythrocytes numbers were $\geq$ 100 cells / mm^3 and in 232 of these samples erythrocyte numbers were $\geq$ 1000 cells / mm^3. This interferes with the measurement of CSF

protein resulting in elevated levels as well as resulting in falsely elevated polymorphonuclear cells and lymphocytes hence affecting the overall interpretation.

3.3 TB culture data

Table 3.6 reflects TB culture data for those patients in this study that had both a mycobacterial culture and an ADA requested on a CSF specimen. Please note that the 5 patients who fell in the 9 – 12 months treatment period for TBM and hence had culture positivity on a previous admission, did have a CSF specimen sent for mycobacterial culture in the current admission were an ADA was requested, but the result of this culture was negative. In only a 115 patients, more than 1 specimen was sent for culture (totaling 141 specimens in addition to the admission specimen that was sent). As mentioned in the introduction, culture yield is affected by the volume and frequency of specimen submitted. Total samples referred from CMJAH to the Mycobacterial Reference Laboratory for 2009 and 2010 totaled 2411. Culture positivity for these specimens was 5.4%. Contamination of the 2411 specimens occurred in 3.7% of cases. The contamination rate for the total number of specimens relevant to this study sent to the laboratory was 2.9%. This is acceptable.

Table 3.6: TB culture data amongst patients used in this study.

Total number of patients with a CSF sample sent for culture	1490
Total number of patients that are culture positive for TB	83
Total number of patients that are culture positive for MOTT	5
Total specimens sent to laboratory	1631
Total patients with more than 1 sample sent	115
Total specimens positive TB	88
Total specimens positive MOTT	5
Total specimens contaminated	47
AFB only positive	4

3.4 Ranges of CSF ADA for categories

The performance of CSF ADA measurement in each of the categories is depicted in Figure 3.2 and 3.3 as well as in Table 3.7. Figure 3.2 emphasises the outlier values in terms of interquartile ranges (IQR), whereas Figure 3.3 reflects the 95% distribution of the values in each of the categories. In addition, the diamond plot in Figure 3.2 reflects the 95% confidence interval of the mean values. The box and whisker plot in Figure 3.3 reflects the median value as well as the first and third IQRs. In Table 3.7 the actual minimum, median and maximum ADA values are given for each of the categories as well as the IQRs. Figure 3.2 and Figure 3.3 as well as Table 3.7 appear on pages 48 to 50. It is important to note at this point that the ADA results follow a non-parametric or non-Gaussian distribution (Figure 3.1) in the total population assessed for this study (n=3548). The bulk of the results are in fact all < 10 U/L.

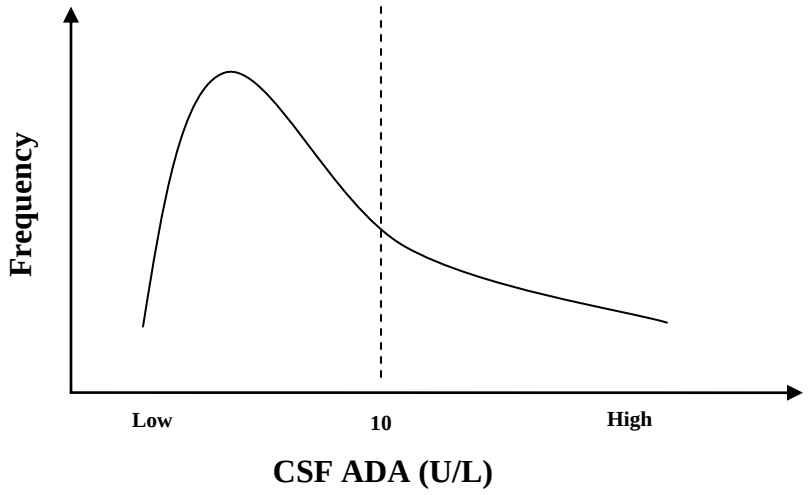


Figure 3.1: Non-parametric / non-Gaussian distribution of CSF ADA results.

The CSF ADA results depicted in Figure 3.2 and Figure 3.3 reflect a considerable overlap, not only in the outliers for each category, but also in each category's 95% distribution of values.

Overlapping in the outliers can be seen in the following categories: 'Suspected bacterial meningitis', 'Suspected meningitis', 'Suspected mixed infection', 'Suspected TBM', 'Confirmed bacterial meningitis', 'Carcinomatous meningitis', 'Confirmed cryptococcal meningitis', 'Medical – related pathology', 'No diagnosis made', 'Other', 'Psychiatric disorders (and psychiatric – related disorders)', 'Surgical – related pathology', 'Confirmed TBM', and 'Confirmed ventriculitis' (Figure 3.2).

Overlapping in the 95% distribution of values can be seen especially in the following categories: 'Suspected bacterial meningitis', 'Suspected mixed infection', 'Suspected TBM', 'Confirmed bacterial meningitis', 'Carcinomatous meningitis', 'Confirmed cryptococcal meningitis', 'Confirmed MOTT meningitis', 'Confirmed TBM' and 'Confirmed ventriculitis' (Figure 3.3).

Of note the 'Confirmed ventriculitis' category had the widest 95% distribution of values (Figure 3.3). This is most likely due to the fact that the ADA assay is picking up ADA-1 from cellular activity of polymorphonuclear cells and lymphocytes in the CSF.

Key to category indications in Figure 3.2 and Figure 3.3 and Table 3.7: ? Bacterial meningitis – Suspected bacterial meningitis; ? Meningitis – Suspected meningitis; ? Mixed infection – Suspected mixed infection; ? Neurocysticercosis – Suspected neurocysticercosis; ? TBM – Suspected TBM; ? Viral meningitis / Encephalitis – Suspected viral meningitis / encephalitis; Bacterial meningitis – Confirmed bacterial meningitis; Brain abscess – Confirmed brain abscess; Cerebral malaria – Confirmed cerebral malaria; Cerebritis – Suspected cerebritis; Cryptococcal meningitis – Confirmed cryptococcal meningitis; Medical – Medical – related pathology; Mixed infection – Confirmed mixed infection; MOTT meningitis – Confirmed MOTT meningitis; Neurosyphilis – Confirmed Neurosyphilis; Psychiatric – Psychiatric disorders (and psychiatric-related disorders); Surgical – Surgical – related pathology; TBM – Confirmed TBM; Ventriculitis – Confirmed ventriculitis; Viral meningitis / Encephalitis – Confirmed viral meningitis / encephalitis.

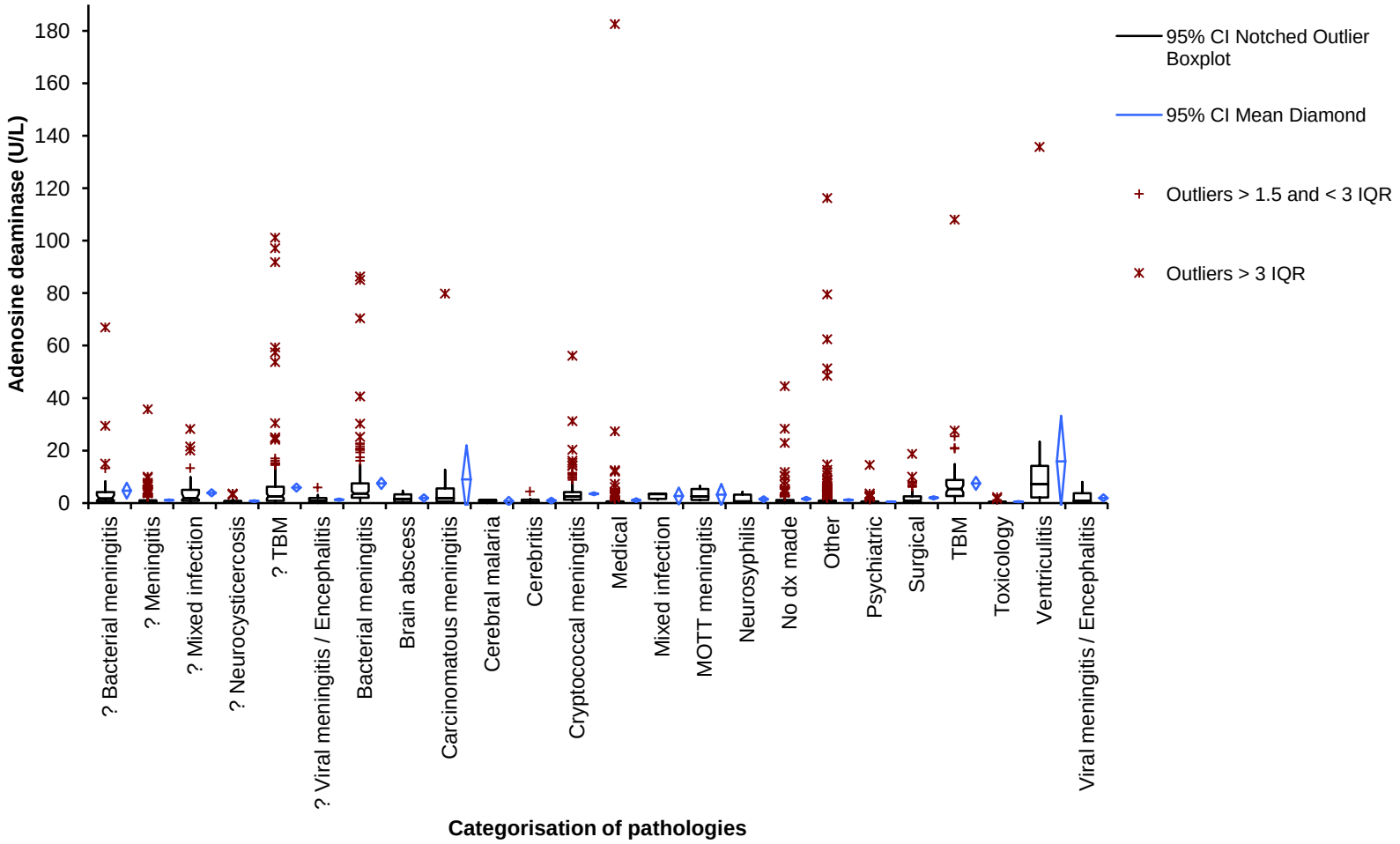


Figure 3.2: CSF ADA values for each of the categories, with emphasis on outliers.

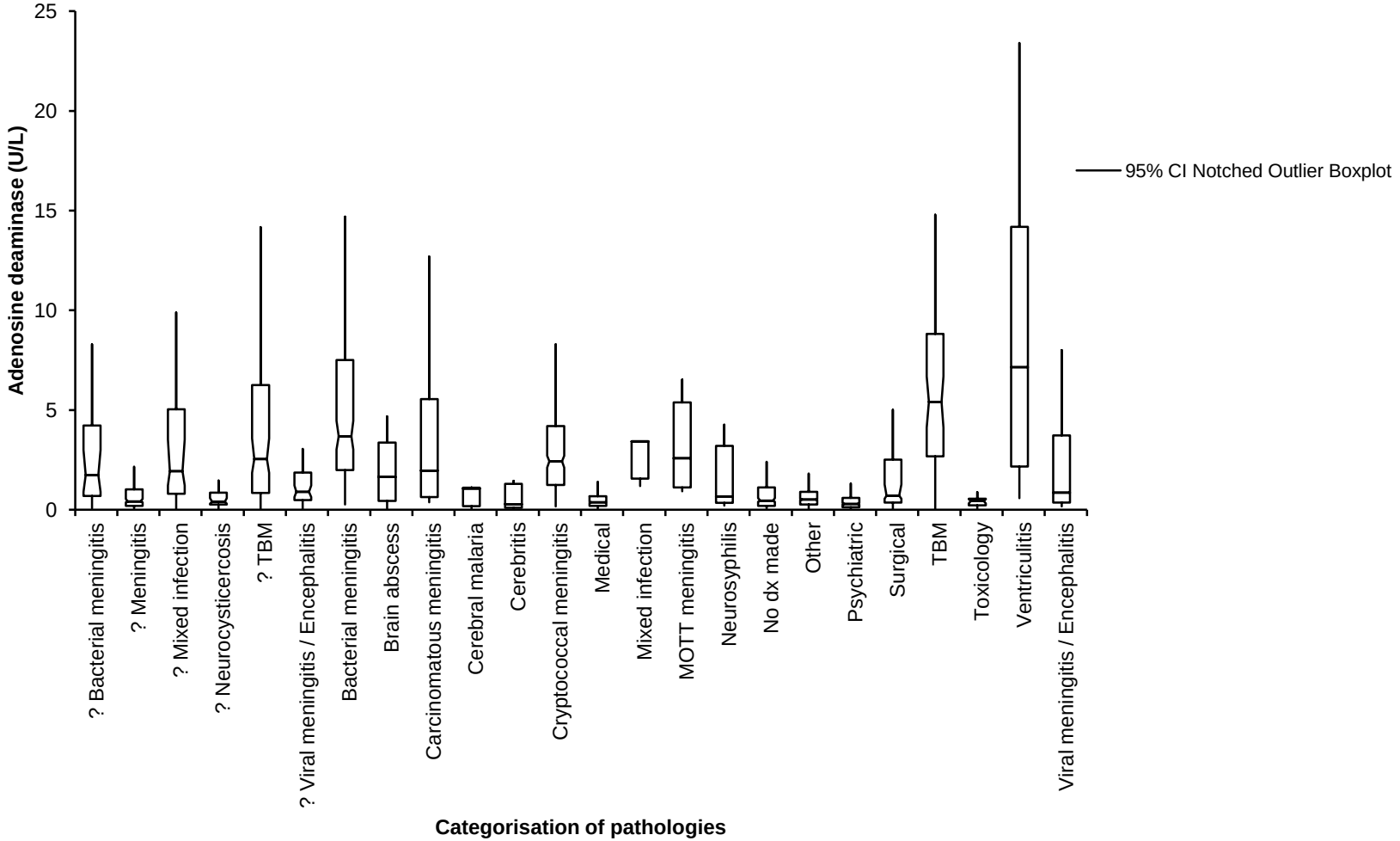


Figure 3.3: CSF ADA values for each of the categories, with emphasis on the 95% distribution of values.

Table 3.7: CSF ADA values (in U/L) for each of the categories.

Categories	n	Minimum	Median	Maximum	IQR
? Bacterial meningitis	48	0.1	1.74	66.9	3.53
? Meningitis	299	0.0	0.42	35.7	0.84
? Mixed infection	62	0.0	1.94	28.2	4.24
? Neurocysticercosis	35	0.0	0.40	3.6	0.59
? TBM	264	0.0	2.54	101.1	5.41
? Viral meningitis / Encephalitis	34	0.0	0.90	5.9	1.38
Bacterial meningitis	134	0.3	3.69	86.3	5.52
Brain abscess	8	0.1	1.64	4.7	2.93
Carcinomatous meningitis	13	0.4	1.96	79.8	4.91
Cerebral malaria	3	0.0	1.07	1.1	0.93
Cerebritis	9	0.0	0.28	4.4	1.20
Cryptococcal meningitis	302	0.2	2.43	56.1	2.96
Medical	525	0.0	0.38	182.5	0.49
Mixed infection	3	1.2	3.42	3.4	1.88
MOTT meningitis	4	0.9	2.59	6.5	4.26
Neurosyphilis	10	0.2	0.67	4.3	2.86
No dx made	168	0.0	0.45	44.5	0.92
Other	1082	0.0	0.51	116.2	0.63
Psychiatric	298	0.0	0.29	14.5	0.48
Surgical	93	0.0	0.71	18.7	2.16
TBM	92	0.0	5.40	108.0	6.14
Toxicology	28	0.0	0.44	2.2	0.33
Ventriculitis	16	0.6	7.15	135.7	12.02
Viral meningitis / Encephalitis	14	0.2	0.86	8.0	3.37

Table 3.8 summarises cases where, in addition to *Mycobacterium tuberculosis*, other organisms were also identified as well as the CSF ADA for each of these cases. As discussed in 2.5.2.1.2.1.8 of this research report, these patients were captured as part of the 'Confirmed TBM' category and excluded from the other categories.

Table 3.8: Mixed infections classified as 'Confirmed TBM'.

Organism identified in addition to *Mycobacterium tuberculosis* with identification method	**Number of cases**	**CSF ADA (U/L)**
***Cryptococcus neoformans* (culture)**	1	4.1
***Cryptococcus neoformans* (CLAT)**	1	6.7
***Streptococcus pneumoniae* (culture)**	2	5.9 and 4.4
Epstein- Barr virus (viral load)	1	6.7
***Mycobacterium intracellulare* (culture)**	1	7.1

As the second organism may have contributed to the CSF ADA result, exclusion of these cases from the 'Confirmed TBM' category resulted in the following: for the remaining 86 of 92 CSF ADA results considered in this category the minimum value was 0.0 U/L, the median value was 5.27 U/L, the maximum value was 108 U/L and the IQR was 7.13 U/L. The median value and IQR did not differ significantly when compared to the total data set (n=92).

In amongst other categories the 27 patients who were found with radiological evidence of cerebritis for the most part had CSF ADA values ≤ 6 U/L.

In the ‘Confirmed cryptococcal meningitis’ category the bulk of the CSF ADA results for patients who were treated for TBM as well were < 7 U/L (one result was measured at 11.3 U/L and one result was measured at 14.1U/L). The bulk of the CSF ADA results for patients who were treated for bacterial meningitis as well were < 7 U/L (one result was measured at 9.7 U/L and one result was measured at 11.1 U/L).

3.5. Common infective intra-cranial pathologies compared

Individually all CSF ADA results in the ‘Confirmed cryptococcal meningitis’ (n=302), ‘Confirmed bacterial meningitis’ (n=134), ‘Confirmed viral meningitis / encephalitis’ (n=14) and ‘Confirmed ventriculitis’ (n=16) categories were compared with the ‘Confirmed TBM’ category (n=92) using the Mann-Whitney test (for independent samples). Statistically significant differences were noted in all cases (p values ≤ 0.0001) except in the comparison between the ‘Confirmed bacterial meningitis’ and ‘Confirmed TBM’ categories (p value = 0.0643) and the ‘Confirmed ventriculitis’ and ‘Confirmed TBM’ categories (p value = 0.4162). As stated previously, this may be accounted for by the polymorphonuclear cell and lymphocyte activity in CSF specimens of patients in the ‘Confirmed bacterial meningitis’ and ‘Confirmed ventriculitis’ categories.

3.6 Statistical comparison of demographic groups regarding CSF ADA results

3.6.1 Total population (n=3548)

The Mann-Whitney and Kruskal-Wallis tests were performed to compare CSF ADA results across all 8 demographic groups that had a CSF ADA requested (Table 3.9). The results did

not differ between these 2 tests. In all comparisons, CSF ADA results were higher in adult than in paediatric patients, higher in male than in female patients, higher in HIV-positive than in HIV-negative patients and higher in patients with CD4 cell counts ≤ 350 x 10^6/L as opposed to patients with CD4 cell counts > 350 x 10^6/L. These differences were statistically significant ($p < 0.05$).

Table 3.9: Statistical comparison of the 8 demographic groups regarding their CSF ADA results (total population, n=3548).

n = 3548	A	P	M	F	HIV-positive	HIV-negative	CD4 ≤ 350 x 10^6/L	CD4 > 350 x 10^6/L
n	3170	365	1721	1824	1970	693	1456	235
Minimum (U/L)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Maximum (U/L)	182.5	135.7	182.5	135.7	116.2	91.8	116.2	35.7
Median (U/L)	0.6	0.5	0.7	0.6	0.9	0.3	1.0	0.6
95% C/I of Median	0.6 to 0.7	0.4 to 0.6	0.6 to 0.7	0.6 to 0.6	0.9 to 1.0	0.3 to 0.4	0.9 to 1.0	0.4 to 0.7
Two-tailed probability	p = 0.0174		p = 0.0066		p < 0.0001		p < 0.0001	

3.6.2 'Confirmed TBM' category (n=92)

The Mann-Whitney and Kruskal-Wallis tests were performed to compare CSF ADA results among all patients in the 'Confirmed TBM' category across the 8 demographic groups (Table 3.10). The results did not differ between these 2 tests. Although the median CSF ADA results in each of the categories performed similarly to that in the total population (n=3548), no statistically significant differences were noted. This may be related to the small numbers

compared (2 paediatric patients, 7 HIV-negative patients, and 4 patients with CD4 cell counts > 350 x 10^6/L).

Table 3.10: Statistical comparison of the 8 demographic groups regarding their CSF ADA results ('Confirmed TBM' category, n=92).

n = 92	A	P	M	F	HIV-positive	HIV-negative	CD4 ≤ 350 x 10^6/L	CD4 > 350 x 10^6/L
n	90	2	47	45	77	7	58	4
Minimum (U/L)	0.0000	0.6000	0.0000	0.4000	0.0000	0.6000	0.0000	2.7000
Maximum (U/L)	108.0000	2.0000	25.4000	108.0000	108.0000	25.4000	108.0000	25.4000
Median (U/L)	5.6500	1.3000	6.7000	4.1000	5.2000	3.6000	4.9500	5.2000
95% C/I of Median	4.1455 to 6.7000		4.5000 to 8.4684	3.3382 to 5.8000	4.1000 to 6.6573	0.6000 to 15.9411	3.9134 to 6.6933	
Two-tailed probability	p = 0.0539		p = 0.0603		p = 0.2248		p = 0.7309	

3.6.3 Comparison of 'Confirmed TBM' CSF ADA results to all CSF ADA results for patients where TB culture on CSF was absent and or negative

In Table 3.11 'Confirmed TBM' CSF ADA results is compared to all CSF ADA results for patients where TB culture on CSF was absent and or negative, i.e. on the total population for this study (n=3548), using the Mann-Whitney test. Similar results ($p < 0.0001$) in the sub-population (n=1490) where both culture and ADA was requested on a CSF sample, were found (results not depicted). Although it would appear that there is some value in CSF ADA measurement to assist in diagnosis of TBM, the non-parametric distribution of the total population's CSF ADA results (n=3548) must be taken into consideration, especially the overlap between the outliers and as well as the 95% distribution of values for the individual categories.

Table 3.11: Statistical comparison of 'Confirmed TBM' CSF ADA results with all CSF ADA results for patients where TB culture on CSF was absent and or negative (in U/L).

	TBM	**Non-TBM**
n	92	3456
Minimum	0.0000	0.0000
Maximum	108.0000	182.5000
Median	5.4000	0.6000
95% Confidence interval of the median	4.1000 to 6.6875	0.6000 to 0.6000
Two-tailed probability	$p < 0.0001$	

Key to this table: TBM – refers to 'Confirmed TBM' patients; Non-TBM – refers to patients where TB culture on CSF was absent and or negative.

3.7 ROC curve analysis

3.7.1 ROC curve analysis performed on all patients that had both an ADA and culture for TB requested on a CSF specimen (n=1490)

ROC curve analysis performed on all patients that had both an ADA and culture for TB requested on a CSF specimen (n=1490) resulted in an AUC of 0.86 (95% CI of 0.82 – 0.89 and a p – value of < 0.0001). Hence the ROC curve generated is statistically significantly different from the line of 'No discrimination' (AUC of 0.5). Please see Figure 3.4 for the ROC plot.

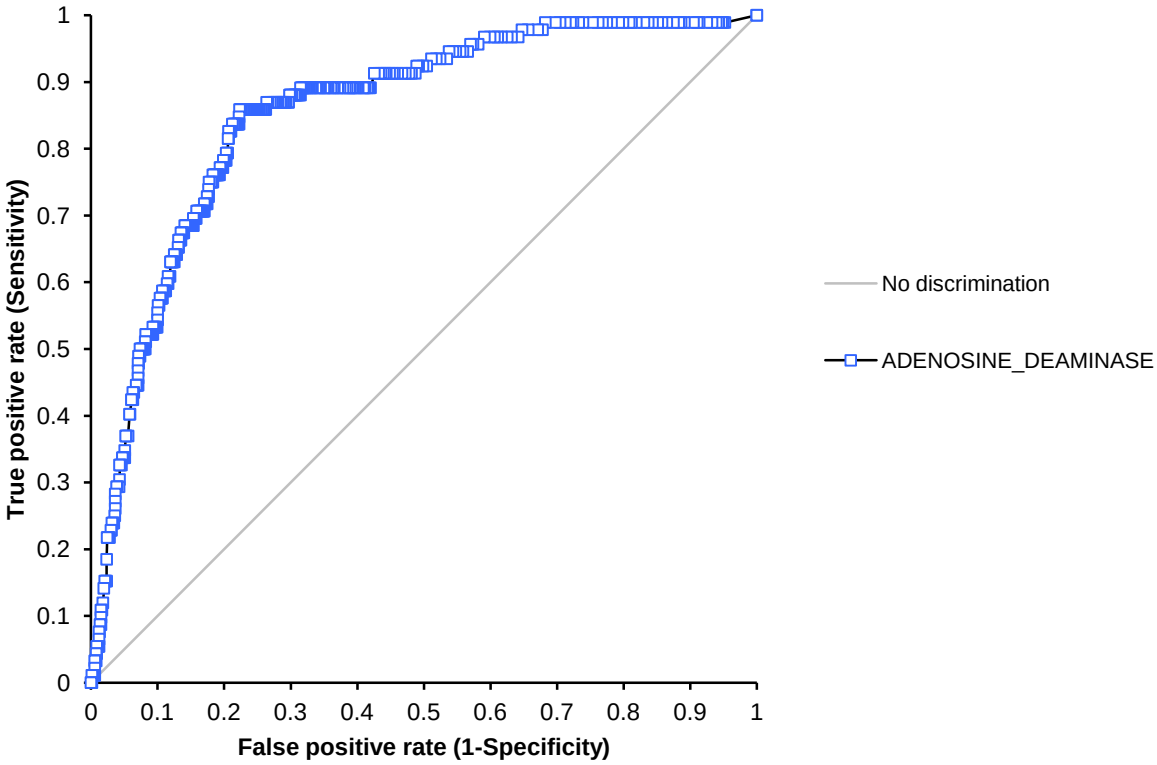


Figure 3.4: ROC plot for all patients that had both an ADA and a culture for TB requested on a CSF specimen.

The optimal cut-off of CSF ADA to be used in the context of suspected TBM patients according to this ROC plot and utilising the Youden index is 2.0 U/L (Sensitivity of 85.9% and Specificity of 77.7%). The derived values and ratios at this cut-off are noted in Table 3.12. It is important to note that, even at this cut-off, 13 cases of confirmed TBM were missed. The exclusion of the 6 cases of mixed infection in the 'Confirmed TBM' category did not result in a different cut-off.

Table 3.12: Derived values and ratios at a CSF ADA cut-off of 2.0 U/L.

ADA	**2.0U/L**
Youden index	0.636
True positive (n)	79
True negative (n)	1086
False positive (n)	312
False negative (n)	13
Sensitivity (%)	85.9
95% confidence interval	77.0-92.3
Specificity (%)	77.7
95% confidence interval	75.4-79.8
Positive predictive value (%)	20
Negative predictive value (%)	99
Positive likelihood ratio	3.85
Negative likelihood ratio	0.18
Diagnostic odds ratio	21.2

Target and actual sensitivities and specificities at different CSF ADA levels on this ROC plot are noted in Table 3.13. As the target sensitivity is increased, the cut-off for CSF ADA falls, with a corresponding drop in specificity. As the target specificity is increased, the cut-off for CSF ADA increases, with a drop in sensitivity.

Table 3.13: Target and actual sensitivities and specificities at different CSF ADA levels.

Target Sensitivity	First cut-off closest the these targets	Actual Sensitivity	Corresponding Specificity
≥ 80%	2.3 U/L	81.5%	79.4%
≥ 85%	2.0 U/L	85.9%	77.7%
≥ 90%	0.8 U/L	91.3%	57.4%
≥ 95%	0.5 U/L	95.7%	43%
Target Specificity	**First cut-off closest the these targets**	**Actual Specificity**	**Corresponding Sensitivity**
≥ 80%	2.4 U/L	80%	78.3%
≥ 85%	3.3 U/L	85%	68.5%
≥ 90%	4.5 U/L	90%	55.4%
≥ 95%	7.3 U/L	95%	33.7%

3.7.2 ROC curve analysis performed on the individual demographic categories for all patients that had both an ADA and culture for TB requested on a CSF

ROC analysis was performed in each of the 8 demographic categories of this population of 1490 patients to assess for any possible differences in the optimal cut-off for CSF ADA (see Table. 3.14). Similarly to the CSF ADA cut-off determined above, cut-offs in the individual demographic categories were low (< 3 U/L). No optimal cut-off could be determined for paediatric patients and HIV-negative patients with suspected TBM.

Table 3.14: ROC curve results for each of the demographic categories in the population of n=1490.

	TBM positive (n)	TBM negative (n)	ROC curve			Sensitivity (%)	Specificity (%)
			AUC	p value	Cut-off (U/L)		
Adult patients	90	1345	0.86	< 0.0001	2.0 / 2.2	86.7 / 85.6	77.8 / 79.0
Paediatric patients	2	53	0.58	< 0.3285	0.6	100.0	41.5
Male patients	47	695	0.86	< 0.0001	2.2 (2.0)	89.4 (89.4)	75.3 (74.1)
Female patients	45	703	0.86	< 0.0001	2.0	82.2	81.1
HIV positive patients	77	846	0.83	< 0.0001	2.2 (2.0)	84.4 (85.7)	74.1 (72.8)
HIV negative patients	7	274	0.88	< 0.0001	0.6	100.0	70.1
Patients with CD4 count ≤ 350 x 10^6/L	58	653	0.83	< 0.0001	2.0	86.2	74.4
Patients with CD4 count > 350 x 10^6/L	4	114	0.93	< 0.0001	2.7	100.0	86.0

Cut-offs represented was based on the Youden index. In adult patients the Youden index performed similar for cut-off of 2.0 U/L and 2.2 U/L. In brackets performance at a cut-off of 2.0 U/L is indicated.

3.8 Prediction rule

Stepwise logistic regression analysis was utilized to attempt to formulate a prediction rule including the CSF ADA result as well as clinical and radiological and other laboratory data. Combining CSF ADA with the established prediction rules mentioned previously failed to produce a specific rule with a high / acceptable AUC and one that can adequately identify positive cases in the population studied. Similarly other combinations of data collected together with CSF ADA did not perform any differently.

For example: the combination of CSF ADA, together with the presence of symptoms for more than 5 days, percentage lymphocytes on CSF, and CSF protein yielded a AUC of 0.748 (95% CI of 0.734 – 0.763) and failed to detect any confirmed TBM cases. Another example: CSF ADA combined with the presence of headache yielded an AUC of 0,901 (95% CI of 0.870 – 0.927), the highest of all combinations, but could only identify 'Confirmed TBM' in 16.67% of cases. This combination is likely to be too non-specific. Other combinations including CSF ADA together with serum sodium (AUC of 0.750; 95% CI of 0.690 – 0.810) and serum chloride (ADA of 0.710; 95% CI 0.650 – 0.780) were also too non-specific and failed to identify adequate numbers of 'Confirmed TBM' cases.

Because of the wide variety of data collected and the fact that in many cases results were not available, it is possible that too few correlations between chosen variables could be made by the statistical program for meaningful analysis.

3.9 Clinical research case definition for TBM

3.9.1 CSF ADA results for the total population of n=3548

CSF ADA results for the clinical research case definition (as defined by Marais, et al., 2010) applied on the total population of n=3548 can be found in Table 3.15. Of note is that although the median CSF ADA for the 'Definite tuberculous meningitis' category is much higher than the other categories, the 'Not tuberculous meningitis' category has a higher maximum CSF ADA result. Again keep the non-parametric distribution of the total population (n=3548) in mind.

Table 3.15: CSF ADA results for the clinical research case definition (in U/L).

Categories	0	1	2	3
n	2839	536	81	92
Minimum ADA	0.0	0.0	0.0	0.0
Median ADA	0.6	0.7	1.1	5.4
Maximum ADA	182.5	101.1	20.0	108.0

Key to this table: 0 – Not tuberculous meningitis, 1 – Possible tuberculous meningitis, 2 – Probable tuberculous meningitis, 3 – Definite tuberculous meningitis.

3.9.2 CSF ADA results of the 'Suspected TBM' group compared to the clinical research case definition.

Patients in the 'Suspected TBM' group were categorised as such based on the evidence as used and interpreted by clinicians in these patients' files. Of the suspected 394 patients, 256 could be reclassified by the clinical research case definition (as defined by Marais, *et al*., 2010) as 'Not tuberculous meningitis', 108 as 'Possible tuberculous meningitis' and 30 as 'Possible tuberculous meningitis'. This reflects the impact of a standardised case definition on the diagnostic process, with better categorisation of patients.

3.10 Specimen integrity

Specimen integrity refers to the comments made by technologists on the appearance of CSF specimens: bloodstained, xanthochromia, turbid and clear and colourless. All CSF ADA requests (n=3548) were re-categorised according to their integrity and are plotted in Figure 3.5 and are also noted in Table 3.16. Of note is the considerable spread of CSF ADA results for the 'Ungraded xanthochromia' category compared to the 'Clear and colourless' category, both in terms of outliers and 95% distribution of values.

Table 3.16: Specimen integrity comments for CSF ADA requests (in U/L).

Integrity	n	Min	Med	Max	IQR
1	13	0.1	0.90	2.1	0.66
2	196	0.0	0.63	10.7	1.29
3	28	0.0	0.89	28.3	2.53
4	81	0.0	10.70	182.5	19.74
5	52	0.0	1.75	101.1	5.21
6	6	0.3	1.61	8.6	2.15
7	11	0.0	3.27	5.8	2.42
8	148	0.0	2.60	19.3	3.09
9	10	0.1	3.14	7.2	2.38
10	3003	0.0	0.57	108.0	1.14

Key to this table: 1 – ungraded bloodstained, 2 – bloodstained +/- to 2+, 3 – bloodstained 3+ to 4+, 4 – ungraded xanthochromia, 5 – xanthochromia +/- to 2+, 6 – xanthochromia 3+ to 4+, 7 – ungraded turbid, 8 – turbid +/- to 2+, 9 – turbid 3+ to 4+, 10 – clear and colourless; Min – Minimum, Med – Median, Max – Maximum, IQR – Interquartile range.

When excluding all specimens that were not clear and colourless (n=545), no change was noted in the ROC curve analysis and the same cut-off of 2.0 U/L using the Youden index was determined. Furthermore the overlap in CSF ADA results for the different categories remained.

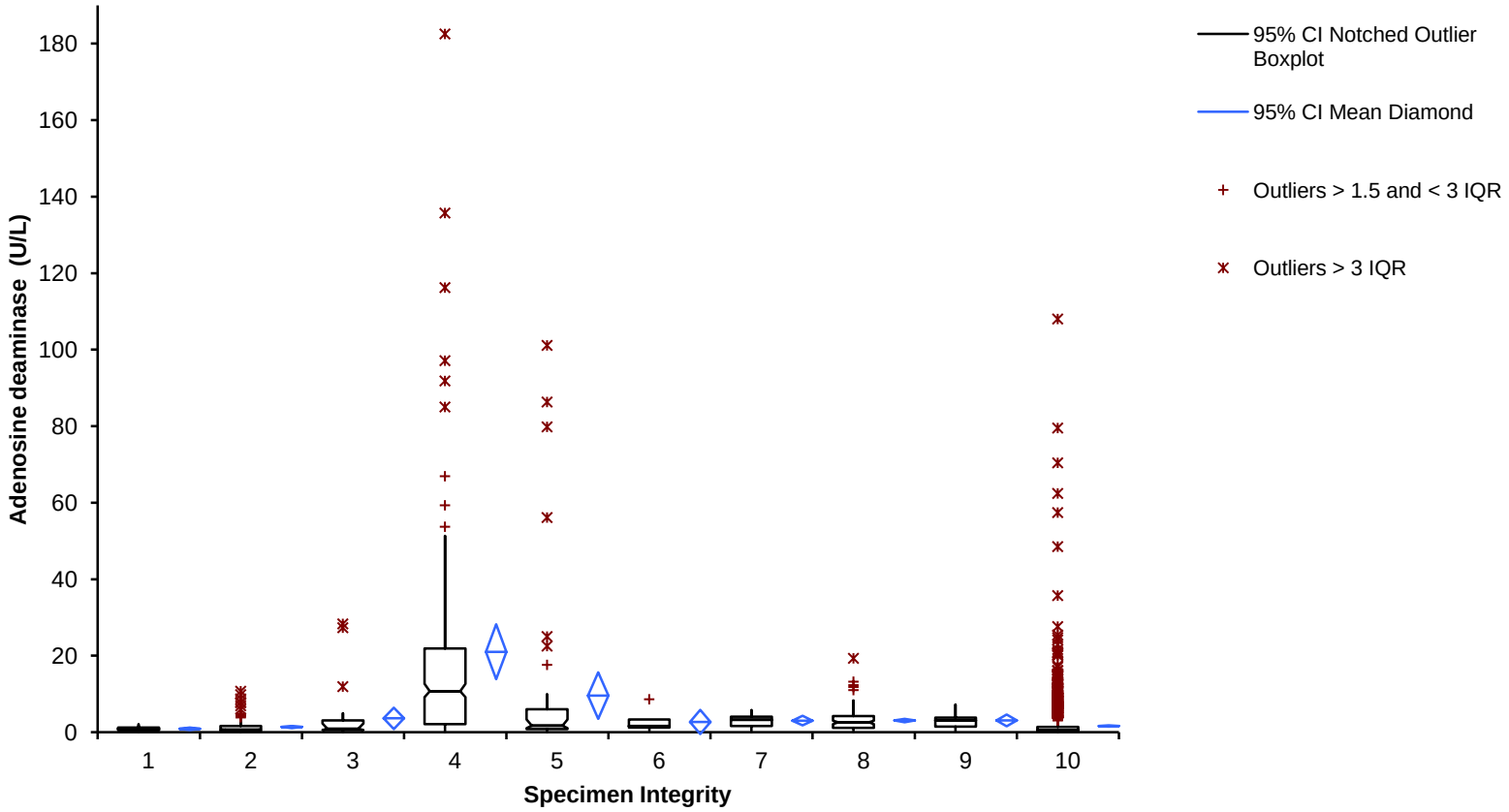


Figure 3.5: CSF ADA values plotted according to the specimen integrity comments for each sample. The 95% distribution of values (including the median, first and third IQRs), outliers and the 95% CI of the means are indicated. <u>Key to this figure</u>: 1 – ungraded bloodstained, 2 – bloodstained +/- to 2+, 3 – bloodstained 3+ to 4+, 4 – ungraded xanthochromia, 5 – xanthochromia +/- to 2+, 6 – xanthochromia 3+ to 4+, 7 – ungraded turbid, 8 – turbid +/- to 2+, 9 – turbid 3+ to 4+, 10 – clear and colourless; IQR – Interquartile range.

The sample integrity comments for the CSF ADA requests for the 92 confirmed TBM patients can be found in Table 3.17. In these specimens the elevation in CSF ADA is less dramatic in those that were bloodstained, xanthochromic, or turbid compared to those specimens that were clear and colourless. However in bloodstained and xanthochromic specimens CSF ADA results did exceed the current clinical decision limit of 6 U/L.

Table 3.17: Specimen integrity comments for CSF ADA requests in the 'TBM category'.

Integrity comment	n	ADA results
Bloodstained +/- to ++	5	ADA = 10.7 U/L and less
Xanthochromia	8	ADA = 14.4 U/L and less
Turbid	1	ADA = 5.5 U/L
Turbid +/- to ++	8	ADA = less than 6 U/L
Clear and colourless	70	65 ADA less than 15 U/L, 4 ADA in the 20 U/L+ range, and 1 outlier at 108 U/L

4.0 DISCUSSION AND CONCLUSION

The diagnosis of TBM is extremely challenging especially in view of the fact that the gold standard for its diagnosis, culture, generally performs so poorly. The primary objective of this study was to determine an appropriate cut-off level for CSF ADA to assist in the diagnosis of TBM and hence to reflect its usefulness in the clinical setting. The investigator aimed to assess its performance in all clinical scenarios where CSF sampling was performed and ADA was requested as part of a patient work-up.

The current CSF ADA cut-off used by clinicians in the CMJAH complex is 6 U/L. The origin of this cut-off is not clear, but potentially may be traced back to research performed by Blake & Berman (1982). The investigator determined in this study through ROC curve analysis that the optimal CSF ADA cut-off for the diagnosis of TBM to be 2.0 U/L (sensitivity of 85.9% with a 95% CI of 77.0% - 92.3% and specificity of 77.7% with a 95% CI of 75.4% - 79.8%). Compared to the literature (Tuon, *et al*., 2010; Xu, *et al*., 2010) this cut-off is unusually low.

Several reasons may account for this difference. The CSF ADA results included in this study follow a non-parametric distribution with the bulk of the results falling in the lower range (3415 results are < 10 U/L). Selection bias may have been introduced in the way the CSF ADA results were chosen i.e. only the CSF ADA result from the first CSF sampling was considered in each admission. Although the inclusion in the 'Confirmed TBM' category was based on culture positivity only and yielded 92 patients, CSF ADA performance was compared to a significantly larger set of results in patients were TB culture of CSF was

negative (n=1398). This may also have skewed the ROC curve analysis. Pre-analytical and analytical factors may also have contributed. These will be discussed later.

At the current CSF ADA cut-off of 6 U/l this ROC curve analysis showed it to have a sensitivity of 44.6% and specificity to be 93.1% (potentially missing 55.4% of TBM patients). Using the optimal cut-off with best sensitivity and specificity was of 2.0 U/L potentially only 14.1% of TBM patients would be missed. Thirteen TBM patients had ADA values less than 2.0 U/L. This is problematic. When looking at the individual demographic groups, no marked difference in the cut-off was noted. Too few cases of TBM were available when assessing the cut-off in paediatric patients, HIV negative patients and patients with CD4 cell counts > 350 x 10^6/L. The cut-off of 2.0 U/L showed a slight improvement in sensitivity for adult patients, male patients and patients with CD4 cell counts ≤ to 350 x 10^6/L. The cut-off of 2.0 U/L showed a slight improvement in specificity for adult patients.

It is clear from the literature that CSF ADA cannot be used as a definitive diagnostic tool for TBM as concluded by Corral, *et al.,* (2004). If it is used for screening purposes the sensitivity should be optimized, but this may result in the cut-off of 2.0 U/L being dropped even further. If ADA is used for rule-out purposes the specificity should be optimized, resulting in an elevation of the cut-off with a considerable loss of sensitivity. As TBM is the most serious complication of extra-pulmonary tuberculosis, it can be argued that both sensitivity and specificity for CSF ADA should be assigned equal importance.

The positive predictive value at a cut-off of 2.0 U/L performed poorly whereas the negative predictive value was excellent. It is important to note that the negative predictive value

performed well at almost every ADA cut-off, the reason for which is unclear. The non-parametric distribution of CSF ADA results as well as the relatively small number of patients in the 'Confirmed TBM' category (n=92) compared to those who did not culture TB on CSF (n=1398) may account for this.

It is important to understand the statistics in terms of the pre-test probability of the disease, in this case TBM. We are living in a country with a high prevalence of TB. However the prevalence of TBM in patients who had a CSF culture and ADA requested in this study (n=1490) was only 6.2%. The likelihood ratios did not add significantly to this pre-test probability for TBM. The positive likelihood ratio performed much lower than that found in the meta-analysis by Xu, *et al*., (2010), but with a similar negative likelihood ratio.

The diagnostic odds ratio is used as an indicator of test accuracy, combining sensitivity and specificity as well as the ratio of the odds of positive results in patients with and without disease. Usually the higher the score achieved, the higher the accuracy of the particular test (Glas, *et al*., 2003). The current study achieved similar results as cited in the meta-analysis by Xu, *et al*., (2010). There is no consensus as to what diagnostic odds ratio is considered as useful for the diagnosis of TBM.

It is important to also note than when confirmed TBM ADA results (Median of 5.4000 U/L; 95% CI of 4.1000 – 6.6875 U/L) were compared with all ADA results for patients where TB culture on CSF was absent and or negative (Median of 0.6000 U/L; 95% CI of 0.6000 – 0.6000 U/L), a statistically significant difference was noted ($p < 0.0001$). But when the ADA results in the individual categories are compared by visual inspection, there is tremendous

overlap regarding each category's 95% distribution of values, as well as their outlier values. The impression is that a positive result doesn't necessarily rule in the diagnosis of TBM nor does a negative result rule it out. Elevations in non-infectious pathologies have also been noted by other researchers (Donald, *et al*., 1986; Petterson, *et al*., 1991).

When comparing ADA values between prominent infective pathologies, the absence of a statistically significant difference between the 'Suspected bacterial meningitis' and 'Confirmed TBM' categories concurred with Malan, *et al*., (1984), Coovadia, *et al*., (1986) and Donald, *et al*., (1986). The absence of a statistically significant difference extended to the 'Confirmed ventriculitis' category when compared to the 'Confirmed TBM' category.

CSF ADA results were statistically significantly different between the 'Viral meningitis / encephalitis' and 'Confirmed TBM' categories. This concurred with the research by Donald, *et al*., (1986) and Eintracht, *et al*., (2000), although the latter investigator specifically looked at the usefulness of the ADA-2 isoenzyme. Coovadia, *et al*., (1986) did not find any difference between the 2 categories.

The statistically significant difference found when comparing CSF ADA results between the 'Confirmed cryptococcal meningitis' and 'Confirmed TBM' categories concurred with work by Rohani, *et al*., (1995). Eintracht, *et al*., (2000) found no difference when specifically looking at the ADA-2 isoenzyme.

Next it is important to reiterate that dual infective pathology was reported in a percentage of patients. In the 'Confirmed TBM' category 6.5% had dual infection confirmed; 2 cases were

cryptococcal meningitis and 2 cases were bacterial meningitis. In amongst the other mixed infections only 3 cases were found. In some cases treatment for dual pathology was commenced in the absence of confirmatory evidence. In the 'Confirmed cryptococcal meningitis' category at least 6.3% were treated for TBM and at least and 3.5% for bacterial meningitis (the ADA for the bulk of these two groups was less than 7.0 U/L). Unnecessary treatment for TBM may cause complications, such as drug-induced hepatitis, although this was not a common occurrence in the population studied (n=26).

It is very interesting to note in this study that when the investigator compared ADA results for the 8 demographic categories for the total population of 3548 patients, in all cases a statistically significant difference existed. The fact that ADA results in adult and paediatric patients were statistically significantly different concurred with the work by Malan, *et al.*, (1984). It was unexpected to find ADA results to be higher in HIV positive patients and patients with CD4 cell counts less than or equal to 350 x 10^6/L as intuitively one would think that the cellular responses in these patients are impaired. Co-morbidities may account for this. When this demographic comparison was performed in the 'Confirmed TBM' category, no difference was found in all cases. This may be due to the small numbers in the 'Confirmed TBM' category. The effect of clinical stage of retroviral disease was not assessed in this study. The response in ADA to treatment in the TBM culture confirmed group was also not assessed in this study.

The secondary objective of this study was to formulate a predictive model for the clinical diagnosis of TBM in the absence of definitive evidence, using ADA together with the clinical, radiological and other laboratory features collected during the study. No optimal combination

could be found. In this retrospective study there was just not enough information available to generate enough correlations for each combination of variables considered. Missing files, inadequate documentation, and absence of certain tests requested may have all contributed to this.

The clinical research case definition as published by Marais, *et al*., (2010) shows a comprehensive and holistic approach for the diagnosis of TBM. It may not necessarily be easily applied in general clinical practice, but the principle of determining the relative weight of evidence gathered for the diagnosis of suspicious can potentially avoid unnecessary treatment. In the 'Suspected TBM' category at least 32% of patients were treated while perhaps only 7.6% required treatment. Missing data may have confounded reclassification of the data according to this clinical research case definition. Outlier CSF ADA results need to be treated with caution.

4.1 Limitations

Several pre-analytical and analytical factors limit adequate interpretation of the results from this study.

No specific specimen collection and handling procedures are noted in the Diazyme kit package insert (Diazyme, United States of America) regarding CSF samples. This may affect stability of the analyte. The investigator did not cite any literature regarding the stability of ADA in CSF samples. The Diazyme kit package insert (Diazyme, United States of America) notes the stability of ADA content in blood as one week at 2-4 ^{o}C (Appendix B). Stability in

sample types other than CSF has been noted in the literature. Giusti noted that the information on stability of ADA in serum is variable, but according to his laboratory's experience it is 5 to 6 days (Giusti, 1974). ADA has been found to be stable in pleural fluid for 6 hours at room temperature and one month at 2-8 ^{o}C (Song, *et al*., 2010). The addition of 5% glycerol and 5% ethylene glycol maintained the stability of ADA in pleural fluid for 21 days at room temperature and 37 ^{o}C and hence allows for shipping of samples at ambient temperatures (Miller, *et al.*, 2004).

No specific reference is made in the Diazyme kit package insert (Diazyme, United States of America) regarding the optimal volume of CSF required to perform the assay. Under the assay procedure section of the Diazyme kit package insert (Diazyme, United States of America) reference is made to sample volume as being 5 μL required to perform the assay not specifying sample type (Appendix B). Feres, *et al*., (2008) mention in their validation of the Diazyme assay for measurement of ADA (Diazyme, United States of America) in CSF that 22 μL was used.

It is very interesting to plot the ADA results in this study in terms of the integrity comments assigned to them (i.e. if the sample was bloodstained, xanthochromic, turbid or clear and colourless). 'Ungraded xanthochromia' accounted for 81 specimens and showed the widest distribution of 95% of their values. Both this category and samples assigned xanthochromia +/- to 2+ (accounting for 52 samples) showed a wide scatter in their outliers, similar to the category for clear and colourless samples (accounting for 3003 samples). Ungerer, *et al*.,

(1992) demonstrated that erythrocytes contribute 100% of ADA-1 and polymorphonuclear cells contribute 70% ADA-1 and 30% ADA-1+CP to the total ADA activity in cell extracts.

The Diazyme kit package insert (Diazyme, United States of America) notes the following regarding interferences: the assay is not affected by interference from serum bilirubin up to 342 μmol/L, haemoglobin up to 0.2 g/dL, triglycerides up to 8.475 mmol/L and ascorbic acid up to 231.12 μmol/L. Specific references to specimen type is not made (Appendix B).

Few researchers have actively investigated for interference in the Diazyme assay (Diazyme, United States of America). Song, *et al*., (2010) found in the measurement of ADA in pleural fluid that haemoglobin up to 0.18 g/dL showed minimal interference, bilirubin concentrations > 300 μmol/L showed a 17% reduction in the ADA results and 10% intralipid reduced the ADA result by 31% (pleural fluid). Delacour, *et al*., (2010) showed in pleural fluid samples that haemoglobin up to 0.29 g/dL showed no interference, but at a level of 0.40 g/dL a positive interference was observed. Furthermore they found a significant and near linear negative interference with bilirubin concentrations > 50 μmol/L. As a negative interference was found was found with the NaOH in which the bilirubin was dissolved, they could not exclude bilirubin interference. Triglycerides up to 5.51 mmol/L showed no interference in the Diazyme assay (Diazyme, United States of America). This was a surprising finding as the value is already quite high and one would expect interference. Finally Delacour, *et al*., (2010) recommended rejection of icteric and highly haemolysed samples.

The effect of sample integrity of CSF samples on assay performance at the CMJAH NHLS Chemical Pathology laboratory has not been formally investigated. From a basic understanding of spectrophotometric principles, the addition of colour from interferents should falsely elevate results. From experience the laboratory has found that equally xanthochromic samples can perform quite differently (one reading low and another high).

The previous assay utilised at the CMJAH NHLS Chemical Pathology laboratory measured total ADA activity by a Non-Giusti method (enzymatic spectrophotometric method) on a Cobas Mira auto-analyser. Eintracht, *et al*., (2000) published a modification of this method for ADA-2 analysis for assisting in the diagnosis of TBM. The current Diazyme assay (Diazyme, United States of America) utilised also measures total ADA (personal communication, Chong Yuan, Diazyme, United States of America). The assay was validated on the Pentra analyser (Horiba Medical Diagnostics, Japan) using the EP-10 Clinical and Laboratory Standards Institute guidelines and no major problems were identified in the validation process. Control material supplied by Diazyme (Diazyme, United States of America) was used for this purpose. The use of the Diazyme assay (Diazyme, United States of America) remained constant over 2009 and 2010.

Prior to availability of calibration material, only calibration factors were used. Despite currently having access to calibration material, the Pentra analyser (Horiba Medical Diagnostics, Japan) software still used a calibration factor. The calibrator material supplied by Diazyme (Diazyme, United States of America) is bovine serum albumin based. This together with 0.9% saline as a zero reference is used for calibration (Appendix B). The calibration records for the two years were not assessed by the investigator. The CSF ADA results were

captured on the Excel spreadsheet as the absolute values recorded by the laboratory information system.

Ideally control material should reflect analyte levels at which clinical decisions are made. Currently this is true for pericardial, peritoneal and pleural fluids, but not for CSF (where the clinical decision limit is set at 6 U/L). The control material supplied by Diazyme (Diazyme, United States of America) is bovine serum albumin-based (Appendix B).

From the foregoing an important question to resolve is how matrix effects interfere with the Diazyme assay. Is it a problem if the assay is calibrated and quality control is performed using bovine serum albumin-based material when CSF samples are run? Diazyme stated that data in this regard was not available (personal communication, Chong Yuan, Diazyme, United States of America). What could be done to resolve this is to spike a normal CSF sample with no cellular activity and CSF protein and glucose in the normal range with serum bovine albumin as well as purified ADA to assess for matrix effects. Similarly the effect of sample viscosity has not been assessed on assay performance.

When assessing the Giusti and Non-Giusti methods for the measurement of ADA it is important to note that one is dealing with enzymatic - based assays where conditions according to temperature and pH may differ. It is important to take cognisance of reagent stability. Specific guidelines for the analyser response in terms of absorbance at different levels of activity have not been found by the investigator. Wavelengths at which measurement occurs differ amongst assays (Tuon, *et al.*, 2010). The calibration curve for the Diazyme assay (Diazyme, United States of America) lies between 0 – 50 U/L. Logically, samples that result

above this level should be diluted into the linear range. The package insert for the Diazyme assay (Diazyme, United States of America) states that dilution should occur for samples with results > 200 U/L (Appendix B). Delacour, *et al*., (2010) performed a linearity study and determined the analytical measurement range to be 0.5 – 120 U/L.

The measurement of ADA in general is complicated by poor standardisation. Giusti and Non-Giusti methods have been documented. Most methods are in-house based. The Diazyme assay (Diazyme, United States of America) is the only commercially available assay. Bota, *et al*., (2001) produced and certified enzyme reference material for ADA-1 to be used to compare laboratories and verify performance of calibration material and quality control material. This group utilised ADA purified from human erythrocytes. A commutability study was only performed on pleural fluid samples clear of haemolysis and turbidity (Bota, *et al*., 2001). The poor standardisation of ADA measurement makes it difficult to interpret the significance of CSF ADA values in the literature. ADA results on pleural fluid between a laboratory in Auckland, New Zealand and a laboratory in Queensland, Australia differed by 45% (personal communication, Dr Song, Labplus, New Zealand). In addition to this, no external quality assurance programmes exist for ADA measurement.

The imprecision of the ADA assay used at the CMJAH NHLS Chemical Pathology Laboratory at a cut-off of 2.0 U/L is not known. A formal imprecision study has not been performed. The data from the EP-10 validation at the lower control level of 10.6 U/L reflected the precision of the assay as follows: within-run standard deviation of 0.172 U/L, total standard deviation of 0.601 U/L, and the total coefficient of variation of 5.8 % (the allowable coefficient of variation was 7.0 %). This performance is acceptable. It is possible that the

imprecision of the assay is poor at a cut-off of 2.0 U/L. This would require further formal investigation.

This study was a secondary data analysis of clinical and laboratory data. Every effort was made to find available data as well as the checking thereof. Unfortunately only the investigator participated in this process. Clinical data obtained from patient files was not viewed again at a later stage after the initial extraction process, due to time constraints. Some files were missing. Not all data required could be sourced from the patient files or the laboratory information system. This may have affected the categorisation process.

As it was not always clear from both the patient files and the laboratory information system what the specimen submitted in a BACTEC bottle was, some cases of TBM may have been missed.

Furthermore less than 10% of the 1490 patients who had a culture and ADA requested on a CSF sample had more than one sample submitted. The bulk of these were TBM patients (n=17) and patients with cryptococcal meningitis (n=41). Consequently some cases of TBM may have been missed.

Regarding the clinical case definition for TBM as proposed by Marais, *et al*., (2010) there was often no plasma glucose result available within 24 hours on either side of the time of CSF sampling, thus this value could not be used in the categorisation process.

4.2 Other issues

The investigator notes the following regarding the entering / capturing of numerical data on the laboratory information system. There may be a difference between the absolute minimum and maximum and reportable minimum and maximum values for particular analytes. These usually take into consideration the analytical range as well as the linearity of the assay concerned, together with the current status of clinical decision limits. This may change as new instruments are introduced in the laboratory. Sometimes limits are arbitrarily chosen, for example when determining CSF glucose.

The effect of erythrocyte contamination on CSF cell count interpretation and CSF protein measurement must also be considered, especially in the absence of a definitive diagnosis for infective agents. It may potentially obscure the picture completely.

Patients aged 5 years or less were excluded from comparison of CD4 cell counts. In these patients both absolute and percentage CD4 cell count were considered when assessing for immunodeficiency. Both were utilised in the clinical setting with equal weighting and it was difficult to include these values in the data analysis.

Although the use of ADA for the diagnosis of TBM adheres to the WHO criteria for population screening (Garg & Dasouki, 2006), the investigator noticed in some patient groups no additional benefit of this practice. An example would be patients with congenital hydrocephalus where ADA was periodically requested and none of these cases ever had culture-confirmed TBM.

As the study aimed to assess the usefulness of CSF ADA for the diagnosis of TBM, the statistical analyses performed in this study were limited to certain infective diagnoses. This was done especially in light of the limitations mentioned previously to prevent over-interpretation of the data. The most remarkable finding in this study is the considerable overlap in the CSF ADA results both in the 95% distribution of values and of outliers between the different infective and non-infective categories. When CSF ADA results were compared between the 'Confirmed TBM' category and those patients where TB culture on CSF results were absent or negative, a statistically significant difference was found ($p < 0.0001$). However, when comparing CSF ADA results between the 'Confirmed TBM' and 'Confirmed bacterial meningitis' categories, no statistically significant difference was found ($p = 0.0643$).

4.3 Conclusion

Several pre-analytic and analytical factors have been mentioned above that affect interpretation of CSF ADA results. No sound conclusions can ever be made regarding CSF ADA cut-off levels, without fully understanding these factors. The effect of sample integrity cannot be underestimated and there is a pressing need for standardisation of CSF ADA measurement. CSF culture remains the gold standard for the diagnosis of TBM. Its poor performance overall, however, makes it a problematic gold standard to which CSF ADA is compared to. A replacement for culture is eagerly awaited. In the investigator's experience there exists misunderstanding and confusion over ADA relevance amongst clinicians. Its adjunctive use in the diagnosis of TBM is still to be fully determined, although the data from this study show that the CSF ADA result may potentially mislead clinicians in the diagnostic process. Careful assessment and weighting of all the available evidence should prove more fruitful. Some data are available in the literature regarding CSF ADA's usefulness in assessing treatment response, although this may not be easily applied in clinical practice. A future prospective multicentre study on a larger cohort, especially once the standardisation of CSF ADA measurement has improved, is advocated.

APPENDICES

Appendix A: Clinical research case definition by Marais, *et al*., 2010.

Table A1: TBM case definition.

Clinical entrance criteria: Symptoms and signs of meningitis including 1 or more of the following: headache, irritability, vomiting, fever, neck stiffness, convulsions, focal neurological deficits, altered consciousness, or lethargy.

Definite TBM	Clinical entry criteria plus 1 or more of the following: AFB seen in the CSF; *Mycobacterium tuberculosis* cultured form the CSF; or a CSF positive commercial NAAT.
Probable TBM	Clinical entry criteria plus a total diagnostic score of 10 or more points (when cerebral imaging is not available) or 12 or more points (when cerebral imaging is available) plus exclusion of alternative diagnoses. At least 2 points should come from CSF or cerebral imaging criteria.
Possible TBM	Clinical entry criteria plus a total diagnostic score of 6-9 points (when cerebral imaging is not available) or 6-11 points (when cerebral imaging is available) plus exclusion of alternative diagnoses. Possible tuberculosis cannot be diagnosed or excluded without doing a lumbar puncture or cerebral imaging.
Not TBM	Alternative diagnosis established, without a definitive diagnosis of TBM or other convincing signs of dual disease.

Table A2: Diagnostic criteria for classification of definite, probable and possible TBM.

Criteria	Diagnostic score
Clinical criteria	**Max score=6**
Symptom duration of more than 5 days	4
Systemic symptoms suggestive of TB (one or more of the following): weight loss (or poor weight gain in children), night sweats, or persistent cough for more than 2 weeks	2
History of recent (within past year) close contact with an individual with pulmonary TB or a positive tuberculin skin test or interferon-gamma release assay (only in children < 10 years of age)	2
Focal neurological deficit (excluding cranial nerve palsies)	1
Cranial nerve palsy	1
Altered consciousness	1
CSF criteria	**Max score=4**
Clear appearance	1
Cells: 10-500 per µL	1
Lymphocytic predominance (>50%)	1
Protein concentration greater than 1 g/L	1
CSF to plasma glucose ratio of less than 50% or an absolute CSF glucose concentration less than 2.2 mmol/L	1
Cerebral imaging criteria	**Max score=6**
Hydrocephalus	1
Basal meningeal enhancement	2
Tuberculoma	2
Infarct	1
Pre-contrast basal hyperdensity	2

Table A2 continued.

Evidence of tuberculosis elsewhere	**Max score=4**
CXR suggestive of active TB: signs of TB=2; military TB=4	**2/4**
CT / Magnetic resonance imaging / ultrasound evidence for TB outside the central nervous system	**2**
AFB identified or *Mycobacterium tuberculosis* culture form another source – i.e. sputum, lymphnode, gastric washing, urine, blood culture	**4**
Positive commercial *Mycobacterium tuberculosis* NAAT from extra-neural specimen	**4**

Appendix B: Package inserts for ADA assay, quality control material and calibrator.

DIAZYME

Diazyme Laboratories
12889 Gregg Court
Poway, CA 92064, USA
Tel: 858-455-4768 / Fax: 858-455-3701
Email: support@diazyme.com
Website: www.diazyme.com

Adenosine Deaminase Assay Kit

Configuration
The Diazyme Adenosine Deaminase reagent is provided in bulk and the following kit configuration:

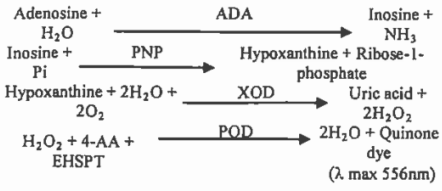
Configuration	Catalog No.	Kit Size
Universal	DZ117A-K	R1: 1 x 50 mL R2: 1 x 25 mL

Note: Calibrators sold separately

Intended Use
Adenosine deaminase (ADA) assay kit is for determination of ADA activity in serum and plasma samples. For Research Use Only in the USA.

Background
ADA is an enzyme catalyzing the deamination reaction from adenosine to inosine. The enzyme is widely distributed in human tissues, especially high in T lymphocytes. Elevated serum ADA activity has been observed in patients with acute hepatitis, alcoholic hepatic fibrosis, chronic active hepatitis, liver cirrhosis, viral hepatitis and hepatoma.[1,2] Increased ADA activity was also observed in patients with tuberculous effusions.[3] Determination of ADA activity in patient serum may add unique values to the diagnosis of liver diseases in combination with ALT or γ-GT (GGT) tests. ADA assay may also be useful in the diagnostics of tuberculous pleuritis.[3]

Assay Principle
The ADA assay is based on the enzymatic deamination of adenosine to inosine which is converted to hypoxanthine by purine nucleoside phosphorylase (PNP). Hypoxanthine is then converted to uric acid and hydrogen peroxide (H_2O_2) by xanthine oxidase (XOD). H_2O_2 is further reacted with N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline (EHSPT) and 4-aminoantipyrine (4-AA) in the presence of peroxidase (POD) to generate quinone dye which is monitored in a kinetic manner. The entire enzymatic reaction scheme is shown below.



One unit of ADA is defined as the amount of ADA that generates one μmole of inosine from adenosine per min at 37°C.

Materials Required but not Provided
Any instrument with temperature control of 37 ± 0.5°C that is capable of reading absorbance accurately at 540nm – 550nm may be used.

Reagent Composition

Active Ingredients	Concentration
Reagent 1	
Tris HCl, pH 8.0	50 mM
4-AA	2 mM
PNP	0.1 U/mL
XOD	0.2 U/mL
Peroxidase	0.6 U/mL
Stabilizers	
Reagent 2	
Tris-HCl, pH 4.0	50 mM
Adenosine	10 mM
EHSPT	2 mM
ADA Control	
Adenosine deaminase (bovine liver) and BSA	

Reagent Preparation
Liquid two-reagent system, ready-to-use for both manual method and automated chemistry analyzers (kinetics). ADA control and calibrator are in lyophilized form, and need to be reconstituted with 1.0 mL of DI water before use. The reconstituted ADA controls and calibrator are stable for 1 week at 2-8°C. Control and calibrator sold separately.

Reagent Stability and Storage
Reagents are stable until their expiration date when stored at 2-8°C.

Specimen Collection and Handling
Serum or heparinized plasma may be assayed. Ideally, venous blood should be collected and handled anaerobically. Do not use citrate or oxalate as anticoagulant.

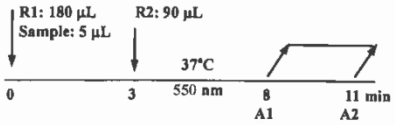
Plasma and serum, after prompt separation from cells or clot, should be kept tightly stoppered. ADA content of blood is stable for 1 week when stored at 2–4°C.

Precautions
1. For Research Use Only in the USA. Not for use in diagnostic procedures.
2. Reagent R1 is light-sensitive. Store in a dark place.
3. Specimens containing human sourced materials should be handled as if potentially infectious using safe laboratory procedures, such as those outlined in Biosafety in Microbiological and Biomedical Laboratories (HHS Publication Number [CDC] 93-8395).

4. Avoid ingestion and contact with skin and eyes. See Material Safety Data Sheet.
5. The reagents contain < 0.1% sodium azide, NaN_3, as preservative. Sodium azide may react with lead and copper plumbing to form highly explosive metal azide. On disposal, flush with a large volume of water to prevent azide buildup.
6. Do not use the reagents after the expiration date labeled on the outer box.
7. Additional safety information concerning storage and handling of this product is provided within the Material Safety Data Sheet for this product.

Assay Procedure

Test Scheme for Chemistry Analyzers



Application sheets for use of Diazyme Adenosine Deaminase Assay on automated clinical chemistry analyzers are available upon request. Please call 858-455-4768 or email: support@diazyme.com.

Calibration
A single calibrator, along with 0.9% saline as a zero reference, should be used as directed to calibrate the procedure.

Quality Control
Diazyme recommends that each laboratory use ADA controls to validate the performance of ADA reagents. ADA controls are available from Diazyme Laboratories (Cat. # DZ117A-Con). If the results from the control falls outside the acceptable limits, the test should not be performed. We recommend that your quality control testing follows federal, state, and local guidelines.

Results
The ADA results are printed out in U/L. Literature cites ADA activity tests in serum samples to be in the range of 0-15 U/L[1-4]. Literature citations show that for pleural fluid[3], values were found to be in the range of 0-30 U/L, and for C.S.F.[4], values were found to be in the range of 0-9 U/L.

Limitations
If the sample ADA activity is greater than 200 U/L, the sample should be diluted with saline before measurement. The result should be multiplied by the dilution factor. Assay is specific for ADA and has no detectable reaction with other nucleosides. The reagent solution should be clear. If turbid, the reagent may have deteriorated.

Analytical Characteristics[5]

Precision
The precision of the Diazyme Adenosine Deaminase Assay was evaluated on the Cobas Mira instrument according to a modified Clinical Laboratory Standards Institute EP5-A guideline. In the study, two serum specimens containing 11 U/L and 30 U/L ADA were tested with 2 runs per day with duplicates over 15 working days.

	Within Run Precision		Run to Run Precision	
	11 U/L	30 U/L	11 U/L	30 U/L
No. of Data Points	30	30	30	30
Mean (U/L)	11.11	30.74	9.63	29.62
SD	0.16	0.45	0.47	0.59
C_V%	1.47	1.45	4.90	2.00

Linearity
The linearity of the procedure is from 0 – 200 U/L.

Interference
Assay is not affected by serum bilirubin up to 30 mg/dL, hemoglobin up to 200 mg/dL, triglycerides up to 750 mg/dL, and ascorbic acid up to 4 mg/dL.

References

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EC REP MDSS Schiffgraben 41 30175 Hannover, Germany

12889 Gregg Court Poway, CA 92064, USA Tel: (858) 455-4768 Fax: (858) 455-3701

7/11/11

DIAZYME	Title: Adenosine Deaminase Calibrator Certificate of Analysis	Page 1 of 1
Doc. #: CQF 4080	Rev: B DCO #: 1553	Effective: 05/01/09

Catalog Number: DZ117A-CAL

Intended Use

The adenosine deaminase calibrator is used for quality control procedures in examining the accuracy and precision of quantitative adenosine deaminase assays. For investigational use only.

Principle

The activity of the adenosine deaminase calibrator is determined by a UV spectrophotometer measuring a change in absorbance at 550 nm resulting from the deamination of adenosine. One unit of adenosine deaminase is defined as the amount of adenosine deaminase that generates one micromole of inosine from adenosine per minute at 37°C.

Characteristics

The adenosine deaminase calibrator is prepared in a bovine serum base, provided in lyophilized powder, and should be stored at –20°C.

Stability

The lyophilized powder (not reconstituted) is stable until the expiry date indicated on the label when stored at -20°C. Once reconstituted, the components of the serum are stable for 1 week at 2-8°C.

Preparation

Open one vial of the adenosine deaminase calibrator carefully to avoid any loss of material and reconstitute with exactly 1 mL of distilled H_2O. Close the vial and let stand for thirty (30) minutes at room temperature, dissolving contents completely by gently swirling or rotating.

Warnings and Precautions

The bovine source material used in this product originated in the USA. The origin of raw materials, type of tissue, and manufacturing process were deemed by the European Directorate of the Quality of Medicines to meet criteria concerning transmission of animal spongiform encephalopathies. Possible infectious agents in the materials have been inactivated. Because no method can offer complete assurance as to the absence of infectious agents, however, this material and all patient samples should be handled as though capable of transmitting infectious disease and disposed of accordingly.

Calibrator Information

Lot Number	Expiration Date	Value (U/L)
ADS00110	2012-03	50.0

Certified by: _____ Date: 2010-04-05

Diazyme Laboratories 12889 Gregg Court, Poway, California 92064 USA
Tel 858-455-4754; Fax 858-455-3701; Web www.diazyme.com; Email: support@diazyme.com

DIAZYME	Title:	Adenosine Deaminase Control Certificate of Analysis	Page 1 of 1
Doc. #: CQF 4021	Rev: E	DCO #: 2527	Effective: 06/14/11

Catalog Number: DZ117A

7/11/11

Intended use
The adenosine deaminase (ADA) control is used for quality control procedures in examining the accuracy of quantitative adenosine deaminase assays. For Research Use Only in the USA.

Characteristics
The adenosine deaminase control is prepared in a bovine serum base, provided in lyophilized powder.

Preparation
Open one vial of the adenosine deaminase control carefully to avoid any loss of material and reconstitute with exactly 1 mL of distilled H_2O. Close the vial and let stand for thirty (30) minutes at room temperature, dissolving contents completely by gently swirling or rotating.

Stability
The lyophilized powder (not reconstituted) is stable until the expiration date indicated on the label when stored at -20°C. Once reconstituted, the components of the serum are stable for 1 week at 2-8°C.

Warnings and Precautions

- For Laboratory Reagent Use Only. Do Not Feed to Cattle or Other Ruminants.
- Product contains highly purified bovine source material from non-BSE countries. The manufacturing facility does not receive, store or process ruminant materials from restricted countries.
- Possible infectious agents in the materials have been inactivated. Because no method can offer complete assurance as to the absence of infectious agents, this material should be handled as though capable of transmitting infectious disease and disposed as biohazard waste or medical waste according to applicable local and national laws.
- Additional safety information concerning storage and handling of this product is provided within the Material Safety Data Sheet for this product. To obtain an MSDS, please contact our customer service department at 858-455-4768.

Control Information

Control	Lot Number	Expiration Date	Value (U/L)
Control 0	ADCL0111A	2013-01	10.7 ± 1.9

Certified by: _____ Date: 7/27/11

Diazyme Laboratories 12889 Gregg Court, Poway, California 92064 USA
Tel: 858-455-4768; Fax 858-455-3701; Web: www.diazyme.com; Email: support@diazyme.com

DIAZYME	Title:	Adenosine Deaminase Control Certificate of Analysis	Page 1 of 1
Doc. #: CQF 4021	Rev: E	DCO #: 2527	Effective: 06/14/11

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7/11/11

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Preparation
Open one vial of the adenosine deaminase control carefully to avoid any loss of material and reconstitute with exactly 1 mL of distilled H_2O. Close the vial and let stand for thirty (30) minutes at room temperature, dissolving contents completely by gently swirling or rotating.

Stability
The lyophilized powder (not reconstituted) is stable until the expiration date indicated on the label when stored at -20°C. Once reconstituted, the components of the serum are stable for 1 week at 2-8°C.

Warnings and Precautions

- For Laboratory Reagent Use Only. Do Not Feed to Cattle or Other Ruminants.
- Product contains highly purified bovine source material from non-BSE countries. The manufacturing facility does not receive, store or process ruminant materials from restricted countries.
- Possible infectious agents in the materials have been inactivated. Because no method can offer complete assurance as to the absence of infectious agents, this material should be handled as though capable of transmitting infectious disease and disposed as biohazard waste or medical waste according to applicable local and national laws.
- Additional safety information concerning storage and handling of this product is provided within the Material Safety Data Sheet for this product. To obtain an MSDS, please contact our customer service department at 858-455-4768.

Control Information

Control	Lot Number	Expiration Date	Value (U/L)
Level I	ADC102110	2013-07	29.8 ± 5.1

Certified by: Taylor L Caldwell Date: 7/21/11

Diazyme Laboratories 12889 Gregg Court, Poway, California 92064 USA
Tel: 858-455-4768; Fax: 858-455-3101; Web: www.diazyme.com; Email: support@diazyme.com

DIAZYME	Title:	Adenosine Deaminase Control Certificate of Analysis	Page 1 of 1
Doc. #: CQF 4021	Rev: E	DCO #: 2527	Effective: 06/14/11

Catalog Number: DZ117A

Intended use
The adenosine deaminase (ADA) control is used for quality control procedures in examining the accuracy of quantitative adenosine deaminase assays. For Research Use Only in the USA.

Characteristics
The adenosine deaminase control is prepared in a bovine serum base, provided in lyophilized powder.

Preparation
Open one vial of the adenosine deaminase control carefully to avoid any loss of material and reconstitute with exactly 1 mL of distilled H_2O. Close the vial and let stand for thirty (30) minutes at room temperature, dissolving contents completely by gently swirling or rotating.

Stability
The lyophilized powder (not reconstituted) is stable until the expiration date indicated on the label when stored at -20°C. Once reconstituted, the components of the serum are stable for 1 week at 2-8°C.

Warnings and Precautions

- For Laboratory Reagent Use Only. Do Not Feed to Cattle or Other Ruminants.
- Product contains highly purified bovine source material from non-BSE countries. The manufacturing facility does not receive, store or process ruminant materials from restricted countries.
- Possible infectious agents in the materials have been inactivated. Because no method can offer complete assurance as to the absence of infectious agents, this material should be handled as though capable of transmitting infectious disease and disposed as biohazard waste or medical waste according to applicable local and national laws.
- Additional safety information concerning storage and handling of this product is provided within the Material Safety Data Sheet for this product. To obtain an MSDS, please contact our customer service department at 858-455-4768.

Control Information

Control	Lot Number	Expiration Date	Value (U/L)
Control 2	ADC20110A	2012-08	137.6 +/- 23.4

Certified by: Daniel Abbeduto Date: 2011-06-21

Diazyme Laboratories 12889 Gregg Court, Poway, California 92064 USA
Tel: 858-455-4768; Fax: 858-455-3701; Web: www.diazyme.com; Email: support@diazyme.com

Appendix C: Ethics clearance certificate.

M110212M110212

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Dr Pieter A Ekermans

CLEARANCE CERTIFICATE	**M110212**
PROJECT	The clinical usefulness of adenosine deaminase measurementin cerebrospinal fluid.
INVESTIGATORS	Dr Pieter A Ekermans.
DEPARTMENT	School of Pathology/Clinical Microb& Infect Dis
DATE CONSIDERED	25/02/2011
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 25/02/2011 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Dr J George

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

Appendix D: CSF cell counts and CSF chemistry.

Table D1: CSF cell counts for the individual categories.

Dx	Polymorphonucleur cells (cells / mm^3)				Lymphocytes (cells / mm^3)				Erythrocytes (cells / mm^3)			
	Min	Med	Max	IQR	Min	Med	Max	IQR	Min	Med	Max	IQR
? BM	0	160.0	10001	399.2	0	11.0	10001	98.3	0	2.0	10001	149.2
? M	0	0.0	400	0.0	0	0.0	640	2.0	0	0.0	10001	12
? MI	0	8.0	640	70.0	0	45.0	590	110.0	0	2.0	10001	59.3
? NCC	0	0.0	2	0.0	0	0.0	8	1.6	0	0.0	10001	67.1
? TBM	0	0.0	560	3.0	0	8.5	4800	51.3	0	2.0	10001	52
? VME	0	0.0	40	0.2	0	15.0	545	33.2	0	0.0	10001	10.1
BM	0	143.5	10001	534.1	0	29.0	9350	97.8	0	10.5	10001	73.8
BA	0	0.0	65	14.6	0	1.0	240	33.8	0	13.0	470	75
CaM	0	0.0	77	5.0	0	7.0	1440	36.7	0	0.0	350	5.3
CeM	0	0.0	10	8.3	0	0.0	0	0.0	15	21.0	32	14.2
Ce	0	0.0	0	0.0	0	0.0	2	0.7	0	0.0	58	3
CCM	0	0.0	880	2.0	0	8.0	2000	42.5	0	0.0	10001	10.8
M	0	0.0	119	0.0	0	0.0	200	1.0	0	0.0	10001	37
MI	0	5.0	50	41.7	0	0.0	2	1.7	0	5.0	25	20.8
MM	0	0.0	0	0.0	0	3.0	125	72.9	0	2.5	200	118.8
NS	0	0.0	80	0.4	0	13.5	70	44.8	0	3.0	10001	253.3
NdM	0	0.0	4160	0.0	0	0.0	880	2.0	0	0.0	10001	33.3
O	0	0.0	10001	0.0	0	0.0	2400	2.0	0	0.0	10001	30
P	0	0.0	13	0.0	0	0.0	35	1.0	0	0.0	10001	30
S	0	0.0	740	0.0	0	1.0	450	8.8	0	30.0	10001	10001
TBM	0	3.0	1430	26.7	0	22.0	960	83.3	0	10.0	10001	64.7
T	0	0.0	50	0.0	0	0.0	90	2.0	0	0.0	1280	36.2
Ve	0	5.0	2160	103.3	0	15.0	880	40.7	0	5.0	60	20
VME	0	0.0	175	0.0	0	13.0	43	23.3	0	1.5	315	20.3

Key to abbreviations used in this table: ? BM – Suspected bacterial meningitis; ? M – Suspected meningitis; ? MI – Suspected mixed infection; ? NCC – Suspected neurocysticercosis; ? TBM – Suspected TBM; ? VME – Suspected viral meningitis / encephalitis; BM – Confirmed bacterial meningitis; BA – Confirmed brain abscess; CaM – Carcinomatous meningitis; CeM – Confirmed cerebral malaria; Ce – Suspected cerebritis; CCM – Confirmed cryptococcal meningitis; Dx – Diagnosis; IQR – Interquartile range; Max – Maximum; Med – Median; M – Medical – related pathology; Min – Minimum; MI – Confirmed mixed infection; MM – Confirmed MOTT meningitis; NS – Confirmed neurosyphilis; NdM – No dx made; O – Other; P – Psychiatric disorders (and psychiatric-related disorders); S – Surgical – related pathology; TBM – Confirmed TBM; T – Toxicology; Ve – Confirmed ventriculitis; VME – Confirmed viral meningitis / encephalitis.

Table D2: CSF chemistry for the individual categories.

Dx	CSF protein (g/L)				CSF glucose (mmol/L)			
	Min	Med	Max	IQR	Min	Med	Max	IQR
? BM	0.1	0.8	17.0	1.3	0.0	2.6	8.9	2.5
? M	0.0	0.3	17.0	0.3	0.1	3.3	19.6	1.1
? MI	0.2	0.9	5.3	1.9	0.1	2.6	43.3	2.2
? NCC	0.1	0.3	7.1	0.2	0.0	3.7	12.9	1.5
? TBM	0.1	1.2	17.0	1.9	0.0	2.7	7.7	1.9
? VME	0.2	0.4	6.1	0.4	2.0	3.0	5.2	0.9
BM	0.0	3.3	17.0	2.5	0.0	0.1	7.9	0.8
BA	0.4	1.1	5.5	1.0	3.0	3.9	7.2	1.9
CaM	0.3	1.5	17.0	3.4	0.1	1.7	5.9	2.5
CeM	0.3	0.4	0.7	0.3	2.9	3.8	4.2	1.1
Ce	0.2	0.3	2.3	0.3	1.1	2.8	3.9	1.0
CCM	0.0	0.8	17.0	0.9	0.0	2.0	6.7	1.5
M	0.0	0.3	17.0	0.3	0.0	3.6	37.7	1.4
MI	0.4	0.8	0.8	0.3	2.2	2.3	2.8	0.5
MM	0.3	0.6	0.9	0.5	0.2	1.0	4.2	2.5
NS	0.2	0.5	2.5	0.8	1.0	3.0	5.0	1.7
NdM	0.1	0.3	17.0	0.4	0.0	3.2	12.6	1.0
O	0.0	0.3	17.0	0.3	0.0	3.3	35.6	1.2
P	0.1	0.3	4.2	0.2	0.3	3.5	10.0	1.1
S	0.0	0.7	7.4	2.1	0.7	3.2	8.6	1.7
TBM	0.1	1.4	5.9	2.3	0.0	1.5	13.6	1.1
T	0.1	0.3	0.7	0.2	2.0	3.8	9.5	1.3
Ve	0.5	2.7	17.0	5.7	0.1	1.8	3.5	2.0
VME	0.2	0.5	3.4	1.5	0.3	3.5	4.7	1.5

Key to abbreviations used in this table: ? BM – Suspected bacterial meningitis; ? M – Suspected meningitis; ? MI – Suspected mixed infection; ? NCC – Suspected neurocysticercosis; ? TBM – Suspected TBM; ? VME – Suspected viral meningitis / encephalitis; BM – Confirmed bacterial meningitis; BA – Confirmed brain abscess; CaM – Carcinomatous meningitis; CeM – Confirmed cerebral malaria; Ce – Suspected cerebritis; CCM – Confirmed cryptococcal meningitis; Dx – Diagnosis; IQR – Interquartile range; Max – Maximum; Med – Median; M – Medical – related pathology; Min – Minimum; MI – Confirmed mixed infection; MM – Confirmed MOTT meningitis; NS – Confirmed neurosyphilis; NdM – No dx made; O – Other; P – Psychiatric disorders (and psychiatric-related disorders); S – Surgical – related pathology; TBM – Confirmed TBM; T – Toxicology; Ve – Confirmed ventriculitis; VME – Confirmed viral meningitis / encephalitis.

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