

**From Manual Microscopy to Automated Cell Counters
for First Line Screening of Body Fluids**

*"But not without a special *body fluid* mode"*

Chérina K. A. Fleming

COLOPHON

The studies described in this thesis were performed at the Department of Clinical Chemistry from the Erasmus University Medical Center in Rotterdam, The Netherlands.

I hereby disclose that this research project was partially supported by Sysmex Corporation (Europe/Japan). Sysmex supplied the evaluated automated analyzers, and the reagents, and paid part of the PhD candidate's salary. Sysmex had no influence on the data analysis, data interpretation and drawn conclusions from the studies described in this thesis. We declare that we have no commercial interest in Sysmex, and there are no conflicts of interest.

Printing of this thesis was sponsored by Sysmex Corporation.

ISBN: 978-94-6169-844-5

Cover design: Emar Thomasa

Layout and printing: Optima Grafische Communicatie (www.ogc.nl)

Copyright © 2016 C. K. A. Fleming, Rotterdam, The Netherlands

All rights reserved. No part of this thesis may be reproduced or transmitted in any form by any means, without prior written permission of the copyright owner.

The **digital version** of this thesis can be downloaded at <https://epubs.ogc.nl/?epub=c.fleming>

**From Manual Microscopy to Automated Cell Counters
for First Line Screening of Body Fluids**

*“But not without a special *body fluid mode*”*

**Van manueel microscopie naar geautomatiseerde cel tellers
voor eerste lijn screening van lichaamsvochten**

*“Maar niet zonder een speciale *body fluid mode*”*

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties

De openbare verdediging zal plaatsvinden op
woensdag 11 mei 2016 om 13:30 uur

door

Chérina Katalina Anaïsa Fleming

geboren te Marigot, Saint Martin F.W.I.

PROMOTIECOMMISSIE:

Promotoren: Prof.dr. J. Lindemans
Prof.dr. R. de Jonge

Overige leden: Prof.dr. A. Verbon
Prof.dr. P.A.E. Sillevius Smitt
Prof.dr. J. Penders

Copromotor: Dr. H. Russcher

**To Douglas, Alma, Berteaux, Corcilio, Kalomo
Shaniqua & Shalisa Fleming**

SUCCESS IS NO ACCIDENT

It is hard work, perseverance, learning, studying, sacrifice and most of all LOVE of what you are doing. –PELE–

CONTENTS

Chapter 1	General Introduction	9
Chapter 2	Clinical relevance and contemporary methods for counting blood cells in body fluids suspected of inflammatory disease	23
Chapter 3	Role of automated peritoneal leukocyte differential counting in diagnostic accuracy in peritoneal dialysis associated peritonitis	57
Chapter 4	Improved software on the Sysmex XE-5000 BF mode for counting leukocytes in Cerebrospinal fluid	73
Chapter 5	Validation of the body fluid module on the new Sysmex XN-1000 for counting blood cells in cerebrospinal fluid and other body fluids	85
Chapter 6	Evaluation of Sysmex XN-1000 high sensitive analysis (hsA) research mode for counting and differentiating cells in cerebrospinal fluid	101
Chapter 7	UF-1000i: validation of the body fluid mode for counting cells in body fluids	119
Chapter 8	Liposomal interference on Sysmex XN-Series body fluid mode	139
Chapter 9	General Discussion	149
Chapter 10	Summary Samenvatting	167 173
Chapter 11	Addendum List of abbreviations List of publications PhD portfolio Acknowledgements About the author	179 181 183 185 187 191

Chapter 1

General Introduction

BODY FLUIDS

Cerebrospinal fluid and Synovial fluid

Body fluids (BFs) can be found in various cavities of the body under normal conditions (i.e., cerebrospinal fluid and synovial fluid), and others during pathological conditions (i.e., serous fluids). Cerebrospinal fluid (CSF) is mainly produced (80%) by the choroid plexuses, circulates through the ventricular system, subarachnoid space and down the spinal cord (1). In adults, CSF production rate is about 500 ml/day, with approximately 150 ml CSF in the central nervous system (CNS) at any given time (2). CSF is crystal clear and colorless, and serves a variety of purposes including protection of the brain from physical trauma, and the removal of waste products. Adult CSF contains no red blood cells (RBCs), and very few ($<5 \times 10^6/L$) white blood cells (WBCs). Roughly 70% of the WBCs are lymphocytes and 30% monocytes (3). The analysis of CSF is a key diagnostic element in the diagnosis of a variety of diseases including inflammatory conditions and infectious or non-infectious, amongst others, involving the CNS. Synovial fluid, also called joint fluid, is an ultra-filtrate of plasma combined with hyaluronic acid, found in synovial joints (i.e., knee and hip). Its main functions is to transport nutrients to the articular cartilage, and to lubricate the joints. Normal synovial fluid is highly viscous. It has a light yellow color, and it contains less than $200 \times 10^6/L$ WBCs in a distribution of roughly 70% monocytes, $<25\%$ lymphocytes, and $<25\%$ neutrophils (4, 5). The number of RBCs varies, and an increase in their number may result from a traumatic tap or hemorrhage (1). The purpose of synovial fluid analysis is to detect arthritis and to place a fluid into one of several categories, in the diagnosis of joint diseases.

Serous fluids

Serous cavities (pleural, peritoneal) normally contain small amounts of fluid, formed by the filtration of plasma that acts as a lubricant to the parietal (cavity wall) and visceral (organ within the cavity) membrane surfaces. An accumulation of fluid in the serous cavities is called an effusion, and based on the underlying pathophysiology, they are classified into transudates or exudates. Transudates are usually the result of a systemic non-inflammatory disease such as congestive heart failure and hypertension, while exudates are associated with disorders such as inflammation, infection and malignancies, involving the organ. Peritoneal effusion (ascites) is defined as an abnormal accumulation of fluid in the peritoneal space. The main cause of ascites is due to liver cirrhosis (80%), followed by cancer (10%), congestive heart failure (3%), tuberculosis (2%) or other causes (6). Ascites fluids normally contain $<300 \times 10^6$ WBC/L with $<25\%$ neutrophils (7).

Pleural effusions, may result from congestive heart failure, bacterial pneumonia, neoplastic diseases or cirrhosis amongst others (8). The majority of cells found in normal

pleural fluids are macrophages (75%). Other cells include lymphocytes (25%), while neutrophils and eosinophils account for less than 2% each (9). Continuous ambulatory peritoneal dialysis (CAPD) fluid is not a biological body fluid, and is used as an alternative to hemodialysis to treat patients with end-stage renal disease. A catheter is inserted into the peritoneal cavity, where dialysate fluid fills the cavity and “dwells” for about 4-6 hours, while gradually removing waste products and extra fluid from the bloodstream. Fluid is then drained out, and replaced with fresh fluid. This procedure is repeated about 4 times a day. CAPD fluid normally contains $<50 \times 10^6$ WBC/L with a majority (80%) of cells being mononuclear cells (MN) (10).

WHITE BLOOD CELLS (WBCs)

Activation of the immune system is a coordinated effort, and may be detected by an increased number of markers including WBCs. WBCs are an important part of the body's defense against microbial invaders. They attack and engulf infected or dead cells, and they play an immediate and delayed role with their response. Neutrophils (10 to 15 μm) are primarily involved in the first line defense against infection/inflammation, particularly bacterial infection; eosinophils (10 to 14 μm) are mostly encountered in anti-parasitic and allergic reactions; lymphocytes (8 to 10 μm) are involved in recognizing foreign particles such as viruses and antigens, and monocytes (15 to 20 μm) play multiple roles in immune function including their transformation into highly phagocytic macrophages. Neutrophils together with eosinophils and basophils form the polymorphonuclear (PMNs) cells, whereas the combination of lymphocytes and monocytes are called MNs.

Apart from WBCs, other cells can be seen in a variety of BFs. These include lining cells (mesothelial, leptomeningeal, synoviocyte), phagocytic cells (histiocytes/macrophages, erythrophages, siderophages, lipophages), malignant cells (blasts, lymphoma cells and non-hematopoietic malignant cells), but also, bacteria, fungi and yeast cells (11). Mesothelial cells form the lining of serous cavities. These cells are large (12 - 30 μm), have a large round nucleus, and can appear in single or clustered forms. Non-hematopoietic malignant cells (i.e., adenocarcinoma, breast carcinoma, primary brain tumors) are also large, with large nucleoli, and are known to form tight clusters. Differentiating between mesothelial cells and malignant cells can be difficult because reactive mesothelial cells can be large, and may cluster together to resemble malignant cells (1). The (unexpected) finding of malignant cells in BFs, especially in patients with unknown malignant diseases, is of utmost interest, and should always be reported.

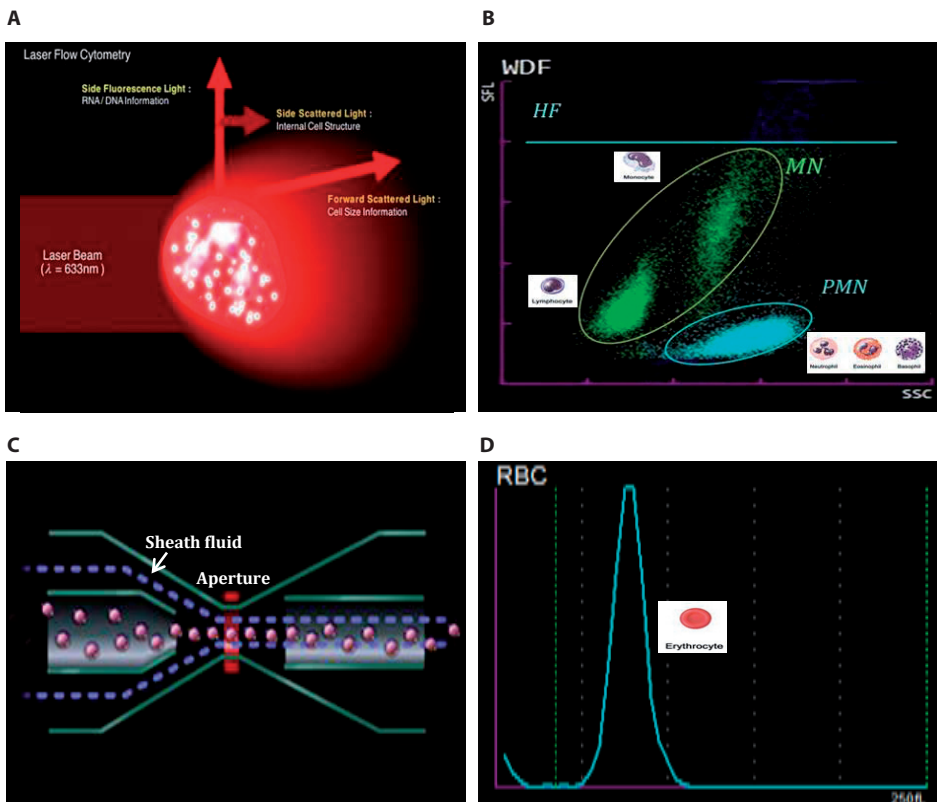


Figure 1 Principles adapted by Sysmex hematology analyzers to determine WBCs and RBCs. **(A)** Fluorescent flow cytometry principle, used to count and differentiate WBCs. **(B)** WBC differential (WDF) scattergram, with the various WBC cell types indicated. Within the MN cluster, the clusters for lymphocytes (lower left) and monocytes (upper right) are clearly visible; within the PMN cluster eosinophils (not present in this example) will appear as a separate cluster on the right of the neutrophil cluster that is clearly visible in this picture. The HF cluster represents cells with high nucleic acid content and large size such as macrophages, mesothelial and tumour cells. **(C)** Hydrodynamic focussing impedance technique, used to count RBCs. **(D)** RBC histogram. In graph B, the x-axis (SSC) represents the side scattered light and the y-axis (SFL) represents the side fluorescent light signal. MN= monocytes + lymphocytes and PMN= neutrophils+ eosinophils+ basophils. HF= high fluorescent cells.

RELEVANCE OF CELL COUNTS

Changes in the cellular components of BFs are a reflection of disease pathogenesis and disease stage. For example, *bacterial meningitis*, a life threatening infection of the CNS, is characterized by an elevated WBC ($>1000 \times 10^6/L$) and PMN predominance ($>50\%$) in CSF. The less severe *viral meningitis* is characterized by CSF WBCs between 10 and $1000 \times 10^6/L$ with a lymphocytic pleocytosis (14) (**Table 1**). The number of RBCs in CSF can aid in distinguishing between a traumatic tap and a subarachnoid hemorrhage (18).

Table 1 WBC counts in normal state and in various disease states (inflammatory and infectious) in a variety of BF. CSF= Cerebrospinal fluid, PMN= polymorphonuclear, MN= mononuclear

Normal state				Disease state			Refs	
WBC ($\times 10^6/L$)	PMN %	MN %	WBC ($\times 10^6/L$)	PMN %	MN %			
CSF	≤ 5	< 2	> 90	Bacterial Meningitis	> 1000	> 50	(1, 12-14)	
				Viral Meningitis	10 - 1000			> 80
				Fungal Meningitis	20 - 2000			> 50
				Eosinophilic Meningitis		$> 10\%EO$ or $> 10 EO/\mu L$		
CAPD fluid	< 50	12	> 80	Peritonitis		> 50	(10, 15, 16)	
				Eosinophilic peritonitis	> 100	$> 10\%EO$ or $> 40 \times 10^6 EO/L$		
Pleural fluid	1716	< 10	25	Transudate	< 1000		(8, 9)	
				Exudate	> 1000			
Peritoneal fluid	281	27	-	Spontaneous Bacterial Peritonitis		$\geq 250 \times 10^6/L$	(7, 17)	
Synovial fluid	< 200	< 25	< 80	Non-inflammatory	0 - 5000	< 30	(1, 5)	
				Inflammatory	2000 - 200,000	> 50		
				Infectious	50,000 - 200,000	> 90		
				Hemorrhagic	50 - 10,000	< 50		

Historically, pleural effusions have been dichotomized into *transudates* and *exudates* based on the following criteria: WBC $< 1000 \times 10^6/L$ (transudates) and WBC $> 1000 \times 10^6/L$ (exudates) (19, 20). However, because of the significant overlap when this criteria was used, classification of effusions is currently based on biochemical tests (Light's criteria) (21) and less so on cytological parameters. Although the total WBC count is of limited diagnostic value in distinguishing transudates from exudates, the WBC differential count can narrow the diagnostic possibilities. For example, the finding of neutrophilia heightens suspicion for parapneumonic effusion; whereas, a lymphocytosis ($> 50\%$) profile is indicative of tuberculous effusions and malignancies. Some even suggest that hemorrhagic pleural fluid (RBC $> 10,000 \times 10^6/L$) can predict malignancy (22). Cirrhotic patients with ascites are highly susceptible to *spontaneous bacterial peritonitis* (SBP). According to guidelines, SBP is present when $> 250 \times 10^6$ PMNs/L are counted in ascites (17). The frequent occurrence of *peritonitis* is a major complication of CAPD in patients undergoing dialysis. If the following criteria's are present: cloudy effluent containing more than 100×10^6 WBC/L with $\geq 50\%$ PMNs, and symptoms or signs of peritoneal inflammation, peritonitis is deemed the probable cause (15). In synovial fluids, the WBC and PMN counts are important diagnostic markers in enabling classification between *non-inflammatory*

(WBC: $<5000 \times 10^6/L$, PMN: $<30\%$), *inflammatory* (WBC: $2000-200,000 \times 10^6/L$, PMN: $>50\%$), *septic* (WBC: $50,000-200,000 \times 10^6/L$, PMN: $>90\%$) and *hemorrhagic disorders* (WBC: $50-10,000 \times 10^6/L$, PMN: $<50\%$) (23).

It is important to know that no diagnosis is made solely on the WBC/WBC differential results. However, their role as an important diagnostic parameter has undoubtedly been recognized, and is currently included in many clinical guidelines to help point the way to more specific testing, and possibly empiric treatment when necessary. Inaccurate results may lead to inappropriate diagnosis and therapy; therefore, accurate, precise and rapid laboratory results are of major clinical relevance.

MEASURING CELLS IN BFs

Traditional manual microscopy

The hemocytometer, also referred to as “counting chamber”, is traditionally used to determine the concentration of total WBCs and RBCs in BFs. For WBC differentiation into MNs and PMNs, a stained cytocentrifuged slide is prepared. Cytocentrifugation is a cell preparation system that uses centrifugal forces to deposit cells onto a slide. The slide is stained, and followed by a 100- 200 cell count differentiation (depending on laboratories protocol). The operating parameters (speed, timing, sample volume) generally vary from lab to lab, and this can influence the quality of the slide, and even result in quantitative variations in evaluation studies. The combined techniques are considered the gold standard; however, they have their limitations (24, 25). The hemocytometer is subjective, it is labor intensive, and has a high inter and intra-assay imprecision. Major concerns of the differential method correspond to the preparation of the cytospin slide. Because of the centrifugation, vulnerable cells are lost or obtain aberrant morphology, and macrophages or mesothelial cell clusters can be mistaken for malignancy. Collectively, results obtained from traditional microscopic methods should be viewed skeptically because their potential sources of errors can contribute to misleading results. The above described components emphasize the deficiencies of manual microscopy. However, it has remained the gold standard for BF analysis although, part of this method (hemocytometer) has been abolished decades ago as a reference method for blood samples (26). Because of the significant limitations of the manual method, along with the continuous increase of workload and shortage of skilled personnel (24/7), many laboratories opted for alternative methods by adopting the use of automated hematology analyzers, and more recent, urine analyzers to perform BF cell counts.

Automated analyzers

Up until the mid-1950s, cells in blood samples were routinely counted manually. The first automated cell counter was invented by Wallace Coulter based on electrical impedance principle (27). The Coulter principle revolutionized blood counting, and substantially reduced the time-consuming manual blood cell counts. His principle also laid the groundwork for subsequent development of modern day cell counters which employ similar techniques, but slightly different approaches. The two basic principles applied are “flow cytometry” and “electric impedance” technology. The basic principle of flow cytometry is the passage of fluorescently labelled cells in single file (hydrodynamic focusing) through a flow cell that is being intersected by a laser light. Photodetectors collect and measure the light in different wavelength ranges and scatter wavelengths by the use of specific optical filters. To this classic method, fluorescent dyes has been added to newer devices (28). Cells are then categorized based on the combination of sideward scattered light (cell granularity), forward scattered light (size of the cell) and fluorescent light (RNA/DNA content). In impedance technique, cells are sized and counted by detecting and measuring changes in electrical resistance when a particle passes through a small aperture. The change in voltage generates a pulse and the number of pulses is proportional to the number of cells counted. The size of the voltage pulse is also directly proportional to the volume or size of the cell. Most instruments generate two types of data graphic display for laboratory review: scattergrams and histograms. Histogram depicts the relative number of cells plotted against cell size, and scattergrams provide information on the WBC differential cells.

Hematology analyzers were originally intended for measuring cells in whole blood. However, WBCs in blood samples are at least 1000 times higher than that of BF samples, rendering these analyzers (using standard blood software) less suitable for BF analysis due to their high imprecision in the lower concentration range, and due to misclassification of tissue cells (i.e., mesothelial cells counted as WBCs) (29, 30). Currently there are a number of manufacturers on the market with instruments (hematology and urine) suitable for BF cell counting (31). Each analyzer uses either impedance, flow cytometry, digital imaging flow cytometry or a combination of these technologies. Since their introduction, efficiency and productivity have significantly improved by reducing turn-around-time, increasing precision and accuracy, and eliminating inter-observer variability compared to traditional manual methods (32-38).

Evolution of Sysmex’s automated cell counters for BF analysis

Sysmex Corporation (Kobe, Japan) is currently the market leader in Europe for BF analysis. In 1999, Sysmex launched its fully automated hematology analyzer, the XE-2100, initially developed for the analysis of whole blood samples. A few years later, this analyzer was

FDA cleared for measuring a wide variety of BFs with the exception of CSF due to its high background limits (LoQ WBC: $50 \times 10^6/L$). In 2007 Sysmex launched the XE-5000, a fully automated hematology analyzer, which contains unique software tailored for BFs analysis (the body fluid mode). It allows instant quantification of CSF, serous fluids, synovial fluids and CAPD without any pretreatment, and counts three times more cells than the XE-2100, resulting in improved precision at the lower concentration range (LoQ: $10 \times 10^6/L$). Sysmex's latest hematology analyzer, the XN-Series, was launched in 2011. It also contains a BF mode, measures a variety of BFs and counts two times more cells than the XE-5000 to increase precision (LoQ: $5 \times 10^6/L$). These analyzers adapted the combination of fluorescent flow cytometry with a semiconductor laser and impedance technique to measure the following cellular parameters: size, volume, granularity, surface area, and fluorescent signal. This data is used to determine the total nucleated cell count (TNC), RBC, WBC, MN, PMN, and high fluorescent (HF-BF) cells such as macrophages, malignant and mesothelial cells. These HF-BF cells are found just above the MN cluster, and are not included in the WBC (differential) count. However, they are included in the TNC (**Figure 1**). In 2014, Sysmex released new BF software for its urine analyzer UF-1000i, initially developed for measuring urine samples. The UF-1000i BF mode identifies cells based on forward scatter, fluorescence and adaptive cluster analysis, and reports a TNC, WBC and RBC count.

OVERALL RESEARCH QUESTIONS

Since their introduction in clinical laboratories, the use of automated cell counters has led to major improvements in BF analysis, and gained increasing, though still limited acceptance as an alternative to manual microscopy. The current limitations of BF modes applies to automated analyzers: lack of precision in the low ($<20 \times 10^6/L$) counting range, inability to detect malignant cells, interferences by non-cellular particles (bacteria, lipids, crystals) leading to spurious WBC and/or RBC results, and their inability to flag abnormality (33, 35, 38, 39). Therefore, in this thesis we investigated whether the currently available automated analyzers (by Sysmex) containing a dedicated BF mode are, *I*) sufficiently precise to measure blood cells in non-malignant BFs in clinically relevant ranges, *II*) sufficiently accurate in the WBC and WBC differential count, *III*) able to discriminate normal from infectious/inflamed samples (diagnostic test accuracy), *IV*) capable of detecting and flagging interfering particles, and *V*) suitable for replacing manual microscopy.

SCOPE OF THESIS

To date, there is an ongoing discussion about the use of automated cell counters versus conventional microscopy for body fluid cell counts. In **chapter 2**, a review of the literature is presented on the clinical relevance of blood cell counts in BFs and contemporary methods for measuring them in samples suspected of inflammatory diseases. Currently, there remains a discussion about which diagnostic parameter (WBC or PMN) best predicts peritonitis in dialysis patients. To this end, **chapter 3** describes a retrospective study using the automated XE-5000 WBC count and differential results in effluent dialysate samples for distinguishing between infectious and non-infectious peritonitis.

In 2007, Sysmex launched the XE-5000 BF mode, and this analyzer was evaluated extensively in our laboratory and also by others. The two main limitations resulted from its evaluation include high imprecision in the low concentration range and overestimation of CSF PMNs. Consequently, Sysmex developed new software to solve these issues. With the new software, gating algorithms for separation of unknown particles such as cell debris or fragments in the WBC/PMN count has been improved. Therefore, in **chapter 4** we evaluated this new software in comparison with the current software on the XE-5000 in CSF samples. Next, in 2011 Sysmex released the XN-Series which is their latest hematology analyzer. The XN-BF mode has several new features compared to the XE-5000 BF mode. It is able to count four times more WBCs than the XE-5000, aspirates less sample volume (88 μL) compared to the XE-5000 (130 μL), and offers open or closed tube sampling for BFs. In **chapter 5**, we compared the XN-1000 BF mode with the hemocytometer for counting cells in CSF, CAPD and serous fluids. Because of the continued demand for low imprecision in the low concentration range and high accuracy in CSF samples, Sysmex developed a new high sensitive Analysis mode (hsA) on the XN-Series, specifically for counting cells in CSF, which we evaluated in **chapter 6**. The XN-hsA mode has several new features such as it provides a 4-part differential count, uses flow cytometry technique for counting RBCs, aspirates more sample volume (180 μL) than the XN-BF mode and it counts twice as many cells, which in turn increases its precision and finally has new gating algorithms to detect and flag abnormal cells in CSF samples. Optical urine sediment analyzers are also being used for BF analysis, although they are not designed and certified for this application. At present, the Sysmex UF-1000i analyzer contains a urine mode and a dedicated BF mode. The BF mode contains new gating and modified algorithms to enhance cell counting and decrease interference with cell fragments (observed when measuring BF in the urine mode). This brings us to **chapter 7**, where we report on a validation study between the UF-1000i and reference methods for counting cells in serous fluids.

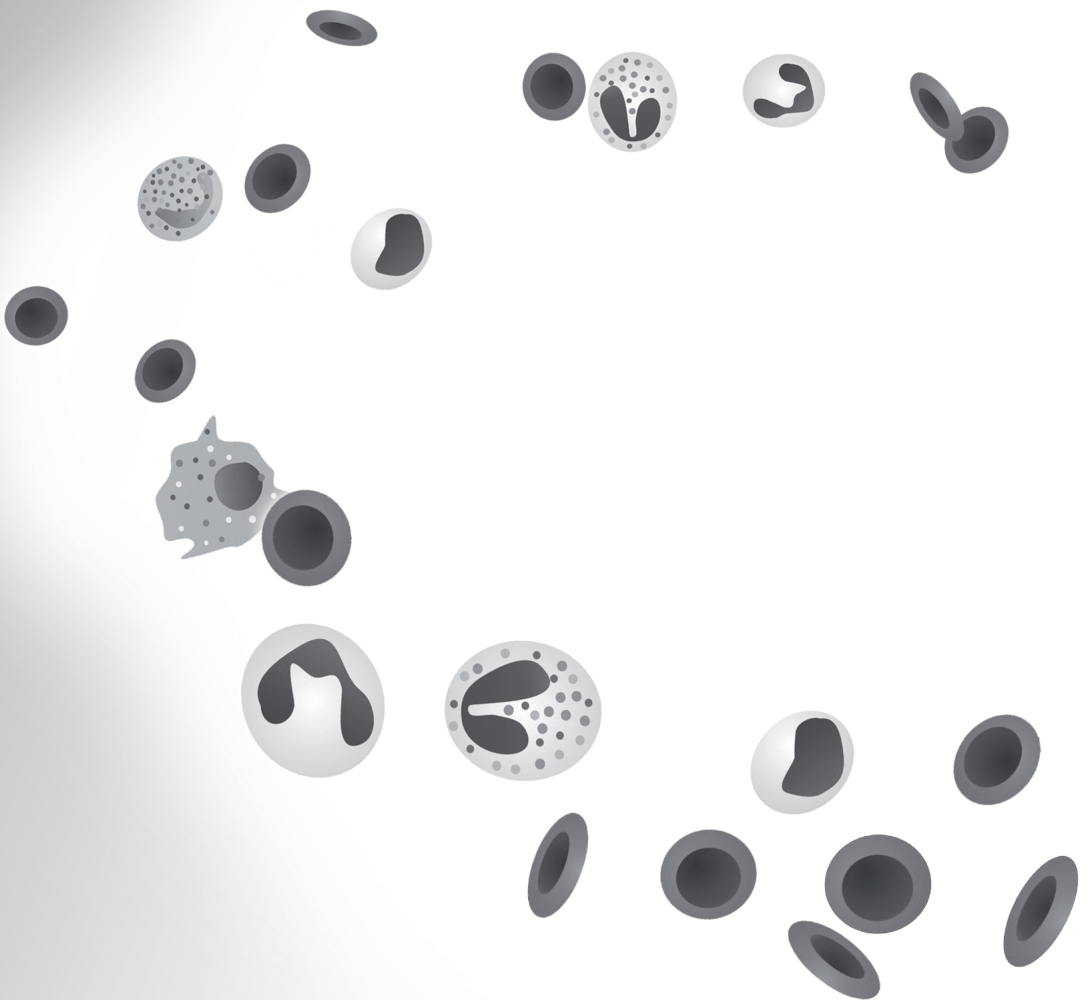
Disadvantages of automated analyzers include potential interfering factors from non-cellular particles including liposomal particles. Patients with neoplastic meningitis

are treated with the chemotherapeutic drug DepoCyt (cytarabine encapsulated in liposomal particles). The active drug is uniformly distributed throughout the CSF by its sustained release, and is detectable up to 14 days after administration. Interferences of DepoCyt particles are of concern because reporting falsely elevated WBC counts can have potential adverse clinical consequences in patients with neoplastic meningitis. In **chapter 8**, the interference of liposomal particles on the XN-1000 BF mode is studied. Lastly, **chapter 9** discusses the main findings of this thesis in a broader perspective, and gives suggestions for future research.

REFERENCES

1. Kjeldsberg C, Knight J. Body fluids. Laboratory Examination of Amniotic Cerebrospinal, Seminal, Serous, & Synovial Fluids: A textbook Atlas. Chicago, America 1986.
2. Jerrard DA, Hanna JR, Schindelheim GL. Cerebrospinal fluid. J Emerg Med. 2001;21:171-8.
3. Jurado R, Walker K. Cerebrospinal fluid. In: Walker K, Hall W, Hurst J, editors. Clinical Methods: The history, Physical, and Laboratory Examinations. 3rd ed. Boston 1990.
4. Mundt L, Shanahan A. Graff's Textbook of Urinalysis and Body fluids, 2nd Edition. Lippincott Williams & Wilkins, a Wolters Kluwer business 2010.
5. Brannan SR, Jerrard DA. Synovial fluid analysis. J Emerg Med. 2006;30:331-9.
6. Moore KP, Wong F, Gines P, Bernardi M, Ochs A, Salerno F, et al. The management of ascites in cirrhosis: report on the consensus conference of the International Ascites Club. Hepatology. 2003;38:258-66.
7. Bar-Meir S, Lerner E, Conn HO. Analysis of ascitic fluid in cirrhosis. Digestive Diseases and Sciences. 1979.
8. Light RW, Macgregor MI, Luchsinger PC, Ball WC, Jr. Pleural effusions: the diagnostic separation of transudates and exudates. Ann Intern Med. 1972;77:507-13.
9. Noppen M. Normal volume and cellular contents of pleural fluid. Curr Opin Pulm Med. 2001;7:180-2.
10. Ludlam HA, Price TN, Berry AJ, Phillips I. Laboratory diagnosis of peritonitis in patients on continuous ambulatory peritoneal dialysis. J Clin Microbiol. 1988;26:1757-62.
11. Szamosi D, Bautista JM, Cornbleet J, Glasser L, Rothe G, Sandhaus L, et al. Clinical and Laboratory Standards Institute. *Body fluid Analysis for Cellular Composition; Approved guideline. CLSI document H56-A*. 2006;26.
12. Greenlee JE. Approach to diagnosis of meningitis. Cerebrospinal fluid evaluation. Infect Dis Clin North Am. 1990;4:583-98.
13. Seehusen DA, Reeves MM, Fomin DA. Cerebrospinal fluid analysis. Am Fam Physician. 2003;68:1103-8.
14. A. K. Cerebrospinal fluid and other body fluids. In: J H, editor. Clinical diagnosis and management by laboratory methods. Philadelphia: WB Saunders; 1979. p. 635-79.
15. Piraino B, Bailie GR, Bernardini J, Boeschoten E, Gupta A, Holmes C, et al. Peritoneal dialysis-related infections recommendations: 2005 update. Perit Dial Int. 2005;25:107-31.
16. Chandran PK, Humayun HM, Daugirdas JT, Nawab ZM, Gandhi VC, Ing TS. Blood eosinophilia in patients undergoing maintenance peritoneal dialysis. Arch Intern Med. 1985;145:114-6.
17. Rimola A, García-Tsao G, Navasa M, Piddock LJV, Planas R, Bernard B, et al. Diagnosis, treatment and prophylaxis of spontaneous bacterial peritonitis: a consensus document. Journal of Hepatology. 2000;32:142-53.
18. Gorchynski J, Oman J, Newton T. Interpretation of traumatic lumbar punctures in the setting of possible subarachnoid hemorrhage: who can be safely discharged? Cal J Emerg Med. 2007;8:3-7.
19. Pettersson T, Riska H. Diagnostic value of total and differential leukocyte counts in pleural effusions. Acta Med Scand. 1981;210:129-35.
20. Leuallen EC, Carr DT. Pleural Effusion. New England Journal of Medicine. 1955;252:79-83.
21. Light RW. Useful tests on the pleural fluid in the management of patients with pleural effusions. Curr Opin Pulm Med. 1999;5:245-9.
22. Rashmi KS, O.; Hiremath,S.; Basavaraj,H.;. Cells in pleural fluid and their value in differential diagnosis. Journal of cytology. 2008;25:138-43.

23. Ropes MW, Rossmeis EC, Bauer W. The Origin and Nature of Normal Human Synovial Fluid. *J CLIN INVEST.* 1940;19:795-9.
24. Dux R, Kindler-Rohrborn A, Annas M, Faustmann P, Lennartz K, Zimmermann CW. A standardized protocol flow cytometric analysis of cells isolated from cerebrospinal fluid. *J NEUROL SCI.* 1994;121:74-8.
25. De Jonge R, Brouwer R, Van Rijn M, Van Acker BAC, Otten HJAM, Lindemans J. Automated analysis of pleural fluid total and differential leukocyte counts with the Sysmex XE-2100. *Clin Chem Lab Med.* 2006;44:1367-71.
26. DeNicola DB. Advances in hematology analyzers. *Top Companion Anim Med.* 2011;26:52-61.
27. Kratz A, Brugnara C. Automated Hematology Analyzers: State of the Art. *Clinics in Laboratory Medicine.* [doi: 10.1016/j.cll.2014.11.004].35:xiii-xiv.
28. Brown M, Wittwer C. Flow Cytometry: Principles and Clinical Applications in Hematology. *Clinical Chemistry.* 2000;46:1221-9.
29. Yang D, Zhou Y, Chen B. Performance evaluation and result comparison of the automated hematology analyzers Abbott CD 3700, Sysmex XE 2100 and Coulter LH 750 for cell counts in serous fluids. *Clin Chim Acta.* 2013;419:113-8.
30. Brown W, Keeney M, Chin-Yee I, Johnson K, Lantis K, Finn W, et al. Validation of body fluid analysis on the coulter LH 750. *Lab Hematol.* 2003;9:155-9.
31. Fleming C, Russcher H, Lindemans J, de Jonge R. Clinical relevance and contemporary methods for counting blood cells in body fluids suspected of inflammatory disease. *Clin Chem Lab Med.* 2015;53:1689-706.
32. Barnes PW, Esby CS, Shimer G. An evaluation of the utility of performing body fluid counts on the coulter LH 750. *Lab Hematol.* 2004;10:127-31.
33. De Jonge R, Brouwer R, De Graaf MT, Luitwieler RL, Fleming C, De Frankrijker-Merkestijn M, et al. Evaluation of the new body fluid mode on the Sysmex XE-5000 for counting leukocytes and erythrocytes in cerebrospinal fluid and other body fluids. *Clin Chem Lab Med.* 2010;48:665-75.
34. de Jonge R, Brouwer R, Smit M, de Frankrijker-Merkestijn M, Dolhain RJEM, Hazes JMW, et al. Automated counting of white blood cells in synovial fluid. *Rheumatology (UK).* 2004;43:170-3.
35. Sandhaus LM. Validation of sysmex XE-5000 cell counts for cerebrospinal fluids (CSFS) with normal cell counts. *Int J Lab Hematol.* 2012;34:132.
36. Paris A, Nhan T, Cornet E, Perol JP, Malet M, Troussard X. Performance evaluation of the body fluid mode on the platform Sysmex XE-5000 series automated hematology analyzer. *Int J Lab Hematol.* 2010;32:539-47.
37. Keuren JFW, Hoffmann JJML, Leers MPG. Analysis of serous body fluids using the CELL-DYN Sapphire hematology analyzer. *Clin Chem Lab Med.* 2013;51:1285-90.
38. Boer K, Deufel T, Reinhoefer M. Evaluation of the XE-5000 for the automated analysis of blood cells in cerebrospinal fluid. *Clin Biochem.* 2009;42:684-91.
39. Perne A, Hainfellner JA, Womastek I, Haushofer A, Szekeres T, Schwarzinger I. Performance evaluation of the sysmex XE-5000 hematology analyzer for white blood cell analysis in cerebrospinal fluid. *Arch Pathol Lab Med.* 2012;136:194-8.



Chapter 2

Clinical relevance and contemporary methods for counting blood cells in body fluids suspected of inflammatory disease

Chérina Fleming, Henk Russcher, Jan Lindemans, Robert de Jonge

ABSTRACT

In many inflammatory diseases, the cellular components in body fluids (CSF, serous fluids) are increased, rendering essential diagnostic information. The diagnostic value of the total WBC and differential count has been evaluated extensively over the years, and a remarkable amount of knowledge has been gained; yet, there is a great deal of clinical uncertainty whether the diagnosis should be based solely on these variables. In some diseases such as peritonitis, the total WBC and differential count has high sensitivity; whereas, in differentiating pleural effusions, it lacks the sensitivity required to be clinically useful. Nevertheless, many guidelines consider these test as cornerstone parameters, and in combination with clinical variables, they can successfully guide clinical decision making in initiating or postponing a treatment course for infection and/or inflammatory diseases while awaiting culture results. Although other methods are available for detecting and differentiating WBCs in body fluids, manual microscopy is still considered the gold standard despite its many limitations. During the last decade, automated analyzers have become a popular method for first line screening. Continued progress in their design has led to major improvements including their speed, improved accuracy and lower variability compared with microscopy. Disadvantages of this method include high imprecision in low ranges (depending on the method) and interfering factors. In a time where automation is at the front line in clinical laboratories, it is essential the results obtained are precise, accurate and reproducible. This review provides an overview of the relevance for cell counting in a variety of diagnostic body fluids, and highlights the current technologies used.

INTRODUCTION

Body fluids (Cerebrospinal fluids, serous fluids, synovial fluids) contain a variety of nucleated cells including white blood cells (WBCs), nucleated red blood cells (NRBC), lining cells (mesothelial cells, synoviocytes, ependymal cells etc.) and other non-hematopoietic cells (malignant cells) which are reported as the total nucleated cell count (TNC) (1). Various inflammatory diseases are characterized by the presence of abnormal numbers of WBCs in body fluids (BFs) other than blood; therefore, the total WBC and the differential (lymphocytes, monocytes, eosinophils, neutrophils) count is of substantial diagnostic value in these conditions, and may guide therapy. The diagnostic value of WBCs in various BFs has been studied extensively, but results remain controversial. Nonetheless, the role of WBCs as important diagnostic parameters has undoubtedly been recognized, and is currently included in many clinical diagnostic guidelines. Improper techniques and inaccurate results may lead to the under or over-diagnosis of threatening inflammatory disorders such as meningitis and peritonitis. Currently, three methodologies prevail to generate data for counting and differentiating cells in BFs. These methods are manual microscopy, automated flow cytometry, and automated impedance technology. Traditional manual microscopy is the gold standard; however, in the last decade, automated analyzers using flow cytometry and/or impedance technology have become mainstream in clinical laboratories. To date, there is an ongoing discussion about the use of automated cell counters versus conventional microscopy; however, each technique comes with its own limitations. In selecting between methodologies, many factors such as technical capability, sample volume, type of body fluid and patient category come into play. Each laboratory should make a choice based on its own situation; however, profound knowledge is required of the advantages and disadvantages of each available technology.

The first part of this review focusses on the diagnostic significance of the total WBC and differential count in various BFs during inflammatory diseases. The second part describes the advantages and disadvantages of the different techniques for measuring these cells in BFs.

METHODS

To identify relevant articles, a literature search was conducted in Pubmed and Medline up to May 2014 using varied Mesh and free-text terms such as "cerebrospinal fluid", "synovial", "CAPD", "peritoneal dialysis", "ascites fluid", "pleural fluid", "erythrocyte", "leukocyte", "neutrophil", "PMN", "MN", "hematology guidelines", "hematology analyzers",

“automated analyzers”, “meningitis”, “peritonitis”, and “cell count”. The number of initial hits was reduced to 143 by including only full research articles and reviews written in English, and are based on human studies with no restrictions to the year of publication. Furthermore, the reference list of relevant individual papers was examined for other articles of interest.

BODY FLUIDS

Cerebrospinal fluid (CSF)

CSF is located within the cerebral ventricles and subarachnoid spaces of the brain. The composition (WBC, glucose, protein etc.) of CSF in the ventricles is different compared to the lumbar region in physiological and in pathological conditions (2). The majority of CSF samples sent to the laboratory for cellular analysis are from patients suspected of an infectious or inflammatory disease of which meningitis is the main suspect. Meningitis, which is mainly caused by bacteria and viruses, is the most common and severe infection affecting the CNS. Microbiological culture technique is the gold standard for confirmation of the causative organism; however, results are usually delayed (up to 72 hours for bacteria, and 6 weeks for *Mycobacterium*). In approximately 80% of the cases, culture results will reveal the causative agent; however, in patients receiving antibiotics prior to CSF analysis, results may be obscured, and the test sensitivity decreases by ~20% (3). This prompted the search for other rapid and reliable markers to predict and differentiate between bacterial and non-bacterial meningitis, and to aid in reducing the administration of unnecessary antibiotics. Early reports in the 1970s indicated the total WBC and differential counts to be helpful in distinguishing bacterial meningitis from other meningitis (4). WBC counts $>1000 \times 10^6/L$ and $>50\%$ polymorphonuclear (PMN) cells were seen in more than half of the cases with bacterial compared to viral meningitis (4-7). In a large retrospective study, the group with bacterial meningitis resulted in a higher WBC count (median: $1195 \times 10^6/L$ vs. $100 \times 10^6 WBC/L$) and %PMN (median: 86% vs. 33%) compared to the viral meningitis group (7). However, in 45% of the bacterial cases, the WBC count was $<1000 \times 10^6/L$, and even $<250 \times 10^6/L$ at times. The authors concluded that no single parameter could rule out the presence of bacterial meningitis, nor could it differentiate between bacterial and viral meningitis because of the wide range of overlapping results between the two diseases. However, the combined use of the predictors (age, CSF-blood glucose ratio, month of the year, and CSF PMN count) could predict the likelihood of bacterial versus viral meningitis with optimal diagnostic accuracy in the test sample (AUC: 0.968) and the validation set (AUC: 0.985) (7). The diagnostic accuracy of this prediction model was confirmed in a retrospective validation study (n=500) (8), but these authors proposed a new diagnostic model (AUC close to 1)

based on 4 different independent variables (CSF protein, CSF PMN, blood glucose and blood WBC). These findings evoked the interest of other groups to validate the various diagnostic prediction models, and also to introduce new self-computed models that showed varying degrees of sensitivity (77-98%) and specificity (49-98%) for predicting bacterial meningitis in neonates, children and adults (9-14). Unfortunately, a large variability between studies in the methodological section (i.e. study design, laboratory criteria, patient inclusion criteria, and isolated pathogen) was found, which made it difficult to interpret and compare the results of the different models. All prediction models for differentiating bacterial from viral meningitis include a combination of parameters (WBC, PMN, protein, LDH, CRP, lactate, age, month, glucose, etc.) as opposed to one single parameter (2, 15-20).

Apart from bacteria and viruses, meningitis can be caused by fungi and parasites. Fungal meningitis predominantly affects immune compromised, and in particular HIV/AIDS patients, and is usually caused by *Cryptococcus neoformans* (21). In CSF, WBCs range from as low as 20 to a maximum of $2000 \times 10^6/L$, with a preponderance (>50%) of lymphocytes (22). Tuberculous meningitis represents about 1% of all tuberculosis cases and remains the most lethal form of extra-pulmonary tuberculosis (23). The WBC findings were similar to those described in fungal meningitis, ranging from 10 to $3900 \times 10^6/L$, with a predominance of lymphocytes (80%); however, it was not unusual to find patients with PMN predominance (24-27). Eosinophilic meningitis accounts for less than 2% of all meningitis cases, and is mainly correlated with parasitic and fungal infections or allergic reactions to shunt material. Eosinophilic meningitis is defined by the presence of $>10 \times 10^6$ eosinophils/L, or when >10% of total WBCs are eosinophils (28, 29). In summary, the evaluation of the total WBC and differential count in CSF is an important aspect of laboratory diagnosis of meningitis. Some are of the opinion that the WBC and differential counts are indispensable diagnostic parameters (5), while others tend to deny this (6). The majority of proposed guidelines use the traditional combination of CSF parameters including an elevated WBC count ($>1000 \times 10^6/L$) with a predominance of PMNs (**Table 1**), elevated protein and decreased glucose to be characteristic of bacterial meningitis (30). Conversely, WBCs between $10 - 1000 \times 10^6/L$ with a lymphocytic pleocytosis, normal to elevated protein and normal glucose is more representative of viral meningitis (31). Of note, in the acute stage of viral meningitis, WBCs can be $>1000 \times 10^6/L$ with neutrophilic predominance (32); however, as the disease progresses, the balance shifts, and lymphocytes start to predominate (33). Similar to viral meningitis, WBCs in tuberculous and fungal meningitis range between $10 - 1000 \times 10^6/L$ with lymphocytic predominance (34). Importantly, when interpreting biochemical and cytological CSF results, CSF collection site (ventricular drainage or LP) should be considered, because CSF composition varies between the two especially during CNS infection/inflamma-

Table 1 overview of studies evaluating the diagnostic accuracy of WBC counts in inflammatory disorders and the current guidelines.

Fluid type	Study Criteria (Ref)	Counting method	No. patients included	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	ROC	Current Guidelines	Ref
CSF	BM									
	WBC >1500 ⁽⁶⁾	manual	710	51	99	-	-	-	WBC: 1000 – 10,000	(3)
	WBC >1000			61	98	-	-	-	PMN: 80-90%	
Pleural	WBC >1000 ⁽⁵⁶⁾								Exudate: WBC: >1000	(66)
	WBC <1000	manual	150	-	-	-	-	-	Transudate: WBC: <1000	
Ascites	WBC >1000 ⁽⁴⁸⁾		10	100	89	63	100	-		
	WBC >500 ⁽⁴⁷⁾		46	91	88	-	-	-		(53)
	PMN >500 ⁽⁴⁹⁾	manual	14	86	98	92	96	95	PMN: ≥250	
	PMN >250 ⁽⁵²⁾		6	100	94	67	100	-		
	WBC >2,000 ⁽⁷²⁾			84	84	-	-	-	WBC: 2000 – 50,000	
SF	PMN >70%		100	75	92	-	-	94	PMN: >50%	(74)
	WBC >50,000 ⁽⁷³⁾	manual	44	72	92	-	-	-	WBC: >50,000	
	PMN >80%			93	59	-	-	-	PMN: >75%	
CAPD	WBC >100 ⁽⁸⁷⁾		48	-	-	-	-	-	WBC: >100	(95)
	PMN >50%	manual							PMN: >50%	

BM= bacterial meningitis, SBP= spontaneous bacterial meningitis, WBC= white blood cells, PMN=polymorphonuclear cells, Ref= references, PPV= positive predictive value, NPV=negative predictive value, ROC= Receiver Operating Curve

tion, and can produce higher or lower results (35). Based on the reviewed literature, no single finding (WBC or differential) was absolutely conclusive in differentiating bacterial from non-bacterial conditions; therefore, should not be used as a sole criterion. However, when clinical condition will not allow waiting for confirmation by microbiological culture results, the CSF WBC and differentials (relative proportion of PMN to MN) can give the best indication of the degree and type of inflammation, and may guide initial treatment choices.

Reference range CSF

To distinguish normal from abnormal, the reference range must be known. Normal CSF WBC reference values have been mentioned in many scientific publications from old textbooks to recent references (36-38). In 1931, normal CSF WBCs were reported to be $<2.6 \times 10^6/L$ (39). Presently, there is a general consensus that WBCs in CSF is age-dependent, and normal adult CSF should contain $<5 \times 10^6 WBC/L$ (40), infants $<7 \times 10^6 WBC/L$ (41) and neonates $<30 \times 10^6 WBC/L$ (42), based on manual microscopic analysis. There are only three peer-reviewed papers providing reference values for automated cell counters in CSF. By using the Sysmex XE-5000 BF mode, our group (43) reported normal CSF ($n=87$) reference ranges to be $<7 \times 10^6 WBC/L$ in adult patients who underwent spinal anesthesia before an orthopedic, gynecological, surgical or urological surgery; whereas, Sandhaus et al. (44) found values ranging between 20 to $30 \times 10^6 WBC/L$ ($n=200$) in patients with (non) hematological malignancies, cerebral hemorrhage and suspected infection/inflammation disorders. Differences in the patients included may be responsible for the observed discrepancy. The adult patients included in our study had no history of neurological diseases; whereas, Sandhaus et al. (44) included samples from both children and adults with known CNS disorders. More recently, new reference ranges for CSF samples ($n=80$) of neurologically healthy patients undergoing orthopedic surgery were measured on the Advia 2120i, and were reported to be less than $7 \times 10^6/L$ (45), which are in concordance with our study (43). In summary, interpretation of automated CSF WBC counts requires established reference values. Though, ethical considerations prohibit subjecting healthy individuals to invasive procedures solely for research purposes; consequently, normative values are determined from diagnostic LPs, preferentially from patients with non-inflammatory disorders. The current reference ranges referenced by manufacturers and textbooks were established decades ago using manual microscopy. However, with many laboratories transitioning from manual to automated methods, old reference ranges should be verified because higher reference ranges have been reported using automated methods (43-45).

Ascites

Ascites fluid is defined as an abnormal accumulation of fluid in the peritoneal space. The most common cause of ascites is liver cirrhosis which accounts for 80%, and the remaining 20% may be caused by congestive heart failure, tuberculosis, cancer or other causes. Spontaneous bacterial peritonitis (SBP) is defined as an infection of pre-existing ascites without any obvious intra-abdominal source of infection (46). Bar-Meir et al. (47) reported the total WBC and differential count to be of paramount importance in diagnosing SBP in cirrhotic patients. Because culture results were not available within 24 hours, and guidelines to diagnose SBP were lacking at that time, this group recommended treating patients suspected of SBP if one of the following criteria were present: $WBC > 1000 \times 10^6/L$ (sensitivity 89%) or $PMN \geq 500 \times 10^6/L$ (sensitivity 87%). Moreover, this group proposed treating patients with symptoms compatible with SBP if the WBCs are $> 500 \times 10^6/L$ (sensitivity 91%) or the PMNs $> 250 \times 10^6/L$ (sensitivity 92%). Several studies investigated the proposed criteria, and compared them with the gold standard (positive bacterial culture). The ascites PMN count had the highest diagnostic test accuracy ($> 92\%$) for SBP, and could potentially be used in diagnosis (48-50). Sensitivity varied from 86 to 100% and specificity between 93 and 98% when PMNs were $> 500 \times 10^6/L$ (49, 50). Further attempts to increase the diagnostic ability of the PMN count led to higher sensitivity (90 to 100%) and slightly lower specificity (86 to 100%) when the cut-off criterion was set to $\geq 250 \times 10^6$ PMN/L (51, 52). In summary, $PMN \geq 250 \times 10^6/L$ shows optimal sensitivity to detect SBP; whereas, $PMN > 500 \times 10^6/L$ is more specific (Table 1). Taking the severity of this disease, coupled with the high mortality rate if left untreated, the number of false negatives should be at a minimum (high sensitivity). In 2000, a panel of experts formed by the International Ascites Club suggested the best criteria for diagnosing SBP is based on $\geq 250 \times 10^6$ PMN/L in ascitic fluid, which should immediately initiate empiric antibiotic treatment (53).

Pleural fluid

Pleural effusions result from an excessive accumulation of fluid in the pleural cavity, and are associated with a wide variety of disorders. When pleural effusions are discovered, the primary question is whether it is a transudate or an exudate. Common causes of exudates include malignancy, inflammation and infection; whereas, congestive heart failure and osmotic pressure are amongst the many diseases causing transudative effusions (54). A WBC count $\geq 1000 \times 10^6/L$ was three times more frequent in infected compared to non-infected fluids (55). Similar findings were reported by Light et al. (56) who noticed a higher tendency for WBCs to be $< 1000 \times 10^6/L$ in transudative effusions compared to exudative effusions ($WBC > 1000 \times 10^6/L$); although, these differences alone were not significant enough to be of diagnostic value (Table 1). The same authors (57) reported the WBC differential count to be quite helpful in the differential diagnosis. For example,

the presence of PMNs ($\geq 50\%$) in pleural fluids is suggestive of acute pleural inflammation. In the same way, the presence of lymphocytes ($>50\%$) was predominantly found in tuberculous exudates, but this was also a trademark for malignant exudates (58-60). The presence of $>10\%$ eosinophils is correlated with eosinophilic pleural effusion, and found in a wide range of diseases including infection, drug reactions and malignancy (61).

In summary, controversial results regarding the diagnostic value of WBCs in pleural effusions remain. Several authors (58, 62) consider the total WBC and differential count to be useful, while others (63-66) concluded these test are of limited diagnostic value. However, none of the reported studies have assessed the diagnostic accuracy (sensitivity/specificity) of the WBC and differential count. Based on the above literature, there is insufficient evidence supporting the use of WBCs as the sole diagnostic criterion in pleural fluid; however, in combination with biochemical parameters, this data can provide additional information in assisting the separation of transudates (WBC: $<1000 \times 10^6/L$) from exudates (WBC: $>1000 \times 10^6/L$). Furthermore, the WBC differential count can be used to narrow down the diagnostic possibilities in exudative effusions. During the past four decades, Light's criteria have been the most common criteria used for differentiating pleural effusions. It relies on the measurement of biochemical parameters solely (protein and LDH levels) in pleural fluid and serum, and not on cellular parameters (56). The validity of Light's criteria proved to be robust, and has a high (90%) diagnostic accuracy (67).

Synovial fluid

Synovial fluid (SF) is an ultra-filtrate of plasma combined with hyaluronic acid secreted by joint tissue. The analysis of SF is strongly recommended as a vital tool in diagnosing patients with joint effusions, and to classify the fluid as non-inflammatory, inflammatory or septic (68, 69). The SF WBCs and PMNs were recommended as diagnostic parameters to differentiate between various forms of inflammatory disorders (70, 71). Shmerling et al. (72) prospectively studied 100 SFs, and found the WBC count and %PMN to be significantly higher in the inflammatory group (median: 16100×10^6 WBC/L, 90%PMN) compared with the non-inflammatory group (median: 400×10^6 WBC/L, 13%PMN). The diagnostic accuracy obtained from the ROC curve for %PMN (AUC=0.94) was beyond that of the WBC (AUC=0.91) and the LDH (AUC=0.81) in classifying the process as inflammatory or non-inflammatory. Furthermore, they reported the %PMN to be more specific (92%) than the WBC count (84%) in diagnosing inflammatory arthritis (72). Various studies concluded that the WBC count alone is insufficient to reliably discriminate between infectious and inflammatory arthritis because of the tremendous amount of overlap between the groups (73-76). As an example, one study showed 70% of patients with culture-confirmed infections had a WBC count $>50,000 \times 10^6/L$ together with a preponderance of PMN ($>90\%$); whereas, 26% of their patients with inflammatory diseases (gout

and rheumatoid arthritis) had WBC counts in the same range (77). In patients with knee or hip prosthetic joint infections (PJI), the criteria for SF WBC and differential count are different compared to patients without prostheses. One study reported a cut-off value of $>50,000 \times 10^6$ WBC/L (specificity: 99%, sensitivity: 36%) and $>90\%$ neutrophils (specificity: 85%, sensitivity: 89%) to be highly indicative for knee PJI, which is similar to non-prosthetic infections (78). However, a more recent study reported lower cut-off values for WBC (1590×10^6 /L, sensitivity: 0.89, specificity: 0.91, AUC: 0.99) and neutrophils (65%, sensitivity: 0.89, specificity: 0.86, AUC: 0.95) in diagnosing PJI with higher sensitivity compared to previous criteria (79). In summary, evidence regarding the use of the WBC and differential count in SF analysis in literature is mixed. The findings of total WBC and its differential in SFs varied markedly, but in general, the majority of textbooks and studies emphasize that the combination of SF WBCs and PMNs are important diagnostic markers in the immediate discrimination of non-inflammatory, inflammatory and infectious disorders. However, the WBC alone and the %PMN alone are limited in distinguishing among the specific disease categories within inflammatory groups because of the wide range of overlapping results. Some suggest the SF WBC count to be the better predictive marker in inflamed joints; while others consider the %PMN to be more sensitive than the WBC count, especially in the case of septic arthritis (73, 75, 77). Today, many textbooks and publications quote the following traditional classification system composed by the American Rheumatism Association: normal (WBC: $<200 \times 10^6$ /L, PMN: $<25\%$), non-inflammatory (WBC: $<2,000 \times 10^6$ /L, PMN: $<25\%$), inflammatory (WBC: $2,000-50,000 \times 10^6$ /L, PMN: $>50\%$) and infectious (WBC: $>50,000 \times 10^6$ /L, PMN: $>75\%$) (74, 80).

Continuous ambulatory peritoneal dialysis fluid

Patients with end stage renal diseases may be treated with continuous ambulatory peritoneal dialysis (CAPD). Peritonitis, a major complication of CAPD, occurs in 10 to 25% of patients undergoing dialysis, and it is the most frequent cause of technique failure leading to significant morbidity (81). In the past, diagnosis of peritonitis was predominantly based on clinical symptoms and microbiology results. This view shifted in the early 80's when Rubin et al. (82) and others (83-86) reported that the WBC and PMN counts were early indicators of peritonitis. In 43 infected CAPD patients, the WBC count exceeded 100×10^6 /L, and the PMNs accounted for more than 50% in all but one patient (PMN: 37%) (87). In this study, the %PMN in uninfected patients never exceeded 50%, indicating that the %PMN was a better predictive marker for discriminating infected from non-infected, even when the WBCs are $<100 \times 10^6$ /L. Similar results were observed in a retrospective study by our group (unpublished data). In our study, the neutrophilic PMN count was a better predictor of infectious peritonitis compared to the WBC count in 64 infected-related peritonitis cases. Fungal peritonitis (FP) is rare, but is coupled to a high morbidity and mortality rate, and accounts for 3 to 6% of all peritonitis episodes

(88). The clinical symptoms of FP are similar to those of bacterial peritonitis, which makes diagnosis difficult. In a large number ($n=804$) of peritonitis episodes, ~6% were caused by fungi, and WBCs ranged from 150 to $9000 \times 10^6/L$ with a preponderance of PMNs, which is a similar finding for bacterial peritonitis (89). Given the lack of specificity in accurately differentiating bacterial from fungal peritonitis based on cellular analysis, culture results together with clinical judgment remain important parameters in confirming FP (89). Eosinophilic peritonitis (EP) is usually benign. It is either caused by an allergic reaction to some constituents of the peritoneal dialysis system or from intraperitoneal air introduced at the time of catheter placement (90). Treatment regimen includes clinical observation without antibiotic treatment (91). In 1967, Lee and Schoen (92) were the first to describe the presence of elevated eosinophils in a patient undergoing peritoneal dialysis with consistent negative culture results. EP was later defined as either an eosinophilic count greater than 10% of the WBC count, provided the absolute eosinophilic count exceeded $40 \times 10^6/L$ (93), or when the absolute eosinophilic count was $>100 \times 10^6/L$ (94). Eosinophils should always be reported when present. In summary, a review of the literature revealed surprisingly few studies reporting on the diagnostic accuracy of the WBC and PMN count in peritonitis; however, all studies report the WBC, PMN and eosinophilic count to be of paramount importance in diagnosing dialysate peritonitis, and should be included in the diagnostic work-up. According to the International Society for Peritoneal Dialysis, the effluent WBC ($>100 \times 10^6/L$) and PMN ($>50\%$) count, together with patient symptoms, are supportive of early peritonitis diagnosis (Table 1). Also, when more than 100×10^6 eosinophils/L, or $>10\%$ of effluent WBCs are eosinophils, EP should be considered (95).

LABORATORY TECHNIQUES FOR HEMOCYTOMETRIC ANALYSIS

Pre-analytical phase

The pre-analytical phase (specimen collection, transport and processing) of BFs has a great importance on the analysis technique, and can affect the reliability of the results. In compliance with CSLI H56- A guidelines (1), CSF samples should be collected in plain tubes and transported at room temperature to the laboratory immediately. The time delay between CSF collection and laboratory entry should be to a minimum to minimize/avoid cell loss which could result in erroneous WBC/differential counts. One study showed that up to 40% of lymphocytes decayed in native CSF after 90 minutes; moreover, 90% of monocytes and neutrophils were lost after the same time-frame (96). Other BFs (serous fluids, synovial fluids, CAPD) are collected in anticoagulant tubes (EDTA tubes) to prevent cell clumping and to prolong cell stability; however, samples should be administered to the laboratory immediately after collection.

Manual microscopy

The hemocytometer, also referred to as the counting chamber, is the most frequently used method for determining WBCs and RBCs in BFs. The Neubauer-Improved and Fuchs-Rosenthal chambers are the most common, and both utilize the same basic principles, but differ in dimensions (counting grids and depth). The hemocytometer consists of a special optical glass slide with a rectangular indentation that creates a chamber. The addition of Samson or Türk staining reagent may facilitate the total WBC and differential count (66). Accuracy of this device strongly depends on a number of variables including correct loading of sample volume, appropriate dilutions and the number of squares and cells counted. Other methodological factors contributing to the disadvantage of this technique include: high imprecision in samples with low cellularity, the process is tedious, and variability between technicians is high. Variation coefficients for hemocytometers were reported to be as high as 45% (44); moreover, variability between technicians is even more pronounced, and range from 2.5 to 116% for WBCs ($>300 \times 10^6/L$) and up to 141% for RBCs ($>300 \times 10^6/L$) (97, 98). However, if this technique is performed by an experienced observer, disadvantages previously mentioned will be minimized or eliminated.

Stained cytocentrifugation is considered the best preparative method for differentiating cells in BFs (99-101). Major concerns with this procedure are the loss of cells during centrifugation (96, 102), aberrant cell morphology due to the forces of centrifugation (103), and clustering of macrophages or mesothelial cells (which can be mistaken for malignancy, especially in the case of pleural effusions and ascites fluids) (104). To overcome these difficulties, studies recommend specimens to be fresh and processed within a few hours to limit cellular degradation (103), immediate addition of a serum containing medium to CSF tubes to protect against degradation and cellular distortion (105), and to carefully validate centrifugation speed and processing time because these can influence the quality of the slide (104). Apart from manual differentiation, BF cytocentrifuged slides can be automatically analyzed using the BF module on the Cellavision digital microscopy system (DM96) (106). The DM96 is FDA cleared, and enables automatic recognition and pre-classification of eosinophils, neutrophils, lymphocytes and macrophages and other cells. The DM96 accurately pre-classified cells in 90% of the CSF cases and in 83% of the other BFs, and is recommended as a reliable and accurate system (106). In summary, the combination of hemocytometry and stained cytocentrifugation is considered the gold standard for counting and differentiating cells in BFs despite their disadvantages. The reasons are: 1) in small laboratories cell counts are sporadic and automation is not justified, or 2) automated counters still misidentify specific cell types. The results of the gold standards must be interpreted in the context of the method used, specimen type, and technical problems should be considered.

Automated hematology analyzers

Nowadays, automated hematology analyzers are employed for counting blood cells in CSF, pleural fluid, ascites fluid, CAPD, pericardial fluid and synovial fluid amongst others. These machines are designed to be faster, more precise and easier to use compared to traditional manual methods. Many manufactures have adapted flow cytometry and impedance technique for counting and differentiating cells. The principles of these methods are well known and are available in various textbooks and publications (107). To briefly summarize, flow cytometry relies on 3 main properties: size of the particle (forward scatter), granularity or internal complexity of the cell (side scatter) and DNA/RNA binding (fluorescence intensity). After sample incubation and staining with a fluorescence marker, light signals are collected and digitized for computer analysis, and displayed as scattergrams or histograms to provide information about cell populations within a sample. The impedance method requires cells to pass through an aperture where an electrical current is passing. This results in a change in the electrical resistance, which is proportional to the cell volume. With these analyzers, a fixed volume of sample is first mixed and incubated with specific reagents, followed by counting. Automated analyzers aspirates more sample volume compared to the counting chamber. This leads to more cells being counted to enhance precision and accuracy. Common difficulties encountered with these analyzers are: high imprecision in the low range, inability to detect malignant cells and interference from non-cellular particles (crystals, bacteria, fat globules and yeast) which leads to false increased WBC or RBC results (108, 109).

At present, there are a variety of manufacturers (Abbott, Beckman Coulter, Siemens, and Sysmex) on the market with analyzers suited for routine BF analysis, and the majority is FDA cleared (see **Table 2** for summary of the manufacturer's performance specification for each analyzer).

Advia analyzers (Siemens Healthcare Diagnostics, Deerfield, IL, USA)

The ADVIA 120/2120 has a dedicated CSF application. This semi-automated instrument uses direct flow cytometry to count and differentiate cells according to their size and granularity. First, samples are manually pretreated with special CSF reagent to fix and sphere cells, followed by a minimum incubation period of 4 minutes. The following CSF parameters are reported: RBC, WBC, 3-part differential (neutrophils, lymphocytes, monocytes), PMN and mononuclear (MN) cells. The eosinophil count is available as research parameter. For serous fluids and peritoneal dialysis fluids, the total nucleated cell (TNC) and RBC counts are reported. The ADVIA does not provide morphology flags to indicate the presence of abnormal cells. Comparison studies between conventional microscopy and the ADVIA CSF assay showed acceptable results in CSF, serous fluids and synovial fluids (110-119). Common difficulties observed amongst studies were *i*) absence of morphological flagging (making it difficult to detect abnormality in samples), *ii*)

Table 2 Overview of Manufacturer's specification for automated analyzers.

Manufacturer	Model	Analysis Principles	Volume (µL)	Throughput samples/hr	Reportable Parameters	Linearity WBC×10 ⁹ /L	Linearity RBC×10 ⁹ /L	Carry over (%)	Precision WBC (CV%)	Precision RBC (CV%)	FDA Cleared BFs
Symx	XE-5000 BF mode	Fluorescence Flow cytometry Impedance	130	38	WBC, RBC MN, PMN TC-BF	0 – 10,000	0 – 5,000	-	-	-	CSF Peritoneal-dialysate Serous fluids Synovial fluids
	XN-Series BF mode		88	40				≤ 0.3	≤ 7.5	≤ 4.00	
Siemens	120/2120 CSF mode	Flow cytometry Peroxidase staining	175	120	WBC, RBC MN, PMN Neut, lymph Mono	0 – 5,000	0 – 1,500	< 0.4	≤ 15	≤ 10	CSF Peritoneal-dialysate Serous fluids Synovial fluids
	2120 BF mode				TNC, RBC	20 - 400	100 – 6760				
Abbott	CELL-DYN 3500	Flow cytometry Impedance	130	90	WBC, RBC Neut, lymph Baso, Mono EO	0.4-250	0.22 – 750	-	≤ 2.7	≤ 1.5	no -
	CELL-DYN Sapphire		117	105							
Beckman Coulter	LH 750 CSF mode	Impedance	200	110	WBC/TNC RBC	0 – 3,570	0 – 129,000	< 0.64	≤ 4.4	≤ 6.6	CSF Serous fluids Synovial fluids
Symx	UF-1000i/ BF mode	Flow cytometry	800	100	WBC, RBC, EC, Bacteria	1 - 5,000	1 – 5,000	≤ 0.1	≤ 10	≤ 10	no -
	UF-1000i/ BF mode		550	28	WBC, TNC RBC	2 – 10,000	5 – 9999.9	< 0.01	≤ 30	≤ 30	
Iris	iQ200 BF mode	Digital Imaging Auto-recognition	500	-	TNC, RBC	0-10,000	0 – 10,000	-	-	-	CSF Peritoneal-dialysate Serous fluids Synovial fluids

Specification information was taken from the online published food and drug administration (FDA) decision summary reports 510(k) and from manufacturer's online websites. TNC= Total Nucleated Count, EC= epithelial cells, Neut=neutrophils, Mono=monocytes, Lymph= lymphocytes, Baso= basophil, WBC= white blood cells, RBC=Red blood cells. Values are taken from online publications of the FDA

presence of high RBC ($>1500 \times 10^6/L$) counts, (leading to falsely increased WBCs because of overflow in the fixed WBC clusters), and *iii*) incorrect classification of malignant cells, macrophages, atypical lymphocytes and degenerated neutrophil cells (leading to imprecise differential counts). In samples containing $<20 \times 10^6$ WBC/L, the differential count is not reported (111). The best correlations ($r=0.98$) were observed when RBCs were low ($<100 \times 10^6/L$) and the WBCs were high ($\geq 100 \times 10^6/L$) (112). The ADVIA CSF reagent (mixture of formaldehyde and glutaraldehyde) causes cells to shrink and appear smaller in size (112). Because flow cytometry is partially based on the detection of cell size, this phenomenon may hamper the differential results; however, this is yet to be confirmed by other studies. Synovial fluids are highly viscous in nature. Pretreatment of these fluids with hyaluronidase is necessary to prevent a strong negative bias on the ADVIA because of the polymerization in the acidic environment (116). In summary, the ADVIA CSF assay shows promising results for BF analysis making it a suitable analyzer for preliminary evaluation. Some recommend limited use of the Advia for analyzing CSF samples (112) while others promote their use (111). An overview of the Advia's advantages, disadvantages and recommendations based on published literature is summarized in **Table 3**.

Sysmex analyzers BF mode (Sysmex Corporation, Kobe, Japan)

Sysmex analyzers (XE-5000 and XN-Series) are fully automated and contain dedicated BF modes. While the total WBC and its differentials are determined by forward and sideward scattered light (size and inner complexity) and fluorescence intensity (DNA/RNA content) measurements, the RBCs are measured by electrical impedance. The XE-5000 reports RBC, WBC, MN/PMN and (TC-BF). High fluorescence body fluid (HF-BF) cells are reported as research parameter. The HF-BF cluster can consist of macrophages, mesothelial and malignant cells, and are not included in the WBC differential count in contrast to most other BF modes of other manufacturers. Apart from these parameters, the XN-Series reports a 4 part differential (lymphocytes, monocytes, eosinophils and neutrophils) as research parameters. Both analyzers generate one flag ("WBC Abn Scattergram") in the presence of abnormality.

XE-5000 BF mode

The XE-5000 is the most commonly evaluated analyser for BFs analysis. Good results were demonstrated for analysing CSF, serous, CAPD and synovial fluids; although, the results were not always superior (43, 44, 119-128). The most encountered problems were associated with high imprecision of the XE-5000 in the low WBC range ($<20 \times 10^6/L$), and the inability to detect or specifically flag malignant cells. The XE-5000 is shown to have slightly higher WBC counts than manual methods, especially in the lower concentration range (121). More pathological cell counts ($>5 \times 10^6/L$) were detected by the XE-5000 (sensitivity 100%, specificity 75%) compared to manual counts (120, 126). This could be

Table 3 Summary of the advantages, disadvantages and recommendations of automated analyzers.

Counting method	Advantages	Disadvantages	Recommendations	Refs
Hemocytometry (Fuchs-Rosenthal & Neubauer chamber)	<ul style="list-style-type: none"> - Inexpensive - Low sample volume - TNC/WBC/RBC - Reduced TAT - No sample preparation - Low sample volume - Low detection limit (~5×10⁶/L) → XN-BF - 2-part diff (MN/PMN) - 4-part diff (research parameters) - Extended counting (count more cells) - 1 flag to notify abnormality - Commercially QC material available 	<ul style="list-style-type: none"> - High imprecision - High inter-observer variability - Time consuming - Lowest reportable value RBC: 1000×10⁹/L - Overestimation of PMNs in low range (WBC <20×10⁶/L) → XE-BF - High imprecision in the low WBC range (CSF) → XE-BF - Unable to detect malignant cells 	<ul style="list-style-type: none"> - Use only in doubt of results analyzed on automated analyzers and from samples suspected of malignancy (66, 98, 111) - Critically review scattergrams and histograms to detect abnormality and follow up with manual microscopy (43, 44, 119-122, 125, 126, 128, 130) - Not to analyze samples from Oncology patients - Do not report Diff when WBC <10×10⁹/L → XE-BF - Pretreat SFs with hyaluronidase - Samples should be diluted when RBCs 1500×10⁶/L or use special hemolysis reagent - Not to analyze samples from Oncology patients (110-112, 115-118) - Cytopsin slide in the presence of abnormality - Reports WBC Diff only when WBCs 20×10⁶/L - Only report counts greater than the detection limits - Do not analyze samples from Oncology patients (131-134) - Run blank sample after blood sample to eliminate carryover 	
Sysmex (XE-5000 BF-mode & XN-Series BF-mode)	<ul style="list-style-type: none"> - Longer cell stability in samples - Low detection limit (~2×10⁷/L) - 3-part diff (lymph, mono, neutro) - Eosinophils (research parameter) - Detection of Cryptococcus in CSF samples (distinct cytogram pattern) - Commercially QC material available 	<ul style="list-style-type: none"> - Samples containing ↑ RBCs or incomplete lysis of RBCs leads to false elevated WBCs - Do not provide morphology flags - Requires manual predilution - Incorrect classification of malignant cells and atypical lymphocytes - High imprecision in the low range (CSF) 		
Siemens (Advia 120/2120 CSF mode & BF mode)	<ul style="list-style-type: none"> - 5-part diff (lympho, mono, neutro, eo, baso) - Extended count mode (count more cells) - Possible to detect malignant cells by using monoclonal antibodies - 3 flags to notify WBC abnormality 	<ul style="list-style-type: none"> - Detection limits WBC 50×10⁶/L and RBCs 3000×10⁶/L - Overestimation PMNs in serous fluids - No flagging for abnormality - Not FDA cleared 		
Abbott (CELL-DYN 3500 & CELL-DYN Sapphire)				

Table 3 Summary of the advantages, disadvantages and recommendations of automated analyzers. (continued)

Counting method	Advantages	Disadvantages	Recommendations	Refs
Beckman Coulter (LH 750 BF mode)	<ul style="list-style-type: none"> - Reduced TAT - WBC (TNC)/RBC 	<ul style="list-style-type: none"> - Do not report diff counts - Overestimation of PMNs in serous fluids - Detection limit WBC ($>200 \times 10^6/L$) and RBC ($>10,000 \times 10^6/L$) - No detection of malignant cells 	<ul style="list-style-type: none"> - Restricts CSF and peritoneal fluids analysis - Only analyze samples greater than detection limits - Pretreat SFs with hyaluronidase 	(98, 136, 137)
Symex (urinalysis) (UF-1000i & UF-1000i BF mode)	<ul style="list-style-type: none"> - Report semi-quantitative bacteria counts (urine mode) - Reports WBC/RBC/TNC (BF mode) 	<ul style="list-style-type: none"> - Do not report Diff counts - Requires high sample volume - Overestimation WBCs in serous fluids (BF mode) - Not FDA cleared 	<ul style="list-style-type: none"> - Critically review scatterplots to detect Abnormality especially in serous fluids 	(144, 145, 147)
Iris (urinalysis) (iQ200 BF-mode)	<ul style="list-style-type: none"> - Reduced TAT - Reports RBC/TNC 	<ul style="list-style-type: none"> - Semi-automated (requires 2 manual dilutions) - Decreased WBC counts in serous fluids due to cell Clumping - Do not report DIFF counts 	<ul style="list-style-type: none"> - Critically review plots to detect cell clumping - Dilute samples based on appearance 	(135, 139-141)

TAT= turnaround time, SF= synovial fluid, Diff= differential, QC= quality control, TNC= total nucleated count

partially explained by the imprecision of both methods in the lower range (44) or the slightly higher reference values for automated methods (43). Another explanation might be the presence of cell debris or interfering fragments, causing the overestimation of WBCs/PMNs on the XE-5000. This phenomenon was more pronounced in CSF samples with low cell counts (WBC: $<20 \times 10^6/L$) and in samples taken from ventricular drainage systems (43, 121). To solve this problem, Sysmex upgraded its software on the XE-5000 by improving gating algorithms; however, a positive bias remained for PMNs with low WBCs (127). The lower limit of quantitation (LoQ) is defined as “the lowest concentration in a sample that can be quantitatively determined with acceptable imprecision (coefficient of variation: 20%)” (129). The LoQ of the XE-5000 is $\sim 10 \times 10^6$ WBCs/L (43). Because the XE-5000 lacks precision in the lower range, it was recommended that samples with low WBCs ($5 - 20 \times 10^6/L$) be manually recounted (121), and in samples containing $<10 \times 10^6$ WBCs/L, the differential count should not be reported (43). The accuracy of the “WBC Abnormal Scattergram flag” was evaluated in CSF samples and showed poor sensitivity (60%) but good specificity (96%) (44). Of the eight false negative cases, 4 cases had lymphocytosis with few reactive lymphocytes, 3 cases had neural tissue, and 1 case was suspected for malignancy. The HF-BF count should not be used as a diagnostic test (sensitivity: 75%, specificity: 73%) for identification or exclusion of neoplastic meningitis (125). In cases where a high percentage of HF cells are incidentally present, further evaluation by manual microscopy is necessary (43, 121, 122). RBCs are counted in a separate RBC channel using impedance technique on the XE-5000. Unlike the Advia CSF mode, blood stained fluids do not interfere with the WBC/differential count on Sysmex analyzers. However, the lowest reportable value for RBCs on the XE-5000 is $1000 \times 10^6/L$.

XN- BF mode

The XN-BF mode is relatively new, which explains the low quantity of published data. We evaluated this mode and found good agreement with manual microscopy for counting cells in CSF, serous and CAPD samples (130). The XN-BF mode overestimated the CSF PMN count compared to microscopy. This could possibly be explained by the fact that cells break down during cytopspin preparation, and because CSF WBCs are known to decay easily due to their fragile nature. Other fluids (CAPD, Ascites and Pleura fluid), investigated in the same study did not show an overestimation of PMNs. Furthermore, the sensitivity (100%) and specificity (97%) on the XN-BF mode were good for predicting abnormality in CSF (WBC: $>6 \times 10^6/L$) (130). In summary, Sysmex analyzers have proven to be accurate and sensitive for processing BF samples while offering advantages such as cost effectiveness and faster turnaround times. Literature reveals contrasting data on the analysis of CSF on these analyzers. Although similar results were found in studies, the interpretations and recommendations differed. An overview of the advantages, disadvantages and recommendations, based on published literature are summarized in Table 3.

CELL-Dyn (Abbott diagnostics, Abbott Park, IL, USA)

The CELL-DYN Sapphire hematology analyzer and its predecessor, the CELL-DYN 3500, are fully automated and primarily designed for measuring cells in blood samples. They do not contain any application for separate BF analysis. Nevertheless, investigators have evaluated their performance for counting cells in BFs. The CELL-DYN analyzers enumerate and differentiate WBCs by dual technologies (multi-angle polarized scatter separation combined with impedance method). The total WBC and a 5-part differential (lymphocytes, monocytes, neutrophils, basophils and eosinophils) are reported. RBCs on the CELL-DYN 3500 are measured by impedance technique; whereas, on the CELL-DYN Sapphire, they are measured by impedance as well as optically. Three flags are generated when WBC abnormality is suspected in blood.

The CELL-DYN 3500 should not be used for counting cells in CSF (113, 131) because CSF samples containing high numbers of lymphocytes and atypical lymphocytes resulted in false elevated WBCs on the 3500 (131). Abbott's newer generation, the CELL-DYN Sapphire, was evaluated for counting cells in CSF and serous fluids (132). No significant differences were found when concentrations exceeded the detection limits ($>50 \times 10^6$ WBC/L and $>3000 \times 10^6$ RBC/L); however, samples below these values differed significantly. Furthermore, the Sapphire significantly overestimated the PMN count, and underestimated the MNC count in serous fluids compared to manual microscopy. Apparently, macrophages and mesothelial cells are clustered as PMNs, making the automated differential counts unreliable in these fluids. To overcome this problem, new software with optimized gating algorithms specifically for BF analysis was developed, and its performance was evaluated in serous fluids. Results were greatly improved between microscopy and the optimized method compared with the standard algorithm. Despite the use of the improved software, the Sapphire was unable to correctly identify monocytic cells (133). The Sapphire has a high diagnostic ability (sensitivity and specificity $>90\%$) to distinguish normal from abnormal samples in serous and CAPD samples; whereas, for CSF samples (cut off limit WBC: $>5 \times 10^6$ /L), sensitivity was poor (45%) (132). This could be expected because of analyzers high detection limits. Furthermore, the ability of the Sapphire to detect malignant cells resulted in low sensitivity (20%) but high specificity (94%). The Sapphire correctly flagged 2 out of the 7 samples flagged for malignancy, but failed to flag 8 other samples with metastatic cells. However, when an immunophenotypic assay (using monoclonal antibodies) on the Sapphire was used, sensitivity increased to 75% (134). In summary, CELL-DYN analyzers are easy to use, and with exception to CSF samples, they are suitable as a screening tool for BF analysis. Like other hematology analyzers, the Sapphire is unable to detect or generate a flag for the presence of malignant cells. However, unlike other analyzers, this instrument offers the possibility to detect such cells by using specific monoclonal antibodies. An overview

of advantages, disadvantages and recommendations based on published literature are summarized in Table 3.

LH 750 (Beckman Coulter, Miami, FL, USA)

The LH 750 is fully automated and contains a separate BF application. The LH 750 is based on impedance technology combining direct current (size of cells), conductivity (internal structure) and light scatter (cell surface and granularity) to count and report WBC/TNC and RBC counts.

The LH 750 reports inaccurate results when concentrations are below the manufacturer's detection limits (WBC: $200 \times 10^6/L$ and RBC: $10,000 \times 10^6/L$). However, when samples contained counts greater than the reportable limits, correlation coefficients improved and no statistical differences were observed between automated and manual method (98, 135-137). Because ~ 90% of CSF samples have WBC counts lower than the detection limits of this analyzer, it is recommended not to use this instrument for CSF and peritoneal fluid analysis, and to analyze these samples manually instead to ensure accuracy (98, 135). The LH 750 was able to discriminate normal from abnormal samples with high sensitivity (>85%) in serous fluids and synovial fluids (135). For CSF samples, the LH 750 misclassified 64% of samples according to clinical threshold (WBC: $>5 \times 10^6/L$) (135). The LH 750 was compared with the FC500 flow cytometry (Beckman Coulter) for counting WBCs (differential) in ascitic fluids (138). The methods correlated well for WBCs ($r=0.99$) and moderately for neutrophils ($r=0.82$). In the majority of cases, the LH 750 counted higher neutrophils than flow cytometry. It seems as if macrophages were being misclassified as neutrophils on the LH 750, which led to the overestimation. In summary, the LH 750 BF application has been proven to be accurate and precise in measuring all types of BF samples containing $>200 \times 10^6$ WBC/L and $>10,000 \times 10^6$ RBC/L. An overview of advantages, disadvantages and recommendations, based on published literature, are summarized in Table 3.

Other analyzers

Apart from hematology analyzers, urinalysis analyzers such as the Sysmex UF-Series and Iris iQ200 have grown in popularity for BF analysis.

iQ200 (Iris Diagnostics, Chatsworth, CA)

The Iris iQ200 is a semi-automated analyzer with a BF module. The TNC and RBC counts are determined by using flow cell digital imaging and auto particle recognition. Comparative studies between the automated iQ200 and microscopy showed good to excellent correlation (0.84 to 0.99) for WBCs and RBCs in various BFs including CSF (139-141). Some concluded that the precision of the iQ200 may not be adequate for CSF analysis because of its detection limit (35×10^6 WBC/L)(140), while others report the iQ200 to

have high precision in the lower range, deeming it acceptable for CSF analysis (139, 141). Furthermore, WBCs in serous fluids were falsely decreased on the iQ200 because of nucleated cell clumping in these samples (139).

Sysmex UF-1000i

The Sysmex UF-Series is fully automated, and uses hydrodynamic focusing and flow cytometry technique to count particles. It reports WBCs and RBCs along with other urinalysis parameters including bacteria and yeast counts. In 2014, Sysmex urinalysis division launched its first application for BF analysis on the UF-1000i. It employs the same techniques as the urine mode, but contains new gating's and modified algorithms to enhance cell counting. The UF-1000i BF mode reports TNCs, WBCs and RBCs.

Good agreement between the UF-1000i urine mode and manual microscopy for RBCs and WBCs was found (142-146). We observed that the optical RBC count on the UF outperformed the impedance count on the XE analyzers (unpublished data). The UF-1000i urine mode contains improved algorithms (ability to classify lymphocytes as WBCs) compared to its predecessor (UF-100) (142). This solved the problem of WBC underestimation in the presence of elevated lymphocytes on the UF-100 (145). Furthermore, the diagnostic accuracy of the UF-1000i for differentiating between pathological and normal CSF (cut-off: 4×10^6 WBC/L) showed 100% sensitivity and 84% specificity (144). The UF-1000i BF mode was evaluated in our lab using CAPD and serous fluids (147). Agreement between the UF-BF mode and the counting chamber was acceptable. The UF overestimated the WBCs in serous fluids. A possible explanation could be the presence of macrophages and mesothelial cells which are being counted as WBCs. In addition, the diagnostic accuracy of the UF-BF showed excellent sensitivity (100%) and a specificity of 78% and 83%, respectively to detect WBCs in CAPD fluid ($>100 \times 10^6$ /L) and ascites ($>250 \times 10^6$ /L). In summary, the analysis of various BFs on urinalysis analyzers showed satisfactory agreement for rapid and accurate screening of total WBCs and RBCs. Like hematology analyzers, conflicting evidence remain for measuring CSF samples on these analyzers because of their detection limits. Compared to hematology analyzers and manual methods, urinalysis analyzes require far more sample volume, which can be problematic for CSF. However, CSF samples containing low volumes can be manually prediluted (1:5) and measured on Sysmex UF-1000i urine mode (145). Naturally, the LoQ will increase accordingly with the dilution factor and accuracy is also compromised, which hampers counting in CSF with low WBC counts. Results obtained by urinalysis analyzers are mostly restricted to WBCs and RBCs. This limits their use because most guidelines rely on the combination of WBC and differential counts to differentiate between infectious and non-infectious diseases. Despite the fact that these analyzers do not report a differential count, they have the ability to detect and semi-quantitate bacteria and yeast cells. A future possibility to report bacteria counts along with the

total WBC and differential count simultaneously would be meaningful for clinicians in the early diagnosis of peritonitis and bacterial meningitis.

Man versus Machine in the diagnosis of inflammation

The hematology and urinalysis analyzers reviewed in this study showed promising results when compared with manual microscopy (**Table 4**). Different studies confirmed many of their benefits such as improved proficiency and workflow productivity, and recommend them as suitable alternatives for conventional microscopy (Table 3). With this in mind, one can predict that it is only a matter of time before all labs make the leap from manual to automated methods for BF analysis to diagnose infection and inflammation. However, there are still some unresolved issues regarding these analyzers. First, the most common literature discussions pertain to the inability of some analyzers to accurately count cells in the lower concentration ranges. Samples containing low WBCs $<30 \times 10^6/L$ can be problematic for some of these analyzers due to their relative high LoQ (Table 4) which limits their utility for CSF analysis. Analyzers such as the CELL DYN and LH 750 using impedance technology have high background counts which prevent or hinder accuracy in detecting low cell counts (Table 2). In contrast, a LoQ of 5×10^6 WBC/L for all fluids on the Sysmex XN-Series BF mode, and 2×10^6 WBC/L for CSF on the Advia 120/2120 probably make these analyzers more suitable for low WBC counts in CSF. An ongoing challenge will be for manufacturers to develop analyzers with extremely low detection limits and high precision, especially for CSF. Second, automated analyzers are unable to reliably differentiate between hematological and non-hematological cells. Serous fluids are characterized by their unique composition of biological matrix, and by the presence of cellular elements such as macrophages and mesothelial cells. Though these cells are generally larger (15-21 μm) than WBCs (10-20 μm), there is a window of overlap which causes them to be mistakenly counted as WBCs. The presence of macrophages and mesothelial cells in serous fluids can lead to falsely elevated WBCs, and furthermore, mistakenly assigned to MNs and/or PMNs (43, 132, 138, 147). The acquisition of these false positive data can result in misdiagnosis, and have adverse implications for therapy in SBP for example. Unfortunately, not all analyzers report TNC and WBC counts separately (Table 2), and often, non-WBCs (included in the TNC count) are counted as WBCs or the TNC and WBC count are similar. The Sysmex BF-mode on the XE and XN-Series analyzers excludes HF-BF cells such as macrophages and mesothelial cells from the WBC differential count, limiting their positive interference (43). Other analyzers, containing dedicated BF modes such as the Beckman 750, Siemens 2120i BF mode and the Iris iQ200, reports the WBC (TNC) count, hence the TNC count is equal to the WBC count for these analyzers, which is not always the case. According to CSLI H56-A guidelines (1), the TNC should include all nucleated cells including nucleated WBCs, NRBCs, lining cells and other non-hematological cells. In the majority of cases, it's the WBC/WBC differential

Table 4 Published results on performance evaluations on automated analyzers for CSF analysis.

	Number of samples in study	Parameters	Slope, r	Sen/Spec (%)	Linearity WBCx10 ⁶ /L	Linearity RBCx10 ⁶ /L	LoQ WBCx10 ⁶ /L	Carry over WBC%	Ref
XE-5000 BF mode		WBC	1.15*						
	69	RBC	-	-	0 – 13,000	0 – 5,000	10	<0.17	(43)
		PMN	2.40*						
		MIN	1.08						
XN-1000 BF mode		WBC	1.06*, r=0.96						
	67	RBC	0.99, r=0.99	100/97	0 – 12,000	0 – 8,000	5	<0.05	(130)
		PMN	1.48*, r=0.99						
		MIN	1.04, r=0.93						
ADVIA 120 CSF assay		WBC	0.83, r=0.98						
	60	RBC	-	-	0 – 9,850	0 – 20,000	2 ≈	-	(118)
		PMN	0.83, r=0.98						
		MIN	0.83, r=0.98						
CELL-DYN 3500		WBC	1.11*, r=0.92						
	73	RBC	-	-	5- 900	-	-	-	(131)
		WBC	1.04, r=1.00						
		RBC	1.06, r=1.00	97/45	10 – 900	3,000-90,000	50	-	(132)
CELL-DYN Sapphire		PMN	0.87, r=1.00						
	30	MIN	0.89, r=1.00						
		WBC	0.89, r=0.99		200 – 3,570	10,000-129,000	200	<0.64	(137)
	148	RBC	1.05, r=0.84						
LH 750		WBC	0.93*, r=0.99	100/84	4- 278	-	-	<0.3	(144)
	77	RBC	r=0.88						
UF-1000i		WBC	r=0.91						
	256	RBC	1.10, r=0.99						
UF-100		WBC	0.81, r=0.99						
	66	RBC			0 – 2,558	0-43,829	35	0	(139)

The r values demonstrate the correlation between automated analyzers and manual microscopy. * = significant. LoQ = lower limit of quantitation at a CV of 20%, ≈ CV = 30%. For the LH750, results are for samples correlating above the detection limits. Results were considered normal or abnormal based on counting chamber (WBCs < 6x10⁶/L), Ref = references

count that reflects different stages of the inflammation/infection; therefore reporting accurate results for the WBC count is of utmost importance. Manufacturers should improve this interference in the WBC count to be able to deliver reliable and fast total WBC and differential counts to aid the diagnosis of inflammation. Furthermore, all automated analyzers could not accurately identify or flag malignant cells. This is clearly reflected in the low sensitivity values found (125). These cells, if present, were being misclassified, mainly as lymphocytes. In light of these limitations we suggest *i*) malignant body fluids should always be analyzed by manual microscopy, and *ii*) doubtful automated results of inflamed BFs such as abnormal scatterplots, suspected interferences or many large cells should always be followed up by manual/digital microscopy to reveal the cause. The diagnostic accuracy of automated analyzers to differentiate between normal and abnormal conditions showed good results. Though the sensitivity was nearly always 100%, the specificity ranged between 70 - 85%. This means patients with negative results can be spared antibiotic treatment; however, there is a higher chance of reporting false positive results compared with manual counts. In summary, it is our experience, in common with those of others, that automated analyzers are suitable for BF analysis of suspected inflammatory disease by providing preliminary rapid results for clinical action which is likely to benefit patients' care. Deciding on which method to use is dependent on the laboratory's preference, experience and an assessment of the advantages and disadvantages each analyzer has to offer.

REFERENCES

1. Szamosi D, Bautista J, Cornbleet P, Glasser L, Rothe G, Sandhaus L, et al. Body fluid analysis for cellular composition; Approved guideline. H56-A. 2006.
2. Krieg A. Cerebrospinal fluid and other body fluids. In: Henry J, editor. *Clinical Diagnosis and Management by Laboratory Methods*. Philadelphia: Saunders, WB; 1979. p. 635-79.
3. Tunkel AR, Hartman BJ, Kaplan SL, Kaufman BA, Roos KL, Scheld WM, et al. Practice guidelines for the management of bacterial meningitis. *Clin Infect Dis*. 2004;39:1267-84.
4. Karandanis D, Shulman JA. Recent survey of infectious meningitis in adults: review of laboratory findings in bacterial, tuberculous, and aseptic meningitis. *South Med J*. 1976;69:449-57.
5. Nye FJ. The value of initial laboratory investigations in the management of meningitis. *J Infect*. 1983;7:31-8.
6. Lindquist L, Linne T, Hansson LO, Kalin M, Axelsson G. Value of cerebrospinal fluid analysis in the differential diagnosis of meningitis: a study in 710 patients with suspected central nervous system infection. *Eur J Clin Microbiol Infect Dis*. 1988;7:374-80.
7. Spanos A, Harrell FE, Jr, Durack DT. Differential diagnosis of acute meningitis: An analysis of the predictive value of initial observations. *JAMA*. 1989;262:2700-7.
8. Hoen B, Viel JF, Paquot C, Gerard A, Canton P. Multivariate approach to differential diagnosis of acute meningitis. *Eur J Clin Microbiol Infect Dis*. 1995;14:267 - 74.
9. Nigrovic LE, Kuppermann N, Macias CG, Cannavino CR, Moro-Sutherland DM, Schremmer RD, et al. Clinical prediction rule for identifying children with cerebrospinal fluid pleocytosis at very low risk of bacterial meningitis. *JAMA*. 2007;297:52-60.
10. Dubos F, Korczowski B, Aygun DA, Martinot A, Prat C, Galetto-Lacour A, et al. Distinguishing between bacterial and aseptic meningitis in children: European comparison of two clinical decision rules. *Arch Dis Child*. 2010;95:963-7.
11. Nigrovic LE, Kuppermann N, Malley R. Development and Validation of a Multivariable Predictive Model to Distinguish Bacterial From Aseptic Meningitis in Children in the Post-Haemophilus influenzae Era. *Pediatrics*. 2002;110:712-9.
12. McKinney WP, Heudebert GR, Harper SA, Young MJ, McIntire DD. Validation of a clinical prediction rule for the differential diagnosis of acute meningitis. *J Gen Intern Med*. 1994;9:8-12.
13. Jaeger F, Leroy J, Duchene F, Baty V, Baillet S, Estavoyer JM, et al. Validation of a diagnosis model for differentiating bacterial from viral meningitis in infants and children under 3.5 years of age. *Eur J Clin Microbiol Infect Dis*. 2000;19:418-21.
14. Leblebicioglu H, Esen S, Bedir A, Gunaydin M, Sanic A. The validity of Spanos and Hoen's models to differential diagnosis of meningitis. *Eur J Clin Microbiol Infect Dis*. 1996;15:252 - 4.
15. Smith G, Kjeldsberg C. Cerebrospinal, Synovial and Serous body fluids. In: Henry JB, editor. *Clinical Diagnosis and Management by Laboratory Methods* 20th ed. Philadelphia: WB Saunders; 2001. p. 403- 24.
16. Deisenhammer F, Bartos A, Egg R, Gilhus NE, Giovannoni G, Rauer S, et al. Guidelines on routine cerebrospinal fluid analysis. Report from an EFNS task force. *Eur J Neurol*. 2006;13:913-22.
17. Ross D, Rosegay H, Pons V. Differentiation of aseptic and bacterial meningitis in postoperative neurosurgical patients. *J Neurosurg*. 1988;69:669-74.
18. Powers WJ. Cerebrospinal fluid lymphocytosis in acute bacterial meningitis. *Am J Med*. 1985;79:216-20.

19. Viallon A, Desseigne N, Marjollet O, Biryńczyk A, Belin M, Guyomarch S, et al. Meningitis in adult patients with a negative direct cerebrospinal fluid examination: value of cytochemical markers for differential diagnosis. *Crit Care*. 2011;15:R136.
20. Landaas S, Von der Lippe B. Chemical analyses for early differential diagnosis between bacterial and viral meningitis. *Scand J Clin Lab Invest*. 1985;45:525-9.
21. Wadhwa A, Kaur R, Bhalla P. Profile of central nervous system disease in HIV/AIDS patients with special reference to Cryptococcal infections. *Neurologist*. 2008;14:247-51.
22. Anderson NE, Willoughby EW. Chronic Meningitis without Predisposing Illness—A Review of 83 Cases. *Q J Med*. 1987;63:283-95.
23. Thwaites GE, van Toorn R, Schoeman J. Tuberculous meningitis: more questions, still too few answers. *The Lancet Neurology*. 2013;12:999-1010.
24. Marx GaCE. Tuberculous Meningitis: Diagnosis and Treatment Overview. *Tuberc Res Treat*. 2011;2011:798764.
25. Jeren T, Beus I. Characteristics of cerebrospinal fluid in tuberculous meningitis. *ACTA CYTOL*. 1982;26:678-80.
26. Kumar R, Singh SN, Kohli N. A diagnostic rule for tuberculous meningitis. *Arch Dis Child*. 1999;81:221-4.
27. Luma HN, Tchaleu BC, Ngahane BH, Temfack E, Doualla MS, Halle MP, et al. Tuberculous meningitis: presentation, diagnosis and outcome in hiv-infected patients at the douala general hospital, cameroon: a cross sectional study. *AIDS Res Ther*. 2013;10:16.
28. Miller MA, Menowsky M, Leeson K, Leeson B. Eosinophilic meningitis: what's the "diff"? *Am J Emerg Med*. 2014;32:107.e5-7.
29. Graeff-Teixeira C, da Silva AC, Yoshimura K. Update on eosinophilic meningoencephalitis and its clinical relevance. *CLIN MICROBIOL REV*. 2009;22:322-48.
30. Greenlee JE. Approach to diagnosis of meningitis. *Cerebrospinal fluid evaluation*. *Infect Dis Clin North Am*. 1990;4:583-98.
31. Seehusen DA, Reeves MM, Fomin DA. Cerebrospinal fluid analysis. *Am Fam Physician*. 2003;68:1103-8.
32. Straussberg R, Harel L, Nussinovitch M, Amir J. Absolute neutrophil count in aseptic and bacterial meningitis related to time of lumbar puncture. *Pediatr Neurol*. 2003;28:365-9.
33. Bonadio WA. The cerebrospinal fluid: physiologic aspects and alterations associated with bacterial meningitis. *Pediatr Infect Dis J*. 1992;11:423-31.
34. Jerrard DA, Hanna JR, Schindelheim GL. Cerebrospinal fluid. *J Emerg Med*. 2001;21:171-8.
35. Sommer JB, Gaul C, Heckmann J, Neundorfer B, Erbguth FJ. Does lumbar cerebrospinal fluid reflect ventricular cerebrospinal fluid? A prospective study in patients with external ventricular drainage. *Eur Neurol*. 2002;47:224-32.
36. Kestenbaum LA, Ebberson J, Zorc JJ, Hodinka RL, Shah SS. Defining cerebrospinal fluid white blood cell count reference values in neonates and young infants. *Pediatrics*. 2010;125:257-64.
37. Ahmed A, Hickey SM, Ehrett S, Trujillo M, Brito F, Goto C, et al. Cerebrospinal fluid values in the term neonate. *Pediatr Infect Dis J*. 1996;15:298-303.
38. Portnoy JM, Olson LC. Normal cerebrospinal fluid values in children: another look. *Pediatrics*. 1985;75(3):484-7.
39. McDiarmid N. The Value of Routine Examination of the Cerebrospinal Fluid: Report on the Examination of 3,200 Fluids. *J Neurol Psychopathol*. 1931;s1-11:247-54.
40. Jurado R, Walker K. Cerebrospinal fluid. In: Walker K, Hall W, Hurst J, editors. *Clinical Methods: The history, Physical, and Laboratory Examinations*. 3rd ed. Boston 1990.

41. Bonadio WA, Stanco L, Bruce R, Barry D, Smith D. Reference values of normal cerebrospinal fluid composition in infants ages 0 to 8 weeks. *Pediatr Infect Dis J*. 1992;11:589-91.
42. Sarff LD, Platt LH, McCracken GH, Jr. Cerebrospinal fluid evaluation in neonates: comparison of high-risk infants with and without meningitis. *J Pediatr*. 1976 Mar;88(3):473-7.
43. De Jonge R, Brouwer R, De Graaf MT, Luitwieler RL, Fleming C, De Frankrijker-Merkestijn M, et al. Evaluation of the new body fluid mode on the Sysmex XE-5000 for counting leukocytes and erythrocytes in cerebrospinal fluid and other body fluids. *Clin Chem Lab Med*. 2010;48:665-75.
44. Sandhaus LM, Ciarlini P, Kidric D, Dillman C, O'Riordan M. Automated cerebrospinal fluid cell counts using the sysmex XE-5000: Is it time for new reference ranges? *Am J Clin Pathol*. 2010;134:734-8.
45. Bremell D, Mattsson N, Wallin F, Henriksson J, Wall M, Blennow K, et al. Automated cerebrospinal fluid cell count — New reference ranges and evaluation of its clinical use in central nervous system infections. *Clin Biochem*. 2014;47:25-30.
46. Link BC, Ziske CG, Schepke M, Schmidt-Wolf IGH, Sauerbruch T. Total ascitic fluid leukocyte count for reliable exclusion of spontaneous bacterial peritonitis in patients with ascites. *Eur J Gastroenterol Hepatol*. 2006;18:181-6.
47. Bar-Meir S, Lerner E, Conn HO. Analysis of ascitic fluid in cirrhosis. *Dig Dis Sci*. 1979.
48. Yang CY, Liaw YF, Chu CM, Sheen I. White count, pH and lactate in ascites in the diagnosis of spontaneous bacterial peritonitis. *Hepatology*. 1985;5:85-90.
49. Garcia-Tsao G, Conn HO, Lerner E. The diagnosis of bacterial peritonitis: comparison of pH, lactate concentration and leukocyte count. *Hepatology*. 1985;5:91-6.
50. Stassen WN, McCullough AJ, Bacon BR, Gutnik SH, Wadiwala IM, McLaren C, et al. Immediate diagnostic criteria for bacterial infection of ascitic fluid. Evaluation of ascitic fluid polymorphonuclear leukocyte count, pH, and lactate concentration, alone and in combination. *Gastroenterology*. 1986;90:1247-54.
51. McGibbon A, Chen GI, Peltekian KM, van Zanten SV. An evidence-based manual for abdominal paracentesis. *Dig Dis Sci*. 2007;52:3307-15.
52. Conn HO. Acidic ascitic fluid: a leap forward (or a step?). *Hepatology*. 1982;2:507-10.
53. Rimola A, García-Tsao G, Navasa M, Piddock LJV, Planas R, Bernard B, et al. Diagnosis, treatment and prophylaxis of spontaneous bacterial peritonitis: a consensus document. *J Hepatol*. 2000;32:142-53.
54. Collins TR, Sahn SA. Thoracocentesis. Clinical value, complications, technical problems, and patient experience. *CHEST*. 1987;91:817-22.
55. Paddock FK. The Diagnostic Significance of Serous Fluids in Disease. *N Engl J Med*. 1940;223:1010-5.
56. Light RW, Macgregor MI, Luchsinger PC, Ball WC, Jr. Pleural effusions: the diagnostic separation of transudates and exudates. *Ann Intern Med*. 1972;77:507-13.
57. Light RW, Erozan YS, Ball WC, Jr. Cells in pleural fluid. Their value in differential diagnosis. *Arch Intern Med*. 1973;132:854-60.
58. Pettersson T, Riska H. Diagnostic value of total and differential leukocyte counts in pleural effusions. *Acta Med Scand*. 1981;210:129-35.
59. Epstein DM, Kline LR, Albelda SM, Miller WT. Tuberculous pleural effusions. *CHEST*. 1987;91:106-9.
60. Light RW. Clinical practice. Pleural effusion. *N Engl J Med*. 2002;346:1971-7.
61. Krenke R, Nasilowski J, Korczynski P, Gorska K, Przybylowski T, Chazan R, et al. Incidence and aetiology of eosinophilic pleural effusion. *Eur Respir J*. 2009;34:1111-7.
62. Rashmi K, Shashikala O, Hiremath S, Basavaraj H, Shashikala O, Hiremath S, et al. Cells in pleural fluid and their value in differential diagnosis. *J Cytol*. 2008;25:138-43.

63. Dines DE, Pierre RV, Franzen SJ. The value of cells in the pleural fluid in the differential diagnosis. *Mayo Clin Proc.* 1975;50:571-2.
64. Storey DD, Dines DE, Coles DT. Pleural effusion. A diagnostic dilemma. *JAMA.* 1976;236:2183-6.
65. Leuallen EC, Carr DT. Pleural Effusion. *N Engl J Med.* 1955;252:79-83.
66. Kjeldsberg CR, Knight JA. *Body Fluids: Laboratory Examination of Amniotic, Cerebrospinal, Seminal, Serous & Synovial Fluids : a Textbook Atlas 2nd edition.* Chicago: American Society of Clinical Pathologists Press; 1986
67. Joseph J, Badrinath P, Basran GS, Sahn SA. Is the pleural fluid transudate or exudate? A revisit of the diagnostic criteria. *Thorax.* 2001;56:867-70.
68. Freemont AJ. Microscopic analysis of synovial fluid—the perfect diagnostic test? *Ann Rheum Dis.* 1996;55:695-7.
69. Freemont A. Role of cytological analysis of synovial fluid in diagnosis and research. *Ann Rheum Dis.* 1991;50:120-3.
70. Ropes MW, Rossmeisil EC, Bauer W. The Origin and Nature of Normal Human Synovial Fluid. *J CLIN INVEST.* 1940;19:795-9.
71. Hollander JL, Reginato A, Torralba TP. Examination of synovial fluid as a diagnostic aid in arthritis. *Med Clin North Am.* 1966;50:1281-93.
72. Shmerling RH, Delbanco TL, Tosteson AN, Trentham DE. Synovial fluid tests. What should be ordered? *JAMA.* 1990;264:1009-14.
73. Baran S, Price C, Hak DJ. Diagnosing joint infections: synovial fluid differential is more sensitive than white blood cell count. *Eur J Orthop Surg Traumatol.* 2014;24:1469-74.
74. McCutchan HJ, Fisher RC. Synovial leukocytosis in infectious arthritis. *Clin Orthop Relat Res.* 1990;257:226-30.
75. Brannan SR, Jerrard DA. Synovial fluid analysis. *J Emerg Med.* 2006;30:331-9.
76. Dougados M. Synovial fluid cell analysis. *Baillieres Clin Rheumatol.* 1996;10:519-34.
77. Krey PR, Bailen DA. Synovial fluid leukocytosis. A study of extremes. *Am J Med.* 1979;67:436-42.
78. Spangehl MJ, Masri BA, O'Connell JX, Duncan CP. Prospective analysis of preoperative and intraoperative investigations for the diagnosis of infection at the sites of two hundred and two revision total hip arthroplasties. *J Bone Joint Surg Am.* 1999;81:672-83.
79. Dinneen A, Guyot A, Clements J, Bradley N. Synovial fluid white cell and differential count in the diagnosis or exclusion of prosthetic joint infection. *Bone Joint J.* 2013;95-B:554-7.
80. Horowitz DL, Katzap E, Horowitz S, Barilla-LaBarca ML. Approach to septic arthritis. *Am Fam Physician.* 2011;84:653-60.
81. Warady BA, Bakkaloglu S, Newland J, Cantwell M, Verrina E, Neu A, et al. Consensus guidelines for the prevention and treatment of catheter-related infections and peritonitis in pediatric patients receiving peritoneal dialysis: 2012 update. *Perit Dial Int.* 2012;32:532-86.
82. Rubin J, Rogers WA, Taylor HM, Everett ED, Prowant BF, Fruto LV, et al. Peritonitis During Continuous Ambulatory Peritoneal Dialysis. *Ann Intern Med.* 1980;92:7-13.
83. Gokal R, Francis DMA, Goodship THJ, Bint AJ, Ramos JM, Ferner RE, et al. PERITONITIS IN CONTINUOUS AMBULATORY PERITONEAL DIALYSIS: Laboratory and Clinical Studies. *The Lancet.* 1982;320:1388-91.
84. Ludlam HA, Price TN, Berry AJ, Phillips I. Laboratory diagnosis of peritonitis in patients on continuous ambulatory peritoneal dialysis. *J Clin Microbiol.* 1988;26:1757-62.
85. Hurley RM, Muogbo D, Wilson GW, Ali MA. Cellular composition of peritoneal effluent: response to bacterial peritonitis. *Can Med Assoc J.* 1977;117:1061-2.

86. Williams P, Pantalony D, Vas SI, Khanna R, Oreopoulos DG. The value of dialysate cell count in the diagnosis of peritonitis in patients on continuous ambulatory peritoneal dialysis *Perit Dial Int.* 1981;1:59-62.
87. Flanigan MJ, Freeman RM, Lim VS. Cellular response to peritonitis among peritoneal dialysis patients. *Am J Kidney Dis.* 1985;6:420-4.
88. Matuszkiewicz-Rowinska J. Update on fungal peritonitis and its treatment. *Perit Dial Int.* 2009;29 S161-5.
89. Bibashi E, Memmos D, Kokolina E, Tsakiris D, Sofianou D, Papadimitriou M. Fungal Peritonitis Complicating Peritoneal Dialysis during an 11-Year Period: Report of 46 Cases. *Clin Infect Dis.* 2003;36:927-31.
90. Daugirdas JT, Leehey DJ, Popli S, Hoffman W, Zayas I, Gandhi VC, et al. Induction of peritoneal fluid eosinophilia and/or monocytosis by intraperitoneal air injection. *Am J Nephrol.* 1987;7:116-20.
91. Oh SK, H.; Kang, JM.; Lim, S.; Park, H.; Jung, S.; Lee, K. Eosinophilic Peritonitis in a Patient with Continuous Ambulatory Peritoneal Dialysis CAPD FAU - Oh, Se Yong FAU - Kim, Hyang FAU - Kang, Jeung Mook FAU - Lim, Sung Ho FAU - Park, Hyun Duk FAU - Jung, Soo Suk FAU - Lee, Kyu Beck. *Korean J Intern Med.* 2004;19:121-3.
92. Lee S, Schoen I. Eosinophilia of peritoneal fluid and peripheral blood associated with chronic peritoneal dialysis. *AM J CLIN PATHOL.* 1967;47:638-40.
93. Chandran PK, Humayun HM, Daugirdas JT, Nawab ZM, Gandhi VC, Ing TS. Blood eosinophilia in patients undergoing maintenance peritoneal dialysis. *Arch Intern Med.* 1985;145:114-6.
94. Chan MK, Chow L, Lam SS, Jones B. Peritoneal eosinophilia in patients on continuous ambulatory peritoneal dialysis: a prospective study. *AM J KIDNEY DIS.* 1988;11:180-3.
95. Piraino B, Bailie GR, Bernardini J, Boeschoten E, Gupta A, Holmes C, et al. Peritoneal dialysis-related infections recommendations: 2005 update. *Perit Dial Int.* 2005;25:107-31.
96. Dux R, Kindler-Röhrborn A, Annas M, Faustmann P, Lennartz K, Zimmermann CW. A standardized protocol for flow cytometric analysis of cells isolated from cerebrospinal fluid. *J Neurol Sci.* 1994;121:74-8.
97. de Jonge R, Brouwer R, Smit M, de Frankrijker-Merkestijn M, Dolhain RJEM, Hazes JMW, et al. Automated counting of white blood cells in synovial fluid. *Rheumatology (UK).* 2004;43:170-3.
98. Barnes PW, Esby CS, Shimer G. An evaluation of the utility of performing body fluid counts on the coulter LH 750. *Lab Hematol.* 2004;10:127-31.
99. Rabinovitch A, Cornbleet PJ. Body fluid microscopy in US laboratories: Data from two College of American Pathologists surveys, with practice recommendations. *ARCH PATHOL LAB MED.* 1994;118:13-7.
100. Whitmore EL, Hochberg F, Wolfson L, Royalty J, Taft PD. Quantitative cyto centrifugation in the evaluation of cerebrospinal fluid. *ACTA CYTOL.* 1982;26:847-50.
101. Seyfert S, Kabbeck-Kupijai D, Marx P, Kossmehl G. Cerebrospinal fluid cell preparation methods. An evaluation. *ACTA CYTOL.* 1992;36:927-31.
102. Steele RW, Marmer DJ, O'Brien MD, Tyson ST, Steele CR. Leukocyte survival in cerebrospinal fluid. *J Clin Microbiol.* 1986;23:965-6.
103. Strokes BL, W. Principles of cyto centrifugation. *laboratory medicine.* 2004;35:434- 37.
104. Fleury-Feith J, Escudier E, Pocholle MJ, Carre C, Bernaudin JF. The effects of cyto centrifugation on differential cell counts in samples obtained by bronchoalveolar lavage. *ACTA CYTOL.* 1987;31:606-10.
105. De Graaf MT, De Jongste AHC, Kraan J, Boonstra JG, Smitt PAES, Gratama JW. Flow cytometric characterization of cerebrospinal fluid cells. *Cytometry Part B Clin Cytometry.* 2011;80 B:271-81.

106. Riedl JA, Dinkelaar RB, van Gelder W. Automated morphological analysis of cells in body fluids by the digital microscopy system DM96. *J CLIN PATHOL.* 2010;63:538-43.
107. Brown M, Wittwer C. Flow Cytometry: Principles and Clinical Applications in Hematology. *Clin Chem.* 2000;46:1221-9.
108. Shen PUF, Blair JL. Cholesterol crystals causing falsely elevated automated cell count. *Am J Clin Pathol.* 2006;125:358-63.
109. Omuse G, Makau P. Interference of cerebrospinal fluid white blood cell counts performed on the Sysmex XT-4000i by yeast and bacteria. *Int J Lab Hematol.* 2013;35:e5-e7.
110. Mahieu S, Vertessen F, Van der Planken M. Evaluation of ADVIA 120 CSF assay (Bayer) vs. chamber counting of cerebrospinal fluid specimens. *Clin Lab Haematol.* 2004;26:195-9.
111. Aune MW, Becker JL, Brugnara C, Canfield W, Dorfman DM, Fiehn W, et al. Automated Flow Cytometric Analysis of Blood Cells in Cerebrospinal Fluid: Analytic Performance. *Am J Clin Pathol.* 2004;121:690-700.
112. Strik H, Luthe H, Nagel I, Ehrlich B, Bahr M. Automated cerebrospinal fluid cytology: limitations and reasonable applications. *Anal Quant Cytol Histol.* 2005;27:167-73.
113. Kleine TO, Nebe CT, Lower C, Lehmitz R, Kruse R, Geilenkeuser WJ, et al. Modifications of haematology analyzers to improve cell counting and leukocyte differentiating in cerebrospinal fluid controls of the Joint German Society for Clinical Chemistry and Laboratory Medicine. *Cytometry Part A.* 2009;75:688-91.
114. Conner BD, Lee YCG, Branca P, Rogers JT, Rodriguez RM, Light RW. Variations in pleural fluid WBC count and differential counts with different sample containers and different methods. *Chest.* 2003;123:1181-7.
115. Danise P, Maconi M, Rovetti A, Avino D, Di Palma A, Gerardo Pirofalo M, et al. Cell counting of body fluids: Comparison between three automated haematology analyzers and the manual microscope method. *Int J Lab Hematol.* 2013;35:608-13.
116. Froom P, Diab A, Barak M. Automated evaluation of synovial and ascitic fluids with the advia 2120 hematology analyzer. *Am J Clin Pathol.* 2013;140:828-30.
117. Lippi G, Cattabiani C, Benegiamo A, Gennari D, Pavesi F, Caleffi A, et al. Evaluation of white blood cell count in peritoneal fluid with five different hemocytometers. *Clin Biochem.* 2013;46:173-6.
118. Tanada H, Ikemoto T, Masutani R, Tanaka H, Takubo T. Evaluation of the automated hematology analyzer ADVIA 120 for cerebrospinal fluid analysis and usage of unique hemolysis reagent. *Int J Lab Hematol.* 2013;36:83-91.
119. Zur B, Eichhorn L, Albers E, Stoffel-Wagner B. Evaluation of 2 hematology analyzers in body fluid mode versus flow cytometry immunophenotyping of mainly neurosurgical cerebrospinal fluid samples. *J Neurol Surg A Cent Eur Neurosurg.* 2012;73:93-8.
120. Perne A, Hainfellner JA, Womastek I, Haushofer A, Szekeres T, Schwarzinger I. Performance evaluation of the sysmex XE-5000 hematology analyzer for white blood cell analysis in cerebrospinal fluid. *Arch Pathol Lab Med.* 2012;136:194-8.
121. Boer K, Deufel T, Reinhoefer M. Evaluation of the XE-5000 for the automated analysis of blood cells in cerebrospinal fluid. *Clin Biochem.* 2009;42:684-91.
122. Paris A, Nhan T, Cornet E, Perol JP, Malet M, Troussard X. Performance evaluation of the body fluid mode on the platform Sysmex XE-5000 series automated hematology analyzer. *Int J Lab Hematol.* 2010;32:539-47.
123. Kleine TO, Nebe CT, Lower C, Geilenkeuser WJ, Dorn-Beineke A. Cell analysis in cerebrospinal fluid (CSF) using Sysmex(registered trademark) hematology analyzers XT-4000i and XE-5000:

- Evaluation with CSF controls of the Joint German Society for Clinical Chemistry and Laboratory Medicine (DGKL). *Cytometry Part A*. 2012;81:255-64.
124. Noguera Velasco JA, Martinez Villanueva M, Martinez Hernandez P. About the clinical performance of the sysmex XE-500 for automated cerebrospinal fluid cell counts. *Am J Clin Pathol*. 2011;136:156-7.
 125. Zimmermann M, Otto C, Gonzalez JB, Prokop S, Ruprecht K. Cellular origin and diagnostic significance of high-fluorescent cells in cerebrospinal fluid detected by the XE-5000 hematology analyzer. *Int J Lab Hematol*. 2013;35:580-8.
 126. Zimmermann M, Ruprecht K, Kainzinger F, Heppner FL, Weimann A. Automated vs. manual cerebrospinal fluid cell counts: a work and cost analysis comparing the Sysmex XE-5000 and the Fuchs-Rosenthal manual counting chamber. *Int J Lab Hematol*. 2011;33:629-37.
 127. Fleming C, Brouwer R, Lindemans J, De Jonge R. Improved software on the sysmex xe-5000 body fluid mode for counting wbc and pmn in cerebrospinal fluid. *Clin Chem Lab Med*. 2012;34:113.
 128. Williams JE, Walters J, Kabb K. Gaining efficiency in the laboratory - automated body fluid cell counts: Evaluation of the body fluid application on the sysmex XE-5000 hematology analyzer. *Lab Med*. 2011;42:395-401.
 129. Armbruster DA, Pry T. Limit of blank, limit of detection and limit of quantitation. *Clin Biochem Rev*. 2008;29:549-52.
 130. Fleming C, Brouwer R, Lindemans J, De Jonge R. Validation of the body fluid module on the new sysmex xn-1000 for counting blood cells in cerebrospinal fluid and other body fluids. *Clin Chem Lab Med*. 2012;34:112-3.
 131. Ziebig R, Lun A, Sinha P. Leukocyte counts in cerebrospinal fluid with the automated hematology analyzer CellDyn 3500 and the urine flow cytometer UF-100. *Clin Chem*. 2000;46:242-7.
 132. De Smet D, Van Moer G, Martens GA, Nanos N, Smet L, Jochmans K, et al. Use of the cell-dyn sapphire hematology analyzer for automated counting of blood cells in body fluids. *Am J Clin Pathol*. 2010;133:291-9.
 133. Keuren JFW, Hoffmann JJML, Leers MPG. Analysis of serous body fluids using the CELL-DYN Sapphire hematology analyzer. *Clin Chem Lab Med*. 2013;51:1285-90.
 134. Keuren JF, Hoffmann JJ, Leers MP. Immunological screening for tumor cells in serous body fluids has added value with the CELL-DYN Sapphire. *Clin Chem Lab Med*. 2014;52:253-8.
 135. Glasser L, Murphy CA, Machan JT. The clinical reliability of automated cerebrospinal fluid cell counts on the beckman-coulter LH750 and iris iQ200. *Am J Clin Pathol*. 2009;131:58-63.
 136. Yang D, Zhou Y, Chen B. Performance evaluation and result comparison of the automated hematology analyzers Abbott CD 3700, Sysmex XE 2100 and Coulter LH 750 for cell counts in serous fluids. *Clin Chim Acta*. 2013;419:113-8.
 137. Brown W, Keeney M, Chin-Yee I, Johnson K, Lantis K, Finn W, et al. Validation of body fluid analysis on the coulter LH 750. *Lab Hematol*. 2003;9:155-9.
 138. Van De Geijn GJM, Van Gent M, Van Pul-Bom N, Beunis MH, Van Tilburg AJP, Njo TL. A new flow cytometric method for differential cell counting in ascitic fluid. *Cytometry Part B Clin Cytometry*. 2014 Epub ahead of print.
 139. Walker TJ, Nelson LD, Dunphy BW, Anderson DM, Kickler TS. Comparative evaluation of the iris iQ200 body fluid module with manual hemacytometer count. *Am J Clin Pathol*. 2009;131:333-8.
 140. Butch AW, Wises PK, Wah DT, Gornet TG, Fritsche HA. A multicenter evaluation of the Iris iQ200 automated urine microscopy analyzer body fluids module and comparison with hemacytometer cell counts. *Am J Clin Pathol*. 2008;129:445-50.

141. Goubard A, Marzouk M, Canoui-Poitrine F, Launay S, Le Thuaut A, Legrand P. Performance of the Iris iQ(registered trademark)200 elite analyser in the cell counting of serous effusion fluids and cerebrospinal drainage fluids. *J Clin Pathol.* 2011;64:1123-7.
142. Van Acker JT, Delanghe JR, Langlois MR, Taes YE, De Buyzere ML, Verstraete AG. Automated flow cytometric analysis of cerebrospinal fluid. *Clin Chem.* 2001;47:556-60.
143. Yamanishi H, Imai N, Ohmine T, Nishiyama M, Suehisa E, Kanakura Y, et al. Urine flow cytometer quantification of leukocytes in samples containing a large proportion of lymphocytes. *Clin Biochem.* 2006;39:857-9.
144. Buoro S, Ottomano C, Esposito SA, Gherardi P, Alessio MG, Crippa A, et al. Analytical and clinical evaluation of sysmex UF1000i for automated screening of cerebrospinal fluids. *J Med Biochem.* 2013;33:191-6.
145. Nanos NE, Delanghe JR. Evaluation of Sysmex UF-1000i for use in cerebrospinal fluid analysis. *Clin Chim Acta.* 2008;392:30-3.
146. Buoro S, Gustinetti R, Dominoni P, Alessio MG, Filisetti P, Luca MG, et al. Analytical evaluation of Sysmex UF-1000i for flow cytometric analysis of peritoneal fluid. *Clin Biochem.* 2012;45:1263-5.
147. Fleming C, Brouwer R, van Alphen A, Lindemans J, de Jonge R. UF-1000i: validation of the body fluid mode for counting cells in body fluids. *Clin Chem Lab Med.* 2014;52:1781-90.

Chapter 3

Role of automated peritoneal leukocyte differential counting in diagnostic accuracy in peritoneal dialysis associated peritonitis

**Adriaan van Alphen, Chérina Fleming, Marike Wabbijn, Robert de Jonge,
Marien Fieren**

Under preparation

ABSTRACT

Objective

Dialysate white blood cell (WBC) differential counting is essential to the diagnosis of infectious peritoneal dialysis associated peritonitis. The standard method of WBC counting is time consuming, suffers from substantial variability and is seldom available 24 hours a day. These problems may be overcome using automated hemocytometric analysis.

Methods

A single center, retrospective analysis was performed after introduction of dialysate WBC differential counting using automated hemocytometric analysis. From 2008 to 2012 all effluent dialysate samples that were taken because of suspected peritonitis were analyzed.

Results

From 59 cases with WBC over $100 \times 10^6/L$ and neutrophilic polymorph nucleated cells (nPMN) over 50%, 51 cases were culture-positive and 8 cases culture-negative. In 2 cases with WBC over $100 \times 10^6/L$ and positive culture, nPMN fraction was slightly below 50%. In 3 cases cultures were positive while WBC was smaller than $100 \times 10^6/L$. nPMN fraction was 31%, 48% and 82%. Values for WBC and nPMN rose above $100 \times 10^6/L$ and 50% respectively. In addition to these 64 infection-related peritonitis cases, there were 5 cases where WBC was higher than $100 \times 10^6/L$ while nPMN was smaller than 50%. In these cases dialysate culture remained sterile and all episodes recovered without antibiotic treatment.

Conclusions

The immediate availability of WBC differential counting is essential to distinguish infectious from non-infection related peritonitis.

INTRODUCTION

Infectious peritonitis is still a major complication of Peritoneal Dialysis (PD), accounting for significant morbidity (1-3). Immediate treatment is mandatory even before the results of bacteriological cultures are known. In the majority of cases patients present with cloudy effluent caused by an increased white blood cell (WBC) count. These white blood cells typically consist for more than 50% of neutrophilic polymorphonuclear cells (nPMN's) (4). However, in some cases effluent leukocytosis ($>100 \times 10^6$ cells/L) predominantly consists of eosinophilic granulocytes or mononuclear cells when differential counting is performed. Such peritonitis episodes are usually not caused by bacterial or fungal infections and do not require antibiotic treatment (5, 6). On rare occasions, typically in the initial stages of infectious peritonitis, total effluent WBC count does not exceed 100×10^6 cells/L while more than 50% of WBC are PMN's (7). Immediate availability of differential counting is essential in detecting these cases of infectious peritonitis. However, in various studies on PD-related peritonitis information on WBC differential counting is missing. Often increased percentage of nPMN's is not mentioned as a diagnostic criterion for infectious peritonitis, suggesting that WBC differential counting may not have been routinely performed (8-12).

Microscopic analysis has been the gold standard of WBC differential counting. However this method is time consuming, requires skilled personnel, lacks precision and may not be available around the clock. These difficulties can be overcome using automated hemocytometric analysis. Previously we showed that even when total WBC count is low, this method enables highly accurate WBC differential counting in body fluids including PD dialysate (13). Since the implementation of automated hemocytometric analysis at the Erasmus MC, results of PD effluent WBC counting became available within one hour after sampling. We conducted a single center, retrospective analysis of the data with special emphasis on the question whether WBC differential counting is a valuable tool in diagnosing infectious peritonitis.

Patients

From March 2008 until March 2012, all effluent dialysate samples from patients treated in Erasmus University Medical Centre with CAPD or APD were analyzed. Patients were included in the analysis when they reported clinical signs that raised suspicion of peritonitis, when abnormalities in the peritoneal effluent leucocyte count were found or when cultures taken from the peritoneal effluent proved positive for pathogens. In our center patients are instructed to report to the PD unit if abdominal discomfort or turbidity of effluent raises even the slightest suspicion of peritonitis. Initial examination includes WBC differential counting. Results of differential counting are available to the nephrologist within one hour. Most patients are living at fairly short distances but some

patients reside in locations that are remote from our hospital, up to a distance of 100 km. If total WBC count is more than $100 \times 10^6/L$ with more than 50% nPMN's, a diagnosis of infectious peritonitis is made² whereupon empiric treatment with intraperitoneal administration of Vancomycin 2000 mg/week and Ceftazidim 250 mg/2L is immediately started. Before administration of antibiotics a specimen of peritoneal effluent is collected in blood culture bottles (Bactec^R Plus, aerobic and anaerobic; Becton Dickinson and Co., Sparks, Maryland, USA). These samples are cultured for five days. Antibiotic treatment is continued for 2 or, in some cases, for 3 weeks. Antibiotic regimen is adjusted according to sensitivity testing. When the response of peritonitis to treatment with proper antibiotics is insufficient, it is policy to remove the catheter to avoid protracted courses of antibiotic treatment. Treatment failure is distinguished in refractory, relapsing, recurrent and repeat peritonitis². Relapsing peritonitis was not counted as a separate episode of peritonitis.

In our center, PD training is started 2 weeks after surgical catheter implantation. Our catheter implantation protocol dictates that the peritoneal catheter is flushed weekly with approximately 300 ml saline. When patients in the meantime feel abdominal pain, abdominal fluid is liberally taken for WBC counting and culture. As abdominal complaints are rather common after catheter implantation, it is our practice not to start treatment with antibiotics unless bacteriological cultures are positive. Often leukocytes found in these samples are predominantly comprised of nPMN's. Since differences in dwell time or flush/dwell volume may interfere with the results of differential counting we subsequently monitored dialysate samples from the empty abdomen when peritoneal cavity was flushed according to protocol. Dialysate samples were taken from fluid obtained after flushing the peritoneal cavity. In total 42 samples were collected from 30 patients.

MATERIALS AND METHODS

The Sysmex XE-5000 (Sysmex, Etten-Leur, The Netherlands) is an automated hematology analyzer equipped with both a whole blood mode and a body fluid (BF) mode. The XE-5000 BF mode is based on two techniques; hydrodynamic focusing and fluorescent flow cytometry. The RBCs are counted in the RBC channel using hydrodynamic focusing and the WBCs, MNs and PMNs are enumerated in the DIFF channel using fluorescent flow cytometry. The DIFF channel identifies and clusters each cell depending on 3 characteristics: the size of the cell (forward scatter), inner complexity of the cell (side scatter) and DNA/RNA content (fluorescence intensity). Additionally, the DIFF channel also reports high fluorescence body fluid (HF-BF) cells such as macrophages and mesothelial cells. These HF-BF cells are found just above the MN cluster at fluorescence discriminator >200 channels, and are not included in the WBC (differential) count, but are included in the

total nucleated cell count (TC-BF). Apart from the WBC, RBC, MN and PMN (absolute and percentile) counts displayed, the HF-BF and eosinophil counts are also available as research parameters. By subtracting the eosinophil count from PMN count, nPMN count can be calculated. The XE-5000 has a variation of coefficient (CV) of 3% and 30% for absolute WBC counts of $200 \times 10^6/L$ and $5 \times 10^6/L$, respectively.

Patient samples

CAPD samples were measured immediately after arrival in the laboratory in the open BF mode on the Sysmex XE-5000 using 130 μL sample volume. Prior to sample analysis, CAPD fluids were mixed using an automated mixer and no sample pretreatment was necessary. When $>100 \times 10^6/L$ HF-BF were present, a cytospin slide was prepared for microscopic analysis. Everyday two levels (low & high) of BF quality controls were measured prior to sample analysis. To eliminate carry-over, Sysmex has incorporated an automatic rinse cycle after every measurement. In this study, software version 00-06 of the XE-5000 was used. After technical validation in the Sysmex Information System (SIS), results were directly available for the physicians (**Figure 1**). Next, clinical validation was performed in the Laboratory Information System (LIS). When noted from the scattergram, presence of eosinophils was also reported to the clinician.

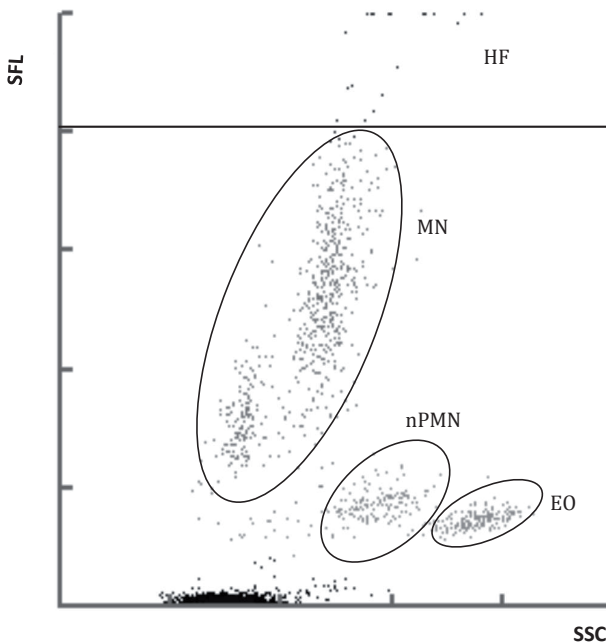


Figure 1 A white blood cell scattergram. Clusters are identified depending on 3 characteristics: side scatter (SSC), fluorescence intensity (SFL) and forward scatter. In the presented sample mononuclear cells (MN), neutrophilic polymorphonuclear cells (nPMN), eosinophils and high fluorescent cells (HF) are recognized.

RESULTS

During the 3-years observation period 69 new episodes of suspected peritonitis were evaluated. 51 episodes presented with all three criteria for peritonitis: $>100 \times 10^6$ WBC/L, $>50\%$ nPMN's and positive culture. As shown in **Figure 2**, 8 episodes were culture negative but were considered due to infection because of increased WBC count with nPMN predominance. Five culture negative episodes were probably not caused by infection because of low WBC count with nPMN paucity. In the initial dialysate sample of 5 patients with positive culture, WBC count was $<100 \times 10^6$ /L or nPMN fraction was $<50\%$.

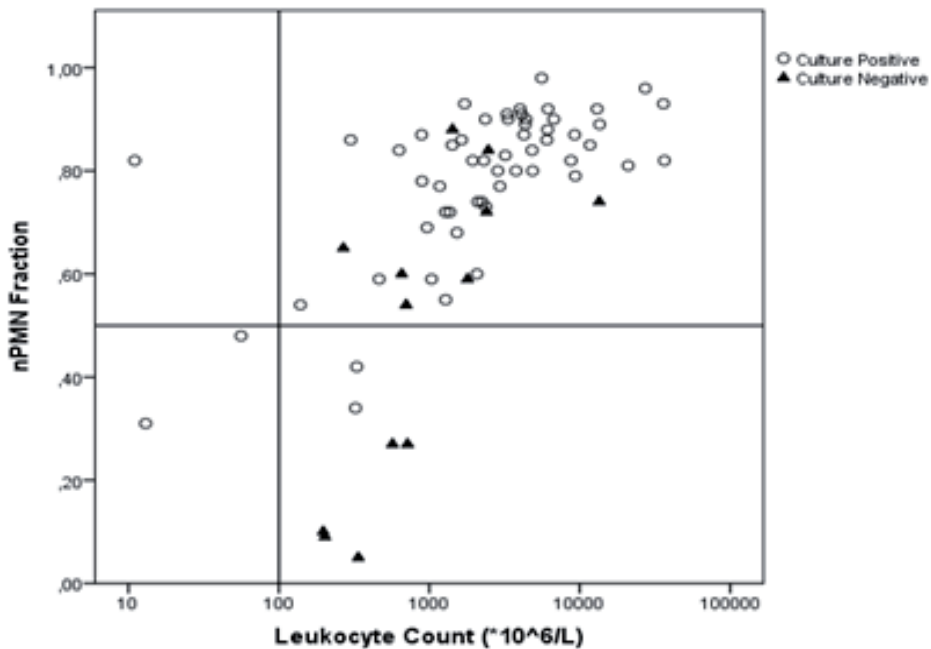


Figure 2 nPMN fraction was plotted against WBC count in peritoneal dialysate samples obtained at initial presentation of 56 culture positive and 13 culture negative episodes of peritonitis. Average fractions of proven infectious peritonitis, culture negative probably infectious peritonitis and culture negative improbably infectious peritonitis are 0.78, 0.70 and 0.16 respectively.

It should be noted that in 2 cases of culture-proven peritonitis with increased WBC count the first sample contained less than 50% nPMN's: In one case nPMN percentage was 43%, in the other case 35%. However, nPMN fraction rose to more than 50% the following day in both cases (**Table 1**). In contrast, nPMN's were repeatedly far lower than 50% in the obviously non- infection related cases. Overall rate of proven and probable infectious peritonitis was one episode every 23.6 treatment-months. The results of cultures are depicted in **Figure 3**.

Table 1 summarizes all episodes that did not conform to all three criteria for infectious peritonitis. Culture results, WBC differential counting and outcome are presented in 5 distinct categories. Categories are labeled according to presence of WBC > 100 × 10⁶/L, nPMN percentage > 50% and culture result.

Category	Causative microorganism	nPMN (10 ⁶ /L)	WBC (10 ⁶ /L)	nPMN %	Outcome	Comments
Episodes of culture negative infectious peritonitis						
A	culture negative	9957	13463	74	recovery	
	culture negative	1051	1795	59	recovery	
	culture negative	174	268	65	recovery	
	culture negative	2069	2468	84	recovery	
	culture negative	1738	2402	72	refractory	catheter removal
	culture negative	394	655	60	recovery	
	culture negative	376	701	54	recovery	
	culture negative	1247	1425	88	recovery	
Episodes of culture negative infectious peritonitis with WBC > 100 × 10⁶/L and PMN < 50%						
B	<i>Corynebacterium</i>	138	329	42	transplantation	1 day later: nPMN/WBC: 216/474 (46%)
	<i>Klebsiella pneumoniae</i>	111	323	34	recovery	1 day later: nPMN/WBC: 5065/5731 (88%)
Episodes of culture negative infectious peritonitis with WBC < 100 × 10⁶/L and PMN > 50%						
C	<i>E.Coli</i>	9	11	82	refractory	1 day later: nPMN/WBC: 4681/4997 (82%)
Episodes of culture negative infectious peritonitis with < 100 × 10⁶/L and PMN < 50%						
D	<i>Micrococcus Luteus</i>	27	56	48	relapsing	7 days later: nPMN/WBC: 1987/2646 (75%)
	<i>S. species</i>	4	13	31	recovery	3 days later: nPMN/WBC: 244/460 (53%)
Episodes of non-nPMN dominated peritonitis with repeated negative cultures						
E	culture negative	196	716	27	recovery	recovery after recovery diarrhea
	culture negative	18	202	9	recovery	recovery after recovery systemic viral infection
	culture negative	151	569	27	recovery	resolved after discontinuing icodextrin
	culture negative	19	197	10	recovery	eosinophilic PMN/WBC: 103/197
	culture negative	17	337	5	recovery	eosinophilic PMN/WBC: 30/337

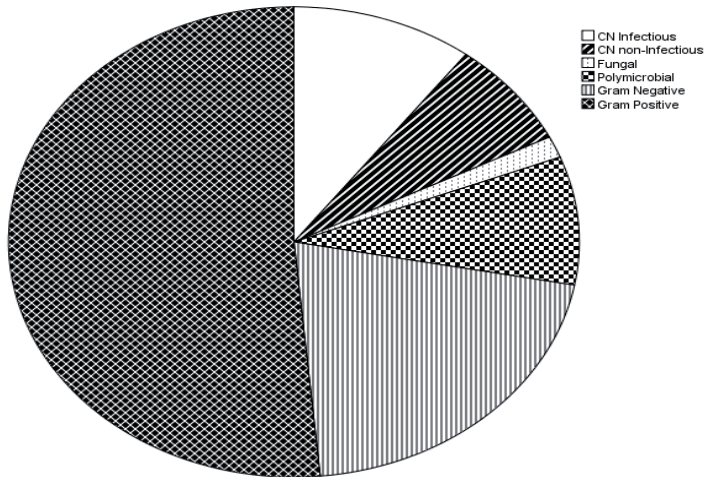


Figure 3 Results of microbial cultures of 69 episodes of suspected peritonitis, represented as relative proportions. CN= culture negative.

Using clinical follow-up as gold standard, sensitivity and specificity was calculated. nPMN fraction in the peritoneal effluent could predict the presence of infectious peritonitis with a sensitivity, specificity, positive predictive value and negative predictive value of 0.94, 1.0, 1.0 and 0.56 respectively. When WBC alone was used these values were 0.95, 0, 0.92 and 0 (**Table 2**). Additionally, we investigated the value of differential WBC counting when fluid was used to flush an empty peritoneal cavity. 42 samples were analyzed. These samples were taken from 30 patients. When leukocyte count was determined from a flush taken from an empty abdomen, it was more than $100 \times 10^6/L$ in 36% of all samples (**Figure 4**).

Average leukocyte count was $1063 \times 10^6/L$ (standard deviation, SD: $\pm 2083 \times 10^6/L$). Differential counting yielded average numbers of $516 \times 10^6/L$ (SD: $\pm 1236 \times 10^6/L$), $56 \times 10^6/L$ (SD: $\pm 205 \times 10^6/L$) and $491 \times 10^6/L$ (SD: $\pm 1165 \times 10^6/L$) for neutrophilic, eosinophilic and mononuclear cells respectively. Mononuclear cells were predominant in 52% of samples while nPMN was predominant in 43% of all samples.

In 21% of the samples eosinophilic cell fraction was more than 10%, satisfying the criterion for eosinophilic peritonitis. Antibiotic treatment was not started in any of the patients and none showed signs of infectious peritonitis upon follow-up.

Table 2 A) represents a 2x2 analysis comparing leukocyte count (L) in peritoneal dialysate and presence of infectious peritonitis. **B)** represents the same analysis for fraction of nPMN in the peritoneal dialysate. Clinical follow-up was used as gold standard for the presence (D+) or absence (D-) of infectious peritonitis.

A				B			
	D+	D-	Total		D+	D-	Total
L ≥100×10⁶	61	5	66	nPMN ≥50%	60	0	60
L <100×10⁶	3	0	3	nPMN <50%	4	5	9
Total	64	5	69	Total	64	5	69

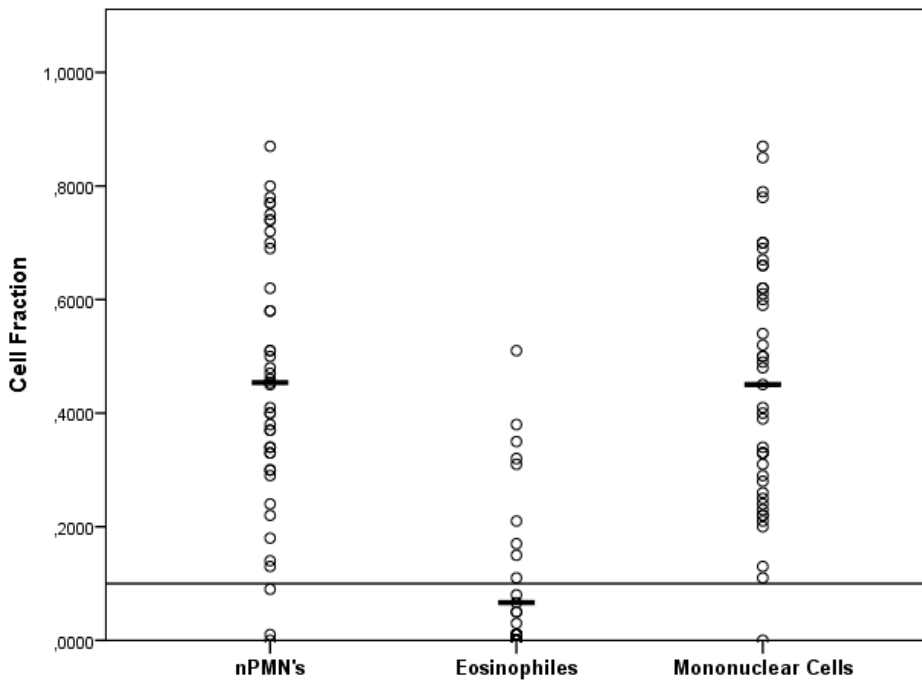


Figure 4 nPMN, eosinophilic and mononuclear cells are represented as a fraction of the total number of cells found in a 300 ml flush taken from a “dry” abdomen in the first two weeks after catheter implantation. Circles represent individual 42 samples taken from 32 patients. Thick horizontal lines represent mean values. The horizontal line is set at 0.1. Ten percent eosinophilic cells in peritoneal fluid is considered indicative for eosinophilic peritonitis.

DISCUSSION

In the present study we found that WBC differential counting is a valuable tool in diagnosing PD- related peritonitis. It especially distinguishes infection-related from non-infection-related peritonitis. Infectious peritonitis is still a major complication of

PD despite substantial improvements following the introduction of “flush before fill” systems at the end of the eighties. In large series peritonitis occurs roughly one time per 20-24 treatment months. The diagnosis of infectious peritonitis appears to be straightforward: increased dialysate leukocyte count with more than 50% nPMN's and positive microbial culture in patients presenting with abdominal discomfort or cloudy effluent dialysate. In the present study we found that in the majority of cases suspect for infectious peritonitis and an increased dialysate WBC, nPMN's were predominant. In 8/69 of these cases bacteriological cultures were negative but they were all considered and treated as infectious PD-related peritonitis. According to most studies, no causative microorganism can be identified in about 10 - 20% of the peritonitis episodes (culture-negative peritonitis), but percentages up to 36% are reported (1, 3, 11, 14-17). Usually in culture-negative peritonitis an intraperitoneal infectious cause is still assumed and antibiotic treatment is continued. Culture-negative episodes of peritonitis are likely associated with suboptimal culture techniques or recent use of antibiotics (6).

In 5/69 cases with increased WBC in the dialysate we found that less than 50% of WBC were nPMN's. Repeated cultures were negative and all these cases recovered without antibiotic treatment. Remarkably, most observational studies give no detailed information on WBC differential counting in culture-negative peritonitis and several studies do not mention increased nPMN counts as a criterion for the diagnosis of infection-related peritonitis (8, 11, 18). It is therefore likely that in these studies cases of non-nPMN-dominated peritonitis were regarded and treated as infection-related culture-negative peritonitis. Such peritonitis episodes are usually not related to microbial infection but rather attributable to other causes including inflammation inducing substances in the dialysate, juxtaperitoneal visceral inflammation such as renal allograft rejection or intestinal viral infections (5, 19, 20). A number of outbreaks of so-called sterile peritonitis have been reported that were attributable to contamination of fresh dialysis fluid with high concentrations of the microbial cell wall product peptidoglycan (19, 21). Initially, such cases of peritonitis were treated as infection-related peritonitis (19). The fact that the 5 non-nPMN-dominated episodes of peritonitis in the present study recovered without antibiotic treatment confirmed the absence of an infectious cause. Noticeably, in 2 of these cases leukocytosis predominantly consisted of MN and developed a few weeks after recovery from proven infectious peritonitis. It is tempting to speculate that even some time after full recovery from an intra-peritoneal infection the host defense system is still in a primed state (22). This primed state might cause an increased mononuclear reaction to non-infectious stimuli. We observed one patient who used Icodextrin but developed non-PMN-dominated leukocytosis after recovering from infectious peritonitis. Leukocytosis resolved after cessation of Icodextrin.

In 3 cases dialysate samples were examined because of abdominal pain, prior to manifest clinical peritonitis. In spite of low WBC counts ($< 100 \times 10^6/L$), cultures turned out

to be already positive while nPMN percentages were 31%, 48% and 82% respectively. Although samples are normally taken from dialysate that has a dwell time of at least 2 hours, dwell times are usually far shorter than the length of an overnight dwell. The fact that initial samples are taken from the abdomen at presentation might account for these low WBC counts. However, a delayed WBC reaction to infection has also been reported earlier (7).

When a sensitivity analysis was performed on the current data set, nPMN fraction yielded better positive and negative predictive value than leukocyte count alone. However the analysis was hampered by the low number of true negative cases in the current data set. This shortcoming is possibly caused by incomplete registration of negative cases. Patients who presented to our clinic but in whom peritonitis was deemed unlikely because of absence of cultures or because leukocyte count was smaller than $100 \times 10^6/L$, might not have been registered. More accurate determination of sensitivity and specificity would require a prospective analysis including all incident cases presenting at the clinic.

We also investigated the value of WBC differential counting in fluid obtained from an empty peritoneal cavity during the post catheter implantation period. Almost half these samples showed predominance of nPMN while none of the patients developed peritonitis. These findings are in line with previously reported predominance of neutrophilic leukocytes in overnight effluent during the first few days after initiation of peritoneal dialysis (23). nPMN predominance in peritoneal fluid not diluted by dialysis fluid is at variance with the findings in liver cirrhosis associated ascites. In cirrhosis patients mononuclear cells are predominant, suggesting that the presence of a PD catheter in an empty abdomen may be causing nPMN predominance in a significant number of cases (24). Obviously, when the catheter is in situ but peritoneal cavity is empty, predominance of nPMN's in peritoneal fluid is not indicative of infection.

In this study the number of non-infection-related peritonitis cases was close to the number of culture-negative peritonitis cases that were considered as infectious because of nPMN predominance. Increased WBC counts that were not dominated by nPMN's led us to suspect a non-infectious cause which was confirmed by the course of the peritonitis episodes. Early recognition of non-infection related peritonitis is crucial to avoid unnecessary treatment with antibiotics. When such peritonitis does not respond to therapy, it may be taken as a refractory culture-negative infectious peritonitis, which could result in protracted antibiotic courses or catheter removal. Occasionally, in proven infection-related peritonitis, nPMN percentage was less than 50% at initial presentation. In these cases PMN percentage was either only slightly lower than 50% or rose to high values in the following samples. Therefore, we feel that closely monitoring of WBC differential counting and culture results rather than starting immediate antibiotic treatment can be considered in peritonitis cases that are not nPMN dominated at initial presenta-

tion. On the other hand, relatively high nPMN percentages with normal WBC counts at first presentation may be the very first sign of an infectious peritonitis. We recommend monitoring these patients closely so that treatment with antibiotics, if applicable, can be timely started. The immediate availability of dialysate WBC differential counting provides essential diagnostic information for suspected peritonitis cases. However, as an exception to the rule, in samples of peritoneal fluid taken from an empty abdomen, nPMN predominance is not indicative for an infectious peritonitis.

In conclusion, the immediate availability of highly precise dialysate WBC counting can substantially contribute to diagnostic accuracy of PD-related peritonitis. Although the diagnosis of infection-related peritonitis is usually straightforward, some cases pose a diagnostic challenge, especially concerning the question whether an episode of peritonitis is caused by infection or not.

REFERENCES

1. Davenport A: Peritonitis remains the major clinical complication of peritoneal dialysis: the London, UK, peritonitis audit 2002-2003. *Perit Dial Int*, 29: 297-302, 2009
2. Li PK, Szeto CC, Piraino B, Bernardini J, Figueiredo AE, Gupta A, Johnson DW, Kuijper EJ, Lye WC, Salzer W, Schaefer F, Struijk DG: Peritoneal dialysis-related infections recommendations: 2010 update. *Perit Dial Int*, 30: 393-423, 2010
3. Mactier R: Peritonitis is still the achilles' heel of peritoneal dialysis. *Perit Dial Int*, 29: 262-266, 2009
4. Flanigan MJ, Freeman RM, Lim VS: Cellular response to peritonitis among peritoneal dialysis patients. *Am J Kidney Dis*, 6: 420-424, 1985
5. de Freitas DG, Gokal R: Sterile peritonitis in the peritoneal dialysis patient. *Perit Dial Int*, 25: 146-151, 2005
6. Rocklin MA, Teitelbaum I: Noninfectious causes of cloudy peritoneal dialysate. *Semin Dial*, 14: 37-40, 2001
7. Betjes MG, Tuk CW, Visser CE, Zemel D, Krediet RT, Arisz L, Beelen RH: Analysis of the peritoneal cellular immune system during CAPD shortly before a clinical peritonitis. *Nephrol Dial Transplant*, 9: 684-692, 1994
8. Chow KM, Szeto CC, Cheung KK, Leung CB, Wong SS, Law MC, Ho YW, Li PK: Predictive value of dialysate cell counts in peritonitis complicating peritoneal dialysis. *Clin J Am Soc Nephrol*, 1: 768-773, 2006
9. Geerse DA, Rutherford P, Bogers JC, Konings CJ: Sterile peritonitis associated with the use of amino-acid solution in eight peritoneal dialysis patients. *Perit Dial Int*, 31: 90-91, 2011
10. Os I, Gudmundsdottir H, Draganov B, von der Lippe E: Sterile peritonitis associated with amino acid-containing dialysate—a single center experience in Norway. *Perit Dial Int*, 31: 103, 2011
11. Ruger W, van Ittersum FJ, Comazzetto LF, Hoeks SE, ter Wee PM: Similar peritonitis outcome in CAPD and APD patients with dialysis modality continuation during peritonitis. *Perit Dial Int*, 31: 39-47, 2011
12. Mangram AJ, Archibald LK, Hupert M, Tokars JI, Silver LC, Brennan P, Arduino M, Peterson S, Parks S, Raymond A, McCullough M, Jones M, Wasserstein A, Kobrin S, Jarvis WR: Outbreak of sterile peritonitis among continuous cycling peritoneal dialysis patients. *Kidney Int*, 54: 1367-1371, 1998
13. de Jonge R, Brouwer R, de Graaf MT, Luitwieler RL, Fleming C, de Frankrijker-Merkestijn M, Sillevius Smitt PA, Boonstra JG, Lindemans J: Evaluation of the new body fluid mode on the Sysmex XE-5000 for counting leukocytes and erythrocytes in cerebrospinal fluid and other body fluids. *Clin Chem Lab Med*, 48: 665-675, 2010
14. Brown MC, Simpson K, Kerssens JJ, Mactier RA: Peritoneal dialysis-associated peritonitis rates and outcomes in a national cohort are not improving in the post-millennium (2000-2007). *Perit Dial Int*, 31: 639-650, 2011
15. Fahim M, Hawley CM, McDonald SP, Brown FG, Rosman JB, Wiggins KJ, Bannister KM, Johnson DW: Culture-negative peritonitis in peritoneal dialysis patients in Australia: predictors, treatment, and outcomes in 435 cases. *Am J Kidney Dis*, 55: 690-697, 2010
16. Ghali JR, Bannister KM, Brown FG, Rosman JB, Wiggins KJ, Johnson DW, McDonald SP: Microbiology and outcomes of peritonitis in Australian peritoneal dialysis patients. *Perit Dial Int*, 31: 651-662, 2011
17. Hildebrand A, Komenda P, Miller L, Rigatto C, Verrelli M, Sood AR, Sathianathan C, Reslerova M, Eng L, Eng A, Sood MM: Peritonitis and exit site infections in First Nations patients on peritoneal dialysis. *Clin J Am Soc Nephrol*, 5: 1988-1995, 2010

18. Szeto CC, Kwan BC, Chow KM, Law MC, Pang WF, Chung KY, Leung CB, Li PK: Recurrent and relapsing peritonitis: causative organisms and response to treatment. *Am J Kidney Dis*, 54: 702-710, 2009
19. Boer WH, Vos PF, Fieren MW: Culture-negative peritonitis associated with the use of icodextrin-containing dialysate in twelve patients treated with peritoneal dialysis. *Perit Dial Int*, 23: 33-38, 2003
20. Yalavarthy R, Teitelbaum I: Sterile peritonitis with monocytic predominance secondary to acute rejection of a previously failed renal allograft. *Perit Dial Int*, 27: 466-467, 2007
21. Adam FU, Singan M, Ozelsancak R, Torun D, Ozdemir FN, Haberal M: Icodextrin-associated sterile peritonitis: a recent outbreak in Turkey. *Perit Dial Int*, 27: 598-599, 2007
22. Fieren MW, Van den Bemd GJ, Bonta IL: Endotoxin-stimulated peritoneal macrophages obtained from continuous ambulatory peritoneal dialysis patients show an increased capacity to release interleukin-1 beta in vitro during infectious peritonitis. *Eur J Clin Invest*, 20: 453-457, 1990
23. Fijen JW, Struijk DG, Krediet RT, Boeschoten EW, de Vries JP, Arisz L: Dialysate leucocytosis in CAPD patients without clinical infection. *The Netherlands journal of medicine*, 33: 270-280, 1988
24. Flessner MF, Credit K, Richardson K, Potter R, Li X, He Z, Hoskins G, Henegar J: Peritoneal inflammation after twenty-week exposure to dialysis solution: effect of solution versus catheter-foreign body reaction. *Perit Dial Int*, 30: 284-293, 2010

Chapter 4

Improved software on the Sysmex XE-5000 BF mode for counting leukocytes in Cerebrospinal fluid

Chérina Fleming, Rob Brouwer, Jan Lindeman, Robert de Jonge

ABSTRACT

Objective

Previously, we showed that the Sysmex XE-5000 body fluid mode using software version 06 (v06) is accurate for counting WBC and RBC in various body fluids. However, a significant positive bias for counting WBC and PMN in CSF samples was noted, especially in the lower counting area. Sysmex recently released software version 10 (v10) with improved gating/cluster analysis. The aim of this study was to establish Sysmex's new software v10 as an improvement in comparison with the old software v06.

Methods

CSF samples (n=138) were routinely collected, measured and compared for WBC, PMN and MN on the XE-5000 software v06 and v10 with manual microscopy (Fuchs-Rosenthal chamber and stained cytopsin slides).

Results

Bland-Altman analysis showed good agreement between software v10 and manual methods for counting WBC (mean bias +4.7) and MN (mean bias -4.3). However, higher cell counts (mean bias +10.7) were noted for counting PMN on the XE-5000 software v10 compared to reference. Analysis of the data between software v06 and manual methods demonstrated similar results for counting MN cells as software v10. Conversely, higher counts for WBC (mean bias +9.9) and PMN (mean bias +17.3) were found with software v06 compared to reference. Additionally, v10 showed better agreements in the lower CSF concentration range (WBC: $\leq 20 \times 10^6/L$) for counting WBC and PMN compared to v06; although, a slight positive bias still remained.

Conclusion

The v10 software for the XE-5000 BF mode demonstrated superior performance with WBC and PMN counts in CSF in comparison with v06 software.

INTRODUCTION

Cellular analysis of body fluids can provide clinicians with useful diagnostic and treatment effect information. Normally, CSF samples contain very few white blood cells (WBC). The presence of elevated WBC (differential) can be an early indication of a severe infection or diseases such as meningitis, encephalitis or other neurological disorders. In recent years, there has been an increase in demand for rapid and accurate automated hemocytometric analyzers for testing diverse body fluids in routine diagnostic settings. In 2006, Sysmex introduced the XE-5000 fully automated hematology analyzer that was cleared by the United States Food and Drug Administration (FDA) in 2007. It contains a whole blood mode and a dedicated Body Fluid (BF) mode. The XE-5000 BF mode uses two techniques: sheath flow impedance and fluorescence flow cytometry for cell identification and clustering. While sheath flow impedance technique is used for counting red blood cells (RBC) in the RBC channel, total WBC, polymorphonuclear (PMN), mononuclear (MN) and high fluorescent body fluid (HF-BF) cells are enumerated in the DIFF channel using fluorescent flow cytometry. This technique is based on the combination of side scatter (inner complexity of the cell) and fluorescence intensity (DNA/RNA content). The introduction of a dedicated BF mode on the XE-5000 has advantages such as lower imprecision, higher accuracy, faster turn-around time, and it is more cost effective than the gold standard (1-3). However, the high imprecision on the XE-5000 in low WBC concentrations ($\leq 20 \times 10^6/L$) remains a major challenge (1, 4, 5). This is particularly critical for CSF samples that normally contain very few cells. Previously, our group demonstrated that the counting of PMN in CSF samples on the XE-5000 BF mode using software v06 resulted in a significant positive proportional bias ($y=2.4x-1.4$) compared to stained cytospin slides (1). Moreover, the overestimation of PMN counts was most evident in CSF samples with low WBC counts ($< 20 \times 10^6/L$). There was no specific explanation for the large positive bias in this lower concentration range; however, we speculated that some cell debris or fragments between the x-axis and the PMN/MN clusters were counted as PMN. As a response, Sysmex recently released new software, v10, to improve the counting of leukocytes in body fluids on the XE-5000 BF mode. In v10, amongst others, the gating algorithm for separation of unknown particles such as cell debris or fragments in the WBC/PMN count has been improved. The aim of this study was to evaluate the counting of WBC, MN and PMN in CSF samples on the XE-5000 using the new software v10 by comparing the results with conventional manual microscopy as the reference method and the old software v06. We focused on CSF samples with low cell counts.

MATERIALS AND METHODS

Patient samples

This study was performed on 138 randomly selected CSF samples which entered the laboratory routinely from various in and out-patient departments of our university hospital. Informed consent from patients was not required because material used was that which remained following routine analysis. Because blinded material was used, we do not have any information on patient disease or patient treatment. Body fluid collection and analysis for cellular composition were performed in compliance with the CLSI H56-A guidelines. All samples were collected in plain tubes and analysed within one hour upon laboratory entry.

Study design

All samples were first subjected to requested routine cellular analysis on the XE-5000 using software v06, followed by measurements on the research XE-5000 (Sysmex Corporation, Kobe, Japan) using software v10. Quality control was performed daily, and no sample pretreatment was necessary prior to sample analysis. By setting manual microscopy analysis as the reference method, total WBC counts were determined by using Fuchs-Rosenthal counting chambers. Total WBCs were first stained with Samson reagent (fuchsine/acetic acid/phenol), followed by counting in the Fuchs-Rosenthal chamber using a 400-fold magnification. For each sample, 256 squares of the Fuchs-Rosenthal chamber were counted. Total WBC counts were calculated as follows: Total number of cells in 256 squares divided by 2.88. Manual differentiation of WBC into PMN (neutrophils, basophils and eosinophils) and MN (lymphocytes and monocytes) were determined in May-Grünwald Giemsa stained cytopsin slides. An experienced technician classified 200 cells using light microscopy under oil immersion at 400-fold magnification. A more detailed description of the methods can be found in our previous publications (8).

Statistical Analysis

Statistical analysis was performed using Analyse-it for Microsoft Excel software version 2.21. Agreement between automated and microscopic data was determined by Passing-Bablok regression analysis and Bland and Altman difference plots. Only the absolute values of measurements were used for data analysis. Statistical significance was based on the 95% Confidence Intervals (CI). A significant proportional or constant bias was noted when the 95% CI of the slope did not encompass 1, and the 95% CI of the intercept did not encompass 0, respectively.

RESULTS

Accuracy software v10

Passing-Bablok regression and Bland-Altman analysis data for counting total WBC, MN and PMN cells between the XE-5000 software v10 and v06 with the manual reference methods are summarized in **Table 1 and 2**. Analysis of the 138 randomly selected CSF samples showed good agreement for counting WBC ($y=1.04x+0.32$, $n=138$, $R^2=0.98$; mean bias: $+4.7 \times 10^6/L$; **Figure 1A**) between software v10 and the manual method; although, a slight significant positive bias was evident. Of the 138 CSF samples, 92 samples were used for WBC differentiation. Forty-six samples were excluded from the differential study due to insufficient sample volume for cytopsin preparation. Good agreement was found for MN ($y=0.91x+0.29$, $n=92$, $R^2=0.93$; mean bias: $-4.3 \times 10^6/L$; **Figure 1B**); although, the XE-5000 software v10 counted significantly less MN cells. Reasonable agreement, but a significant positive bias, was observed for PMN ($y=1.35x+0.33$, $n=92$, $R^2=0.87$; mean bias: $+10.7 \times 10^6/L$; **Figure 1C**; Table 1 and 2).

Table 1 Comparison between the XE-5000 version 10 and version 06 with manual microscopy.

Software version 10 vs. Manual reference method				
	Regression line	CI Slope	CI Intercept	R²
WBC	$y=1.04x + 0.32$ (n=138)	1.00 – 1.06	-0.06 – 0.50	0.97
PMN	$y=1.35x + 0.27$ (n=91)	1.16 – 1.51	0.00 – 0.50	0.87
MN	$y=0.91x + 0.27$ (n=91)	0.88 – 0.96	-0.27 – 0.96	0.93
Software version 06 vs. Manual reference method				
	Regression line	CI slope	CI intercept	R²
WBC	$y=1.16x + 0.32$ (n=138)	1.09 – 1.06	0.29 – 1.00	0.96
PMN	$y=1.66x + 0.41$ (n=91)	1.50 – 1.92	0.00 – 0.97	0.82
MN	$y=0.95x + 0.80$ (n=91)	0.88 – 1.03	0.32 – 1.52	0.88

CI: 95% Confidence Interval (Passing-Bablok)

Table 2 Bland-Altman analysis between the XE-5000 version 10 and 06 and manual reference methods.

Software version 10 vs. Manual microscopy			Software version 06 vs. Manual microscopy		
	Bias	95% CI mean bias		Bias	95% CI mean bias
WBC	4.7	1.7 to 7.6	WBC	9.9	5.8 to 14.0
PMN	10.7	4.5 to 16.9	PMN	17.3	8.0 to 26.6
MN	-4.3	-7.7 to -0.8	MN	-3.5	-8.1 to 1.0

CI: 95% Confidence Interval (Passing-Bablok)

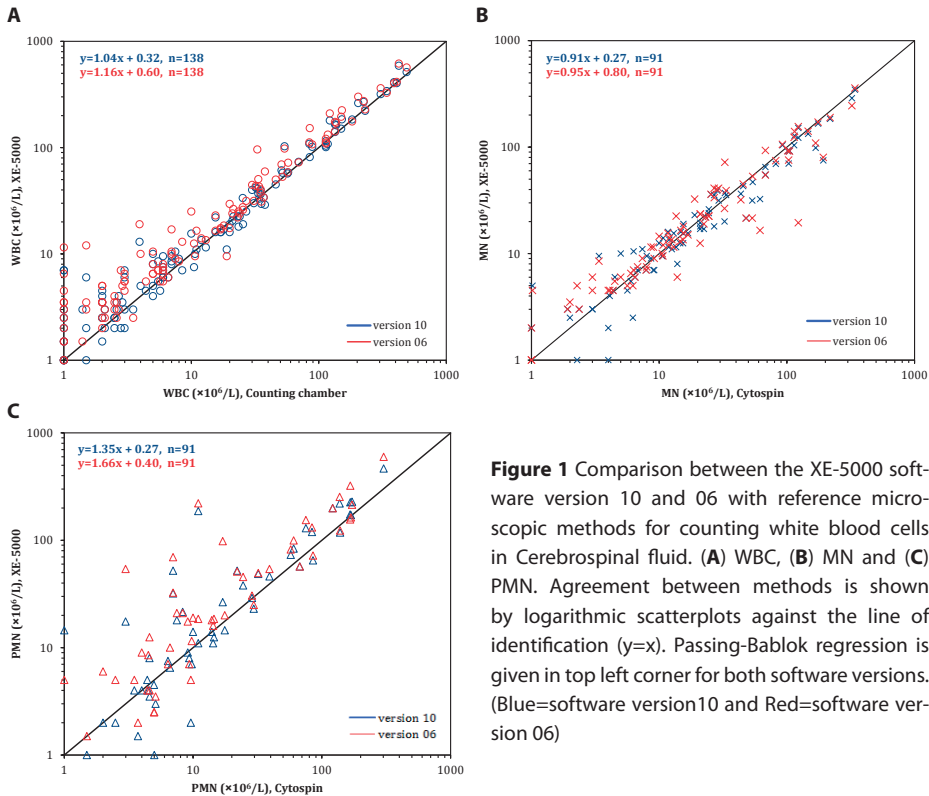


Figure 1 Comparison between the XE-5000 software version 10 and 06 with reference microscopic methods for counting white blood cells in Cerebrospinal fluid. (A) WBC, (B) MN and (C) PMN. Agreement between methods is shown by logarithmic scatterplots against the line of identification ($y=x$). Passing-Bablok regression is given in top left corner for both software versions. (Blue=software version 10 and Red=software version 06)

Accuracy software v06

By utilizing the same CSF samples, we compared results from the XE-5000 software v06 with the manual reference counts. As shown in Table 1, acceptable agreement for counting total WBC ($y=1.16x+0.60, n=138, R^2=0.96$; mean bias: $+9.9 \times 10^6/L$; Figure 1A) and PMN ($y=1.68x+0.45, n=96, R^2=0.92$; mean bias: $+17.3 \times 10^6/L$; Figure 1C) between methods was found; although, a significant positive bias was observed for both parameters. Excellent agreement for counting MN ($y=0.95x+0.90, n=92, R^2=0.88$; mean bias: $-3.5 \times 10^6/L$; Figure 1B) was noted. These results for software v06 are in keeping with our previous findings using the same software (1). If we compare software v10 with v06, the strong positive bias for WBC and PMN observed in software v06 was significantly reduced in software v10; although, a positive bias for PMN was still present (Table 1 and 2).

Accuracy low WBC concentrations

As a result of the large significant positive bias found primarily in CSF samples with low cell counts, separate regression analyses were performed on samples with WBC reference counts $\leq 20 \times 10^6/L$ to study the performance of software v10 more thoroughly (1). Excellent agreement was found between the new software and reference methods for

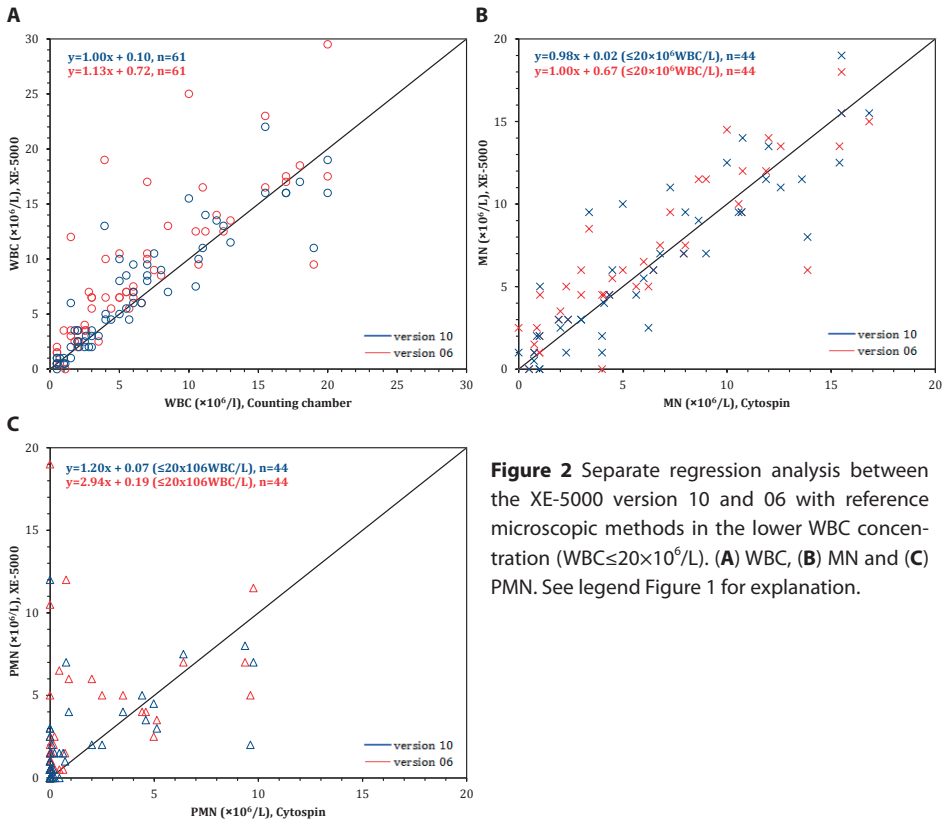


Figure 2 Separate regression analysis between the XE-5000 version 10 and 06 with reference microscopical methods in the lower WBC concentration ($WBC \leq 20 \times 10^6/L$). (A) WBC, (B) MN and (C) PMN. See legend Figure 1 for explanation.

counting WBC ($y=1.00x + 0.10$, [CI slope: 0.95 to 1.14, CI intercept: 0.21 to 0.34], $n=61$; **Figure 2A**) and MN ($y=0.98x + 0.02$, [CI slope: 0.86 to 1.12, CI intercept: 0.62 to 0.47], $n=44$; **Figure 2B**) with low cell counts. PMN counts showed reasonable agreement ($y=1.20x + 0.07$, [CI slope: 0.88 to 4.76, CI intercept: 0.00 to 0.46], $n=44$; **Figure 2C**), despite the fact that a slight significant positive bias was still present. Re-evaluation of the data, $\leq 20 \times 10^6 WBC/L$, using software v06 resulted in the following regression analysis: WBC ($y=1.13x + 0.72$, [CI slope: 1.00 to 1.38, CI intercept: 0.19 to 0.50], $n=61$; Figure 2A), MN ($y=1.00x + 0.67$, [CI slope: 0.88 to 1.15, CI intercept: 0.14 to 1.28] $n=44$; Figure 2B) and PMN ($y=2.94x + 0.19$, [CI slope: 1.18 to 12.50, CI intercept: 0.06 to 0.50], $n=44$; Figure 2C) that was in line with our previous report using the same software that showed a large and significant positive bias for WBC, and particularly, PMN. In line with the results for the whole concentration range, the positive bias for WBC and PMN has improved significantly in the low counting area with software v10. The positive bias for WBC was not significant in the low counting range using software v10, whereas for PMN, a significant bias remained.

DISCUSSION

Sysmex released new software v10 to improve the counting of leukocytes on the XE-5000 BF mode. Software v10 includes improved gating algorithms for excluding cell debris and/or fragments from the WBC/PMN cluster in body fluid analysis. In this study, we show that the counting of total WBC and PMN in CSF samples has been improved on software v10 compared to software v06; although, a significant positive bias for PMN remained in comparison with the manual method. Because normal CSF samples contain very few cells, it is important for WBC counts to be accurate, especially in the lower concentration range, around the cut-off upper limit of normal. Consequently, we focused on CSF samples $\leq 20 \times 10^6/L$ WBC to analyze the new software performance. Excellent agreement was found in the low range for both WBC and MN in software v10. Conversely, PMN still showed a significant positive bias; although, its magnitude was decreased with software v10 compared to software v06. It is a constant trend that the BF mode on the XE-5000 counts more cells in CSF samples compared to conventional microscopy (1,2,4). Furthermore, the overestimation of PMN is especially noted in samples containing very low WBC counts ($\leq 20 \times 10^6/L$) (1, 4). In a previous study, we demonstrated that the functional detection limit of the XE-5000 is $\sim 10 \times 10^6/L$. The lower the WBC count, the higher the imprecision. In the lower WBC range, the mean count of PMN determined by the XE-5000 software v10, was 2 (range: 0-12) and the median was 1.

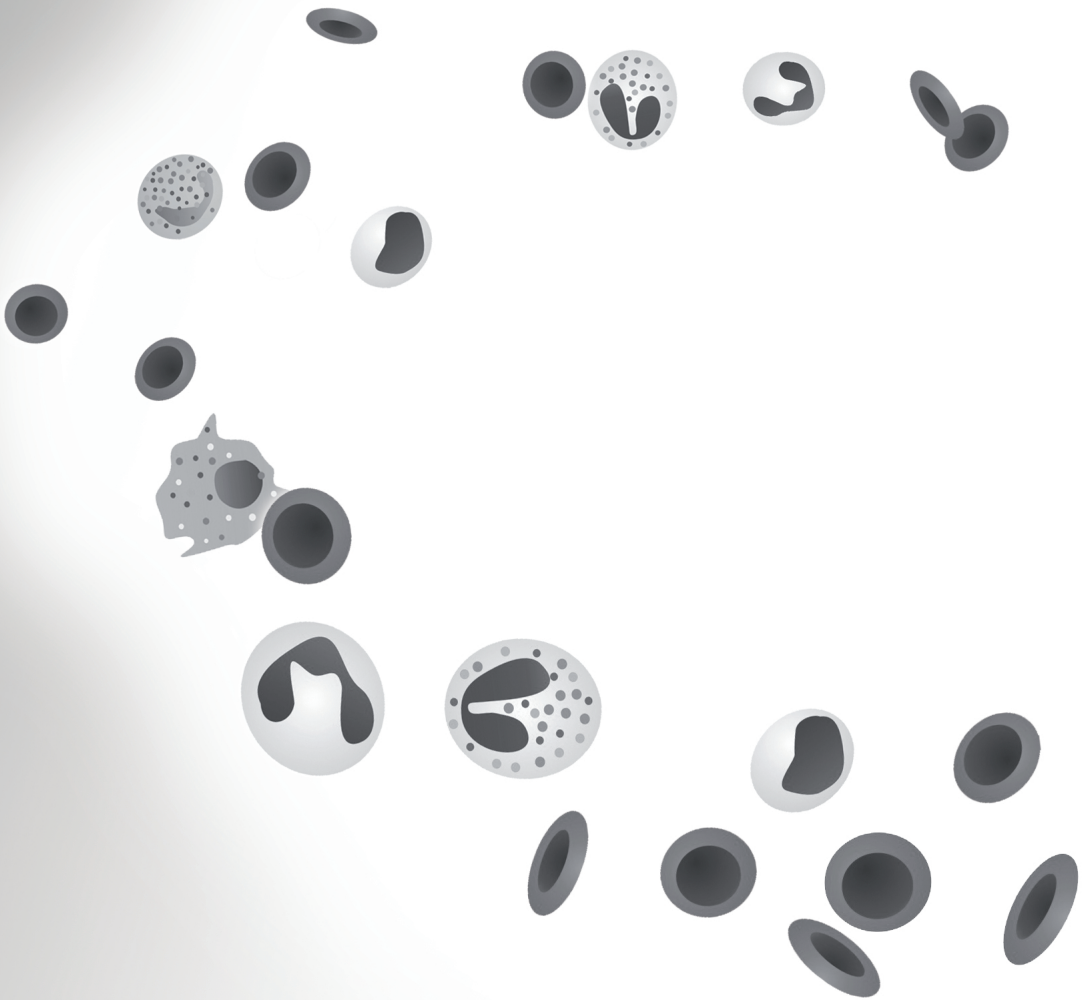
Interestingly, ventricular CSF samples showed a significant slope for WBC counting in contrast to lumbar punctures (2). CSF samples collected from ventricular drainage systems are prone to contain cell debris. This is possibly due to postoperative sample contamination or drainage installation systems (2,4). Since our study was blinded, we were unable to differentiate between CSF samples collected from lumbar punctures or from ventricular drainage systems. Future studies should include differentiating between lumbar and ventricular CSF samples, especially in lower concentration ranges, to examine if the positive bias observed using the XE-5000 is more pronounced in CSF ventricular samples due to the presence of cell debris in these samples. Though, one would expect less interference using software v10 as a result of the improved gating algorithms for excluding cell debris.

In this study, differentiation of WBC into PMN and MN cells were examined by stained cytopsin slides. However, this method contains a known pitfall. During preparation of cytopsin slides, cells were transferred to polylysine coated slides by cytocentrifugation. But according to Dux et al., roughly 17% of cells are lost after each centrifugation step in native CSF samples. Moreover, 90% of monocytes and granulocytes deteriorate after 90 min at room temperature (6). The duration from CSF sample collection to sample analysis is not always recorded. CSF samples may remain unattended for too long at room temperature in the operating room or during transport to the laboratory, thereby

delaying sample analysis (7). In native CSF, cells decrease rapidly as a result of cell lysis due to lower concentrations of albumin and lipids. Currently, mostly plain tubes are used to collect CSF samples in the majority of Clinical laboratories. However, previous studies demonstrated that the immediate addition of a serum containing medium to CSF tubes can stabilize cells for 5-24 hours after sampling (6-7). Possibly in the future, standardized specific tubes containing unique substances will be developed and introduced on the market for collecting and stabilizing cells in CSF. Taken together, a) the imprecision of the XE-5000 and also the manual reference method in very low cell counts, b) CSF samples from drainage systems which usually contain cell debris and c) the rapid cell loss in native CSF, may have contributed to the positive bias. Future studies should investigate this more thoroughly. Additionally, we compared the data between both software versions. Software v06 was set as reference method. Excellent agreement between both software versions were found for counting WBC ($y=0.93x-0.37$, [CI slope: 0.90 to 1.96, CI intercept: -0.75 to 0.07], $n=138$) and MN ($y=0.97x-0.03$, [CI slope: 0.95 to 1.00, CI intercept: -0.50 to 0.15], $n=138$) cells. Software v06 significantly counted more PMN ($y=0.84x-0.38$, [CI slope: 0.78 to 0.89, CI intercept: -0.44 to -0.17], $n=138$) than software v10. Collectively, agreements for counting WBC and PMN in CSF were better with software v10 compared to software v06. However, despite the improvement on software v10, a significant positive bias remained for PMN. In the future, continued efforts should be made by Sysmex to enhance precision in lower concentration ranges and to exclude cell debris or fragments from WBC/PMN counts.

REFERENCES

1. de Jonge R, Brouwer R, de Graaf MT, Luitwieler RL, Fleming C, de Frankrijker-Merkestijn M, et al. Evaluation of the new body fluid mode on the Sysmex XE-5000 for counting leukocytes and erythrocytes in cerebrospinal fluid and other body fluids. *Clin Chem Lab Med*. 2010;48:665-75.
2. Boer K, Deufel T, Reinhoefer M. Evaluation of the XE-5000 for the automated analysis of blood cells in cerebrospinal fluid. *Clin Biochem*. 2009;42:684-91.
3. Zimmermann M, Ruprecht K, Kainzinger F, Heppner FL, Weimann A. Automated vs. manual cerebrospinal fluid cell counts: a work and cost analysis comparing the Sysmex XE-5000 and the Fuchs–Rosenthal manual counting chamber. *Int J Lab Hematol*. 2011;33:629-37.
4. Perné A, Hainfellner JA, Womastek I, Haushofer A, Szekeres T, Schwarzinger I. Performance Evaluation of the Sysmex XE-5000 Hematology Analyzer for White Blood Cell Analysis in Cerebrospinal Fluid. *Arch Pathol Lab Med*. 2012;136:194-8.
5. Sandhaus LM, Ciarlini P, Kidric D, Dillman C, O’Riordan M. Automated Cerebrospinal Fluid Cell Counts Using the Sysmex XE-5000. *Am J Clin Pathol*. 2010;134:734-8.
6. Dux R, Kindler-Rohrborn A, Annas M, Faustmann P, Lennartz K, Zimmermann CW. A standardized protocol for flow cytometric analysis of cells isolated from cerebrospinal fluid. *Journal of the Neurological Sciences*. 1994;121:74-8.
7. Graaf MTd, Broek PDMvd, Kraan J, Bent RLLMJvd, Boonstra JG, Schmitz PIM, et al. Addition of serum-containing medium to cerebrospinal fluid prevents cellular loss over time. *J Neurol*. 2011;258:1507-12.
8. Fleming C, Brouwer R, Lindemans J, deJonge R. Validation of the body fluid module on the Sysmex XN-1000 for counting blood cells in cerebrospinal fluid and other fluids. *Clin Chem Lab Med* 2012; Epub ahead of print.



Chapter 5

Validation of the body fluid module on the new Sysmex XN-1000 for counting blood cells in cerebrospinal fluid and other body fluids

Chérina Fleming, Rob Brouwer, Jan Lindemans and Robert de Jonge

ABSTRACT

Objective

We evaluated the body fluid (BF) module on the new Sysmex XN-1000 for counting blood cells.

Methods

One hundred and eighty seven BF samples (73 CSF, 48 CAPD, 46 ascites, and 20 pleural fluid) were used for method comparison between the XN-1000 and manual microscopy (Fuchs-Rosenthal chamber and stained cytospin slides) for counting RBCs and WBCs (differential).

Results

Good agreement was found for counting WBCs ($y=1.06x+0.09$, $n=67$, $R^2=0.96$) and mononuclear cells (MNs) ($y=1.04x-0.01$, $n=40$, $R^2=0.93$) in CSF. However, the XN-1000 systematically counted more polymorphonuclear cells (PMNs) ($y=1.48x+0.18$, $n=40$, $R^2=0.99$) compared to manual microscopy. Excellent correlation for RBCs $>1\times 10^9/L$ ($y=0.99x+116.56$, $n=26$, $R^2=0.99$) in CSF was found. For other fluids (CAPD, ascites and pleural fluid), excellent agreement was found for counting WBCs ($y=1.06x+0.26$, $n=109$, $R^2=0.98$), MNs ($y=1.06x-0.41$, $n=93$, $R^2=0.96$), PMNs ($y=1.06x+0.81$, $n=93$, $R^2=0.98$) and RBCs ($y=1.04x+110.04$, $n=43$, $R^2=0.98$). By using BF XN-check, the lower limit of quantitation (LoQ) for WBC was defined as $5\times 10^6/L$. Linearity was excellent for both the WBCs ($R^2=0.99$) and RBCs ($R^2=0.99$) and carryover never exceeded 0.05%.

Conclusion

The BF module on the XN-1000 is a suitable tool for fast and accurate quantification of WBC (differential) and RBC counts in CSF and other BFs in a diagnostic setting.

INTRODUCTION

Analysis of white blood cells (WBCs) and red blood cells (RBCs) in body fluids (BF) are important for detecting signs of organ injury or infection which can cause fluid formation in several diseases (1). For example, the presence of elevated WBCs (differential) and/or RBCs in cerebrospinal fluid (CSF) may aid in the diagnosis of meningitis, encephalitis, brain abscess, multiple sclerosis and intracerebral hemorrhage (2). Diseases such as tuberculosis, congestive heart failure or malignancies can be distinguished by the separation of pleural effusion into transudates or exudates (3). Classification of these effusions strongly depends on lactic dehydrogenase (LDH) and protein levels in concert with total WBC count (4,5). Cirrhotic patients with ascites are susceptible to spontaneous bacterial peritonitis (SBP). According to the clinical diagnostic guidelines, SBP is present when more than $250 \times 10^6/L$ PMN cells are counted in ascites (6). Peritonitis is most common in patients with end stage renal disease, treated with continuous ambulatory peritoneal dialysis (CAPD). When $>100 \times 10^6$ WBC/L and $\geq 50\%$ PMNs are present in CAPD fluid, peritonitis is suspected (7); therefore, rapid laboratory results are of important clinical relevance.

Manual differential counting has been the "gold standard" for the determination of WBC and RBC in fluids for decades (8). However, this method is labor-intensive, and manual counting is hampered by high inaccuracy, imprecision and high inter-observer variability (9). As a result, many modernized laboratories have replaced the conventional manual differential count with automated hemocytometric analyzers for initial screening and detection of hematologic abnormalities in fluids (10). Analysis of BFs on automated hematology analyzers such as the Cell-Dyn Sapphire (Abbott) and the ADVIA 2120 (Siemens) are capable of detecting RBCs, WBCs and WBC differential with certain limitations (9,11,12). In 2006, Sysmex introduced a dedicated BF mode on the XE-5000 automated hematology analyzers (13), and recently launched the XN-Series which contains an improved BF module compared to the XE-5000 at the 2011 IFCC-WorldLab congress in Berlin.

In this study, we evaluated the BF module on the new XN-1000 analyzer for counting RBCs and WBCs (differential) in BFs, and compared them with manual counts. In addition, the precision profile, carryover and linearity were assessed.

MATERIALS AND METHODS

Patient material

Collectively, 187 BF samples were prospectively collected and used for method comparison. The BF samples included 73 CSF, 46 ascites, 20 pleural and 48 CAPD. Informed consent from patients was not required, because material used, was that which remained

following routine analysis. Because blinded material was used, we do not have any information on patient disease nor patient treatment. However, we do know that these patients were both in and out-patients from various departments in our university hospital. Therefore, our results can be generalized to a broad patient population. All fluids were collected in ethylenediaminetetraacetic acid (EDTA) anticoagulant tubes except CSF samples which were collected in plain tubes. Body fluid collection and analysis for cellular composition were performed in compliance with the CLSI H56-A guideline. The majority of samples were analysed within one hour upon laboratory entry.

Automated method: XN-1000 BF module

RBC, WBC and WBC differential counts of all fluids were first measured in the open BF mode on the routine XE-5000 (Sysmex, Etten-Leur, The Netherlands) upon arrival in the laboratory for routine analysis. Next, samples were determined on the XN-1000 (Sysmex Corporation, Kobe, Japan) BF open mode in duplicate. No sample pre-preparation was necessary prior to sample analysis. Every day, two levels (low & high) of BF XN-check were measured prior to sample analysis. While the RBCs are counted in the RBC channel using sheath flow impedance technology the WBC, MN and PMN cells are determined by flow cytometry in the DIFF channel. The DIFF channel combines forward scatter (size of the cell) with side scatter (inner complexity of the cell) and the fluorescence intensity (DNA/RNA content) to identify and cluster each cell (14). This channel also reports high fluorescence body fluid cells (HF-BF) such as macrophages and mesothelial cells. These HF-BF cells are found just above the MN cluster at fluorescence discriminator >200 channels, and are not included in the WBC (differential) count; however, they are included in the total nucleated cell count (TC-BF). If the number of HF-BF cells present exceeds a preset cut-off value, the "WBC abnormal scattergram" flag will be generated. This cut-off value is user-definable. Additionally, if non-cellular particles such as bacteria or cell debris are present, leading to erroneous clusters, the "WBC abnormal scattergram" flag will be automatically generated. Furthermore, the XN-1000 BF module has several new features compared to the XE-5000 BF mode. It is able to count 4x more WBCs than the XE-5000, aspirates less sample volume (88 μL) compared to the XE-5000 (130 μL), and offers open or closed tube sampling in fluids and blood as well as a micro-tube adapter for easy handling of pediatric tubes.

Manual Method

For manual microscopy, the Fuchs-Rosenthal hemocytometer was used to count RBCs and total WBCs in BFs. While the RBCs were counted unstained, WBCs were first stained with Samson reagent (fuchsin/ acetic acid/phenol) followed by counting in the Fuchs-Rosenthal chamber using a 400-fold magnification. If samples measured on the XE-5000 presented high WBCs ($>200 \times 10^6/\text{L}$), dilutions with phosphate-buffered saline (PBS) pH

7.4, containing 0.01 mol/L phosphate, were prepared accordingly. For each sample, 256 squares of the Fuchs-Rosenthal chamber were counted, except for CSF samples with WBC $<30 \times 10^6/L$ where 2×256 squares were enumerated. Total WBC counts were calculated as follows:

Total number of cells in 256 squares $\times 10/9$ (Samson dilution factor) \times dilution factor.

3.2 (total volume counting chamber in μL)

RBCs were calculated in a similar fashion except for the Samson reagent dilution factor. Manual differentiation of WBCs into PMNs (neutrophils, basophils and eosinophils) and MNs (lymphocytes and monocytes) was determined by cytopsin slides. The polylysine cytopsin slides were prepared by using the Rotofix 32 cytocentrifuge (Hettich, Germany) with the following settings: 5 minutes at $85 \times g$. Like the counting chamber, if more than $200 \times 10^6/L$ WBCs were present in fluids, samples were first diluted according to protocol to prepare cytopsin slides. After centrifugation, slides were May-Grünwald Giemsa stained using the Sysmex SP-100 slide-stainer unit (Sysmex, Etten-Leur, The Netherlands). An experienced technician, scoring 200 cells, reviewed all slides using light microscopy under oil immersion at 400-fold magnification. In several cases, preparation of the cytopsin slide failed or not enough material remained; therefore, those samples were excluded from the differential evaluation. The reference method was considered the microscopic counting of RBC, total WBC and WBC differential for all BFs.

Precision

Between-run precision was assessed by analyzing two levels (low and high) of Sysmex BF XN-check 20x over 18 days. Serially diluted BF XN-check was used to determine the WBC within-run precision and precision profile. The serially diluted BF XN-check was measured 10 times consecutively on the XN-1000 instrument in the QC analysis mode. The mean WBC count of each dilution was plotted against the coefficient of variation (%CV). The point where the CV exceeded 20% was arbitrarily defined as the lower limit of quantitation (LoQ), and served as means for comparing analyzers (13).

Accuracy

CSF, pleural fluid, ascites and CAPD fluids were used for method comparison studies. Samples were first measured on the automated analyzers, followed by manual counts as described in the method section. There were two exclusion criteria during this study: 1) presence of the "WBC abnormal scattergram" flag or 2) more than 5% broken cells were found in either the counting chamber or cytopsin slides. For RBCs, the XN-1000 is only capable of reporting RBC counts $\geq 1000 \times 10^6/L$ ($1 \times 10^9/L$). XN-1000 counts lower and higher than 500×10^6 RBC/L are reported as $0 \times 10^9/L$ and $1 \times 10^9/L$ respectively. Because of this, regression analysis was performed only on counts $> 1 \times 10^9/L$ for CSF and other fluids.

Linearity

Samples with high WBC/RBC cell counts were serially diluted with PBS to relevant concentrations. All samples were measured 5 times, and the means were compared with the expected cell counts.

Sample carryover

Carryover was determined by measuring samples with high cell counts in triplicate (H1, H2, H3), followed by 3 consecutive measurements of a blank (Cellpack; L1, L2, L3). Carryover was calculated as follows: $[(L1-L3)/(H3-L3)] \times 100\%$ as recommended by the International Council for Standardization in Haematology (15).

Statistics

For all statistical analysis, the absolute values of the measurements were used, unless mentioned otherwise. Analyse-it for Microsoft Excel software version 2.21 and Microsoft Excel 2003 were used for data analysis. Passing-Bablok regression analysis was used for method comparison and linearity studies. Statistical significance was based on the 95% Confidence Intervals (CI). A significant proportional or constant bias was noted when the CI of the slope did not encompass 1, and the CI of the intercept did not encompass 0 respectively.

RESULTS

Precision

The WBC (within-run) precision profile was determined by using serially diluted BF XN-check, depicted in **Figure 1**. A CV of 20% was attained at 5×10^6 WBC/L (LoQ). The between-run precision was always $<7.5\%$ for WBC, MN and PMN for both BF XN-check levels (**Table 1**).

Accuracy

From a total of 187 BF samples (73 CSF, 48 CAPD, 46 ascites, 20 pleural fluid) collected for method comparison study, only 133 BF samples were used. Fifty-four samples (33 CSF, 10 CAPD, 8 ascites, 3 pleural fluid) were excluded from this evaluation because of either missing slides (due to insufficient sample volume $n=24$), the presence of the "WBC abnormal scattergram" flag ($n=4$) or when more than 5% broken cells were found in both the counting chamber or cytospin slides ($n=26$). Furthermore, no technical difficulties were encountered during this study.

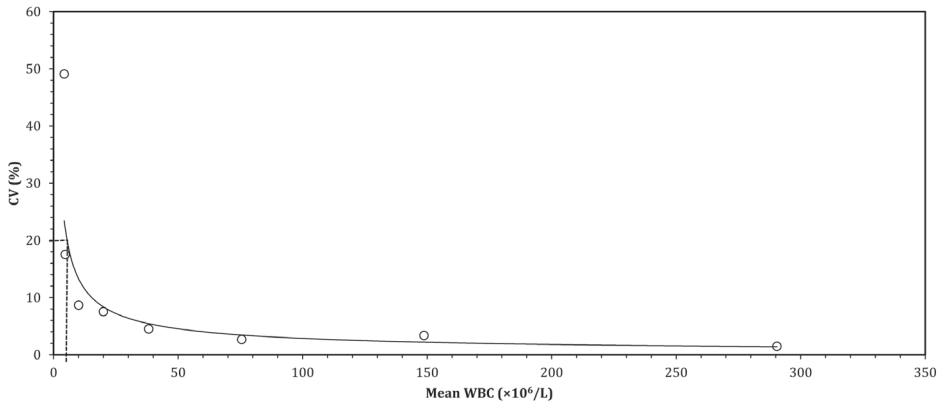


Figure 1 Precision profile (lower limit of quantitation) for total WBC count on the XN-1000 BF module using serially diluted BF XN-check material. Each point represents the CV of a 10-fold measurement.

Table 1 Between-run precision.

	Sample	Mean ($\times 10^6/L$)	SD	CV (%)
WBC	BF XN-check I	84.0	4.4	5.2
MN	BF XN-check I	32.0	2.4	7.4
PMN	BF XN-check I	52.1	3.8	7.2
WBC	BF XN-check II	309.2	9.9	3.2
MN	BF XN-check II	123.0	4.9	4.0
PMN	BF XN-check II	186.2	6.7	3.6

CV (%): coefficient of variation

Cerebrospinal fluid

Correlation between the XN-1000 and Fuchs-Rosenthal chamber for counting WBCs in CSF is depicted in **Figure 2A**. Good agreement ($y=1.06x+0.09$, $n=67$, $R^2=0.96$) was found between methods for counting WBCs in CSF, albeit the small significant positive bias (Table 2). There was one outlier present in the higher WBC concentration range. The XN-1000 reported $4970 \times 10^6/L$ WBCs; whereas, the counting chamber enumerated $2694 \times 10^6/L$ WBCs. We could not find any obvious errors in the manual or automated analysis. Because of the large discrepancy, the data was re-analysed, excluding this data point. The re-analysed data resulted in a similar regression ($y=1.05x+0.07$, [CI slope: 1.02 to 1.11, CI intercept: -0.20 to 0.45] $n=66$, $R^2=0.99$), and the significant positive bias remained.

Normally, white blood cells in CSF range from $0-6 \times 10^6/L$ in adults as we have established using the XE-5000 (13). Separate regression analysis was performed in the lower WBC ($<30 \times 10^6/L$) range, and excellent agreement ($y=1.00x+0.20$, $n=45$, $R^2=0.94$) was

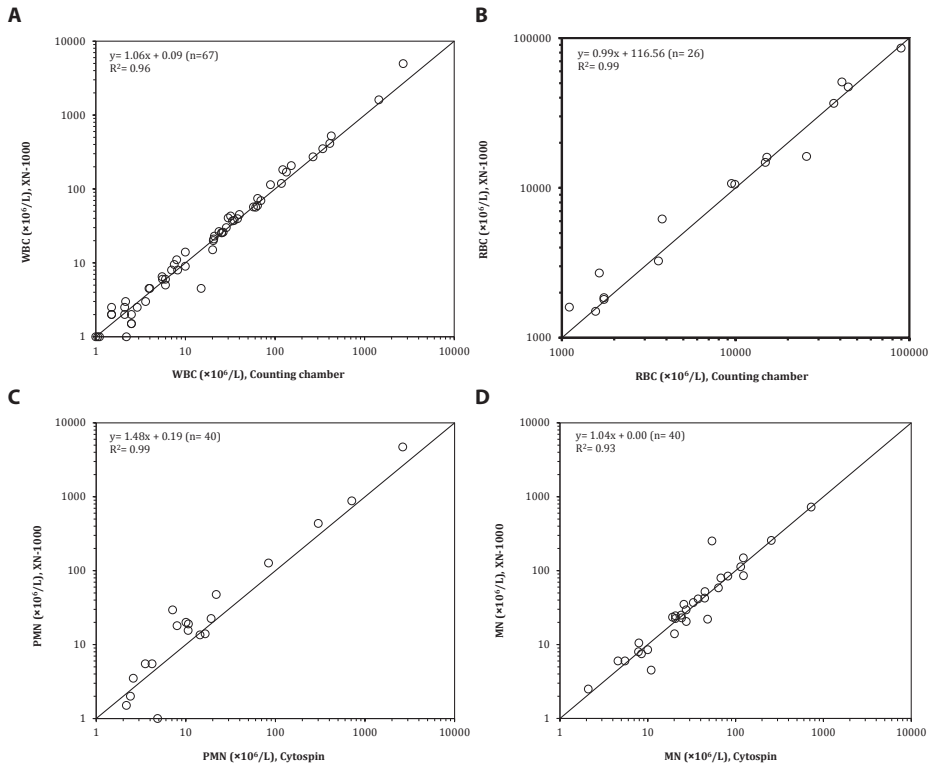


Figure 2 Comparison between the XN-1000 and the reference microscopic methods for counting blood cells in cerebrospinal fluid. **(A)** WBC, **(B)** RBC, **(C)** PMN and **(D)** MN. Agreement between methods is shown by logarithmic scatterplots against the line of identification (Passing-Bablok regression is given in top left corner). The minimal reportable unit for RBC counts on the XN-1000 BF-module is $1000 \times 10^6/L$.

observed (**Table 2**). For differential WBC in CSF, a significant positive bias ($y=1.48x+0.18$, $n=40$, $R^2=0.99$; **Figure 2C**) was observed for PMN and good agreement ($y=1.04x-0.01$, $n=40$, $R^2=0.93$; **Figure 2D**) was found for MN. Comparison between the counting chamber and the XN-1000 BF module for counting RBCs $>1 \times 10^9/L$ in CSF ($y=0.99x+116.56$, $n=26$, $R^2=0.99$; **Figure 2B**) showed excellent agreement.

Other fluids

Regression analysis for total and differential WBC counts in other fluids is shown in **Figure 3**. A slight positive, but significant proportional bias was found for total WBC counts in ascites, CAPD fluid and pleural fluid between the automated and reference methods ($y=1.06x-0.26$, [CI slope: 1.04 to 1.09, CI intercept: -3.63 to 0.59], $n=109$, $R^2=0.96$; **Figure 3A**). Good agreement was also found for both PMNs ($y=1.06x+0.81$, [CI slope: 1.00 to 1.13, CI interval: 0.05 to 1.49] $n=93$, $R^2=0.98$; **Figure 3C**) and MNs ($y=1.06x-0.41$, [CI slope: 1.01 to 1.11, CI intercept: -2.38 to 1.99] $n=93$, $R^2=0.96$; **Figure 3D**) in other fluids (CAPD,

Table 2 Method comparison between the XN-1000 and manual reference methods

Fluid	Regression line	CI Slope	CI Intercept	R ²
CSF _(WBC)	y=1.06x + 0.09 (n=67)	1.02 - 1.12	-0.23 - 0.41	0.96
CSF _(WBC <30×10⁶/L)	y=1.00x + 0.20 (n=45)	0.97 - 1.07	0.07 - 0.50	0.94
CSF _(MNC)	y=1.04x - 0.00 (n=40)	0.99 - 1.14	-0.84 - 0.48	0.93
CSF _(PMN)	y=1.48x + 0.19 (n=40)	1.27 - 1.79	0.00 - 0.45	0.99
Ascites _(WBC)	y=1.06x - 2.37 (n=43)	1.01 - 1.14	-12.24 - 2.71	0.99
Ascites _(MNC)	y=1.03x - 2.89 (n=38)	0.96 - 1.10	-10.70 - 3.67	0.97
Ascites _(PMN)	y=1.10x + 2.11 (n=38)	1.00 - 1.18	-0.14 - 3.33	0.99
CAPD _(WBC)	y=1.10x - 0.64 (n=46)	1.05 - 1.15	-2.93 - 0.33	0.97
CAPD _(MNC)	y=1.17x - 1.19 (n=38)	1.11 - 1.25	-3.32 - 0.01	0.72
CAPD _(PMN)	y=1.04x + 0.25 (n=38)	0.98 - 1.11	-0.22 - 0.94	0.97
Pleural _(WBC)	y=1.08x - 37.15 (n=20)	1.03 - 1.14	-92.30 - 2.78	0.99
Pleural _(MNC)	y=1.05x - 8.99 (n=17)	0.95 - 1.22	-69.08 - 55.00	0.97
Pleural _(PMN)	y=1.02x + 8.95 (n=17)	0.74 - 1.45	-17.87 - 29.91	0.85

CI= 95% confidence Interval (Passing-Bablok).

ascites, pleural fluid) between the XN-1000 and manual method, despite the small positive proportional bias.

The regression analysis for the individual fluids is presented in Table 2. The trends were similar compared to the combined analysis in Figure 3. In general, a slight positive bias was observed for WBC, PMN and MN in ascites, CAPD fluid and pleural fluid. The R² values were >0.97 with exception to MN counts in CAPD fluid and PMNs in pleural fluid (Table 2). The MN count in CAPD fluid showed a larger and significant positive bias (y=1.17x-1.19, n=38, R²=0.72) compared to the other fluids. In pleural fluid, the PMNs showed good agreement, but a lower correlation of R²=0.85 was found. Separate regression analysis was performed in the lower <10×10⁶/L and higher >10×10⁶/L WBC counts, and the results were comparable to the combined analysis (data not shown). Excellent correlation was found between the XN-1000 and the reference method for counting RBCs >1×10⁹/L in other fluids (y=1.04x+110.04, n=43, R²=0.98; **Figure 3B**).

Linearity

Linearity for WBCs (R²=0.99) in both the low and high concentration ranges was excellent on the XN-1000 (**Figure 4A and 4B**). However, linearity in the very low WBC range (5- 12×10⁶/L) showed higher discrepancies. The mean bias in this concentration range was -22.68%. Despite the negative bias in the lower concentration range, an average recovery of 81.8% was observed (Figure 4A). Linearity for RBC (R²=0.99) was also excellent, despite the increasing differences in the lower concentration range 0- 200×10⁶/L (**Figure 4C**).

Sample carryover

Carryover on the XN-1000 BF module was negligible for both WBC and RBC in all BF. It never exceeded 0.05% (Table 3).

Table 3 Carryover for the XN-1000 body fluid module.

Sample	Mean ($\times 10^6/L$)				Carryover (%)			
	WBC	MN	PMN	RBC	WBC	MN	PMN	RBC
CSF-1	15,421	2,062	13,359	900	0.03	0.00	0.03	0.00
CSF-2	9,562	2,269	7,293	1,704,200	0.04	0.04	0.04	0.00
CSF-3	271	77	194	153,200	-0.04	0.00	-0.53	0.00
Ascites	1,891	1,181	714	5,333	0.00	-0.03	0.05	0.00
Pleural	2,471	2,358	63	26,400	-0.06	0.03	-3.11	0.00

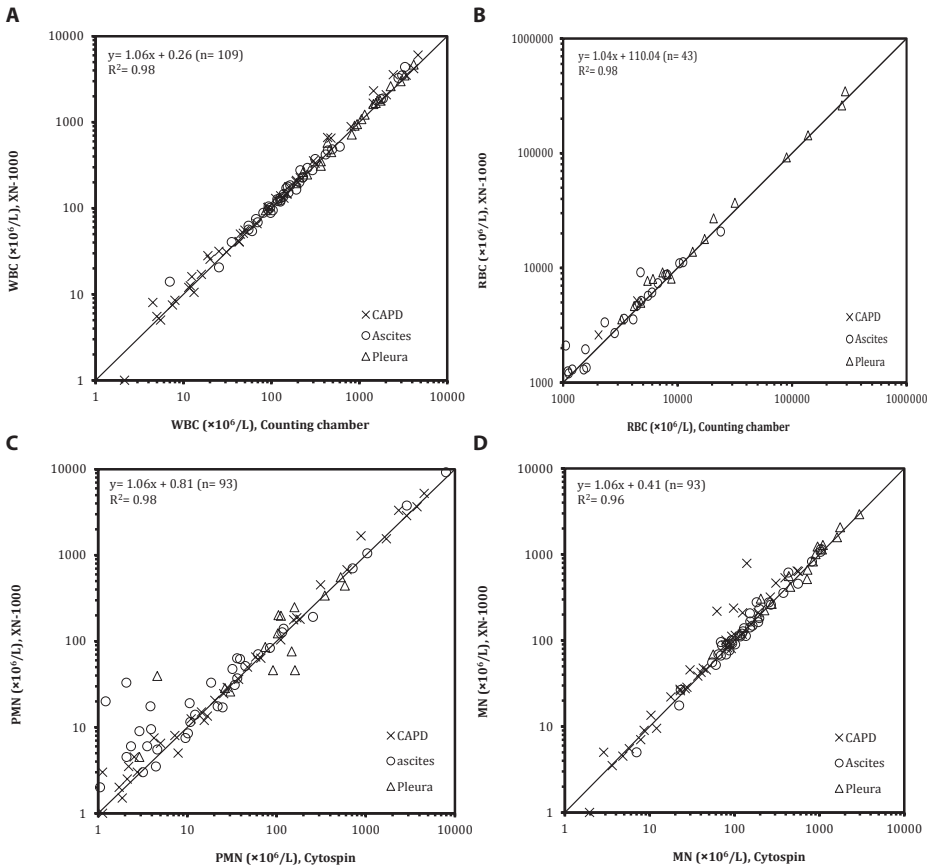


Figure 3 Comparison between the XN-1000 and the reference microscopic methods for counting various (CAPD, ascites, pleural fluid) body fluids. The data from these fluids are represented in one graph, with a different symbol for each fluid. (A) WBC, (B) RBC, (C) PMN and (D) MN.

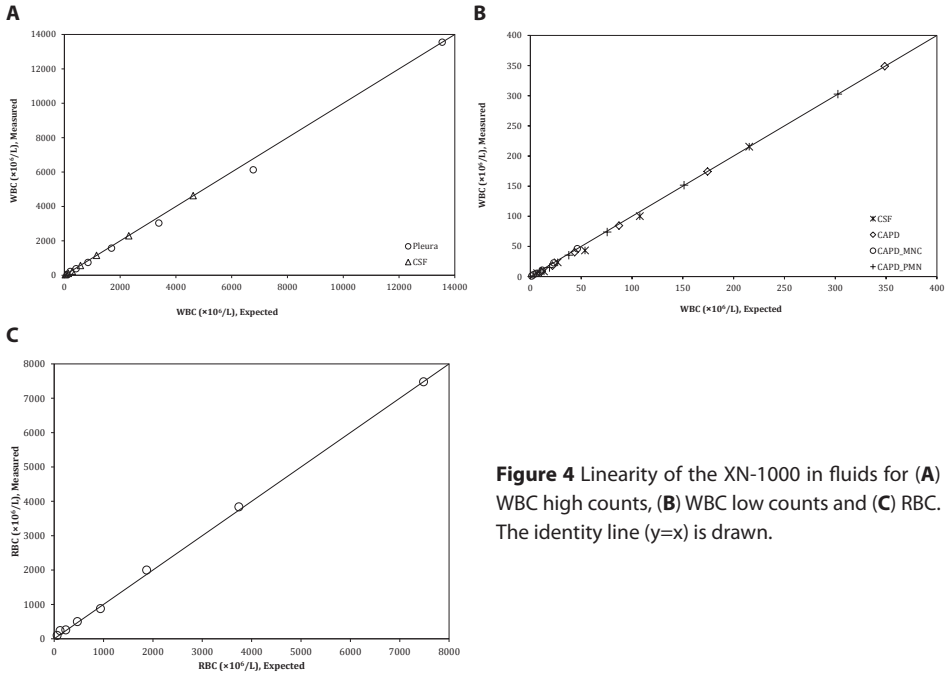


Figure 4 Linearity of the XN-1000 in fluids for (A) WBC high counts, (B) WBC low counts and (C) RBC. The identity line ($y=x$) is drawn.

DISCUSSION

The aim of this evaluation study was to compare the counting of blood cells in various BF_s ($n=187$) between the BF module on the new Sysmex XN-1000 automated analyzer and the manual microscopic methods. Additionally, the precision profile, carryover and linearity were determined. Our results indicate good agreement between the XN-1000 and the reference methods for counting WBC and RBC, and the precision profile have improved significantly in comparison to the XE-5000. Carryover was negligible and linearity was good.

Previously, counting of red and white blood cells was only determined by manual microscopic hemocytometer counting. However, this method comes with many limitations such as high imprecision, inaccuracy and inter-observer variability, and it is time-consuming (9). Because laboratory assessment of BF_s is an essential part of disease diagnosis and follow-up, rapid accurate results are very important. Presently, most automated hematology analyzers, including the new Sysmex XN-Series, have adopted the use of flow cytometry technology for identifying and quantifying cells. This technology enhances precision, and is less time consuming (9).

Comparison between the automated and manual reference methods showed excellent correlation for counting WBCs, RBCs and MNs in CSF. However, a significant positive

proportional bias for PMNs in CSF was observed between methods (Figure 2D, Table 2). The systematic increase of PMNs in CSF samples observed on the XN-1000 BF module is in line with our previous findings on the XE-5000 BF mode ($y=2.4x-1.4$) (13); although, its magnitude is decreased on the XN-1000. Several explanations for the discrepancy observed between automated and reference manual methods are: 1) cell debris that appears just below the PMN clustering is being included in the PMN algorithm, 2) there are loss of cells during centrifugation in cytopsin slide preparation; whereby, fewer PMNs are enumerated, and 3) the fact that cell numbers decrease rapidly as a result of cell lysis in native CSF due to lower concentrations of albumin and lipids (13,16). Furthermore, the duration from CSF collection to sample analysis is not always recorded. CSF samples may remain unattended for too long at room temperature either in the operating room or during transport to the laboratory, thereby delaying sample analysis (17). Previous studies have shown that immediate addition of a serum containing medium to CSF tubes can stabilize CSF cells for 5-24 hours after sampling (17,18). According to Dux et al., roughly 17% of cells are lost after each centrifugation step in native CSF samples. Moreover, 90% of monocytes and granulocytes deteriorated after 90 minutes at room temperature (18). We speculated that cell stability could have caused the increased PMN count on the XN-1000 because it occurred over the whole concentration range (hence, it was proportional); Table 2. When CSF samples entered the laboratory, they were first measured on the automated analyzers and then counted manually. The XN-1000 counts total WBCs, MNs and PMNs in one process using the same technique as opposed to the manual reference method which uses two techniques. Firstly, after Samson staining, total WBCs are microscopically counted in the Fuchs-Rosenthal counting chamber. Next, MN and PMN cells are microscopically differentiated by cytopsin slides after cytocentrifugation, followed by May-Grünwald Giemsa staining as described in the method section. During the time-interval between total WBC count and WBC differential, and during the course of centrifugation, cells may have decayed in native CSF, resulting in differences between the automated and manual methods that we observed in this study. In conclusion, cellular decay during prolonged analysis, centrifugation and the ghost gating algorithm could have caused the observed discrepancy in the CSF PMN count. Future studies should investigate the stability of cells in CSF. Additionally, the manufacturer should improve the clustering algorithm for PMN in CSF.

To investigate how well the XN-1000 is at predicting abnormality, we looked at the clinical cut-off value for CSF ($0-6 \times 10^6/L$). The XN-1000 BF module proved to be highly sensitive (100%) and specific (97.6%) for CSF specimens (data not shown). Of the 67 CSF samples, only one false positive result was found.

All other fluids (CAPD, ascites, pleural fluid) showed excellent agreement between the XN-1000 and reference manual methods for counting RBC and WBC (differential), which is in keeping with our previous findings on the XE-5000 (13). Samples such as pleural

and ascites fluids usually contain macrophages or mesothelial cells. These cells are classified as HF-BF cells on the XN-1000, and are excluded from the WBC (differential) count. However, the presence of HF-BF cells in CSF is nearly always abnormal and should be followed up by manual microscopy.

The XN-1000, using automated hemocytometry, offers several advantages over manual counting for routine clinical laboratories: *i*) fast turn-around-time also during night shifts and emergency situations, *ii*) reproducible and accurate results, *iii*) small sample volume (88 μL) necessary for analysis and *iiii*) no sample pre-treatment, reducing pre-analytical time. However, a critical aspect of the XN-1000 is its disability to differentiate malignant cells (blast or lymphoma cells) from other large cells (mesothelial and macrophages). Depending on the cell, these malignant cells will be clustered into either the HF-BF fraction or the MN cluster. There is no specific flag to alarm users of the presence of malignant cells. However, the "WBC abnormal scattergram" flag will be generated if HF-BF cells are present. A cut-off value can be manually set by the operator. Every user should have a follow-up protocol in the presence of "HF-BF flagging" on how to proceed. When abnormal clustering or blast cells are suspected, or in case of doubt, manual review of a cytospin slide should follow automated counting. In the future, more specific flags/action messages could be developed by the manufacturer in order to help the laboratory in differentiating malignant from non-malignant (HF-BF) cells in BF samples. Compared to the LoQ of the XE-5000 ($10 \times 10^6/\text{L}$), the XN-1000 has improved significantly ($5 \times 10^6/\text{L}$). This is an excellent improvement with regard to CSF, where the blood cell counts are normally very low. The between-run precision was also excellent.

Linearity and Carryover

Sample carryover and/or cross contamination is a problem that can affect accuracy and precision of a method; therefore, it is important that these interfering factors are minimal or absent between measurements; else, this may lead to erroneous results which, in turn, can lead to incorrect clinical prognosis or treatment of patients. This is especially critical for CSF samples because of their low clinical threshold values. To eliminate carryover, Sysmex has incorporated an automatic rinse cycle after every measurement. If BF samples measured on the XN-1000 contains $>1000 \times 10^6/\text{L}$ WBC, a background check is automatically executed. A low background count of only 0 or $1 \times 10^6/\text{L}$ WBC is accepted in the BF module. However, if the background check exceeds these numbers, automatically extra rinse cycles are carried out until acceptable WBC values are acquired. The XN-1000 is able to alternate between the WB (whole blood) and BF mode within 120 seconds (depending on the number of rinse cycles) with few to zero cells in the background. Carryover on the XN-1000 between modes and sample analysis was insignificant and never exceeded 0.05%. The linearity test best reflects the accuracy and precision of any given method. According to the manufacturer's specification, the BF module on the XN-1000

is linear from 0 to $10,000 \times 10^6$ WBC/L. The XN-1000 presented excellent linearity for WBC counts within the relevant clinical ranges for all fluids (Figure 4A and 4B).

In conclusion, the data presented in this study demonstrates that the BF module on the new Sysmex XN-1000 is rapid, reliable and accurate for counting RBC and WBC (differential) in CSF and other BF's with some limitations.

REFERENCES

1. Paris A, Nhan T, Cornet E, Perol JP, Malet M, Troussard X. Performance evaluation of the body fluid mode on the platform Sysmex XE-5000 series automated hematology analyzer. *Int J Lab Hematol* 2010;32:539-47.
2. de Graaf MT, de Jongste AH, Kraan J, Boonstra JG, Smitt PA, Gratama JW. Flow cytometric characterization of cerebrospinal fluid cells. *Cytometry B Clin Cytom* 2011;80:271-81.
3. Light RW, Macgregor MI, Luchsinger PC, Ball WC, Jr. Pleural effusions: the diagnostic separation of transudates and exudates. *Ann Intern Med* 1972;77:507-13.
4. Joseph J, Badrinath P, Basran GS, Sahn SA. Is the pleural fluid transudate or exudate? A revisit of the diagnostic criteria. *Thorax* 2001;56:867-70.
5. Light RW, Erozan YS, Ball WC. Cells in Pleural Fluid. *Arch Intern Med* 1973;132:854-60.
6. Angeloni S, Nicolini G, Merli M, Nicolao F, Pinto G, Aronne T, et al. Validation of automated blood cell counter for the determination of polymorphonuclear cell count in the ascitic fluid of cirrhotic patients with or without spontaneous bacterial peritonitis. *Am J Gastroenterol* 2003;98:1844-8.
7. Li PK, Szeto CC, Piraino B, Bernardini J, Figueiredo AE, Gupta A, et al. Peritoneal Dialysis-Related Infections Recommendations: 2010 Update. *Perit Dial Int* 2010;30:393-423.
8. Rappaport ES, Helbert B, Beissner RS, Trowbridge A. Automated Hematology: Where We Stand. *Southern Med J* 1988;81:365-70.
9. Aune MW, Becker JL, Brugnara C, Canfield W, Dorfman DM, Fiehn W, et al. Automated Flow Cytometric Analysis of Blood Cells in Cerebrospinal Fluid. *Am J Clin Path* 2004;121:690-700.
10. Jung S, Chae H, Lim J, Oh EJ, Kim Y, Park YJ, et al. Differential blast counts obtained by automated blood cell analyzers. *Korean J Lab Med* 2010;30:540-6.
11. De Smet D, Van Moer G, Martens GA, Nanos N, Smet L, Jochmans K, et al. Use of the Cell-Dyn Sapphire Hematology Analyzer for Automated Counting of Blood Cells in Body Fluids. *Am J Clin Path* 2010;133:291-9.
12. Zur B, Eichhorn L, Albers E, Stoffel-Wagner B. Evaluation of 2 Hematology Analyzers in Body Fluid Mode versus Flow Cytometry Immunophenotyping of Mainly Neurosurgical Cerebrospinal Fluid Samples. *Cen Eur Neurosurg*. Epub ahead of print.
13. de Jonge R, Brouwer R, de Graaf MT, Luitwieler RL, Fleming C, de Frankrijker-Merkestijn M, et al. Evaluation of the new body fluid mode on the Sysmex XE-5000 for counting leukocytes and erythrocytes in cerebrospinal fluid and other body fluids. *Clin Chem Lab Med* 2010;48:665-75.
14. Linssen J, Jennissen V, Hildmann J, Reisinger E, Schindler J, Malchau G, et al. Identification and quantification of high fluorescence-stained lymphocytes as antibody synthesizing/secretory cells using the automated routine hematology analyzer XE-2100. *Cytometry B Clin Cytom* 2007;72:157-66.
15. England JM, Rowan RM, Assendelft OW, Bull BS, Coulter WH, McLaren CE. Guidelines for the evaluation of blood cell analyzers including those used for differential leucocyte and reticulocyte counting and cell marker applications. *Clin Lab Haematol* 1994;16:157-74.
16. Aune MW, Sandberg S. Automated counting of white and red blood cells in the cerebrospinal fluid. *Clin Lab Haematol* 2000;22:203-10.
17. Graaf MT, Broek PD, Kraan J, Bent RL, Boonstra JG, Schmitz PI, et al. Addition of serum-containing medium to cerebrospinal fluid prevents cellular loss over time. *J Neurol* 2011;258:1507-12.
18. Dux R, Kindler-Röhrborn A, Annas M, Faustmann P, Lennartz K, Zimmermann CW. A standardized protocol for flow cytometric analysis of cells isolated from cerebrospinal fluid. *J Neuro Sciences* 1994;121:74-8.

Chapter 6

Evaluation of Sysmex XN-1000 high sensitive analysis (hsA) research mode for counting and differentiating cells in cerebrospinal fluid

**Chérina Fleming, Henk Russcher, Rob Brouwer, Jan Lindemans,
Robert de Jonge**

ABSTRACT

Objective

Counting cells in cerebrospinal fluid (CSF) using automated analyzers is generally problematic due to low precision at low cell numbers. To overcome this limitation, Sysmex developed the high sensitive analysis (hsA) research mode specifically for counting cells in fluids that contain low cell counts. We evaluated this mode by counting RBCs, WBCs and differentiated WBCs in CSF samples.

Methods

We analyzed 248 CSF samples using the XN-hsA mode and compared these results with results obtained using the manual counting method. We also evaluated the linearity, detection limits, carryover and precision of the XN-hsA mode.

Results

Using the XN-hsA mode, the lower limit of quantification for RBCs and WBCs was 10 and $2 \times 10^6/L$, respectively. Comparing the two methods revealed good agreement with respect to WBCs ($y=1.08x + 0.52$), RBCs ($y=1.07x + 0.00$), lymphocytes ($y=1.00x + 0.00$), neutrophils ($y=1.05x + 0.00$) and monocytes ($y=0.88x + 0.07$). Regression analysis for samples containing low WBCs ($<10 \times 10^6/L$) and low RBCs ($<50 \times 10^6/L$) also had good agreement, although, a slight positive bias was found for RBCs. Linearity was good ($r^2 \geq 0.99$) for all parameters evaluated. Carryover was negligible and never exceeded 0.04%.

Conclusion

The XN-hsA research mode provides reliable cell counts in CSF samples, even in samples containing low numbers of WBCs and RBCs.

INTRODUCTION

In recent years, automated analyzers have become increasingly available for counting cells in body fluids (BFs) and are widely replacing more traditional methods such as the use of a manual hemocytometer (i.e., counting chamber). With some sample types, automated BF cell counting analyzers provide improved accuracy and precision, reduced inter-observer variability, and shorter turnaround times (1-4). However, high imprecision in the low cell concentration ranges has limited the use of automated cell counting in cerebrospinal (CSF) samples (5-8).

Previously, we reported the advantages and disadvantages associated with using either the Sysmex XE-5000 or XN-1000 body fluid (BF) mode compared with manual microscopy (8, 9). Although both analyzers yielded good agreement and performance, limitations were observed, including *i*) significant proportional bias for PMNs in CSF, *ii*) limited flagging possibilities and *iii*) high imprecision with respect to measuring RBCs at concentrations below $1000 \times 10^6/L$.

To overcome these limitations, Sysmex Corporation developed a new research mode called the high sensitive analysis (hsA) mode; this new analysis mode also provides additional quantitative cytological information in a CSF analysis. With this new research mode, Sysmex claims to have improve precision in the lower WBC and RBC concentration ranges. This research mode also provides a 4-part WBC differential count (lymphocytes, monocytes, eosinophils and neutrophils) and uses new gating algorithms to detect and flag pathological cells. This research mode is currently not commercially available, but will be released in the near future. The purpose of this study was to evaluate whether the XN-hsA research mode is feasible for clinical use in measuring CSF samples. In addition, we evaluated the linearity, detection limits, precision and carryover of this analysis mode.

MATERIALS AND METHODS

CSF samples

A total of 248 CSF samples were collected for this study. Approval was obtained from our institute's medical ethics committee, and the committee waived the need to obtain informed consent. The samples were collected from women 127 (51.2%) and 121 men (48.8%) and were obtained by lumbar puncture (64%), ventricular drainage (35%), or an unknown sampling method (1%). The patients were either in-patients or out-patients at various departments in our university hospital. Body fluid was collected and analyzed for cellular composition in accordance with the CLSI H56-A guidelines (10). All CSF samples were collected in plain tubes and mixed by gentle inversion before measure-

ment. The samples were first measured using the XN-3000 BF mode (Sysmex, Etten-Leur, the Netherlands) for routine diagnostics, followed by measurements using the XN-1000 hsA research mode (Sysmex, Kobe, Japan) and manual counting using a hemocytometer (Fuchs-Rosenthal). For the differentiation of WBCs, May-Grünwald Giemsa stained cytopsin slides were prepared, stored, then later analysed using the CellaVision Digital Cell Morphology (DM96) system (Sysmex, Etten-Leur, the Netherlands). All samples, except the cytopsin slides, were analysed in the laboratory within one hour.

Automated analysis using the XN-1000 hsA research mode

The XN-Series analyzer is a fully automated hematology analyzer equipped with a dedicated body fluid mode; this mode has been described in detail previously (9). The XN-hsA research mode uses fluorescence flow cytometry and impedance measurements to count the number of WBCs and RBCs in CSF samples. To measure RBCs, both the optical (for lower concentration ranges) and impedance (for higher concentration ranges) techniques can be used. In the WDF channel, the total WBC and differential WBC counts are reported based on flow cytometry measurements (e.g., side scatter, forward scatter and DNA/RNA fluorescence). The following values are reported: RBC count, WBC count, total nucleated cells (TNC) count, and the absolute numbers and relative percentages of lymphocytes, monocytes, neutrophils, eosinophils, PMNs and MNs. In addition, new software algorithms have been incorporated in the WDF channel for detecting and flagging cell types: activated lymphocytes and plasma cells (the Atypical Lymphocytes flag), activated monocytes and cell-phages (the Atypical Monocytes flag), and large cells such as malignant cells (the High Fluorescent [HF] flag); these are reported as research parameters (**Figure 1**). Finally, the XN-hsA research mode flags the presence of RBC abnormalities (the RBC Abnormal Scattergram flag) and/or WBC abnormalities (the WBC Abnormal Scattergram flag).

Prior to analysis using the XN-hsA research mode, the samples require no preparation. Changing from the whole-blood mode to the XN-hsA research mode takes approximately 4 minutes, including automatic rinse cycles and a background measurement after changing to the hsA research mode.

Manual microscopic method

RBCs, WBCs and TNCs were counted manually using the Fuchs-Rosenthal counting chamber (400-fold magnification). RBCs were counted unstained, whereas WBCs were stained with Samson reagent (fuchsin/acetate/phenol). If any samples measured using the XN-3000 BF mode contained $>200 \times 10^6/L$, they were diluted with 0.01 mol/L phosphate-buffered saline (PBS) pH 7.4. If the automated count reported $>30 \times 10^6$ cells/L, 256 squares in the counting chamber were counted; in contrast if less than 30×10^6 cells/L were reported, two chambers (i.e., 2 x 256 squares) were counted in order to

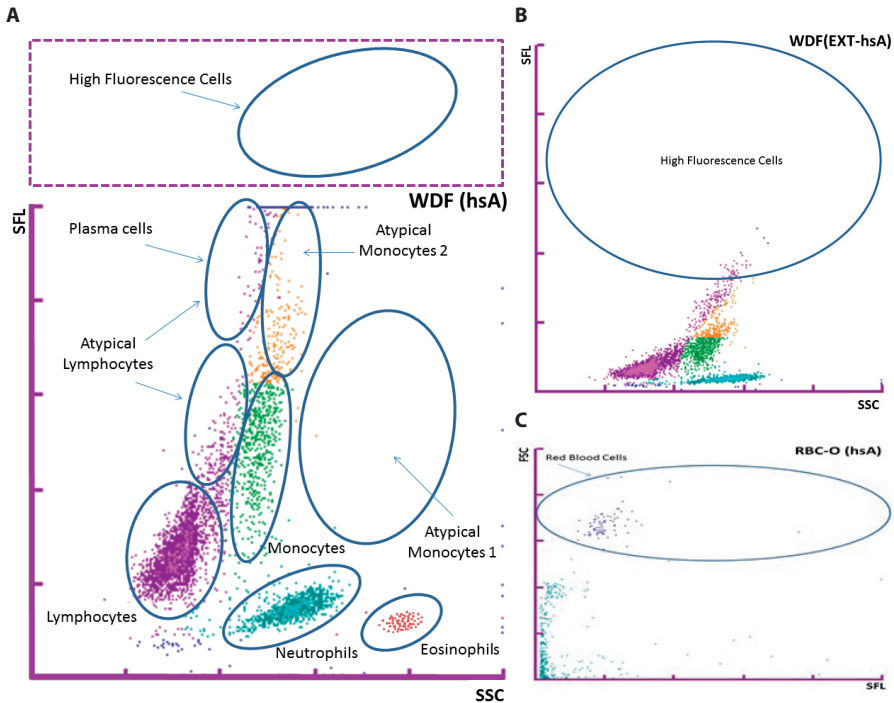


Figure 1 WDF scattergram and the RBC fluorescent (RBC-O) channel of the XN-hsA research mode; the areas used for gating are indicated. (A) WDF scattergram, with the various cell types indicated, (B) Expanded WDF scattergram showing the presence of high fluorescence cells (C) RBC-O channel. In graph A & B, the x-axis (SSC) represents the side scattered light and the y-axis (SFL) represents the side fluorescence light signal. In graph C, the x-axis (SFL) represents the side fluorescence light signal, and the y-axis (FSC) represents the forward scattered light.

increase precision and the average of these two counts was calculated. To minimize inter-observer variability, one technician performed all of the chamber counts.

Polylysine cytopsin slides were prepared for measuring the WBC differential using a Rotofix 32 cytocentrifuge (Hettich, Germany) for 5 minutes at 85 x g. Samples in which the cell concentration was $>200 \times 10^6/L$ were first diluted in accordance with the protocol for the counting chamber. After centrifugation, the slides were stained with May-Grünwald-Giemsa using an SP-10 slide staining unit (Sysmex, Etten-Leur, the Netherlands). All slides were pre-classified on the DM96 system using BF software; the automated DM96 uses digital image technology to examine cells in blood samples and other body fluids. The DM96 was configured to analyze 200 cells (if present) and then create a 10x overview. Post-classification and verification were performed by one experienced technician. The WBCs were classified into the following five categories: lymphocytes, monocytes, neutrophils, eosinophils and basophils. All "other cells" observed (bacteria, cell-phages, plasma cells, activated lymphocytes, smudge cells and artifacts) were classified separately. The following formula was used to calculate the absolute dif-

ferential counts (the absolute WBC count on the hsA mode \times % DM96 DIFF)/ 100%. The results obtained from this combined technique was used as the reference for the WBC differential. In our laboratory, DM96 is used exclusively for routine analysis of peripheral blood samples, not for routine analysis of BFs.

Precision

Between-run precision was determined by analyzing one level of Sysmex XN CHECK BF L2 twenty times over 20 days in the BF QC-mode. To determine the within-run precision and the precision profile, serially diluted XN CHECK BF was measured ten consecutive times in the hsA research mode. The mean value obtained from each dilution was plotted against the coefficient of variation (% CV). The Limit of Quantitation (LoQ) was defined as the point at which CV exceeded 20%, and this limit served as the benchmark for comparing analyzers (8, 11). However, the XN CHECK BF does not include eosinophils; therefore, the LoQs for eosinophils, neutrophils and PMNs were analyzed using serially diluted fresh human blood.

Detection limits

The Limit of Blank (LoB) was estimated by measuring 60 blank samples (CELL-PACK reagent) in accordance with the CSLI EP-17-A protocol (11, 12), and was calculated using the following formula: $\text{average}_{(\text{blank})} + 1.645 \times \text{standard deviation}$. In addition, the Limit of Detection (LoD) was calculated using six CSF samples containing extremely low WBC and RBC counts; these samples were measured ten consecutive times on different days. The LoD was calculated using the formula stated in the EP-17 protocol.

Method comparison

After routine analysis on the XN, the samples were analyzed using the XN hsA research mode, after which the cells were counted using a hemocytometer, and cytopsin smears were prepared; all of these steps were performed within approximately one hour. Some samples had insufficient volume for performing both hemocytometer cell counts and cytopsin smears for manual differential counts. For such samples, only cytopsin smears were prepared, as they required a smaller volume than hemocytometer cell counts (50 μL vs 180 μL , respectively).

Linearity

Samples that contained high WBC/RBC counts were serially diluted with PBS to achieve relevant concentrations. All samples were measured five times, and the mean of these 5 values were compared with the expected cell counts.

Carryover

Carryover was determined by measuring various sample types (CSF, pleura, CAPD) containing high cell counts in triplicate (H1 - H3), followed by three consecutive measurements of a blank (Cell pack L1- L3). Carryover was calculated as a percentage using the following formula: $[(L1-L3)/(H3-L3)] \times 100$ as recommended by the International Council for Standardization in Hematology (13).

Diagnostic test sensitivity

To evaluate the sensitivity of the XN-hsA mode for detecting abnormal WBC counts, each CSF sample was clustered as either normal or abnormal based on published adult criteria (5×10^6 cells/L) for discriminating between normal and pathological samples. The counting chamber was used as the reference method, and both sensitivity and specificity were calculated.

Statistical analysis

For all statistical analysis the absolute cell counts ($\times 10^6/L$) were used, unless mentioned otherwise. Analyze-it for Microsoft Excel version 2.21 (running on Microsoft Excel 2003) was used for data analysis. The Passing-Bablok regression analysis was used to compare methods. Statistical significance was based on the 95% confidence interval (CI); specifically significant proportional or constant bias was reported when the CI of the slope and the CI of the intercept did not include the values 1 and 0, respectively.

Table 1 Lower limits.

	RBC $\times 10^6/L$	WBC $\times 10^6/L$	NEUT $\times 10^6/L$	LYMPH $\times 10^6/L$	MONO $\times 10^6/L$	EO $\times 10^6/L$	MN $\times 10^6/L$	PMN $\times 10^6/L$
LoB	0.5	0.1	0.0	0.0	0.0	0.0	0.1	0.1
LoD	4.3	1.6	1.0	0.8	0.8	0.7	0.9	0.9
LoQ	10.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0

LoB= Limit of Blank, LoD= Limit of detection, LoQ= Limit of Quantitation. NEUT = Neutrophils, LYMPH = lymphocytes, MONO = monocytes, EO = eosinophils, MNs = mononuclear cells, PMNs = polymorphonuclear cells

RESULTS

Precision and detection limits

The detection limits of the hsA mode (LoB, LoD, LoQ) for the various cell types are summarized in **Table 1**. The LoB was $<0.1 \times 10^6/L$ for all WBC types and $0.5 \times 10^6/L$ for RBCs. The LoQ for WBC and WBC differentials is $2 \times 10^6/L$ and for RBCs $10 \times 10^6/L$ (**Table 1** and **Figure 2**). The within-run imprecision for WBCs (differential) and RBCs is shown in **Figure 2**. The mean between-day precision for all parameters was acceptable ($<10\%$) with

the exceptions of monocytes and eosinophils. The CV of the monocytes and eosinophils was difficult to determine due to the extremely low number of these cells in the XN CHECK BF control sample. Moreover, it is important to note that the XN CHECK BF was not developed for the XN-hsA research mode, but was developed for the BF mode, in which the differential count is not reportable (**Table 2**).

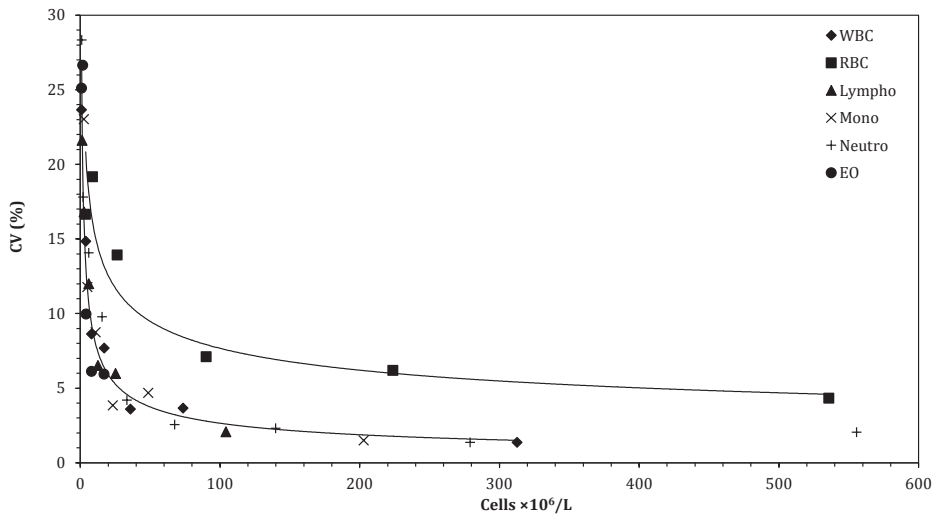


Figure 2 Precision profile (LoQ) of WBCs, RBCs and the indicated WBC types measured using serially diluted XN CHECK BF control sample with the XN hsA research mode. The precision profiles of the lymphocytes, monocytes, eosinophils, and neutrophils are plotted on the line representing the total WBC precision profile. Each point represents the CV of a 10-fold measurement. CV, coefficient of variance; Lympho, lymphocytes; Mono, monocytes; Neutro, neutrophils; EO, eosinophils.

Table 2 Between-day precision.

	Mean ($\times 10^6/L$)	CV (%)
WBC	329	3
RBC	76450	1
MN	137	6
PMN	192	5
Lymphocytes	134	7
Neutrophils	192	5

CV%, coefficient of variation, MN= mononuclear cells, PMN= polymorphonuclear cells

Method comparison

Of the 248 samples collected, 20 (8%) were collected from infants (≤ 1 year of age) and the remaining samples (92%) were collected from patients >1 year (range 1 to 84 years, median age: 41 years). In total, 16 samples (WBC counts ranging from: 1 to $1331 \times 10^6/L$)

were excluded from our analysis because the “WBC Abnormal Scattergram” flag was present on the XN-hsA research mode. The WDF scattergram of these samples appeared as either diffuse (i.e., scattered dots); in some cases, the scattergram was abnormal due to perceived interference from the x-axis. The 16 samples were collected either from a ventricular drain (69%) or lumbar puncture (31%). Collectively, a total of 111 samples were used for comparison between the counting chamber and the XN-hsA research mode, and a total of 179 samples were used for the differential study. The regression plots and analyses are shown in **Figure 3** and **Table 3**. The data show good agreement, albeit a small but significant positive bias between the XN-hsA research mode and hemocytometer for WBCs and RBCs (Table 3 and **Figure 3A & B**). Similarly, neutrophils had a small but significant positive bias towards the XN-hsA mode compared to the manual method (**Figure 3C**), whereas no significant bias was observed for lymphocytes (**Figure 3D**). Fewer monocytes were counted using the XN-hsA mode compared to the reference method (**Figure 3E**). When the proportional WBC differential counts were compared between the XN-hsA research mode and the DM96, the results were similar to when the absolute values were compared (Table 3).

A regression analysis between the XN-hsA research mode and the microscopic method for counting eosinophils could not be performed, as the majority of samples contained few or no eosinophils. We next examined the agreement between the XN-hsA mode and the hemocytometer in the low range of cell counts. We found no significant bias for WBCs at concentrations $<10 \times 10^6/L$ (**Figure 3F**); however, we found a significant posi-

Table 3 Passing-Bablok agreement between the XN-1000 hsA research mode and reference method.

	Regression line	(95% CI)		r	Regression line (%)	(95% CI)		r
		Slope	Intercept			Slope	Intercept	
WBC	y= 1.08x + 0.52 (n=111)	1.03 - 1.13	0.15 - 0.68	0.99	n/a	n/a	n/a	n/a
WBC <10×10⁶/L	y= 1.02x + 0.54 (n=64)	0.93 - 1.21	0.24 - 0.65	0.94	n/a	n/a	n/a	n/a
Lympho	y= 1.00x + 0.00 (n=179)	0.99 - 1.02	-0.13 - 0.05	0.98	y= 0.94x + 4.42 (n=179)	0.89 - 0.98	2.30 - 6.34	0.74
Mono	y= 0.88x + 0.07 (n=179)	0.82 - 0.92	0.00 - 0.19	0.99	y= 1.01x + 1.88 (n=179)	0.92 - 1.10	1.00 - 2.57	0.71
Neutro	y= 1.05x + 0.00 (n=179)	1.03 - 1.08	0.00 - 0.03	1.00	y= 1.03x + 0.01 (n=179)	1.00 - 1.06	0.00 - 0.60	0.93
RBC	y= 1.07x + 0.00 (n=111)	1.03 - 1.09	0.00 - 0.64	0.98	n/a	n/a	n/a	n/a
RBC <50×10⁶/L	y= 1.20x + 0.00 (n=69)	1.05 - 1.51	0.00 - 0.15	0.91	n/a	n/a	n/a	n/a

n/a= not applicable, CI= confidence interval, Lympho = lymphocytes, Mono = monocytes; Neutro = neutrophils

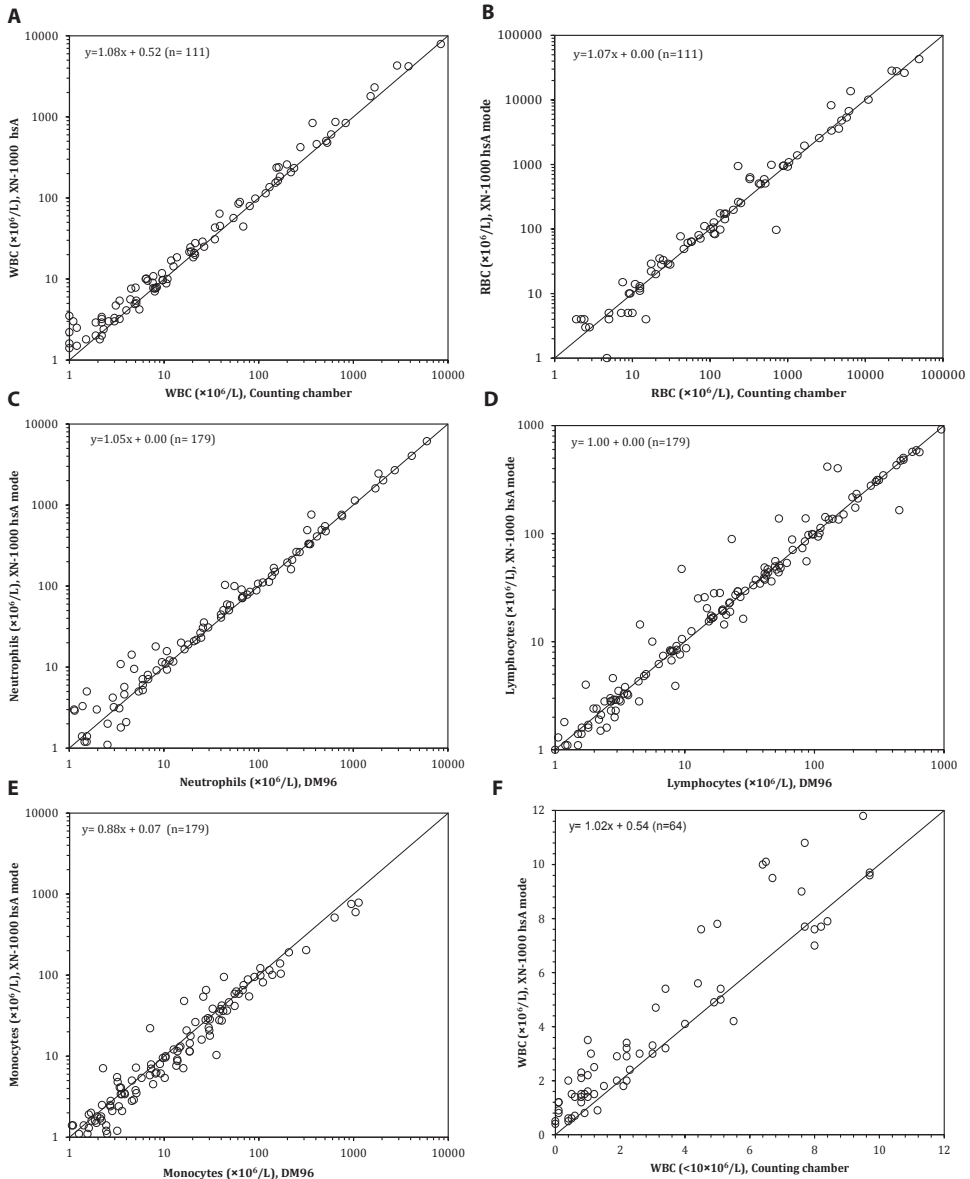


Figure 3 Passing-Bablok regression analysis comparing the XN-1000 hsA research mode (y-axis) with the microscopic methods for counting cell types in CSF samples. (A–E) Regression analyses of total WBCs (A), RBCs (B), neutrophils (C), lymphocytes (D), monocytes (E). (F) Regression analysis of samples containing $< 10 \times 10^6$ WBCs/L. The solid line represents a linear fit of the data points; the regression and sample sizes are shown in the top-left corner of each plot. Figure A–E, agreement between methods is shown by logarithmic scatterplots.

tive bias for the XN-hsA mode for RBCs at concentrations $\leq 50 \times 10^6/L$ (Table 3). Next, we reanalyzed the WBC, RBC and WBC differential results, after including the 16 flagged samples that were originally excluded. Similar results were obtained regardless of whether these 16 flagged samples were included in the analysis.

Linearity

The linearity was evaluated by serially diluting samples with high-concentration samples. Using this approach, the correlation coefficients was excellent ($R^2 \geq 0.999$; **Figure 4**) for total WBCs (range: $0.1 - 7801 \times 10^6/L$), neutrophils (range: $0.08 - 5927 \times 10^6/L$), lymphocytes

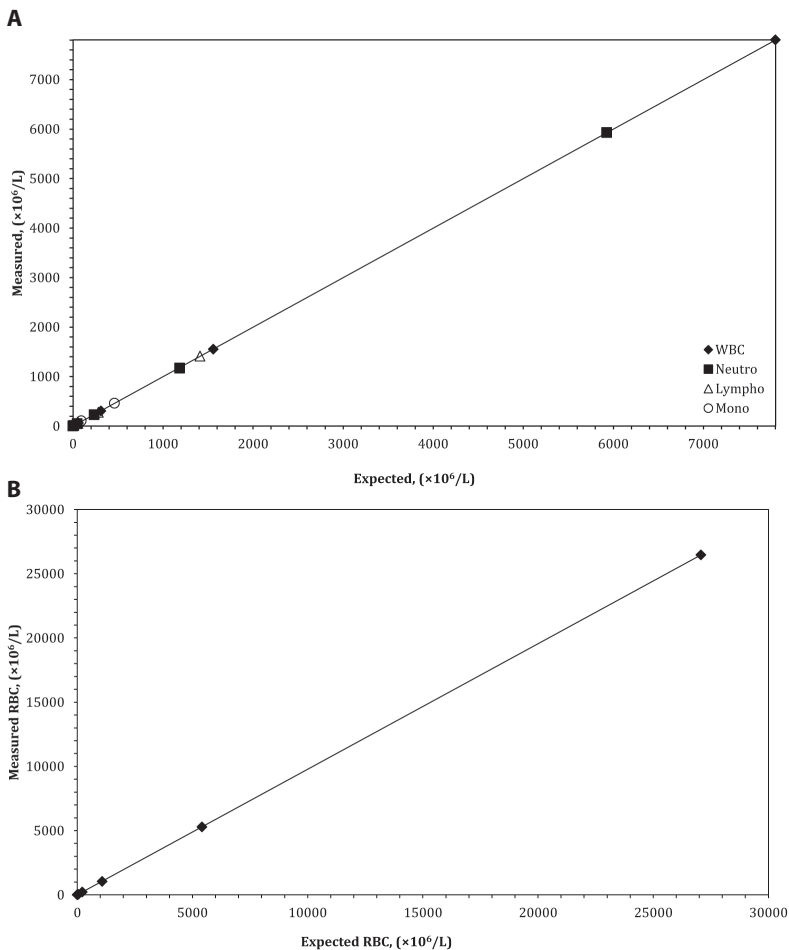


Figure 4 Linearity of the XN hsA research mode for measuring cell types was examined by performing serial dilutions of CSF samples. The actual counts are plotted against the expected (i.e., calculated) values. **(A)** Total WBCs, neutrophils, lymphocytes and monocyte. **(B)** RBCs. The solid line represents a 1:1 relationship ($y=x$).

(range: 0.02 - $1412 \times 10^6/L$) and monocytes (range: 0.0 - $460 \times 10^6/L$) (**Figure 4A**), as well as for RBCs (range: 1.2 - $26,459 \times 10^6/L$); **Figure 4B**. Linearity could not be determined for eosinophils due to the low number of eosinophils in the samples.

Carryover

Next, we calculated the carryover of cells from one measurement to the next. We first measured the BF samples with high counts, after which we measured a blank sample. The results showed that carryover never exceeded 0.04% for any of the parameters measured (data not shown).

Diagnostic sensitivity

Using the results obtained with the hemocytometer as the reference method, we found high overall sensitivity (98%) and specificity (95%) with respect in detecting abnormal WBC counts (**Table 4**). Fifty of the 111 samples (45%) had a normal WBC count ($<6 \times 10^6/L$), and the remaining 61 samples (55%) contained an abnormal WBC count ($\geq 6 \times 10^6/L$). Three samples had a normal range based on the hemocytometer method (mean WBC count was $4.7 \times 10^6/L$) but an abnormal WBC count when measured using the hsA research mode (mean WBC count was $7.3 \times 10^6/L$).

Table 4 Sensitivity/Specificity.

		Counting chamber		
		WBC $<6 \times 10^6/L$	WBC $\geq 6 \times 10^6/L$	Total
XN-hsA mode	WBC $<6 \times 10^6/L$	47	1	48
	WBC $\geq 6 \times 10^6/L$	3	60	63
	Total	50	61	111

DISCUSSION

In recent years, the use of automated analyzers to measure cells in body fluids has become increasingly popular, with the notable exception of CSF. Indeed, many groups recommend against the use of automated analyzers for CSF samples due to lack of agreement in the low concentration ranges, particularly when the concentration is at the border between normal and pathological levels (5, 14-17). The goal of developing the XN-hsA research mode by Sysmex was to obtain reliable cell counts in CSF samples, particularly in lower concentration ranges. This research mode was also developed to provide a 4-part WBC differential count and to flag pathological cells present during an inflammatory response. Here we report the first study to validate this mode, and we report the performance of this mode using CSF specimens. We found low detection

limits for RBCs and WBCs, and good agreement between the results obtained using the hsA mode and the reference methods. Interestingly, a slight significant positive bias was observed for WBCs, RBCs and neutrophils, whereas a negative bias was observed for monocytes. The hsA research mode has a wide linear range, and this linear range spanned the measurement ranges that are relevant to CSF samples with respect to both low and high cell concentrations. Lastly, we found that carryover of the cells from high to low concentrations was insignificant (<0.04 %) for all parameters.

We previously reported that the LoQ for WBCs has improved incrementally with each new generation of Sysmex analyzers (8, 9). For example, the LoQ for the XE-5000 BF mode is approximately $10 \times 10^6/L$; with the newer XN-1000 BF mode, LoQ is approximately $5 \times 10^6/L$. To increase precision further with the hsA research mode, Sysmex improved the counting procedure, yielding an even lower LoQ (WBC: $2 \times 10^6/L$). The LoQ of the XN-hsA research mode is thus considerably lower than the LoQ of the counting chamber (10×10^6 WBCs/L) (8). Moreover, the counting volume of the XN-hsA mode is approximately 7-fold larger than the volume used in a counting chamber (2.9 μ L), thus allowing more cells to be counted with higher precision.

As discussed above, we observed a slight positive bias towards the XN-hsA mode compared to the reference method for total WBCs (8%) and neutrophils (5%). We also observed such bias in our previous study, which yielded higher reference values (8). This could be due in part to methodological errors associated with the counting chamber (e.g., cell lysis, poor reproducibility and lack of precision with relatively low counts) or to the cytospin preparation (which could have led to cell loss and/or aberrant cell morphology). Nevertheless, the results of our study using manual microscopy are suitable for evaluating the performance of the XN-hsA research mode. Even considering the bias, similar results will likely be obtained when measuring a CSF sample manually (e.g., 3×10^6 WBCs/L), or using the automated hsA mode (in this example, yielding 3.2×10^6 WBCs/L), thus leading the practitioner to arrive at the same conclusion, as both values are below the clinical threshold. Moreover, the hsA mode has high sensitivity (98%) and specificity (95%). Taking these factors into consideration, we accept the slight proportional bias of 8%, as this would not likely have any adverse effects with respect to the patient, given that a clinical diagnosis is based on a combination of many factors, including the patient's clinical presentation.

The high sensitivity and specificity of the hsA research mode enabled us to count cells within the normal range for CSF samples. Indeed, only three out of 111 cases were classified incorrectly as abnormal which was likely due to the slight positive bias of the hsA mode compared to manual counting. Consistent with this finding, we previously reported that the reference values for CSF samples measured on Sysmex machines should be increased slightly to $<7 \times 10^6$ WBCs/L(8).

In addition to the total WBC and RBC counts, WBC differential counts can provide critical diagnostic information, particularly with respect to neurological diagnoses. In the field of cytology, clinicians primarily discriminate between viral and bacterial infections based on the relative proportions of lymphocytes and neutrophils. Thus, it is worth noting that the hsA research mode provides a 4-part WBC differential count and has good agreement with respect to counting monocytes, lymphocytes and neutrophils (Table 3 and Figure 3). Eosinophils are usually not present in the CSF; if they are present, this usually indicates a parasitic infection (18). Unfortunately, none of the CSF samples that we collected contained high eosinophil counts (the median eosinophil count using both methods was 0.1×10^6 cells/L); thus, we were unable to determine the reliability of the hsA research mode with respect to counting eosinophils, and this should be examined in future studies.

In our study, we observed atypical lymphocytes in 52 samples using the hsA research mode. Similar results were obtained when using microscopy (i.e., 100% sensitivity, unpublished data). On the other hand, the hsA research mode detected atypical lymphocytes in 38 samples, whereas no atypical lymphocytes were observed using the DM96 method, indicating 70% specificity. None of the CSF samples contained malignant cells; thus, we were unable to determine the performance of the hsA mode with respect to the HF flag. The hematology analyzers that are used today to analyze CSF samples are unable to accurately report or flag pathological cells (19); when present, these cell types are usually misclassified, and no flag is generated to notify user. Therefore, incorporating new flagging will improve the preliminary screening of CSF samples. Future studies should include larger sample sizes in combination with patient diagnoses in order to determine the diagnostic sensitivity of these flags.

To determine the appropriate course of treatment, clinicians must be able to distinguish between a traumatic tap and subarachnoid hemorrhage (SAH). However, there is currently no general consensus regarding how to differentiate between these two conditions, although one study reported that SAH can be safely ruled out (100% sensitivity) by an RBC count of $<500 \times 10^6$ cells/L (20). Therefore, the ability to accurately measure low numbers of RBCs in CSF is clinically important. In addition to using impedance, the hsA research mode also uses flow cytometry to count RBCs. Thus, the hsA research mode can measure RBC concentrations as low as 10×10^6 cells/L. This low LoQ can allow clinicians to use the hsA mode to detect low RBC concentrations in CSF, thus helping to differentiate between a traumatic tap and SAH.

As previously mentioned, preparation of cytopsin slides has several methodological limitations (21). In several cases, no cells were reported using the DM96, whereas cells were reported in these same samples using the hsA mode. Importantly, all of these samples had an extremely low WBC count ($\leq 5 \times 10^6$ cells/L). Given the type of fluid being examined (CSF) and the low number of cells in the sample, it is likely that the low values

reported were due to cell loss during the centrifugation procedure rather than to incorrect identification by the hsA mode.

In conclusion, our evaluation of the XN hsA research mode shows that the performance of this method is acceptable for performing RBC, WBC and WBC differential counts in CSF samples, even in samples that contain relatively low numbers of WBCs and/or RBCs. The new technological advances introduced in the XN hsA research mode (i.e., the ability to report a 4-part WBC differential count, new software algorithms and new flagging possibilities) create new opportunities for automated differentiation between pathological and non-pathological cells by providing more cellular information more rapidly than other automated analyzers currently being used for CSF samples. Importantly, an automated differential analysis using the XN-hsA research mode can be performed in approximately 3.5 minutes, whereas the stained cytopspin slide technique requires approximately 40 minutes from start to finish. Moreover, compared to using the counting chamber, the XN-hsA research mode has lower detection limits, a faster turn-around-time and virtually no intra-observer variability. Although automated analysis cannot completely replace microscopic analyses, it is useful for performing an initial screen of BFs by providing clinically relevant information in a short amount of time and by flagging possible abnormal samples for further examination.

REFERENCES

1. Nanos NE, Delanghe JR. Evaluation of Sysmex UF-1000i for use in cerebrospinal fluid analysis. *Clin Chim Acta*. 2008;392:30-3.
2. De Smet D, Van Moer G, Martens GA, Nanos N, Smet L, Jochmans K, et al. Use of the Cell-Dyn Sapphire hematology analyzer for automated counting of blood cells in body fluids. *Am J Clin Pathol*. 2010;133:291-9.
3. Paris A, Nhan T, Cornet E, Perol JP, Malet M, Troussard X. Performance evaluation of the body fluid mode on the platform Sysmex XE-5000 series automated hematology analyzer. *Int J Lab Hematol*. 2010;32:539-47.
4. Zimmermann M, Ruprecht K, Kainzinger F, Heppner FL, Weimann A. Automated vs. manual cerebrospinal fluid cell counts: a work and cost analysis comparing the Sysmex XE-5000 and the Fuchs-Rosenthal manual counting chamber. *Int J Lab Hematol*. 2011;33:629-37.
5. Barnes PW, Eby CS, Shimer G. An evaluation of the utility of performing body fluid counts on the coulter LH 750. *Lab Hematol*. 2004;10:127-31.
6. Boer K, Deufel T, Reinhoefer M. Evaluation of the XE-5000 for the automated analysis of blood cells in cerebrospinal fluid. *Clin Biochem*. 2009;42:684-91.
7. Zimmermann M, Otto C, Gonzalez JB, Prokop S, Ruprecht K. Cellular origin and diagnostic significance of high-fluorescent cells in cerebrospinal fluid detected by the XE-5000 hematology analyzer. *Int J Lab Hematol*. 2013;35:580-8.
8. de Jonge R, Brouwer R, de Graaf MT, Luitwieler RL, Fleming C, de Frankrijker-Merkestijn M, et al. Evaluation of the new body fluid mode on the Sysmex XE-5000 for counting leukocytes and erythrocytes in cerebrospinal fluid and other body fluids. *Clin Chem Lab Med*. 2010;48:665-75.
9. Fleming C, Brouwer R, Lindemans J, de Jonge R. Validation of the body fluid module on the new Sysmex XN-1000 for counting blood cells in cerebrospinal fluid and other body fluids. *Clin Chem Lab Med*. 2012;50:1791-8.
10. Szamosi D, Bautista JM, Cornbleet J, Glasser L, Rothe G, Sandhaus L, et al. Clinical and Laboratory Standards Institute. *Body fluid Analysis for Cellular Composition; Approved guideline. CLSI document H56-A*. 2006;26.
11. Armbruster DA, Pry T. Limit of blank, limit of detection and limit of quantitation. *Clin Biochem Rev*. 2008 Aug;29 Suppl 1:S49-52.
12. Tholen DW, Linnet K, Kondratovich M, Ambruster DA, Garrett PE, Jones RL, et al. NCCLS. *Protocols for determination of Limits of Detection and Limits of Quantitation; Approved guideline*. NCCLS document EP17-A. 2004;34.
13. Bourner G, De la Salle B, George T, Tabe Y, Baum H, Culp N, et al. ICSH guidelines for the verification and performance of automated cell counters for body fluids. *Int J Lab Hematol*. 2014.
14. Perne A, Hainfellner JA, Womastek I, Haushofer A, Szekeres T, Schwarzingler I. Performance evaluation of the Sysmex XE-5000 hematology analyzer for white blood cell analysis in cerebrospinal fluid. *Arch Pathol Lab Med*. 2012;136:194-8.
15. Brown W, Keeney M, Chin-Yee I, Johnson K, Lantis K, Finn W, et al. Validation of body fluid analysis on the Coulter LH 750. *Lab Hematol*. 2003;9:155-9.
16. Ziebig R, Lun A, Sinha P. Leukocyte counts in cerebrospinal fluid with the automated hematology analyzer CellDyn 3500 and the urine flow cytometer UF-100. *Clin Chem*. 2000;46(2):242-7.
17. Sandhaus LM, Ciarlino P, Kidric D, Dillman C, O'Riordan M. Automated cerebrospinal fluid cell counts using the sysmex XE-5000: Is it time for new reference ranges? *Am J Clin Pathol*. 2010;134(5):734-8.

18. Fishman R. Cerebrospinal Fluid in Diseases of the Nervous System. 2nd ed. Saunders W, editor. Philadelphia 1992.
19. Strik H, Luthe H, Nagel I, Ehrlich B, Bahr M. Automated cerebrospinal fluid cytology: limitations and reasonable applications. *Anal Quant Cytol Histol.* 2005;27:167-73.
20. Gorchynski J, Oman J, Newton T. Interpretation of traumatic lumbar punctures in the setting of possible subarachnoid hemorrhage: who can be safely discharged? *Cal J Emerg Med.* 2007;8:3-7.
21. Dux R, Kindler-Rohrborn A, Annas M, Faustmann P, Lennartz K, Zimmermann CW. A standardized protocol for flow cytometric analysis of cells isolated from cerebrospinal fluid. *J Neurol Sci.* 1994;121:74-8.

Chapter 7

UF-1000*i*: validation of the body fluid mode
for counting cells in body fluids

**Chérina Fleming, Rob Brouwer, Adriaan van Alphen, Jan Lindemans,
Robert de Jonge**

ABSTRACT

Objective

We evaluated the new body fluid mode on the UF-1000*i* urinalysis analyzer for counting total WBC and RBC in Continuous Ambulatory Peritoneal Dialysis (CAPD), ascites and pleural fluids.

Methods

We collected 154 body fluid samples, and compared the results of the UF-1000*i* BF mode with the Fuchs-Rosenthal counting chamber and the XN-1000 BF mode. Linearity, carryover and precision were also assessed.

Results

Method comparison results showed acceptable WBC agreement between UF-1000*i* and chamber ($y=1.27x+3.13$, $n=135$, $r=0.99$) and between UF-1000*i* and XN ($y=1.15x+0.31$, $n=135$, $r=1.00$). Comparison between the UF-1000*i* and both comparison methods showed good agreement for RBC counts. Overall results were better when UF-1000*i* was compared with the XN-1000 than with the Fuchs-Rosenthal chamber. The Lower Limit of Quantitation was defined at 9×10^6 WBC/L and at 25×10^6 RBC/L. Linearity for both WBC ($r=1.00$) and RBC ($r=0.99$) was good. Carryover was negligible, and it never exceeded 0.01%. In one sample, a high discrepancy was observed between WBC results for both automated analyzers and the counting chamber. This discrepancy was due to interfering factors such as bacteria and yeast cells, and it led to a false increased WBC count on both automated systems.

Conclusion

The UF-1000*i* BF mode offers rapid and reliable total WBC and RBC counts for initial screening of CAPD, ascites and pleural fluid, and it can improve the workflow in a routine laboratory; however, when using automated analyzers, the inspection of scattergrams is required to ensure the most accurate results are obtained.

INTRODUCTION

Manual microscopy is considered the gold standard for counting cells in body fluids (BF); however, this method has disadvantages of high imprecision and wide inter-observer variability. Also, it is time and labor consuming which greatly adds to the cost of laboratory services, and it remains challenging to maintain a high quality in many laboratories. Since their introduction in clinical laboratories, the use of automated hemocytometric analyzers has gained increasing acceptance as an alternative method to manual microscopy for initial screening of abnormalities in BF. In several studies, different types of automated cell counters were validated for counting and differentiating cells in BF. The results, when compared with manual microscopy, demonstrated good correlation, higher laboratory productivity, cost effectiveness and faster turn-around times (1-7). Notwithstanding these advantages, the majority of studies encountered a common problem in the analysis of BF on automated cell counters. They have poor precision in the lower concentration range (8-10) which is especially problematic for cerebrospinal fluid; consequently, automated analyzers with higher precision in the lower range are desirable.

The UF-1000i, which was recently launched on the European market, is the first fully automated urinalysis analyzer developed by Sysmex containing a urine mode and a dedicated body fluid mode. The UF-1000i BF mode uses hydrodynamic focusing and flow cytometry for counting and differentiating cells in BF. In comparison with the urine mode, the BF mode contains new gating and modified algorithms to enhance cell counting and decrease interference with cell fragments.

Although previous studies have evaluated the use of the UF-Series urinalysis analyzers for counting blood cells in BF (11-15), we are the first to validate the dedicated BF mode on the UF-1000i for counting red blood cells (RBC), white blood cells (WBC) and total nucleated cells (TNC) in ascites, Continuous Ambulatory Peritoneal Dialysis (CAPD) and pleural fluids, and compare the results with the manual reference method (Fuchs-Rosenthal chamber) and the XN-1000 BF mode.

MATERIALS AND METHODS

Patient material

For this study, 154 body fluid samples from hospitalized patients and out-patients were randomly collected and submitted to our laboratory for routine hemocytometric analysis. The BF samples included 60 ascites, 33 pleural and 61 CAPD samples. All fluids were collected in ethylene diamine tetra acetic acid (EDTA) anticoagulant tubes. This study was performed using blinded material; therefore, we do not have any information on

the patient's diseases and treatment. Additionally, CAPD samples were collected from the Maasstad Hospital, Rotterdam, to increase sample power. Informed consent from patients was waived because material used was that which remained following routine analysis.

Study design

In this study, clinical samples were first measured on the XN-1000 (Sysmex, Etten-Leur, The Netherlands) for routine diagnostics, followed by measurements on the research UF-1000i BF (Sysmex, Kobe Japan) and Fuchs-Rosenthal chamber. Samples were analysed within two hours upon laboratory entry.

UF-1000i BF mode

The UF-1000i body fluid mode uses hydrodynamic focussing and fluorescence flow cytometry techniques for counting cells in body fluids. The UF-1000i BF mode uses the bacteria and sediment channels to report 3 diagnostic parameters including the WBC, RBC and TNC count and 1 research parameter "Large cells" (LC) (**Figure 1**). The TNC count includes all nucleated cells covering WBCs, macrophages and mesothelial cells, and the LC parameter reports "epithelial cells". Unlike the standard urine mode on the UF-Series, the BF mode does not report bacteria counts or yeast cells, and it has no specific flagging for those parameters. Cells are identified and characterized based on the detection of fluorescence (DNA content), forward-scatter light (cell size, particle width and length),

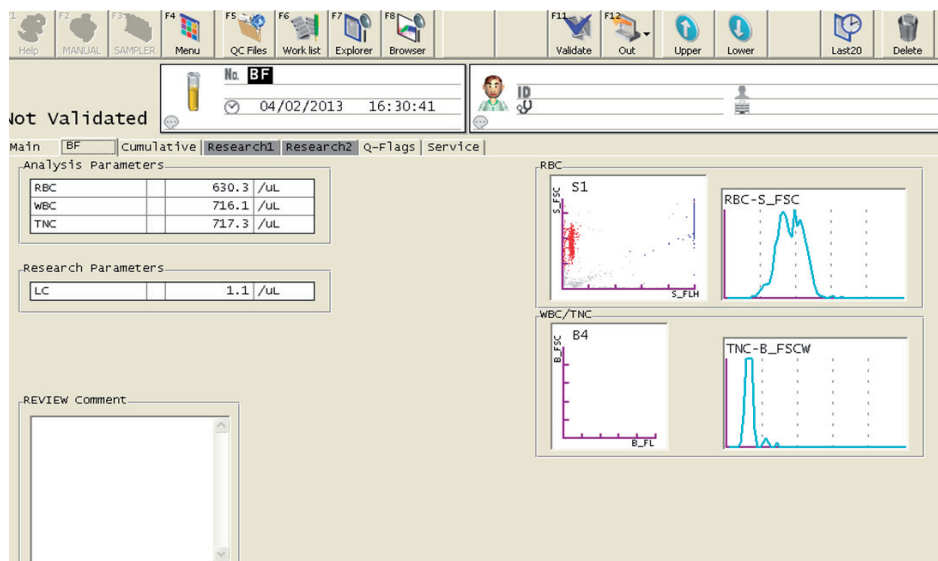


Figure 1 Scattergram and histogram of the UF-1000i body fluid mode. The upper two quadrants depicts the RBCs and the lower two quadrants depicts the WBCs.

and side-scatter light (inner complexity). The RBCs are counted in the sediment channel, and the WBCs and total nucleated cells (TNC) are counted in the bacteria channel after the cells are stained with specific fluorescent dyes. Results are displayed as scattergrams, histograms and in numeric notations/ μL ; additionally, an "ERROR" message is displayed when the scattergrams and histograms are abnormal. Two levels (low & high) of UF II quality-control samples were measured daily, and the total WBC, TNC and RBC counts were used for validation. The UF aspirates 398 μL samples for BF analysis. No sample preparation is needed prior to analysis. It takes about 2 minutes to switch from the urinalysis mode to the BF mode. This change includes automatic rinse cycles and an automatically performed background check once the UF is in BF mode.

XN-1000 BF mode

Every day, two levels (low & high) of XN-BF checks were measured prior to sample analysis. While the RBCs are counted in the RBC channel using sheath flow impedance technology, the WBC (differentiation) is determined by flow cytometry in the DIFF channel. The DIFF channel combines forward scatter (size of the cell) with side scatter (inner complexity of the cell) and the fluorescence intensity (DNA/RNA content) to identify each cell. This channel also reports high fluorescence body fluid cells (HF-BF) such as macrophages and mesothelial cells. These HF-BF cells are found just above the mono-nuclear cluster at fluorescence discriminator >200 channels, and they are not included in the WBC (differential) count; however, they are included in the total nucleated cell count (TC-BF). If non-cellular particles such as bacteria or cell debris are present, and this leads to erroneous clusters, the "WBC abnormal scattergram" flag will be automatically generated. The XN-1000 BF mode has been validated in our laboratory for BF analysis.

Manual Method

For manual microscopy, RBC, WBC and TNC were counted in the Fuchs-Rosenthal counting chamber. While the RBCs were counted unstained, WBCs were first stained with Samson reagent (fuchsine/acetic acid/phenol), and then counted in the Fuchs-Rosenthal chamber using a 400-fold magnification. If samples measured on the XN-1000 revealed high WBC ($>200 \times 10^6/\text{L}$) counts, dilutions with phosphate-buffered saline (PBS) pH 7.4 containing 0.01 mol/L phosphate were prepared accordingly. For each sample, 256 squares of the Fuchs-Rosenthal chamber were counted. Total WBC and RBC counts were calculated as follows: [total number of cells counted in 256 squares $\times 1/3.2 \times 10/9$ (dilution factor Samson reagents for WBC counting) \times dilution factor fluid (for WBC: $>200 \times 10^6/\text{L}$)]. To reduce inter-observer variability, the same technologist performed all microscopic analyses.

Precision

Between and within-run precision were determined by analysing two levels (low and high) of Sysmex UF II CONTROL material. The between-run precision was assessed by analysing the UF II CONTROL material for 20 consecutive days. The within-run precision was determined by measuring UF II CONTROL material 10 times consecutively within a single run. Additionally, the precision profile was determined. An ascites sample was serially diluted and measured 10 times consecutively on the UF-1000i BF mode. The mean WBC and RBC count of each dilution was plotted against the coefficient of variation (%CV). The point where the CV exceeded 20% was arbitrarily defined as the lower limit of quantitation (LoQ) and served as means for comparing analyzers.

Linearity

Samples with high WBC/RBC counts were serially diluted with the analyser's dilution reagent (UF-II PACK SED) to relevant concentrations. All samples were measured 5 times and the means were compared with the expected cell counts.

Accuracy

Ascites, pleural fluid and CAPD fluids were used for method comparison studies. UF-1000i results were compared with both the XN-1000 and the counting chamber results which were considered as the reference methods.

Sample carryover

Carryover was determined by measuring samples with high cell counts in triplicate (H1, H2, H3), followed by 3 consecutive measurements of a blank (UF-II PACK SED; L1, L2, L3). Carryover was calculated as follows: $[(L1-L3)/(H3-L3)] \times 100\%$ as recommended by the International Council for Standardization in Haematology (16).

Diagnostic test accuracy

To evaluate the diagnostic accuracy of detecting bacterial infections, CAPD and ascites samples were categorized into 2 groups (normal and infectious) based on published criteria for CAPD ($>100 \times 10^6$ WBC/L with $>50\%$ neutrophils)(17) and ascites ($>250 \times 10^6$ /L) (18). The counting chamber and the XN-1000 were considered as the reference methods, and the sensitivity and specificity were calculated. Because the UF does not report neutrophils, CAPD samples with a WBC count $>100 \times 10^6$ cells/L were considered abnormal, and a WBC count $>250 \times 10^6$ cells/L was considered abnormal in ascitic fluid. Pleural fluids were not included in the analysis because there are no clear-cut medical cut-off values for the diagnosis of infectious diseases based on cellular analysis.

Statistics

Unless mentioned otherwise, the absolute values of the measurements were used for all statistical analysis. Analyse-it for Microsoft Excel software version 2.21 and Microsoft Excel 2003 was used for data analysis. Passing & Bablok regression analysis was used for method comparison and linearity studies. Statistical significance was based on the 95% Confidence Intervals (CI). A significant proportional or constant bias was noted when the CI of the slope did not encompass 1, and the CI of the intercept did not encompass 0 respectively. The results of the UF-1000i were compared with manual microscopy and the XN-1000.

RESULTS

Precision

The precision profile (LoQ) of the UF-1000i is depicted in **Figure 2**. A CV of 20% was attained at approximately 9×10^6 WBC/L and 25×10^6 RBC/L. The within-run and between-run imprecision was always $< 8.0\%$ for WBC and RBC counts for both low and high levels of UF II CONTROL (**Table 1**).

Table 1 Between-run precision

	Sample	Mean ($\times 10^6$ /L)	SD	CV (%)
WBC	UF II CONTROL L1	39.0	3.0	7.6
RBC	UF II CONTROL L1	41.2	2.0	4.9
WBC	UF II CONTROL L2	795.3	11.8	1.5
RBC	UF II CONTROL L2	206.0	4.5	2.2

CV (%): coefficient of variation

Linearity

Linearity is presented in **Figure 3**. Linearity results were good for WBC ($R^2=1$) and RBC ($R^2=0.99$) on the UF-1000i even in the lower concentration range (**Figure 3A & 3B**). The lack of fit (F) for both samples was < 1.80 . The average recovery for WBC was 91.5% and for RBC 100.3%.

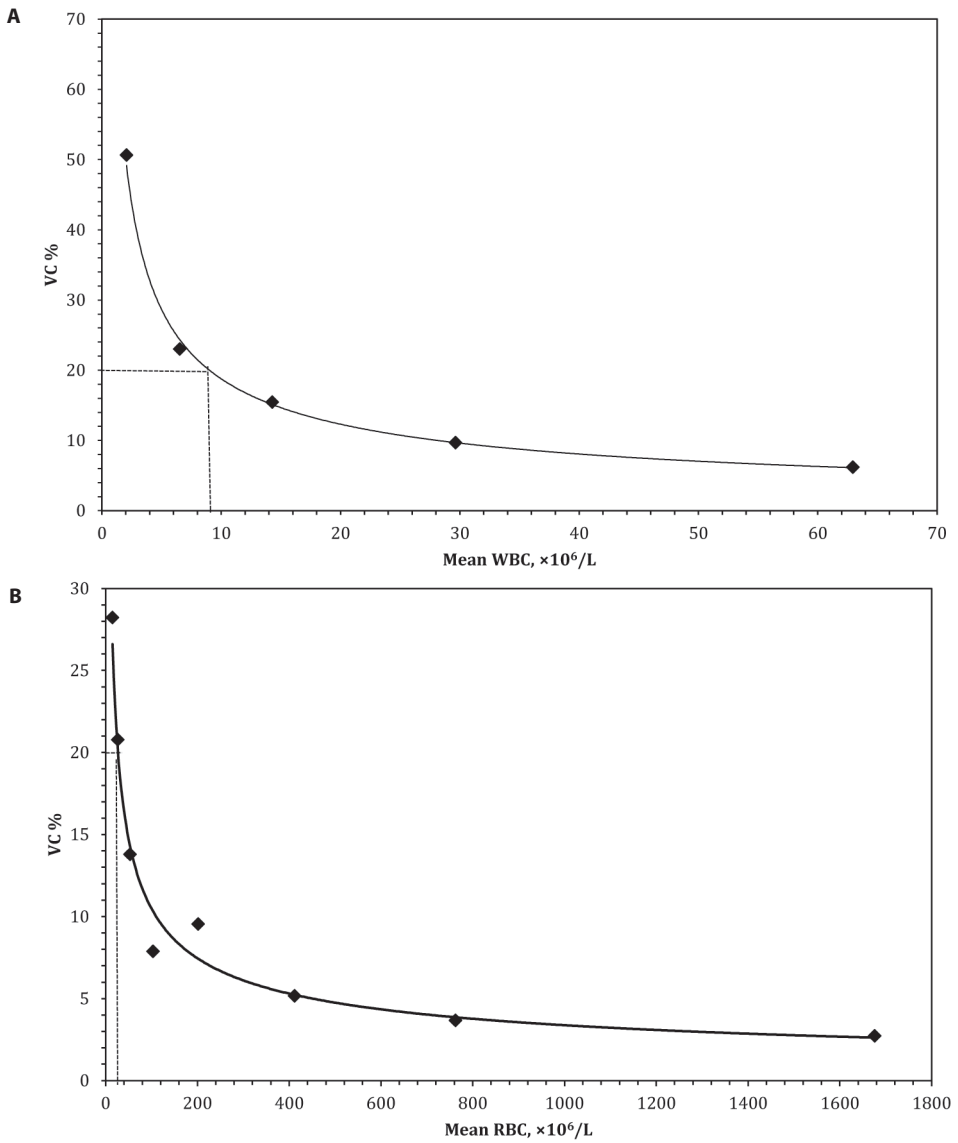


Figure 2 Precision profile (lower limit of quantitation) using a serially diluted ascites fluid on the UF-1000i BF mode. **A)** total WBC count and **B)** total RBC count. Each point represents the CV of a 10-fold measurement.

Accuracy

WBC counts

A total of 154 (60 ascites, 33 pleural and 61 CAPD) BF samples were collected for com-

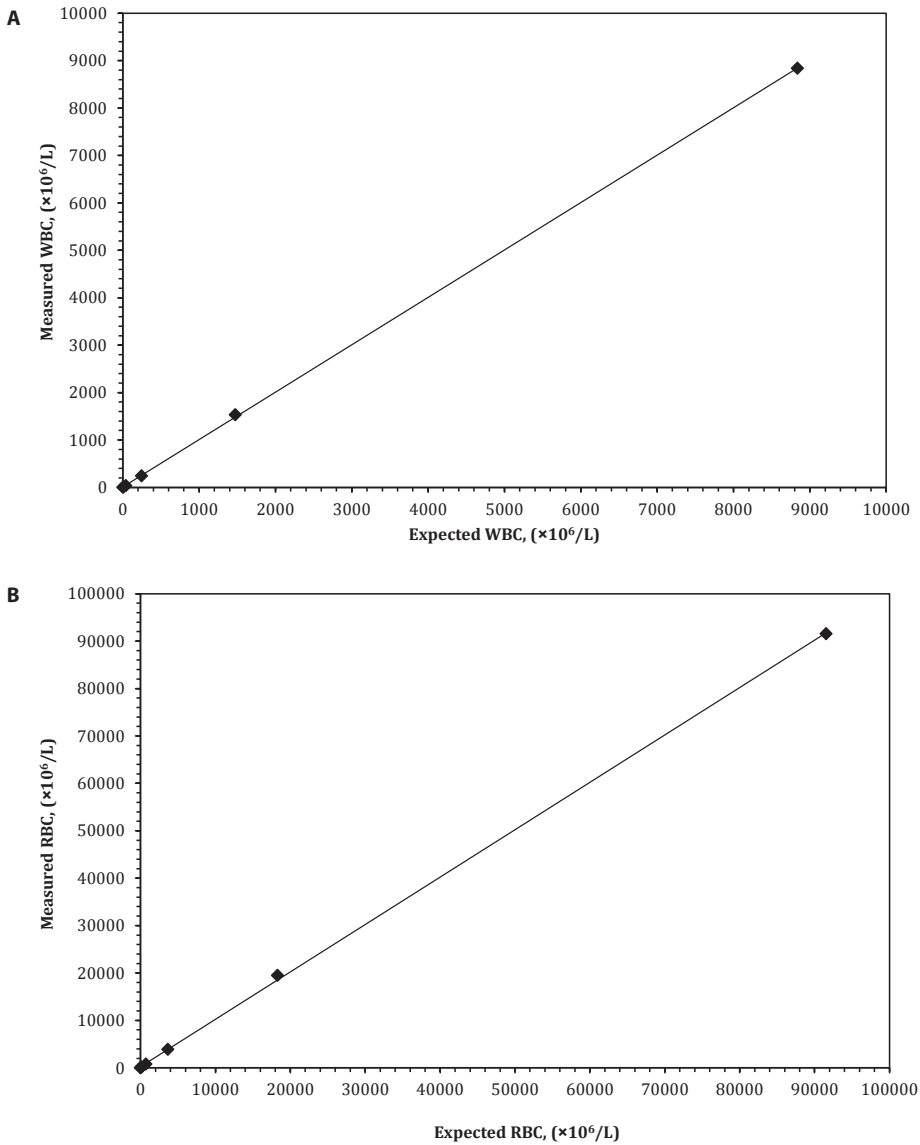


Figure 3 Linearity of the UF-1000i in fluids for (A) WBC counts and (B) RBC counts. The identity line ($y=x$) is drawn.

parison study. Of the 154 samples collected, 19 were excluded from this study when *i*) an "ERROR message" was present on the UF-1000i, and/or there was the presence of the "WBC abnormal scattergram flag" on the XN-1000 ($n=16$), and *ii*) there was missing data from the counting chamber ($n=2$) or analyzers ($n=1$). The WBC count ranged from $2.1 \times 10^6/L$ to $4326 \times 10^6/L$. The results of Passing & Bablok regression analyses

and Pearson correlation coefficients (r) for WBC and RBC counts are shown in **Table 2 & 3**. The WBC count on the UF-1000*i* demonstrated a significant proportional bias for all fluids. Noticeably, the bias was larger when compared with the counting chamber ($y=1.27x+3.13$, $n=135$, $r=0.99$) than with the XN-1000 ($y=1.15x+0.31$, $n=135$, $r=1.00$) **Figure 4 A & B**. Next, WBCs $<1000 \times 10^6/L$ were separately analysed between the UF-1000*i* and counting chamber ($y=1.32x+1.56$, $n=112$, $r=0.96$) and between the UF-1000*i* and XN-1000 ($y=1.17x-0.98$, $n=112$, $r=0.97$) Table 2. Although both regression lines showed a significant (proportional) overestimation of WBCs using the UF-1000*i*, the bias was smaller when compared with the XN-1000 than the counting chamber. Fluids were also analysed individually. The regression lines between UF-1000*i* and chamber counts showed similar proportional bias for pleural fluids ($y=1.32x+10.85$, $n=24$, $r=0.99$), ascites ($y=1.24x+15.11$, $n=54$, $r=0.98$) and CAPD ($y=1.23x+1.65$, $n=57$, $r=1.00$) Table 2.

Furthermore, comparison of the 3 groups separately with the UF-1000*i* and XN-1000 showed smaller but still significant proportional positive bias for pleural fluid ($y=1.15x+20.07$, $n=25$, $r=0.99$), ascites ($y=1.14x+17.59$, $n=54$, $r=1.00$) and CAPD ($y=1.09x-0.40$, $n=57$, $r=1.00$), see Table 2. Apart from the WBC count, the UF-1000*i* also reports a TNC count. The TNC count on the UF-1000*i* was compared with the TNC results obtained from the counting chamber ($y=1.22x+2.63$, $n=135$, $r=0.99$) and the TNC count from the XN-1000 ($y=1.10x-1.30$, $n=135$, $r=1.00$).

Table 2 Passing-Bablok agreement between the UF-1000*i* and reference methods (FR and XN-1000) for WBCs

Fluid	UF vs FR	95%CI			r	UF vs XN	95%CI			r
		slope	intercept				slope	intercept		
All	$y=1.27x+3.13$ $n=135$	1.23 – 1.32	-0.26 - 8.42		0.99	$y=1.15x+0.31$ $n=135$	1.13 – 1.17	-2.03 – 3.63		1.00
Pleura	$y=1.32x+10.85$ $n=24$	1.22 – 1.40	-58.14 - 66.69		0.99	$y=1.15x+20.07$ $n=25$	1.08 – 1.21	-12.31 - 61.42		0.99
Ascites	$y=1.24x+15.11$ $n=54$	1.16 – 1.32	5.76 - 30.95		0.98	$y=1.14x+17.59$ $n=54$	1.11 – 1.17	13.9 - 24.97		1.00
CAPD	$y=1.23x+1.65$ $n=57$	1.22 – 1.33	-0.94 - 2.27		1.00	$y=1.09x-0.40$ $n=57$	1.08 – 1.11	-1.85 - 1.66		1.00
ALL WBC _{<1000}	$y=1.32x+1.56$ $n=112$	1.26 – 1.38	-1.31 – 5.41		0.96	$y=1.17x-0.98$ $n=112$	1.15 – 1.20	-3.33 – 1.98		0.97

FR= Fuchs Rosenthal. CI= Confidence Interval

RBC counts

RBCs counted on the UF-1000*i*, compared with the counting chamber, showed the following regression ($y=1.07x+12.12$, $n=134$, $r=1.00$) over the whole range (**Figure 4C**). To evaluate the performance of the UF-1000*i* in the lower RBC concentration range

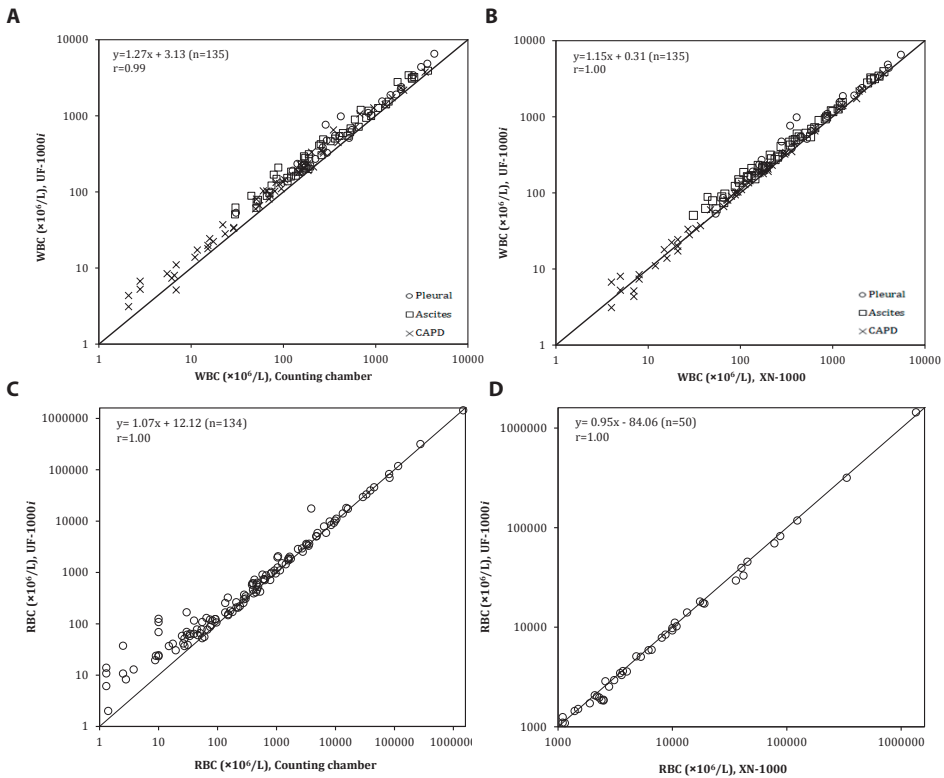


Figure 4 Passing-Bablok regression analysis between the UF-1000i and the comparison methods for counting WBC and RBC in CAPD, ascites and pleural fluids. **(A)** UF-1000i vs counting chamber and **(B)** UF-1000i vs XN-1000. Agreement between methods is shown by logarithmic scatterplots against the line of identification (Passing-Bablok regression of combined fluids is given in top left corner). The data from these fluids are represented in one graph, with a different symbol for each fluid. **(C)** UF-1000i vs counting chamber and **(D)** UF-1000i vs XN-1000. The XN-1000 only reports RBC counts $>1000 \times 10^6/L$. Agreement between methods is shown by logarithmic scatterplots against the line of identification (regression of combined fluids is given in top left corner).

($<1000 \times 10^6/L$) more thoroughly, a separate regression analysis was performed, and this resulted in the following regression: $y=1.18x+8.20$, $n=93$, $r=0.97$ between the UF-1000i and the counting chamber. The XN-1000 only reports RBC counts $\geq 1000 \times 10^6/L$. XN-1000 counts lower and higher than $500 \times 10^6/L$ are reported as $0 \times 10^9/L$ and $1 \times 10^9/L$. Therefore, $RBC >1000 \times 10^6/L$ was used for regression analysis for comparison between the UF-1000i and the XN-1000. This resulted in the regression line $y=0.95x-84.06$, $n=50$, $r=1.00$ (**Figure 4D**). Furthermore, the XN-1000 reports RBC values $<1000 \times 10^6/L$ as research parameters. By using these research values, we compared the UF-1000i and the XN-1000 in the lower concentration range ($<1000 \times 10^6/L$). The XN-1000 counted 19% more RBCs than the UF-1000i ($y=0.81x-64.92$, $n=94$, $r=0.47$) see Table 3.

Interfering factors

In one CAPD sample, both the XN-1000 BF mode ($\text{WBC}=492 \times 10^6/\text{L}$) and the UF-1000i BF-mode ($\text{WBC}=2184 \times 10^6/\text{L}$) overestimated the leucocyte count compared with the counting chamber ($\text{WBC}=3 \times 10^6/\text{L}$). This was due to the presence of yeast and bacteria cells which were clearly observable in the cytospin slide. The XN-1000 BF mode displayed a “WBC abn scattergram flag” because of abnormal clustering. The UF-1000i did not generate a flag; although, the RBC scattergram was abnormal. It displayed grey (unknown) and red (RBC) dots (**Figure 5**).

Table 3 Passing-Bablok agreement between the UF-1000i and reference methods (FR and XN-1000) for RBC counts.

Fluid	UF vs FR	95%CI		r	UF vs XN	95%CI		r
		slope	intercept			slope	intercept	
All	$y=1.07x+12.12$ n=134	1.05 – 1.12	8.56 - 16.02	1.00	$y=0.96x-84.06$ n=50	0.94 – 0.99	-231.55 - 7.31	1.00
Pleura	$y=1.01x+39.03$ n=23	0.98 – 1.14	11.14 - 130.14	0.90	$y=0.96x-247.79$ n=16	0.94 – 1.01	-444.64 - - 85.17	1.00
Ascites	$y=1.05x+29.58$ n=54	1.02 – 1.07	17.15 - 50.70	0.90	$y=0.98x-139.19$ n=25	0.95 – 1.05	-438.34 - 7.17	1.00
CAPD	$y=1.20x+4.89$ n=57	1.09 – 1.30	3.05 - 8.70	1.00	$y=0.94x+60.93$ n=9	0.78 – 0.98	-37.80 - 345.65	1.00
ALL RBC _{<1000}	$y=1.18x+8.20$ n=93	1.11 – 1.25	4.75 – 12.14	0.97	$y=0.81x-64.92$ n=94	0.71 – 0.90	-88.27 - - 33.33	0.47

FR= Fuchs Rosenthal, XN-1000 reports $\text{RBC} > 1000 \times 10^6/\text{L}$

Diagnostic test accuracy

Taking the counting chamber as reference method, 56% (32/57) of CAPD samples had a normal WBC count and 47% (27/57) had an abnormal WBC count ($> 100 \times 10^6/\text{L}$). Of the 56% with a normal WBC count, 22% (7/32) had a WBC count below $100 \times 10^6/\text{L}$ on the counting chamber (with an average count of 82×10^6 WBC/L), while the corresponding UF results were above 100 (with an average count of 116×10^6 WBC/L). A sensitivity of 100% and a specificity of 78% were found.

For ascites fluid, 54% (29/54) were categorized as normal, and 46% (25/54) were grouped as abnormal. Seventeen percent (5/29) of samples grouped as normal according to the counting chamber (average WBC $193 \times 10^6/\text{L}$) resulted in a higher WBC average ($313 \times 10^6/\text{L}$) on the UF. This resulted in an 83% specificity and a 100% sensitivity. Similar results were found when the XN BF mode was chosen as reference method. For CAPD, of the 51% (29/57) samples with a normal WBC count, 14% (4/29) had a WBC below $100 \times 10^6/\text{L}$ on the XN (average count of 99×10^6 WBC/L), while the corresponding UF results were above 100 (average count of 111×10^6 WBC/L). For ascites fluid, 50% (27/54)

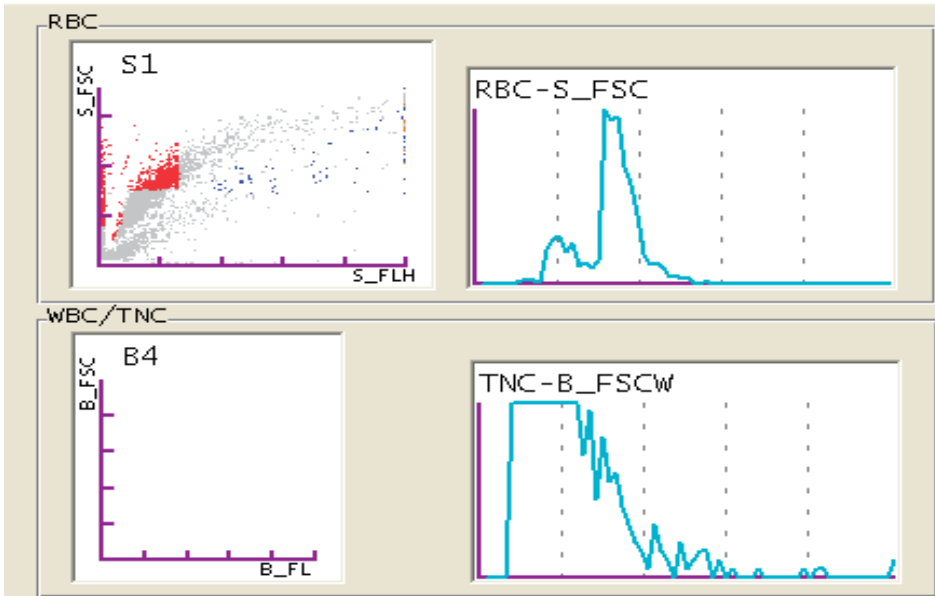


Figure 5 Scattergram of a CAPD sample containing yeast cells and bacteria on the UF-1000i body fluid mode. The upper left quadrant shows the RBC scattergram displaying red dots (RBCs) and grey dots (unknown) and the upper right quadrant displays the RBC histogram. The lower left quadrant shows the WBC/TNC channel displaying an abnormal histogram pattern and the right quadrant the WBC/TNC histogram.

were categorized as normal. Eleven percent (3/27) of samples grouped as normal according to the XN (average WBC: $222 \times 10^6/L$) resulted in a higher WBC average ($289 \times 10^6/L$) count on the UF. Sensitivity of 100% for both CAPD and ascites fluids was found, and specificity of 86% CAPD and 89% ascites fluid was found.

Sample carryover

Carryover on the UF-1000i BF mode was negligible for both WBC and RBC in all body fluids. It never exceeded 0.01%.

DISCUSSION

Laboratory assessment of BFs is an essential part of disease diagnosis and follow-up; therefore, accurate and rapid results are very important. Also, because of sample instability, the rapid production of results has added importance. The UF-1000i, initially developed for urinalysis, now includes a dedicated BF mode for counting WBC, TNC and RBC in BF samples. In this study, the performance of the UF-1000i BF mode was validated, and cell counts were compared with conventional manual microscopy and

the XN-1000 using different BF samples (n=154). The results of this study show acceptable agreement between the UF-1000i and the reference methods for counting WBC, and good agreement for RBC counts. Linearity and precision were good, and carryover was negligible. In general, agreement between the UF-1000i and the XN-1000 was better than agreement between the UF-1000i and the counting chamber for WBC counts (Table 2). The UF-1000i WBC count was 15% higher than the XN-1000 and 27% higher than the counting chamber.

Manual microscopy is considered the gold standard; however, there are various methodological errors that hamper the precision and accuracy of this method. These include pipetting errors, dilution errors and chamber-loading errors. These pre-analytical steps or a combination of them could have led to cellular loss or cell lysis which could have resulted in lower chamber counts. On the other hand, lipids, insufficiently lysed RBC, protein, bacteria, cell debris and fungi are all common interfering factors that can cause an increased WBC and/or RBC count on automated analyzers (19, 20).

The current BF software on the UF-1000i generates 1 message: "ERROR". When interfering factors such as those previously mentioned are present, and they lead to erroneous WBC/RBC clustering, this "ERROR" message will be generated; however, that is not always the case. In the example of the CAPD sample, both the XN-1000 BF mode (WBC=492×10⁶/L) and the UF-1000i/BF-mode (WBC=2184×10⁶/L) overestimated the leucocyte count compared with the counting chamber (WBC=3×10⁶/L) due to the presence of yeast and bacteria cells (Figure 5). A possible explanation for the misclassification of bacteria and yeast cells as WBCs and RBCs on the UF-1000i is the technology used. As previously mentioned in the method's section, the UF-1000i uses flow cytometry that is based on a combination of DNA/RNA signal, cell inner complexity and cell size for cell detection and identification. Both bacteria and yeast cells contain low concentrations of DNA/RNA, and are usually small in size compared to RBCs and WBCs; however, when these cells are aggregated, they can display signals possibly equal to RBCs and WBCs. Other studies reported similar results (13,21). In this case, the UF-1000i should have generated an "ERROR" message alerting the operator of interference; consequently, this sample was eliminated from data analysis because of the exclusion criteria: presence of "WBC abn scattergram flag". For this reason, it remains crucial to review the scattergram and histogram patterns, and in the presence of abnormalities, BF samples should be thoroughly evaluated by microscopic analysis in order to deliver accurate results.

Serous fluids frequently contain non haematological cells such as macrophages, mesothelial cells and malignant cells. These cell types should be excluded from the WBC count, but they should be included in the TNC count. However, this is not always the case. In one example, a pleural sample showed the following results after measurements on the 3 machines: XN-BF: WBC 408×10⁶/L and TNC 954×10⁶/L, Fuchs-Rosenthal: WBC 419×10⁶/L and TNC 949×10⁶/L and UF: WBC 981×10⁶/L and TNC: 987×10⁶/L. Cytospin

analysis resulted in 370% large cells/100 WBC along with the following comment: “a lot of macrophages and mesothelial cells present”. In this example, it is clear the UF included macrophages and mesothelial cells in the WBC count which led to the overestimation of the WBCs. The UF did not provide a correction for the presence of these large cells. According to Conner et al. (22), the WBC count in pleural effusions can aid in fluid aetiology whether it is a transudate (WBC: $<1000 \times 10^6/L$) or an exudate (WBC: $>1000 \times 10^6/L$). In this case, both values were below the medical upper limit value of $1000 \times 10^6/WBC/L$; therefore, it had no critical medical effect on possible diagnosis based of the above mentioned criteria. This same phenomenon, but to a lesser degree, was seen mostly in pleural samples and also in a few ascites samples. From experience, we have noticed that these large cells, due to their high forward scatter and high fluorescent signal, appear in the scattergram to the extreme right when present. Though the TNC count is not requested by physicians, this count is important for operators because it gives an indication of the presence or absence of macrophages, mesothelial or malignant cells. We therefore recommend, when using this mode for measurements of serous fluids, to be aware of the upper phenomenon, and critically review scatterplots. If these cells are suspected, it is necessary to follow up with a microscopic revision.

To assess the diagnostic accuracy of the UF-BF mode in differentiating between infectious and non-infectious CAPD and ascites samples, the sensitivity and specificity were calculated. Comparison with the counting chamber showed that the UF-1000i BF mode is highly sensitive (100%) in detecting abnormal (infectious) WBC counts in CAPD and ascites, but on the other hand, the specificity was 78% and 83%, respectively. When compared with the XN BF, similar results to the counting chamber were found; although, the specificity was slightly better for CAPD (86%) and ascites (89%) than with the counting chamber. Thus, using only the WBC count as a diagnostic criteria, the UF-1000i BF mode displayed 100% sensitivity and good specificity indicating that patients with negative results can be spared antibiotic treatment; however, in some cases, false-positive results are possible.

Microbiology is the gold standard for detecting micro-organisms in fluids; however, results can take up to 72 hours. For example, bacterial meningitis and peritonitis are life threatening diseases coupled with high mortality rates if left untreated; therefore, rapid detection and treatment are important steps in the diagnosis and clinical outcome of patients. Previous studies have evaluated the UF urinalysis series for counting bacteria and yeast cells in body fluids. Penders et al. (13) reported that the bacteria count on the UF-100 was more sensitive than gram staining technique, though caution should be taken when analysing for bacteria on the UF-100 due to high background noise in bacteria channel. Additionally, when the UF WBC count, UF bacteria count and total protein were combined, a sensitivity of 75% and a specificity of 72.2% were found for early prediction of a positive fluid culture. Compared with the UF urine mode, the BF

mode neither generates a bacteria and/or yeast count, nor does it report specific flags. However, these parameters can be of added value to the WBC count to aid clinicians in infectious diagnosis. The next generation of the UF-1000i BF-mode could be improved by *i)* including a WBC differential count to improve the diagnostic test accuracy for infection and *ii)* by better flagging/counting of cells such as yeast, bacteria, mesothelial cells and macrophages. These recommendations for the manufacturer warrants further technical and clinical investigations.

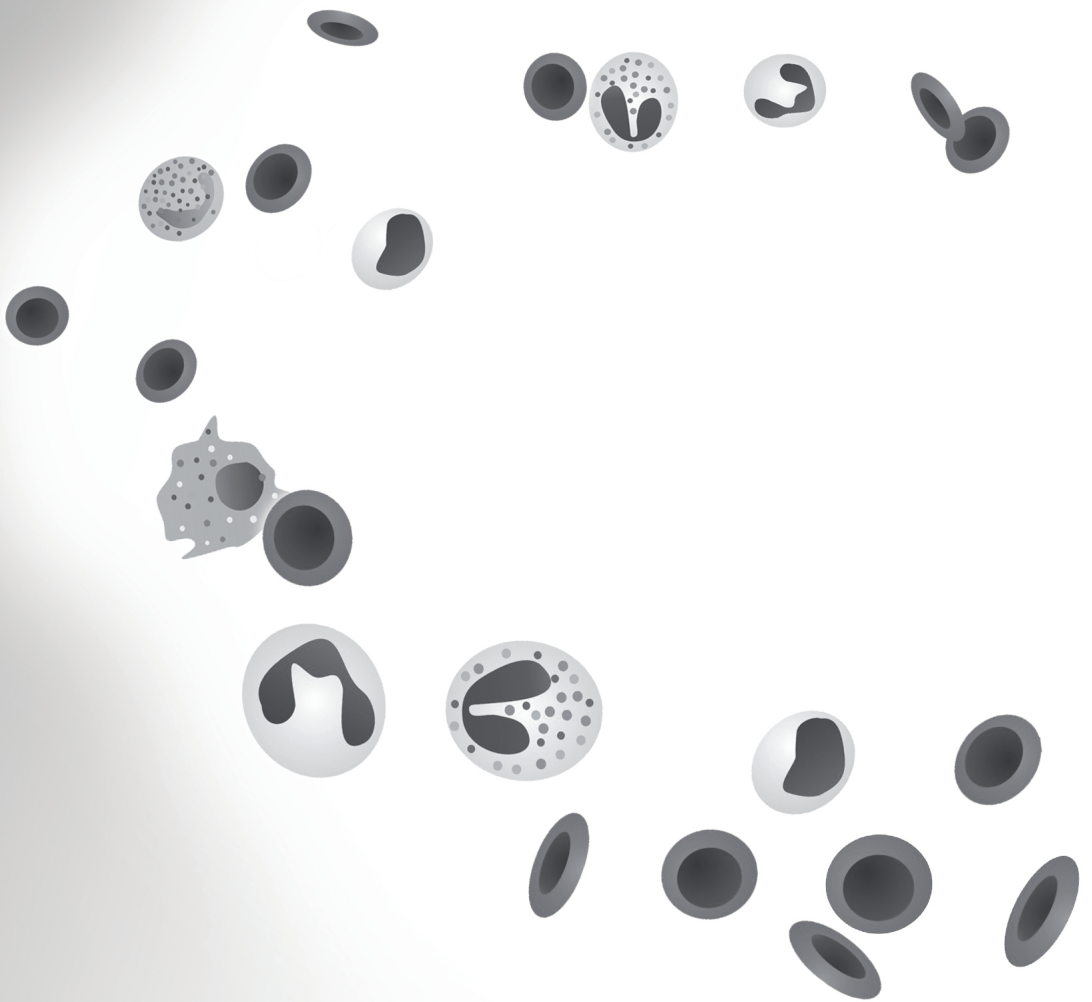
According to the manufacturer's specification, the UF-1000i is linear up to $10,000 \times 10^6$ WBC/L and $100,000 \times 10^6$ RBC/L. This was nicely verified during this study (Figure 3). Furthermore, this wide range limit for WBC and RBC offered on the UF-1000i covers the medically relevant range and minimizes the need for manual dilutions. Carryover on the UF-1000i between modes and sample analysis was insignificant, and it never exceeded 0.01%. To avoid carryover, Sysmex has incorporated an automatic rinse cycle after every measurement. A low background count of only 0 or 1×10^6 WBC/L is accepted in the BF mode. However, if the background check exceeds these numbers, extra rinse cycles are carried out automatically until acceptable WBC and/or RBC values are acquired.

In conclusion, despite some discrepancies compared to the chamber count, the UF-1000i BF mode is sufficiently accurate, and it will eliminate the wide inter-observer variability seen in manual microscopy. Furthermore, the UF-1000i is very useful for initial screening of BF, and it can improve the workflow in the routine laboratory. The ability of the UF-1000i to detect or flag the presence of other cells such as macrophages, mesothelial cells, malignant cells, fungi or bacteria, if present, should be further investigated. Each laboratory must establish its own review and screening criteria in order to report accurate results. Also, scatterplots must be inspected for interfering factors and the presence of large cells in serous fluids. The UF-1000i is not a substitute for manual microscopy; nevertheless, it can substantially reduce the number of samples submitted for microscopy.

REFERENCES

1. de Jonge R, Brouwer R, de Graaf MT, Luitwieler RL, Fleming C, de Frankrijker-Merkestijn M, et al. Evaluation of the new body fluid mode on the Sysmex XE-5000 for counting leukocytes and erythrocytes in cerebrospinal fluid and other body fluids. *Clin Chem Lab Med* 2010;48:665-75.
2. P.W.Barnes CSE, G. Shimer. An evaluation of the utility of performing Body Fluid Counts on the Coulter LH 750. *Lab Hematol* 2004;10:127-31.
3. de Jonge R, Brouwer R, van Rijn M, van Acker BA, Otten HJ, Lindemans J. Automated analysis of pleural fluid total and differential leukocyte counts with the Sysmex XE-2100. *Clin Chem Lab Med* 2006;44:1367-71.
4. De Smet D, Van Moer G, Martens GA, Nanos N, Smet L, Jochmans K, et al. Use of the Cell-Dyn Sapphire Hematology Analyzer for Automated Counting of Blood Cells in Body Fluids. *Am J Clin Path* 2010;133:291-9.
5. Angeloni S, Nicolini G, Merli M, Nicolao F, Pinto G, Aronne T, et al. Validation of automated blood cell counter for the determination of polymorphonuclear cell count in the ascitic fluid of cirrhotic patients with or without spontaneous bacterial peritonitis. *Am J Gastroenterol* 2003;98:1844-8.
6. Zimmermann M, Ruprecht K, Kainzinger F, Heppner FL, Weimann A. Automated vs. manual cerebrospinal fluid cell counts: a work and cost analysis comparing the Sysmex XE-5000 and the Fuchs-Rosenthal manual counting chamber. *Int J of Lab Hematol* 2011;33:629-37.
7. Fleming Chérina RB, Jan Lindemans, Robert deJonge. Validation of the body fluid module on the new Sysmex XN-1000 for counting blood cells in cerebrospinal fluid and other body fluids. *Clin Chem Lab Med* 2012;50:1791-8.
8. Anthony W. Butch P, Porntip K. Wises, David T. Wah, Terrie G. Gornet and Herbert A. F r i t - sche, PhD. A Multicenter Evaluation of the Iris iQ200 Automated Urine Microscopy Analyzer Body Fluids Module and Comparison with Hemacytometer Cell Counts. *Am J Clin Pathol* 2008;129:445-50.
9. Boer K, Deufel T, Reinhoefer M. Evaluation of the XE-5000 for the automated analysis of blood cells in cerebrospinal fluid. *Clin Biochem* 2009;42:684-91.
10. Perné A, Hainfellner JA, Womastek I, Haushofer A, Szekeres T, Schwarzinger I. Performance Evaluation of the Sysmex XE-5000 Hematology Analyzer for White Blood Cell Analysis in Cerebrospinal Fluid. *Arch Pathol Lab Med* 2012;136:194-8.
11. Van Acker JT, Delanghe JR, Langlois MR, Taes YE, De Buyzere ML, Verstraete AG. Automated flow cytometric analysis of cerebrospinal fluid. *Clin Chem* 2001;47:556-60.
12. Yamanishi H, Imai N, Ohmine T, Nishiyama M, Suehisa E, Kanakura Y, et al. Urine flow cytometer quantification of leukocytes in samples containing a large proportion of lymphocytes. *Clin Biochem* 2006;39:857-9.
13. Penders J, Fiers T, Dhondt AM, Claeys G, Delanghe JR. Automated flow cytometry analysis of peritoneal dialysis fluid. *Nephrol Dial Transpl* 2004;19:463-8.
14. Ziebig R, Lun A, Sinha P. Leukocyte counts in cerebrospinal fluid with the automated hematology analyzer CellDyn 3500 and the urine flow cytometer UF-100. *Clin Chem* 2000;46:242-7.
15. Nanos NE, Delanghe JR. Evaluation of Sysmex UF-1000i for use in cerebrospinal fluid analysis. *Clin Chim Acta* 2008;392:30-3.
16. England JM, Rowan RM, Assendelft OW, Bull BS, Coulter WH, McLaren CE. Guidelines for the evaluation of blood cell analyzers including those used for differential leucocyte and reticulocyte counting and cell marker applications. *Clin Lab Haematol* 1994;16:157-74.

17. Keane WF, Bailie GR, Boeschoten E, Gokal R, Golper TA, Holmes CJ, et al. Adult peritoneal dialysis-related peritonitis treatment recommendations: 2000 update. *Perit Dial Int* 2000;20:396-411.
18. Rimola A, Garcia-Tsao G, Navasa M, Piddock LJ, Planas R, Bernard B, et al. Diagnosis, treatment and prophylaxis of spontaneous bacterial peritonitis: a consensus document. International Ascites Club. *J Hepatol* 2000;32:142-53.
19. Soogarun S, Sirimongkolsakul S, Wiwanitkit V, Siritantikorn A, Pimsane K, Srivijarn R. Leukocyte counts in cerebrospinal fluid with the automated hematology analyzer, Technicon H*3. *Clin Lab* 2002;48:623-9.
20. Omuse G, Makau P. Interference of cerebrospinal fluid white blood cell counts performed on the Sysmex XT-4000i by yeast and bacteria. *Int J Lab Hematol* 2013;35:5-7.
21. Langlois MR, Delanghe JR, Steyaert SR, Everaert KC, De Buyzere ML. Automated flow cytometry compared with an automated dipstick reader for urinalysis. *Clin Chem* 1999 ;45:118-22.
22. Conner BD, Lee YCG, Branca P, Rogers JT, Rodriguez RM, Light RW. Variations in pleural fluid wbc count and differential counts with different sample containers and different methods. *Chest* 2003;123:1181-7.



Chapter 8

Liposomal interference on Sysmex XN-Series
body fluid mode

**Chérina Fleming, Michiel de Bruin, Henk Russcher, Jan Lindemans,
Robert de Jonge**

INTRODUCTION

Continued progress in the design of automated analyzers has led to major improvements of cellular analysis in body fluids, mainly because of the sophisticated techniques used. In recent years, a vast majority of laboratories have transitioned from traditional manual microscopy to automated counting of blood cells in body fluids for first line screening. Depending on laboratory defined criteria, a majority of laboratories perform microscopic examination on samples based on flagging by the automated analyser, visual inspection of abnormal scattergram/histogram when counts are below/above a predefined threshold value set by users, or when the requests come from onco-hematology departments. Although it is generally accepted that automated analyzers have improved the quality and turn-around-times of body fluids analysis, difficulties that include potential interfering factors from non-cellular particles remain. These interfering factors can lead to potentially spurious elevated WBC/RBC results (1, 2).

Herein, we report a case of non-cellular particles interfering with the WBC count on the Sysmex XE-5000 BF mode (software version: 00-06C). A 68-year-old female, diagnosed with follicular non-Hodgkin's lymphoma, metastasized to the central nervous system (CNS), was undergoing chemotherapy treatment. CSF samples of this patient were simultaneously sent to our laboratory for cellular analysis, and to the Department of Immunology for immunophenotyping. While the XE-5000 BF mode reported 44×10^6 WBCs/L (MN: 0×10^6 /L, PMN: 44×10^6 /L), immunophenotyping analysis found no (malignant) cells present in the CSF sample. On further investigations, it was revealed that the patient was treated with the chemotherapeutic agent DepoCyt cytarabine approximately 2 weeks prior to CSF lumbar puncture. This prompted us to investigate whether the falsely elevated WBC counts by our automated analyser was due to interference of liposomal particles. Roughly six weeks after, a second CSF sample from the same patient was sent to our laboratory for cellular analysis. After measurement on the XE-5000 BF mode (WBC: 12×10^6 /L, MN: 0×10^6 /L, PMN: 12×10^6 /L), the sample was intercepted for further microscopic analysis. The Fuchs-Rosenthal chamber showed no WBCs present in the CSF sample; although, "round balls" were clearly present. A retrospective review of the patient's CSF laboratory results revealed the presence of WBCs (predominantly PMNs) in 4 CSF samples coinciding with the patient's DepoCyt administration (**Table 1**).

DepoCyt is composed of active cytarabine suspended in aqueous chambers and encapsulated in lipid bi-layers (3). The active drug is uniformly distributed throughout the CSF by its sustained release, and is detectable up to 14 days after administration ($T_{1/2} = 5.8$ days) (4). Depending on the treatment regimen, patients with neoplastic meningitis are treated intrathecally with a standard dose of 50 mg DepoCyt, once every 2 weeks. Interferences of DepoCyt particles are of concern because reporting false elevated WBC

Table 1 Retrospective review of patient's CSF results measured on the XE-5000 BF mode showed falsely elevated counts. CSF samples were collected approximately 2 weeks apart, with exception to sample 4, collected 6 weeks after sample 3.

CSF Sample	WBC ($\times 10^6/L$)	MN ($\times 10^6/L$)	PMN ($\times 10^6/L$)	Eo ($\times 10^6/L$)	WBC Flag
1	18	0	18	9	No
2	45	8	37	2	No
3	44	0	44	41	No
4	12	0	12	0	No

EO= eosinophils (found in research parameters)

counts in patients diagnosed with neoplastic meningitis can have potential adverse clinical consequences; therefore, these results should be interpreted with care.

In 2011, Sysmex launched its new hematology XN-Series analyser which contains a dedicated Body Fluid (BF) mode (5). The XN-Series BF mode utilizes fluorescence flow cytometry technique for counting and differentiating WBCs into mononuclear (MN) and polymorphonuclear (PMN) cells based on forward scattered light (cell size), side scattered light (internal cell structure) and side fluorescence light (DNA/RNA content). In this study, we evaluated the interference of DepoCyt on the Sysmex XN-1000 BF mode because our laboratory switched from XE-Series to XN-Series analyzers.

MATERIALS AND METHODS

Two experiments were performed in this study. In the first experiment, 10 mg/ml DepoCyt (Pacira pharmaceuticals Inc., Mundipharma Pharmaceuticals B.V, the Netherlands) was serially diluted with Sysmex CELLPACK DCL diluent, and measured once on the XN-BF mode. Subsequently, a fresh human blood sample was mixed with DepoCyt in a one to one ratio, and then serially diluted with Sysmex CELLPACK DCL reagent for measurements on the XN-BF mode. The second experiment was similar to the first experiment with the difference that samples were measured in the XN-BF mode using "evaluation software" developed by Sysmex for the detection/flagging of liposomal particles.

RESULTS

In samples containing only DepoCyt, the XN-BF mode (current software: version 00-18) reported falsely elevated WBC counts with a higher accent on the PMN cluster which was overly increased compared to the MN cluster (**Table 2**). We observed a distinct "banana like" pattern in the scattergram of these samples which is depicted in **Figure 1**

Table 2 Serially diluted samples containing only DepoCyt, measured on the XN-BF mode “current software”.

Dilution	Measured WBC ($\times 10^6/L$)	Measured MN ($\times 10^6/L$)	Measured PMN ($\times 10^6/L$)	WBC flag
10mg/ml	35342	194	35148	Yes
5x	1076	96	980	No
10x	292	38	254	No
20x	117	21	96	No
40x	31	8	23	No
80x	9	3	6	No
160x	3	1	2	No
320x	2	1	1	No
640x	1	0	1	No
1280x	0	0	0	No
2560x	0	0	0	No

A & B. Similar results were found when blood samples mixed with DepoCyt were measured in the XN-BF mode. The scattergram of these samples displayed the characteristic “banana-like” pattern along with the falsely increased PMN counts (**Figure 1C**). The “WBC abnormal scattergram” flag was triggered only when the undiluted DepoCyt sample was measured, and displayed a grey “cloud”. This was probably due to the extremely high number of particles being counted (linearity range: 0 to 10,000 cells/ μ L) in the BF mode (Figure 1A). At higher dilutions, the warning flag disappeared, yet the scattergram displayed colors despite the abnormal clustering (Figure 1B and 1D). Stained cytopsin slides were prepared of DepoCyt samples alone, and blood samples mixed with DepoCyt. However, DepoCyt particles were neither visible on slides containing only DepoCyt, nor on blood slides mixed with DepoCyt. When samples, both in DepoCyt alone and in blood mixed with DepoCyt, were counted using the Fuchs-Rosenthal chamber, a heterogeneous mixture of small and large round spherules was present, and each spherule contained a “nucleus” in the center (**Figure 2**).

Results of the second experiment using the XN-BF “evaluation software” are depicted in **Figure 3**. By using serially diluted DepoCyt samples, the “evaluation software” triggered a series of events in 56% (5/9) of the cases; *i*) presence of the WBC abnormal scattergram flag, *ii*) “grey cloud” in the WDF scattergram, *iii*) asterisk marks next to the WBC and Diff results, and *iiii*) a “corrected” WBC count for DepoCyt interference. The “corrected WBC” count is not accurate. It should have been zero in all cases; however, results reported were significantly lower than the reported WBC counts using current software. Nevertheless, the “evaluation software” displayed an abnormal WBC scattergram flag in all samples containing the characteristic “banana-like” pattern (**Figure 3A & B**).

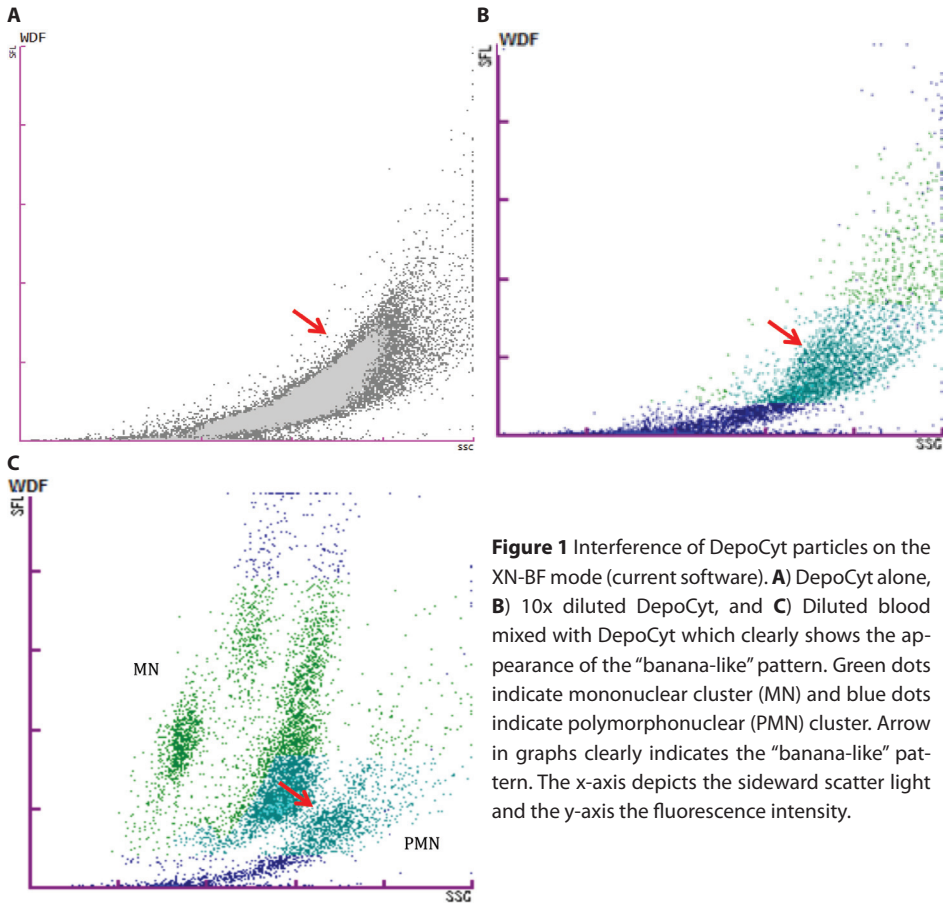


Figure 1 Interference of DepoCyt particles on the XN-BF mode (current software). **A)** DepoCyt alone, **B)** 10x diluted DepoCyt, and **C)** Diluted blood mixed with DepoCyt which clearly shows the appearance of the “banana-like” pattern. Green dots indicate mononuclear cluster (MN) and blue dots indicate polymorphonuclear (PMN) cluster. Arrow in graphs clearly indicates the “banana-like” pattern. The x-axis depicts the sideward scatter light and the y-axis the fluorescence intensity.

The remaining 44% (4/9) of samples were not flagged, and no “banana-like” pattern was visible in scattergram. These samples contained less particles (“corrected” WBC counts of 5 or less WBCs $\times 10^6/L$) because of the higher dilutions ($> 80x$ diluted) (**Figure 3C**).

DISCUSSION

The purpose of this study is to highlight the interference of liposomal particles (DepoCyt) present in samples measured on the XN-BF mode because they can cause incorrect WBC/differential counts by reporting falsely elevated results. We clearly demonstrated that the events appearing predominantly to the right of the WDF scattergram (banana-like pattern) are attributed to DepoCyt particles mimicking WBCs. Furthermore, the majority of particles counted as WBCs were predominantly misclassified as PMNs. A closer look at the cluster showed that mainly the eosinophilic counts (research param-

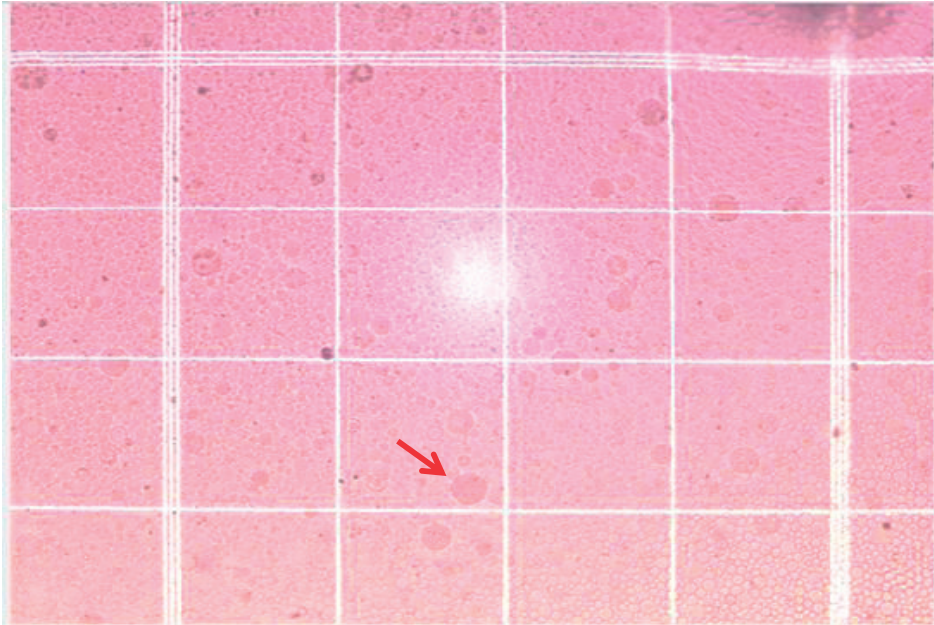


Figure 2 Heterogeneous picture of Samson stained DepoCyt particles (small and large) in the Fuchs-Rosenthal chamber. Arrow depicts spherules with “small round dot” (nucleus) inclusions.

eter) were affected (falsely increased), causing the PMN count to be falsely increased. Extrapolating these results to CSF samples, we predict that CSF samples containing a mixture of both cells and residual DepoCyt (depending on the time frame of CSF collection and amount of residual DepoCyt present) will appear to the extreme right of the scattergram, producing the distinct “banana-like” pattern and false increased PMNs. This was also observed in our case study (Table 1). Like other studies, ours could not detect liposomal particles in the stained cytopsin slides (3, 6). Perhaps, the liposomal particles were destroyed during the May-Grünwald Giemsa staining process or during the centrifugation step. We informed Sysmex Corporation of the above mentioned phenomenon; consequently, Sysmex developed new software algorithms to address this issue. With the new software, three alerts are triggered when liposomal interference is detected 1) WBC abnormal scattergram flag is displayed, 2) asterisk marks next to the WBC/DIFF results, and 3) grey colored WBC scattergram is produced, except in those samples containing very low amounts of particles. The new software displayed a “corrected” WBC count in samples containing only DepoCyt; however, the “corrected” WBC should have been zero. Nevertheless, the new software contains new algorithms to help users identify samples containing interfering liposomal particles (distinct banana-pattern), which should trigger them to proceed with microscopic evaluation in order to report accurate WBC counts. The new software will be released in the near future.

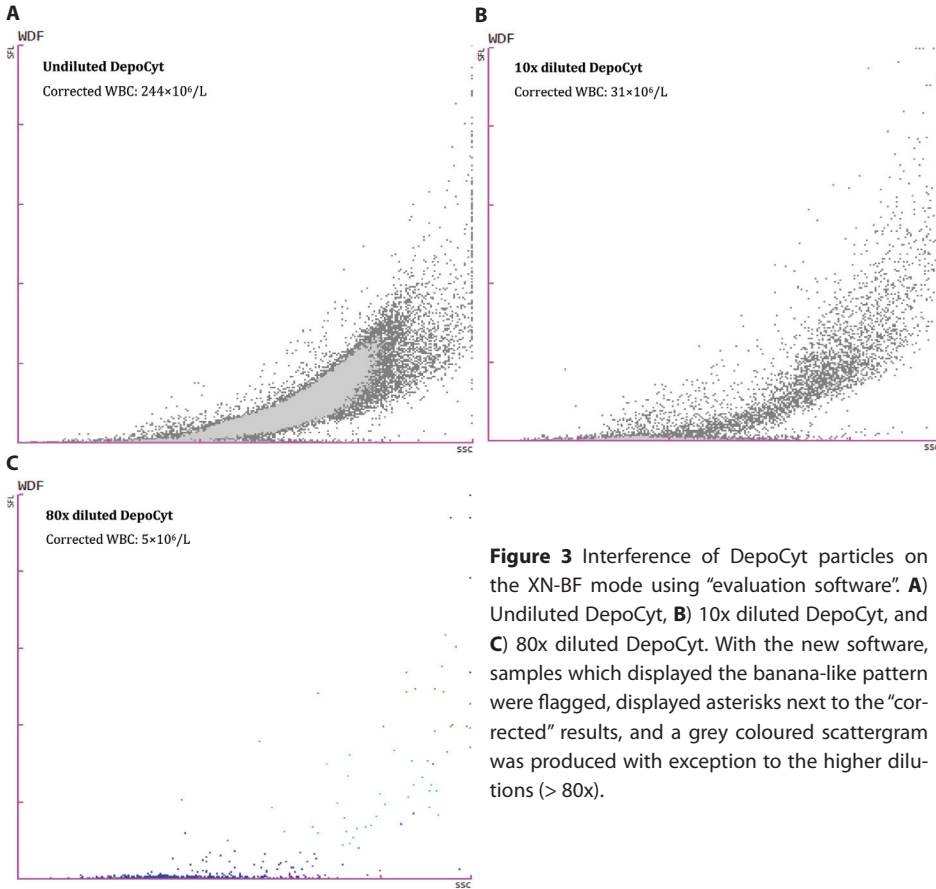


Figure 3 Interference of DepoCyt particles on the XN-BF mode using "evaluation software". **A)** Undiluted DepoCyt, **B)** 10x diluted DepoCyt, and **C)** 80x diluted DepoCyt. With the new software, samples which displayed the banana-like pattern were flagged, displayed asterisks next to the "corrected" results, and a grey coloured scattergram was produced with exception to the higher dilutions (> 80x).

There are few studies reporting on the spurious elevation of CSF's WBCs measured on automated analyzers from patients treated with DepoCyt (4, 6). Stacchini et al. (6), using flow cytometric analysis, demonstrated the presence of liposomal cytarabine particles in CSF samples taken from patients treated with this drug. The flow cytometry forward and side scattergram of these samples displayed high numbers of "atypical" events. These "cells" were both CD45 (WBC) and CD235a (RBC) negative, and microscopic evaluation revealed the presence of spherules of varied diameters in the CSF.

Automated analyzers have been developed to accurately and precisely count cells in CSF and other body fluids; however, they are not error proof. This study reminds us of that fact by clearly showing these non-WBC particles interfere with cell counting, and hamper the reporting of correct results. Liposomal DepoCyt particles are reported to range in size from 3 to 30 μm , which is similar in size to WBCs (10-20 μm). A challenge of hematology analyzers is the discrimination amongst particles which possess similar physical properties, and fall into similar areas of a scattergram, making it, at times, diffi-

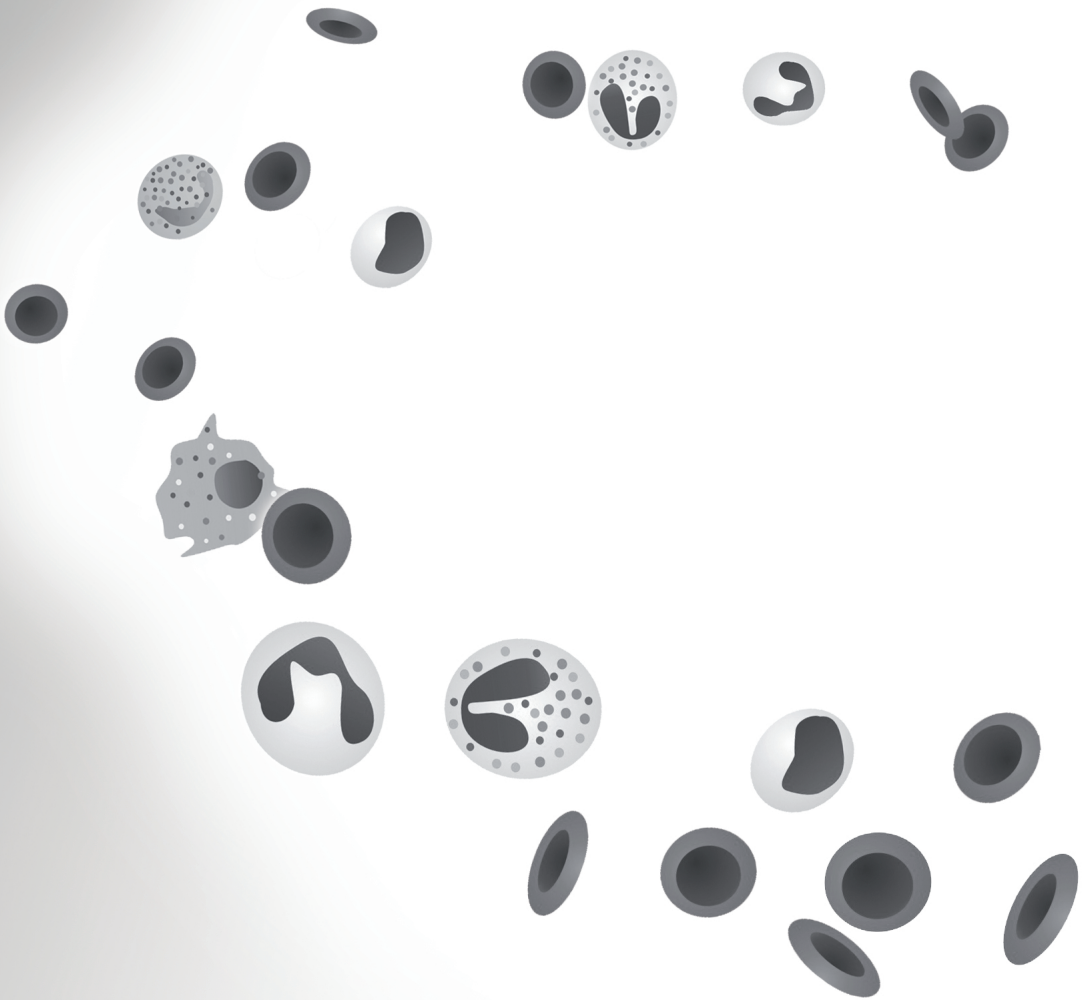
Table 3 Serially diluted samples containing only DepoCyt, measured on the XN-BF mode “evaluation software”.

Dilution	Measured WBC ($\times 10^9/L$)	Measured MN ($\times 10^9/L$)	Measured PMN ($\times 10^9/L$)	WBC flag
10mg/ml	244	29	215	Yes
5x	38	10	28	Yes
10x	31	9	24	Yes
20x	15	2	13	Yes
40x	5	1	4	Yes
80x	5	2	3	No
160x	2	1	1	No
320x	1	1	0	No
640x	0	0	0	No

cult to differentiate one from another. When these non-cellular particles are abundantly present and/or clumped together, their size and structure characteristics will cause the XN-1000 BF mode to falsely count them as WBCs. Based on available evidence, we suggest these types of samples should not be analysed using automated analyzers, but analysed manually until automated analyzers can detect/flag interfering particles such as DepoCyt, and alert users of their presence.

REFERENCES

1. Omuse G, Makau P. Interference of cerebrospinal fluid white blood cell counts performed on the Sysmex XT-4000i by yeast and bacteria. *Int J Lab Hematol.* 2013;35:e5-7.
2. Shen PU, Blair JL. Cholesterol crystals causing falsely elevated automated cell count. *Am J Clin Pathol.* 2006;125:358-63.
3. Mathai S, Jones GL. Spurious pleocytosis. *Blood.* 2010;116:678.
4. Swords R, O’Rafferty C, Giles F, Browne P. Spurious elevation of the cerebrospinal fluid white cell counts in three patients receiving liposomal cytarabine as part of post-remission therapy. *Leukemia.* 2008;22:2277-8.
5. Fleming C, Brouwer R, Lindemans J, de Jonge R. Validation of the body fluid module on the new Sysmex XN-1000 for counting blood cells in cerebrospinal fluid and other body fluids. *Clin Chem Lab Med.* 2012;50:1791-8.
6. Stacchini A, Demurtas A, Aliberti S. Flow cytometric detection of liposomal cytarabine in cerebrospinal fluid of patients treated with intrathecal chemotherapy. *Cytometry B Clin Cytom.* 2012;82:280-2.



Chapter 9

General Discussion

Automated cell counters

In this thesis, the suitability of automated hematology and urine analyzers for the cellular analysis of non-malignant body fluids (BFs) has been thoroughly investigated, especially with respect to their capacity to replace manual microscopy in clinical practice. Manual microscopic analysis of BFs remains problematic in many laboratories. One option to overcome this tedious process is to analyze the samples using an automated cell counter. These cell counters are designed to be faster, more precise and easier to use compared to manual microscopy. However, two-constraints of standard hematology analyzers in BF analysis is the matrix of these fluids being quite different from whole blood, and the high detection limits of these devices. In recent years, several manufacturers including Sysmex Corporation have extended their technological capabilities by inserting dedicated BF applications in their automated cell counters. This major development led to the **overall** aim of this thesis, which is to determine the capability of analyzers with dedicated BF-mode for counting blood cells in non-malignant BFs. More specifically, are these analyzers:

- Sufficiently precise to measure blood cells in clinically relevant ranges?
- Sufficiently accurate in the WBC and WBC differential count?
- Able to discriminate normal from infectious/inflamed samples (diagnostic test accuracy)?
- Capable of detecting and flagging interfering particles?
- Suitable for replacing manual microscopy?

Each chapter ends with a separate discussion; therefore, in this final chapter, an overview of the general observations will be discussed, and suggestions for potential future research will be given.

Are Sysmex's analyzers sufficiently precise to measure blood cells in clinically relevant ranges?

The limit of quantitation (LoQ) is an important indicator of instrument performance, for it describes the smallest concentration of measurand that can be reliably measured by an analytical procedure (1). A review of the results in **Figure 1** clearly shows that the LoQ for WBCs has constantly improved over each new generation of Sysmex's analyzers, especially compared to the XE-2100 whole blood (WB) mode, which was previously used for BF analysis. In fact, the LoQ of the XN-hsA mode is lower than the upheld gold standard (hemocytometer). The good precision can be attributed to Sysmex implementation of extended cell counting, which allows many more cells to be counted. This is especially important for CSF because the upper WBC limit of normal is quite low (adults $5 \times 10^6/L$); whereas, the WBC upper threshold for other body fluids is over $100 \times 10^6/L$, which is far above the LoQ of the Sysmex analyzers in BF mode. Interestingly, the LoQ of all other analyzers capable of BF analysis is higher than those of Sysmex, with exception to the

Advia (LoQ: 2×10^6 WBCs/L; CV=30%) in CSF mode. The Advia shows a similar LoQ to the XN-hsA mode (2-5). Similar to our study (Figure 1), a high imprecision was found with the manual method by others (6-8). One examiner determined the WBC imprecision of the manual count by repeatedly (3 – 7 times) counting cells in the low concentration range, and the results of the CVs ranged from 35% at 5×10^6 /L to 40% at 50×10^6 /L (6). Moreover, manual cell counting is also hampered by the large inter-individual imprecision, that is absent in automated methods. We showed earlier that the inter-individual imprecision of experienced laboratory technicians at WBC counts of 1000×10^6 /L was as high as 21% (9). Using optical measurements for RBCs, the LoQ of the XN-hsA mode (10×10^6 /L) and the UF-BF mode (25×10^6 /L) showed good precision. Compared to Sysmex's analyzers, the Abbott Sapphire, which also uses optical measurements, showed a higher LoQ for RBCs (3000×10^6 /L, CV:20%) (3). The Sapphire employs software of the WB mode rather than dedicated software for BF analysis, and it has a limited precision in the low range, resulting in this high LoQ.

From this perspective, Sysmex's analyzers are more precise at analyzing samples with low and high cellularity because of their low LoQ in both ranges and wide linearity ranges compared to the hemocytometer. One study suggested recounting WBCs when the automated results are between 5 – 20×10^6 /L (10). However, recounting cells using the hemocytometer has limited effect because of the high imprecision of this technique. Because the newest generation of Sysmex's BF modes outperforms the hemocytometer in terms of precision, we recommend automated counting of WBCs in BFs.

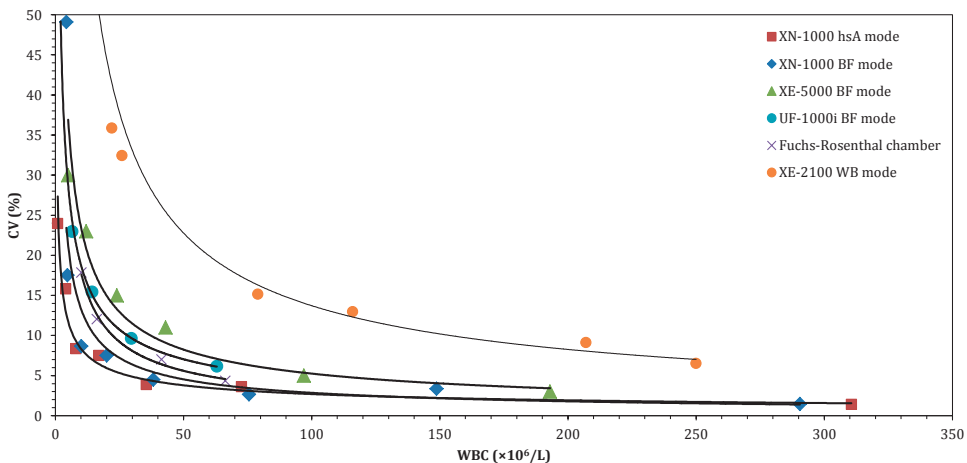


Figure 1: Precision profile of Sysmex automated analyzers containing dedicated BF modes and the reference method. LoQ was determined at CV 20%. LoQ of the XE-5000 BF mode: 10×10^6 /L, UF-BF mode: 9×10^6 /L, XN-BF mode: 5×10^6 /L, XN-hsA mode: 2×10^6 /L and the counting chamber: 10×10^6 /L. The LoQ of the XE-2100 whole blood (WB) mode: 50×10^6 /L.

Are Sysmex's analyzers sufficiently accurate to measure WBCs, WBC differentials and RBCs?

WBCs

Accuracy refers to the degree to which a measured value for a sample agrees with a reference or true value of the measurand (11). Though manual microscopy is considered the gold standard, it does not serve as a reference technique for measuring cells in BFs. It must be kept in mind that a reference method is only the most accurate representation at a given time; therefore, it is dependent on the state of art at a given time and consequently on the method used (12). The concept of WBC counting in BFs was historically created from manual microscopic evaluations, so it seemed natural to use this method as our gold standard. We are well aware of the major limitations of this technique including its high imprecision and inter-operator variability. Throughout this thesis, one technician performed all hemocytometric counting, and another technician performed all cytopsin differential counts to limit variation.

Accuracy was considered acceptable if no significant differences from the gold standard were observed using Passing & Bablok regression, and/or when the proportional bias (slope) was $\leq 20\%$. Generally, Sysmex hematology analyzers produced good results (proportional bias was always less than 10%) for counting WBCs in all fluids compared to the gold standard (**Table 1**). Conversely, the UF-BF mode showed the largest disparity from the true values. Perhaps, this is due to interference from large cells such as macrophages, mesothelial and malignant cells that are present in serous fluids (3). Our results are in line with Buoro et al., (13) which show an overestimation of the WBC count in CSF samples containing high numbers of macrophages. From this point of view, the WBC count on the UF-BF is unreliable in samples containing large cells; therefore, we recommend reporting the TNC instead of the WBC count on this analyzer.

The XE-BF and XN-Series are the only analyzers that exclude large cells from the WBC/WBC differential count. This is achieved by special gating algorithms (cell size and high nucleic acid content) that report these cells separately and limit their positive interference on the WBC count, and in doing so, improve accuracy (14, 15). Other analyzers such as the Beckman LH 750, the Iris iQ200 and the Advia 2120i BF mode report a TNC/WBC count. Consequently, non-WBCs are counted as WBCs, which is confusing for the interpretation (3, 16). The CSLI H56-A guideline defines the TNC count as including all nucleated cells (WBCs, nucleated red blood cells, lining cells and other non-hematopoietic cells); whereas, the WBC count includes only neutrophils, monocytes, lymphocytes, eosinophils and basophils (17). Considering these guidelines, we recommend that in all fluids, the TNC and WBC count be reported separately. This will aid clinicians and laboratory specialist in the interpretation of the results but will also lead to better interpretation and comparison of results from scientific studies. Relevant reference ranges

Table 1 Summary of comparison studies between automated analyzers and the manual methods.

	Body fluid	WBC x10⁹/L	PMN x10⁶/L	MIN x10⁶/L	RBC x10⁶/L	
XE-5000 BF mode	CSF	1.04x + 0.32	1.35x + 0.27	0.91x + 0.27	n/a	Chapter 4
	CSF	1.06x + 0.09	1.48x + 0.19	1.04x - 0.00	0.99x + 116.56*	Chapter 5
	Ascites	1.06x - 2.37	1.10x + 2.11	1.03x - 2.89	1.04x + 110.04*	
	Pleura	1.08x - 37.15	1.02x + 8.95	1.05x - 8.99		
	CAPD	1.10x - 0.64	1.04x + 0.25	1.17x - 1.19		
XN-Series hsA mode	CSF	1.08x + 0.52	Neutro: 1.05x + 0.00 EO: n/a	Lymph: 1.00x - 0.00 Mono: 0.88x + 0.07	1.07x + 0.00	Chapter 6
	Ascites	1.24x + 15.11	n/a	n/a	1.05x + 29.58	Chapter 7
	Pleura	1.32x + 10.85	n/a	n/a	1.01x + 39.03	
	CAPD	1.23x + 1.65	n/a	n/a	1.20x + 4.89	

* = RBC > 1000x10⁶/L, n/a= not applicable; Neutro=neutrophils; Lymph= lymphocytes, Mono=monocytes, EO=eosinophils

are important for the clinical interpretation of results. Already, slightly higher reference ranges has been established by automated analyzers in CSF samples (15, 18); therefore, it would be beneficial for future studies (including large sample sizes) to investigate if current reference ranges are adaptable to automated analyzers, or whether new reference values should be established for all BFs.

WBC differentials

Most clinical guidelines rely on the WBC differential count, which is of substantial value in diagnosing and monitoring treatment in conditions such as meningitis and peritonitis. Both the XE-5000 and the XN-BF mode had a tendency to overestimate the PMN count in CSF samples compared to manual counting (Table 1). This finding has also been documented by others (6, 15, 19-25). Interestingly, overestimation of the PMNs was pronounced in CSF samples but not in other BFs, reflecting a problem with CSF. CSF differs from other BFs by their cellular instability, limited sample volume, low cellularity and the need for prompt results. Consequently, these samples pose the greatest challenge for automated analysis. First, a possible explanation for the observed bias could be the collection site of CSF. CSF samples collected from ventricular drains are prone to cell debris after the installation of drainage systems, and they showed a higher positive bias compared to samples collected by lumbar puncture (10, 21). These particles appeared (interfered) in the gating area of the PMN as randomly scattered events, causing the WBC/PMN to be falsely increased (21). This indicates that the gating algorithms on these analyzers are not optimized for CSF samples containing low cellularity. Second, the lack of agreement could have been a result of the time delay between automated measurements and manual counting. From previous studies, it is known that the proportion of PMNs and MNs in CSF decrease over time, and more so after cyto centrifugation (26, 27). CSF samples in this thesis were analyzed within 2 hours after entering the laboratory. Samples were first measured on the automated analyzers, followed by manual counts. Consequently, cellular decay due to longer pre-analytical time and cyto centrifugation may have caused falsely low cell counts in the manual counting procedure (the comparison method/gold standard) and "overestimation" of the PMN count. Third, the BF mode on Sysmex's analyzers count and differentiate WBCs in one process using the same technique, and do not depend on a centrifugation step; whereas, the hemocytometer requires two separate preparation steps (counting chamber and cytospin). Hence, results of manual microscopy are a ratio of two counts, both of which are derived from a relatively small number of cells (28), which according to Rümke's table (29), lead to wider confidence interval limits. The greater the number of cells counted, the greater the statistical precision of the differential count will be. Conversely, the smaller number of cells that are counted, the less precise the blood cell percentage is. In other words, Sysmex analyzers are superior to manual methods because they count a larger number

of cells, which increases precision and minimizes statistical errors. For this reason, and to overcome technical limitations related to cell loss, it is important to analyze CSF samples immediately after collection. At the same time, centrifugation steps should be kept to a minimum. CSF samples are collected in plain tubes, and if left unattended for too long, cells may deteriorate. Therefore, the development of stabilized tubes to prevent cell loss may be a focus for further research. To obtain a better understanding of the observed bias on the XE-5000 and the XN-BF mode in CSF, future studies should discriminate between CSF samples collected from ventricular systems, and those collected by lumbar puncture. Sysmex should improve its gating algorithms to exclude cell debris or fragments from the differential count.

The overestimation of PMNs in CSF samples seems to be resolved by the use of the XN-hsA mode (Table 1). This can be attributed to the software of the XN-hsA mode which was tailored for CSF samples. It counts more cells, and has new gating algorithms to exclude particles interfering between the x-axis and the neutrophil count. In the XN-hsA mode, each cell type is gated separately (4-part: lymphocytes, monocytes, neutrophils and eosinophils) instead of clustered (2-part: PMN and MN). The new gating has a smaller circumference, which reduces the chances of reporting interferences compared to gating algorithms designed for a broader circumference. Generally speaking, reporting a 2-part differential count limits the information because each cell type has a specific role that is increased or decreased during different stages of disease. In line with the CSLI H56-A guideline, we recommend that a 4-part differential count be reported, and if abnormal cells are present, they should be reported separately (11). For example, in CAPD fluid, a high PMN count due to eosinophils should be treated differently from one with a predominance of neutrophils. In the former, antibiotic treatment is not necessary; whereas, it is crucial in the latter. Reporting a full differential count is already implemented on standard hematology analyzers, so this should be adopted also for BF analysis.

RBCs

The XE-5000 and the XN-1000 BF mode employ impedance technique for counting RBCs, and can reliably measure RBCs $>1000 \times 10^6/L$ (Table 1). This is similar to the BF modes of other analyzers such as the iQ200 and the LH 750, also using impedance technique (3, 16). Impedance technique is perfectly suitable for analysing samples with high counts. However, in samples containing a limited number of cells, this technique may become less accurate because fewer events are counted, and there is an increased influence of background particles (4, 30). To facilitate the counting of RBCs in low concentration ranges, Sysmex uses optical measurements rather than impedance technique on the XN-hsA mode and the UF-1000i BF mode. Comparison studies between the optical mea-

surements and the manual method for RBCs showed good agreement, even in samples containing less than $1000 \times 10^6/L$ (Table 1).

Subarachnoid hemorrhage (SAH) is a life threatening neurosurgical emergency. Distinguishing between a traumatic tap and a SAH is critical for physicians, as both disorders mounts to RBCs in CSF samples. One study showed that the presence of fewer than 500×10^6 RBCs/L excluded the diagnosis of SAH with a 100% negative predictive value (14). Moreover, a traumatic tap obscures the WBC count in CSF evaluation of meningitis. To overcome this, a WBC/RBC ratio of $\leq 1:100$ is used to exclude a traumatic tap with high specificity (100%) and sensitivity (84%) (31). Given this knowledge, analyzers using impedance techniques are less suitable for CSF analysis, but are sufficient for the analysis of bloody effusions (clinical cut-off $>1000 \times 10^6/L$).

In conclusion, the employment of optical methods (UF-BF mode and XN-hsA mode) is necessary to reliably count RBCs in the low range which is especially important for CSF samples. Prospective studies should include optical RBC measurements to evaluate the usefulness of RBCs counts in the low range in order to help distinguish between traumatic tap and SAH.

Malignant cells

Malignant cells are usually investigated by the anatomical pathologist; however, when malignancy is not clinically suspected, and the samples are sent to the routine hematology laboratory, they should be alert and able to detect malignant cells (32). Therefore, having reliable flags or specific cutoffs for abnormal cells could potentially improve the detection rate of malignancy in the routine laboratory, especially in CSF samples where malignant cells may appear in low frequency (33). Malignant cells have extremely variable morphological properties, and can be easily confused with non-malignant cells such as plasma cells and mesothelial cells. Analyzers employing fluorescent flow cytometry technique have been shown to either misclassify malignant cells (i.e., lymphoid blast cells clustered as MNs) or fail to detect them in BFs (19, 33-35). This thesis focused on non-malignant BFs, and it did not investigate the reliability of the high fluorescent (HF-BF) flag. Zimmerman and colleagues investigated the diagnostic accuracy of the HF-BF parameter on the XE-5000 in 65 malignant and 126 non-malignant CSF samples. Their investigation resulted in a sensitivity of 78% and specificity of 74% (33). They found also that low percentages of tumour cells frequently escaped detection by the XE-5000. In a similar study, reasonable sensitivity (87%) and poor specificity (60%) were found for the identification of samples containing malignant cells on the XN-BF mode using the HF-BF parameter in CSF and serous fluids (34). This study reported that non-malignant fluids depicted a high HF-BF value, which is probably caused by tissue cells in serous fluids. Interestingly, high numbers of HF-BF cells were observed in 86% of ventricular CSF samples, in none of which tumour cells were detected by microscopy (10).

Taken together, neither Sysmex's analyzers nor any other analyzers for that matter, are sensitive enough to use for the detection and flagging of malignant cells. While Sysmex's analyzers count significantly more cells than technicians do to create a differential count, they are perhaps still not efficient in detecting malignant cells. As a general rule, we recommend that suspected malignant BFs or samples from hemato-oncology departments should not be analyzed on Sysmex's analyzers. But rather, a microscopic differential count must be performed independently of whether the automated analyzer gives a suspect flag error or not. In future research, it is important for samples suspected of containing malignant cells to be included in the evaluation of the HF-BF parameter on Sysmex's analyzers. These studies should use immunophenotyping flow cytometry as the reference method because this method is considered the gold standard for detecting malignancy.

Are Sysmex's analyzers able to discriminate normal from infectious/inflamed samples?

Diagnostic accuracy relates to the ability of a test to discriminate between health and disease. This discriminative potential can amongst others be quantified by the measures of sensitivity and specificity (36). From our literature review in chapter 2, we observed that only few studies investigated the diagnostic accuracy of the WBC and WBC differential count in the diagnoses of infectious and inflammatory BFs. A majority of studies focused on SBP in ascites fluid and less so on other disorders including peritonitis. In chapter 3, we retrospectively evaluated the diagnostic accuracy of the automated neutrophil and WBC count in 69 effluent dialysates suspected of peritonitis. It was clear that the neutrophilic PMN dominated the WBC count as the better diagnostic marker, especially in culture-negative patients (37). This is not surprising, as neutrophils are the primary WBCs that respond to a bacterial infection, and can be recruited to an infection site within a short time. Some studies evaluated only the WBCs as diagnostic marker for peritonitis (38). However, WBCs can be increased without neutrophilic predominance. This suggests there is another cause of inflammation (i.e., eosinophilic peritonitis). Current guidelines advocate the combination of both WBCs and PMNs as diagnostic parameters for peritonitis. Perhaps, this is due to a lack of evidence supporting which parameter is the best predictor. In fact, we suggest future studies to evaluate the diagnostic accuracy of both parameters for all BFs. As this might be of added value in discriminating between infectious from non-infectious, using automated techniques such as Sysmex's, to increase precision in determining and/or confirming cutoffs; since, current cutoffs were determined on the basis of manual microscopy, and the limitations of this technique are well known.

To assess the ability of Sysmex's analyzers to discriminate normal from infectious/inflamed samples by showing elevated WBC counts, results were compared with those

Table 2 Diagnostic accuracy of the different analyzers in differentiating normal from abnormal fluids shown as Sensitivity/Specificity results. For CSF samples cut-off value $<6 \times 10^6/L$ normal and $WBC > 6 \times 10^6/L$ (abnormal). For CAPD cut-off value normal: $<100 \times 10^6/L$ and ascites normal: $<250 \times 10^6/L$.

	Fluid	No. samples included	Sensitivity (%)	Specificity (%)
XE-5000 BF mode	-	-	-	-
XN-Series BF mode	CSF	67	100	97
XN-Series hsA mode	CSF	111	98	95
UF-1000i BF mode	CAPD	57	100	78
	Ascites	54	100	83

obtained with the hemocytometer. Excellent sensitivity $>98\%$ was found and specificity varied from 78 – 98% (**Table 2**). The lowest specificity was reported by the UF-BF, consequently high false positive rates, which correlate to the overall positive bias, were observed on this device. These results indicate that patients measured on Sysmex's analyzers with negative results can be spared antibiotic treatment, and do not need manual follow-up in the laboratory. On the other hand, patients with false positive results may receive unnecessary antibiotics due to erroneous results of the UF-BF count. In aggregate, the current Sysmex's BF modes display high sensitivity for elevated WBC counts; hence, manual follow up of these samples can be omitted.

Are Sysmex's analyzers capable of detecting and flagging interfering particles?

Suspected flags are usually preprogrammed and generated in the presence of an abnormality by internal algorithms. In the BF mode of the XE-5000, XN-Series and the UF-1000i, only one flag is generated in the presence of abnormality ("WBC abnormal scattergram"). A disadvantage of having only one flag is its lack of specificity. The flag is generated by abnormality in the WBC population, cell distribution and/or in the presence of large cells (HF-BF). The latter is user-definable. It is well known that automated cell counts may be falsely elevated in the presence of medication, incomplete lysis of RBCs, cell debris, crystals and even when bacteria and yeast cells are present (39, 40). In chapter 8, we investigated the interference of DepoCyt, a chemotherapy drug used in the treatment of lymphomatous meningitis on the XN-BF mode using current software and an evaluation software to detect interfering liposomal particles. Results from the current software clearly showed abnormality on the scattergram (banana-like pattern), but failed to flag for these interferences; whereas, the evaluated software displayed the following signs: abnormal flag, grey scattergram and asterick next to the WBC results. We suspect the reason for interference is likely related to the way cells are counted in the

BF mode. Flow cytometry has a tendency to overestimate WBC and/or RBC counts when other particles with the size of WBC/RBC contaminate the sample, and make it difficult to distinguish between the two. In these circumstances, manual microscopy becomes an important quality control strategy to identify this phenomenon. Such problems can be avoided if patients treated with this drug were reported to the laboratory, for this would enable the laboratory to be aware, and take precautions. Until the evaluated software becomes available on the market, we recommend samples displaying a banana-like pattern be followed-up with microscopy.

During our evaluations, we discovered that warning flags were not always generated, even when interferences from bacteria and yeast cells were present (41). Though we did not assess the accuracy of the “abnormal scattergram flag”, one study showed that the “WBC abnormal flag” on the XE-5000 has poor sensitivity (60%) but good specificity (96%) in CSF samples (8). This means a high number of abnormal samples will be missed during the automated screening.

In daily practice, automated BF analysis is used as a screening mode. Only fluids that are suspected of interference or abnormal clustering/analysis are subjected to further manual evaluation. In these circumstances, flagging algorithms that are highly sensitive are necessary to recognize abnormal results. Therefore, the introduction of gating/flags for additional aberrations would make Sysmex’s analyzers of greater value for primary screening of BF samples because they would trigger a turn to the microscope or to a digital microscopy (DM96) system for verification. The DM96 system has separate BF software and enables automated recognition and pre-classification of WBCs (4-part differential and other cells) with good results (42). Because interfering substances such as bacteria, liposomal drugs and cell debris still pose a problem for automated flow cytometry, future studies should always assess their impact when validating these machines. Until that time comes, visual inspection of the BF scattergrams is mandatory in order to select those samples with possible interference for manual follow-up.

Are Sysmex’s analyzers suitable for replacing manual microscopy?

Medical diagnosis and treatments rely on the accurate and timely issuing of high quality laboratory results (43). The Sysmex’s analyzers studied in this thesis showed promising results when compared with microscopy. First, in terms of precision, Sysmex’s automated analyzers are certainly suitable because of the lower imprecision of these analyzers compared to microscopy. Second, the total and differential count on automated analyzers has been standardized, eliminating inter and intra-operator variability, which is still a big limitation of the manual method. Third, the possibility of (preferential) cell loss is minimized on the automated method because analysis time is faster, and no centrifugation step is necessary for cell differentiation, which generates improved (differential) counts. Fourth, the automated counts were highly sensitive in discriminating normal from

infected/inflamed samples based on published cutoff values. Thus, negative samples do not need to be followed up by manual microscopy. Fifth, Sysmex analyzers with an exception to the UF-BF mode, automatically report a WBC differential count. Finally, Sysmex's analyzers are regularly subjected to quality controls to assure their proper functioning; whereas, homocytometers are not subjected to the same checks.

In view of these findings, we believe Sysmex's analyzers (BF mode) can replace manual microscopy to a major extent in screening for infection/inflammation. Though they are superior to microscopy in many aspects (precision, faster, consistency), they are perhaps still not efficient enough in detecting important morphological abnormalities that so far only microscopy can provide. Therefore, the decision about which method to choose for analysis of BFs will depend strongly on the primary clinical question. In the case of malignancy, preferably all samples should be analyzed microscopically. However, in the case of first line screening for infection and inflammation, Sysmex's analyzers are sufficient to deliver accurate results in all fluids, and manual microscopy is only warranted when the "WBC abnormal scattergram" is triggered or when visual inspection of the scattergram shows abnormal clustering. Therefore, the goal of Sysmex's analyzers to replace microscopy has not yet been fully accomplished.

Concluding remarks

Compared to blood samples, automated analysis of BF samples is still in its infancy, but progressive improvements are on the rise. The use of automated analyzers without dedicated BF mode to enumerate cell counts in BFs poses difficulties (high detection limits and overestimation of counts), and is not advocated. However, the Sysmex BF mode achieved good levels of precision and accuracy in cell counting through the examination and identification of thousands of cells in each BF sample. The purpose of the BF mode is to optimize the analysis of BF samples, taking into account the different cellular composition and matrix effects (32). At this point, Sysmex's analyzers (BF mode) emerged as the best solution for first line screening of non-malignant BFs. Limitations of Sysmex's cell counters are often linked to their inability to differentiate cell populations and to eliminate interference in BFs. Also, the recognition and flagging of abnormal cells remains a challenge. In today's clinical laboratory, improved efficiencies and enhanced productivity are essential. Merging hematology/urinalysis analyzers with BFs testing, as done on Sysmex's devices, adds benefits through automation and standardization of test performance which ultimately benefits the patient. The question that now remains is whether the manual method should still be considered the "gold standard" for BFs analysis since Sysmex's analyzers have shown better performance for most applications.

REFERENCES

1. Armbruster DA, Pry T. Limit of blank, limit of detection and limit of quantitation. *Clin Biochem Rev.* 2008;29 Suppl 1:549-52.
2. Tanada H, Ikemoto T, Masutani R, Tanaka H, Takubo T. Evaluation of the automated hematology analyzer ADVIA 120 for cerebrospinal fluid analysis and usage of unique hemolysis reagent. *Int J Lab Hematol.* 2013.
3. De Smet D, Van Moer G, Martens GA, Nanos N, Smet L, Jochmans K, et al. Use of the cell-dyn sapphire hematology analyzer for automated counting of blood cells in body fluids. *Am J Clin Pathol.* 2010;133:291-9.
4. Brown W, Keeney M, Chin-Yee I, Johnson K, Lantis K, Finn W, et al. Validation of body fluid analysis on the coulter LH 750. *Lab Hematol.* 2003;9:155-9.
5. Walker TJ, Nelson LD, Dunphy BW, Anderson DM, Kickler TS. Comparative evaluation of the iris iQ200 body fluid module with manual hemacytometer count. *Am J Clin Pathol.* 2009;131:333-8.
6. Li A, Gronlund E, Brattsand G. Automated white blood cell counts in cerebrospinal fluid using the body fluid mode on the platform Sysmex XE-5000. *Scand J Clin Lab Invest.* 2014;74:673-80.
7. Barnes PW, Esby CS, Shimer G. An evaluation of the utility of performing body fluid counts on the coulter LH 750. *Lab Hematol.* 2004;10:127-31.
8. Sandhaus LM, Ciarlina P, Kidric D, Dillman C, O'Riordan M. Automated cerebrospinal fluid cell counts using the sysmex XE-5000: Is it time for new reference ranges? *Am J Clin Pathol.* 2010;134:734-8.
9. de Jonge R, Brouwer R, Smit M, de Frankrijker-Merkestijn M, Dolhain RJEM, Hazes JMW, et al. Automated counting of white blood cells in synovial fluid. *Rheumatology (UK).* 2004;43:170-3.
10. Boer K, Deufel T, Reinhoefer M. Evaluation of the XE-5000 for the automated analysis of blood cells in cerebrospinal fluid. *Clin Biochem.* 2009;42:684-91.
11. Szamosi D, Bautista JM, Cornbleet J, Glasser L, Rothe G, Sandhaus L, et al. Clinical and Laboratory Standards Institute. *Body fluid Analysis for Cellular Composition; Approved guideline. CLSI document H56-A.* 2006;26.
12. Briggs C. Evaluation of hematology automated In: Kottke-Marchant K, Davis B, editors. *Laboratory Hematology Practice: Blackwell Publishing Ltd; 2012.* p. 96 -102.
13. Buoro S, Apassiti Esposito S, Alessio M, Crippa A, Ottomano C, Lippi G. Automated Cerebrospinal Fluid Cell Counts Using the New Body Fluid Mode of Sysmex UF-1000i. *J Clin Lab Anal.* 2015.
14. Lippi G, Cattabiani C, Benegiamo A, Gennari D, Pavesi F, Caleffi A, et al. Evaluation of the Fully Automated Hematological Analyzer Sysmex XE-5000 for Flow Cytometric Analysis of Peritoneal Fluid. *Journal of Laboratory Automation.* 2013;18:240-4.
15. De Jonge R, Brouwer R, De Graaf MT, Luitwieler RL, Fleming C, De Frankrijker-Merkestijn M, et al. Evaluation of the new body fluid mode on the Sysmex XE-5000 for counting leukocytes and erythrocytes in cerebrospinal fluid and other body fluids. *Clin Chem Lab Med.* 2010;48:665-75.
16. Glasser L, Murphy CA, Machan JT. The clinical reliability of automated cerebrospinal fluid cell counts on the beckman-coulter LH750 and iris iQ200. *Am J Clin Pathol.* 2009;131:58-63.
17. Szamosi D, Bautista J, Cornbleet P, Glasser L, Rothe G, Sandhaus L, et al. Body fluid analysis for cellular composition; Approved guideline. H56-A. 2006.
18. Bremell D, Mattsson N, Wallin F, Henriksson J, Wall M, Blennow K, et al. Automated cerebrospinal fluid cell count - New reference ranges and evaluation of its clinical use in central nervous system infections. *Clin Biochem.* 2014;47:25-30.

19. Paris A, Nhan T, Cornet E, Perol JP, Malet M, Troussard X. Performance evaluation of the body fluid mode on the platform Sysmex XE-5000 series automated hematology analyzer. *Int J Lab Hematol.* 2010;32:539-47.
20. Zur B, Eichhorn L, Albers E, Stoffel-Wagner B. Evaluation of 2 hematology analyzers in body fluid mode versus flow cytometry immunophenotyping of mainly neurosurgical cerebrospinal fluid samples. *J Neurolog Surg Part A Cent Eur Neurosurg.* 2012;73:93-8.
21. Perne A, Hainfellner JA, Womastek I, Haushofer A, Szekeres T, Schwarzingler I. Performance evaluation of the sysmex XE-5000 hematology analyzer for white blood cell analysis in cerebrospinal fluid. *Arch Pathol Lab Med.* 2012;136:194-8.
22. Zimmermann M, Ruprecht K, Kainzinger F, Heppner FL, Weimann A. Automated vs. manual cerebrospinal fluid cell counts: A work and cost analysis comparing the Sysmex XE-5000 and the Fuchs-Rosenthal manual counting chamber. *Int J Lab Hematol.* 2011;33:629-37.
23. Williams JE, Walters J, Kabb K. Gaining efficiency in the laboratory - automated body fluid cell counts: Evaluation of the body fluid application on the sysmex XE-5000 hematology analyzer. *Lab Med.* 2011;42:395-401.
24. Noguera Velasco JA, Martinez Villanueva M, Martinez Hernandez P. About the clinical performance of the sysmex XE-500 for automated cerebrospinal fluid cell counts. *Am J Clin Pathol.* 2011;136:156-7.
25. Seo JY, Lee ST, Kim SH. Performance evaluation of the new hematology analyzer Sysmex XN-series. *Int J Lab Hematol.* 2015;37:155-64.
26. Dux R, Kindler-Rohrborn A, Annas M, Faustmann P, Lennartz K, Zimmermann CW. A standardized protocol flow cytometric analysis of cells isolated from cerebrospinal fluid. *J NEUROL SCI.* 1994;121:74-8.
27. Steele RW, Marmer DJ, O'Brien MD, Tyson ST, Steele CR. Leukocyte survival in cerebrospinal fluid. *J Clin Microbiol.* 1986;23:965-6.
28. Aune MW, Becker JL, Brugnara C, Canfield W, Dorfman DM, Fiehn W, et al. Automated Flow Cytometric Analysis of Blood Cells in Cerebrospinal Fluid: Analytic Performance. *Am J Clin Pathol.* 2004;121:690-700.
29. Rumke CL. The imprecision of the ratio of two percentages observed in differential white blood cell counts: a warning. *Blood Cells.* 1985;11:137-40.
30. Yang D, Zhou Y, Chen B. Performance evaluation and result comparison of the automated hematology analyzers Abbott CD 3700, Sysmex XE 2100 and Coulter LH 750 for cell counts in serous fluids. *Clin Chim Acta.* 2013;419:113-8.
31. Mazor SS, McNulty JE, Roosevelt GE. Interpretation of Traumatic Lumbar Punctures: Who Can Go Home? *Pediatrics.* 2003;111:525-8.
32. Sandhaus LM. Body fluid cell counts by automated methods. *Clin Lab Med.* 2015;35:93-103.
33. Zimmermann M, Otto C, Gonzalez JB, Prokop S, Ruprecht K. Cellular origin and diagnostic significance of high-fluorescent cells in cerebrospinal fluid detected by the XE-5000 hematology analyzer. *Int J Lab Hematol.* 2013;35:580-8.
34. Cho YU, Chi HS, Park SH, Jang S, Kim YJ, Park CJ. Body fluid cellular analysis using the Sysmex XN-2000 automatic hematology analyzer: focusing on malignant samples. *Int J Lab Hematol.* 2015;37:346-56.
35. Labaere D, Boeckx N, Geerts I, Moens M, Van den Driessche M. Detection of malignant cells in serous body fluids by counting high-fluorescent cells on the Sysmex XN-2000 hematology analyzer. *Int J Lab Hematol.* 2015.

36. Irwig L, Bossuyt P, Glasziou P, Gatsonis C, Lijmer J. Designing studies to ensure that estimates of test accuracy are transferable. *BMJ*. 2002;324:669-71.
37. Flanigan MJ, Freeman RM, Lim VS. Cellular response to peritonitis among peritoneal dialysis patients. *AM J KIDNEY DIS*. 1985;6:420-4.
38. Chow KM, Szeto CC, Cheung KK, Leung CB, Wong SS, Law MC, et al. Predictive value of dialysate cell counts in peritonitis complicating peritoneal dialysis. *Clin J Am Soc Nephrol*. 2006;1:768-73.
39. Omuse G, Makau P. Interference of cerebrospinal fluid white blood cell counts performed on the Sysmex XT-4000i by yeast and bacteria. *Int J Lab Hematol*. 2013;35:e5-e7.
40. Shen PUF, Blair JL. Cholesterol crystals causing falsely elevated automated cell count. *Am J Clin Pathol*. 2006;125:358-63.
41. Fleming C, Brouwer R, van Alphen A, Lindemans J, de Jonge R. UF-1000i: validation of the body fluid mode for counting cells in body fluids. *Clin Chem Lab Med*. 2014;52:1781-90.
42. Riedl JA, Dinkelaar RB, Van Gelder W. Automated morphological analysis of cells in body fluids by the digital microscopy system DM96. *J Clin Pathol*. 2010;63:538-43.
43. Plebani M. Clinical laboratories: production industry or medical services? *Clin Chem Lab Med*. 2015;53:995-1004.

Chapter 10

Summary

Samenvatting

SUMMARY

The analysis of body fluids (BFs) including cerebrospinal fluid (CSF), serous fluids, and synovial fluids, provides essential information in the care and treatment of patients. Typically, such a work-up consist of a vast amount of studies from biochemical to microbial to cellular analysis. Of these, the total white blood cell (WBC) and WBC differential count have repeatedly been found useful in the differential diagnosis as well as for monitoring the progression of a disease and its responsiveness to treatment. For example, it is well known that elevated WBCs in CSF can be observed in meningitis and other neurological diseases. In synovial fluid, the WBC and polymorphonuclear (PMN) count play a central role in the decision-making process for placing a fluid into one of several categories, including inflamed or infectious, as part of the diagnosis of joint diseases. In ascites, the PMN count remains the best parameter for making a presumptive diagnosis of spontaneous bacterial peritonitis. Similarly, neutrophilia in pleural effusions is a valid marker for acute inflammation of the pleural space, as can be seen in pneumonia whereas, a predominance of lymphocytes is often seen in tuberculosis and malignancy. Continuous ambulatory peritoneal dialysis (CAPD) is a widely accepted treatment for end-stage renal disease, and peritonitis is a frequent complication thereof. Traditional teaching holds that peritonitis should be highly suspected when WBCs are increased along with a predominance of PMNs.

In most laboratories, WBCs are still counted manually by using the hemocytometer and/or cytocentrifugation, and together, this is considered the gold standard. However, this technique is hampered by the amount of time necessary to obtain results, their dependence on the availability of skilled personnel, and the inherent imprecision of the process. In view of these limitations, automated analyzers, initially developed for whole blood and urine samples, have become popular for first line screening.

Sysmex Corporation is currently the market leader in Europe for BFs analysis. Over the past years, Sysmex launched a number of automated analyzers including the XE-5000, the XN-Series and the UF-1000i. Each contains a unique BF mode using flow cytometry and impedance technique for facilitating the counting of blood cells. Generally speaking, the use of automated cell counters in BF analysis has led to major improvements, and they have gained increasing, though still limited acceptance as an alternative to manual microscopy. The studies in this thesis mainly aimed to investigate whether the currently available automated analyzers by Sysmex are suitable for replacing manual microscopy.

In many inflammatory diseases, the cellular components in BFs are increased, rendering essential diagnostic information. **In chapter 2**, we reviewed the literature on the clinical

significance of the total WBC and WBC differential count in non-malignant BFs during inflammatory diseases, and we investigated and summarized the advantages and disadvantages of the contemporary methods used for measuring these cells. Currently, a discussion remains about which diagnostic parameter (WBC or PMN) best predicts peritonitis in patients treated with peritoneal dialysate fluids. Therefore, in **chapter 3**, we retrospectively evaluated the diagnostic accuracy of the automated neutrophilic PMN and WBC count in 69 effluent dialysates suspected of peritonitis. The neutrophilic count prevailed as the better predictive marker over the WBC count to differentiate infectious from non-infectious peritonitis, especially in culture negative samples.

The following chapters cover the analytic performance of Sysmex analyzers. The XE-5000 BF mode is Sysmex's first hematology analyzer to contain a dedicated BF mode. This analyzer was studied extensively in the past, and results from our laboratory and others showed persistently high imprecision in the low concentration range along with an overestimation of PMNs in CSF samples. Consequently, Sysmex developed new software to correct this problem. In **chapter 4**, we evaluated the new software in 138 randomly collected CSF samples and compared the results with the current software on the XE-5000. This study demonstrated that agreements for counting WBCs and PMNs in CSF were better with the new software compared to the old software. However, despite the improvements, a significant positive bias remained for PMNs on the new software, and no specific causes were identified.

In 2011, Sysmex released their latest hematology analyzer, the XN-Series, which contains an improved BF mode compared to the XE-5000 BF mode. In **chapter 5**, we evaluated the BF mode on the XN-1000 for counting RBCs, WBCs and WBC differential in 73 CSF, 48 CAPD and 66 serous fluids, and compared them with manual counts. Our results show good agreement between methods for all fluids with an exception to PMNs in CSF samples. The XN-1000 systematically counted more PMNs compared to manual microscopy. By using BF XN-check, the lower limit of quantitation (LoQ) for WBCs was defined at $5 \times 10^6/L$. Taking this into account, the XN-1000 BF mode is a suitable tool for fast and accurate quantification of RBCs, WBCs and WBC differential.

Because of the persistent demand for low imprecision in the low concentration range and high accuracy in CSF samples, Sysmex developed a new high sensitive analysis mode (hsA) on the XN-Series, specifically for counting cells in CSF samples. The XN-hsA mode provides a 4-part differential count, uses flow cytometry technique for counting RBCs, and it has new gating algorithms to detect and flag abnormal cells in CSF samples. In **chapter 6**, we evaluated the XN-hsA research mode by measuring 248 CSF samples to determine whether it is fit for clinical use. In addition, we evaluated the linearity, detection limits, precision and carryover of this analysis mode. Using the XN-hsA mode, the

LoQ for RBCs and WBCs was $10 \times 10^6/L$ and $2 \times 10^6/L$, respectively. A comparison of the two methods revealed good agreement for WBCs, RBCs, lymphocytes, neutrophils and monocytes, even in the very low concentration ranges.

Optical urine sediment analyzers are also being used for BFs analysis, although they are not designed and certified for this application. The Sysmex UF-1000i analyzer contains a dedicated BF mode. The BF mode contains new gating and modified algorithms to enhance cell counting and decrease interference from cell fragments. In **chapter 7**, we collected 154 serous fluids, and compared the results of the UF-1000i BF mode with the manual method and the XN-1000 BF mode. Overall results were better when the UF-1000i was compared with the XN-1000 than with the counting chamber. Perhaps, this can be ascribed to the nature of these fluids. Serous fluids frequently contain large cells, which may have been misclassified as WBCs. Comparison between the UF-1000i and both comparison methods showed good agreement for RBCs. The LoQ was defined at 9×10^6 WBC/L and at 25×10^6 RBC/L.

Disadvantages of automated analyzers include potential interfering factors from non-cellular particles including liposomal particles. Patients with neoplastic meningitis are treated with the chemotherapeutic drug DepoCyt (cytarabine encapsulated in liposomal particles). Interferences of DepoCyt are of concern because reporting falsely elevated WBC counts can have potential adverse clinical consequences in patients with neoplastic meningitis. In **chapter 8**, the interference of this drug on the XN-1000 BF mode is studied using current software and an "evaluation software". This software, which is still to be released, was developed by Sysmex for the detection/flagging of liposomal particles. Unlike the current software, the "evaluated software" triggered a battery of warning signs including greying of scattergram, presence of the WBC abnormal scattergram flag, a "corrected WBC" count and asterisks next to WBC counts to alert the user of abnormality; however in the presence of low ($\leq 5 \times 10^6/L$) "WBCs", no alerts were triggered.

In **chapter 9** the results of our studies are discussed and recommendations for future research is given. In conclusion, Sysmex's analyzers are accurate and precise for measuring a variety of BFs, even in the low concentration ranges. These observations brought us to the ultimate question of this thesis whether Sysmex's analyzers can replace manual microscopy to count cells in non-malignant fluids. We conclude that the Sysmex BF mode can replace manual microscopy in first-line screening because of its high sensitivity; however, manual microscopy remains necessary for inspecting samples with abnormal cell clustering ("WBC abnormal scattergram") to identify morphological abnormalities and interfering substances, that might be overlooked by automated analyzers.

SAMENVATTING

Het analyseren van lichaamsvochten, waaronder liquor, pleuravocht, ascitesvocht en synoviaalvocht, levert essentiële informatie op in de zorg voor en behandeling van patiënten. Meestal bestaat zo'n onderzoek uit biochemische, microbiële en cellulaire analyses. De telling van het totale aantal witte bloedcellen (WBC) en de WBC differentiatie zijn herhaaldelijk nuttig gebleken in de differentiële diagnose, als ook voor het monitoren van het verloop van een ziekte en de reactie op de behandeling. Het is bijvoorbeeld algemeen bekend dat een verhoogd aantal WBC's in liquor gevonden wordt bij meningitis en andere neurologische aandoeningen. In synoviaalvocht speelt het aantal WBC's en de polymorphonucleaire cellen (PMN's) een centrale rol in het onderscheid of een gewrichtsaandoening veroorzaakt wordt door een ontsteking of een infectie. In ascites blijft de PMN differentiatie de beste parameter voor het stellen van de vermoedelijke diagnose van een spontane bacteriële peritonitis. Een vergelijkbare situatie doet zich voor in pleuravocht, waarin de aanwezigheid van voornamelijk neutrofielen duidt op een acute ontsteking van de pleurale ruimte, zoals kan worden gezien bij longontsteking, terwijl een verhoogd aantal lymfocyten beter past bij tuberculose of een maligniteit. Continue ambulante peritoneale dialyse (CAPD) is een algemeen aanvaarde behandeling voor nierziekte in het eindstadium en daarbij komt peritonitis vaak als complicatie voor. De klinische indicaties voor het vaststellen van peritonitis zijn verhoogde WBC's in samenhang met een verhoogd aantal PMN's.

In de meeste laboratoria worden WBC's nog steeds handmatig geteld met behulp van een microscoop, een telkamer en/of cytospin, dat in combinatie beschouwd wordt als de gouden standaard. Echter, deze techniek kent een aantal beperkingen, waarvan de inherente onnauwkeurigheid de belangrijkste is. Daarnaast is het een tijdrovende procedure die sterk afhankelijk is van de beschikbaarheid van gekwalificeerd personeel. Met het oog op deze beperkingen worden steeds vaker geautomatiseerde analyzers, oorspronkelijk ontwikkeld voor het meten van bloed- en urinemonsters ingezet voor de eerste screening van lichaamsvochten.

Sysmex Corporation is momenteel de marktleider in Europa voor de geautomatiseerde analyse van lichaamsvochten. In de loop van de afgelopen jaren lanceerde Sysmex een aantal geautomatiseerde analyzers, waaronder de XE-5000, de XN-serie en de UF-1000i, met ieder een unieke "BF mode" (*Body Fluid mode*), die bloedcellen telt door gebruik te maken van flowcytometrie en impedantie techniek. In algemene zin heeft het gebruik van geautomatiseerde celtellers voor lichaamsvochten geleid tot grote verbeteringen en worden zij in toenemende mate geaccepteerd als alternatief voor manuele microscopie. Het onderzoek in dit proefschrift is voornamelijk gericht op de vraag of de mo-

menteel beschikbare geautomatiseerde analyzers van Sysmex geschikt zijn om manuele microscopie volledig te vervangen.

In veel ontstekingsziekten zijn de cellulaire componenten in lichaamsvochten verhoogd aanwezig, hetgeen essentiële diagnostische informatie oplevert. In **hoofdstuk 2** wordt een literatuurstudie naar de klinische betekenis van het totale aantal WBC en de differentiatie daarvan in niet-maligne lichaamsvochten tijdens ontstekingsziekten beschreven. Daarnaast worden de voor- en nadelen van de hedendaagse methoden om deze cellen te meten samengevat. Op dit moment bestaat er nog steeds een discussie over welke diagnostische parameter (WBC of PMN) de aanwezigheid van peritonitis in patiënten die behandeld worden met peritoneale dialyse, het beste voorspelt. Daarom hebben we retrospectief onderzoek gedaan naar de diagnostische nauwkeurigheid van de geautomatiseerde neutrofiële PMN en WBC telling in 69 dialysemonsters verdacht voor peritonitis, en dit is beschreven in **hoofdstuk 3**. De neutrofielen telling blijkt een betere parameter te zijn dan de WBC telling om onderscheid te maken tussen infectieuze peritonitis en niet-infectieuze peritonitis, vooral in monsters waarin de kweek negatief is.

De volgende hoofdstukken behandelen de analytische prestaties van de verschillende Sysmex analyzers. De XE-5000 is de eerste hematologie analyzer van Sysmex die een BF-mode bevatte. Deze analyzer is in het verleden uitgebreid bestudeerd door ons laboratorium en door anderen, waarbij bleek dat in liquor de onnauwkeurigheid in het lage concentratiebereik structureel hoog was en de PMN overschat werd. Om dit probleem op te lossen heeft Sysmex nieuwe software ontwikkeld. In **hoofdstuk 4** hebben we die nieuwe software geëvalueerd door de resultaten van 138 willekeurige liquor monsters te vergelijken met de eerdere software op de XE-5000. Deze studie toont aan dat tellingen van WBC's en PMN's met de nieuwe software beter overeenkomen met de handmatige microscopische tellingen dan met de oude software. Echter, ondanks de verbetering, blijft een aanzienlijke positieve bias voor PMN's bestaan, zonder dat hiervoor een specifieke oorzaak kon worden gevonden.

In 2011 bracht Sysmex hun nieuwste hematologie analyzer op de markt, de XN-serie, die een verbeterde BF-mode bevat ten opzichte van de XE-5000 BF-mode. In **hoofdstuk 5** is de BF-mode op de XN-1000 onderzocht door de telling van RBC's, WBC's en WBC differentiatie in 73 liquoren, 48 CAPD vloeistoffen en 66 sereuze vochten te vergelijken met manuele microscopie. Onze resultaten laten een goede overeenkomst zien tussen de methoden voor alle vloeistoffen, met uitzondering van PMN's in liquoren. De XN-1000 telt systematisch meer PMN's ten opzichte van manuele microscopie. Met behulp van XN BF-controlemateriaal werd de "lower limit of quantitation" (LoQ) voor WBC's vastgesteld op $5 \times 10^6/L$. Wanneer dit in beschouwing wordt genomen, is de BF-mode

van de XN-1000 geschikt voor de snelle en nauwkeurige meting van RBC's, WBC's en WBC differentiatie.

Vanwege de voortdurende vraag naar hoge nauwkeurigheid in het lage concentratiegebied in liquoren, ontwikkelde Sysmex een nieuwe "high sensitive analysis mode" (hsA) op de XN-serie, speciaal voor het tellen van cellen in liquoren. De XN-hsA mode biedt een 4-delige WBC differentiële telling, gebruikt een flowcytometrische techniek voor het tellen van RBC's en heeft nieuwe algoritmes voor het opsporen en signaleren van abnormale cellen in liquoren. In **hoofdstuk 6** hebben we de XN-hsA mode geëvalueerd door het meten van 248 liquoren om te bepalen of deze bruikbaar is voor klinisch toepassing. Bovendien hebben we de lineariteit, detectiegrenzen, precisie en carry-over geëvalueerd. De LoQ van de XN-hsA mode voor RBC's en WBC's is respectievelijk $10 \times 10^6/L$ en $2 \times 10^6/L$. De vergelijking tussen de geautomatiseerde en de manuele methode toont goede overeenkomst voor WBC's, RBC's, lymfocyten, neutrofielen en monocytten, zelfs in het zeer lage concentratiebereik. De lineariteit was goed en bestreek een breed en klinisch relevant gebied voor alle geëvalueerde parameters.

Ook optische urineanalyzers worden gebruikt voor de analyse van lichaamsvochten, hoewel zij niet zijn ontworpen en gecertificeerd voor deze toepassing. De Sysmex UF-1000i analyzer bevat een specifieke BF-mode. Deze BF-mode bevat nieuwe algoritmes om het tellen van cellen te verbeteren en de interferentie van celfragmenten te verminderen. In **hoofdstuk 7**, verzamelden we 154 sereuze vochten die gemeten zijn met de UF-1000i BF-mode en vergeleken met manuele microscopie en de XN-1000 BF-mode. Resultaten waren over het algemeen beter vergelijkbaar tussen de UF-1000i en de XN-1000 dan met gebruik van de telkamer. Misschien kan dit worden toegeschreven aan de aard van deze vochten. Sereuze vochten bevatten vaak grote cellen, die verkeerd geclassificeerd kunnen worden als WBC's. Verder toonde de vergelijking tussen de UF-1000i en de beide andere methoden goede overeenkomst voor het tellen van RBC's. De LoQ's zijn vastgesteld op 9×10^6 WBC/L en 25×10^6 RBC/L.

Een nadeel van geautomatiseerde analyzers is mogelijke interferentie door niet-cellulaire deeltjes als liposomen. Patiënten met neoplastische meningitis worden behandeld met het chemotherapeutische middel DepoCyt (cytarabine ingekapseld in liposomen). Interferentie door DepoCyt deeltjes is zorgwekkend, omdat het rapporteren van vals verhoogde WBC's negatieve klinische consequenties kan hebben voor deze patiënten. In **hoofdstuk 8** is de interferentie van dit medicijn op de XN-1000 BF mode onderzocht met de huidige software en de "evaluatiesoftware". Deze nieuwe software, die nog niet is vrijgegeven, werd ontwikkeld door Sysmex voor de detectie van lipidendeeltjes. In tegenstelling tot de huidige software, kan de "evaluatiesoftware" een serie van waarschuwingssignalen geven om de gebruiker te wijzen op de aanwezigheid van liposomen. Dit blijkt echter niet te gebeuren bij lage ($\leq 5 \times 10^6$ cells/L) tellingen.

In **hoofdstuk 9** bespreken we de resultaten van onze studies en geven we aanbevelingen voor toekomstige onderzoek. Samenvattend zijn Sysmex analyzers nauwkeurig en precies voor het meten van een verscheidenheid aan lichaamsvochten, zelfs in het lage concentratiegebied. Deze observaties leidden tot de uiteindelijke vraag of de nieuwste Sysmex analyzers manuele microscopie volledig kunnen vervangen voor het tellen van cellen in niet-maligne vochten. Hoewel we concluderen dat de Sysmex BF mode uitstekend kan dienen als eerstelijns screening door haar hoge gevoeligheid, blijft manuele microscopie desondanks nog steeds nodig om morfologische afwijkingen, zoals bij maligne cellen, en interferenties, door andersoortige deeltjes waar te nemen, die door automatische analyzers soms over het hoofd worden gezien.

Chapter 11

Addendum

LIST OF ABBREVIATIONS

BF	Body Fluid
CAPD	Continuous Ambulatory Peritoneal Dialysis
CSF	Cerebrospinal fluid
CV	Coefficient of Variation
DIFF	Differential
EDTA	Ethylene-diamine-tetraacetic acid
EO	Eosinophils
HF-BF	High Fluorescent Body Fluid cells
LIS	Laboratory Information System
Lymph	Lymphocytes
MN	Mononuclear cells
Mono	Monocytes
Neutro	Neutrophils
PD	Peritoneal Dialysis
PMN	Polymorphonuclear cells
RBC	Red Blood Cells
WB	Whole blood
WBC	White Blood Cells
SAH	Subarachnoid hemorrhage
SBP	Spontaneous Bacterial Peritonitis
SIS	Sysmex Information System

LIST OF PUBLICATIONS

Fleming C, Russcher H, Lindemans J, de Jonge R. Clinical relevance and contemporary methods for counting blood cells in body fluids suspected of inflammatory disease. Clin Chem Lab Med. 2015; 53:1689-706

Fleming C, Brouwer R, Lindemans J, de Jonge R. Validation of the body fluid module on the new Sysmex XN-1000 for counting blood cells in cerebrospinal fluid and other body fluids. Clin Chem Lab Med. 2012;50:1791-8

Fleming C, de Bruin M, Russcher H, Lindemans J, de Jonge R. Liposomal interference on Sysmex XN-Series body fluid mode. Clin Chem Lab Med. 2016;54: e19 – e23

Fleming C, Brouwer R, Lindemans J, de Jonge R. Improved software on the Sysmex XE-5000 BF mode for counting leukocytes in cerebrospinal fluid. Clin Chem Lab Med. 2013;51: e61-3

de Jonge R, Brouwer R, de Graaf MT, Luitwieler RL, **Fleming C**, de Frankrijker-Merkestijn M, Sillevius Smitt PA, Boonstra JG, Lindemans J. Evaluation of the new body fluid mode on the Sysmex XE-5000 for counting leukocytes and erythrocytes in cerebrospinal fluid and other body fluids. Clin Chem Lab Med. 2010;48:665-75

van Alphen A, **Fleming C**, Wabbijn M, de Jonge R, Fieren M. Role of automated peritoneal leukocyte differential counting in diagnostic accuracy in peritoneal dialysis associated peritonitis. Under preparation

Fleming C, Brouwer R, Russcher H, Lindemans J, de Jonge R. Sysmex XN-1000 high sensitive analysis (hsA) mode for counting and differentiating cells in Cerebrospinal fluid. Am J Clin Pathol. 2016;0:1-9

Fleming C, Brouwer R, van Alphen A, Lindemans J, de Jonge R. UF-1000i: validation of the body fluid mode for counting cells in body fluids. Clin Chem Lab Med. 2014;52:1781-90

PHD PORTFOLIO

Name PhD Student:	Chérina K. A. Fleming
Erasmus MC Department:	Clinical Chemistry
Research School:	Molecular Medicine
PhD period:	November 2011 – November 2014
Promotors:	Prof. dr. J. Lindemans Prof. dr. R. de Jonge
Copromotor:	Dr. H. Russcher

		Workload	
PhD training		Year	ECTS
General courses	Biomedical English Writing and Communication	2012	4.0
	Biostatistics	2012	5.7
	Presentation skills	2012	1.0
	Diagnostic Research	2013	0.9
	Research Integrity	2014	0.3
	Systematic Literature Search and Endnote	2014	
National conferences	Nederlandse Vereniging Klinische Chemie (NVKC), Netherlands (poster presentation)	2012	0.3
	Molecular Medicine (MolMed) PhD days, Rotterdam, Netherlands (poster presentations)	2012/ 2013	0.6
	Post academische onderwijs voor Klinisch Chemici (PAOKC), Utrecht, Netherlands (oral presentation)	2014	0.4
	Sysmex Expert Class Body fluids, Etten-Leur, Netherlands (oral presentation)	2014	0.4
International conferences	International Federation of Clinical Chemistry, Berlin, Germany (poster presentation)	2011 2012	0.3 0.6
	International Standardization and Laboratory Hematology, Nice, France (2 posters presentations)	2012	1.0
	Sysmex User Symposium, Northamptonshire, United Kingdom (oral presentation)		
	Sysmex European Haematology Symposium, Lyon, France (oral presentation)	2012	1.0
	EuroMedLab, Milan, Italy (oral presentation)	2013	1.0
	International Standardization and Laboratory Hematology, The Hague, Netherlands (poster presentations)	2014	0.3
Other Presentations	Several oral presentations during various research meetings and journal clubs at Clinical Chemistry department	2011- 2014	4.0
Teaching Activities	Supervising bachelor student (2 nd year)	2012	0.3
	Supervising MLO student (4 th year)	2013	0.3
	Supervising MLO student final report	2013	0.3

ECTS = European Credit Transfer and Accumulation System
1 ECTS = 28 hours

ACKNOWLEDGEMENTS

I could not have completed this thesis without the help of a lot of people; therefore, I want to thank all those who have, in one way or another, helped me in making this journey a success. In particular, I want to mention a few.

Professor J. Lindemans, beste Jan, dank voor jouw vertrouwen in mij, de mogelijkheid om dit promotieonderzoek te doen en jouw zeer kritische blik op al mijn stukken. Het is voor mij een eer dat je mijn promotor wilde zijn. Bedankt voor de prettige samenwerking.

Professor R. de Jonge, beste Robert, als student tijdens mijn analisten opleiding leerde ik je kennen, waarbij ik je al snel bewonderde om je enorme toewijding voor het vak klinische chemie en het onderzoek. Je hebt mij voor beide vakgebieden enthousiast gemaakt, waarbij jij ook degene bent geweest die mij de kans heeft gegeven beide uit te voeren. Dankzij jouw expertise en kritische blik zijn bijna alle hoofdstukken in dit proefschrift tot stand gekomen en gepubliceerd in mooie tijdschriften. Kan jij je bovenstaand moment nog herinneren? Het was tijdens het congres in Milan, toen de PC crashte aan het begin van mijn presentatie en ik een tijdje verder moest zonder PowerPoint.



Je kwam na afloop direct naar mij toe, om mij gerust te stellen en liet mij weten dat je heel trots op mij was. Dit moment zal ik nooit vergeten! Ik heb veel van jou geleerd maar ook ontzettend veel met je gelachen de afgelopen jaren. Robert, jij was niet alleen mijn promotor, maar ook mijn copromotor, leraar en mijn voorbeeld. Jij hebt een manier van werken waarmee je iets kan bereiken en ik ben blij dat ik veel van heb mogen leren. Ik ben trots dat jij mijn promotor bent en dat ik je eerste promovenda ben waarbij jij in toga staat. Ik vond het erg prettig om met je samen te werken en hoop je nog te zien op mijn kleine maar fijne eiland St. Maarten, bedankt voor alles!

Dr. H. Russcher, beste Henk, halverwege deze promotietraject werd je mijn copromotor. Dank voor je constructieve bijdrage, suggesties en ondersteuning.

De leden van de kleine commissie, prof. dr. A. Verbon, prof. dr. P.A.E. Sillevius Smitt en prof. dr. J. Penders wil ik hartelijk danken voor het positieve beoordelen van mijn proefschrift en jullie bereidheid om plaats te nemen in de kleine commissie.

Daarnaast wil ik de leden van de grote commissie, prof. dr. H. Metselaar, prof. dr. G. Sturk en prof. dr. R. de Man, hartelijk danken voor het plaats nemen in deze commissie.

Lieve Rob, bedankt voor je expertise en enthousiasme op het gebied van diffen in body fluids, maar ook bedankt voor alle Nederlandse uitdrukkingen die je mij hebt geleerd. Ik kan je niet genoeg bedanken voor je hulp, wijze woorden, goede humor en positiviteit. Ik vind je echt een topper!

Graag wil ik alle medewerkers van de afdeling Klinisch Chemie Centrum en Daniel locatie bedanken voor de fijne samenwerking en de belangstelling voor mijn onderzoek. Hierbij wil ik specifiek de analisten van de frontservice en hematologie bedanken voor alle telefoontjes met de boodschap “er is een vocht voor jou binnen”. Met name Evert en Magda, hebben heel erg geholpen bij het in goede banen leiden van de logistiek rondom de vochten. Jullie dank voor de prettige samenwerking en de ondersteuning bij het onderzoek.

Mijn opleiders, Professor Y. de Rijke, Dr. J. Hens en Dr. G. Lansbergen, beste Yolanda, Jacques en Gideon, hartelijk dank voor de aanmoediging en de ruimte die jullie mij gaven om dit proefschrift af te ronden tijdens de opleiding.

Lieve Gigi, collega/vriendin. Jij weet altijd leven in de brouwerij te brengen. Kan jij je nog herinneren toen we werden betrappt door Ron op onze “danza kuduro” performance in de oudbouw? Jij bent echt geweldig! Bedankt voor je steun, gezelligheid en natuurlijk al het lekkere eten – Mi stimabo!

Bertrand, Samira, Pamela, Evert, Pieter, Martin en Marianne. Bedankt voor de gezellige tijd tijdens de uitjes en borrels. Het was geweldig en zal me altijd bijblijven! Bertrand, bedankt voor de grappige discussies/opmerkingen de afgelopen jaren, ik heb enorm met je gelachen. Marianne, bedankt voor de lekkere “mince pies” en ook Samira bedankt voor de Marokkaanse pannenkoeken!

I thank my fellow PhD colleagues Pooja, Maja and Ethan (former). The times we spent together were priceless. Ethan, I thank you for your stimulating discussions, good advice and always supplying me with your wife’s delicious food and good “old” Dutch cheese. Maja, thanks for all the interesting conversations, all your silly moments, and not forgetting all your un-ripe fruits! Pooja, we began our PhD as colleagues, and we ended as friends. After our first dinner at “Luciano’s”, it became evident that it would not be our last. In fact, Luciano’s became our favorite restaurant. I could always count on you to make me laugh after a long day, and keep my company on weekends while writing the last chapters of my thesis. You are truly a wonderful person and I’m glad I had you to

share both the ups and the downs of my PhD. Poo, I hope that I'm around to see you receive that engraved medal from the Rotterdam marathon! Both you and Maja will be finishing your PhD shortly and I wish you both the best of luck!

Dear Dr. Jo Linssen, Hans Kalkman and Nagai Takaaki, I thank you for your fruitful collaboration over the years. I thank you, not only for the continuous discussions, but also in helping me to improve my knowledge in the measurement of blood cells on Sysmex machines. Hans, I could always count on you for your prompt and detailed responses to my mails. Furthermore, I am still amazed of how organized your files were, and your ability to find documents so quickly, even when they were from way back when. Jo, I thank you for sharing your knowledge and I enjoyed your great sense of humor (100+ slides)! Nagai-san, thank you for the nice collaboration despite the language barrier. Though I did not make it to Japan during the years of my PhD studies, I hope to do so in the near future, and I am looking forward to some Japanese karaoke - Campai! I thank the entire team of Sysmex Europe/Japan who in one way or the other contributed to the work of this thesis.

——- Danke schön- Bedankt- Domo arigato- I Thank You ——-

My friends & family,

"True friendship is not being inseparable, it is being separated and nothing changes"

To my friends Micheilla, Renata, Denira, Kalida, Smally and Shanda, thanks for the continuous support, and memorable moments. From now on, I shall be present at gatherings again!

George, thank you for taking the time out to edit my manuscripts! For someone who has no medical background, you sure have learned a lot in 4 years. I also thank you for your constant reminder that "nothing is too difficult for me".

"Every member of a family has a unique role to play in a family unit"

To tanty Alby, tanty Delly, tanty Borchy, tanty Amalie, tanty Drucella, tanty Sady, tanty Ginette, tanty Joyce, tanty Dianne and all my uncles. Thanks for your unconditional love and the values you have passed on to me. I couldn't have asked for a better family to grow up with! To the rest of my family, too numerous to mention, thank you for your constant prayers and support!

"A cousin is a readymade friend for life"

To my cousins Jacky, Daniela, Tara, Tammy, Tamalia, Sharlyne, Denise, Angie, Sharon, Shaki, Cassie, Junita, Karen, Paula, Derick, Alonso, Jerome and Glen. I am grateful for all your support during the happy but also stressful times. Thanks for all the calls, jokes,

text-messages and inspiring quotes. You guys bring out the best in me, and I love you so much. I certainly could not have done this without your support. And yes my family, I have only 3 more years of being a “student”, I swear!

“Sometimes the most ordinary things can be made extraordinary, just by doing them with the right people”

To my paranymphs Sharlyne and Renata. Sharlyne, I have known and loved you all my life, as we differ only 3 months in age. For me there was no other option than to have you stand next to me on this important day. Renata, we have been friends for more than a decade and still counting. I am blessed to have you as my friend and honored to have you stand next to me today.

“I am who I am because of you”

To Mom and Daddy, the sacrifices you’ve made to help your children be successful in life are indescribable. I cannot thank you enough for all the support and love you have given me. Even though you never understood what I was really doing, you were always proud of me. I (we) couldn’t have asked for better parents – I love you to no end!

“The greatest gifts my parents ever gave me were my brothers”

To my brothers, Berteaux, Corcilio and Kalomo, my guardian angels. Thanks for always believing in me and being my number one supporters. Because I have you guys, I will always have a best friend - I love you!

To Danny, mon chéri doudou, thank you for your support and encouragement throughout my studies. You helped me remain stable in times of instability, and helped guide me in moments of confusion. I could never imagine doing this without you - Je t’aime !

To my grandmother, when I think of love, forgiveness, faith, and family, you come to mind. I loved everything about you; your smile, your laugh, your voice and your kindness. I loved sitting with you, and listening to the memories and life lessons you had to share. I could only hope to become half the amazing person you were. I miss you so much and I love you!



Live, Love, Laugh

Chérina

'16

ABOUT THE AUTHOR

Chérina K. A. Fleming was born on August 13th, 1983 in Marigot, St. Martin F.W.I. After receiving her high school diploma in 2002 from the Milton Peters College in St. Maarten, she relocated to the Netherlands for further education. From 2002 to 2006, she attended the Hogeschool Rotterdam where she obtained her Bachelor's Degree in Applied Sciences. During the third and fourth year of the Bachelor's programme, she did an internship at the department of Clinical Chemistry in the Erasmus Medical Center where her admiration for Clinical Chemistry became pronounced, and this motivated her to pursue a career as a Clinical Chemist. Her journey in becoming a Clinical Chemist began in 2006 when she worked in the department of Clinical Chemistry in Erasmus Medical Center as a research technician on automated cell counters manufactured by Sysmex Corporation for counting cells in body fluids. She completed that assignment in 2007, and in the same year, she continued her education at the Utrecht University where she later received her Master Degree in Biomedical Sciences in 2010. Shortly after, she returned to the Department of Clinical Chemistry at the Erasmus Medical Center where she continued her investigation in automated cell counters for body fluids analysis. Under the supervision of Prof. dr. J. Lindemans, Prof. dr. R. de Jonge and Dr. H. Russcher, and in collaboration with Sysmex Corporation, the groundwork of her PhD research project materialized into the studies described in this thesis. From December 2014, she combined her PhD research work with training to become a clinical chemist in the department of Clinical Chemistry at the Erasmus MC and at the Groene Hart hospital. She is scheduled to complete her training in Clinical Chemistry in 2018.

