RESEARCH REPORT

Validation of the Cerebrospinal Fluid (CSF) Module of the Siemens ADVIA® 2120i for Automated Cell Counts of Cerebrospinal Fluid

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DECLARATION

I declare that this research report, *Validation of the Cerebrospinal Fluid (CSF) Module of the Siemens ADVIA® 2120i for Automated Cell Counts of Cerebrospinal Fluid*, is my own, original work. All the sources that I used or quoted have been indicated and acknowledged by means of complete references. This work has not been submitted before for any other degree or at any other institution.

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ABSTRACT

Introduction: The majority of haematology analysers routinely utilised for whole blood specimens are now equipped with cerebrospinal fluid (CSF) and/or body fluid modules. These automated cell counters are steadily replacing manual microscopy for CSF cell counts.

This switch is attributed to the automated analysers' higher throughput, improved turnaround times, superior precision and higher accuracy compared to manual microscopy.

Methods: 46 routine CSF samples were analysed using the ADVIA2120i CSF Module to acquire a red cell count (RCC), white cell count (WCC) and a four-part WCC differential. Results were compared against the mean of two CSF cell counts performed by manual microscopy using the Improved Neubauer chamber haemocytometer. Quantitative method comparison was performed using EP Evaluator® software version 8.0.0 and Microsoft Excel®. The ADVIA2120i was, furthermore, assessed for precision, linearity and carryover.

Results: The ADVIA 2120i meets all acceptable claims in terms of precision, and demonstrated acceptable accuracy for RCC, mononuclear and polymorphonuclear cells, compared with the reference method. Linearity results showed that the ADVIA2120i produced results that were proportional after serial dilutions. All parameters were deemed to have acceptable carryover.

Conclusions: The ADVIA2120i offers rapid, precise and accurate RCC and WCC for both normal and abnormal cell counts. Our results show that the ADVIA2120i is a suitable alternative to manual microscopy for CSF cell counts and is deemed fit for purpose.

KEYWORDS

Cerebrospinal fluid, manual microscopy, ADVIA2120i, red cell counts, white cell counts

LIST OF ABBREVIATIONS AND ACRONYMS

ALL	Acute lymphoblastic leukaemia/lymphoma
CLSI	Clinical Laboratory Standards Institute
CNS	Central nervous system
CNS-IPI	Central Nervous System International Prognostic Index
CNS	Central nervous system
CSF	Cerebrospinal fluid
CV	Coefficient of variation
DNA	Deoxyribonucleic acid
DLBCL	Diffuse large B cell lymphoma
ECAM	Epithelial cell adhesion molecule
ICSH	International Council for Standardization in Haematology
IgH	Immunoglobulin heavy chain
IPI	International Prognostic Index
miRNA	MicroRNA
NHLS	National Health Laboratory Services
PCNSL	Primary central nervous system lymphoma
PCR	Polymerase chain reaction
PMN	Polymorphonuclear
RBC	Red blood cells
RCC	Red cell count
RNA	Ribonucleic acid

RT-PCR	Reverse-transcription polymerase chain reaction
WBC	White blood cells
WCC	White cell count

LIST OF UNITS

µL	Microliter
g/mL	Grams per millilitre
mg	Milligrams
mg/dL	Milligrams per decilitre
mL	Milliliters
mm^2	Millimeters squared
mmHG	Millilitres mercury
RBC/L	Red blood cells per liter
U/mL	Units per milliliter
WBC/L	White blood cells per liter
WCC/µL	White cell count per microliter
Wt	Weight unit

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LITERATURE REVIEW

1. CEREBROSPINAL FLUID

Cerebrospinal fluid (CSF) is a non-viscous, clear fluid that surrounds the brain and spinal cord. It functions predominantly as a shock absorber between the brain and the skull, to protect the CNS. Suspension of the brain in CSF, furthermore, provides buoyancy, which lowers the brain's weight from 1 400–1 500 grams to 25–50 grams.[1] Buoyancy prevents the base of the brain from being subjected to excessive pressure, which may impede blood supply.[2] In addition, CSF also circulates key nutrients (glucose, proteins, lipids and electrolytes) that are required by neurons for basic function.[3] Waste products diffuse into the CSF and are removed as CSF is resorbed through arachnoid granulations into venous circulation.[3,4]

In normal adults, CSF is produced primarily by the choroid plexus, which lines the lateral, third, and fourth ventricles of the brain.[4] Extrachoroidal production of CSF from extracellular fluid, cerebral capillaries and ependymal epithelium, constitutes the minor source. The rate of CSF production is equal to the rate of CSF reabsorption, maintaining volumes between 125 mL and 150 mL in normal adults.[5] This wellregulated equilibrium between production and reabsorption rates maintains the normal supine CSF pressure of 8 mmHg to 15 mmHg, and ± 20 mmHg when erect.[3]

Once secreted, CSF is distributed rostrally over the convexities of the brain and to the villous sites of absorption, and caudally to the spinal subarachnoid space.[4] In the cranial subarachnoid space, CSF flows towards the arachnoid villi for reabsorption. The reabsorbed CSF is transported to the kidneys and liver, where it is filtered and excreted.[5] In the spinal subarachnoid space, CSF is absorbed by the epidural venous plexus and spinal nerve sheaths, and drained into the lymphatic system.[4]

2. LUMBAR PUNCTURE

A lumbar puncture, also known as a spinal tap, is an invasive, sterile procedure that involves insertion of a hollow needle into the subarachnoid space of the lumbar spine. It can be performed for both diagnostic and therapeutic reasons. The procedure should

be carried out by a competent clinician, to minimise potential complications. Post-dural puncture headache is the most common complication encountered, occurring in 30% of patients.[6] Other complications include lower back pain, cranial neuropathies , nerve root irritation, infections, brain tissue herniation and spinal haematomas.[7] An appropriate understanding of the contraindications, the anatomy and sterile technique is, thus, essential. Complications, though rare, are potentially life-threatening. It is due to these complications and the difficulties in acquiring CSF samples that these specimens should be regarded as precious samples.[8,9]

Nevertheless, lumbar punctures are routinely performed in clinical practice, to assess CSF.[10] The chemical analysis and cell counts in these CSF samples provides essential information for the diagnosis of various CNS diseases.[8] CSF analysis is indispensable as a diagnostic and therapeutic tool, as seen in Table 1.

Table 1: Indications for a lumbar puncture, adapted from Ellenby et al.[9]

Indications for lumbar puncture
For diagnostic purposes
Viral, bacterial or fungal meningitis
Encephalitis
Inflammatory conditions: Multiple sclerosis and Guillain-Barré syndrome
CNS malignancy diagnosis and monitoring
For therapeutic purposes
Administration of anaesthetic drugs
Lower-body analgesia
Antibiotic administration for ventriculitis
Intrathecal chemotherapy for CNS leukaemia and lymphomas

Normal CSF is acellular, however, up 5 to WCC/µL and 5 RCC/µL can occur in normal adults without it being considered pathological. In neonates, counts of up to 20 WCC/µL are considered normal.[10]

During infection or malignant infiltration of the CNS, the cellular constituents in the CSF are altered.[10] If WCCs are present, the differential WCC count in CSF normally comprises 70% lymphocytes and 30% monocytes.[10] More than three PMN/µL, as seen, inter alia, with infection, inflammation or leukaemic infiltration, is always regarded as abnormal in adults.[10] In turn, an increase in the RCC is indicative of the

presence of subarachnoid haemorrhage, intracerebral haemorrhage or a traumatic spinal tap.[45]

Acute bacterial meningitis has been found to be approximately 10 times more common in developing countries than in developed counterparts. It is almost always fatal in the absence of appropriate management, with survival heavily dependent on accurate diagnosis and early administration of antibiotics.[46]

In 2001, Berkely et al. conducted a prospective study on 905 children undergoing lumbar puncture at a rural district hospital in Kenya over a period of one year. These authors assessed the value of non-laboratory tests that were normally available at the hospital for laboratory assessment of CSF. They found that, without adequate and reliable laboratory resources, the diagnosis of childhood acute bacterial meningitis was missed in up to a third of cases. Accurate leucocyte counting and glucose measurement were found to be superior to CSF culture facilities, which were expensive and difficult to maintain.[47] In addition, CSF culture has a long turn-around time, whilst CSF cell counts and chemistry offer immediate results that may aid clinicians with treatment decisions.

The number of requests for CSF analysis at the Universitas Microbiology Department has increased substantially. In 2016, a total of 2 780 samples were analysed, and it increased to 3 720 samples analysed in 2017.[48] This increase has proven to be a challenge for the Department, due to critical staff shortages and the lack of microbiology technologists for an after-hours service. The result is an increased turnaround time, sample degradation (evident within two hours after collection)[49] and, ultimately, a delay in service delivery.

3. CEREBROSPINAL FLUID IN HAEMATOLOGY

Malignant CNS infiltration is a potentially fatal complication that is a result of haematological and non-haematological malignancies. These malignancies may be primary neural or extraneural in origin.[11]

Primary neural malignancies include primary CNS lymphoma (PCNSL) and solid tumours, namely, germ cell tumours, ependymoma, medulloblastoma and neuroectodermal tumors.[12,13] PCNSL is an aggressive, acquired immunodeficiency

syndrome (AIDS)-defining non-Hodgkin lymphoma that accounts for 2.2%[14] of all CNS tumours. PCNSL presents a diagnostic challenge, as it involves critical areas within the CNS, and the diagnosis relies on histopathology of brain biopsies from these sites. Obtaining biopsy specimens may be complicated by bleeding, with devastating consequences, and biopsies often yield limited tissue. These diagnostic limitations have led to the use of less invasive diagnostic approaches to assess malignant cells in CSF.[14]

Metastases to the CNS from other primary sites constitute extraneural CNS malignancies, which include haematological malignancies, such as leukaemia and lymphoma and a myriad of non-haematological solid tumours. In the era of effective treatment protocols and improved imaging, the incidence of CNS metastases has risen, consequent to prolonged survival and early detection respectively.[15]

Non-haematological solid tumours account for 67-80% of all CNS metastases. Tumors with a predilection for CNS infiltration include carcinomas of the lung (50%), breast (20%), melanoma (10%) and colon (5%). Up to 15% of CNS metastases may present with no known primary.[16]

Haematological malignancies with a high propensity for the CNS include aggressive non-Hodgkin lymphomas, such as intravascular diffuse large B cell lymphoma (DLBCL), acute lymphoblastic leukaemia/lymphoma (ALL), the blastoid variant of mantle cell lymphoma, and Burkitt lymphoma.[15]

The incidence of CNS infiltration is dependent on the site of the primary malignancy. Non-haematological malignancies with a high propensity for CNS infiltration (67-80% of all metastases) include carcinomas of the lung (50%) and breast (20%); melanoma (10%), and 15% presenting with no known primary.[16]

CNS infiltration in patients with leukaemia and lymphoma confers a poor prognosis, with a median survival of three months.[11,17] One of the reasons for the poor prognosis is the development of neoplastic meningitis, a rapidly progressive and fatal condition.[13] Signs and symptoms are dependent on the location of the disease within the CNS, and include headache, cranial nerve palsies, radicular pain, neck and back pain, altered level of consciousness and focal weakness or loss of sensation; seizures can occur, but are rare.[18,19]

ALL accounts for approximately 25% of all childhood malignancies. Although CNS directed therapies form part of the standard chemotherapeutic regimens, CNS relapse is still observed in approximately 2–8% of leukaemia patients. The CNS is the most common site for relapse consequent to the blood-brain barrier.[20]

Although not as well understood as the bone marrow microenviroment, the CNS leukaemia niche provides a microenviroment suitable for CNS relapse. Once the leukaemic cells breach the blood-brain barrier, they may escape the effects of chemotherapy (poor penetration, resulting in inadequate therapeutic levels) and immune surveillance. If leukaemic cells persist in the CNS, relapse could also occur.[20] The risk factors for CNS involvement in ALL are listed in Table 2.

Table 2: Risk factors for CNS involvement in ALL[17,21,22]

Tumour characteristics	T-cell phenotype in paediatric ALL and a mature B cell phenotype in adult ALL
	Hyperleucocytosis
	High proliferative index (>14% lymphoblasts in cell cycle [S or G2/M phase])
	Blast expression of CD56
Biochemical findings	Elevated □2-microgobulin levels
	Lactate dehydrogenase levels >600 U/L
Cytogenetics	Philadelphia (Ph) chromosome positivity
	Complex karyotype (>5 chromosomal abnormalities)
	t(8:14)
	t(4:11)
	Low hypodiploidy/near triploidy

Based on cytomorphological identification of CSF leukaemic blasts and the absolute WCC, patients can be stratified according to risk for CNS recurrence (see Table 3).[21] Patients with CNS2 and CNS3 are at increased risk of CNS relapse.[22]

Table 3: Classification of CNS involvement[23]

CNS1	No leukaemic blasts
CNS2	CSF: <5 WCC/µL with leukaemic blasts
CNS3	CSF: >5 WCC/µL with leukaemic blasts

Most cases of non-Hodgkin lymphoma with CNS involvement (approximately 4.2%) occur post chemotherapy with disease relapse. Leptomeningeal spread and, to a lesser extent, haematogenous spread to the cerebral parenchyma, are the most common means of metastases. In the cerebral parenchyma, lymphoma cells form nodular deposits within brain parenchyma via the Virchow-Robin spaces.[24] These spaces are continuations of the subarachnoid space that accompany vessels entering the brain parenchyma.[25] High expression of CD56, a neural cell adhesion molecule, by lymphoma cells has been implicated in the spread of lymphoma cells to the CNS. It is postulated that the CD56 molecule enables the interaction between lymphoma cells and cells in the CNS.[26] The specific cellular and molecular pathways responsible for mobilisation of lymphoma cells to the CNS, however, remain to be fully elucidated.

In aggressive non-Hodgkin lymphomas, risk factors for CNS infiltration include the following:[17,22]

- Concordant bone marrow involvement;
- Involvement of the kidneys, adrenals, breast, paranasal sinus, testes or orbital cavities;
- Raised lactate dehydrogenase levels and the involvement of multiple extranodal sites;
- Double expression of dominant acting oncogenes *MYC* and B-cell lymphoma 2 (*BCL2*); and
- Advanced stage with a high International Prognostic Index (IPI) score.

The risk for lymphoma CNS involvement can be predicted using the Central Nervous System International Prognostic Index (CNS-IPI) tool. It was originally developed by Schmitz et al. to predict the risk of CNS relapse/progression in patients with DLBCL

treated with R-CHOP. R-CHOP, comprising rituximab, cyclophosphamide, doxorubicin, vincristine and prednisolone, is the standard chemotherapeutic regimen used for most of the aggressive non-Hodgkin B-cell lymphomas.[27] In addition to the standard variables used for the IPI, the CNS-IPI assesses involvement of the adrenal glands and kidneys. Patients are then classified into different risk categories. Patients with DLBCL that fall into the low- and intermediate-risk groups and have a CNS relapse risk <5%, whereas those in the high-risk group have a >10% risk of CNS relapse.[27]

Table 4: IPI for non-Hodgkin lymphomas[55]

Variable	Risk group: (Score) [% 5-year overall survival]
Age >60 years	Low: (0–1) [73%]
LDH	Low-intermediate (2) [51%]
Eastern Cooperative Oncology Group performance status >1	High-intermediate: (3) [43%]
Ann- Arbor Stage III or IV	High: (4 or 5) [26%]
Involvement of more than one extranodal site	

Furthermore, as an immune-privileged site (sites with limited regeneration capacity that are adapted to restricting potentially harmful immune-mediated inflammation),[28] the CNS is protected by a blood-brain barrier. The blood-brain barrier is formed from tight junctions between endothelial cells and is the main reason for chemotherapy failure in the case of CNS malignancies.[29] This failure is due to failure of most chemotherapeutic agents to reach desirable therapeutic levels within the CNS. To overcome this barrier, high-dose chemotherapy, and intrathecal and intra-arterial routes for chemotherapy administration are advocated. Intrathecal methotrexate (12–15 mg flat dose) is the preferred chemotherapeutic agent, administered together with standard chemotherapy.[14] Cranial radiation, intrathecal cytarabine and systemic chemotherapy that crosses the blood-brain barrier, such as methotrexate, cytarabine or ifosfamide, may serve as alternatives for prophylaxis.[29]

4. DIAGNOSTIC TOOLS FOR CEREBROSPINAL FLUID ANALYSIS

The blood-brain barrier prevents the release of tumour-secreted markers into the blood stream, thus, making blood analysis inferior to CSF analysis for the diagnosis of CNS malignancies.[12]

The current, most widely utilised tools for assessing CNS infiltration by malignant cells include clinical neurological examination, neuroimaging of the neuroaxis, and CSF cytomorphology. These three modalities can be used concurrently. Clinical signs and symptoms of CNS infiltration may present late in the disease course, and are not specific for CNS infiltration.[15] Neuroimaging techniques, such as magnetic resonance imaging, are sensitive, but have low specificity, depending on the location and type of malignancy.[15]

CSF cytomorphology and a few of the current methodologies used for CSF analysis will be discussed below.

4.1. Cerebrospinal fluid cytomorphology

This is considered the current gold standard for diagnosing primary and metastatic malignancies of the CNS.[13] It involves centrifugation of the specimen, staining with May-Grunwald-Giemsa stain and microscopic visualisation for the presence of malignant cells.[13] Although highly specific (>95%), the test lacks sensitivity (<50%), and false negatives remain a concern.[30] This method is also labour-intensive and requires experienced staff to execute.[31]

Sensitivity can be improved by obtaining sample volumes of at least 10.5 mL, performing a second collection if the first is negative, obtaining CSF from as close to the tumour as possible, and timeous sample processing.[13] In patients with CNS malignancies, up to 45% will have a negative cytological examination with the first lumbar puncture. With a second tap, the yield is increased to 80%, but repeated lumbar punctures after the first two are not beneficial.[15]

Although in some instances cytomorphology can distinguish benign cells from malignant cells, combination with other modalities, such as immunohistochemistry,

flow cytometry and polymerase chain reaction (PCR), can improve the diagnostic sensitivity of CSF involvement by monoclonal and neoplastic cells.[15]

4.2. Flow cytometry

Flow cytometry is a laser-based technique that categorises cells based on their size, complexity and fluorochrome-labelled cell surface markers. It currently serves as a useful adjunct to CSF cytomorphology for the diagnosis and staging of haematological malignancies. Flow cytometry enables rapid acquisition and processing of diagnostic data.[13] It has a higher sensitivity, requiring as low as 0.01% malignant cells (of total leucocytes) for detecting an abnormal cell population, compared to the 5% tumour cells required for cytomorphology.[13,21] Up to 80% of cases with CSF infiltration by lymphoma cells are detected on the first CSF sample.[15] The higher sensitivity makes flow cytometry valuable for detecting occult or subclinical CNS involvement during maintenance therapy and clinical follow-up, especially when cytomorphology is negative.[11] Identifying abnormal cells within the CSF that have an immunophenotype similar to that of the malignant population in the peripheral blood or bone marrow aspirate samples, supports the diagnosis of concurrent CNS infiltration with the systemic lymphoma diagnosis.[15]

Flow cytometry can, furthermore, assess clonality of B-cells, based on the cells expressing either lambda or kappa surface light chains.[32] Immunophenotyping helps to distinguish indolent non-Hodgkin lymphomas from normal lymphocytes, and to detect abnormal patterns of antigen expression on neoplastic cells. This is useful for both diagnosis and assessment of therapeutic response.[33] A well accepted limitation of CSF flow cytometry is the possible diagnostic uncertainty accompanying traumatic lumbar punctures, where CSF is contaminated with peripheral blood. In this setting, false positives due to contamination of the CSF by peripheral lymphoma cells can complicate staging.[15]

4.3. Immunocytochemistry

Immunocytochemistry makes use of peroxidase or cytokeratin-labelled monoclonal antibodies to detect the expression of antigens on paraffin-embedded cells. This modality can be applied to the CSF cytospin.[13,34] CSF cytospin involves gentle and controlled centrifugation of CSF to produce a monolayer of preserved cells. The cells

are flattened onto a slide, which enhances the visibility of the nuclear material.[34] Immunohistochemistry has a sensitivity of 89–95% and a specificity of 89–100% for the detection of leptomeningeal haematological metastases.[32] At lower cell counts, immunocytochemistry should be used in conjunction with cytomorphology to improve sensitivity and to distinguish between reactive and neoplastic lymphocytes in CSF.[15]

Detecting high-density antigens with immunocytochemistry yields similar results to flow cytometry, however, when antigens are expressed at low density, immunocytochemistry may be more reliable.[32] Since the advent of flow cytometry, using immunocytochemistry to detect haematological malignancies in the CSF has declined. It is, however, still very useful for detecting leptomeningeal metastases of solid tumours, in combination with cytomorphology.[32]

4.4. CellSearch

CellSearch is a technology approved by the United States' Food and Drug Administration, which is currently utilised to follow up patients with metastatic epithelial cell adhesion molecule (ECAM) expressing tumours, such as melanoma, breast, prostate, colorectal and lung carcinomas.[35]

It is a semi-automated cell analysis method that enumerates circulating tumour cells present in 7.5 mL whole blood. Ferrofluid nanoparticles, with antibodies attached, then bind to ECAM, after which the circulating tumour cells are magnetically separated from the rest of the cells. Cytokeratin monoclonal antibodies, which are specific to epithelial cells, are attached to a magnetic plate and CD45 is added to identify any leukocytes that may have contaminated the sample. 4', 6' diamino-2-phenylindole (DAPI) stain is also utilised to highlight the nuclei of both circulating tumour cells and leukocytes.[35,36]

Compared to cytomorphology, CellSearch technology has better sensitivity and a specificity of 90%.[35] Growing interest in its use for detecting CSF malignancies has led to research in this field, and further publications are eagerly awaited.[12]

4.5. Polymerase chain reaction

Cytomorphology may not be able to distinguish between reactive lymphocytes and malignant cells. Therefore, a definitive diagnosis requires evaluation of clonality.[37] CSF analysis with PCR makes use of primers to amplify specific tumour cell-derived

deoxyribonucleic acid (DNA).[32] In B-cell malignancies, PCR analysis amplifies the immunoglobulin heavy chain gene in the third complementarity determining region (CDR3).[32] The presence of a clonally rearranged CDR3 is found in 80–95% of B-cell lymphomas, and is the molecular hallmark of malignant B-lymphocytes. In the peripheral blood and bone marrow aspirate samples, this is the preferred method for diagnosis, assessment of treatment efficacy and detection of minimal residual disease.[15,32]

Galoin et al. utilised semi-nested PCR to carry out multiple runs on a single CSF sample to detect rearranged immunoglubulin heavy chain (IgH) genes in a clonal B-cell population. The primers used were specific to CDR2 and CDR3 regions of the heavy chain variable regions. Although multiple runs are required, PCR was able to detect clonal populations in CSF samples, thus, making it a more sensitive diagnostic tool for leptomeningeal metastases than cytomorphology. Galoin et al., thus, proved that assessment of IgH gene rearrangement, when used in conjunction with cytology and immunocytochemistry, provided more accurate results than cytomorphology alone.[37]

Reverse-transcription polymerase chain reaction (RT-PCR) enables the amplification of small amounts of ribonucleic acid (RNA) into larger amounts of complementary DNA, thus, requiring only limited amounts of neoplastic cells to be present in CSF. RTPCR, however, requires appropriate primers for the type of RNA being assayed, which limits its use as a screening tool. RT-PCR that uses probes specifically for clonal immunoglobulin gene rearrangements, is able to distinguish between polyclonal reactive and monoclonal neoplastic cells.[17]

4.6. Cerebrospinal fluid microRNA

MicroRNAs (miRNAs) are non-coding, small regulatory RNA molecules that are 19 to 25 nucleotides long. They inhibit gene expression by preventing translation and promoting degradation of target messenger RNA. They can function as tumour suppressors or oncogenes, and play critical roles in apoptosis, proliferation and cell differentiation.[12,15] miRNAs are deregulated in malignancies and have recently been utilised as diagnostic and prognostic markers.[12] miRNAs are stable in body fluids, including CSF. Their analysis provides a non-invasive diagnostic alternative to brain biopsies for patients with suspected CNS infiltration.[12]

Detection of CSF miRNA by means of RT-PCR has been shown to have diagnostic value for diagnosing PCNSL with overexpression of miR-21, miR-19 and miR-92a. Using this modality, Baraniskin et al. were able to distinguish between patients with PCNSL and patients with other neurologic disorders with 95.7% sensitivity and 96.7% specificity.[38]

4.7. Cerebrospinal spinal biomarker

Proteonomic analysis of CSF has led to the discovery of several protein markers within the CSF, of which expression is upregulated in CNS lymphomas. These protein biomarkers include antithrombin chemokine (C-X-C motif) ligand 13 **(**CXCL 13), C-XC chemokine receptor type 5 (CXCR5), β2-microglobulin and soluble IL-2 receptor (sIL-2R).[17]

Antithrombin is a serine protease inhibitor that has been linked to neovascularisation in CNS lymphomas.[12] Increased levels (>1.2 g/ml) are suggestive of CNS lymphoma and are associated with poorer chemotherapy responses and lower overall survival rates .[12] Levels higher than 1.2 g/mL detect CNS lymphoma with a sensitivity of 75% and a specificity 98.7%.[12]

CXCL13 is a chemokine secreted by follicular dendritic cells in the lymph node germinal centre that mediates chemotaxis of B-cells to the germinal centre through its cognate receptor CXCR5.[39] Rubenstein et al. found elevated levels of CXCL13 in CSF of CNS lymphoma patients compared to control patients.[12] Elevated levels of the chemokine receptor CXCR5, and its ligand CXCL13, are responsible for lymphoma tropism to the CNS and are reflective of an increased tumour burden.[17] β2-microglobulin is a cell surface protein associated with the human leucocyte antigen (HLA) class I. It is expressed on all nucleated cells and its elevation is reflective of increased cell turnover. Elevated CSF β2-microglobulin levels are associated with CNS involvement by leukaemia or lymphoma, and were found to be elevated in up to

50% of patients with these disorders.[15] sIL-2R is a cytokine receptor released from activated T-cells. It is reflective of immune activity and elevated levels in blood are an important marker of acute lymphoblastic leukaemia/lymphoma.[40] In CSF, elevated levels (>10 U/mL) of soluble IL-2 receptor, when used in conjunction with positive cytomorphology and elevated CSF WCC, are suggestive of CNS involvement with

ALL.[17] At values of ≥10 U/mL of CSF sIL2-R, the sensitivity and specificity were 89.5% and 89.6% respectively for ALL.[40]

Current diagnostic modalities for leptomeningeal metastases lack sensitivity, particularly in early disease states.[13] The additional use of the new diagnostic approaches mentioned in Section 4 allow for early detection of CNS infiltration and, consequently, earlier management.

5. MANUAL CEREBROSPINAL FLUID ANALYSIS

Historically, CSF analysis has been conducted through manual microscopy by means of haemocytometers (counting chambers) and is considered the gold standard for CSF analysis.[41] The commercially available chambers include the Improved Neubauer, Burker, Thomas and Fuchs-Rosenthal chambers.[31]

The Microbiology Department of the NHLS at Universitas Hospital utilises the Improved Neubauer chamber.[42] The chamber consists of a thick glass plate that has two raised, square-shaped platforms (see Figure 1). The ruled area on each counting chamber (9 mm^2) is divided into nine large (1 mm^2) squares. The four large corner squares are subdivided into 16 equally-sized squares and the large centre square is subdivided into 25 equally-sized squares. Each of these 25 squares is subdivided further into 16 squares (Figure 2).[43]

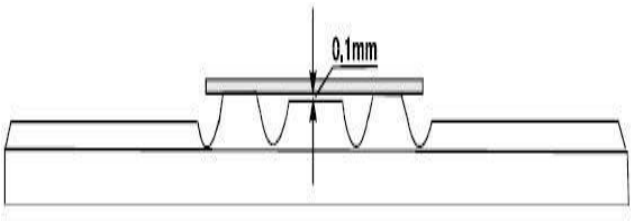

Figure 1: Improved Neubauer chamber with coverglass[43]

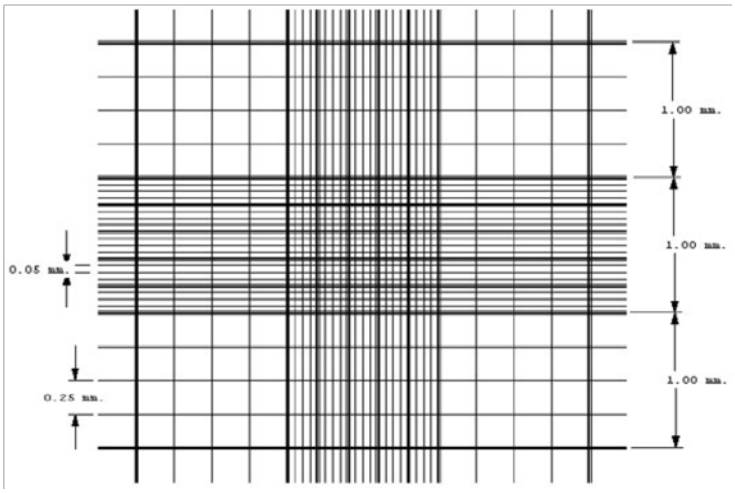

Figure 2: Improved Neubauer chamber calibrated coverglass[43]

A calibrated coverglass that covers the central area of the chamber controls the depth of the field precisely, so that each large (1 mm^2) square encloses a volume of 0.1 µL. To avoid inaccurate results, the calibrated coverglass must be used to perform cell counts.[43] Once a specimen is deemed acceptable for evaluation (as per standard operating procedure of the Microbiology Department),[44] crystal violet stain is added to the CSF sample. The mixture is allowed to stand for one minute before it is loaded onto the counting chamber. The counting chamber is, furthermore, allowed to stand for another minute on the microscope stage, to allow cells to settle. Using the 40 x dry objective, the different cell lines are counted within the four large (4 x 16) squares.[42]

The total of each cell type is then calculated as follows:[42]

$$\text{PMN or lymph} = \frac{\text{Dilution x Depth of counting chamber x Number of cells counted}}{\text{Number of squares counted}}$$

$$\text{Erythrocytes} = \frac{\text{Depth of counting chamber x Number of cells}}{\text{Number of squares counted}}$$

After the total for each cell type has been determined, the correlating number of cells per microliter is determined, as per Table 5.

Table 5: Conversion of cells counted to cells/µl[34]

Cerebrospinal Fluid Cell Counts							
Undiluted RCC				**1:2 Dilution of WCC (PMN and Lymph)**			
Low count: Count 4 big squares		**High count: Count 1 big square**		**Low count: Count 4 big squares**		**High count: Count 1 big square**	
RCC Counted	**RCC/µl**	**RCC Counted**	**RCC/µl**	**WCC Counted**	**WCC/ µl**	**WCC Counted**	**WCC/ µl**
1	3	1	10	1	5	1	20
2	5	2	20	2	10	2	40
3	7	3	30	3	15	3	60
4	10	4	40	4	20	4	80
5	13	5	50	5	25	5	100
6	15	6	60	6	30	6	120
7	17	7	70	7	35	7	140
8	20	8	80	8	40	8	160
9	23	9	90	9	45	9	180
10	25	10	100	10	50	10	200
11	28	20	200	11	55	20	400
12	30	25	250	12	60	25	500
13	33	30	300	13	65	30	600
14	35	35	350	14	70	35	700
15	38	400	400	15	75	40	800
16	40	50	500	16	80	50	1 000
17	43	60	600	17	85	60	1 200
18	45	70	700	18	90	70	1 400
19	8	80	800	19	95	80	1 600
20	50	90	900	20	100	90	1 800
25	63	100	1 000	25	126	100	2 000

30	75	150	1 500	30	150	150	3 000
40	100	200	2 000	40	200	200	4 000

Although considered the gold standard for CSF cell counts, manual microscopy is subject to several shortcomings and, as a result, the last decade has favoured and streamlined the move towards automated cell counters. Shortcomings associated with manual microscopy include:[31,49]

- High imprecision (at lower cell counts) and high inter-observer variability;
- Labour-intensive methodology with prolonged turnaround times (30 to 45 minutes to complete);
- It is subjective and technique-dependent; and
- Inability to differentiate mononuclear cells into lymphocytes and monocytes.

6. AUTOMATED CEREBROSPINAL FLUID ANALYSIS

In most clinical laboratories, automated haematology analysers are utilised to analyse full blood counts on whole blood samples. Many of these analysers are now equipped with CSF and/or body fluid modules that are able to perform CSF and body fluid cell counts.

Although the automated method on the ADVIA2120i is not currently utilised in South Africa to analyse CSF and other body fluids, several international publications have supported its use.[31,32,49,50] The Siemens ADVIA120 and ADVIA2120 (Siemens Healthcare Diagnostics, Deerfield, IL, USA), Sysmex XE-5000 (Sysmex Corporation, Kobe, Japan), Abbott CELL-DYN Sapphire (Abbott Diagnostics, Abbott Park, IL, USA) and the Beckman Coulter LH750 (Beckman Coulter, Miami, FL, USA) are among the instruments currently utilised for automated body fluid cell counts.[31]

The automated haematology analysers all work on the same basic principles. After the specimen is incubated with a manufacturer-specific reagent, it is analysed using either flow cytometry, electrical impedance, or both. With the electrical impedance principle (Coulter principle), a stream of cells is passed through an aperture over which an electrical current is applied. The amount of current displaced results in a variation in electrical resistance that is directly proportional to the cell size.[31] Flow cytometry, of

which the basic principle is explained above, categorises cells based on their size (forward side scatter), complexity (side scatter) and DNA/RNA binding

(fluorescence).[31,32] The light signals are organised into a digital form and displayed as scattergrams. The scattergrams below (Figure 3) are from the ADVIA2120i, which utilises flow cytometry for cell characterisation.[31,51]

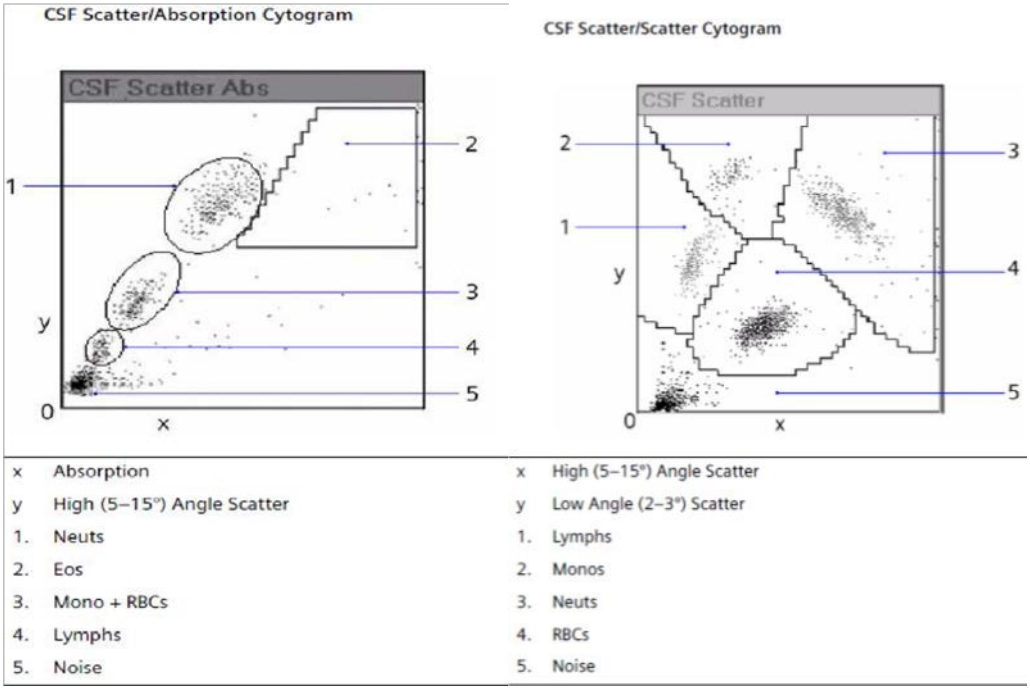

Figure 3: CSF scatter/scatter and CSF scatter/absorption cytograms[51]

Like the manual method, automated systems provide a RCC, a total WCC and a WCC differential count; however, the automated method is able to separate the mononuclear component further, into lymphocytes and monocytes.[52]

Fleming et al. compared these analysers to conventional manual microscopy for assessment of CSF and body fluid cell counts in inflammatory diseases. They conclude that the automated methods provide a rapid and more precise diagnosis. The accuracy and high precision is a result of the aspiration of larger volumes by the analysers than of the volumes required for manual microscopy. Consequent to their higher limits of quantification, particularly with the Sysmex XE-5000, automated analysers are less precise at lower cell counts. Another shortcoming is the inability of the analysers to detect neoplastic cells, which they erroneously classify as lymphocytes.[31]

Aune et al. had similar favourable outcomes, proving that the ADVIA120 compared well to manual microscopy. As the ADVIA120 does not provide morphology flagging, the authors suggest that cytomorphology be used in conjunction with automated analysis to ascertain the presence or absence of neoplastic cells, particularly for oncology specimens.[49]

7. CEREBRAL SPINAL FLUID CELL COUNT REFERENCE RANGES

Reference ranges for CSF cell counts are based primarily on manual microscopy techniques. Reference ranges in healthy individuals are age-dependent, with normal adult CSF containing <5 x10^6 WCC/L, that of infants <7 x10^6 WCC/L, and neonates <30 x10^6 WCC/L.[52]

Very few studies assessing the reference ranges of the automated methods have been conducted. The establishment of new reference ranges for the Sysmex XE-5000 was assessed by two separate studies, by De Jonge et al. and Sandhaus et al. Using a cohort of 87 normal adult patients undergoing spinal anaesthesia, De Jonge et al. report normal reference ranges for RCC 0 x 10^6/L, WCC and mononuclear <7 x 10^6/L, and polymorphonuclear cells <3 x 10^6/L.[50]

Sandhaus et al. had different findings from De Jonge et al. Sandhaus et al. found the normal reference range for WCC to be between 20 x10^6 WCC/L and 30 x10^6 WCC/L. They conclude that this range was due to a decrease in linearity between the results of the Sysmex XE-5000 and the manual method at cell counts <20 x10^6 WCC/L.[31]

At a CSF RCC of 250 cells/L, good correlation between counts were obtained for the ADVIA2120i and manual microscopy.[53] Bremell et al. calculated new ADVIA2120i specific reference ranges as per CLSI C28-A3 guidelines. Their normal reference ranges were <5 x 10^6 WCC/L, <4 x 10^6 lymphocytes/L, <3 x 10^6 polymorphonuclear cells/L, and <3 x 10^6 monocytes/L.[53]

At present, there is no uniformity for reference ranges between haematology analysers. This hinders acceptance of automated cell counts in clinical practice. For standardisation of reference ranges, multi-institutional studies with clear sample inclusion criteria are needed, to compare the different haematology analysers currently approved for CSF analysis.[52]

8. SIEMENS HEALTHCARE: ADVIA2120I

Universitas Hospital Service Laboratory installed two ADVIA2120i instruments in 2015. These instruments are bench-top haematology analysers, and are currently utilised for routine whole blood specimens only.
The ADVIA2120i is semi-automated, with three aspiration modes, namely, the rackbased auto sampler, manual closed tube sampler, and manual open tube sampler. The rack-based system has a throughput of 120 samples per hour in the full blood count (FBC) /differential mode and 74 samples per hour when reticulocyte analysis is performed. The ADVIA2120i is equipped with a CSF module that provides rapid, automated analysis of CSF samples.[51,54]

Analysing CSF with the ADVIA2120i involves mixing and incubating the CSF sample with CSF reagent, which spheres and fixes the cells. Incubation requires a minimum of four minutes, up to a maximum incubation time of four hours. Once incubated, the sample is aspirated directly into the ADVIA2120i system. The cells are detected and enumerated based on light scatter and absorbance measurements. A scatter versus scatter, and scatter versus absorbance cytogram is displayed with the thresholds, and results are automatically calculated for each sample (see Figure 2.3).[51]

Reportable parameters include:

- WCC
- Full four-part differential count, which includes the absolute and percentage values for neutrophils, lymphocytes, monocytes and eosinophils; and
- RCC

We aim to validate the use of the Siemens ADVIA2120i for CSF analysis within NHLS laboratories countrywide. The instrument will be evaluated for precision and accuracy against our currently utilised method, which is manual microscopic counts.

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MANUSCRIPT FOR PUBLICATION

This chapter will be submitted to the *International Journal of Laboratory Haematology* (IJLH) for possible publication. The article that was submitted followed the author guidelines of the journal (see Appendix I).

Title Page

Running title: Man versus Machine

Full title: Validation of the Cerebrospinal Fluid (CSF) Module of the Siemens ADVIA® 2120i for Automated Cell Counts of Cerebrospinal Fluid

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Abstract

Introduction: The majority of haematology analysers routinely utilised for whole blood specimens are now equipped with cerebrospinal fluid (CSF) and/or body fluid modules. These automated cell counters are steadily replacing manual microscopy for CSF cell counts.

This change is attributed to the automated counters' higher throughput, improved turnaround times, superior precision and higher accuracy, compared to manual microscopy.

Methods: In total, 46 consecutive routine CSF samples were analysed using the ADVIA2120i CSF Module to obtain a red cell count (RCC), white cell count (WCC) and a four-part WCC differential. Results were compared against the mean of two CSF cell counts performed by manual microscopy using the Improved Neubauer chamber haemocytometer. Quantitative method comparison was performed using EP Evaluator® software Version 8.0.0 and Microsoft Excel®. The ADVIA2120i was assessed further for precision, linearity and carryover.

Results: The ADVIA2120i meets all the claims in terms of precision and demonstrated acceptable accuracy for RCC and WCC, specifically including lymphocytes and polymorphonuclear (PMN) cell counts, when compared with the reference method. Linearity results show that the ADVIA2120i produced results that were proportional after serial dilutions. All parameters were deemed to have acceptable carryover.

Conclusions: The ADVIA2120i offers rapid, precise and accurate RCC and WCC for both normal and abnormal cell counts. Our results show that the ADVIA2120i is a suitable alternative to manual microscopy for CSF cell counts and is deemed fit for purpose.

Keywords: ADVIA2120i, Cerebrospinal fluid, manual microscopy, red cell counts, white cell counts

1. INTRODUCTION

Analysis of cerebrospinal fluid (CSF) cell counts provides critical information required for the diagnosis of various inflammatory and infective conditions, as well as for monitoring the response to therapeutic measures.[1-3] The value of CSF cell counts is underscored further by its usefulness for diagnosing central nervous system (CNS) infiltration by primary neural and extraneural malignancies.[4]

As an adjunct to the other, albeit more sophisticated and more expensive, diagnostic modalities utilised for CSF analysis, such as flow cytometry, CSF cytomorphology, immunohistochemistry, CellSearch, polymerase chain reaction (PCR), microRNA and CSF biomarkers; simple and relatively inexpensive CSF cell counts enable early detection of CNS malignant infiltration and, consequently, earlier management.[5]

Normal CSF is acellular, however, up to five white cell count (WCC) $x10^6$/L and five red cell count (RCC) $x10^6$/L can occur in normal adults, without it being considered pathological. The WCC differential in CSF normally comprises 70% lymphocytes and 30% monocytes. More than five PMN $x10^6$/L, as seen with, inter alia, infection, inflammation or leukaemic infiltration, is always regarded as abnormal in adults.[2] An increase in the RCC count is indicative of the presence of subarachnoid haemorrhage, intracerebral haemorrhage or a traumatic spinal tap.[3]

For decades, and even currently, manual microscopy has been regarded as the gold standard for CSF cell counts.[6] However, this conventional method is currently falling out of favour, as a result of its shortcomings, namely, increased turnaround times, labour intensiveness, technique dependence, high inaccuracy, inter-observer variability, requirement of 24-hour availability of experienced microbiology staff, and inability to differentiate mononuclear cells into lymphocytes and monocytes.[7] To overcome these shortcomings, there has been a paradigm shift, towards automation of CSF cell counts in most modern laboratories worldwide.[16]

Although the ADVI2120i is not currently clinically utilised for CSF and other body fluids (BF) analysis in South Africa, the majority of new automated haematology analysers are equipped with CSF and/or body fluids modules, and their use is steadily gaining favour. The Siemens ADVIA120 and 2120i (Siemens Healthcare Diagnostics, Tarrytown, NY, USA), Sysmex XE-5000 (Sysmex Corporation, Kobe, Japan), Abbott

CELL-DYN Sapphire (Abbott diagnostics, Abbott Park, IL, USA) and Beckman Coulter LH750 (Beckman Coulter, Miami, FL, USA) are among the instruments currently equipped and validated for automated body fluid cell counts.[6,8,9]

The automated method has the advantages of being faster, having improved precision, and being easier to use than the traditional manual microscopy method.[7] The accuracy and high precision of the automated method is a result of the aspiration of larger volumes by the analysers, compared to the volumes required for manual microscopic counts.[6]

A disadvantage of the automated method is a loss of precision at lower cell counts. This is most likely consequent to the automated method's higher limits of quantification, particularly with the Sysmex XE-5000. Another shortcoming is the inability of the analysers to detect neoplastic cells, which are erroneously classified as lymphocytes.[6] Consequently, supplementary cytomorphology analysis should be mandatory for all oncology specimens.[10]

In this study, we evaluated the CSF Module of the ADVIA2120i for CSF cell counts (RCC, WCC and differential) and compared them to the reference method (PMN, lymph and RCC manual microscopic counts). The ADVIA2120i was also assessed for precision, linearity, accuracy and carryover.

2. MATERIALS AND METHODS

We conducted a single-centre study at the National Health Laboratory Service (NHLS) Universitas Hospital Service Laboratory (Bloemfontein, Free State, South Africa).

Approval for the study was obtained from the Health Sciences Research Ethics Committee (Faculty of Health Sciences, University of the Free State, Bloemfontein, South Africa) [HSREC117/2017(UFS)HSD2017/1179].

2.1. STUDY SAMPLES

In total, 46 consecutive CSF samples that had been received for routine CSF analysis at the NHLS Universitas Hospital Service Laboratory were included. Only samples with at least 600 µL of CSF remaining after all required microbiological and/or cytological tests had been performed were included. Samples were excluded as per rejection

criteria of the standard operating procedures of the Microbiology Department for the reference method.[11]

These were namely:

- Specimens collected in wrong tubes
- Insufficient specimen
- Specimens in leaky containers

To account for sample degradation (which is evident within two hours after collection), the automated method was performed within five minutes of the manual method.

2.2. REFERENCE MANUAL MICROSCOPY METHOD

Manual CSF microscopic cell counts were conducted as per standard operating procedure of the Microbiology Department of the NHLS Universitas Service Laboratory.[11] The Improved Neubauer counting chamber (Neubauer Improved, Marienfeld, Germany) was used for the RCC and WCC. The WCC was differentiated further into polymorphonuclear cells (PMN) and mononuclear cells. Mononuclear cells could not be differentiated further into lymphocytes or monocytes, and were, therefore, reported as lymphocytes.

Cell counting was performed using 30 µL of CSF. The initial cell count was performed by a senior microbiology technologist. The cell counts were then verified by a haematologist. A mean of the two results was used, which was then compared to the automated ADVIA2120i cell counts. The cell count obtained was then converted to cells/µL using the following equation:

$$\text{PMN or lymph} = \frac{\text{Dilution x Depth of counting chamber x Number of cells counted}}{\text{Number of squares counted}}$$

$$\text{Erythrocytes} = \frac{\text{Depth of counting chamber x Number of cells}}{\text{Number of squares counted}}$$

2.3. ADVIA2120I CSF MODULE

The ADVIA2120i CSF module is a semi-automated method, specifically for CSF samples.[12,13] Calibration is not required for this CSF module,[12] and internal quality control was conducted once daily before the first sample was analysed.

For analysis of CSF with the ADVIA2120i, 300 µL of thoroughly mixed CSF sample was added to 300 µL of CSF Reagent [ADVIA2120i CSF (Prod. No.T01-4610-01)] in a labelled test tube. The prepared mixture was mixed by gently inverting the tubes five times and leaving them at room temperature for a minimum of four minutes and a maximum of four hours.[12]

Once in CSF mode, and once acceptable background counts had been obtained, CSF samples were aspirated directly into the ADVIA2120i system using the manual opentube mode. The cells were detected and enumerated based on light scatter and absorbance measurements. Scatter versus scatter and scatter versus absorbance cytograms were generated for each sample and results were calculated automatically.

Reportable parameters include WCC, RCC and a four-part differential count, which included the absolute and percentage values for neutrophils, lymphocytes, monocytes and eosinophils.

2.4. PERFORMANCE PARAMETERS

2.4.1. Precision

As per the Clinical and Laboratory Standards Institute (CLSI) EP15A3E guideline, precision was determined by running five replicates of the low and high controls per day for five days; values were, thus, used for precision evaluation. Simple total precision with means, standard deviations and coefficient of variation (CV) were calculated. Precision was accepted if it was equal to or less than that claimed by the manufacturer or a CV less than 18.89% for Level 1 and less than 33.3% for Level 2, as reported by Tanada et al.[21]

2.4.2. Accuracy

Accuracy of the ADVIA2120i CSF method was evaluated by regression analysis (Deming and regular), comparing 46 automated results for absolute CSF RCC, CSF PMN and CSF lymphocytes with the mean reference CSF manual counts. Comparison was conducted using the slopes, intercepts and confidence intervals, as well as percentage bias, bias plots and Bland-Altman plots.

The accuracy of the automated method was deemed acceptable if the slopes were within 0.95 to 1.05 and intercepts between ±1% of Deming or regular regression analysis, provided the 95% confidence intervals for the slopes and intercepts included 1.00 and 0.00 respectively. This was conducted as per the CLSI EP09-A2-IR guideline.

The quality of the data was assessed by evaluating the result ranges and correlation coefficient. A correlation coefficient value of >0.90 was deemed acceptable.[12]

4.2.3. Linearity

Cell counts for the high control (ADVIA2120i TESTpoint CSF Controls (PN T03-448501)) were utilised to demonstrate linearity as per the standard operating procedure of the NHLS (Protocol for Instrument Evaluation: GPQ0007). The samples were serially diluted with phosphate-buffered saline to relevant dilutions of 3.13%, 6.25%, 12.50%, 25%, 50% and 100%.

4.2.4. Carryover

The high control (ADVIA2120i TESTpoint CSF Controls (PN T03-4485-01)) was measured in triplicate (H1, H2, H3), followed by three consecutive measurements of saline/blank (L1, L2, L3). Carryover was calculated according to the International Council for Standardization in Haematology (ICSH): (L1-L3)/(H3-L3)] x100%.[14]

A value of less than one was deemed acceptable.[12]

2.5. STATISTICAL ANALYSIS

Statistical analyses were performed using the EP Evaluator Version 8.0.0 and Microsoft Excel®.

3. RESULTS

3.1. Descriptive statistics

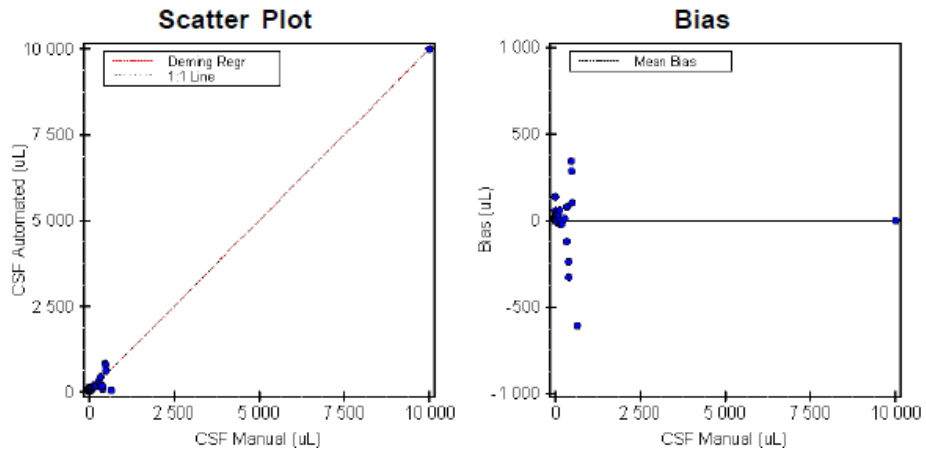
Insert table 1

3.2. Precision

Insert table 2

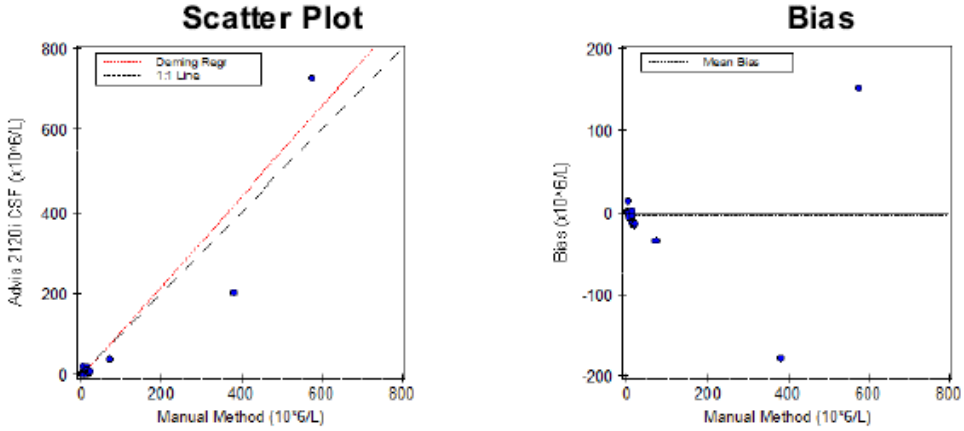
3.3. Accuracy

RCC



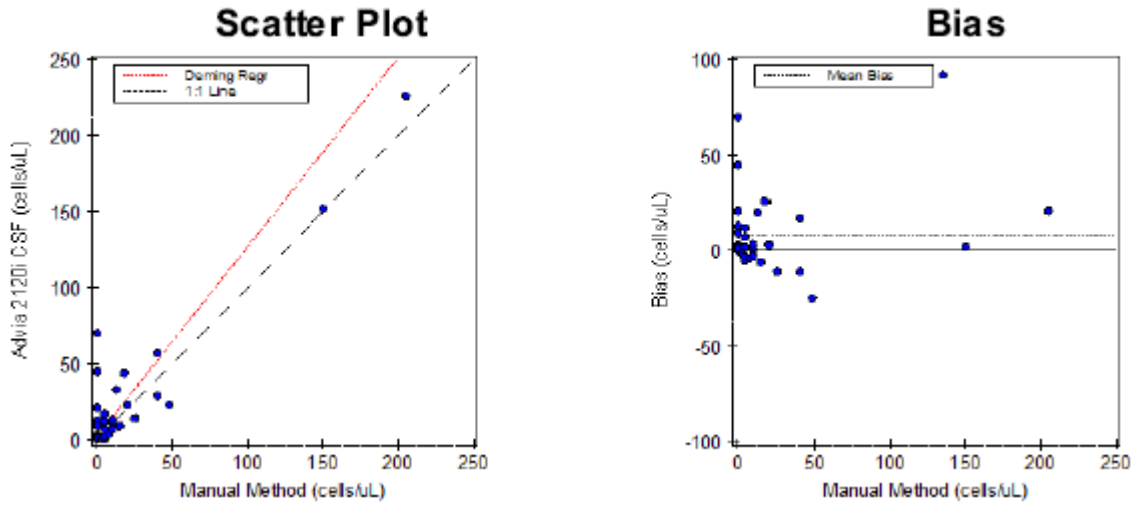
Slope: Deming**:** 1,000 (0,986 to 1,015); Regular 0,999 (0,985 to 1,013). **Intercept:** Deming**:**-0,8 (-42,2 to 40,5); Regular: 0,2 (-41,1 to 41,6)

PMN



Slope: Deming: 1.106 (0.993 to 1.219); Regular: 1.042 (0.931 to 1.153). **Intercept**: Deming: -5.2 (-17.2 to 6.7); Regular: -3.5 (-15.3 to 8.3)

Lymphocytes



Slope: Deming: 1.247 (1.093 to 1.401) Regular: 1.139 (0.989 to 1.289). **Intercept:** Deming: 2.5 (-4.9 to 9.8); Regular: 4.6 (-2.5 to 11.8).

Figure 1: Method comparison of CSF RCC, PMN and lymphocytes; ADVIA2120 vs manual microscopy (N=46).

3.4 Linearity

After serial dilutions, the ADVIA2120i CSF module displayed acceptable linearity throughout a broad range of clinically significant values, with R^2 approaching 1 for all parameters. Analytical ranges were as follows: RCC 4.5 – 166 X 10^6 cells/L; WCC 2.5 – 89 X 10^6 cells/L; PMN 1 – 42 X 10^6 cells/L and lymphocytes 1 – 36 X 10^6 cells/L

3.5 Carryover

All parameters were deemed to have acceptable carryover.

4. DISCUSSION

This was the first evaluation of the ADVIA2120i CSF module for CSF cell counts at our centre, and in South Africa. In the present study, we found the ADVIA2120i CSF

module easy to operate; it required no prior calibration and internal quality control procedures could be performed effortlessly. The smooth transition from whole blood to the CSF module, rapid acquisition of acceptable background counts, and preparation of the sample – it took less than four minutes – are additional features in favour of the ADVIA2120I instrument. Furthermore, the virtually immediately available raw data printout provides more rapid results than the relatively longer turnaround times of the manual counting method.

The ADVIA2120i showed exceptional performance for RCC and PMN counts, with the RCC obtaining a slope of 1.000 on Deming regression. The lymphocytes, however, only met acceptance criteria on the regular regression regarding the slope, with an overall positive bias of 7.5, which demonstrates the increased sensitivity of the automated method as a plausible explanation. The increased sensitivity is most likely due to the higher volumes (300 µL) aspirated with the automated method, compared to the 30 µL of the manual method.[11,12] Discrepant results pertaining to the lymphocyte count are expected due to variations in methodology. With manual microscopy, one value is provided for all mononuclear cells whilst the automated method is able to separate the mononuclear component further, into lymphocytes and monocytes.

The instrument demonstrated acceptable precision, carryover and linearity for all three parameters.

Automated CSF cell counts, as mentioned, are not limited to the ADVIA2120i platform; various other manufacturers also offer CSF and BF modules. In this regard, it might be pertinent to point out that the ADVIA2120i does not come equipped with a morphology flagging function within its CSF module, whilst the Sysmex XE-5000, Sysmex XN-series and the Abbott CELL-DYN3500 have single and 3-flagging functions.[9,15] However, although equipped with flagging functions, the performance (with regard to CSF analysis) in clinical diagnostics for oncology samples has been disappointing.[8,9,16] The ADVIA2120i does provide a flagging function for Cryptococcus infection in CSF.[6] Although not specifically evaluated in this study, there was one study sample with positive Cryptococcus latex agglutination tests, which were not flagged by the instrument, nor by the manual microscopy method. In our setting of a relatively high Cryptococcus meningitis prevalence, this could be a valuable topic for a future study.[17]

A practical drawback of the ADVIA2120i is the volume of CSF required (300 µL) for automated CSF cell counts. This may have resulted in the exclusion of many patient samples during our evaluation, due to insufficient sample volumes as specified in our inclusion criteria. To implement the automated method in clinical diagnostics, would require informing clinicians about minimum sample volume requirements.
Although the aim of the study was to evaluate the performance characteristics of the ADVIA2120i CSF module, we foresee a potential stumbling block for implementation of this platform in clinical diagnostics. Very few studies are available on reference ranges for automated methods. Two separate studies, by De Jonge et al. and Sandhaus et al, assessed the establishment of new reference ranges for the Sysmex XE-5000.[16,18] Using a cohort of 87 normal adult patients undergoing spinal anaesthesia, De Jonge et al. report normal reference ranges for RCC as 0 x 10^6/L, MN as <7 x 10^6/L and PMN as <3 x 10^6/L.[18] Bremell et al. calculated new ADVIA2120i specific reference ranges as CLSI C28-A3 guidelines. The reference ranges obtained were WCC as <5 x 10^6/L, lymphocytes as <4 x 10^6/L, PMN as <3 x 10^6 and monocytes <3 x 10^6/L.[19] Thus, at present, there is no uniformity for CSF cell count reference ranges between haematology analysers. Multi-institutional studies with clear sample inclusion criteria are needed to compare the different haematology analysers currently approved for CSF analysis, in order to standardise reference ranges.[16]

A possible limitation of our study is that it did not specifically evaluate the effect of high RCC or traumatic spinal taps on the WCC. This has previously been reported as a clear limitation of the ADVIA120.[10] RCC more than 1 500 x 10^6/L might falsely elevate white cell and neutrophil counts; the same authors advise diluting the sample to a maximum of 1:10.[10] This is not a recommendation in the ADVIA2120i manual, but as four of our study samples had exceptionally high RCC (>10 000 x 10^6/L), it could have been of value to determine whether dilution of these samples affected the white cell and neutrophil counts. Another limitation of our study is that it did not evaluate unprepared sample stability over time, as very little recent data is available on this.[6,20] However, the automated method is probably superior to the manual method in this regard, as samples are analysed in a fraction of the time, which may result in less sample degradation.

Due to the fact that the ADVIA CSF automated module is not widely utilised, another possible limitation to be expected is the availability of quality control materials as well

as the availability of participants for inter-laboratory and external quality assessment programmes.

Although cost comparisons between the automated and manual methods were not the focus of this study, this will need to be evaluated by future studies. Such an analysis will have to take into account the labour and expertise required for each of the methods, the time needed to complete analysis, reagent costs and availability of afterhours expertise. These factors would also have to be seen in conjunction with the lower overall patient medical costs that accompany the rapid, accurate and precise results offered by the automated method.

CONCLUSION

The results of our study indicate that, when compared with the reference method, the ADVIA2120i meets all acceptable claims in terms of precision and demonstrated acceptable accuracy. On performance characteristics alone, the CSF module can be declared fit for purpose. Implementation of this platform at the NHLS Universitas Hospital Service Laboratory will only be considered after evaluation of method-specific reference ranges in the clinical setting, and formal cost analysis.

CONFLICT OF INTEREST

The authors declare that there are no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ACKNOWLEDGEMENTS

The authors acknowledge the immense help received from the Microbiology Department, especially from Miss Pelonemi Leeuw, who assisted in the manual CSF cell counts. We are grateful to Dr Anneke van Marle for taking the time to verify the manual CSF cell counts. We acknowledge Mrs Ina Freitag and Mr Lance Hildebrande, for their assistance with our statistical analysis. The authors are also grateful to the authors, editors and publishers of all articles, journals and books from which the literature for this article was reviewed and discussed. The authors thank Siemens Healthineers South Africa for generously sponsoring all reagents and control material used in this evaluation.

5. Tables

Table 1: Descriptive statistics

CSF Parameter	Statistical Parameter	ADVIA2120i (x10^6)	Manual Microscopy (x10^6)
RCC	Range (mean)	0 – 115 752 (6 038.80)	0 - 10 000 (977.60)
	Standard deviation	22 225.54	2 820. 54
PMN	Range (mean)	0 – 77 506 (3 388.08)	0- 575 (39.43)
	Standard deviation	13 828.12	118.19
Lymphocytes	Range (mean)	0 – 8 227 (387.36)	0 – 743 (54.54)
	Standard deviation	1 394.49	131.58

Table 2. Simple precision for WCC and RCC

	Within-run		Between-run		Manufacturer claim (first line)	Tanada et al[5] (second line)
	Normal control mean CV (%)	High control mean CV (%)	Normal control mean CV (%)	High control mean CV (%)	CV (%)	CV (%)
WCC	17.6	9.8	22.2	11.1	≤ 11.2	Normal control ≤18.89 and High control ≤ 33.3
RCC	17.4	6.6	30.3	10.8	≤ 31.7	Normal control ≤18.89 and

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APPENDIX A: PERMISSION LETTERS

These letters were addressed to:

1. Head: Department of Haematology and Cell Biology, UFS: Prof. MJ Coetzee
2. Acting head: Department of Microbiology, UFS: Dr D Goedhals
3. Head and business unit manager, NHLS Universitas: Prof. H Pieters

To whom it may concern:

With this letter I, Dr Kalambi-Matengu, Department of Haematology and Cell Biology, would like to obtain your approval for performing the study:

Validation of the Cerebrospinal Fluid (CSF) Module of the Siemens ADVIA2120i for Automated Cell Counts of Cerebrospinal Fluid

The aim of this study is to validate the fully automated Siemens ADVIA2120i for cerebrospinal fluid (CSF) analysis. The study will take place at the National Health Laboratory Services (NHLS) Universitas Academic Business Unit. The time needed for the study is sixteen months and it is to be completed in January 2019.

The goal of this study is to validate and implement the use of the fully automated ADVIA2120i for the analysis of cerebrospinal fluid. The new fully automated method will improve turn-around time for cerebrospinal requests with improved precision.

This study is also performed in partial fulfilment of the requirements for the degree Magister Medicinae in Haematology.

All the institutions will be acknowledged in any presentation or publication.

Yours sincerely

……

Dr EN Kalambi-Matengu

(Cell No: 0769332717)

ANNEXURE B: ETHICS APPROVAL LETTER

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE

IRB nr 00006240
REC Reference nr 230408-011
IORG0005187
FWA00012784

01 November 2017

KALAMBI-MATENGU, ESTHER N
DEPT OF HAEMATOLOGY AND CELL BIOLOGY
FACULTY OF HEALTH SCIENCES
UFS

Dear Kalambi-Matengu, Esther N

HSREC 117/2017 (UFS-HSD2017/1179)
PRINCIPAL INVESTIGATOR: KALAMBI-MATENGU, ESTHER N
PROJECT TITLE: VALIDATION OF THE CEREBROSPINAL FLUID (CSF) MODULE OF THE SIEMENS ADVIA® 2120I FOR AUTOMATED CELL COUNTS OF CEREBROSPINAL FLUID

APPROVED

1. You are hereby kindly informed that the Health Sciences Research Ethics Committee (HSREC) approved this project at the meeting held on 31 October 2017 after all conditions were met.
2. The Committee must be informed of any serious adverse event and/or termination of the study
3. The Committee must be informed of any serious adverse event and/or termination of the study.
4. Any amendment, extension or other modifications to the protocol must be submitted to the HSREC for approval.
5. A progress report should be submitted within one year of approval and annually for long term studies.
6. A final report should be submitted at the completion of the study.
7. Kindly use the **HSREC NR** as reference in correspondence to the HSREC Secretariat.
8. The HSREC functions in compliance with, but not limited to, the following documents and guidelines: The SA National Health Act. No. 61 of 2003; Ethics in Health Research: Principles, Structures and Processes (2015); SA GCP(2006); Declaration of Helsinki; The Belmont Report; The US Office of Human Research Protections 45 CFR 461 (for non-exempt research with human participants conducted or supported by the US Department of Health and Human Services- (HHS), 21 CFR 50, 21 CFR 56; CIOMS; ICH-GCP-E6 Sections 1-4; The International Conference on Harmonization and Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH Tripartite), Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines, Constitution of the HSREC of the Faculty of Health Sciences.

Yours faithfully

DR SM LE GRANGE
CHAIR: HEALTH SCIENCES RESEARCH ETHICS COMMITTEE

Health Sciences Research Ethics Committee
Office of the Dean: Health Sciences
T: +27 (0)51 401 7795/7794 | E: ethicsfhs@ufs.ac.za
Block D, Dean's Division, Room D104 | P.O. Box/Posbus 339 (Internal Post Box G40) | Bloemfontein 9300 | South Africa
www.ufs.ac.za

APPENDIX C: AUTHOR GUIDELINES, INTERNATIONAL JOURNAL OF LABORATORY HAEMATOLOGY

The manuscript should be double-spaced with 30mm margins. Manuscripts must be numbered consecutively in the following sequence: Title Page; Abstract (if required); Main Body of Text; Acknowledgements; Reference List; Tables and Figure caption List.

1. Title page

The title page should contain the authors name(s), initials and place of work. In addition, the name and full postal address including e-mail, of the author who will deal with correspondence and proofs should be supplied. The full title should be accompanied by a short running title where the title exceeds 47 letters and spaces. In the top right hand corner, the number of manuscript pages and illustrations should be marked. Five keywords should be supplied after the Summary.

2. Abstract

A structured abstract of no more than 250 words is required for original articles, subdivided into the following sequential sections: Introduction; Methods; Results; Conclusion.

3. Main body of text

The text of original and review articles should be divided into the following sections: Introduction, Materials and methods, Results, Discussion, Tables, Footnotes and Figure legends (including magnifications).

4. Acknowledgments

Acknowledgements should be submitted on a separate sheet.

5. References

All references should be numbered consecutively in order of appearance and should be as complete as possible. In text citations should cite references in consecutive order using Arabic superscripts numerals. Sample references follow:

Journal article:

1. King VM, Armstrong DM, Apps R, Trott JR. Numerical aspects of pontine, lateral reticular, and inferior olivary projections of two paravermal cortical zones of the cat cerebellum. J Comp Neurol 1998;390:537.551.

Book:

2. Voet D, Voet JG. Biochemistry. New York: John Wiley & Sons; 1990.1223 p.

Please note that journal title abbreviations should conform to the practices of Chemical Abstracts.

For more information about AMA reference style - AMA Manual of Style

6. Tables and figures

The preferred position of figures and tables in the text should be indicated in the left-hand margin. Tables should include only essential data. Each table should be typewritten on a separate sheet and must be numbered consecutively with Arabic numerals, e.g. Table 1, and given a short caption. Vertical rules should not be used. Units should appear in parentheses in the column headings and not in the body of the table. All abbreviations should be defined in a footnote.

All tables and figures that are reproduced from a previously published source must be accompanied by a letter of permission from the publisher or copyright owner.

APPENDIX D: RESEARCH PROTOCOL

1. SUMMARY IN LAY TERMS

Cerebrospinal fluid (CSF) is the clear, colourless liquid that surrounds and protects the brain and spinal cord. During infection or malignant infiltration of the central nervous system (CNS), the cellular constituents of the CSF are altered. These alterations in cell counts offer valuable diagnostic clues, which are essential for patient management decisions. Currently, analysis of CSF is through manual microscopy, which is labour intensive and has a high turnaround time. We set out to validate the use of the ADVIA®2120i haematology analyser (automated method) for the analysis of white blood cell (WBC) and red blood cell (RBC) counts in CSF samples. This procedure has the potential to reduce staff workload and turnaround time, and to increase precision.

2. INTRODUCTION

Cerebrospinal fluid is the clear**,** colourless fluid that fills and surrounds the brain ventricles and cranial and spinal arachnoid spaces. It circulates within the subarachnoid space, between the pia mater and arachnoid mater. Here it functions to provide hydromechanical protection (by acting as a shock absorber), and regulates brain interstitial fluid, which is essential for brain development and neuronal functioning. In addition, it may serve an immunological function, analogous to the lymphatic system.[1,2]

CSF is predominantly produced by the choroid plexus (80%) and, to a lesser extent, by the ependymal lining of the ventricles, in the interstitial space. Absorption of CSF is primarily by the arachnoid villi that extend into the dural venous sinus. A balance between production and absorption maintains mean volumes of 150 ml and pressures of 10 mmHg–15 mmHg in the normal adult subarachnoid space.[2]

Normal CSF is acellular, however, up to five WBC/mm^3 and five RBC/mm^3 can occur in normal adults without it being considered pathological. In neonates, counts of up to 20 WBC/mm^3 are considered normal.

If present, the WBC differential in CSF is normally 70% lymphocytes and 30% monocytes. Monocytes are assigned polymorphonuclear (PMN) cells, on account of

their nuclear shape and lobularity, as opposed to the monomorphic nuclei of lymphocytes. More than three PMN/mm^3, as seen with infection, inflammation or leukaemic infiltration, is always regarded as abnormal in adults.[3] In turn, an increase in the RBC count is indicative of the presence of subarachnoid haemorrhage, intracerebral haemorrhage, or a traumatic spinal tap.[4] A traumatic tap refers to the introduction of a small volume of systemic blood into the dural space, with subsequent collection into the sample tube. Traumatic taps occur in approximately 20% of lumbar punctures.[3]

A lumbar puncture, also known as a spinal tap, is an invasive diagnostic procedure that involves inserting a hollow needle into the subarachnoid space of the lumbar spine. Sterile and precise technique is essential. The procedure should be performed by a competent clinician, to minimise potential complications, which may include spinal headaches, introduction of infections, spinal epidural haematomas and herniations. It is due to these complications, and the difficulty of acquiring CSF samples, that these specimens should be regarded as precious samples.[1]

Nevertheless, lumbar punctures are routinely performed in clinical practice to assess cerebrospinal fluid.[3] The chemical analysis and cell counts in these CSF samples can provide important information that is essential for the diagnosis of various CNS diseases. CSF analysis is indispensable for diagnosing meningitis (viral, bacterial and fungal), encephalitis, neurological disorders (e.g., Guillain-Barré syndrome) and the diagnosis and monitoring of leukaemia and lymphoma with CSF involvement.[1]

Table 2.1: Typical CSF findings in various types of meningitis[5]

Parameter	Normal	Bacterial	Viral	Fungal	Tubercular
Opening pressure	<180	200–500	N/A	>250 (Cryptococcus)	N/A
WBC count (mm^3)	0 –5	100–20 000	5–500	20–2 000	5–2 000
WBC differential	No predominance	>50% PMN	Lymph: >50% PMN: <20%	Lymph: >50%	Lymph: >80%
Protein (mg/dL)	15–50	100–500	30–150	40–150	50–300

Glucose (mg/dL)	50–80	<40	30–70	30–70	<40

Historically, CSF analysis has been conducted through manual microscopy using haemocytometers (counting chambers), which is considered the gold standard for CSF analysis. The two commercially available chambers most frequently used are the Neubauer-Improved and Fuchs-Rosenthal chambers. Variation coefficients for haemocytometers are as high as 45%.[6]

As a result of the various shortcomings of manual microscopy, a move towards automated cell counters has been favoured and streamlined in the last decade.[5]

Shortcomings associated with manual microscopy include

- High imprecision (at lower cell counts) and inter-observer variability;
- Labour-intensive methodology, with long turnaround times; and
  Skilled personnel required.

The haemocytometer currently utilised by the laboratory of the Department of Microbiology at the Universitas Hospital, Bloemfontein, South Africa, is the NeubauerImproved chamber.[7]

Automated haematology analysers are routinely used in the clinical laboratory to perform full blood count test sets on whole blood. Many of these automated analysers are now equipped with a CSF and/or body fluid module, which is able to perform cell counts on CSF and other body fluids. These instruments have the advantages of being faster, having improved precision, and being easier to use than the traditional manual microscopy method.[8]

The number of requests for CSF analysis at the Universitas Hospital Microbiology Department has increased substantially. In 2016 alone, a total of 2 780 samples were analysed, which increased to 3 251 samples in 2018. This demand for CSF analysis has proven to be a burden on the Department, which is experiencing a critical staff shortage, and does not have microbiology technologists available after hours. The consequence is a long turnaround time for analysis, sample degradation (which is evident within two hours after collection) and, ultimately, a failure in service delivery.

Consequently, the aim was to validate the onboard CSF module of the Siemens ADVIA® 2120i. Automated analysers have the advantage of 24-hour availability, lower imprecision and higher throughput.[9]

In 2015, Universitas Hospital Service Laboratory installed two ADVIA2120i instruments, which are currently only utilised for routine analysis of whole blood specimens. The ADVIA2120i has a CSF module that provides rapid, automated analysis of CSF samples. When using the ADVIA2120i CSF Assay, the CSF sample is mixed with ADVIA2120i CSF Reagent, which spheres and fixes the cells. After a minimum of four minutes (a maximum incubation time of four hours is permissible), the prepared sample is aspirated directly into the ADVIA2120i system. The cells are detected and enumerated, based on light scatter and absorbance measurements. Scatter versus scatter and scatter versus absorbance cytograms are displayed, and the thresholds and results are calculated automatically for each sample (Figure 1.1).[9]

Reportable parameters include

- WBC count;
- Full four-part differential count, which includes the absolute and percentage values for neutrophils, lymphocytes, monocytes and eosinophils; and RBC count.

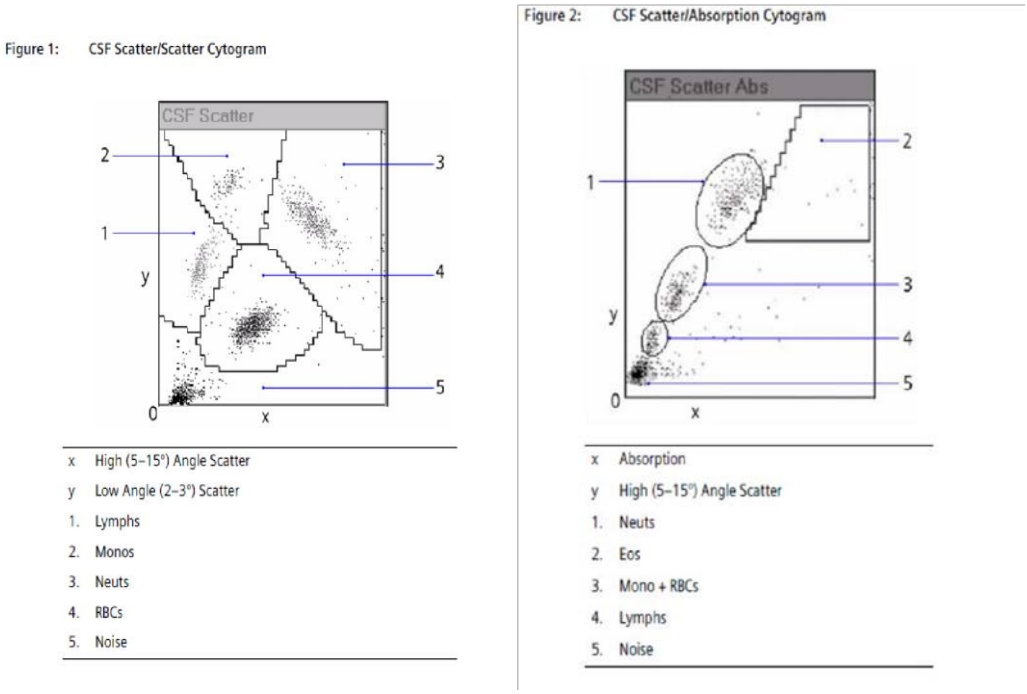

Figure 1: CSF scatter/scatter and CSF scatter/absorption cytograms[9]

3. AIM OF THE STUDY

We aimed to validate the use of the Siemens ADVIA2120i for CSF analysis at National Health Laboratory Services (NHLS) laboratories countrywide. The instrument was evaluated for precision and accuracy against the method currently utilised, namely, manual microscopy, which served as the reference method.

4. METHODOLOGY

4.1. Study design

This was an observational descriptive study.

4.2. Sample size

We obtained 46 samples of human cerebrospinal fluid with a wide range of normal and pathological blood cell counts.

4.3. Sample acquisition

The study samples consisted of 46 consecutive CSF specimens, received for routine microbiological analysis at the NHLS of Universitas Hospital Service Laboratory.

Inclusion criteria: All samples submitted for CSF cell count analysis with a minimum volume of 1 600 µL.

Exclusion criteria: CSF samples were excluded according to the rejection criteria of the standard operating procedures of the Microbiology Department. In addition, if there was insufficient specimen for the purposes of this study, it was excluded.

4.4. Measurement

All laboratory tests and data collection were conducted by the researcher at the NHLS Universitas Hospital Service Laboratory.

A unique study number was assigned to each study sample. This number, together with the laboratory results, was entered onto the data collection sheet by the researcher.

To account for sample degradation, specimens were prepared and analysed on the ADVIA2120i within five minutes of manual microscopy.
Figure 4.1 provides a brief summary of the steps of the validation process.

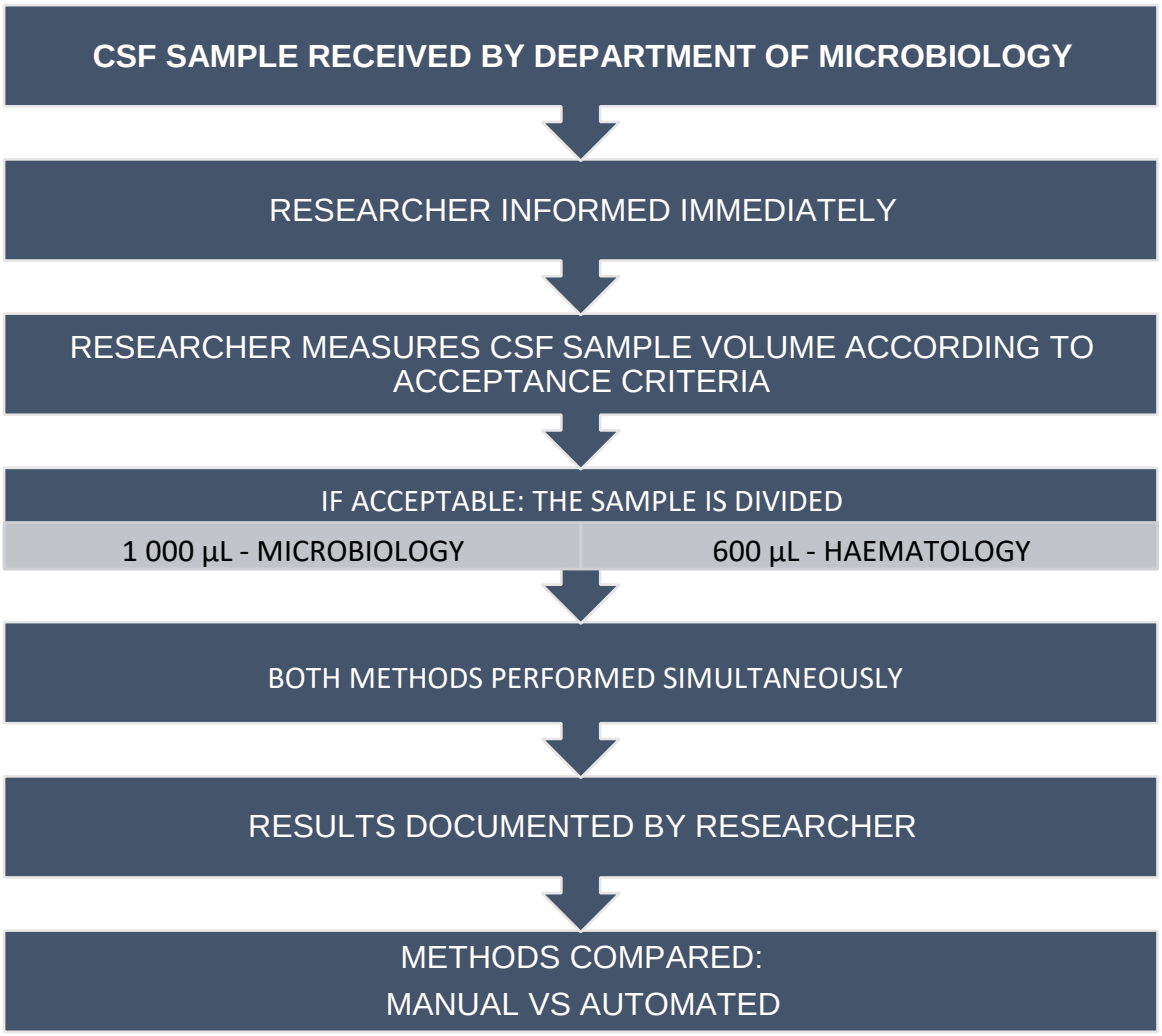

Figure 2: Flowchart illustrating CSF research protocol

Manual microscopy involved the following:

- Manual microscopic cell count was determined in the Department of Microbiology laboratory.
- Manual microscopic cell count was carried out by a single experienced microbiology technologist. Then, a haematologist repeated the microscopic cell count on the same counting chamber, and the mean of the two generated cell counts was entered on the data capture sheet.
- The procedure was conducted as per standard operating procedure (MIC0337).

- The manual microscopy cell counts acted as the reference against which the results from the ADVIA2120i were compared.

The automated method involved the following steps:

- All CSF study samples were collected from the Department of Microbiology (see Figure 1.2), and prepared and analysed by the researcher.
- CSF analysis on the ADVIA2120i was done in the CSF mode using the manual open-tube mode.

  CSF reagent contains:

  - Formaldehyde, 1.85% (Wt)
  - Glutaraldehyde, 0.28% (Wt)
  - Buffer
  - Surfactants

- 300 µL of thoroughly mixed CSF sample was added to 300 µL of CSF reagent [ADVIA2120i CSF (Prod. No.T01-4610-01)] in a labelled test tube.
- The prepared mixture was vortexed for five seconds and incubated at room temperature for five minutes.
- Once the ADVIA2120i had been switched from whole blood mode to CSF mode, the tubular systems were washed during the initialisation cycle, to obtain an acceptable flow cell background count. Doing so prevented residual cells or particles from the whole blood influencing CSF results.
- Acceptable background values are:

  - CSF R (RBC) Count = 0
  - CSF phytohaemagglutanin (WBC) = 0
  - CSF phytohaemagglutanin (WBC)Total ≤10

- Once acceptable background counts had been obtained, internal quality control was done. If the sample passed the internal quality control, the sample was

analysed. If acceptable background counts were not obtained, aspiration of a 25% bleach solution, followed by two aspirations of distilled water or saline, is recommended.

- Due to volume constraints of the study samples, an aspiration error meant that only samples with adequate volume could be rerun. Samples with insufficient volumes were excluded.
- The results obtained were then entered on the data collection sheet, for statistical comparison to the standard method.

4.4.1. Accuracy

Accuracy/trueness refers to extent of conformance of a test result to the accepted reference value.[10] Manual microscopy was used as the reference method, to which the automated analyser results were compared.

4.4.2. Bias

Bias is the measure of deviation from trueness, and was calculated according to the Clinical Laboratory Standards Institute (CLSI) EP15 protocol.[9]

4.4.3. Precision

As per the CLSI EP15A3E guideline, precision was determined by running five replicates of the low and high controls per day for five days, to obtain 25 values for precision evaluation. Simple total precision, with means, standard deviations and coefficient of variation (CV), was calculated according to the CLSI EP15A3E guideline. Precision was accepted if it was equal to or less than that claimed by the manufacturer (see Table 1.1).

4.4.4. Linearity

Linearity is defined as the ability of the assay to provide results that are directly proportional to the concentration of the analyte in a sample. Due to streamline volume constraints, Level 2 (high control) control's WBC/RBC was utilised to demonstrate linearity. The samples were serially diluted with phosphate buffered saline to relevant dilutions; this assessed the analyser's ability to provide results proportional to the concentration of cells in a sample.[11]

4.4.5. Sample carryover

Sample carryover is defined as the contamination of a sample by the sample analysed immediately before it. Three different lots (H1, H2, H3) of the high control (Level 2) were measured in triplicate, followed by three consecutive measurements of a saline/blank (L1, L2, L3). Carryover was calculated according to the International Council for Standardization in Haematology (ICSH): [(L1-L3)/(H3-L3)] x100%.[11]

4.4.6. Possible measurement and methodology errors

Manual microscopy:

- Intra-observer variation: To minimise intra-observer variation, manual cell counts were carried out as per NHLS Universitas Hospital Service Laboratory Department of Microbiology standard operating procedures (MIC0337).
- Inter-observer variation: To minimise inter-observer variation, all manual microscopy cell counts were conducted by the same technologist, who has proven, longstanding competence in this method. A haematologist with comparable competence in this method then repeated the microscopic cell count on the same counting chamber. The mean of the two cell counts that were generated was entered on the data capture sheet.

Automated method:

- Sample degradation: The researcher was notified immediately when specimens that met the inclusion criteria were received at the Department of Microbiology. Specimens were prepared immediately, to be analysed concomitantly by microscopy and the automated method, so as to avoid discrepancies between the two methods as a result of sample degradation.
- Intra-observer variability: Intra-observer variability was minimised by preparing the samples as per Siemens CSF assay recommendations.
- Inter-observer variability: All samples analysed on the ADVIA2120i were prepared and analysed by the researcher, thereby minimising inter-observer variability. All data was collected by the researcher and recorded on the data collection sheet, thereby minimising inter-observer data collection variation.
- Quality control: To ensure validity, expiry dates on the reagent were confirmed and recorded before use. Quality control of the CSF assay was performed using ADVIA2120i TESTpoint CSF controls (PN T03-4485-01) containing both Level

1 and Level 2 controls. Control materials were assayed as per manufacturer recommendations. If the instrument did not pass its internal quality control checks, study samples were not analysed on the instrument until such issues had been addressed and resolved. The ADVIA2120i CSF assay does not require calibration. Accurate cell counts were derived through correct RBC gain adjustment, which eliminates the risk of calibration errors.

- Accuracy (trueness): Adequate rinsing of the ADVIA2120i when switching from whole blood mode to CSF mode was conducted as per manufacturer recommendations. Doing so ensured acquisition of acceptable background counts, and minimised possible errors in the cell counts obtained. To avoid spuriously low cell counts as a result of the cells in the CSF settling over time and/or adherence of RBCs or PMNs to the tubes, the samples were vortexed for 5 minutes, as per manufacturer recommendations.

4.5. Pilot study

A pilot study was performed in October 2017 pending approval for the study by the Ethics Committee of the University of the Free State. The pilot study consisted of the results generated from the first five study samples received. The results were included in the final results, as no adjustments were required.

4.6. Data analysis

Data was reviewed and processed by the researcher and study leader with the assistance of the Quality Control Office of NHLS Universitas Hospital Service Laboratory. The results of the various assays are described using basic descriptive statistics, with ranges, medians, means, CVs and standard deviations.

Quantitative method comparison was performed using EP Evaluator® software, based on the CLSI EP09 protocol and CLSI proposed standard H26-P2. The quality of the data was assessed by evaluating the result ranges and correlation coefficient (R). An R value of >0.90 was deemed acceptable.

Comparison of the two methods was done using the slopes, intercepts and confidence intervals of regression models (Deming and regular) and percentage bias, as well as the bias plot and Bland-Altman plot. For the precision experiment, simple total precision with means, standard deviations and CVs was calculated according to the CLSI EP15 protocol.

Acceptance criteria:

- Automated assay was accepted if precision was equal to or less than that claimed by the manufacturer (see Figure 1.3) or CV of 18.89% for Level 1 and less than 33.3% for Level 2, as reported by Tanada et al.[12]
- The automated assay was considered to be of acceptable accuracy if slopes were within 0.95 to 1.05 and intercepts between ±1% with Deming or regular regression analysis, provided the 95% confidence intervals for the slopes and intercepts included 1.00 and 0.00 respectively.

Precision of CSF WBC Counts (n=65)

Parameter	Mean	SD	CV
WBC	44.6	5.0	11.2

Precision of CSF RBC Counts (n=65)

Parameter	Mean	SD	CV
RBC	12.9	4.1	31.7

Precision of CSF WBC Differential (n=65)

Parameter	Mean	SD	CV
% Neut	12.5	3.2	25.5
% Lymph	74.9	5.1	6.9
% Mono	8.3	3.2	39.3
% MN	83.2	3.6	4.3
% PMN	16.8	3.6	21.2
# Neut	4.8	1.1	23.6
# Lymph	33.8	4.0	11.7
# Mono	4.1	1.2	30.1
# MN	37.9	4.7	12.4
# PMN	6.6	2.0	29.9

Figure 3: Manufacturer precision claims

5. IMPLEMENTATION OF FINDINGS

By validating the ADVIA2120i for CSF analysis, this study will enable the NHLS Universitas Hospital Service Laboratory to overcome the challenges posed by current staff shortages. Improved turnaround time and precision will ensure that clinicians are provided with the best quality results in a timely manner.

6. TIME SCHEDULE

The study was conducted over a period of 16 months, as indicated by Table 1.2.

Table 1: Time schedule of study

Component of study	Date
Protocol submission to ethics committee	18 August 2017
Protocol approval/amendments	September 2017
Pilot study	May 2018
Data collection	May 2018 to December 2018
Data analysis	December 2018
Writing of article	December 2018 and January 2019
Submission of article	January 2019

7. BUDGET

The reagents and control materials used in the study were funded by Siemens Healthcare (Pty) Ltd. The other materials required, as listed below, were funded by the Department of Haematology and Cell Biology of the University of the Free State.

Table 2: Budget for materials

Material	Price
Pipette	R161.80
Gloves	R53.50
TOTAL	**R215.30**

8. ETHICAL ASPECTS

This study was subject to approval by the Ethics Committee of the University of Free State and the Human Tissue Authority. The study was executed in accordance with the Declaration of Helsinki (1964), as amended in Tokyo (2008).

Permission to perform the study was obtained from the following persons:

- Head: Department of Haematology and Cell Biology, UFS: Prof. MJ Coetzee
- Acting head: Department of Microbiology, UFS: Dr D Goedhals
- Head and business unit manager, NHLS Universitas, Prof. H Pieters

Data that was collected will remain confidential, as all samples were allocated unique study numbers when entered on the data collection sheet. After completion of testing,

the samples were stored according to Department of Microbiology protocols, and discarded accordingly.

Samples used in this study were obtained from routine specimens submitted for CSF analysis. As a result, no consent from individual patients was required. The results of the study has no implication for patient management.

Regardless of the outcome of the results obtained on the ADVIA2120i, the clinical doctors were provided with the results of the manual microscopic cell count, as per routine practice. Should abnormalities have been found in a participant's CSF results, the study team would have ensured that the treating physician was informed and that the participant received appropriate treatment.

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APPENDIX E: DATA COLLECTION SHEET

			MANUAL MICROSCOPY			ADVIA®2120i			
DATE	LABORATORY NUMBER	STUDY NUMBER	PMN: NEUTROPHILS AND MONOCYTES	LYMPHOCYTES	ERYTHROCYTES	IQC P: PASS F:FAIL	PMN: NEUTROPHILS , MONOCYTES & EOSINOPHILS	LYMPHOCYTES	ERYTHROCYTES

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CHAPTER 2: MANUSCRIPT FOR PUBLICATION This chapter will be submitted to the International Journal of Laboratory Haematology (IJLH) for possible publication. The article that was submitted followed the author guidelines of the journal (see Annexure 3). Title Page Running title: Man versus Machine Full title: Validation of the Cerebrospinal Fluid (CSF) Module of the Siemens ADVIA® 2120i for Automated Cell Counts of Cerebrospinal Fluid Authors 1. Esther Kalambi-

Matengu, MBChB 2. Leriska Haupt, MB ChB, MMed (Haem) Department of Haematology and Cell Biology, Faculty of

Health Sciences, University of the Free State, Bloemfontein National Health Laboratory Service and 3. Prof Yacoob Coovadia, MB ChB, FF Path (Microbiology) Department of Medical Microbiology, Faculty of Health Sciences, University of the Free State, Bloemfontein National Health Laboratory Service Abstract Introduction: Haematology analysers routinely utilised for whole blood specimens are now equipped with cerebrospinal fluid (CSF) and/or body fluid modules. These automated cell counters are steadily replacing manual microscopy for CSF cell counts. This change is attributed to the automated counters' higher throughput, improved turnaround times, superior precision and higher accuracy, compared to manual microscopy. Methods: In total, 46 consecutive routine CSF samples were analysed using the ADVIA2120i CSF Module to obtain a red cell count (RCC), white cell count (WCC) and a four-part WCC differential. Results were compared against the mean of two CSF cell counts performed by manual microscopy using the Improved Neubauer chamber haemocytometer. Quantitative method comparison was performed using EP Evaluator® software Version 8.0.0 and Microsoft Excel®. The ADVIA2120i was assessed further for precision, linearity and carryover. Results: The ADVIA2120i meets all the acceptable claims in terms of precision and demonstrated acceptable accuracy for RCC and WCC, specifically including lymphocytes and polymorphonuclear (PMN) counts, when compared with the reference method. Linearity results show that the ADVIA2120i produced results that were proportional after serial dilutions. All parameters were deemed to have acceptable carryover. Conclusions: The ADVIA2120i offers rapid, precise and accurate RCC and WCC for both normal and abnormal cell counts. Our results show that the ADVIA2120i is a suitable alternative to manual microscopy for CSF cell counts and is deemed fit for purpose. Keywords: ADVIA2120i, Cerebrospinal fluid, manual microscopy, red cell counts, white cell counts 2.1 INTRODUCTION Analysis of cerebrospinal fluid (CSF) cell counts provides critical information required for the diagnosis of various inflammatory and infective conditions, as well as for monitoring the response to therapeutic measures.1-3 The value of CSF cell counts is underscored further by its usefulness for diagnosing central nervous system (CNS) infiltration by primary neural and extraneural malignancies.4 As an adjunct to the other, albeit more sophisticated and more expensive, diagnostic modalities utilised for CSF analysis, such as flow cytometry, CSF cytomorphology, immunohistochemistry, CellSearch, polymerase chain reaction (PCR), microRNA and CSF biomarkers; simple and relatively inexpensive CFS cell counts enable early detection of CNS malignant infiltration and, consequently, earlier management.5 Normal CSF is acellular, however, up to five WCC x106/L and five RCC x106/L can occur in normal adults, without it being considered pathological. The WCC differential in CSF normally comprises 70% lymphocytes and 30% monocytes. More than five PMN x106/L, as seen with, inter alia, infection, inflammation or leukaemic infiltration, is always regarded as abnormal in adults.2 An increase in the RCC count is indicative of the presence of subarachnoid haemorrhage, intracerebral haemorrhage or a traumatic spinal tap.3 For decades, and even currently, manual microscopy has been regarded as the gold standard for CSF cell counts.6 However, this conventional method is currently falling out of favour, as a result of its shortcomings, namely, increased turnaround times, labour intensiveness, technique dependence, high inaccuracy, inter-observer variability, requirement of 24-hour availability of experienced microbiology staff, and inability to differentiate mononuclear cells into lymphocytes and monocytes.7 To overcome these shortcomings, there has been a paradigm shift, towards automation of CSF cell counts in most modern laboratories in first-world countries. Although not currently clinically utilised for CSF and other body fluids analysis in South Africa, the majority of new automated haematology analysers are equipped with CSF and/or body fluids modules, and their use is steadily gaining favour. The Siemens ADVIA120 and 2120i (Siemens Healthcare Diagnostics, Tarrytown, NY, USA), Sysmex XE- 5000 (Sysmex Corporation, Kobe, Japan), Abbott CELL-DYN Sapphire (Abbott diagnostics, Abbott Park, IL, USA) and Beckman Coulter LH750 (Beckman Coulter, Miami, FL, USA) are among the instruments currently equipped and validated for automated body fluid cell counts.6,8,9 The automated method has the advantages of being faster, having improved precision, and being easier to use than the traditional manual microscopy method.7 The accuracy and high precision of the automated method is a result of the aspiration of larger volumes by the analysers, compared to the volumes required for manual microscopic counts.6 A disadvantage of the automated method is a loss of precision at lower cell counts. This is most likely consequent to the automated method's higher limits of quantification, particularly with the Sysmex XE-5000. Another shortcoming is the inability of the analysers to detect neoplastic cells, which are erroneously classified as lymphocytes.6 Consequently, supplementary cytomorphology analysis should be mandatory for all oncology specimens.10 In this study, we evaluated the CSF Module of the ADVIA2120i for CSF cell counts (RCC, WCC and differential) and compared them to the reference method (PMN, lymph and RCC manual microscopic counts). The ADVIA2120i was also assessed for precision, linearity, accuracy and carryover. 2.2 MATERIALS AND METHODS We conducted a single-centre study at the National Health Laboratory Service (NHLS) Universitas

Hospital Service Laboratory. Approval for the study was obtained from the Health Sciences Research Ethics Committee [HSREC117/2017 (UFS)- HSD2017/1179]. 2.3 STUDY SAMPLES In total, 46 consecutive CSF samples that had been received for routine CSF analysis at the NHLS Universitas Hospital Service Laboratory were included. Only samples with at least 600 µL of CSF remaining after all required microbiological and/or cytological tests had been performed were included. Samples were excluded as per rejection criteria of the standard operating procedures of the Microbiology Department for the reference method.11 2.4 REFERENCE MANUAL MICROSCOPY METHOD Manual CSF microscopic cell counts were conducted as per standard operating procedure of the Microbiology Department of the NHLS Universitas Service Laboratory.11 The Improved Neubauer counting chamber (Neubauer Improved, Marienfeld, Germany) was used for the RCC and WCC. The WCC was differentiated further into PMN and mononuclear cells. Mononuclear cells could not be differentiated further into lymphocytes or monocytes, and were, therefore, reported as lymphocytes. Cell counting was performed using 30 µL of CSF. The initial cell count was performed by a senior microbiology technologist. The cell counts were then verified by a haematologist. A mean of the two results was used, which was then compared to the automated ADVIA2120i cell counts. The cell count obtained was then converted to cells/µL using the following equation: Dilution x Depth of counting chamber xNumber of cells counted PMN

or lymph = Number of squares counted Depth of counting chamber xNumber of cells Erythrocytes = Number of squares counted 2.5
ADVIA2120I CSF MODULE The ADVIA2120i CSF module is a semi-automated method, specifically for CSF samples.12,13 Calibration is not required for this CSF module,12 and internal quality control was conducted once daily before the first sample was analysed. For analysis of CSF with the ADVIA2120i, 300 µL of thoroughly mixed CSF sample was added to 300 µL of CSF Reagent [ADVIA2120i CSF (Prod. No.T01-4610-01)] in a labelled test tube. The prepared mixture was mixed by gently inverting the tubes five times and leaving them at room temperature for a minimum of four minutes and a maximum of four hours.12 Once in CSF mode, and once acceptable background counts had been obtained, CSF samples were aspirated directly

into the ADVIA2120i system using the manual open- tube mode. The cells were detected and enumerated based on light scatter and absorbance measurements. Scatter versus scatter and scatter versus absorbance cytograms were generated for each sample and results were calculated automatically. Reportable parameters include WCC, RCC and a four-part differential count, which included the absolute and percentage values for neutrophils, lymphocytes, monocytes and eosinophils. 2.6 PERFORMANCE PARAMETERS 2.6.1 Precision As per the Clinical and Laboratory Standards Institute (CLSI) EP15A3E guideline, precision was determined by running five replicates of the low and high controls per day for five days; values were, thus, used for precision evaluation. Simple total precision with means, standard deviations and coefficient of variation (CV) were calculated. Precision was accepted if it was equal to or less than that claimed by the manufacturer or a CV less than 18.89% for Level 1 and less than 33.3% for Level 2, as reported by Tanada et al.12 2.6.2 Accuracy Accuracy of the ADVIA2120i CSF method was evaluated by regression analysis (Deming and regular), comparing 46 automated results for absolute CSF RCC, CSF PMN and CSF lymphocytes with the mean reference CSF manual counts. Comparison was conducted using the slopes, intercepts and confidence intervals, as well as percentage bias, bias plots and Bland-Altman plots. The accuracy of the automated method was deemed acceptable if the slopes were within 0.95 to 1.05 and intercepts between ±1% of Deming or regular regression analysis, provided the 95% confidence intervals for the slopes and intercepts included 1.00 and 0.00

respectively. This was conducted as per the CLSI EP09-A2-IR guideline. The quality of the data was assessed by evaluating the result ranges and correlation coefficient. A correlation coefficient value of >0.90 was deemed acceptable.12 2.6.3 Linearity Cell counts for the high control (ADVIA2120i TESTpoint CSF Controls (PN T03-4485- 01)) were utilised to demonstrate linearity as per the standard operating procedure of the NHLS (Protocol for Instrument Evaluation: GPQ0007). The samples were serially diluted with phosphate-buffered saline to relevant dilutions of 3.13%,

6.25%, 12.50%, 25%, 50% and 100%. 2.6.4 Carryover The high control (ADVIA2120i TESTpoint CSF Controls (PN T034485-01)) was measured in triplicate (H1, H2, H3), followed by three consecutive measurements of saline/blank (L1, L2, L3). Carryover was calculated according to the International Council for Standardization in Haematology (

ICSH): (L1-L3)/(H3-L3)] x100%.14 A value of less than one was deemed acceptable.12 2.7 STATISTICAL ANALYSIS Statistical analyses were performed using the EP Evaluator Version 8. 0.0 and Microsoft Excel®. 2.8 RESULTS Table 2.1: Descriptive statistics CSF Parameter Statistical Parameter ADVIA2120i Manual Microscopy RCC Range (mean) 0 – 115 752 (6 038.80) 0 - 10 000 (977.60) Standard deviation 22 225.54 2 820. 54 PMN Range (mean) 0 – 77 506 (3 388.08) 0- 575 (39.43) Standard deviation 13 828.12 118.19 Lymphocytes Range (mean) 0 – 8 227 (387.36) 0 – 743 (54.54) Standard deviation 1 394.49 131.58 2.8.1 Precision Simple precision for WCC and RCC were calculated, and revealed the following: WCC: Within-run precision for WCC had a mean CV of 17.6% for the normal control and a mean CV of 9.8 % for the high control. Between-run precision for the normal control revealed a mean CV of 22.2%, and 11.1% for the high control. RCC: Within-run precision revealed a mean CV of 17.4 % for the normal control and a mean CV of 6.6%. Between-run precision for the normal control revealed a mean CV of 30.3%, and 10.8% for the high control. 2.8.2 Accuracy The method comparison results shown in Table 2 show acceptable agreement for all three parameters. Table 2.2: Summary of the Deming and regular regression for method comparison of CSF RCC, PMN and lymphocytes; ADVIA2120 vs manual microscopy (N=46) 2.8.3 Linearity The ADVIA2120i CSF module displayed acceptable linearity throughout a broad range of clinically significant values (Figure 3.1). Red Cell Count (x 106 cells/ L) White Cell Count (x 106 cells/ L) 200 100 150 R^2 = 0.9983 80 R^2 = 0.995 RCC 100 WCC 60 40 50 20 0 0 0% 50% 100% 150% 0% 50% 100% 150% Dilution Dilution PMN (x 106 cells/L) Lymphocytes (x 106 cells/L) 50 PMN 40 R^2 = 0.9802 Lymphocytes 40 30 R^2 = 0.997 30 20 20 10 10 0 0 0% 50% 100% 150% 0% 50% 100% 150% Dilution Dilution Each point represents the mean of a duplicate sample. Analytical ranges: RCC 4.5 – 166 X 106 cells/L; WCC 2.5 – 89 X 106 cells/ L; PMN 1 – 42 X 106 cells/L; lymphocytes 1 – 36 X 106 cells/L Figure 2.1: Linearity graphs showing absolute counts for WCC, RCC, PMN and lymphocytes 2.8.4 Carryover All parameters were deemed to have acceptable carryover (Table 3.3). Table 2.3: Summary of carryover experiment results Parameter RCC PMN Lymphocytes Carryover 0.37 0.00 0.00 2.9 DISCUSSION This was the first evaluation of the ADVIA2120i CSF module for CSF cell counts at our centre, and in South Africa. In the present study, we found the ADVIA2120i CSF module easy to operate; it required no prior calibration and internal quality control procedures could be performed effortlessly. The smooth transition from whole blood to the CSF module, rapid acquisition of acceptable background counts, and preparation of the sample – it took less than four minutes – are additional features in favour of the ADVIA2120I instrument. Furthermore, the virtually immediately available raw data printout provides more rapid results than the relatively longer turnaround times of the manual counting method. The ADVIA2120i showed exceptional performance for RCC and PMN counts, with the RCC obtaining a slope of 1.000 on Deming regression. The lymphocytes, however, only met acceptance criteria on the regular regression regarding the slope, with an overall positive bias of 7.5, which demonstrates the increased sensitivity of the automated method as a plausible explanation. The increased sensitivity is most likely due to the higher volumes (300 µL) aspirated with the automated method, compared to the 30 µL of the manual method.11,12 The instrument also demonstrated acceptable precision, carryover and linearity for all three parameters. Automated CSF cell counts, as mentioned, are not limited to the ADVIA2120i platform; various other manufacturers also offer CSF and BF modules. In this regard, it might be pertinent to point out that the ADVIA2120i does not come equipped with a morphology flagging function within its CSF module, whilst the Sysmex XE-5000, Sysmex XN-series and the Abbott CELL-DYN3500 have single and 3-flagging functions.9,15 However, although equipped with flagging functions, the performance (with regard to CSF analysis) in clinical diagnostics for oncology samples has been disappointing.8,9,16 The ADVIA2120i does provide a flagging function for Cryptococcus infection in CSF.6 Although not specifically evaluated in this study, there was one study sample with positive Cryptococcus latex agglutination tests, which were not flagged by the instrument, nor by the manual microscopy method. In our setting of a relatively high Cryptococcus meningitis prevalence, this could be a valuable topic for a future study.17 A practical drawback of the ADVIA2120i is the volume of CSF required (300 µL) for automated CSF cell counts. This may have resulted in the exclusion of many patient samples during our evaluation, due to insufficient sample volumes as specified in our inclusion criteria. To implement the automated method in clinical diagnostics, would require informing clinicians about minimum sample volume requirements. Although the aim of the study was to evaluate the performance characteristics of the ADVIA2120i CSF module, we foresee a potential stumbling block for implementation of this platform in clinical diagnostics. The ADVIA2120i acquires higher cell counts than the manual method, which means currently used manual reference ranges do not transcend to the automated method. Very few studies are available on reference ranges for automated methods. Two separate studies, by De Jonge et al. and Sandhaus et al, assessed the establishment of new reference ranges for the Sysmex XE-5000.16,18 Using a cohort of 87 normal adult patients undergoing spinal anaesthesia, De Jonge et al. report normal reference ranges for RCC as 0 x 106/L, MN as <7 x 106/L and PMN as <3 x 106/L.18 Bremell et al. calculated new ADVIA2120i- specific reference ranges as CLSI C28-A3 guidelines. The reference ranges obtained were WCC as <5 x 106/L, lymphocytes as <4 x 106/L, PMN as <3 x 106 and monocytes <3 x 106/L.19 Thus, at present, there is no uniformity for CSF cell count reference ranges between haematology analysers. Multi-institutional studies with clear sample inclusion criteria are needed to compare the different haematology analysers currently approved for CSF analysis, in order to standardise reference ranges.16 A possible limitation of our study is that it did not specifically evaluate the effect of high RCC or traumatic spinal taps on the WCC. This has previously been reported as a clear limitation of the ADVIA120.10 RCC more than 1 500 x 106/L might falsely elevate white cell and neutrophil counts; the same authors advise diluting the sample to a maximum of 1:10.10 This is not a recommendation in the ADVIA2120i manual, but as four of our study samples had exceptionally high RCC (>10 000 x 106/L), it could have been of value to determine whether dilution of these samples affected the white cell and neutrophil counts. Another limitation of our study is that it did not evaluate unprepared sample stability over time, as very little recent data is available on this.6,20 However, the automated method is probably superior to the manual method in this regard, as samples are analysed in a fraction of the time, which may result in less sample degradation. Although cost comparisons between the automated and manual methods were not the focus of this study, this will need to be evaluated by future studies. Such an analysis will have to take into account the labour and expertise required for each of the methods, the time needed to complete analysis, reagent costs and availability of after- hours expertise. These factors would also have to be seen in conjunction with the lower overall patient medical costs that accompany the rapid, accurate and precise results offered by the automated method. 2.10 CONCLUSION The results of our study indicate that, when compared with the reference method, the ADVIA2120i meets all acceptable claims in terms of precision and demonstrated acceptable accuracy. On performance characteristic alone, the CSF module can be declared fit for purpose. Implementation of this platform at the NHLS Universitas Hospital Service Laboratory will only be considered after evaluation of method-specific reference ranges in the clinical setting, and formal cost analysis. 2.11 ACKNOWLEDGEMENTS The authors acknowledge the immense help received from the Microbiology Department, especially from Miss Pelonemi Leeuw, who assisted in the manual CSF cell counts. We are grateful to Dr Anneke van Marle for taking the time to verify the manual CSF cell counts. We acknowledge Mrs Ina Freitag and Mr Lance

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CHAPTER 1: LITERATURE REVIEW 1.1 CEREBROSPINAL FLUID CSF is a non-viscous, clear fluid that surrounds the brain and spinal cord. It functions predominantly as a shock absorber between the brain and the skull, to protect the CNS. Suspension of the brain in CSF, furthermore, provides buoyancy, which lowers the brain's weight from 1 400–1 500 grams to 25–50 grams.1 Buoyancy prevents the base of the brain from being subjected to excessive pressure, which may impede blood supply.2 In addition, CSF also circulates key nutrients (glucose, proteins, lipids and electrolytes) that are required by neurons for basic function.3 Waste products diffuse into the CSF and are removed as CSF is resorbed through arachnoid granulations into venous circulation.3,4 In normal adults, CSF is produced primarily by the choroid plexus, which lines the lateral , third, and fourth ventricles of the brain.4 Extrachoroidal production of CSF from extracellular fluid, cerebral capillaries and ependymal epithelium, constitutes the minor source. The rate of CSF production is equal to the rate of CSF reabsorption, maintaining volumes between 125 mL and 150 mL in normal adults.5 This well- regulated equilibrium between production and reabsorption rates maintains the normal supine CSF pressure of 8 mmHg to 15 mmHg, and ± 20 mmHg when erect.3 Once secreted, CSF is distributed rostrally over the convexities of the brain and to the villous sites of absorption, and caudally to the spinal subarachnoid space. 4 In the cranial subarachnoid space, CSF flows towards the arachnoid villi for reabsorption. The reabsorbed CSF is transported to the kidneys and liver, where it is filtered and excreted.5 In the spinal subarachnoid space, CSF is absorbed by the epidural venous plexus and spinal nerve sheaths, and drained into the lymphatic system. 4 1.2 LUMBAR PUNCTURE A lumbar puncture, also known as a spinal tap, is an invasive, sterile procedure that involves insertion of a hollow needle into the subarachnoid space of the lumbar spine. It can be performed for both diagnostic and therapeutic reasons. The procedure should be carried out by a competent clinician, to minimise potential complications. Postdural puncture headache is the most common complication encountered, occurring in 30% of patients.6 Other complications include lower back pain, cranial neuropathies , nerve root irritation, infections, brain tissue herniation and spinal haematomas.7 An appropriate understanding of the contraindications, the anatomy and sterile technique is, thus, essential. Complications, though rare, are potentially life-threatening. It is due to these complications and the difficulties in acquiring CSF

samples that these specimens should be regarded as precious samples.8,9 Nevertheless, lumbar punctures are routinely performed in clinical practice, to assess CSF.10 The chemical analysis and cell counts in these CSF samples provides essential information for the diagnosis of various CNS diseases.8 CSF analysis is indispensable as a diagnostic and therapeutic tool, as seen in Table 2.1. Table 1.1: indications for a lumbar puncture, adapted from Ellenby et al.9

Indications for lumbar puncture For diagnostic purposes Viral, bacterial or fungal meningitis Encephalitis Inflammatory conditions: Multiple sclerosis and Guillain-Barré syndrome CNS malignancy diagnosis and monitoring For therapeutic purposes Administration of anaesthetic drugs Lower- body analgesia Antibiotic administration for ventriculitis Intrathecal chemotherapy for CNS leukaemia and lymphomas 1.3 CEREBROSPINAL FLUID IN HAEMATOLOGY Malignant CNS infiltration is a potentially fatal complication that is a result of haematological and non-haematological malignancies. These malignancies may be primary neural or extraneural in origin.11 Primary neural malignancies include primary CNS lymphoma (PCNSL) and solid tumours, namely, germ cell tumours, ependymoma, medulloblastoma and neuroectodermal tumors.12,13 PCNSL is an aggressive, acquired immunodeficiency syndrome (AIDS)-defining nonHodgkin lymphoma that accounts for 2.2% of all CNS tumours. PCNSL presents a diagnostic challenge, as it involves critical areas within the CNS, and the diagnosis relies on histopathology of brain biopsies from these sites. Obtaining biopsy specimens may be complicated by bleeding, with devastating consequences, and biopsies often yield limited tissue. These diagnostic limitations have led to the use of less invasive diagnostic approaches to assess malignant cells in CSF.14 Metastases to the CNS from other primary sites constitute extraneural CNS malignancies, which include haematological malignancies, such as leukaemia and lymphoma and a myriad of non-haematological solid tumours. In the era of effective treatment protocols and improved imaging, the incidence of CNS metastases has risen, consequent to prolonged survival and early detection respectively.15 Non-haematological solid tumours account for 67-80% of all CNS metastases. Tumors with a predilection for CNS infiltration include carcinomas of the lung (50%), breast (20%), melanoma (10%) and colon (5%). Up to 15% of CNS metastases may present with no known primary.16 Haematological malignancies with a high propensity for the CNS include aggressive non-Hodgkin lym phomas , such as intravascular diffuse large B cell lymphoma (DLBCL), acute lym phoblastic leukaemia /lymphoma (ALL), the blastoid variant of mantle cell lymphoma, and Burkitt lymphoma. 15 The most common site for CNS metastases is the leptomeninges.15 Malignant cells invade the CSF through the arterial circulation, associated lymphatics, direct extension from the tumour, or along the endoneural and perineural routes, to eventually reach the subarachnoid space.12,13 Once in the CSF, malignant cells disseminate along the meningeal surface and are deposited onto the leptomeninges.15 The incidence of CNS infiltration is dependent on the site of the primary malignancy. Non-haematological malignancies with a high propensity for CNS infiltration (67-80% of all metastases) include carcinomas of the lung (50%) and breast (20%); melanoma (10%), and 15% presenting with no known primary.16 CNS infiltration in patients with leukaemia and lymphoma confers a poor prognosis, with a median survival of three months. 11,17 One of the reasons for the poor prognosis is the development of neoplastic meningitis, a rapidly progressive and fatal condition.13 Signs and symptoms are dependent on the location of the disease within the CNS, and include headache, cranial nerve palsies, radicular pain, neck and back pain, altered level of consciousness and focal weakness or loss of sensation; seizures can occur, but are rare.18,19 ALL accounts for approximately 25% of all childhood malignancies. Although CNS- directed therapies form part of the standard chemotherapeutic regimens, CNS relapse is still observed in approximately 2–8% of leukaemia patients. The CNS is the most

common site for relapse consequent to the blood-brain barrier.20 Although not as well understood as the bone marrow microenviroment, the CNS leukaemia niche provides a microenviroment suitable for CNS relapse. Once the leukaemic cells breach the blood-brain barrier, they may escape the effects of chemotherapy (poor penetration, resulting in inadequate therapeutic levels) and immune surveillance. If leukaemic cells persist in the CNS, relapse could also

occur.20 The risk factors for CNS involvement in ALLare listed in Table 2.3. Table 1.2: Risk factors for CNS involvement in ALL17,21,22 Tumour characteristics T-cell phenotype in paediatric ALL and a mature B cell phenotype in adult ALL Hyperleucocytosis Highproliferative index (>14% lymphoblasts in cell cycle [S or G2/M phase]) Blast expression of CD56 Biochemical findings Elevated ?2-microgobulin levels Lactate dehydrogenase levels >600 U/L Cytogenetics Philadelphia (Ph) chromosome positivity Complex karyotype (>5 chromosomal abnormalities) t(8:14) t(4:11) Low hypodiploidy/near triploidy Based on cytomorphological identification of leukaemic blasts and the absolute WCC, patients can be stratified according to risk for CNS recurrence. 21 Table 1.3: Classification of CNS involvement23 CNS1 No leukaemic blasts CNS2 CSF: <5 WCC/µL with leukaemic blasts CNS3 CSF: >5 WCC/µL with leukaemic blasts Patients with CNS2 and CNS3 are at increased risk of CNS relapse. 22 Most cases of non-Hod g kin l y m phoma with CNS involvement (approximately 4.2%) occur post chemotherapy with disease relapse. Leptomeningeal spread and, to a lesser extent, haematogenous spread to the cerebral parenchyma, are the most common means of metastases. In the cerebral parenchyma, lymphoma cells form nodular deposits within brain parenchyma via the Virchow-Robin spaces.24 These spaces are continuations of the subarachnoid space that accompany vessels entering the brain parenchyma.25 High expression of CD56, a neural cell adhesion molecule, by lymphoma cells has been implicated in the spread of lymphoma cells to the CNS. It is postulated that the CD56 molecule enables the interaction between lymphoma cells and cells in the CNS.26 The specific cellular and molecular pathways responsible for mobilisation of lymphoma cells to the CNS, however, remain to be fully elucidated. In aggressive non-Hodgkin lymphomas, risk factors for CNS infiltration include the following:17,22 ? Concordant bone marrow involvement; ? Involvement of the kidneys, adrenals, breast, paranasal sinus, testes or orbital cavities; ? Raised lactate dehydrogenase levels and the involvement of multiple extranodal sites; ? Double expression of dominant acting oncogenes Myc and B-cell lymphoma 2 (BCL-2) ; and ? Advanced stage with a high International Pro g nostic Index (IPI) score.

The risk for lymphoma CNS involvement can be predicted using the Central Nervous System International Prognostic Index (CNS-IPI) tool. It was originally developed by Schmitz et al. to predictthe risk of CNS relapse/progression in patients with DLBCL treated with R-CHOP. R-CHOP, comprising rituximab, cyclophosphamide, doxorubicin , vincristine and prednisolone, is the standard chemotherapeutic regimen used for most of the aggressive non-Hodgkin B-cell lymphomas. 27 In addition to the standard variables used for the IPI, the CNS-IPI assesses involvement of the adrenal glands and kidneys. Patients are then classified into different risk categories. Patients with DLBCL that fall into the low- and intermediate-risk groups and have a CNS relapse risk <5%, whereas those in the high-risk group have a >10% risk of CNS relapse. 27 Table 1.4: IPI for non-Hodgkin lymphomas14 Variable Risk group: (Score) [% 5year overall survival] Age >60 years Low: (0–1) [73%] LDH Lowintermediate (2) [51%]Eastern Cooperative Oncolo g y Group performance status >1High-intermediate: (3) [43%] Ann- Arbor Stage III or IV Hi g h: (4 or 5) [26%] Involvement of more than one extranodal site

Furthermore, as an immune-privileged site (sites with limited regeneration capacity that are adapted to restricting potentially harmful immunemediated inflammation),28 the CNS is protected by a blood-brain barrier. The blood-brain barrier is formed from ti g ht j unctions between endothelial cells and is the main

reason for chemotherapy failure in the case of CNS malignancies.29 This failure is due to
failure of most chemotherapeutic agents to reach desirable therapeutic levels within the
CNS. To overcome this barrier, high-dose chemotherapy, and intrathecal and intra-arterial routes for chemotherapy administration are advocated. Intrathecal methotrexate (12– 15 mg flat dose) is the preferred chemotherapeutic

agent, administered together with standard chemotherapy.14 Cranial radiation, intrathecal cytarabine and systemic chemotherapy that crosses the blood-brain barrier, such as methotrexate, cytarabine or ifosfamide, may serve as alternatives for prophylaxis.29 1.4 DIAGNOSTIC TOOLS FOR CEREBROSPINAL
FLUID ANALYSIS The blood-brain barrier prevents the release of tumour-secreted markers into the blood stream, thus, making blood analysis inferior to CSF analysis for the diagnosis of CNS malignancies.12 The current, most widely utilised tools for assessing CNS infiltration by malignant cells include clinical neurological examination, neuroimaging of the neuroaxis, and CSF cytomorphology. These three modalities can be used concurrently. Clinical
signs and sym ptoms of CNS infiltration may present late in the disease course, and
are not specific for CNS infiltration.15 Neuroima g in g techniq ues , such as ma g
netic resonance imaging, are sensitive, but have low specificity, depending on the location and type of malignancy.15 CSF cytomorphology and a few of the current methodologies used for CSF analysis will be discussed below. 1.4.1 Cerebrospinal fluid cytomorphology This is considered the current gold standard
for diagnosing primary and metastatic malignancies of the CNS.13 It involves centrifugation of the specimen, staining with MayGrunwald-Giemsa stain and microscopic visualisation for the presence of malignant cells.13 Although highly specific (>95%), the test lacks sensitivity (<50%), and false negatives remain
a concern.30 This method is also labour-intensive and requires experienced staff
to execute.31 Sensitivity can be improved by obtaining sample volumes of at least
10.5 mL, performing a second collection if the first is negative, obtaining CSF from
as close to the tumour as possible, and timeous sample processing.13 In patients
with CNS malignancies, up to 45% will have a negative cytological examination with
the first lumbar puncture. With a second tap, the yield is increased to 80%, but repeated lumbar punctures after the first two are not beneficial.15 Identification of cells on microscopy does not differentiate between benign and malignant cells, thus, combining other modalities, such as immunohistochemistry, flow cytometry and polymerase chain reaction (PCR), is required to identify monoclonal and neoplastic cells.15 1.4.2 Flow c y tometr y
Flow c y tometr y is a laser-based technique that categorises cells based on their size,
complexity and fluorochrome -labelled cell surface markers. It currently serves
as a useful adjunct to CSF cytomorphology for the diagnosis and staging of haematological malignancies. Flow cytometry enables rapid acquisition and processing of diagnostic data.13 It has a higher sensitivity, requiring as low as
0.01% malignant cells (of total leucocytes) for detecting an abnormal cell population, compared to the 5% tumour cells required for cytomorphology.13,21 Up to
80% of cases with CSF infiltration by lymphoma cells are detected on the first CSF sample.15 The higher sensitivity makes flow cytometry valuable for detecting
occult or subclinical CNS involvement during maintenance therapy and clinical follow-
up, especially when cytomorphology is negative. 11 Identifying abnormal cells
within the CSF that have an immunophenotype similar to that of the malignant population in the peripheral blood or bone marrow aspirate samples, supports
the diagnosis of concurrent CNS infiltration with the systemic lymphoma diagnosis.15 Flow cytometry can, furthermore, assess clonality of B- cells,
based on the cells expressing either lambda or kappa surface light chains.32 Immunophenotyping helps to distinguish indolent non-Hodgkin lymphomas
from normal lymphocytes, and to detect abnormal patterns of antigen expression on neoplastic cells. This is useful for both diagnosis and assessment of therapeutic response.33 A well accepted limitation of CSF flow cytometry is the possible diagnostic uncertainty accompanying traumatic lumbar punctures, where CSF is

contaminated with peripheral blood. In this setting, false positives due to contamination of the CSF by peripheral lymphoma cells can complicate staging.15 1.4.3 Immunocytochemistry

Immunocytochemistry makes use of peroxidase or cytokeratin-labelled monoclonal antibodies to detect the expression of antigens on paraffinembedded cells. This modality can be applied to the CSF cytospin.13,34 CSF cytospin involves gentle and controlled centrifugation of CSF to produce a monolayer of preserved cells. The cells are flattened onto a slide, which enhances the visibility of the nuclear material.34 Immunohistochemistry has a sensitivity of 89–95% and a specificity of 89 –100% for the detection of leptomeningeal haematological metastases.32 At lower cell counts, immunocytochemistry should be used in conjunction with cytomorphology to improve sensitivity and to distinguish between reactive and neoplastic lymphocytes in CSF. 15 Detecting high-density antigens with immunocytochemistry yields similar results to flow cytometry, however, when antigens are ex pressed at low density, immunocytochemistry may be more reliable. 32 Since the advent of flow cytometry, using immunocytochemistry to detect haematological malignancies in the CSF has declined. It is, however, still very useful for detecting leptomeningeal metastases of solid tumours, in combination with cytomorphology.32 1.4.4 CellSearch CellSearch is a technology approved by the United States' Food and Drug Administration, which is currently utilised to follow up patients with metastatic epithelial cell adhesion molecule (ECAM) expressing tumours, such as breast, prostate, colorectal and lung carcinomas.35 It is a semi- automated cell analysis method that enumerates circulating tumour cells present in 7.5 mL whole blood. Ferrofluid nanoparticles, with antibodies attached, then bind to ECAM, after which the circulating tumour cells are magnetically separated from the rest of the cells.Cytokeratin monoclonal antibodies , which are s pecific to epithelial cells, are attached to a magnetic plate and CD45 is added to identify any leukocytes that may have contaminated the sample. 4', 6' diamino-2-phenylindole (DAPI) stain is also utilised to hi g hli g ht the nuclei of both circulating tumour cells and leukocytes. 35,36 Compared to cytomorphology, CellSearch technology has better sensitivity and a specificity of 90%.35 Growing interest in its use for detecting CSF malignancies has led to research in this field, and further publications are eagerly awaited.12 1.4.5 Polymerase chain reaction Cytomorphology may not be able to distinguish between reactive lymphocytes and malignant cells. Therefore, a definitive diagnosis requires evaluation of clonality.37 CSF analysis with PCR makes use of primers to amplify specific tumour cell-derived deoxyribonucleic acid (DNA).32 In B- cell malignancies, PCR analysis amplifies the immunoglobulin heav y chain g ene in the third complementarity determining region (CDR3). 32 The presence of a clonally rearranged CDR3 is found in 80–95% of B-cell lymphomas, and is the molecular hallmark of malignant B-lymphocytes. In the peripheral blood and bone marrow aspirate samples, this is the preferred method for diagnosis, assessment of treatment efficacy and detection of minimal residual disease. 15,32 Galoin et al. utilised semi-nested PCR to carry out multiple runs on a single CSF sample to detect rearranged immunoglubulin heavy chain (IgH) genes in a clonal B- cell population. The primers used were specific to CDR2 and CDR3 regions of the heavy chain variable regions. Although multiple runs are required, PCR was able to detect clonal populations in CSF samples, thus, making it a more sensitive diagnostic tool for leptomeningeal metastases than cytomorphology. Galoin et al., thus, proved that assessment of IgH gene rearrangement, when used in conjunction with cytology and immunocytochemistry, provided more accurate results than cytomorphology alone.37 Reverse-transcri ption polymerase chain reaction (RT-PCR) enables the amplification of small amounts of ribonucleic acid (RNA) into larger amounts of complementary DNA, thus, requiring only limited amounts of neoplastic cells to be present in CSF. RT- PCR, however, requires appropriate primers for the type of RNA being assayed, which limits its use as a

screening tool. RT- PCR that uses probes specifically for clonal immunoglobulin gene rearrangements, is able to distinguish between polyclonal reactive and monoclonal neoplastic cells.17 1.4.6 Cerebrospinal fluid microRNA MicroRNAs (miRNAs) are non-

coding, small regulatory RNA molecules that are 19 to 25 nucleotides long. They inhibit gene expression by preventing translation and promoting degradation of target messenger RNA. They can function as tumour suppressors or oncogenes, and play critical roles in apoptosis, proliferation and cell differentiation.12,15 miRNAs are deregulated in malignancies and have recently been utilised as diagnostic and prognostic markers.12 miRNAs are stable in body fluids, including CSF. Their analysis provides a non-invasive diagnostic alternative to brain biopsies for patients with suspected CNS infiltration.12 Detection of CSF miRNA by means of RT- PCR has been shown to have diagnostic value for diagnosing PCNSL with overexpression of miR-21, miR-19 and miR-92a. Using this modality, Baraniskin et al. were able to distinguish between patients with PCNSL and patients with other neurologic disorders with 95.7% sensitivity and 96.7% specificity.38 1.4.7 Cerebrospinal spinal biomarker Proteonomic analysis of CSF has led to the discovery of several protein markers within the CSF, of which expression is upregulated in CNS lymphomas. These protein biomarkers include antithrombin chemokine (C-X-C motif) ligand 13 (CXCL 13), C-X- C chemokine receptor type 5 (CXCR5), β2microglobulin and soluble IL-2 receptor (sIL-2R). 17 Antithrombin is a serine protease inhibitor that has been linked to neovascularisation in CNS lymphomas.12 Increased levels (>1.2 g/ml) are suggestive of CNS lymphoma and are associated with poorer chemotherapy responses and lower overall survival rates .12 Levels higher than 1.2 g/mL detect a CNS lymphoma with a sensitivity of 75% and a specificity 98.7%.12 CXCL13 is chemokine secreted by follicular dendritic cells in the lymph node germinal centre that mediates chemotaxis of B- cells to the germinal centre through its cognate receptor CXCR5.39 Rubenstein et al. found elevated levels of CXCL13 in CSF of CNS lymphoma patients compared to control patients.12 Elevated levels of the chemokine receptor CXCR5, and its ligand CXCL13, are responsible for lymphoma tropism to the CNS and are reflective of an increased tumour burden.17 β2-microglobulin is a cell surface protein associated with the human leucocyte antigen (HLA) class I. It is expressed on all nucleated cells and its elevation is reflective of increased cell turnover. Elevated CSF β2-microglobulin levels are associated with CNS involvement by leukaemia or lymphoma, and were found to be elevated in up to 50% of patients with these disorders.15 sIL2R is a cytokine receptor released from activated T-cells. It is reflective of immune activity and elevated levels in blood are an important marker of acute lymphoblastic leukaemia/lymphoma.40 In CSF, elevated levels (>10 U/mL) of soluble IL-2 receptor, when used in conjunction with positive cytomorphology and elevated CSF WCC, are suggestive of CNS involvement with ALL.17 At values of ≥10 U/mL of CSF sIL2-R , the sensitivity and specificity were 89.5% and 89.6% respectively for ALL.40 Current diagnostic modalities for leptomeningeal metastases lack sensitivity, particularly in early disease states.13 The additional use of the new diagnostic approaches mentioned in Section 2.4 allow for early detection of CNS infiltration and, consequently, earlier management. 1.5 MANUAL CEREBROSPINAL FLUID ANALYSIS Historically, CSF analysis has been conducted through manual microscopy by means of haemocytometers (counting chambers) and is considered the gold standard for CSF analysis.41 The commercially available chambers include the Improved Neubauer, Burker, Thomas and Fuchs-Rosenthal chambers.31 The Microbiology Department of the NHLS at Universitas Hospital utilises the Improved Neubauer chamber.42 The chamber consists of a thick glass plate that has two raised, square-shaped platforms (see Figure 2.1). The ruled area on each counting chamber (9 mm2) is divided into nine large (1 mm2) squares. The four large corner sq uares are subdivided into 16 equally-sized squares and the large centre sq uare is

subdivided into 25 equally-sized squares. Each of these 25 squares is subdivided further into 16 squares (Figure 2.1).43 Figure 1.1: Improved Neubauer chamber calibrated coverglass43 Figure 1.2: Improved

Neubauer chamber with coverglass43 A calibrated coverglass that covers the central area of the chamber controls the depth of the field precisely, so that each large (1 mm2) square encloses a volume of 0.1 µL. To avoid inaccurate results, the calibrated coverglass must be used to perform cell counts.43 Once a specimen is deemed acceptable for evaluation (as per standard operating procedure of the Microbiology Department),44 crystal violet stain is added to the CSF sample. The mixture is allowed to stand for one minute before it is loaded onto the counting chamber. The counting chamber is, furthermore, allowed to stand for another minute on the microscope stage, to allow cells to settle. Using the 40 x dry objective, the different cell lines are counted within the four large (4 x 16) squares.42 The total of each cell type is then calculated as follows:42 Dilution x Depth of counting chamber xNumber of cells counted PMN or lymph = Number of squares counted Depth of counting chamber xNumber of cells Erythrocytes = Number of squares counted After the total for each cell type has been determined, the correlating number of cells per microliter is determined, as per Table 2.6. Table 1.5: Conversion of cells counted to cells/µl34 Cerebrospinal Fluid Cell Counts Undiluted RCC 1:2 Dilution of WCC (PMN and Lymph) Low count: Count 4 big squares High count: Count 1 big square Low count: Count 4 big squares High count: Count 1 big square RCC Counted RCC/µl RCC Counted RCC/µl WCC Counted WCC/ µl WCC Counted WCC/ µl 1 3 1 10 1 5 1 20 2 5 2 20 2 10 2 40 3 7 3 30 3 15 3 60 4 10 4 40 4 20 4 80 5 13 5 50 5 25 5 100 6 15 6 60 6 30 6 120 7 17 7 70 7 35 7 140 8 20 8 80 8 40 8 160 9 23 9 90 9 45 9 180 10 25 10 100 10 50 10 200 11 28 20 200 11 55 20 400 12 30 25 250 12 60 25 500 13 33 30 300 13 65 30 600 14 35 35 350 14 70 35 700 15 38 400 400 15 75 40 800 16 40 50 500 16 80 50 1 000 17 43 60 600 17 85 60 1 200 18 45 70 700 18 90 70 1 400 19 8 80 800 19 95 80 1 600 20 50 90 900 20 100 90 1 800 25 63 100 1 000 25 126 100 2 000 30 75 150 1 500 30 150 150 3 000 40 100 200 2 000 40 200 200 4 000 Normal CSF is acellular, however, up 5 to WCC/µL and 5 RCC/µL can occur in normal adults without it being considered pathological. In neonates, counts of up to 20 WCC/µL are considered normal.10 During infection or malignant infiltration of the CNS, the cellular constituents in the CSF are altered.10 If WCCs are present, the differential WCC count in CSF normally comprises 70% lymphocytes and 30% monocytes.10 More than three PMN/µL, as seen, inter alia, with infection, inflammation or leukaemic infiltration, is always regarded as abnormal in adults.10 In turn, an increase in the RCC is indicative of the presence of subarachnoid haemorrhage, intracerebral haemorrhage or a traumatic spinal tap.45 Acute bacterial meningitis has been found to be approximately 10 times more common in developing countries than in developed counterparts. It is almost always fatal in the absence of appropriate management, with survival heavily dependent on accurate diagnosis and early administration of antibiotics.46 In 2001, Berkely et al. conducted a prospective study on 905 children undergoing lumbar puncture at a rural district hospital in Kenya over a period of one year. These authors assessed the value of non-laboratory tests that were normally available at the hospital for laboratory assessment of CSF. They found that, without adequate and reliable laboratory resources,the diagnosis of childhood acute bacterial meningitis was missed in up to a third of cases. Accurate leucocyte counting and glucose measurement were found to be superior to CSF culture facilities, which were expensive and difficult to maintain. 47 The number of requests for CSF analysis at the Universitas Microbiology Department has increased substantially. In 2016 , a total of 2 780 samples were analysed, and it increased to 3 720 samples analysed in 2017.48 This increase has proven to be a challenge for the Department, due to critical staff shortages and the lack of microbiology technologists for an after- hours service. The result is an increased turnaround time, sample degradation (evident within two

hours after collection)49 and, ultimately, a delay in service delivery. Although considered the gold standard for CSF cell counts, manual microscopy is

subject to several shortcomings and, as a result, the last decade has favoured and streamlined the move towards automated cell counters. Shortcomings associated with manual microscopy include:31,49 ? ? High imprecision (at lower cell counts) and high inter- observer variability; Labour-intensive methodology with prolonged turnaround times (30 to 45 minutes to complete); ? ? It is subjective and technique-dependent; and Inability to differentiate mononuclear cells into lymphocytes and monocytes. 1.6 AUTOMATED CEREBROSPINAL FLUID ANALYSIS In most clinical laboratories, automated haematology analysers are utilised to analyse full blood counts on whole blood samples. Many of these analysers are now equipped with CSF and/or body fluid modules that are able to perform CSF and body fluid cell counts. Although the automated method is not currently utilised in South Africa to analyse CSF and other body fluids, several international publications have supported its use.31,32,49,50 The Siemens ADVIA120 and ADVIA2120 (Siemens Healthcare Dia g nostics, Deerfield, IL, USA), Sysmex XE-5000 (Sysmex Corporation, Kobe , Ja pan), Abbott CELL-DYN Sapphire (Abbott Diagnostics, Abbott Park, IL, USA) and the Beckman Coulter LH750 (Beckman Coulter, Miami, FL, USA) are among the instruments currently utilised for automated body fluid cell counts.31 The automated haematology analysers all work on the same basic principles. After the specimen is incubated with a manufacturerspecific reagent, it is analysed using either flow cytometry, electrical impedance, or both. With the electrical impedance principle (Coulter principle), a stream of cells is passed through an aperture over which an electrical current is applied. The amount of current displaced results in a variation in electrical resistance that is directly proportional to the cell size. 31 Flow cytometry, of which the basic principle is explained above, categorises cells based on their size (forward side scatter), complexity (side scatter) and DNA/RNA binding (fluorescence).31,32 The light signals are organised into a digital form and displayed as scattergrams. The scattergrams below (Figure 2.3) are from the ADVIA2120i, which utilises flow cytometry for cell characterisation.31,51 Figure 1.3: CSF scatter/scatter and CSF scatter/absorption cytograms51 Like the manual method, automated systems provide a RCC, a total WCC and a WCC differential count; however, the automated method is able to separate the mononuclear component further, into lymphocytes and monocytes.52 Fleming et al. compared these analysers to conventional manual microscopy for assessment of CSF and body fluid cell counts in inflammatory diseases. They conclude that the automated methods provide a rapid and more precise diagnosis. The accuracy and high precision is a result of the aspiration of larger volumes by the analysers than of the volumes required for manual microscopy. Consequent to their higher limits of quantification, particularly with the Sysmex XE-5000, automated analysers are less precise at lower cell counts. Another shortcoming is the inability of the analysers to detect neoplastic cells, which they erroneously classify as lymphocytes.31 Aune et al. had similar favourable outcomes, proving that the ADVIA120 compared well to manual microscopy. As the ADVIA120 does not provide morphology flagging, the authors suggest that cytomorphology be used in conjunction with automated analysis to ascertain the presence or absence of neoplastic cells, particularly for oncology specimens.49 1.7 CEREBRAL SPINAL FLUED CELL COUNT REFERENCE RANGES Reference ranges for CSF cell counts are based primarily on manual microscopy techniques. Reference ranges in healthy individuals are age-dependent, with normal adult CSF containing <5 x106 WCC/L, that of infants <7 x106 WCC/L, and neonates <30 x 106 WCC/L.52 Very few studies assessing the reference ranges of the automated methods have been conducted. The establishment of new reference ranges for the Sysmex XE-5000 was assessed by two separate studies, by De Jonge et al. and Sandhaus et al. Using a cohort of 87 normal adult patients undergoing spinal anaesthesia, De Jonge et al. report normal reference ranges for RCC 0 x 106/L, WCC and mononuclear

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<7 x 106/L, and polymorphonuclear cells <3 x 106/L.50 Sandhaus et al. had different findings from De Jonge et al. Sandhaus et al. found the normal reference range for WCC to be between 20 x106 WCC/L and 30 x106 WCC/L. They conclude that this range was due to a decrease in

linearity between the results of the Sysmex XE-5000 and the manual method at cell counts <20 x106 WCC/L.31 At a CSF RCC of 250 cells/L, good correlation between counts were obtained for the ADVIA2120i and manual microscopy.53 Bremell et al. calculated new ADIVA2120i specific reference ranges as per CLSI C28-A3 guidelines. Their normal reference ranges were <5 x 106 WCC/L, <4 x 106 lymphocytes/L, <3 x 106 polymorphonuclear cells/L, and <3 x 106 monocytes/L.53 At present, there is no uniformity for reference ranges between haematology analysers. This hinders acce ptance of automated cell counts in clinical practice. For standardisation of reference ranges, multi-institutional studies with clear sample inclusion criteria are needed, to compare the different haematology analysers currently approved for CSF analysis.52 1.8 SIEMENS HEALTHCARE: ADVIA2120I Universitas Hospital Service Laboratory installed two ADVIA2120i instruments in 2015. These instruments are bench-top haematology analysers, and are currently utilised for routine whole blood specimens only. The ADVIA2120i is semiautomated, with three aspiration modes, namely, the rack- based auto sampler, manual closed tube sampler, and manual open tube sampler. The rack-based system has a throughput of 120 samples per hour in the full blood count (FBC) /differential mode and 74 samples per hour when reticulocyte analysis is performed. The ADVIA2120i is equipped with a CSF module that provides rapid, automated analysis of CSF samples. 51,54 Analysing CSF with the ADVIA2120i involves mixing and incubating the CSF sample with CSF reagent, which spheres and fixes the cells. Incubation requires a minimum of four minutes, up to a maximum incubation time of four hours. Once incubated, the sample is aspirated directly into the ADVIA2120i system. Thecells are detected and enumerated based on li g ht scatter and absorbance measurements. A scatter versus scatter, and scatter versus absorbance cytogram is displayed with the thresholds, and results are automaticall y calculated for each sample (see Figure 2.3).51 Reportable parameters include: ? WCC; ? Full four-part differential count, which includes the absolute and percentage values for neutrophils, lymphocytes, monocytes and eosinophils; and ? RCC. We aim to validate the use of the Siemens ADVIA2120i for CSF analysis within NHLS laboratories countrywide. The instrument will be evaluated for precision and accuracy against our currently utilised method, which is manual microscopic counts.