AUS DEM

INSTITUT FÜR LABORATORIUMSMEDIZIN DES KLINIKUMS DER

LUDWIG-MAXIMILIANS-UNIVERSITÄT (LMU) MÜNCHEN

DIREKTOR: PROF. DR. MED. DANIEL TEUPSER

BIOMARKER DER INFLAMMATION

KUMULATIVE HABILITATIONSSCHRIFT

FÜR DAS FACH

LABORATORIUMSMEDIZIN DER

MEDIZINISCHEN FAKULTÄT

VORGELEGT VON

DR. MED. MATHIAS BRÜGEL

2020

Inhaltsverzeichnis

1. Einleitung

Die Inflammation stellt einen zentralen Schutzmechanismus bei der Abwehr von Pathogenen sowie bei der Reparatur geschädigter Gewebe dar. Im Rahmen inflammatorischer Prozesse transmigrieren leukozytäre Zellen aus der Blutbahn in die betreffenden Gewebe, ein Prozess der sehr präzise durch Mediatoren wie beispielsweise Zytokine und Eicosanoide kontrolliert wird (1) (2) (3). Diese Mediatoren werden am Ort der Infektion oder der Gewebeschädigung durch gewebsständige Zellen sowie Leukozyten und Endothelzellen gebildet und steuern die weitere inflammatorische Reaktion, indem sie zum einen systemische inflammatorische Prozesse wie beispielsweise die Akut-Phase-Reaktion induzieren, zum anderen aber auch deren Intensität limitieren (4) (5).

Viele der im Rahmen der inflammatorischen Reaktion stattfindenden Prozesse spiegeln sich auch im Blut wider. Daher wird eine differenzierte Darstellung mit Hilfe labordiagnostischer Methoden angewandt, um inflammatorische Erkrankungen beispielsweise mit infektiösem, traumatischem, autoimmunologischem oder malignem Hintergrund zu erkennen und in der Folge eine adäquate Therapieinitialisierung und -Steuerung zu ermöglichen. Hierbei spielen sowohl zytologische Analysen als auch labordiagnostische Bestimmungen von sezernierten Mediatoren oder von im Rahmen der Akut-Phase-Reaktion verstärkt gebildeten Proteinen eine entscheidende Rolle (4) (6).

Die wissenschaftliche Untersuchung der inflammatorischen Reaktion, die Standardisierung der in der Abbildung der inflammatorischen Reaktion angewandten labordiagnostischen Methoden sowie die Identifikation neuer Biomarker ist für die Vertiefung des pathophysiologischen Verständnisses sowie die verbesserte Diagnostik verschiedener inflammatorischer Erkrankungen von großem Interesse.

2. Teilprojekte und Bedeutung der Arbeiten für das Fachgebiet

2.1 Vergleichende Validierung von Hämatologie-Analysensystemen

In der labordiagnostischen Basisversorgung im Umfeld Inflammation kommt der Blutbild-Diagnostik mit Bestimmung der Leukozytenzahl und der qualitativen und quantitativen Differenzierung verschiedener Leukozytensubpopulationen eine entscheidende Rolle zu. Entsprechende Veränderungen erlauben oftmals bereits den Nachweis einer Inflammation sowie erste ätiologische Rückschlüsse. Die Blutbild-Analytik beruht in modernen Laboratorien auf der Anwendung automatisierter Hämatologie-Analysensysteme, die vielfach mit komplexen Messtechnologien wie beispielsweise der Fluoreszenz-basierten Laser-Durchflußzytometrie oder auch mit Digital-Imaging-Modulen ausgestattet sind. Neben der Quantifizierung von Leukozyten und leukozytären Subpopulationen haben diese Systeme weiterhin eine zentrale Bedeutung für die Identifizierung von pathologischen Blutproben mit Nachweis von unreifen Vorläuferzellen des weißen und roten Blutbildes (sog. Flagging), deren Nachweis häufig mit inflammatorischen Erkrankungen assoziiert ist (7) (8). Diese Proben werden dann der Mikroskopie oder weiterführenden Analysemethoden zugeführt.

Basierend auf dem hohen diagnostischen Stellenwert dieser Analysensysteme in der Blutbild-basierten Darstellung eines inflammatorischen Hintergrundes bei verschiedenen Erkrankungen war ein Fokus dieser Habilitationsarbeit die vergleichende Validierung verschiedener in der Laboratoriumsmedizin eingesetzter automatisierter Hämatologie-Analyzer.

2.1.1 Vergleichende Validierung von Laser-Durchflußzytometrie basierten Hämatologie-Analysensystemen

Im Rahmen dieser Arbeit wurden auf der Laser-Durchflußzytometrie basierende, Top-Level-Hämatologie-Analyzer (Abbott Cell-Dyn Sapphire, Beckman DxH 800, Siemens Advia 2120i, Sysmex XE-5000, Sysmex XN-2000) einer Validierung an einem unselektierten Kollektiv von 349 Routine-Proben in einem klinischen Setting unterzogen. Vergleichbare Studien mit Interinstrumenten-Vergleichen, insbesondere bei Anwendung von unselektierten Routine-Kollektiven sowie bei Durchführung im klinischen Setting, sind kaum verfügbar. Der Fokus lag zum einen in der vergleichenden Validierung des automatisierten kleinen Blutbildes, des Differentialblutbildes sowie der Bestimmung von Normoblasten (NRBC) als Vorstufen des roten Blutbildes, zum anderen in der vergleichenden Prüfung der Flagging-Qualität der verschiedenen Systeme.

Wie in Abbildung 1 dargestellt, zeigte der Interinstrumenten-Vergleich der genannten Systeme eine gute Übereinstimmung der Parameter des kleinen Blutbildes inklusive der insbesondere im Rahmen der Entzündungsdiagnostik relevanten Leukozytenzahl. Höhere Variabilitäten waren für die Quantifizierung von Thrombozyten nachweisbar sowie für die im Rahmen der Anämie-Abklärung bedeutsamen Erythrozytenindices (9).

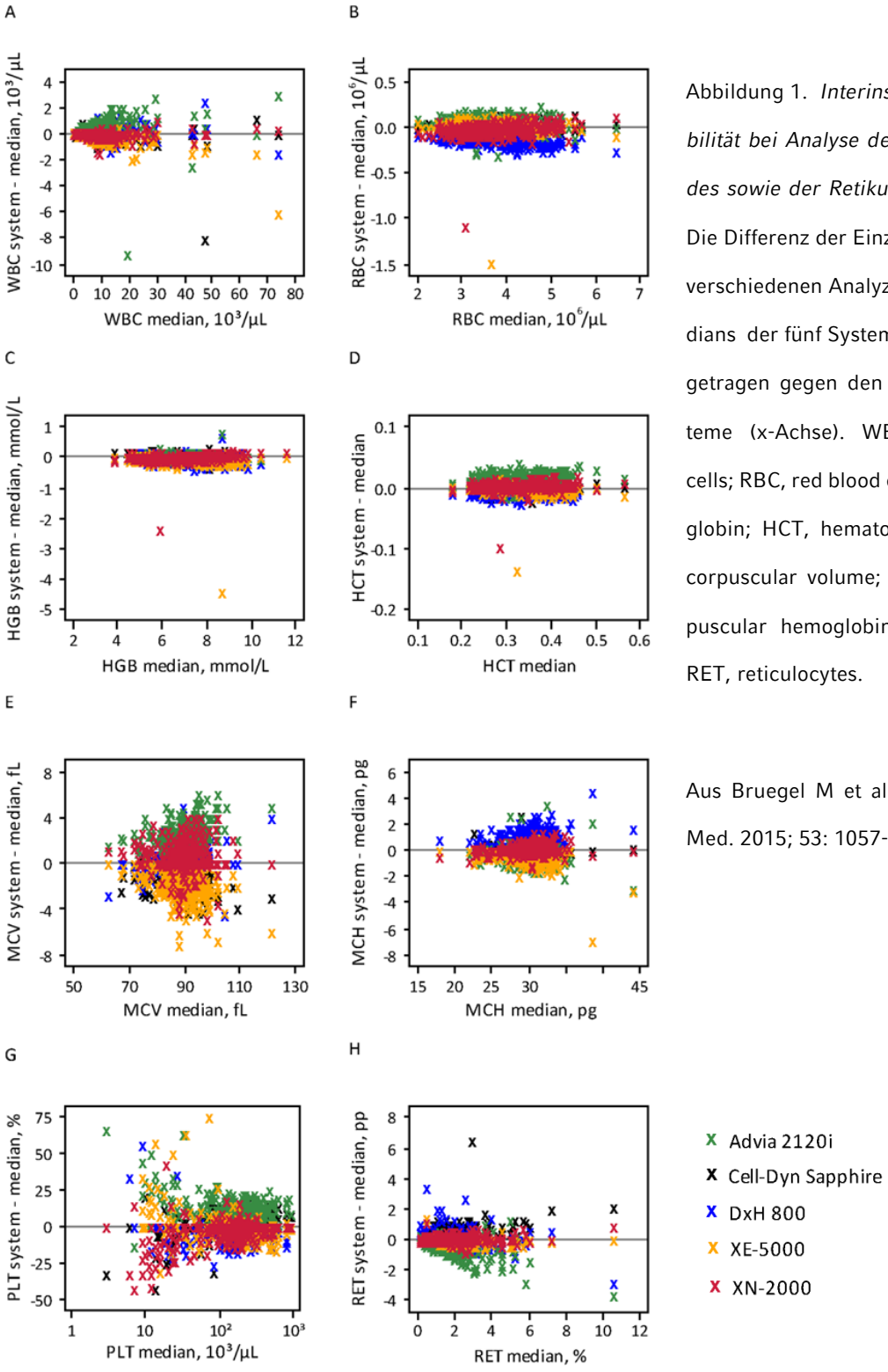


Abbildung 1. *Interinstrumenten-Variabilität bei Analyse des kleinen Blutbildes sowie der Retikulozyten (n = 349).* Die Differenz der Einzelmessungen der verschiedenen Analyzer sowie des Medians der fünf Systeme (y-Achse) aufgetragen gegen den Median der Systeme (x-Achse). WBC, white blood cells; RBC, red blood cells; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; PLT, platelets, RET, reticulocytes.

Aus Bruegel M et al., Clin Chem Lab Med. 2015; 53: 1057-1071

Die Parameter des Differentialblutbildes wurden sowohl im Interinstrumenten-Vergleich als auch gegenüber der manuellen Mikroskopie als Goldstandard verglichen. Auch hier zeigte sich insbesondere in der Zuordnung der im Rahmen der Entzündungsdiagnostik bedeutendsten leukozytären Subpopulationen der neutrophilen und eosinophilen Granulozyten sowie der lymphatischen Zellen eine gute Übereinstimmung (Abb. 2) (9).

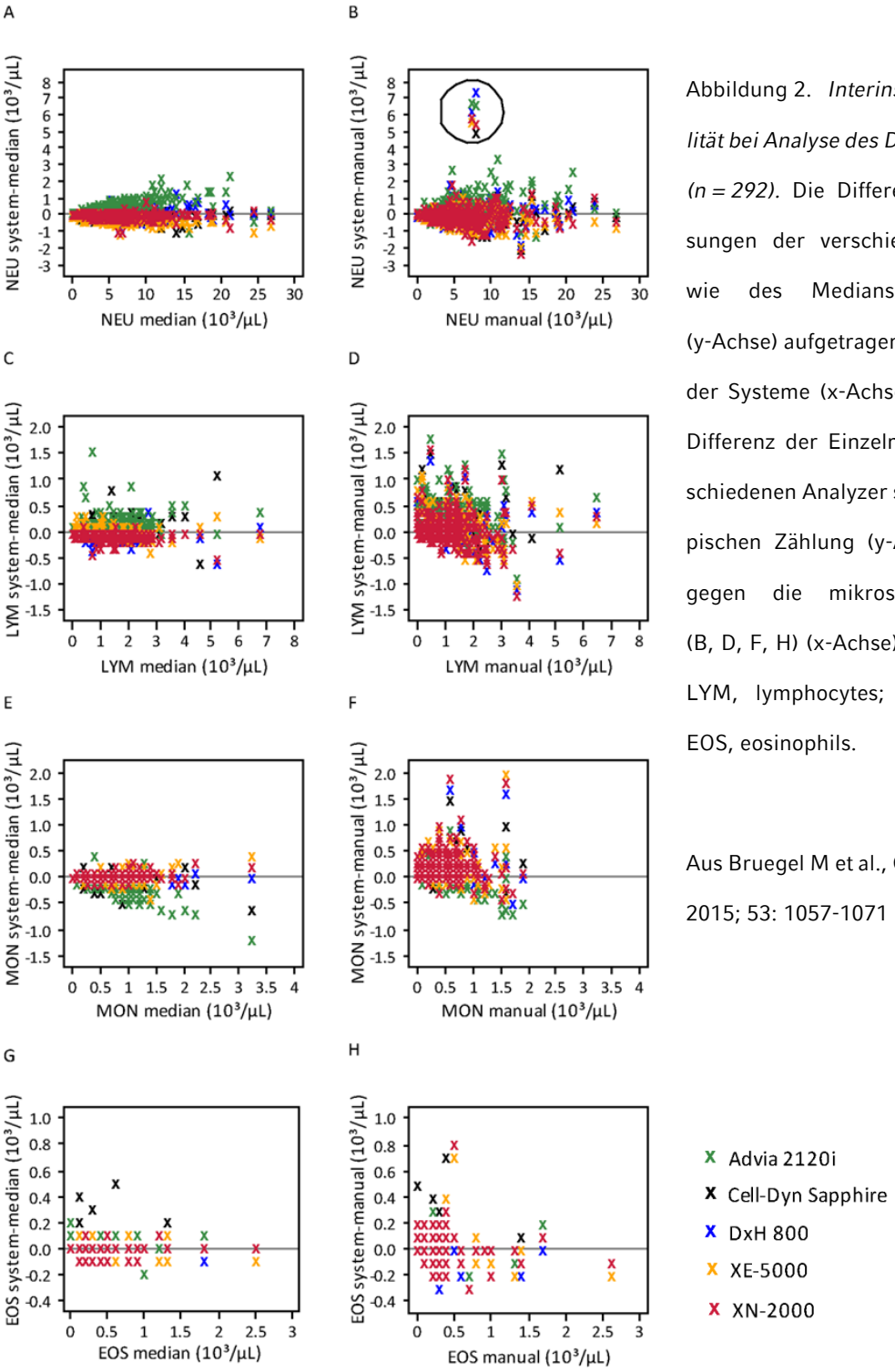


Abbildung 2. *Interinstrumenten-Variabilität bei Analyse des Differentialblutbildes (n = 292).* Die Differenz der Einzelmessungen der verschiedenen Analyzer sowie des Medians der fünf Systeme (y-Achse) aufgetragen gegen den Median der Systeme (x-Achse) (A, C, E, G). Die Differenz der Einzelmessungen der verschiedenen Analyzer sowie der mikroskopischen Zählung (y-Achse) aufgetragen gegen die mikroskopische Zählung (B, D, F, H) (x-Achse). NEU, neutrophils; LYM, lymphocytes; MON, monocytes; EOS, eosinophils.

Aus Bruegel M et al., Clin Chem Lab Med. 2015; 53: 1057-1071

Rote kernhaltige Vorstufen (NRBC) sind bei schweren inflammatorischen Erkrankungen nachweisbar, eine Quantifizierung kann beispielsweise eine prognostische Einordnung unterstützen (10). Der Interinstrumenten-Vergleich der automatisierten NRBC-Bestimmung ergab hohe Variabilitäten insbesondere in höheren Konzentrationsbereichen. Ähnliche Ergebnisse konnten für die vergleichende Prüfung mit dem mikroskopischen Nachweis gezeigt werden (Abb. 3) (9).

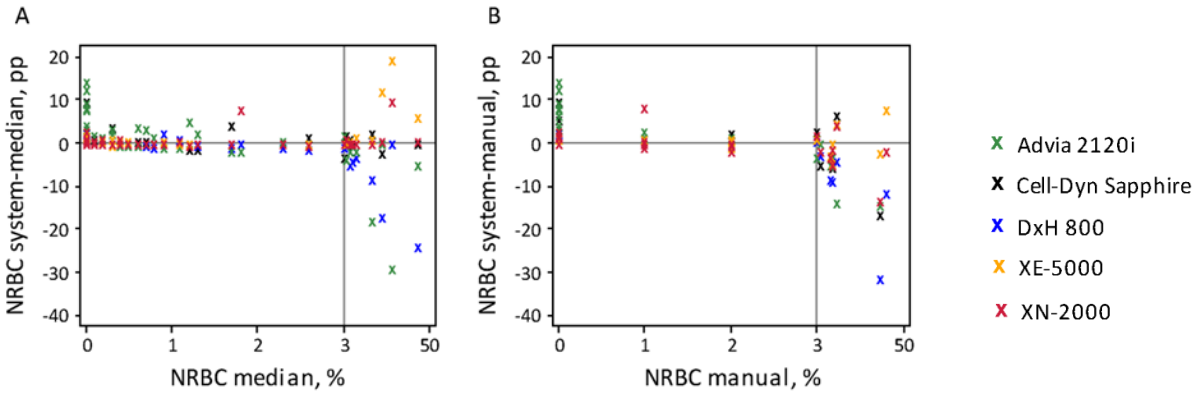


Abbildung 3. *Interinstrumenten-Variabilität bei Analyse von roten kernhaltigen Vorstufen (NRBC).* (A) Die Differenz der Einzelmessungen der verschiedenen Analyzer und dem Median der fünf Systeme (y-Achse) aufgetragen gegen den Median der Systeme (x-Achse). (B) Die Differenz der Einzelmessungen aus automatisierter und mikroskopischer Analyse (y-Achse) aufgetragen gegen die manuelle Zählung (x-Achse). NRBC, nucleated red blood cells.

Aus Bruegel M et al., Clin Chem Lab Med. 2015; 53: 1057-1071

Erhebliche qualitative Unterschiede zeigten die verschiedenen Hämatologie-Analyzer in der Funktionalität, pathologische Proben mit unreifen leukozytären Vorstufen oder abnormen Lymphozyten, die beispielsweise bei schweren Entzündungskonstellationen nachweisbar sind, zu identifizieren. Wie in Tabelle 1 dargestellt ergaben sich im Interinstrumenten-Vergleich für den automatisierten Nachweis von blastären Zellen Sensitivitäten zwischen 65 und 97 %, für den Nachweis von abnormen Lymphozyten zwischen 56 und 80 % sowie für den Nachweis von unreifen Granulozyten zwischen 35 und 82 %.

Instrument flagging	Pathological samples in microscopy (n)	Instrument	True positives (n)	Sensitivity 95% CI (%)	False positives (n)	Specificity 95% CI (%)
Blasts	34	Sapphire	26	76 (59 - 89)	21	93 (90 - 96)
		DxH 800	25	74 (56 - 87)	15	95 (92 - 97)
		Advia 2120i	22	65 (46 - 80)	12	97 (94 - 98)
		XE-5000	22	65 (46 - 80)	6	98 (96 - 99)
		XN-2000	33	97 (85 -100)	14	96 (93 - 98)
Variant lymphocytes	25	Sapphire	14	56 (35 - 76)	18	94 (91 -97)
		DxH 800	16	64 (43 -82)	18	94 (91 -97)
		Advia 2120i	18	72 (51 - 88)	40	88 (84 - 91)
		XE-5000	20	80 (59 - 93)	17	95 (92 - 97)
		XN-2000	20	80 (59 - 93)	14	95 (93 - 98)
Immature granulocytes	90	Sapphire	49	54 (44 - 64)	24	91 (87 - 94)
		DxH 800	60	67 (56 - 76)	16	94 (90 - 96)
		Advia 2120i	35	39 (29 - 50)	11	96 (93 - 98)
		XE-5000	72	80 (70 - 88)	21	92 (88 - 95)
		XN-2000	82	91 (83 - 96)	35	86 (82 - 90)
Left shift	76	Sapphire	39	51 (40 - 63)	13	95 (92 - 97)
		DxH 800	64	84 (74 - 92)	27	90 (86 - 93)
		Advia 2120i	39	51 (40 - 63)	14	95 (92 - 97)
		XE-5000	38	50 (38 - 62)	1	99 (98 -100)
		XN-2000	36	47 (36 - 59)	7	97 (95 - 99)
Platelet clumps	7	Sapphire	4	57 (18 - 90)	8	98 (96 - 99)
		DxH 800	6	86 (42 -100)	7	98 (96 - 99)
		Advia 2120i	4	57 (18 - 90)	6	98 (96 - 99)
		XE-5000	4	57 (18 - 90)	8	98 (96 - 99)
		XN-2000	4	57 (18 - 90)	4	99 (97 -100)
Blasts and/or variant lymphocytes	57	Sapphire	42	74 (60 - 84)	16	95 (91 - 97)
		DxH 800	46	81 (68 - 90)	15	95 (92 - 97)
		Advia 2120i	44	77 (64 - 87)	18	94 (90 - 96)
		XE-5000	43	75 (62 - 86)	11	96 (93 - 98)
		XN-2000	55	96 (88 -100)	18	94 (90 - 96)
Blasts and/or variant lymphocytes and/or immature granulocytes	103	Sapphire	70	68 (58 - 77)	29	88 (84 - 92)
		DxH 800	80	78 (68 - 85)	29	88 (84 - 92)
		Advia 2120i	66	64 (54 - 73)	26	89 (85 - 93)
		XE-5000	88	85 (77 - 92)	30	88 (83 - 92)
		XN-2000	101	98 (93 -100)	54	78 (72 - 83)

Tabelle 1. *Interinstrumenten-Vergleich der Flagging-Qualität.* CI, Konfidenz-Intervall; n, Anzahl

Aus Bruegel M et al., Clin Chem Lab Med. 2015; 53: 1057-1071

2.1.2 Vergleichende Validierung eines Digital-Imaging basierten Hämatologie-Analysensystems

Moderne Hämatologie-Analyzer basieren auf der Laser-Durchflußzytometrie als zentrale Methodik in der Zellidentifizierung- und Klassifikation. Das cobas m 511-System (Roche Diagnostics) stellt eine Geräte-Neuentwicklung dar, in der die automatisierte Blutbildbestimmung methodisch ausschließlich auf digitaler Morphologie beruht (11). Die Evaluierung dieses innovativen Analysensystems erfolgte im Rahmen einer Multicenter-Studie unter Einbeziehung von insgesamt 2546 Patientenproben an zwei europäischen Zentren (Institut für Laboratoriumsmedizin, Klinikum der Ludwig-Maximilians-Universität (LMU) München; Department of Clinical Chemistry, Erasmus MC, University Medical Center, Rotterdam, Niederlande) sowie an zwei Zentren in den USA (TriCore Reference Laboratories, Albuquerque, NM; Virtua Hospital, Voorhees, NJ). Neben einer allgemeinen Leistungsprüfung lag der Studienfokus zum einen in der vergleichenden Validierung des Digital-Imaging basierten Analyzers gegenüber der Mikroskopie als Goldstandard in der hämatologischen Diagnostik, zum anderen gegenüber dem Sysmex XN-System als derzeit qualitativ hochwertigster Vertreter der Laser-Durchflußzytometrie basierten Hämatologie-Analyzer. Wie in Abbildung 4 dargestellt konnte eine sehr gute Übereinstimmung der Parameter des kleinen Blutbildes und des Differentialblutbildes gegenüber dem Sysmex XN gezeigt werden. Auch zeigte sich eine sehr gute Übereinstimmung der automatisierten NRBC-Bestimmung zwischen klassischem und Digital-Imaging basiertem System (11).

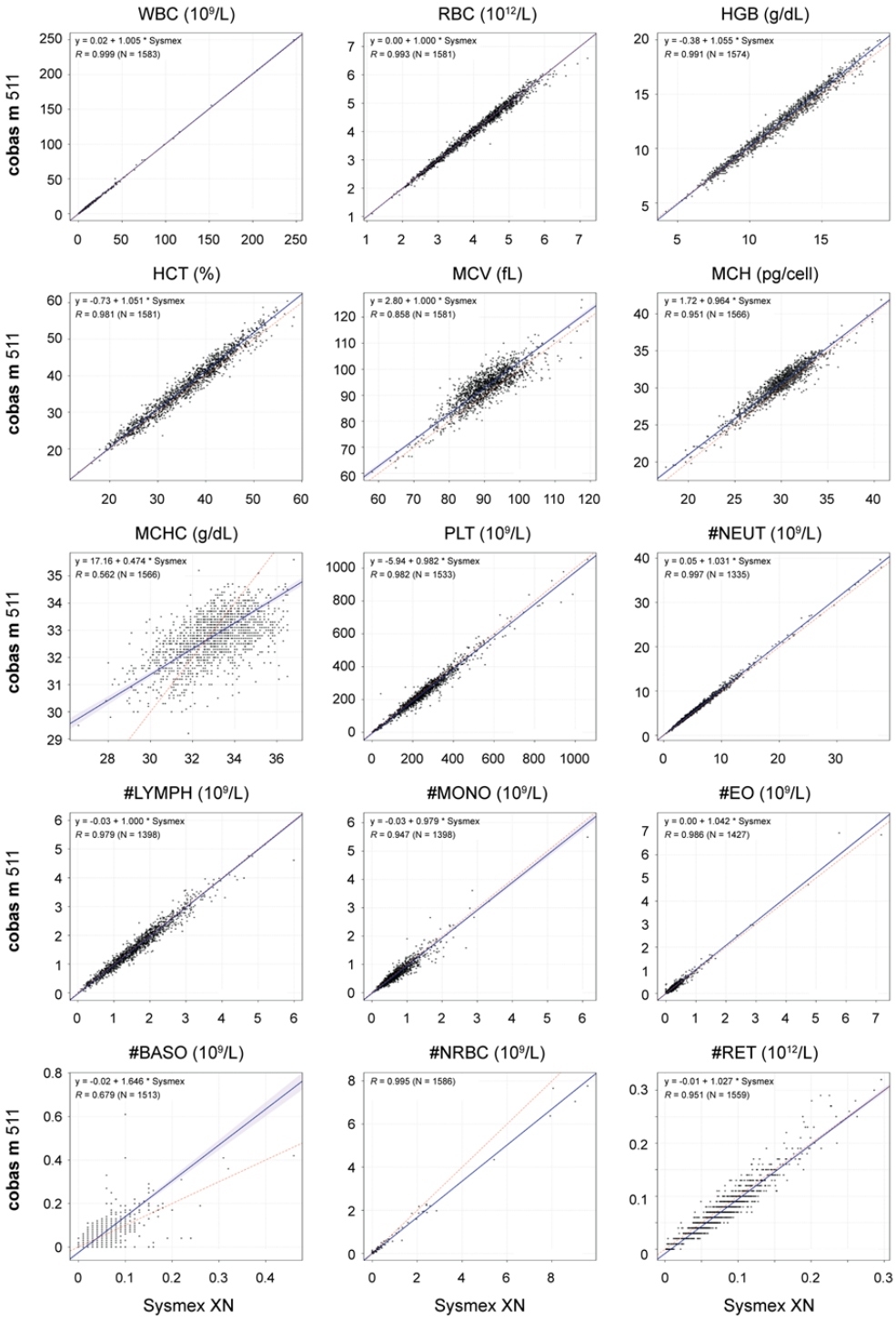


Abbildung 4. *Interinstrumenten-Vergleich von cobas m 511 und Sysmex XN.* Passing-Bablok-Regressionen für Parameter des kleinen Blutbildes sowie des Differential-Blutbildes. WBC, white blood cells; RBC, red blood cells; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelets; NEUT, neutrophils; LYMPH, lymphocytes; MONO, monocytes; EO, eosinophils; BASO, basophils; NRBC, nucleated red blood cells; RET, reticulocytes.

Aus Bruegel M et al., Int J Lab Hematol. 2018; 40: 672-682

Die Qualität dieses meßtechnologisch sehr innovativen Systems in der Erkennung von pathologischen Proben war außerordentlich gut. Wie in Tabelle 2 dargestellt, konnten durch das cobas m 511-System sämtliche Proben mit mikroskopischem Nachweis von blastären Zellen (n = 76) korrekt identifiziert werden, die Sensitivität für die Erkennung von unreifen Zellformen der granulozytären Reihe sowie von abnormen Lymphozyten lag bei 87 bzw. 93 % (11).

	Sample size (N)	TP	FP	FN	TN	Sensitivity (%)	Specificity (%)	Efficiency (%)
cobas m 511 automated flags								
Any message or combination	439	118	10	9	302	92.9	96.8	95.7
"Suspect blasts?"	439	76	5	0	358	100.0	98.6	98.9
"Suspect immature granulocytes?"	439	53	10	8	368	86.9	97.4	95.9
"Suspect variant lymphocytes?"	439	13	8	1	417	92.9	98.1	97.9
"Suspect left shift?"	439	27	4	46	362	37.0	98.9	88.6

Tabelle 2. *Flagging-Qualität des cobas m 511 Hämatologie-Analyzers.* N, number; TP, true positive; FP, false positive; FN, false negative; TN, true negative

Aus Bruegel M et al., Int J Lab Hematol. 2018; 40: 672-682

2.1.3 Stellenwert der Validierung von Hämatologie-Analysensystemen im Interinstrumenten-Vergleich

Bei der Validierungsstudie der Laser-Durchflußzytometrie basierten Hämatologie-Analyzer handelt es sich um den umfassendsten Gerätevergleich, der nach vorliegendem Kenntnisstand im Rahmen eines klinischen Settings durchgeführt wurde. Die Ergebnisse zeigen eine weitgehend gute Übereinstimmung im Bereich des kleinen Blutbildes sowie des Differentialblutbildes. Erhebliche Unterschiede ergeben sich jedoch für die Flagging-Qualität der untersuchten Analysensysteme. Die Identifizierung von abnormalen Patientenproben durch automatisierte Analyzer ist von zentraler Bedeutung, da entsprechende Proben in der Folge im Rahmen der labordiagnostischen Routinediagnostik einer mikroskopischen Prüfung sowie weiterführender Diagnostik wie beispielsweise einer zellulären Oberflächentypisierung

unterzogen werden. Die Ergebnisse der Validierungsstudie zeigen sehr deutlich die Erforderlichkeit, die variable Qualität von Hämatologie-Analysensystemen in die Entwicklung von Algorithmen im Rahmen der klinischen Labordiagnostik einzubeziehen.

Im Rahmen der Validierung der Laser-Durchflußzytometrie basierten Systeme konnte für das Sysmex XN-System sowohl die höchste analytische Qualität als auch das beste Flagging-Verhalten gezeigt werden, so dass dieses in der Folge in der Routinediagnostik des Instituts für Laboratoriumsmedizin am Klinikum der Universität München in der Routinediagnostik etabliert wurde.

Im Rahmen der Validierungsstudie des cobas m 511 konnte eine überaus gute Übereinstimmung zwischen dem Sysmex XN-System als hochqualitativer Vertreter der Laser-Durchfluß-zytometrie basierten Hämatologie-Analyzer sowie dem Digital-Imaging basierten System gezeigt werden. Die Qualität des cobas m 511-Systems in der Identifikation von abnormalen Patientenproben war ausgesprochen gut. Die im Rahmen dieser Validierungsstudie erhobenen Daten waren Grundlage für die Zulassung des cobas m 511 Analysensystems sowohl durch die europäische (EMEA) als auch die amerikanische (FDA) Zulassungsbehörde.

2.2 Eicosanoide als Biomarker der Inflammation

Eicosanoide sind Lipidmediatoren, die insbesondere durch die Phospholipase (PL)A_2 enzymatisch aus in Phospholipidmembranen vorhandener Arachidonsäure (AA) freigesetzt werden. Die weitere enzymatische Metabolisierung über Cyclooxygenase (COX) und Lipoxygenase vermittelte Reaktionen ermöglicht die Bildung eines breiten Spektrums an verschiedenen Eicosanoiden (Abb. 5) mit sehr verschiedenen und zum Teil auch gegenläufigen physiologischen und pathophysiologischen Effekten (12).

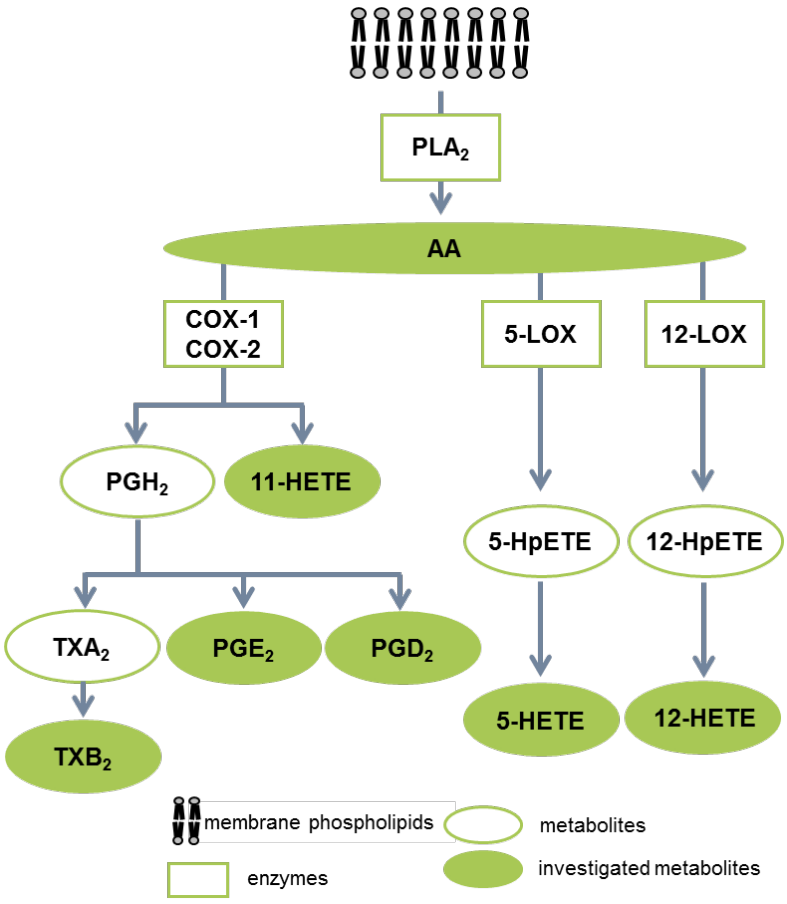


Abbildung 5. *Der Arachidonsäure-Metabolismus.* Darstellung der enzymatischen Arachidonsäure-Freisetzung sowie deren weiteren enzymatischen Metabolisierung. PLA_2, phospholipase A_2; AA, arachidonic acid; COX, cyclooxygenase; LOX, lipoxygenase; PG, prostaglandin; TX, thromboxane; HETE, hydroxyeicosatetraenoic acid; HpETE, hydroxyperoxyeicosatetraenoic acid.

Aus Suhr AC, Bruegel M et al., J Chromatogr B Analyt Technol Biomed Life Sci. 2016; 1022: 173-182

Basierend auf deren vergleichbar den Zytokinen übergeordneter immunmodulatorischer Eigenschaften gelten Eicosanoide als potentielle Biomarker für inflammatorische Erkrankungen. Limitationen in der Anwendung als Biomarker sind die sehr kurze Halbwertszeit, die schnelle Degradation und die pulsatile Freisetzung mit der Folge sehr niedriger und damit schwer nachweisbarer Konzentrationen dieser Mediatoren in Blut. Weiterhin sind verschiedene Eicosanoide strukturell sehr ähnlich, so dass eine korrekte Quantifizierung nur bei Anwendung komplexer Analysemethoden wie der Kombination aus Chromatographie und Tandem-Massenspektrometrie (LC-MS/MS) möglich ist. Aus diesen Gründen wurden Veränderungen des AA-Metabolismus bei verschiedenen entzündlichen Erkrankungen und deren diagnostisches Potential bisher erst in Ansätzen untersucht.

2.2.1 Etablierung eines Vollblutaktivierungs-Modells für die Prüfung der Eicosanoid-Antwort bei Patienten mit Sepsis

Um die Eicosanoid-Antwort trotz der pulsatilen Sekretion und der kurzen Halbwertszeit dieser Mediatoren bei verschiedenen inflammatorischen Erkrankungen analysieren zu können, wurde im Rahmen dieser Arbeit ein *in-vitro* Lipopolysaccharid (LPS)-Vollblutaktivierungs-Modell entwickelt und standardisiert (13). Dabei wird heparinisiertes Vollblut von Probanden oder Patienten mit LPS aktiviert, nach Zentrifugation in der Folge im Zellunterstand die Genexpression von Zielgenen des AA-Metabolismus mittels quantitativer PCR, im Zellüberstand korrespondierende Mediatoren mittels LC-MS/MS, analysiert.

In der klinischen Anwendung mit Fokus auf die Sepsis als Extremvariante einer entzündlichen Reaktion konnte eine differentielle Eicosanoid-Antwort bei Sepsis-Patienten im Vergleich zu gesunden Probanden auf Mediatorenebene gezeigt werden (Abb. 6) (13).

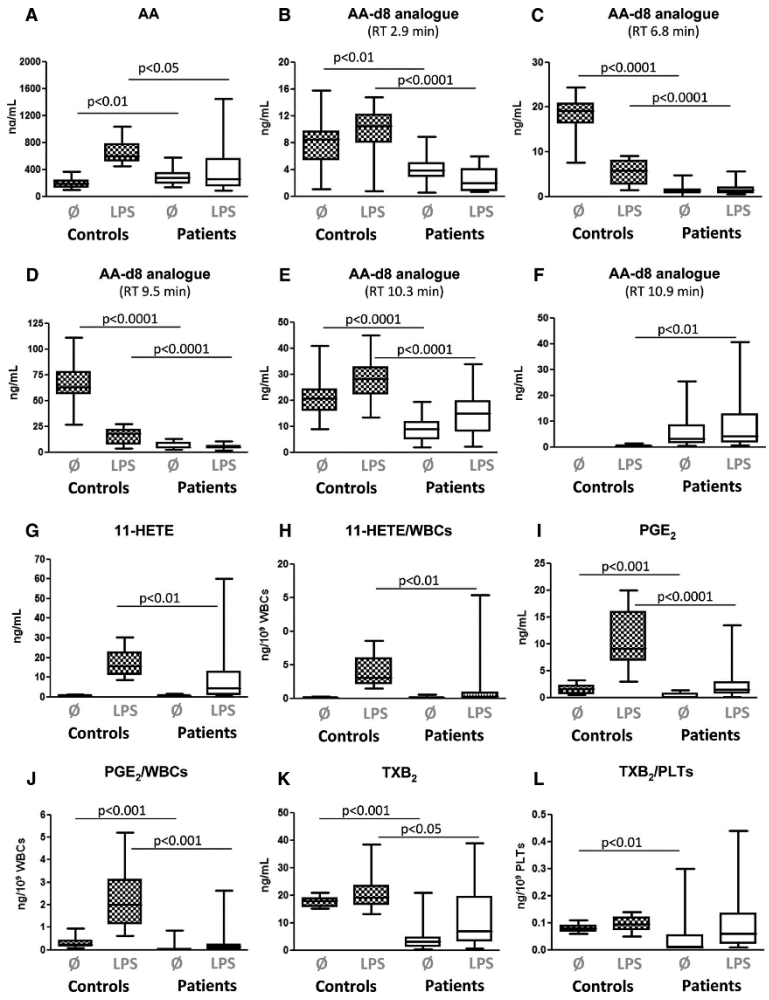


Abbildung 6. *Differentielle Eicosanoid-Antwort bei Gesunden und Patienten mit Sepsis.* Vollblut von Gesunden (n = 15, gepunktet) und Sepsis-Patienten (n = 25, farblos) wurde für 24h ohne/mit LPS (1 mg/ml) inkubiert. Die AA-Metabolite wurden mittels LC-MS/MS im Überstand nach Zentrifugation analysiert. LPS, lipopolysaccharide; AA, arachidonic acid; HETE, hydroxyeicosatetraenoic acid; PG, prostaglandin; WBCs, white blood cells; TX, thromboxane; PLTs, platelets.

Aus Bruegel M et al., Crit Care Med. 2012; 40: 1478-1486

Wie in Abbildung 7 dargestellt war eine reduzierte LPS-vermittelte Freisetzung von AA und COX abhängiger Metabolite mit der Schwere und Prognose der Erkrankung assoziiert, was auf eine potentielle diagnostische Bedeutung der Eicosanoid-Antwort im Vollblutaktivierungs-Modell in der Risiko-Einschätzung bei Patienten mit Sepsis hinweist (13).

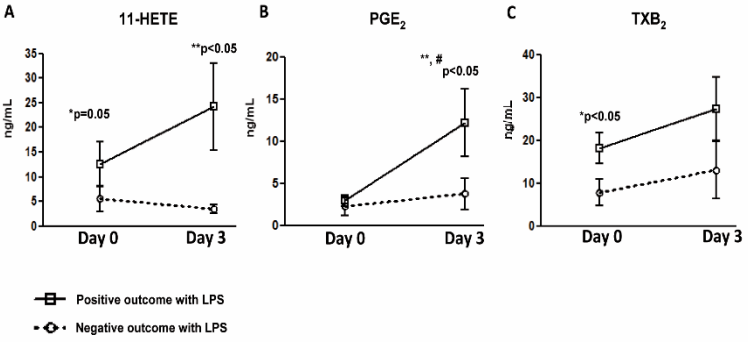


Abbildung 7. *Differentielle LPS-vermittelte Eicosanoid-Antwort bei Sepsis-Patienten mit positivem (n = 13) und negativem Outcome (n = 12).*
HETE, hydroxyeicosatetraenoic acid; PG, prostaglandin; TX, thromboxane.

Aus Bruegel M et al., Crit Care Med. 2012; 40: 1478-1486

Auf Ebene der Genexpression konnte bei Patienten mit Sepsis eine reduzierte Induzierbarkeit der COX-2 mRNA Expression gezeigt werden (Abb. 8) (12).

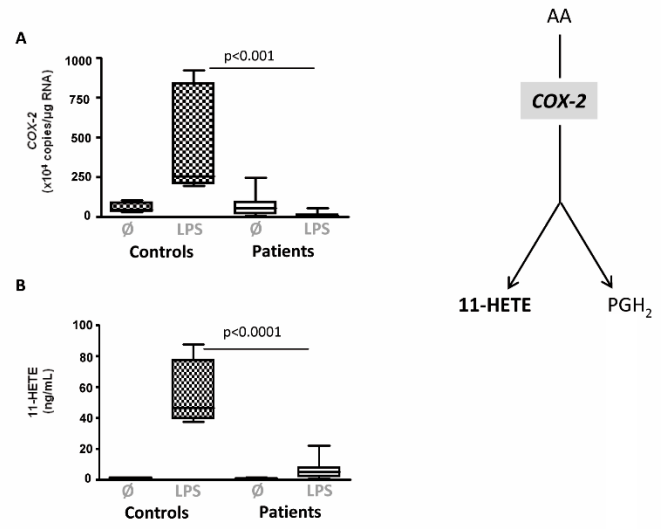


Abbildung 8. *COX-2 Genexpression bei Gesunden (n = 3) und Patienten mit Sepsis (n = 10)*. COX, cyclooxygenase; LPS, lipopolysaccharide; AA, arachidonic acid; HETE, hydroxyeicosatetraenoic acid; PG, prostaglandin.

Aus Bruegel M et al., Crit Care Med. 2012; 40: 1478-1486

2.2.2 Etablierung einer optimierten LC-MS/MS Methode für die Bestimmung von Eicosanoiden

Für eine fortführende Prüfung des diagnostischen Stellenwerts von Eicosanoiden im klinischen Umfeld der Inflammation wurde eine neue auf LC-MS/MS basierte Analysemethode für die Bestimmung von Eicosanoiden aus biologischen Flüssigkeiten entwickelt, die auf der Anwendung von ferromagnetischen Partikeln in der Probenvorbereitung beruht. Vorteile dieser Neuentwicklung gegenüber bereits bestehenden LC-MS/MS basierten Methoden für die Eicosanoid-Analytik ist zum einen die reduzierte Probenbearbeitungszeit als Voraussetzung für eine Anwendung im Rahmen größerer Patienten-Kohorten, zum anderen die Generierung von bereits sehr gut aufgereinigten und damit für die LC-MS/MS-Analytik geeigneten Extrakten sowie der generische Charakter der Methode, der eine große Einsatzbreite auch für eine Vielzahl an Analyten mit verschiedenen physikochemischen Eigenschaften zulässt. Die Methode wurde etabliert für die parallele Bestimmung von AA sowie zentraler Vertreter des COX- (11-Hydroxyeicosatetraen-Säure (HETE), Prostaglandin (PG)E_2, PGD_2, Thromboxan (TX)B_2) und LOX-Pathways (5-HETE, 12-HETE) (14).

Die Prüfung der analytischen Methodenqualität zeigte eine sehr gute Linearität über den gesamten Kalibrationsbereich, Prüfungen zu Intra- und Interassay-Präzision (<15 % für sämtliche Analyte und Kontrollen) sowie zu Matrix-Effekten und Wiederfindung (74 - 95 %) ergaben gute Ergebnisse. In den Experimenten zur Präanalytik konnte eine Stabilität der untersuchten Analyte über einen Monat in Ethanol oder Plasma bei –80°C gezeigt werden. Dreimalige Ein- und Auftauzyklen sowie eine dreitägige Lagerung bei -4°C hatten keine Effekte auf die jeweilige Analyt-Konzentration. Die Funktionalität der neu entwickelten Analysemethode wurde im Anschluss in einem Proof-of-Principle-Ansatz zur Prüfung der Eicosanoid-Antwort bei gesunden Individuen (n = 5) bei Anwendung des standardisierten LPS-Vollblutaktivierungs-Modells geprüft (Tabelle 3). Hier konnte eine signifikante Induktion der Eicosanoid-Antwort nach LPS-Vollblutaktivierung gezeigt werden, wobei die Intensität bei den verschiedenen Individuen erhebliche Unterschiede zeigte (14).

	Baseline (no incubation, no LPS)	**Stimulated** (24 hours incubation with LPS)
TXB_2		
Donor 1	< 0.10*	4.68
Donor 2	< 0.10*	10.5
Donor 3	< 0.10*	5.70
Donor 4	< 0.10*	5.00
Donor 5	< 0.10*	4.63
PGE_2		
Donor 1	< 0.10*	0.62
Donor 2	< 0.10*	10.4
Donor 3	< 0.10*	1.40
Donor 4	< 0.10*	2.74
Donor 5	< 0.10*	5.41
5-HETE		
Donor 1	< 0.05*	0.19
Donor 2	0.06	0.29
Donor 3	< 0.05*	0.29
Donor 4	0.06	0.50
Donor 5	< 0.05*	0.32
11-HETE		
Donor 1	< 0.49*	0.81
Donor 2	< 0.49*	2.94
Donor 3	< 0.49*	1.27
Donor 4	< 0.49*	1.31
Donor 5	< 0.49*	1.66
12-HETE		
Donor 1	0.66	19.8
Donor 2	< 0.49*	46.7
Donor 3	< 0.49*	19.3
Donor 4	< 0.49*	22.6
Donor 5	< 0.49*	18.7
AA		
Donor 1	151	417
Donor 2	383	787
Donor 3	263	568
Donor 4	372	770
Donor 5	228	839

Tabelle 3. *Eicosanoid-Antwort (LC-MS/MS) bei gesunden Individuen (n = 5) bei Anwendung des standardisierten LPS-Vollblutaktivierungs-Modells.* LPS, lipopolysaccharide; <*, below lower limit of quantification.

Aus Suhr AC, Bruegel M et al., J Chromatogr B Analyt Technol Biomed Life Sci. 2016; 1022: 173-182

2.2.3 Stellenwert der Etablierungsarbeiten zur Prüfung der Eicosanoid-Antwort

Das im Rahmen dieser Arbeit entwickelte und standardisierte LPS-Vollblutaktivierungs-Modell erlaubt eine differentielle Prüfung der Eicosanoid-Antwort sowohl auf Metabolitenebene als auch auf Ebene der Genexpression. Im Rahmen einer ersten klinischen Anwendung konnte ein diagnostischer Stellenwert der Prüfung der Eicosanoid-Antwort bei Patienten mit Sepsis gezeigt werden. Die im Rahmen dieser Arbeit erfolgte Etablierung einer optimierten LC-MS/MS basierten Analysemethode für die Bestimmung von Eicosanoiden aus biologischen Flüssigkeiten wird eine Bestätigung dieser Daten in klinischen Studien mit größeren Patientenzahlen ermöglichen. Fortführend wird die Eicosanoid-Antwort aktuell bei weiteren klinischen Anwendungen, z.B. bei Patienten mit und ohne koronarer Herzerkrankung, untersucht.

3. Zusammenfassung

Ziel dieser Arbeit war die Standardisierung der in der Labordiagnostik der inflammatorischen Reaktion angewandten Methoden sowie die Identifikation neuer Biomarker. In einem ersten klinisch orientierten Teil wurden Vergleichsstudien verschiedener Hämatologie-Analysensysteme als Basissysteme für die Darstellung einer inflammatorischen Reaktion durchgeführt. Aufgrund der bisher nur sehr eingeschränkten Verfügbarkeit entsprechender Vergleichsdaten ist die im Rahmen dieser Arbeit gefundene variable Qualität der verschiedenen Systeme für die Entwicklung gerätespezifischer Algorithmen im Rahmen der klinischen Diagnostik von großer Bedeutung. In einem zweiten grundlagenorientierten Teil konnte ein potentieller Stellenwert von Markern des AA-Metabolismus in der diagnostischen Einordnung inflammatorischer Erkrankungen gezeigt werden. Fortführend wurde eine optimierte LC-MS/MS basierte Analysemethode für die Bestimmung von Eicosanoiden aus biologischen Flüssigkeiten entwickelt, die eine Prüfung des diagnostischen Stellenwertes in klinischen Studien mit größeren Patientenzahlen ermöglicht.

4. Literatur

(1) Schett G, Elewaut D, McInnes IB, Dayer JM, Neurath MF. How cytokine networks fuel inflammation: Toward a cytokine-based disease taxonomy. Nat Med. 2013; 19:822-824.

(2) Esser-von Bieren J. Immune-regulation and –functions of eicosanoid lipid mediators. Biol. Chem. 2017; 398: 1177-1191.

(3) von Vietinghoff S, Ley K. Homeostatic regulation of blood neutrophil counts. J Immunol. 2008; 181:5183-8.

(4) Hunter CA, Jones SA. IL-6 as a keystone cytokine in health and disease. Nat Immunol. 2015; 16:448-57.

(5) Gilroy DW, Bishop-Bailey D. Lipid mediators in immune regulation and resolution. Br J Pharmacol. 2019; 176:1009-1023.

(6) Blumenreich MS. The White Blood Cell and Differential Count. Clinical Methods: The History, Physical, and Laboratory Examinations. 3rd edition. Boston: Butterworths; 1990. Chapter 153.

(7) Tan BT, Nava AJ, George TI. Evaluation of the Beckman Coulter UniCel DxH 800, Beckman Coulter LH 780, and Abbott Diagnostics Cell-Dyn Sapphire hematology analyzers on adult specimens in a tertiary care hospital. Am J Clin Pathol. 2011; 135:939-951.

(8) Meintker L, Ringwald J, Rauh M, Krause SW. Comparison of automated differential blood cell counts from Abbott Sapphire, Siemens Advia 120, Beckman Coulter DxH 800, and Sysmex XE-2100 in normal and pathologic samples. Am J Clin Pathol. 2013; 139:641-650.

(9) Bruegel M, Nagel D, Funk M, Fuhrmann P, Zander J, Teupser D. Comparison of five automated hematology analyzers in a university hospital setting: Abbott Cell-Dyn Sapphire, Beckman Coulter DxH 800, Siemens Advia 2120i, Sysmex XE-5000, and Sysmex XN-2000. Clin Chem Lab Med. 2015; 53:1057-1071.

(10) Purtle SW, Horkan CM, Moromizato T, Gibbons FK, and Christopher KB. Nucleated red blood cells, critical illness survivors and postdischarge outcomes: a cohort study. Crit Care. 2017; 21: 154.

(11) Bruegel M, George TI, Feng B, Allen TR, Bracco D, Zahniser DJ, Russcher H. Multicenter evaluation of the cobas m 511 integrated hematology analyzer. Int J Lab Hematol. 2018; 40:672-682.

(12) Brock TG, Peters-Golden M. Activation and regulation of cellular eicosanoid biosynthesis. ScientificWorldJournal. 2007; 7:1273-1284.

(13) Bruegel M, Ludwig U, Kleinhempel A, Petros S, Kortz L, Ceglarek U, Holdt LM, Thiery J, Fiedler GM. Sepsis-associated changes of the arachidonic acid metabolism and their diagnostic potential in septic patients. Crit Care Med. 2012;40:1478-1486.

(14) Suhr AC, Bruegel M (gleichberechtigter Co-Autor), Maier B, Holdt LM, Kleinhempel A, Teupser D, Grimm SH, Vogeser M. Ferromagnetic particles as a rapid and robust sample preparation for the absolute quantification of seven eicosanoids in human plasma by UHPLC-MS/MS. J Chromatogr B Analyt Technol Biomed Life Sci. 2016;1022:173-182.

5. Originalarbeiten der kumulativen Habilitationsschrift

1. **Bruegel M**, George TI, Feng B, Allen TR, Bracco D, Zahniser DJ, Russcher H. Multicenter evaluation of the cobas m 511 integrated hematology analyzer. Int J Lab Hematol. 2018; 40: 672-682.

2. Suhr AC*, **Bruegel M***, Maier B, Holdt LM, Kleinhempel A, Teupser D, Grimm SH, Vogeser M. Ferromagnetic particles as a rapid and robust sample preparation for the absolute quantification of seven eicosanoids in human plasma by UHPLC-MS/MS. J Chromatogr B Analyt Technol Biomed Life Sci. 2016; 1022: 173-182.
   *Authors contributed equally to this work

3. **Bruegel M**, Nagel D, Funk M, Fuhrmann P, Zander J, Teupser D. Comparison of five automated hematology analyzers in a university hospital setting: Abbott Cell-Dyn Sapphire, Beckman Coulter DxH 800, Siemens Advia 2120i, Sysmex XE-5000, and Sysmex XN-2000. Clin Chem Lab Med. 2015; 53: 1057-1071.

4. **Bruegel M**, Ludwig U, Kleinhempel A, Petros S, Kortz L, Ceglarek U, Holdt LM, Thiery J, Fiedler GM. Sepsis-associated changes of the arachidonic acid metabolism and their diagnostic potential in septic patients. Crit Care Med. 2012; 40: 1478-1486.

Received: 29 March 2018 | Revised: 25 May 2018 | Accepted: 11 June 2018

DOI: 10.1111/ijlh.12903

ORIGINAL ARTICLE

WILEY ISLH International Journal of Laboratory Hematology

Multicenter evaluation of the cobas m 511 integrated hematology analyzer

Mathias Bruegel MD[1] | Tracy I. George MD[2] | Bo Feng MD[3] | Timothy R. Allen[4] | Dan Bracco[4] | David J. Zahniser PhD[4] | Henk Russcher PhD[5]

[1]Institute of Laboratory Medicine, Ludwig-Maximilians-University of Munich, Munich, Germany

[2]Department of Pathology, University of New Mexico, Albuquerque, New Mexico

[3]Department of Pathology and Laboratory Medicine, Virtua Voorhees Hospital, Voorhees, New Jersey

[4]Roche Diagnostics, Westborough, Massachusetts

[5]Department of Clinical Chemistry, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands

Correspondence: Henk Russcher, Department of Clinical Chemistry, Erasmus MC, University Medical Center Rotterdam, Room Na410, Wytemaweg 80, 3015 CN, Rotterdam, The Netherlands (h.russcher@erasmusmc.nl).

Present address
Tracy I. George Department of Pathology, University of Utah, Salt Lake City, Utah

Funding information
This study and editorial support for the preparation of this manuscript were supported by Roche Diagnostics.

The copyright line for this article was changed on 14 September after original online publication

Abstract

Introduction: The cobas m 511 integrated hematology analyzer conducts a complete blood count (CBC), white blood cell (WBC) differential, reticulocyte count, and nucleated red blood cell count using automated digital microscopy. This multicenter study validated the analytical performance of the cobas m 511 system.

Methods: Repeatability, reproducibility, carryover, mode-to-mode comparison, cytomorphology, WBC clinical sensitivity, and method comparison were analyzed at four clinical sites using residual whole blood clinical samples (n = 2546) and fresh whole blood from healthy volunteers (n = 480). For WBC clinical sensitivity, the cobas m 511 system automated CBC and WBC differential, system flags, cobas m 511 images, and stained cobas m 511 slides were compared with manual microscopy. Sysmex® XN analyzers were used for interinstrument method comparison.

Results: Repeatability and reproducibility results showed low variability. There was no significant sample carryover and no difference between open/closed modes. The overall percentage agreement of morphology assessments with manual microscopy (n = 163 samples) was 95.6% for cobas m 511 images and 95.7% for cobas m 511 slides. The sensitivity and specificity for detecting distributional and/or morphological abnormalities were 94.4% and 74.6% for cobas m 511 automated differential, and 95.9% and 73.3% for cobas m 511 image assessment, compared with a manual 400-cell reference differential (n = 439 samples). Some discordance was seen for monocytes and basophils. Correlations between cobas m 511 and Sysmex XN system data were very good (Pearson's $R \geq 0.95$ for most CBC parameters).

Conclusion: The cobas m 511 system performs robustly in the clinical laboratory and is suitable for routine clinical use.

KEYWORDS

cobas m 511 system, digital morphology, integrated hematology analyzer, microscopy, multicenter evaluation

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.
© 2018 The Authors *International Journal of Laboratory Hematology* Published by John Wiley & Sons Ltd

1 | INTRODUCTION

Current automated hematology analyzers for the quantitative analysis of complete blood counts (CBC), white blood cell (WBC) differentials, reticulocytes, and nucleated red blood cell (NRBC) counts are methodically based on electrical impedance and optical or fluorescence flow cytometry.[1-3] Abnormal cases are flagged for review, necessitating preparation of a blood smear and a manual microscopic morphological differential count to identify the abnormalities.[4] As manual microscopy is time consuming, requires experienced medical staff, and is subject to significant variance, automated digital imaging systems were developed to address these issues for routine hematology diagnostics.[4-6]

The cobas m 511 integrated hematology analyzer (Roche Diagnostics Operations Inc., Boston, MA, USA) combines a slide maker, slide stainer, digital image-based cell locator, cell counter, and cell classifier in one system. Unlike existing impedance- or flow cytometry-based automated hematology analyzers, all analyses with the cobas m 511 system are performed using microscope slides. Slides are prepared using a precision printing method from ethylenediaminetetraacetic acid (EDTA)-anticoagulated whole blood and stained automatically.[7] The CBC, automated differential, reticulocyte, and NRBC counts are performed using digital morphologic analysis. Stained cells are classified, enumerated, and analyzed using computer image analysis, providing an image-based assessment of cell type and morphology with flags for abnormalities. A viewing station enables manual verification and reclassification of the results, and the classification of unclassified abnormal WBCs.

This study validated the analytical performance of the cobas m 511 integrated hematology analyzer in a multicenter clinical laboratory setting.

2 | MATERIALS AND METHODS

The study was conducted from June 2016 to January 2017. Sites started at different timepoints and the study duration at each site averaged 14 weeks including the reviews of the slides and images.

2.1 | Instrumentation and instrument setting

A nominal 1 μL of EDTA-anticoagulated whole blood was automatically printed on DigiMAC3® slides (Roche) by the cobas m 511 system using Bloodhound® technology. Slides were stained automatically with a modified Romanowsky stain and with a supravital stain on a separate slide for analysis of reticulocytes. These stains were modified for high-speed application and provide consistency across all cobas m 511 systems. The system then digitally identified and counted the red blood cells (RBCs), WBCs, platelets (PLTs), and NRBCs on the microscope slide using low-magnification (×10) multispectral imaging. Randomized locations of between 600 and 700 WBCs were recorded, imaged using high-magnification (×50) multispectral imaging, and an automated WBC differential performed using computer algorithms. Unclassified cells that did not fall into the five normal WBC types were flagged and presented in the viewing station for review. RBC and PLT indices were also measured during the high-magnification analysis.

The cobas m 511 system was evaluated at two European (Department of Clinical Chemistry, Erasmus MC, University Medical Center, Rotterdam, the Netherlands; Institute of Laboratory Medicine, University Hospital, LMU Munich, Germany) and two US sites (TriCore Reference Laboratories, Albuquerque, NM, USA; Virtua Hospital, Voorhees, NJ, USA) sites. Sysmex XN analyzers (XN-10, XN-20; Sysmex, Kobe, Japan) were used for interinstrument method comparison. Default settings for test and flagging algorithms were used for all cobas m 511 systems.

2.2 | Samples and evaluation protocol

Samples were comprised of residual whole blood clinical samples (n = 2546 in total) and fresh whole blood samples from healthy volunteers (n = 480 in total). Sample totals were obtained from collecting approximate equal proportions from the four clinical sites. Residual whole blood samples were collected randomly or selected based on specific laboratory results. These samples were provided with birth year, sex, sample draw time, and laboratory results from the comparative instrument, when required. Fresh whole blood samples were collected from healthy volunteers after they had signed an informed consent and completed a questionnaire to verify health status. These volunteers were males and females aged ≥18 years, and sample collection was in accordance with the Clinical Laboratory Standards Institute (CLSI) EP28-A3c guideline.[8] All samples were collected in standard K2-EDTA collection tubes (Sarstedt, Nümbrecht, Germany; Becton Dickinson, Franklin Lakes, NJ, USA) and processed within 8 hours of venipuncture. Standard blood smears were prepared according to site-specific methods within 2 hours of running on automated analyzers. Dedicated study coordinators at each site performed all instrument-related analytical evaluations and managed the image and slide reviews by study technologists.

The study protocol was approved by each site's Ethics Committee or Institutional Review Board.

The analytical performance assessments of the cobas m 511 system comprised: whole blood repeatability, reproducibility using DigiMAC3® controls (Roche), carryover, mode-to-mode comparison, morphology, WBC clinical sensitivity, and method comparison (Table 1).

2.3 | Whole blood repeatability

Repeatability evaluated within-run precision using whole blood samples according to the CLSI EP05-A3 guideline[9] and CLSI H26-A2 standard.[10] The repeatability of reticulocyte (RET) related parameters (%RET, #RET, RET-HGB) was not evaluated.

Overall, 144 residual whole blood samples were selected for WBC, RBC, hemoglobin (HGB), and PLT parameters at targeted low, middle, and high ranges generally encountered in the laboratory. Forty-eight samples at medical decision levels for anemia (n = 12),

TABLE 1 Analytical performance assessments of the cobas m 511 conducted in this study

Analysis	Sample size (minimum required)	Sample type	Parameters evaluated
WBC clinical sensitivity			
Part I: Method-specific reference ranges	120	Healthy donor samples.	Neutrophils; Lymphocytes; Monocytes; Eosinophils; Basophils; Abnormal WBCs
Part II: Clinical sensitivity evaluation	110	Samples with targeted abnormal WBC differential findings.	
Morphology	40	Samples with WBC, RBC, and/or PLT morphologic abnormalities.	Morphology characteristics of WBCs, RBCs, and PLTs
Carryover	12	Samples with high WBC, RBC, and PLT counts. Samples with a high WBC count and blast percentage.	WBCs; RBCs; PLTs; Blasts
Mode-to-mode comparison	40	Samples randomly selected from routine population Samples with leukopenia.	All reportable parameters
Repeatability	48	Samples that span the measuring range for WBCs, RBCs, HGB, and PLTs. Samples at medical decision levels.	All reportable parameters
Reproducibility	1 tube per level	DigiMAC3 L1, L2, and L3 controls.	All reportable parameters
Method comparison	400	Normal and abnormal samples selected from the laboratory's routine population.	All reportable parameters

HGB, hemoglobin; PLT, platelets; RBC, red blood cell; WBC, white blood cell.

thrombocytopenia (n = 12), severe leukopenia (n = 12), and elevated NRBCs (n = 12) were also evaluated. Samples were processed 31 consecutive times over 35 minutes on the cobas m 511 system. The mean, standard deviation (SD), and coefficient of variance (%CV) were calculated for each sample separately, and the range and mean of the samples means, and repeatability derived by a mixed model analysis, were determined for each parameter.

2.4 | Reproducibility

Reproducibility of results was determined using three levels of DigiMAC3® quality control materials in a multiday assessment according to CLSI EP05-A3 guideline.[9] The same lot was used at each site to minimize lot-to-lot bias.

The assessment used a 4 × 5 × 2 × 3 design, that is four clinical sites, 5 days, two runs per day, and three replicates per run. Within-run precision (repeatability), between-run precision (same day), between-day precision, between-laboratory precision, and reproducibility (total precision) were calculated. For each parameter and control level, the mean, SD, and %CV of the components of precision were determined (with 95% CI of the SD and %CV for repeatability and reproducibility). Low SD and %CV results are indicative of good reproducibility.

2.5 | Carryover

Carryover was assessed at each site for high target value (HTV) samples containing high numbers of WBCs, RBCs, and PLTs according to CLSI H26-A2 standard.[10] Samples with high numbers of blasts were also assessed. The HTV samples were characterized by WBC counts ≥90 × 10^9/L, RBC counts ≥6.20 × 10^{12}/L, and PLT counts ≥900 × 10^9/L. The blast samples had WBC counts ≥20 × 10^9/L and ≥30% blast cells. One of these four sample types was assessed on each test day: Three HTV replicates were run on the cobas m 511 system followed immediately by three low target value (LTV) replicates (filtered serum). There was a minimum of 1-day separation between the test days for each different sample type. The assessment resulted in 12 samples per site (including three blast samples per site) and 48 samples combined from all sites. Individual site results were averaged by sample type. By design, the cobas m 511 system rejects a LTV sample at the low-magnification imaging stage due to insufficient cells (less than approximately 0.4 × 10^9/L). Rejected cobas m 511 slides from all LTV samples from all sites were retrieved and assessed microscopically by a single hematology expert for evidence of carryover, determined for each cell type:

$$\text{Carryover\%} = \frac{\text{total \# cells of interest on serum slides (cobas m 511 slide results)}}{\text{\# cells per μL in high taget sample (Sysmex XN result)}} \times 100\%$$

2.6 | Mode-to-mode comparison

Mode-to-mode evaluations assessed whether consistent results were obtained when using the closed (automated) and open (manual) modes on the cobas m 511 system.

From all sites combined, 145 random samples and sixteen leukopenic samples were collected (n = 161 in total). Bias between the closed and open mode results was assessed per the CLSI EP09-A3 guideline.[11] Bias was calculated at high and low critical bias limits defined either at generally recognized medical decision points or at the extremes of the reference interval. If a parameter had both an absolute and a proportional bias limit, the bias was calculated at the crossover point where they were equal. A Passing-Bablok regression model was used for most parameters, except those with many valid results of zero (ie, %EO, #EO, %BASO and #BASO) in which case a Deming regression model was used. The #NRBC parameter had a limited number of valid, nonzero results, so bias was calculated as the mean difference between open and closed modes, and no regression lines were estimated with CI derived using a paired *t* test. %NRBCs were excluded from this analysis due to very low prevalence.

2.7 | Morphology

Cellular morphology was assessed to ensure that morphological characteristics that were present on blood smears could be identified by a trained technologist using the matched cobas m 511 images and slides.

From all sites combined, 163 residual blood samples were collected and processed on the cobas m 511 system. The cobas m 511 images, cobas m 511 slides, and the corresponding blood smear from each sample were randomized and split between two technologists at each site. Each technologist performed a 100-cell differential with morphology review on the set of cobas m 511 images, cobas m 511 slides, and blood smears from each sample to minimize inter-reviewer bias. Overall agreement for each morphological characteristic compared with the 100-cell reference differential was calculated for each test method (cobas m 511 images or slides) using the total number of results that agreed (A) or disagreed (D) within ± one grade of the blood smear result:

$$\text{Agreement (\%)} = \frac{A_{total}}{A_{total} + D_{total}} \times 100\%$$

2.8 | WBC clinical sensitivity

Consistent with the CLSI H20-A2 standard,[12] WBC clinical sensitivity was assessed in two parts. Part one determined method-specific reference ranges for the cobas m 511 automated differential, images, and slides using 480 samples from normal healthy donors. Part two investigated the sensitivity, specificity, and efficiency of the cobas m 511 system for detecting distributional and morphological abnormalities in 439 mostly abnormal residual samples (granulocytosis with left shift, monocytosis, eosinophilia, lymphocytosis, atypical/reactive/variant lymphocyte forms, lymphopenia, immature granulocytes, blasts, and NRBCs) compared with a manual 400-cell reference differential (combined from two technologists). The goal was to determine whether the cobas m 511 automated WBC differential, system messages, cobas m 511 images, and cobas m 511 slides generated results that were consistent with the manual microscopy. The primary review mechanism for the cobas m 511 system is the images; however, the cobas m 511 slides were also studied to ensure they could be used, if needed, for additional review by the laboratory.

Two qualified technologists at each site each reviewed one-half of the cobas m 511 images and cobas m 511 slides for WBC differentials. Three corresponding blood smears per sample were also produced for the reference differential. Both technologists performed a 200-cell WBC differential on one of these smears and combined their results to create the 400-cell reference differential. A third technologist or laboratory physician with special knowledge in hematology microscopy reviewed the third blood smear if the difference in results of the two technologists exceeded the 99% Fisher exact limits. In these cases, the two results which were closest in agreement were used to derive the 400-cell reference differential. Technologists were blinded to the matching of the cobas m 511 images, cobas m 511 slides, and blood smears during the WBC clinical sensitivity analyses.

For the distributional abnormalities, the five normal WBC types from the cobas m 511 automated differential, images, and slides were evaluated vs method-specific reference ranges established in this study in normal healthy donors consistent with the CLSI H20-A2 standard. Results exceeding the upper and lower limits of the reference range were considered abnormal (positive). The cobas m 511 system messages, images, and slides were also reviewed against the 400-cell reference differential for morphological abnormalities, comprising immature and/or abnormal WBCs. Results exceeding a predefined threshold were considered abnormal (positive). Thresholds for the reference method were blasts >1%, variant lymphocytes >10% (containing lymphocytes suspected to be reactive and to be malignant), immature granulocytes >2%, and left shift (band neutrophils) >5%.

It was expected that distributional and morphologic abnormalities seen on the cobas m 511 system should also be present on the corresponding reference 400-cell reference differential. The sensitivity, specificity, and efficiency of the different cobas m 511 system modalities compared with the 400-cell reference differential were calculated.

2.9 | Method comparison

Method comparison assessed correlation and bias of results obtained on the cobas m 511 system vs the Sysmex XN-10 or XN-20 automated hematology analyzer. Sample processing was randomized and occurred over 2 weeks to minimize sampling bias and to capture the routine populations of each laboratory. From all sites combined, 1591 residual whole blood samples were analyzed. Correlation and bias between results were determined according to CLSI EP09-A3 guideline[11] using either a Passing-Bablok or Deming regression model as described for the mode-to-mode comparison.

2.10 | Data analysis and statistics

Microsoft Excel 2013 (Microsoft Corp., Redmond WA, USA) and R statistical software version 3.3.1 (The R Foundation for Statistical computing Vienna, Austria) were used for the statistical analysis of anonymized data.

The cobas m 511 system reports 26 parameters. For the method comparison and mode-to-mode assessments, only results that were valid from both assessments (instruments or modes) were used in the statistical analysis. If one or more individual parameters were considered invalid, the remaining valid parameters were reported and included in the dataset. All results and conclusions are from combined data from all four clinical sites unless stated otherwise.

3 | RESULTS

3.1 | Whole blood repeatability

The cobas m 511 system demonstrated high repeatability for most of the testing parameters (Table 2), with relatively lower repeatability seen for basophil and NRBC counts.

TABLE 2 Whole blood repeatability results

Parameter (units)	Sample range	Samples/ observations (N/N)	Range of sample means	Repeatability %CV (95% CI)
WBC (10^9/L)	All	144/4436	(1.98, 130.75)	1.93 (1.89, 1.97)
	<4.0 × 10^9/L	17/520	(1.98, 3.95)	3.01 (2.84, 3.21)
	≥4.0 × 10^9/L	127/3916	(4.00, 130.75)	1.85 (1.81, 1.90)
RBC (10^{12}/L)	All	144/4436	(1.92, 6.40)	0.84 (0.82, 0.86)
HGB (g/dL)	All	144/4436	(5.98, 20.26)	1.09 (1.06, 1.11)
HCT (%)	All	144/4436	(18.39, 65.15)	0.99 (0.97, 1.01)
MCV (fL)	All	144/4436	(66.36, 109.80)	0.65 (0.63, 0.66)
MCH (pg)	All	144/4436	(20.16, 36.19)	0.62 (0.61, 0.64)
MCHC (g/dL)	All	144/4436	(30.22, 34.47)	0.43 (0.42, 0.44)
RDW (%)	All	144/4436	(12.23, 27.18)	1.82 (1.78, 1.86)
RDW-SD (fL)	All	144/4436	(40.26, 83.41)	1.72 (1.68, 1.76)
PLT (10^9/L)	All	143/4405	(14.74, 936.84)	2.73 (2.67, 2.79)
	<150 × 10^9/L	31/950	(14.74, 149.61)	3.24 (3.10, 3.40)
	≥150 × 10^9/L	112/3455	(152.71, 936.84)	2.59 (2.53, 2.65)
MPV (fL)[a]	All	141/4347	(8.76, 14.91)	1.58 (1.55, 1.62)
#NRBC (10^9/L)	All	144/4436	(0.00, 1.32)	SD: 0.009 (0.009, 0.009)
	<0.25 × 10^9/L	142/4374	(0.00, 0.20)	SD: 0.007 (0.007, 0.007)
	≥0.25 × 10^9/L	2/62	(0.28, 1.32)	SD: 0.048 (0.041, 0.059)
#NEUT (10^9/L)[b]	All	142/4365	(0.87, 49.70)	3.08 (3.02, 3.15)
#LYMPH (10^9/L)[b]	All	142/4365	(0.21, 116.87)	7.52 (7.36, 7.68)
#MONO (10^9/L)[b]	All	142/4365	(0.03, 7.32)	SD: 0.132 (0.129, 0.135)
#EO (10^9/L)[b]	All	142/4365	(0.00, 0.87)	SD: 0.050 (0.049, 0.051)
#BASO (10^9/L)[b]	All	142/4365	(0.00, 0.25)	SD: 0.028 (0.028, 0.029)
WBC (10^9/L)	All (leukopenic samples)	12/371	(0.21, 2.03)	4.51 (4.21, 4.87)
RBC (10^{12}/L)	All (anemia samples)	12/371	(2.33, 3.78)	0.81 (0.76, 0.87)
HGB (g/dL)		12/371	(7.58, 9.96)	1.14 (1.06, 1.23)
HCT (%)		12/371	(22.95, 31.24)	1.06 (0.98, 1.14)
PLT (10^9/L)	All (thrombocytopenia samples)	12/372	(3.48, 51.55)	6.96 (6.48, 7.50)
#NRBC (10^{12}/L)	All (NRBC samples)	12/366	(0.40, 39.19)	1.90 (1.77, 2.05)

#, count; BASO, basophils; CI, confidence interval; CV, coefficient of variance; EO, eosinophils; HCT, hematocrit; HGB, hemoglobin; LYMPH, lymphocytes; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MONO, monocytes; MPV, mean platelet volume; NEUT, neutrophils; NRBC, nucleated red blood cells; PLT, platelets; RBC, red blood cells; RDW, red cell distribution width; RDW-SD, red cell distribution width standard deviation; SD, standard deviation; WBC, white blood cells.

[a]Only samples with ≥20 × 10^9/L PLT are used for calculation of repeatability of MPV.

[b]Only samples with ≥2.0 × 10^9/L WBC are used for calculation of repeatability of differential parameters.

Data for percentage parameters are not shown. Data are from all sites combined.

TABLE 3 Clinical sensitivity analysis of white blood cells

	Sample size (N)	TP	FP	FN	TN	Sensitivity (%)	Specificity (%)	Efficiency (%)
cobas m 511 automated differential								
Any distributional abnormality or combination of distributional abnormalities	355[a]	272	17	16	50	94.4	74.6	90.7
cobas m 511 images								
Any morphological or distributional abnormality, or combination thereof	426	351	16	15	44	95.9	73.3	92.7
cobas m 511 slides								
Any morphological or distributional abnormality, or combination thereof	438	332	17	45	44	88.1	72.1	85.8
cobas m 511 automated flags								
Any message or combination[b]	439	118	10	9	302	92.9	96.8	95.7
"Suspect blasts?"	439	76	5	0	358	100.0	98.6	98.9
"Suspect immature granulocytes?"	439	53	10	8	368	86.9	97.4	95.9
"Suspect variant lymphocytes?"	439	13	8	1	417	92.9	98.1	97.9
"Suspect left shift?"	439	27	4	46	362	37.0	98.9	88.6

FN, false negative; FP, false positive; TN, true negative; TP, true positive.

[a]Sample size for automated differential is lower because if the cobas m 511 system flagged the results with differential not measurable the system did not report results.

[b]Excluding left shift samples based on high variability seen in technologist identification of this occurrence during manual review and the limited clinical value of the left shift flagging.

The reference method was a 400-cell manual differential derived from combined results of two technologists at each site.

Data are from all sites combined.

TABLE 4 Method comparison of the cobas m 511 system vs Sysmex XN

Parameter (units)	N	Pearson's *R*	Sample range	Intercept (95% CI)	Slope (95% CI)
WBC (10^9/L)	1583	0.999	(0.04, 247.04)	0.02 (−0.01, 0.04)	1.005 (1.000, 1.009)
RBC (10^{12}/L)	1581	0.993	(1.15, 7.21)	0.00 (−0.03, 0.00)	1.000 (1.000, 1.007)
HGB (g/dL)	1574	0.991	(4.20, 21.20)	−0.38 (−0.46, −0.30)	1.055 (1.048, 1.063)
HCT (%)	1581	0.981	(13.50, 66.00)	−0.73 (−1.07, −0.41)	1.051 (1.041, 1.060)
MCV (fL)	1581	0.858	(58.20, 119.20)	2.80 (−0.50, 5.34)	1.000 (0.972, 1.037)
MCH (pg)	1566	0.951	(17.58, 40.75)	1.72 (1.17, 2.22)	0.964 (0.947, 0.983)
MCHC (g/dL)	1566	0.562	(26.59, 36.80)	17.16 (16.27, 18.13)	0.474 (0.444, 0.500)
RDW (%)	1581	0.910	(10.70, 29.40)	2.72 (2.41, 3.02)	0.852 (0.830, 0.875)
RDW-SD (fL)	1581	0.900	(32.00, 97.10)	7.89 (6.75, 8.93)	0.893 (0.868, 0.918)
PLT (10^9/L)	1533	0.982	(1.00, 1061.00)	−5.94 (−7.75, −4.08)	0.982 (0.973, 0.990)
MPV (fL)	1415	0.818	(8.00, 13.00)	−0.10 (−0.20, 0.52)	1.000 (0.937, 1.000)
#NRBC (10^9/L)	1586	0.995	(0.00, 9.59)	N/A	N/A
#NEUT (10^9/L)	1335	0.997	(0.37, 37.66)	0.05 (0.03, 0.08)	1.031 (1.025, 1.038)
#LYMPH (10^9/L)	1398	0.979	(0.02, 5.99)	−0.03 (−0.04, −0.02)	1.000 (0.993, 1.012)
#MONO (10^9/L)	1398	0.947	(0.01, 6.14)	−0.03 (−0.04, −0.01)	0.979 (0.957, 1.000)
#EO (10^9/L)	1427	0.986	(0.00, 7.17)	−0.00 (−0.01, −0.00)	1.042 (1.033, 1.051)
#BASO (10^9/L)	1513	0.679	(0.00, 0.46)	−0.02 (−0.03, −0.02)	1.646 (1.556, 1.736)
%RET (%)	1567	0.968	(0.05, 12.93)	−0.23 (−0.26, −0.20)	1.048 (1.029, 1.066)
#RET (10^{12}/L)	1559	0.951	(0.00, 0.42)	−0.01 (−0.01, −0.01)	1.027 (1.010, 1.047)
HGB-RET (pg)	1465	0.789	(16.23, 45.00)	−1.66 (−3.19, −0.26)	1.107 (1.063, 1.153)

#, count; BASO, basophils; CI, confidence interval; EO, eosinophils; HCT, hematocrit; HGB, hemoglobin; HGB-RET, reticulocyte hemoglobin; LYMPH, lymphocytes; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MONO, monocytes; MPV, mean platelet volume; NEUT, neutrophils; NRBC, nucleated red blood cells; PLT, platelets; RBC, red blood cells; RDW, red cell distribution width; RDW-SD, red cell distribution width standard deviation; RET, reticulocytes; WBC, white blood cells.
Data for percentage parameters are not shown. Data are from all sites combined.

3.2 | Reproducibility

Table S1 shows the results of various components of precision calculated using three levels of DigiMAC3® controls. The SD or %CV results for all parameters indicate that the cobas m 511 system produces reproducible results.

3.3 | Carryover

The mean percent carryover was below 0.01% for WBCs, RBCs, and PLTs and below 0.001% for blasts indicating virtually no carryover between samples, including blast cases.

3.4 | Mode-to-mode comparison

The results of the mode-to-mode comparison demonstrated satisfactory bias and correlation with Pearson's *R* values ranging from 0.674 to 0.997 for CBC and differential parameters (Table S2; %NRBCs were excluded from this analysis).

3.5 | Morphology

The overall percent agreement between the results obtained with the viewing station and the gold standard manual microscopy 100-cell reference differential was 95.6% for WBC, RBC, and PLT characteristics. For cobas m 511 slide microscopy, the overall percent agreement was 95.7%. In general, this demonstrates that the results from the cobas m 511 images and slides compare well with results obtained by technologists in routine practice using a blood smear.

3.6 | WBC clinical sensitivity

The cobas m 511 system assessments correlated well with the manual 400-cell reference differential method for detecting abnormal distribution of WBCs and the presence of morphological abnormalities. The sensitivity, specificity, and efficiency results vs the gold standard manual differential method were 94.4%, 74.6%, and 90.7%, respectively, for the automated differential and 95.9%, 73.3%, and 92.7%, respectively, for the manual image review on the viewing station (Table 3). Slightly lower values were seen for cobas m 511 slides vs the reference method. A lower efficiency was observed for monocytes with all cobas m 511 system modalities (Table S3).

The manual differential count identified 76 samples with blasts, 14 samples with variant lymphocytes, and 61 samples with immature granulocytes (IGs) as the most clinically relevant abnormal cell types. Performance testing of the cobas m 511 automated flags for the respective cell types in comparison with the manual 400-cell

reference differential count as reference revealed an overall sensitivity and specificity of 92.9% and 96.8%, respectively (Table 3). In particular, all 76 blast-positive samples identified by manual differential count were correctly flagged by the cobas m 511 system. Five other samples were flagged for the presence of blast cells by the cobas m 511 system that were not detected by manual differential count. The system also correctly flagged 13 of 14 samples with variant lymphocytes detected with manual differential count and flagged a further eight samples that were not verified by microscopy.

3.7 | Method comparison

Interinstrument comparison between the cobas m 511 and Sysmex XN systems revealed excellent correlations with Pearson's *R* values ≥0.95 for most CBC parameters (WBC, RBC, and PLT counts, HGB, hematocrit [HCT], and MCH; Table 4 and Figure 1), reticulocyte counts ($R = 0.95$), and NRBC counts ($R = 1.00$). The cobas m 511 system consistently revealed slightly higher values for HGB and HCT (slope 1.055 and 1.051, respectively). Pearson's *R* values for automated neutrophil, lymphocyte monocyte, and eosinophil counts (absolute numbers) were all ≥0.94, whereas the *R* value for absolute basophil counts was lower (0.68). Ten outlier samples from one site were excluded from the analysis after the study was completed because it had been discovered they had been rerun on the Sysmex XN system in open mode after sitting for an extended period. Improper hand mixing is thought to be the reason for the outlier values.

4 | DISCUSSION

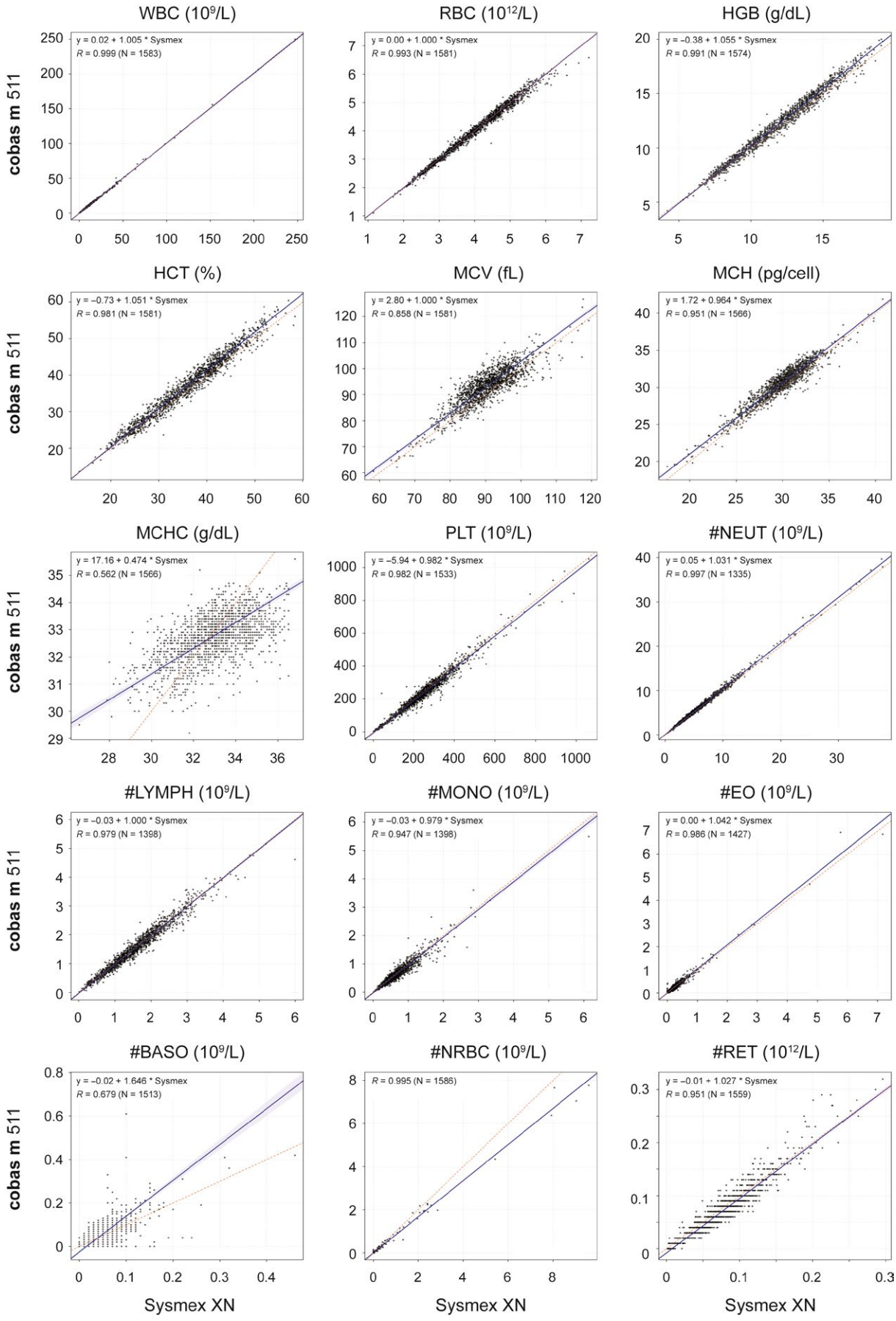
The cobas m 511 system is the first fully automated slide-based hematology analyzer that uses digital microscopy to provide numerical and cell morphology results simultaneously. This multicenter evaluation validated the performance characteristics of the cobas m 511 system. Comparison of cobas m 511 automated blood counts, automated system flags, images, slides, and morphology with standard manual microscopy—and interinstrument comparison with the Sysmex XN hematology analyzer—revealed a high *R* value representing excellent agreement. These results validate the performance of the cobas m 511 system and indicate it is suitable for routine clinical use.

The basic performance characteristics of the cobas m 511 system such as repeatability, reproducibility, carryover, and comparison between closed and open modes all demonstrated acceptable results. This demonstrates the robustness of the cobas m 511 system to provide consistent and precise results in routine diagnostic settings over time regardless of location, system, and operator.

All cobas m 511 system modalities, automated counts, automated system flags, review on viewing station, and review using cobas m 511 slides, were validated in WBC clinical sensitivity testing as compared to manual microscopy as standard method. The overall agreement between cobas m 511 automated analysis of neutrophil, lymphocyte, and eosinophil counts with microscopy was high. This indicates that the cobas m 511 system delivers reliable results for these cell types when compared to the 400-cell reference differential, thereby automating much of the blood analysis process. Comparable results were also observed in previous studies comparing differential blood counts from flow cytometry-based hematology analyzers with microscopy.[3,13,14] The lower correlation of basophil counts between automated and manual counts is largely due to statistical uncertainty, due to low basophil counts in normal samples and the lack of samples with higher basophil counts. Poor correlations between basophil counts are seen in most comparative studies of impedance and flow-based analyzers.[3,13,14]

The main function of routine hematology analyzers is to correctly identify samples containing pathological cell types with a need for further evaluation. A key requirement is that the rate of false-positive flags is low to reduce unnecessary reviews. The automated flagging of samples for the presence of blast cells and variant lymphocytes with the cobas m 511 system was very good, especially considering the cobas m 511 system is a first-of-a-kind instrument. The system identified samples with blast cells detected by microscopy with a sensitivity of 100% and a specificity of 99%. Recent publications show significant differences for blast cell flagging quality between different hematology analyzers with sensitivities ranging from 97% to 100% for the Sysmex XN system to 72% for the Beckman DxH 800 system and specificities of 93%-98%.[3,15] The cobas m 511 system automatically flagged samples for the presence of variant lymphocytes with a sensitivity of 93% and a specificity of 98% compared with microscopy, although numbers of positive samples were low (n = 14). For comparison, existing hematology analyzers demonstrated a sensitivity for variant lymphocyte flagging in the 80% range (for the Sysmex XN system), and specificities of 95%.[3,15] The cobas m 511 system correctly flagged IG-containing samples with a sensitivity of 87% and a specificity of 97%, compared with standard microscopy. For current instruments, a sensitivity in the range of 90% was demonstrated for flagging IG-containing samples (Sysmex XN system), while specificities in the range of 86% have been shown.[3,15] The sensitivity for "suspect left shift" was low

FIGURE 1 Correlation plots from method comparison analysis. Figures show Passing-Bablok regression fits except for those parameters with a large number of valid results of zero (%NRBC, #NRBC, %EO, #EO, %BASO and #BASO) which are represented by Deming regression fits. Data for percentage parameters, MPV, HGB-RET, RDW-CV, and RDW-SD are not shown. Dashed lines indicate line of identify (y = x); gray-shaded regions indicate 95% confidence bounds (calculated with the bootstrap [quantile] method for Passing-Bablok regression fits or the analytical method for Deming regression fits). Data are from all sites combined. Corresponding bias plots are shown in Figure S1. #, count; BASO, basophils; EO, eosinophils; HCT, hematocrit; HGB, hemoglobin; LYMPH, lymphocytes; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MONO, monocytes; MPV, mean platelet volume; NEUT, neutrophils; NRBC, nucleated red blood cells; PLT, platelets; RBC, red blood cells; RET, reticulocytes; WBC, white blood cells


WBC (10⁹/L)
y = 0.02 + 1.005 * Sysmex
R = 0.999 (N = 1583)
RBC (10¹²/L)
y = 0.00 + 1.000 * Sysmex
R = 0.993 (N = 1581)
HGB (g/dL)
y = −0.38 + 1.055 * Sysmex
R = 0.991 (N = 1574)
HCT (%)
y = −0.73 + 1.051 * Sysmex
R = 0.981 (N = 1581)
MCV (fL)
y = 2.80 + 1.000 * Sysmex
R = 0.858 (N = 1581)
MCH (pg/cell)
y = 1.72 + 0.964 * Sysmex
R = 0.951 (N = 1566)
MCHC (g/dL)
y = 17.16 + 0.474 * Sysmex
R = 0.562 (N = 1566)
PLT (10⁹/L)
y = −5.94 + 0.982 * Sysmex
R = 0.982 (N = 1533)
#NEUT (10⁹/L)
y = 0.05 + 1.031 * Sysmex
R = 0.997 (N = 1335)
#LYMPH (10⁹/L)
y = −0.03 + 1.000 * Sysmex
R = 0.979 (N = 1398)
#MONO (10⁹/L)
y = −0.03 + 0.979 * Sysmex
R = 0.947 (N = 1398)
#EO (10⁹/L)
y = 0.00 + 1.042 * Sysmex
R = 0.986 (N = 1427)
#BASO (10⁹/L)
y = −0.02 + 1.646 * Sysmex
R = 0.679 (N = 1513)
#NRBC (10⁹/L)
R = 0.995 (N = 1586)
#RET (10¹²/L)
y = −0.01 + 1.027 * Sysmex
R = 0.951 (N = 1559)
cobas m 511
Sysmex XN

(37.0%, Table 2), likely reflecting how the technologists defined left shift using manual microscopy (based on band counts), and the typically poor inter- and intraobserver reproducibility.[16]

The widely used Sysmex XN hematology analyzer was selected for interinstrument comparison for automatically generated numerical and differential results.[3,13-15] This comparison revealed excellent agreement between the cobas m 511 and Sysmex XN systems for most CBC parameters, WBC differential, reticulocytes, and NRBC analyses, despite the two systems using widely different technologies. A lower correlation was seen for mean corpuscular volume (MCV; $R = 0.86$) and mean corpuscular hemoglobin concentration (MCHC; $R = 0.56$). The low correlation for MCHC is not uncommon as the typical data range for this parameter is very limited which causes low R values. A recent study comparing the performance of hematology analyzers that use somewhat similar technologies demonstrated good correlations for CBC, but poorer or low agreement for reticulocyte and NRBC counts.[3]

An overall adequate agreement to standard manual microscopy was demonstrated for results achieved by review using the cobas m 511 viewing station or microscopy using cobas m 511 slides. Results of WBC, RBC, and platelet morphology assessment indicate a general agreement between the evaluation of morphology on the cobas m 511 images and the manual blood smear. Monocyte counts identified using the viewing station and cobas m 511 slide microscopy were lower than those seen with standard blood smears, and a lower agreement between eosinophil counts generated by cobas m 511 slides and standard microscopy was observed. This may be caused by the low numbers of these types of cells found in most samples. Further experience will help to clarify these results.

Several potential areas where the cobas m 511 system would be useful can be envisaged. First, automated analysis combined with digital morphology would be valuable for mid-size laboratories or for laboratories with high volumes of pathological samples with a need for microscopic review. The high analytical and flagging quality of the cobas m 511 system in relation to standard microscopy and to the Sysmex XN system presented here need to be verified in subsequent studies; however, the results suggest that the cobas m 511 system would be a valuable additional system in larger laboratories to augment flow cytometry-based systems for combined high throughput analysis. The small blood sample volume (30 μL) required by the cobas m 511 system may be useful for analyzing pediatric samples, although these were not evaluated in this study. A key strength of the cobas m 511 system compared with flow cytometry-based systems is the ability to instantly (and remotely) view and sort all the stored cell images on a cobas m 511 viewing station. The clinical value of this viewing station for digital morphology compared with existing digital imaging systems must be further validated. A potential limitation of the cobas m 511 system compared with flow cytometry-based instruments is the relatively low sample throughput, 60 samples per hour, due to the need to print and stain the slides. This may be mitigated, however, by the system's ability to enable review of potentially abnormal cases on a real-time basis.

In conclusion, the novel cobas m 511 slide-based automated hematology analyzer demonstrated very good agreement with the Sysmex XN analyzer for most parameters, despite the different technologies used. Agreement with standard manual microscopy was also very good, and basic quality performance characteristics of the cobas m 511 system were all well within acceptable limits. These results indicate that the slide-based cobas m 511 system is suitable for clinical use and may reduce laborious manual microscopy in routine hematology diagnostics.

CONFLICT OF INTEREST

Tracy I. George and Henk Russcher have received consultancy fees from Roche. Timothy R. Allen, Dan Bracco, and David J. Zahniser are employees of Roche Diagnostics and received stock options. David J. Zahniser is a coinventor of the cobas m 511 system; he holds several patents related to the technology and receives royalty payments through his partnership in Cell Imaging Systems, LLC, the company at which the technology was developed. All other authors have no conflict of interests to declare.

AUTHOR CONTRIBUTION

Mathias Bruegel, Tracy I. George, Bo Feng, and Henk Russcher performed the research. David J. Zahniser, Dan Bracco, and Timothy R. Allen designed the research study and analyzed the data. Dan Bracco coordinated the running of the study. All authors drafted and critically revised the paper and approved the final version.

REFERENCES

1. Sandhaus LM. Body fluid cell counts by automated methods. *Clin Lab Med*. 2015;35:93-103.
2. Verbrugge SE, Huisman A. Verification and standardization of blood cell counters for routine clinical laboratory tests. *Clin Lab Med*. 2015;35:183-196.
3. Bruegel M, Nagel D, Funk M, Fuhrmann P, Zander J, Teupser D. Comparison of five automated hematology analyzers in a university hospital setting: Abbott Cell-Dyn Sapphire, Beckman Coulter DxH 800, Siemens Advia 2120i, Sysmex XE-5000, and Sysmex XN-2000. *Clin Chem Lab Med*. 2015;53:1057-1071.
4. Da Costa L. Digital image analysis of blood cells. *Clin Lab Med*. 2015;35:105-122.
5. Park SH, Park C-J, Choi M-O, et al. Automated digital cell morphology identification system (CellaVision DM96) is very useful for leukocyte differentials in specimens with qualitative or quantitative abnormalities. *Int J Lab Hematol*. 2013;35:517-527.
6. Smits SM, Leyte A. Clinical performance evaluation of the CellaVision Image Capture System in the white blood cell differential on peripheral blood smears. *J Clin Pathol*. 2014;67:168-172.
7. Winkelman JW, Tanasijevic MJ, Zahniser DJ. A novel automated slide-based technology for visualization, counting, and characterization of the formed elements of blood: a proof of concept study. *Arch Pathol Lab Med*. 2017;141:1107-1112.
8. Clinical Laboratory Standards Institute. *EP28-A3c: Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory*, 3rd edn. Wayne, PA: Clinical and Laboratory Standards Institute; 2010.

9. Clinical Laboratory Standards Institute. *EP05-A3: Evaluation of Precision of Quantitative Measurement Procedures*, 3rd edn. Wayne, PA: Clinical and Laboratory Standards Institute; 2014.
10. Clinical Laboratory Standards Institute. *H26-A2: Validation, Verification, and Quality Assurance of Automated Hematology Analyzers*, 2nd edn. Wayne, PA: Clinical and Laboratory Standards Institute; 2010.
11. Clinical Laboratory Standards Institute. *EP09-A3: Measurement Procedure Comparison and Bias Estimation Using Patient Samples*, 3rd edn. Wayne, PA: Clinical and Laboratory Standards Institute; 2013.
12. Clinical Laboratory Standards Institute. *H20-A2: Reference Leukocyte (WBC) Differential Count (Proportional) and Evaluation of Instrumental Methods*, 2nd edn. Wayne, PA: Clinical and Laboratory Standards Institute; 2007.
13. Tan BT, Nava AJ, George TI. Evaluation of the Beckman Coulter UniCel DxH 800, Beckman Coulter LH 780, and Abbott Diagnostics Cell-Dyn Sapphire hematology analyzers on adult specimens in a tertiary care hospital. *Am J Clin Pathol*. 2011;135:939-951.
14. Meintker L, Ringwald J, Rauh M, Krause SW. Comparison of automated differential blood cell counts from Abbott Sapphire, Siemens Advia 120, Beckman Coulter DxH 800, and Sysmex XE-2100 in normal and pathologic samples. *Am J Clin Pathol*. 2013;139:641-650.
15. Hotton J, Broothaers J, Swaelens C, Cantinieaux B. Performance and abnormal cell flagging comparisons of three automated blood cell counters: Cell-Dyn Sapphire, DxH-800, and XN-2000. *Am J Clin Pathol*. 2013;140:845-852.
16. Cornbleet PJ. Clinical utility of the band count. *Clin Lab Med*. 2002;22:101-136.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Bruegel M, George TI, Feng B, et al. Multicenter evaluation of the cobas m 511 integrated hematology analyzer. *Int J Lab Hem*. 2018;40:672–682. https://doi.org/10.1111/ijlh.12903

Journal of Chromatography B, 1022 (2016) 173–182

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Ferromagnetic particles as a rapid and robust sample preparation for the absolute quantification of seven eicosanoids in human plasma by UHPLC–MS/MS

Anna Catharina Suhr*,[1], Mathias Bruegel[1], Barbara Maier, Lesca Miriam Holdt, Alisa Kleinhempel, Daniel Teupser, Stefanie H. Grimm, Michael Vogeser

Institute of Laboratory Medicine, Hospital of the Ludwig-Maximilians-University Munich, Marchioninistrasse 15, 81377 Munich, Germany

ARTICLE INFO

Article history:
Received 4 September 2015
Received in revised form 24 February 2016
Accepted 19 March 2016
Available online 21 March 2016

Keywords:
Magnetic particles
Eicosanoids
Sample preparation
Liquid chromatography
Mass spectrometry
Validation

ABSTRACT

We used ferromagnetic particles as a novel technique to deproteinize plasma samples prior to quantitative UHPLC–MS/MS analysis of seven eicosanoids [thromboxane B_2 (TXB_2), prostaglandin E_2 (PGE_2), PGD_2, 5-hydroxyeicosatetraenoic acid (5-HETE), 11-HETE, 12-HETE, arachidonic acid (AA)]. A combination of *ferromagnetic particle enhanced deproteination* and subsequent *on-line solid phase extraction* (on-line SPE) realized quick and convenient semi-automated sample preparation—in contrast to widely used manual SPE techniques which are rather laborious and therefore impede the investigation of AA metabolism in larger patient cohorts.

Method evaluation was performed according to a protocol based on the EMA guideline for bioanalytical method validation, modified for endogenous compounds. Calibrators were prepared in ethanol. The calibration curves were found to be linear in a range of 0.1–80 ng mL^{-1} (TXB_2, PGE_2, PGD_2), 0.05–40 ng mL^{-1} (5-HETE, 11-HETE), 0.5–400 ng mL^{-1} (12-HETE) and 25–9800 ng mL^{-1} (AA). Regarding all analytes and all quality controls, the resulting precision data (inter-assay 2.6 %–15.5 %; intra-assay 2.5 %–15.1 %, expressed as variation coefficient) as well as the accuracy results (inter-assay 93.3 %–125 %; intra-assay 91.7 %–114 %) were adequate. Further experiments addressing matrix effect, recovery and robustness, yielded also very satisfying results.

As a proof of principle, the newly developed LC–MS/MS assay was employed to determine the capacity of AA metabolite release after whole blood stimulation in healthy blood donors. For this purpose, whole blood specimens of 5 healthy blood donors were analyzed at baseline and after a lipopolysaccharide (LPS) induced blood cell activation. In several baseline samples some eicosanoids levels were below the Lower Limit of Quantification. However, in the stimulated samples all chosen eicosanoids (except PGD_2) could be quantified.

These results, in context with those obtained in validation, demonstrate the applicability of ferromagnetic particles for the sample preparation for eicosanoids in human plasma. Thus, we conclude that *ferromagnetic particle enhanced deproteination* is a promising novel tool for sample preparation in LC–MS/MS, which is of particular interest for automation in clinical mass spectrometry, *e.g.* in order to further address eicosanoid analysis in larger patient cohorts.

© 2016 Elsevier B.V. All rights reserved.

1. Introduction

In the constantly growing field of metabolomics, the lipidomics section plays an important role, addressing a large group of lipid mediators. Among these mediators are the oxylipins, which include polyunsaturated fatty acids (PUFA) and their various metabolites, also referred to as eicosanoids. These lipid mediators are synthesized from arachidonic acid (AA) and other polyunsaturated fatty acids by cyclooxygenases (COXs), lipoxygenases (LOXs) and cytochrome P450 monooxygenases (CYPs), but they might also be generated through the non-enzymatic oxidation of unsaturated fatty acids [1].

Several oxylipins affect processes in health and disease, such as inflammation, coagulation, immune response and smooth muscle cell tonus, with partial opposing effects [2]. Thus, a quantita-

* Corresponding author.
E-mail address: anna.suhr@med.uni-muenchen.de (A.C. Suhr).
[1] Both authors contributed equally to this work.

http://dx.doi.org/10.1016/j.jchromb.2016.03.022
1570-0232/© 2016 Elsevier B.V. All rights reserved.

tive multiparametric analysis of these compounds might have implications for the understanding of disease pathologies in various disciplines, such as neurology, cardiology, oncology, or pulmonology [3], as well as to determine the potential implementation of these compounds as biomarkers. However, the disease-associated changes and the diagnostic potential of these parameters have been poorly investigated so far, due to analytical difficulties and chemical and biological complexities. Astarita et al. recently reported in their review that the analysis of eicosanoids bears three major difficulties: low concentrations in human fluids, limited stability, and a large number of isomeric species [4]. Further preanalytical variables, *e.g.* possible *ex vivo* formation of eicosanoids, have to be taken into account [5].

Various assays based on different techniques have been developed. In recent years, liquid chromatography tandem mass spectrometry (LC–MS/MS) has become the most widely used technique for the quantification of eicosanoids, as the selectivity of this method and the less labour-intensive sample preparation are advantageous compared with previously used immunoassays or gas chromatography mass spectrometry (GC–MS) [6]. Some comprehensive profiling methods for the metabolomics of oxylipins have been previously described [6–11]. Solid phase extraction (SPE) has been used for sample preparation in the majority of LC–MS/MS methods, as this technique facilitates analyte enrichment and provides relatively clean extracts. Nevertheless, SPE is relatively expensive and requires multiple time-consuming steps, which makes this approach unsuitable for larger study cohorts.

Deproteination by means of ferromagnetic particles could be a promising alternative for this purpose, since it is a rapid procedure suitable for a large number of samples [12]. Additionally there is no need for centrifugation, vacuum, or pressure - steps usually impeding automation - and it can be combined with on-line SPE. A major advantage of *ferromagnetic particle enhanced deproteination* is the generic character of this technique. In contrast to liquid-liquid extraction or solid-phase extraction, the chemistry of the analyte is of minor importance since the particles primarily bind to the denatured proteins. Thus, this technique is a promising approach for multi-analyte panels involving molecules with a broad variability of physico-chemical properties, such as eicosanoids. Due to all these facts and our previous experience with this technique [12] we were encouraged to employ *ferromagnetic particle enhanced deproteination* to more challenging multiparametric LC–MS/MS assays for endogenous compounds.

The central aim of this study was the evaluation of *ferromagnetic particle enhanced deproteination* as a suitable tool in the analysis of eicosanoids. Thromboxane B_2 (TXB_2), prostaglandin E_2 (PGE_2), PGD_2, 11-hydroxyeicosatetraenoic acid (11-HETE), 12-HETE, 5-HETE, and AA were selected as parameters of interest in the present study as they are central representatives of COX or LOX pathway (Fig. 1. Additionally, these analytes were identified as potential biomarkers in sepsis, as an extreme type of inflammatory disease, making them particularly suitable for further investigation in large scale clinical studies [13–15].

Due to low abundance, pulsatile secretion and short half-life, a reliable quantification of eicosanoids in whole blood is very demanding. Therefore, it is often recommended to analyse eicosanoids in urine [5]. However, quantitation in urine is highly affected by urine concentration and in many patient groups, *e.g.* sepsis patients who are of particular interest for eicosanoid analysis, urine production is reduced or completely missing. Hence, in order to deal with these issues, eicosanoids were analyzed after lipopolysaccharide (LPS) whole blood activation as a model of inflammation [16]. A comparable application of whole blood stimulation assays was used for the evaluation of cytokine release [17]

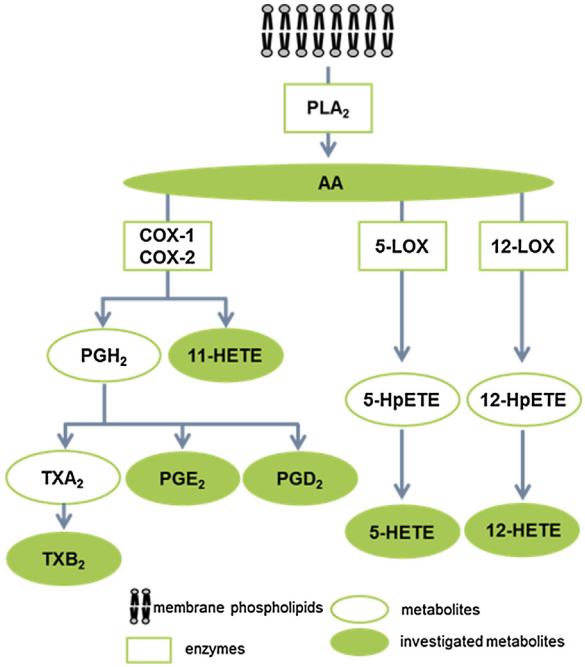


Fig. 1. Schematic representation of established metabolites and corresponding enzymes associated with arachidonic acid (AA) metabolism: released from membrane phospholipids, AA is the general precursor of eicosanoids and might also play an active role in inflammatory disease. 11-HETE directly reflects the activity of COX, thus representing a major eicosanoid pathway. Additionally this compound is differentially released in severe inflammatory diseases, such as sepsis. TXB_2, PGD_2 and PGE_2 represent important COX-associated downstream pathways. Together with 5-HETE and 12-HETE (representing 5-LOX and 12-LOX pathway), these compounds play a pathogenic role in milder inflammations, *e.g.*, occurring in cardiovascular disease.
(COX-1, cyclooxygenase 1; COX-2, cyclooxygenase 2; 5-HETE, 5-hydroxyeicosatetraenoic acid; 11-HETE, 11-hydroxyeicosatetraenoic acid, 12-HETE, 12-hydroxyeicosatetraenoic acid; 5-HpETE, 5-hydroperoxyeicosatetraenoic acid; 12-HpETE, 12-hydroperoxyeicosatetraenoic acid; 5-LOX, 5-lipoxygenase; 12-LOX, 12-lipoxygenase; PGD_2, prostaglandin D_2; PGE_2, prostaglandin E_2; PGH_2, prostaglandin H_2; PLA_2, phospholipase A_2; TXA_2, thromboxane A_2; TXB_2, thromboxane B_2).

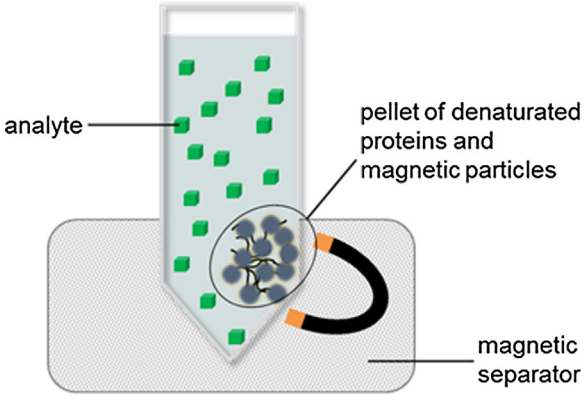


Fig. 2. Scheme of *ferromagnetic particle enhanced deproteination* using the magnetic separator.

and as well by our group for evaluation of eicosanoids in the field of sepsis [15].

As a proof of principle approach, to verify the applicability of the method to clinical samples, the novel LC–MS/MS assay including *ferromagnetic particle enhanced deproteination* was employed to investigate the capacity of AA metabolite release after whole blood stimulation in healthy blood donors.

2. Experimental

2.1. Materials

TXB_2, PGD_2, PGE_2, 5-HETE, 11-HETE, 12-HETE, AA, TXB_2-d_4, PGD_2-d_4, PGE_2-d_4, 5-HETE-d_8, 12-HETE-d_8, AA-d_8, and licofelone were obtained from Cayman Chemicals (Ann Arbor, Michigan, USA). Acetylsalicylic acid, ascorbic acid, and butylhydroxytoluol were purchased from Sigma-Aldrich (Steinheim, Germany). The ferromagnetic particles (MagSi-TOX^{PREP} Type I) were obtained from Magna Medics (Geleen, The Netherlands). Acetonitrile (ACN), methanol (MeOH), water, and acetic acid were of ULC/MS grade and were obtained from Biosolve (Valkenswaard, The Netherlands). HPLC-grade ethanol (EtOH) was purchased from Merck (Darmstadt, Germany).

2.2. Preparation of primary standard and internal standard solutions

Stock solutions were prepared in EtOH at a nominal concentration of 50 mg L^{-1} for 11-HETE, 5000 mg L^{-1} for AA and 100 mg L^{-1} for the remaining analytes. Aliquots of the stock solutions were sealed in glass vials under nitrogen and stored at −80°C.

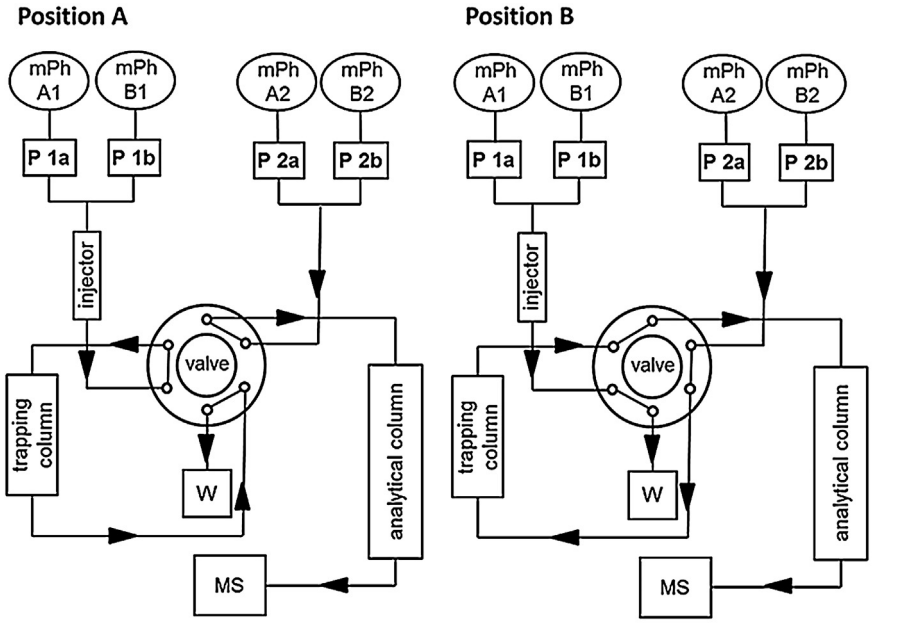
A master calibration solution (TXB_2 990 ng mL^{-1}, PGD_2 990 ng mL^{-1}, PGE_2 979 ng mL^{-1}, 5-HETE 490 ng mL^{-1}, 11-HETE 490 ng mL^{-1}, 12-HETE 4,900 ng mL^{-1}, and AA 245,000 ng mL^{-1}) was used to prepare eight multi-calibrators (Cal) by serial dilution in EtOH (see Table 1 for exact concentrations).

Deuterated analogs of the analytes were combined to an ethanol based *internal standard working solution*, resulting in the following concentrations: 12.3 ng mL^{-1} for TXB_2-d_4, PGD_2-d_4, and PGE_2-d_4; 24.5 ng mL^{-1} for 5-HETE-d_8 and 12-HETE-d_8; and 1960 ng mL^{-1} for AA-d_8. Because there was no commercially available stable isotope labeled analog for 11-HETE, 12-HETE-d_8 was used.

Table 1
Concentrations (ng mL^{-1}) of calibrators and controls.

	TXB_2	PGE_2	PGD_2	5-HETE	11-HETE	12-HETE	AA
Cal 1	0.099	0.098	0.099	0.049	0.049	0.490	24.5
Cal 2	0.198	0.196	0.198	0.098	0.098	0.980	49.0
Cal 3	0.396	0.392	0.396	0.196	0.196	1.960	98.0
Cal 4	0.990	0.979	0.990	0.490	0.490	4.90	245
Cal 5	2.48	2.45	2.48	1.23	1.23	12.3	613
Cal 6	9.90	9.79	9.90	4.90	4.90	49.0	2450
Cal 7	39.6	39.2	39.6	19.6	19.6	196	9800
Cal 8	79.2	78.3	79.2	39.2	39.2	392	(19600)
QC L	0.297	0.294	0.297	0.147	0.147	1.47	73.5
QC M	2.97	2.94	2.97	1.47	1.47	14.7	735
QC H	29.7	29.4	29.7	14.7	14.7	147	7350

Cal, calibrator; QC, quality control; L, low; M, medium; H, high; () excluded after validation.



Solvents delivered by pumps 1a/b

time [min]	solvent flow [mL/min]	mobile phase A1 [%]	mobile phase B1 [%]
0	2.0	100	0
0.6	2.0	100	0
0.7	0.1	100	0
4.8	0.1	100	0
4.9	0.1	0	100
5.1	2.0	0	100
6	2.0	0	100
6.2	2.0	100	0
7.5	2.0	100	0

Solvents delivered by pumps 2a/b

time [min]	solvent flow [mL/min]	mobile phase A2 [%]	mobile phase B2 [%]
0	0.5	60	40
2.5	0.5	60	40
2.7	0.5	30	70
3.7	0.5	30	70
3.8	0.5	10	90
5.8	0.5	10	90
6	0.5	60	40
7.5	0.5	60	40

Fig. 3. Schematic plan of the UHPLC–MS/MS system and corresponding solvent flows (mPh, mobile phase; MS, mass spectrometer; P, pumps; W, waste).

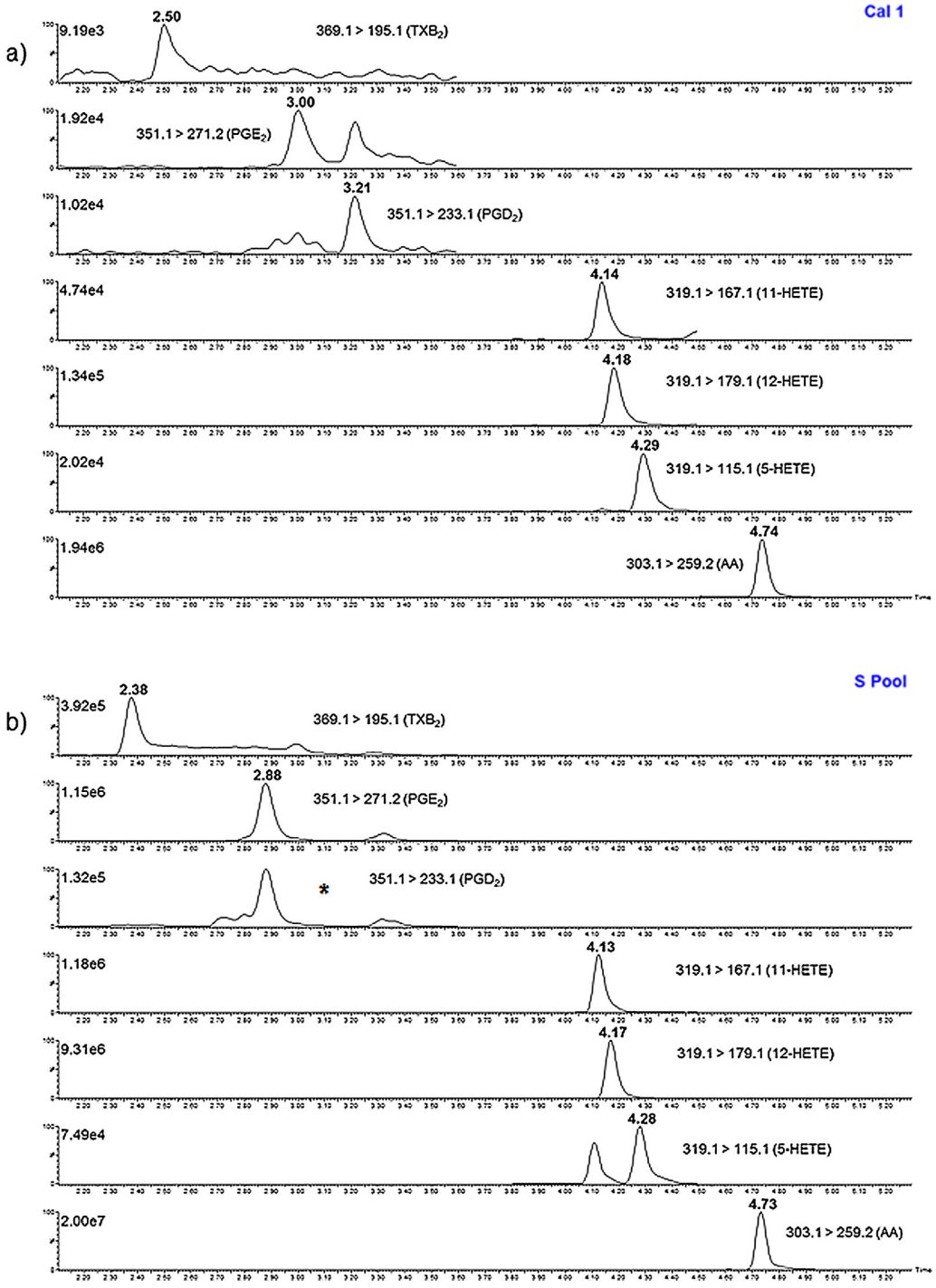


Fig. 4. Exemplary chromatograms of (a) Calibrator 1 (Cal 1), representing the LLOQ, and of (b) the stimulated pool employed as quality control (S Pool). (*, not detected).

2.3. In vitro *stimulation of human whole blood*

For the *in vitro* stimulation we used our previously described whole blood stimulation assay [15]: Briefly, 1 mL of heparinized whole blood was transferred into 6-well cell culture plates (tissue culture plate 6-well, Sarstedt, Nümbrecht, Germany), mixed with 0.5 mL RPMI 1640 medium (Biochrom, Berlin, Germany) containing 1% (m/v) penicillin/streptomycin (both Gibco life technologies, Darmstadt, Germany). A part of this mixture was spiked with LPS (from *Escherichia coli*; Sigma-Aldrich, St. Louis, USA). Whole blood medium mixture without LPS was immediately centrifuged at 4000 *g* for 10 min, serving as baseline. The aliquots containing LPS (final concentration of 100 ng mL^{-1}) were subsequently incubated at 37 °C and 5% CO_2. After 24 h of incubation, these aliquots were centrifuged as described above. All resulting supernatants were stored at −80 °C until quantitative analysis by UHPLC–MS/MS.

2.4. *Quality control samples*

Two quality control (QC) materials were used, ethanol and plasma. Three QC levels were spiked in ethanol at low (QC L), medium (QC M), and high (QC H) concentrations within the calibration range. The exact concentrations are summarized in Table 1.

The second QC type was based on the authentic matrix. Therefore a pool of heparinized whole blood from blood donors was used for *in vitro* stimulation as described in Section 2.3. The resulting baseline sample (Baseline Pool, "B Pool") and the resulting sample which was stimulated with LPS (Stimulated Pool, "S Pool") were each used as authentic matrix QCs.

2.5. Sample preparation

A 100 μL aliquot of the sample (calibrator, QC, Pool, or unknown) was transferred into a 1.5 mL Safe-Lock Tubes™ (Eppendorf, Hamburg, Germany) and mixed with 25 μL of *internal standard working solution* for 5 min on a horizontal shaker. The ferromagnetic bead suspension was re-suspended by vortexing vigorously. Subsequently, 40 μL of the ferromagnetic bead suspension was added to the sample, and the tube was vortexed again. To denature the proteins, 300 μL of ACN were added to the sample-bead mixture, and the tube was vortexed at high speed for at least 10 s to facilitate the binding of the denatured proteins to the surface of the particles. When the tube was placed on the magnetic separator (Magna Medics) the ferromagnetic particles together with the previously bound proteins were magnetically attracted to the permanent magnet integrated in this separator forming a pellet at the tube wall facing the magnet. This is illustrated by Fig. 2. After 1 min, 200 μL of the resulting supernatant were carefully transferred to a 2.0 mL Safe-Lock Tube™ (Eppendorf) without disturbing the pellet. Subsequently, the obtained supernatant was dried in a heat block (40°C) under a gentle stream of nitrogen. After resolving the residue in 100 μL of MeOH/H_2O (50/50, v/v), 85 μL of this mixture were transferred to a brown glass vial containing a micro-insert (both Chromatographie Handel Müller, Fridolfing, Germany) and placed into the autosampler.

2.6. UHPLC–MS/MS

The UHPLC–MS/MS system comprised a Xevo TQ-S and an Acquity UPLC, including an autosampler, a switching valve, a column oven and two pairs of pumps (all Waters, Milford, Massachusetts, USA). Mass Lynx V4.1 (Waters) software was used to control the system.

Fig. 3 shows a scheme of the entire configuration with the trapping column (Oasis HLB Direct Connect 20 μm, 2.1 × 30 mm, Waters), the analytical column (Acquity UPLC BEH Shield RP18 1.7 μm, 2.1 × 100 mm, Waters) and the two potential switching positions.

The column oven for the analytical column was maintained at 55°C. The mobile phases for the trapping column were A1, H_2O/MeOH (90/10, v/v) and B1, ACN. For the analytical column, the mobile phases were A2, 0.025 % (v/v) acetic acid in H_2O (pH∼3.5) and B2, ACN/MeOH (75/25, v/v). Details of the gradients are provided in Fig. 3. A total of 20 μL of the sample, prepared as described in Section 2.5, was injected into the UHPLC system in position A, where the analytes were retained on the trapping column (online SPE). The valve was switched to position B after 0.7 min, and by back-flushing the trapping column the analytes were eluted onto the analytical column for chromatographic separation. After 5.0 min, the valve was switched back to position A, and both columns were re-equilibrated. The entire run time, including re-equilibration, was 7.5 min.

Ionization was performed by electrospray in the negative mode with a capillary voltage of −2.0 kV. The cone voltage was 40 V, the source temperature was 150°C, the desolvation temperature was 600°C, the cone gas flow was 150 L/h, and the desolvation gas flow was 1000 L/h. The MS parameters, particularly the collision energy (see Table 2), were optimized for all analytes using post-column infusion of neat solutions. Multiple reaction monitoring (MRM) was employed: for each analyte two mass transitions were recorded, and for each internal standard one mass transition was recorded. Detailed information is provided in Table 2. To achieve an acceptable dwell time, the MRMs were scheduled.

The quantification method (Waters QuanLynx™ based) included a linear regression with a weighting function of $1/x^2$ and the exclusion of the origin. The analytes were quantified *via* the quotient of the area of the first transition (quantifier) and the area of the internal standard. The second transition (qualifier) was used to confirm the retention time of the analyte but did not contribute to the quantification results. The peaks were smoothed using the mean smoothing algorithm with two iterations and a smoothing width of three.

2.7. Evaluation of analytical performance

We based our evaluation protocol on the *Guidelines of bioanalytical method validation* of the European Medicines Agency (EMA) [18] and in part, also on the CLSI (Clinical and Laboratory Standards Institute) guideline (*Liquid Chromatography-Mass Spectrometry Methods; Approved Guideline, CLSI document C62-A*) [19].

The ethanol-based controls (QC L, QC M, and QC H) and the plasma pools (B Pool and S Pool) were employed as quality controls in several experiments, as detailed below.

2.7.1. Linearity

Aliquots of the eight calibrators were processed as described in Section 2.5 and injected at the beginning of each batch. The data analysis was performed as previously described in Section 2.6, and the correlation coefficient and the slope of the calibration curves were monitored during the validation period.

2.7.2. Accuracy and precision

The accuracy and the precision were assessed with one aliquot of each QC in five independent series (inter-batch). Correspondingly, for intra-batch accuracy and precision, five aliquots of each QC were individually processed and measured in one run. To determine the sole precision of the UHPLC–MS/MS method, multiple injections (n = 5) of one and the same processed aliquot of QC L and of QC H respectively were performed.

Since the nominal concentrations of the eicosanoids in the matrix-based controls B Pool and S Pool are not available due to the unknown endogenous amount of the respective target analytes, only the precision values were accessed for this type of QC.

2.7.3. Stability

The stability of the eicosanoids in plasma as well as in ethanol was investigated for up to one month at different storing conditions in various container materials, *i.e.* polypropylene and glass. In detail, 1.5 mL Safe-Lock Tubes™ (Eppendorf), Matrix™ 0.5 mL ScrewTop Tubes for bio-banking (Thermo Scientific, Waltham, Massachusetts, USA), and 1.5 mL brown glass vials (Chromatographie Handel Müller) were employed to investigate analyte stability at −80°C. For storage at room temperature (+20°C) comprising light exposure as well as for storage in the refrigerator (+4°C) exclusively the 1.5 mL Safe-Lock Tubes™ (Eppendorf) were used.

Furthermore the stability of the processed samples was examined in the autosampler, *e.g.*, during a night batch at +8°C. The post-preparation stability at +20°C was also assessed.

Additionally, the stability of the intermediates of the sample preparation was investigated, using the deproteinated supernatant and the dried extracts, respectively.

2.7.4. Robustness and ruggedness

The dilution integrity was assessed for values inside the calibration range (S Pool) and for those outside the calibration range, as

Table 2
MS/MS Parameters for the eicosanoids and corresponding internal standards.

Analyte	Corresponding internal standard	Retention time [min]	Precursor ion [*m*/*z*]	Quantifier transition		Qualifier transition		Dwell time [s]
				Product ion [*m*/*z*]	CE [eV]	Product ion [*m*/*z*]	CE [eV]	
TXB_2	TXB_2-d_4	2.53	369.1	195.1	14	169.1	16	0.032
PGE_2	PGE_2-d_4	3.03	351.1	271.2	16	315.2	12	0.032
PGD_2	PGD_2-d_4	3.23	351.1	233.1	12	271.2	16	0.032
5-HETE	5-HETE-d_8	4.32	319.1	115.1	14	203.2	16	0.028
11-HETE	12-HETE-d_8	4.17	319.1	167.1	14	149.1	20	0.028
12-HETE	12-HETE-d_8	4.20	319.1	179.1	14	208.2	14	0.028
AA	AA-d_8	4.76	303.1	259.2	14	205.2	14	0.080
TXB_2-d_4		2.51	373.1	173.1	16			0.032
PGE_2-d_4		3.03	355.1	275.2	16			0.032
PGD_2-d_4		3.23	355.1	275.2	16			0.032
5-HETE-d_8		4.29	327.1	116.1	16			0.028
12-HETE-d_8		4.18	327.1	184.1	14			0.028
AA-d_8		4.75	311.1	267.2	14			0.080

CE, collision energy.

recommended in CLSI C62-A [19]. To simulate the latter case, an extra high ethanol-based control containing more than five times the amount of eicosanoids of Cal 8 was prepared. The *extra high QC* and the S Pool were processed and analyzed in triplicate without dilution. Subsequently, the samples were diluted with MeOH/H_2O (50/50, v/v) before and after sample preparation; *i.e.*, three aliquots of both QCs were diluted prior to sample preparation; three other aliquots were processed normally as previously described, and the resolved residues were subsequently diluted.

The influence of different column lots was investigated by analyzing the same set of calibrators and controls twice on the same day. For each run a different lot of the analytical column was employed.

An injection of MeOH after the highest calibrator was used to assess the carry-over. By injecting the calibrators from highest to lowest the influence of potential carry-over on quantification accuracy was further investigated.

Additionally, the inter-operator variability was tested. For this purpose, the first operator, performing the evaluation experiments, and a second operator with no previous experience handling magnetic particles both prepared the same defined set of control samples (n = 10) and the results were compared regarding precision and accuracy.

2.7.5. Matrix effect and recovery

The experiments described in the following were based on the recommendation of CLSI guideline C62-A [19], with slight modifications.

Experiments for the recovery and the matrix effect were performed according to Matuszewski et al. [20] using pre- and post-spiking in seven different lots of plasma. The matrix effect was calculated in consideration of the respective baseline value ($c_{baseline}$) of eicosanoids present in each plasma lot. The baseline values were determined by analyzing unspiked samples (n = 5 for each lot).

$$\text{Recovery} = 100\% \times c_{\text{plasma pre}}/c_{\text{plasma post}} \quad (1)$$

$$\text{Matrix effect} = 100\% \times (c_{\text{plasma post}} - c_{\text{baseline}})/c_{\text{solvent}} \quad (2)$$

According to the EMA *Guideline on bioanalytical method validation*, we also calculated the *IS normalised matrix factor* (MF_{norm}) using the Eqs. (3) and (4):

$$MF = \text{Area}_{\text{matrix}}/\text{Area}_{\text{solvent}} \quad (3)$$

$$MF_{norm} = MF_{\text{analyte}}/MF_{IS} \quad (4)$$

As recommended in the guideline, we subsequently calculated the variation coefficient of the MF_{norm} for each analyte.

Furthermore, we performed post-column infusion according to Bonfiglio et al. [21]. Processed, unspiked plasma samples (containing only the baseline value of eicosanoids) were injected onto the column, while a solution of all analytes and all internal standards was infused directly into the mass spectrometer *via* T-tubing using a syringe pump. The different matrix lots used were either anticoagulated with heparin or EDTA, respectively.

A matrix mixing experiment referring to the CLSI guideline C62-A was the third part of the matrix effect determination. We mixed the S Pool with MeOH/H_2O (50/50, v/v) in different proportions (25/75, 50/50, 75/25, v/v), *e.g.*, 25 μL of MeOH/H_2O mixture and 75 μL of S Pool. Based on the previously determined concentrations of the analytes in undiluted S Pool samples, the theoretical concentrations resulting from dilution were calculated. After LC–MS/MS analysis of the dilutions, the determined concentrations of the analytes were plotted against their respective calculated theoretical concentrations. A linear regression of the plot was performed. Moreover the matrix mixing experiment was repeated with a second lot of stimulated plasma obtained from a different blood donor.

2.8. In vitro *stimulation of specimen from healthy blood donors*

As a proof of principle approach this assay was applied to investigate the release capacity of the targeted eicosanoids (TXB_2, PGE_2, PGD_2, 5-HETE, 11-HETE, 12-HETE, AA) in healthy blood donors (n = 5) using the aforementioned whole blood activation model (Section 2.3). Therefore, whole blood of healthy blood donors was stimulated according to Section 2.3 and subsequently analyzed according to Sections 2.5 and 2.6.

3. Results and discussion

3.1. Method development

3.1.1. Calibration

Unfortunately, it was not possible to generate calibrators in the authentic matrix for two reasons. First, eicosanoid-free plasma was not commercially available. Also, several attempts to reduce the endogenous eicosanoid levels using physical approaches, *e.g.*, UV light, were not successful. A baseline value of relevant height was inevitable in authentic matrix, at least in the case of AA.

Secondly, we observed that after spiking with a mixture of the seven target eicosanoids, the resulting peak areas for PGD_2, 5-HETE, 11-HETE, and 12-HETE were markedly higher in plasma compared to corresponding peak areas in ethanol. The effect could not be easily explained, neither by the endogenous level nor by different solubilities, since ethanol is one of the optimal sol-

vents for eicosanoids [6]. The oxidation of AA to HETEs through oxygen from the surrounding atmosphere was also excluded, as this effect should also occur in ethanol or water. Consequently, this phenomenon was further investigated in spiking experiments at different concentration levels. Therefore each eicosanoid was individually spiked into plasma and ethanol, respectively. This experiment revealed that the sole addition of AA to plasma, led to increased concentrations of several other metabolites in a concentration-dependent manner (see Supplementary material, Fig. S1). All the other metabolites individually spiked did not affect the concentrations of other measured eicosanoids included in the assay. Tsikas et al. described in their review the formation of eicosanoids during and after blood sampling in whole blood samples [5]. However we did not expect this phenomenon to occur in plasma since it is free of platelets and other cells able to produce eicosanoids.

In the next step, we investigated the influence of antioxidants (butylhydroxytoluol, ascorbic acid) and inhibitors of COX and/or LOX (acetylsalicylic acid, licofelone) on this distorting formation of eicosanoids induced by the addition of AA to plasma. With the exception of ascorbic acid (at a final concentration of 1%, m/v) no reduction of the formation of PGD_2, 11-HETE, 12-HETE, and 5-HETE caused by AA spiking was observed (see Supplementary material, Fig. S2). Since even high concentrations of ascorbic acid could not suppress completely this adverse formation of other target eicosanoids, we decided to prepare the calibrators in a surrogate matrix, *i.e.* ethanol, to obtain reliable nominal concentrations.

3.1.2. Sample preparation

The main aim of high-throughput capability for the UHPLC–MS/MS method focused on a sample preparation involving a rapid, effective deproteination step in combination with an on-line SPE procedure involving a trapping column.

During our method development preliminary experiments with *ferromagnetic particle enhanced deproteination* demonstrated reproducible results for eicosanoids. Nevertheless, the high percentage of acetonitrile, necessary for protein precipitation, in the resulting supernatant caused a suboptimal peak shape. The dilution of the supernatant with water, a common approach to reduce the organic content prior to LC–MS/MS analysis, was in our case no option due to the low biological abundance of eicosanoids. Therefore, the supernatant was evaporated under a stream of nitrogen, and the residue was resolved in a different solvent. The temperature during evaporation and the resolving mixture were optimized, with respect to recovery and peak shape: good results were achieved for 40°C and a mixture of 50 % methanol in water.

Although application of on-line SPE has been described in the field of eicosanoid analysis for about thirty years [22], this technique has been rarely employed for these substances so far. Korecka et al. [23] and Willenberg et al. [24] both used on-line SPE for sample preparation, however, either investigated a rather small analyte panel of only two [23] respectively three [24] eicosanoids. Kita et al. combined on-line SPE with a manually, time and labour intensive off-line SPE step [25]. Kortz et al. employed a combination of manual protein precipitation and on-line SPE on a trapping column for sample preparation [11]. Unfortunately, this approach was accompanied with limited recovery rates for some analytes.

A combination of *ferromagnetic particle enhanced deproteination* and on-line SPE seemed a promising alternative to the sample preparation techniques described so far for eicosanoids. This approach combines a fast sample preparation with clean extracts suitable for LC–MS/MS analysis and at the same time, the generic character allows an application to a broad variety of analytes. This assumption was strengthened by our results for matrix effect and recovery for the chosen seven eicosanoids (TXB_2, PGE_2, PGD_2, 5-HETE, 11-HETE, 12-HETE, AA; for further information, see Section 3.2.5).

Table 3
Results for accuracy and precision.

Sample	LLOQ	QC L	QC M	QC H	B Pool	S Pool
TXB_2						
Target concentration [ng mL^{-1}]	0.099	0.297	2.97	29.7	(<0.099)*	(~4.90)
Accuracy inter-assay [%]	100	104	104	109		
Accuracy intra-assay [%]	99.4	103	102	105		
CV inter-assay [%]	19.9	10.4	4.8	4.0		5.2
CV intra-assay [%]	11.4	11.2	4.9	3.8		2.5
CV injection [%]	9.3	4.2	nd	4.8	nd	nd
PGE_2						
Target concentration [ng mL^{-1}]	0.098	0.294	2.94	29.4	(<0.098)*	(~6.72)
Accuracy inter-assay [%]	98.5	108	105	113		
Accuracy intra-assay [%]	98.7	101	101	104		
CV inter-assay [%]	8.3	8.5	6.7	6.8		6.7
CV intra-assay [%]	8.9	5.2	4.6	5.3		5.2
CV injection [%]	6.2	8.0	nd	1.8	nd	nd
PGD_2						
Target concentration [ng mL^{-1}]	0.099	0.297	2.97	29.7	(<0.099)*	(<0.099)*
Accuracy inter-assay [%]	99.8	101	104	111		
Accuracy intra-assay [%]	88.9	94.3	95.7	96.8		
CV inter-assay [%]	8.7	6.1	2.9	5.3		
CV intra-assay [%]	7.4	7.6	6.3	3.9		
CV injection [%]	11.4	5.1	nd	1.6	nd	nd
5-HETE						
Target concentration [ng mL^{-1}]	0.049	0.147	1.47	14.7	(~0.097)	(~0.398)
Accuracy inter-assay [%]	101	110	95.6	125		
Accuracy intra-assay [%]	108	94.0	91.7	97.4		
CV inter-assay [%]	9.2	12.7	15.2	15.5	18.7	14.6
CV intra-assay [%]	7.9	13.1	15.1	9.9	5.5	6.1
CV injection [%]	9.5	5.3	nd	1.3	nd	nd
11-HETE						
Target concentration [ng mL^{-1}]	0.049	0.147	1.47	14.7	(<0.049)*	(~1.61)
Accuracy inter-assay [%]	95.9	104	95.0	117		
Accuracy intra-assay [%]	96.3	93.3	92.8	99.1		
CV inter-assay [%]	5.2	7.0	9.9	12.2		14.6
CV intra-assay [%]	11.2	7.3	11.5	5.7		4.6
CV injection [%]	3.8	2.6	nd	1.6	nd	nd
12-HETE						
Target concentration [ng mL^{-1}]	0.490	1.47	14.7	147	(~1.57)	(~50.9)
Accuracy inter-assay [%]	99.5	107	107	114		
Accuracy intra-assay [%]	100	103	102	106		
CV inter-assay [%]	6.6	5.4	6.0	2.8	5.5	5.8
CV intra-assay [%]	3.0	3.0	3.7	2.5	3.9	2.6
CV injection [%]	3.3	0.9	nd	2.6	nd	nd
AA						
Target concentration [ng mL^{-1}]	24.5	73.5	735	7350	(~627)	(~933)
Accuracy inter-assay [%]	96.3	119	116	93.3		
Accuracy intra-assay [%]	98.9	114	111	92.6		
CV inter-assay [%]	8.7	2.8	2.6	2.7	9.0	3.0
CV intra-assay [%]	0.7	4.0	3.4	6.4	3.1	3.0
CV injection [%]	0.5	0.6	nd	1.8	nd	nd

LLOQ, Lower Limit of Quantification; QC, quality control; L, low; M, medium; H, high; CV, coefficient of variation; (~x), approximate concentration determined over 5 series; nd, not determined; *, below LLOQ.

Consequently, we can state that *ferromagnetic particle enhanced deproteination* in combination with on-line SPE is a reliable semi-automated sample preparation, that is a time-saving alternative to off-line SPE in eicosanoid analysis. Additionally, this technique is also presumed to have less co-precipitation issues, compared to currently used protein depletion by centrifugation.

3.1.3. UHPLC–MS/MS method

We described an UHPLC–MS/MS method for the absolute quantification of seven eicosanoids of particular interest in human plasma. Other LC–MS/MS methods have included additional analytes, but these methods primarily focus on relative quantification, as typically employed in metabolomics. However, the aim of the

Table 4
Results of LC–MS/MS analysis of specimen from 5 healthy blood donors at baseline and after whole blood stimulation (all data are presented in ng mL^{-1}).

	Baseline (no incubation, no LPS)	Stimulated (24 h incubation with LPS)
TXB_2		
Donor 1	<0.10*	4.68
Donor 2	<0.10*	10.5
Donor 3	<0.10*	5.70
Donor 4	<0.10*	5.00
Donor 5	<0.10*	4.63
PGE_2		
Donor 1	<0.10*	0.62
Donor 2	<0.10*	10.4
Donor 3	<0.10*	1.40
Donor 4	<0.10*	2.74
Donor 5	<0.10*	5.41
PGD_2		
Donor 1	<0.10*	<0.10*
Donor 2	<0.10*	<0.10*
Donor 3	<0.10*	<0.10*
Donor 4	<0.10*	<0.10*
Donor 5	<0.10*	<0.10*
5-HETE		
Donor 1	<0.05*	0.19
Donor 2	0.06	0.29
Donor 3	<0.05*	0.29
Donor 4	0.06	0.50
Donor 5	<0.05*	0.32
11-HETE		
Donor 1	<0.49*	0.81
Donor 2	<0.49*	2.94
Donor 3	<0.49*	1.27
Donor 4	<0.49*	1.31
Donor 5	<0.49*	1.66
12-HETE		
Donor 1	0.66	19.8
Donor 2	<0.49*	46.7
Donor 3	<0.49*	19.3
Donor 4	<0.49*	22.6
Donor 5	<0.49*	18.7
AA		
Donor 1	151	417
Donor 2	383	787
Donor 3	263	568
Donor 4	372	770
Donor 5	228	839

LPS, lipopolysaccharide; *, below Lower Limit of Quantification.

present study was absolute quantification using a set of eight calibrators analyzed at the beginning of every batch and a deuterated internal standard for every analyte (if commercially available), as recommended in a recently published review [4]. Moreover, deuterated internal standards play a crucial role when the calibration matrix is different from the authentic matrix of the unknown samples, as in our method.

As many isomers are present among the eicosanoids, selectivity is an essential aspect for the development of UHPLC–MS/MS methods [26]. Unlike HETEs, which are isomeric but can be discriminated through mass spectrometry due to different fragmentation patterns [27], the prostaglandins E_2 and D_2 have to be chromatographically separated. We separated PGE_2 and PGD_2 to baseline, as shown for Cal 1 in Fig. 4a. An exemplary chromatogram for an authentic matrix control, the S Pool, is shown in Fig. 4b (unfortunately the PGD_2 level was still below the LLOQ; for further information see below).

Although only 100 μL of plasma were used and throughout the sample preparation the original eicosanoid level was diluted to a final concentration of approximately 40% from its origin, we realized an LLOQ of 0.1 ng mL^{-1} (5.4 fmol on column) for TXB_2, of 0.1 ng mL^{-1} (5.7 fmol on column) for PGD_2 and PGE_2, and of 0.05 ng mL^{-1} (3.1 fmol on column) for 5-HETE and 11-HETE, respectively. Notably, the LLOQs for 12-HETE and AA were not based on the technical detection limits of this method but were selected as lower calibration limits based on the biological occurrence of these eicosanoids (12-HETE 0.5 ng mL^{-1} and AA 24.5 ng mL^{-1}), as previously reported [7].

Regarding authentic matrix samples, TXB_2, PGE_2, all HETEs and AA could be quantified in our pool originating from stimulated whole blood (S Pool). Levels of 5-HETE, 12-HETE, and AA were actually high enough to be quantified in the baseline pool originating from whole blood without stimulation (B Pool). For detailed concentrations see Table 3.

3.2. Evaluation of analytical performance

There is a lack of official guidelines for the validation of LC–MS/MS methods addressing endogenous compounds, such as eicosanoids. Various approaches to cope with these problems were published in recent years [28–30].

The validation protocol for the assay described herein was designed based on the *Guidelines of bioanalytical method validation* by the EMA [18]. However, this guideline addresses xenobiotics, not endogenous compounds such as eicosanoids. Hence, to generate a validation protocol suitable for the intended endogenous analytes, we modified the protocol referring to the published recommendations mentioned above.

3.2.1. Linearity

Our method was linear ($R^2 > 0.98$ for 5-HETE and AA; $R^2 > 0.99$ for all other analytes) for the whole calibration range (see Table 1).

In case of AA, the highest calibrator, Cal 8, was excluded because the detector response was no longer linear at this concentration. However, the resulting upper limit of quantification (ULOQ) of 9800 ng mL^{-1} (Cal 7) should be sufficient to quantify the majority of biological samples according to previous studies [7,15].

3.2.2. Accuracy and precision

For all five controls (QC L, QC M, QC H, B Pool, and S Pool), the resulting values for accuracy and precision (inter- and intra-assay) were within the limits (±15 %) of the EMA guideline. For 5-HETE, the results were slightly higher but remained under ±20 %. Regarding the lower limit of quantification (LLOQ), the results for all analytes were within the limits of the EMA guideline (±20 %). The detailed values for all analytes and QCs are provided in Table 3.

3.2.3. Stability

The results of the stability experiments showed that the eicosanoids were stable at −80°C for at least one month in ethanol and plasma. Marked differences (> ±15 %) between Eppendorf Safe-Lock Tubes™, Thermo Scientific Matrix™ Tubes (both polypropylene), and glassware were not observed. The only exception is 5-HETE in stimulated plasma (S Pool): over four weeks a decrease of approximately 34 % was observed when stored in Matrix™ Tubes (n = 3) [compared with storage in Safe-Lock Tubes™ (n = 3)]. In further studies, the stability at −80°C should be investigated over a longer time period.

The data also showed that the analyte concentrations are neither impaired after three freeze and thaw cycles (−80°C), after storage for three days at +4°C nor after standing for one hour on the bench (room temperature, light) prior to sample preparation.

The readily processed samples are stable in the described glass vials for at least 72 h in the autosampler (at +8°C) and for 13 h at room temperature.

The data obtained from stability experiments using the sample preparation intermediates suggests that the best point to interrupt

– if necessary – would be after the deproteination step. Without bias, the obtained supernatant might be stored in the refrigerator for up to four hours prior to continuing the sample preparation.

3.2.4. Robustness and ruggedness

The dilution experiment, employing the *extra high QC* (based on EtOH) and the S Pool, showed good accuracy values for pre-preparation and post-preparation dilution. Based on these findings, we selected post-preparation dilution as standard procedure in case an unknown sample having a concentration above the ULOQ might occur. This approach provides more reliability, as the internal standard is already present, and it is additionally more convenient.

Carry over (peak area of the analyte in a blank injection following the highest calibrator divided by the peak area of the analyte in the highest calibrator) was < 0.05 % for all analytes, except AA (1.2 %).

The EMA calculates a second value: the peak area of a blank injection following the highest calibrator is divided by the peak area of the lowest calibrator. Due to the wide calibration ranges of this method, this second value was approximately 20–30 % for the HETEs and 230 % for AA. If this value exceeds 20 %, the EMA recommends arranging the samples based on the concentration (when possible) or otherwise to inject a blank after high concentrations. Nevertheless, the reversed order of the calibrators starting with Cal 8 (highest calibrator) resulted in a calibration with similar parameters and equal quality as the usual curve starting with Cal 1 (lowest calibrator).

The *inter-operator variability* was within the usual variation determined through precision experiments. Also, the use of a different column lot did not affect the results.

3.2.5. Matrix effect and recovery

Notably, the pre-/post-spiking experiments (see Section 2.7.5) according to Matuszewski [20] were performed for AA alone and in parallel for the remaining metabolites to prevent bias through the AA induced formation of PGD_2, 5-HETE, 11-HETE, and 12-HETE in plasma, as described in Section 3.1.1.

No relevant matrix effect was observed (98 % TXB_2, 101 % PGE_2, 98 % PGD_2, 107 % 5-HETE, 102 % 11-HETE, 94 % 12-HETE, and 109 % for AA). The variation coefficient of the *IS normalised matrix factor* (MF_{norm}) ranged from 1 to 7 %, which is clearly below the limit of the EMA (15 %).

Also recovery showed satisfying results: 90 % TXB_2, 95% PGE_2, 74% PGD_2, 93 % 5-HETE, 92 % 11-HETE, 91 % 12-HETE, and 93 % for AA.

Regarding the post-column infusion of blank plasma samples, no ion suppression or ion enhancement was observed, compared to a solvent injection. Because eicosanoids are endogenous compounds present in every plasma sample, a slight increase in the signal resulting from infusion was observed when endogenous AA (having relatively high natural abundance) from the blank plasma sample was eluted from the column.

In a third experiment, we employed matrix mixing to investigate whether a different amount of matrix influences the accuracy: plotting the determined and calculated theoretical concentrations of eicosanoids for the different mixing ratios resulted in a linear relationship with good correlation coefficients for both matrix lots ($R^2 > 0.99$).

The theoretical assumed compensation of matrix effects by the internal standard can be confirmed by these findings. This compensation is very important in LC–MS/MS methods, particularly when constructing the calibration curve in a matrix different from the authentic matrix of the samples.

3.3. In vitro *stimulation of specimen from healthy blood donors*

As a proof of principle approach our newly developed analytical LC–MS/MS assay employing *ferromagnetic particle enhanced deproteination* was used to investigate the release capacity of the targeted eicosanoids (TXB_2, PGE_2, PGD_2, 5-HETE, 11-HETE, 12-HETE, AA) in healthy blood donors (n = 5) using the whole blood activation model described in Section 2.3.

As shown in Table 4, target mediators were not detectable at baseline, with the exception of AA and for some donors also for 5-HETE and 12-HETE. It is noteworthy that the baseline pool (B Pool) used for validation was characterized by markedly higher endogenous levels for 5-HETE and 12-HETE (see also Table 3), compared to the found baseline concentrations of 5-HETE and 12-HETE in the unstimulated specimens of our five healthy blood donors, which were below or only slightly above the respective LLOQ. These obvious differences potentially indicate a broad variability of 5-HETE and 12-HETE at baseline among healthy people. However, further studies are necessary to underpin this assumption.

As expected, LPS whole blood activation resulted in a relevant increase of eicosanoids (compare Table 4). The intensity of the eicosanoid release markedly differed between the individual blood donors. As in the S Pool used during validation, PGD_2 was unfortunately not detected in stimulated samples of the 5 blood donors.

These results show that the newly developed multi-analyte LC–MS/MS assay employing *ferromagnetic particle enhanced deproteination* described herein is suitable for analysis of samples generated with the described whole blood activation model.

4. Conclusion

To the best of our knowledge, the herein presented study is the first to describe *ferromagnetic particle enhanced deproteination* in combination with on-line SPE for sample clean-up of eicosanoids in human plasma samples. This novel approach to semi-automated sample preparation was found convenient and rugged enabling reliable analyses of demanding analytes in complex biological matrices.

Prospectively, the complete automation of the sample preparation might be possible. Actually, *ferromagnetic particle enhanced deproteination* could be performed using a liquid handling system and also the evaporation step could be integrated with the appropriate instrumentation. Based on our promising validation data for eicosanoids we are convinced that this innovative approach will facilitate automation in UHPLC–MS/MS.

Due to its rather generic character and based on our validation results for eicosanoids as a demanding model group of endogenous analytes, we believe *ferromagnetic particle enhanced deproteination* is a very promising tool for the analysis of a broad variety of target analytes, including endogenous analytes as well as xenobiotica.

Acknowledgment

This work was funded by the Hans Fischer Society Munich, Munich, Germany.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2016.03.022.

References

[1] J. Ecker, Profiling eicosanoids and phospholipids using LC–MS/MS: principles and recent applications, J. Sep. Sci. 35 (2012) 1227–1235.

[2] E. Ricciotti, G.A. FitzGerald, Prostaglandins and inflammation, Arterioscler. Thromb. Vasc. Biol. 31 (2011) 986–1000.
[3] D. Balgoma, A. Checa, D.G. Sar, S. Snowden, C.E. Wheelock, Quantitative metabolic profiling of lipid mediators, Mol. Nutr. Food Res. 57 (2013) 1359–1377.
[4] G. Astarita, A.C. Kendall, E.A. Dennis, A. Nicolaou, Targeted lipidomic strategies for oxygenated metabolites of polyunsaturated fatty acids, Biochim. Biophys. Acta 1851 (2015) 456–468.
[5] D. Tsikas, A.A. Zoerner, Analysis of eicosanoids by LC–MS/MS and GC–MS/MS: a historical retrospect and a discussion, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. (2014), http://dx.doi.org/10.1016/j.jchromb.2014.03.017.
[6] Y. Wang, A.M. Armando, O. Quehenberger, C. Yan, E.A. Dennis, Comprehensive ultra-performance liquid chromatographic separation and mass spectrometric analysis of eicosanoid metabolites in human samples, J. Chromatogr. A 1359 (2014) 60–69.
[7] D.D. Shinde, K.B. Kim, K.S. Oh, N. Abdalla, K.H. Liu, S.K. Bae, J.H. Shon, H.S. Kim, D.H. Kim, J.G. Shin, LC–MS/MS for the simultaneous analysis of arachidonic acid and 32 related metabolites in human plasma: basal plasma concentrations and aspirin-induced changes of eicosanoids, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 911 (2012) 113–121.
[8] J. Yang, K. Schmelzer, K. Georgi, B.D. Hammock, Quantitative profiling method for oxylipin metabolome by liquid chromatography electrospray ionization tandem mass spectrometry, Anal. Chem. 81 (2009) 8085–8093.
[9] X. Zhang, N. Yang, D. Ai, Y. Zhu, Systematic metabolomic analysis of eicosanoids after omega-3 polyunsaturated Fatty Acid supplementation by a highly specific liquid chromatography-tandem mass spectrometry-based method, J. Proteome Res. 14 (2015) 1843–1853.
[10] Y. Huang, G. Chen, X. Liu, Y. Shao, P. Gao, C. Xin, Z. Cui, X. Zhao, G. Xu, Serum metabolomics study and eicosanoid analysis of childhood atopic dermatitis based on liquid chromatography-mass spectrometry, J. Proteome Res. 13 (2014) 5715–5723.
[11] L. Kortz, J. Dorow, S. Becker, J. Thiery, U. Ceglarek, Fast liquid chromatography-quadrupole linear ion trap-mass spectrometry analysis of polyunsaturated fatty acids and eicosanoids in human plasma, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 927 (2013) 209–213.
[12] K. Konig, S.F. Goethel, V.M. Rusu, M. Vogeser, Deproteination of serum samples for LC–MS/MS analyses by applying magnetic micro-particles, Clin. Biochem. 46 (2013) 652–655.
[13] E.M. Smyth, T. Grosser, M. Wang, Y. Yu, G.A. FitzGerald, Prostanoids in health and disease, J. Lipid Res. 50 (Suppl) (2009) S423–S428.
[14] V. Capra, M. Back, S.S. Barbieri, M. Camera, E. Tremoli, G.E. Rovati, Eicosanoids and their drugs in cardiovascular diseases: focus on atherosclerosis and stroke, Med. Res. Rev. 33 (2013) 364–438.
[15] M. Bruegel, U. Ludwig, A. Kleinhempel, S. Petros, L. Kortz, U. Ceglarek, L.M. Holdt, J. Thiery, G.M. Fiedler, Sepsis-associated changes of the arachidonic acid metabolism and their diagnostic potential in septic patients, Crit. Care Med. 40 (2012) 1478–1486.
[16] A. Margalit, K.L. Duffin, P.C. Isakson, Rapid quantitation of a large scope of eicosanoids in two models of inflammation: development of an electrospray and tandem mass spectrometry method and application to biological studies, Anal. Biochem. 235 (1996) 73–81.
[17] W. Ertel, J.P. Kremer, J. Kenney, U. Steckholzer, D. Jarrar, O. Trentz, F.W. Schildberg, Downregulation of proinflammatory cytokine release in whole blood from septic patients, Blood 85 (1995) 1341–1347.
[18] E.M. Agency, Guideline on Bioanalytical Method Validation, 2011, http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf, accessed February 2014.
[19] CLSI, Liquid Chromatography-Mass-Spectrometry Methods; Approved Guideline, CLSI Document C62-A, Clinical and Laboratory Standards Institute, Wayne, PA, 2014.
[20] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC–MS/MS, Anal. Chem. 75 (2003) 3019–3030.
[21] R. Bonfiglio, R.C. King, T.V. Olah, K. Merkle, The effects of sample preparation methods on the variability of the electrospray ionization response for model drug compounds, Rapid. Commun. Mass Spectrom. 13 (1999) 1175–1185.
[22] W.S. Powell, Precolumn extraction and reversed-phase high-pressure liquid chromatography of prostaglandins and leukotrienes, Anal. Biochem. 164 (1987) 117–131.
[23] M. Korecka, C.M. Clark, V.M. Lee, J.Q. Trojanowski, L.M. Shaw, Simultaneous HPLC–MS-MS quantification of 8-iso-PGF(2alpha) and 8,12-iso-iPF(2alpha) in CSF and brain tissue samples with on-line cleanup, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 878 (2010) 2209–2216.
[24] I. Willenberg, A.K. Meschede, N.H. Schebb, Determining cyclooxygenase-2 activity in three different test systems utilizing online-solid phase extraction-liquid chromatography-mass spectrometry for parallel quantification of prostaglandin E(2), D(2) and thromboxane B(2), J. Chromatogr. A 1391 (2015) 40–48.
[25] Y. Kita, T. Takahashi, N. Uozumi, T. Shimizu, A multiplex quantitation method for eicosanoids and platelet-activating factor using column-switching reversed-phase liquid chromatography-tandem mass spectrometry, Anal. Biochem. 342 (2005) 134–143.
[26] L. Kortz, J. Dorow, U. Ceglarek, Liquid chromatography-tandem mass spectrometry for the analysis of eicosanoids and related lipids in human biological matrices: a review, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. (2014), http://dx.doi.org/10.1016/j.jchromb.2014.01.046.
[27] R.C. Murphy, R.M. Barkley, K. Zemski Berry, J. Hankin, K. Harrison, C. Johnson, J. Krank, A. McAnoy, C. Uhlson, S. Zarini, Electrospray ionization and tandem mass spectrometry of eicosanoids, Anal. Biochem. 346 (2005) 1–42.
[28] D. Tsikas, A proposal for comparing methods of quantitative analysis of endogenous compounds in biological systems by using the relative lower limit of quantification (rLLOQ), J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 877 (2009) 2244–2251.
[29] R. Houghton, C. Horro Pita, I. Ward, R. Macarthur, Generic approach to validation of small-molecule LC–MS/MS biomarker assays, Bioanalysis 1 (2009) 1365–1374.
[30] N.C. van de Merbel, Quantitative determination of endogenous compounds in biological samples using chromatographic techniques, TrAC Trends Anal. Chem. 27 (2008) 924–933.

Mathias Bruegel*, Dorothea Nagel, Manuela Funk, Petra Fuhrmann, Johannes Zander and Daniel Teupser

Comparison of five automated hematology analyzers in a university hospital setting: Abbott Cell-Dyn Sapphire, Beckman Coulter DxH 800, Siemens Advia 2120i, Sysmex XE-5000, and Sysmex XN-2000

DOI 10.1515/cclm-2014-0945
Received September 24, 2014; accepted December 7, 2014; previously published online January 13, 2015

Abstract

Background: Various types of automated hematology analyzers are used in clinical laboratories. Here, we performed a side-by-side comparison of five current top of the range routine hematology analyzers in the setting of a university hospital central laboratory.
Methods: Complete blood counts (CBC), differentials, reticulocyte and nucleated red blood cell (NRBC) counts of 349 patient samples, randomly taken out of routine diagnostics, were analyzed with Cell-Dyn Sapphire (Abbott), DxH 800 (Beckman Coulter), Advia 2120i (Siemens), XE-5000 and XN-2000 (Sysmex). Inter-instrument comparison of CBCs including reticulocyte and NRBC counts and investigation of flagging quality in relation to microscopy were performed with the complete set of samples. Inter-instrument comparison of five-part differential was performed using samples without atypical cells in blood smear (n=292). Automated five-part differentials and NRBCs were additionally compared with microscopy.
Results: The five analyzers showed a good concordance for basic blood count parameters. Correlations between instruments were less well for reticulocyte counts, NRBCs, and differentials. The poorest concordance for NRBCs with microscopy was observed for Advia 2120i (Kendall's τ_b=0.37). The highest flagging sensitivity for blasts was observed for XN-2000 (97% compared to 65%–76% for other analyzers), whereas overall specificity was comparable between different instruments.
Conclusions: To the best of our knowledge, this is the most comprehensive side-by-side comparison of five current top of the range routine hematology analyzers. Variable analyzer quality and parameter specific limitations must be considered in defining laboratory algorithms in clinical practice.

Keywords: complete blood count; differential; flagging quality; hematology analyzer; inter-instrument comparison.

Introduction

Automated hematology analyzers are used for quantitative high-throughput analysis of complete blood counts (CBC), differentials, reticulocyte and nucleated red blood cell (NRBC) counts. They are also used for identification of pathological patient samples labeled by different flagging alerts for subsequent microscopic evaluation. A primary demand for hematology analyzers is to minimize the number of samples for which time- and personnel-intensive microscopic review is needed without increase of false-negative results. Therefore, all top level instruments combine different techniques, such as impedance and flow cytometry, some of them additionally use fluorescence techniques for optimized cell classification [1, 2]. Only few published studies have performed side-by-side testing of different hematology analyzers equipped with different techniques or different analytical algorithms and only few studies investigated a larger number of

***Corresponding author: Mathias Bruegel,** Institute of Laboratory Medicine, Ludwig-Maximilians-University Munich, Marchioninistrasse 15, 81377 Munich, Germany,
Phone: +49 89 4400 73209, Fax: +49 89 4400 78888,
E-mail: mathias.bruegel@med.uni-muenchen.de
Dorothea Nagel, Manuela Funk, Petra Fuhrmann, Johannes Zander and Daniel Teupser: Institute of Laboratory Medicine, Ludwig-Maximilians-University Munich, Munich, Germany

Bereitgestellt von | Deutsche Vereinte Gesellschaft für Klinische Chemie und Laboratoriumsmedizin e.V. (DGKL)
Angemeldet
Heruntergeladen am | 10.03.20 16:27

instruments in parallel [1–3]. Therefore, data about the diagnostic performance of different measurement technologies are scarce. Moreover, some of the previous evaluation studies were biased towards currently available instrumentation in that laboratory or towards selected patient groups, because testing was performed with flagged samples from routine analysis [2] or with samples from patients in a hematology setting [4].

The aim of our study was to perform an unbiased side-by-side comparison of five current top of the range routine hematology analyzers in the setting of a university hospital central laboratory, regarding inter-instrument comparison of CBC, differential, reticulocyte and NRBC counts and flagging quality.

Materials and methods

Evaluation protocol and specimens

Evaluation was performed during a two-month period, in which all five hematology analyzers were placed in the Institute of Laboratory Medicine, Hospital of Ludwig-Maximilians-University Munich. The XE-5000 (Sysmex, Kobe, Japan) was available in the laboratory as routine instrument, the Cell-Dyn Sapphire (Abbott, Santa Clara, USA), the DxH 800 (Beckman Coulter, Miami, USA), the Advia 2120i (Siemens Healthcare Diagnostics, Eschborn, Germany) and the XN-2000 (Sysmex, Kobe, Japan) were provided by the different companies. Inter-instrument comparisons, comparisons to manual microscopy and comparisons of flagging quality were designed following relevant aspects in International Council for Standardization in Haematology (ICSH) [5] or Clinical and Laboratory Standards Institute (CLSI) guidelines [6] or guidelines given by the International Consensus Group for Hematology Review [7]. Two technicians experienced in hematology were familiarized with each of the different analyzers by the companies during a 1-week period. Specimens were obtained from anonymized leftovers of K_2EDTA whole blood samples collected with the Sarstedt monovette system (Sarstedt, Nuembrecht, Germany) and submitted for routine blood count testing. Samples were stored at room temperature and all analyses in this study were completed within 6 h after sample entry into our laboratory. To permit unbiased testing of the five instruments, a total of 349 unselected study samples were taken randomly out of routine diagnostics without knowledge of clinical background or of results from potential prior blood count analysis. Up to 20 samples were randomly selected, processed each day and run on the instruments in auto-mode, samples without sufficient sample volume for analysis with the five analyzers were excluded. Blood smears were prepared from all samples with May-Gruenwald-Giemsa staining within 2 h of running on the XE-5000 as routine instrument. Specimens were analyzed on each instrument within a 4-h period, rotating the order of system application every day. Blood smear analysis was done by two experienced hematology technicians each performing a 200-cell manual differential as recommended by ICSH and CLSI guidelines [5, 6]. Potential observer dependent differences between the two technicians were evaluated with a set of 30 randomly collected patient samples, and no significant observer bias was noted. Following the International Consensus Group for Haematology Review, blood smears were assessed to be positive for abnormal cells in case of presence of >0% blasts or presence of immature granulocytes (IGs) with ≥2% metamyelocytes or ≥1% myelocytes/promyelocytes [7]. Left shift was defined as a band count ≥8% in blood smear. The manual NRBC count was expressed as number per 100 white blood cells (WBCs), samples were considered to be positive when optical microscopy showed ≥1 NRBC per 100 WBCs. A positive blood smear for variant lymphocytes was assessed in case of presence of ≥1% plasma cells, ≥5% atypical lymphocytes with suspected activation characteristics or ≥2% abnormal lymphocytes with suspected malignant characteristics. To investigate a relationship between flagging sensitivity and concentration of abnormal cells, flagging sensitivity for presence of blasts and variant lymphocytes as clinically most relevant warning massages was further assessed using a cut-off of ≥5% blasts or variant lymphocytes as criteria for positive blood smear.

In a sub-study, platelet (PLT) counts were additionally determined in 30 EDTA blood samples using an anti-CD61 based immunological method which is available in the Cell-Dyn Sapphire, in addition to conventional PLT testing.

Instrumentation, flagging parameters and instrument setting

The investigated hematology analyzers utilize different technologies for determination of CBC, differential, reticulocytes and NRBCs (Table 1) [4, 8–10]. Flagging messages for presence of immature cells, atypical lymphocytes and PLT clumps are similar between the five different instruments. As special features, DxH 800 further differentiates blasts with neutrophil, lymphocyte or monocyte character. Sysmex XE and XN instruments differentiate immature cells into blasts or blasts/abnormal lymphocytes and pathological lymphocytes into abnormal lymphocytes with suspected malignant characteristics or atypical lymphocytes with suspected activation characteristics. In the XN, a white progenitor cell (WPC) channel further allows discrimination between blast cells and abnormal lymphocytes. In the present study, differentiated blast flags in DxH 800 were not considered and the two lymphocyte flags in Sysmex instruments were assessed together as variant lymphocytes to ensure homogeneity with other instruments. Default settings for flagging notes and most favorable test algorithms were used for all three instruments. The fluorescence PLT (PLT-F) channel was used as principal PLT testing in XN-2000.

Testing of required sample volume

For inter-instrument comparison of required sample volumes, different volumes of unselected K_2EDTA routine whole blood samples (500 µL, 300 µL, 200 µL, 150 µL) were transferred into four different tube types [2.7 mL and 1.2 mL K_2EDTA Sarstedt monovettes, 1.0 mL Siemens EZEE-nest tubes (Siemens, Munich, Germany), 1.5 mL Sarstedt micro tubes] and subsequently determined on different hematology analyzers. Any liquid (dissolved EDTA) contained in these tubes was discarded prior to adding the described volumes of anticoagulated blood. Blood count results of this experiment were otherwise not used in our study.

Bereitgestellt von | Deutsche Vereinte Gesellschaft für Klinische Chemie und Laboratoriumsmedizin e.V. (DGKL)
Angemeldet
Heruntergeladen am | 10.03.20 16:27

Table 1 Measurement technologies of different haematology analyzers.

Parameter	Instrument				
	Cell-Dyn Sapphire	DxH 800	Advia 2120i	XE-5000	XN-2000
Hemoglobin	Photometry	Photometry	Photometry	Photometry	Photometry
RBC count	Impedance/Light scatter	Impedance	Light scatter	Impedance	Impedance
PLT count	Impedance/Light scatter/Ab based	Impedance	Light scatter	Impedance/Light scatter/Fluorescence	Impedance/Light scatter/Fluorescence
WBC count	Light scatter/Fluorescence	Impedance	Light scatter/Peroxidase	Impedance/Light scatter/Fluorescence	Light scatter/Fluorescence
5-part differential	Light scatter/Fluorescence	Impedance/Conductivity/Light scatter	Light scatterPeroxidase	Light scatter/Fluorescence	Light scatter/Fluorescence
Reticulocytes	Light scatter/Fluorescence	Impedance/Conductivity/Light scatter	Light scatter	Light scatter/Fluorescence	Light scatter/Fluorescence
NRBC count	Light scatter/Fluorescence	Impedance/Conductivity/Light scatter	Light scatter/Peroxidase	Light scatter/Fluorescence	Light scatter/Fluorescence

Ab, antibody; NRBC, nucleated red blood cell; PLT, platelet; RBC, red blood cell; WBC, white blood cell.

Data analysis and statistics

The complete set of randomly selected patient samples (n=349) was used for inter-instrument comparison of CBC parameters, NRBC and reticulocyte counts between different hematology analyzers. As blasts and variant lymphocytes are not included in the automated differential, inter-instrument comparison of five-part differential was performed using a subgroup of 292 samples without blasts or variant lymphocytes in blood smear. Automated five-part differentials of the sample subgroup and NRBC counts of the complete set of samples generated by the different analyzers were further compared with results of manual microscopy.

Analysis of correlation and regression analysis was done for measurements from each analyzer against the median of all five analyzers. For CBC parameters, differentials and reticulocyte counts method comparison was performed using Passing-Bablok regression [11]. Confidence intervals for slopes and intercepts were calculated to test the hypotheses slope=1 and intercept=0. The values 1 for slope and 0 for intercept not being enclosed in the respective interval indicated significant deviation. Regression equations could not be calculated for NRBCs, because for some instruments resulting slope was zero or infinity. As some investigated parameters showed significant outliers, correlations were calculated by means of Kendall's concordance coefficient τ_b [12], which is less sensitive to outliers than the commonly used correlation coefficient by Pearson. For comparison of automated NRBC counts with manual counts, automated NRBC values below 1% were set to zero. By slightly modifying the method of Bland and Altman, differences between single measurements and the median of all five analyzers were plotted against the median of all five analyzers [13]. Systematic deviation (bias) was estimated by the mean value of the differences. These differences not always corresponded to a Gaussian distribution. In addition to standard deviations, 95% limits of agreement were therefore calculated by the 2.5th and 97.5th percentile as a measure of scatter, which define the range containing 95% of the distribution. Thus, 95% limits of agreement are a measure of the degree to which an analyzer differs from the median of all instruments. We used absolute differences for all parameters except PLT counts, where a clear tendency of proportional bias could be observed and thus percentage differences were more appropriate. Furthermore, values for PLT counts were displayed on a logarithmic scale.

For inter-instrument comparison of flagging quality, all 349 samples were included, and results of microscopic evaluation were compared to flagging alerts of different analyzers. Flagging sensitivity and specificity were calculated by the number of true positives, true negatives, false positives and false negatives. Statistical analysis was performed using SAS (Version 9.3, SAS Institute Inc., Cary, NC, USA).

Results

Inter-instrument comparison of blood count parameters and reticulocyte counts (n=349)

Inter-instrument comparisons of directly analyzed CBC parameters showed good correlations with correlation coefficients $\tau_b \geq 0.93$ for all analyzers (Table 2,

Bereitgestellt von | Deutsche Vereinte Gesellschaft für Klinische Chemie und Laboratoriumsmedizin e.V. (DGKL)
Angemeldet
Heruntergeladen am | 10.03.20 16:27

Table 2 Inter-instrument comparison of blood counts, reticulocyte and NRBC counts (n=349) and comparison of automated NRBCs or PLTs to microscopy or CD61 (n=30).

	System	τ_b	b	a	Mean	SD	95% limits of agreement
			Regression to median				Differences to median
WBC, $10^3/\mu L$	Sapphire	0.98	1.00	0.00	−0.02	0.502	−0.70–0.60
	DxH 800	0.98	1.00	−0.00	0.03	0.309	−0.50–0.70
	Advia 2120i	0.97	**1.05**	−0.01	0.43	0.712	−0.17–1.70
	XE-5000	0.98	**0.98**	−0.02	−0.23	0.425	−1.00–0.08
	XN-2000	0.99	1.00	0.00	−0.05	0.187	−0.34–0.30
RBC, $10^6/\mu L$	Sapphire	0.98	1.00	0.00	0.01	0.037	−0.06–0.09
	DxH 800	0.97	**0.97**	−0.03	−0.13	0.049	−0.24 to −0.03
	Advia 2120i	0.96	1.00	**0.06**	0.06	0.062	−0.05–0.16
	XE-5000	0.97	1.00	0.00	0.01	0.087	−0.05–0.08
	XN-2000	0.96	**1.03**	−0.11	−0.02	0.078	−0.12–0.09
HGB, mmol/L	Sapphire	0.98	1.00	**0.10**	0.06	0.067	−0.06–0.19
	DxH 800	0.96	1.00	0.00	−0.03	0.113	−0.25–0.12
	Advia 2120i	0.98	1.00	0.00	0.01	0.070	−0.06–0.12
	XE-5000	0.96	1.00	**−0.10**	−0.07	0.249	−0.25–0.06
	XN-2000	0.98	1.00	0.00	−0.01	0.142	−0.12–0.12
HCT	Sapphire	0.96	1.00	0.00	−0.00	0.005	−0.01–0.01
	DxH 800	0.95	1.00	−0.01	−0.01	0.005	−0.02–0.00
	Advia 2120i	0.93	**1.04**	0.00	0.02	0.008	0.00–0.03
	XE-5000	0.96	1.00	−0.00	−0.00	0.008	−0.01–0.00
	XN-2000	0.96	**1.02**	−0.00	0.00	0.007	−0.01–0.01
MCV, fL	Sapphire	0.90	1.00	−1.20	−1.26	0.981	−3.30–0.00
	DxH 800	0.91	1.00	0.00	0.52	1.074	−1.50–3.00
	Advia 2120i	0.90	**1.08**	**−4.54**	2.35	1.145	0.20–5.00
	XE-5000	0.89	0.99	−0.74	−1.86	1.180	−4.50–0.00
	XN-2000	0.90	1.00	0.00	0.45	1.199	−2.40–3.10
MCH, pg	Sapphire	0.90	1.00	0.00	0.16	0.373	−0.40–1.10
	DxH 800	0.85	**1.10**	**−1.93**	0.95	0.588	0.00–2.10
	Advia 2120i	0.84	1.00	−0.50	−0.51	0.590	−1.40–0.40
	XE-5000	0.87	1.00	**−0.40**	−0.46	0.572	−1.50–0.20
	XN-2000	0.92	1.00	0.00	0.09	0.330	−0.50–0.80
PLT, $10^3/\mu L$	Sapphire	0.96	**1.07**	**−2.96**	3.44	7.840	−18.18–14.42
	DxH 800	0.96	**0.94**	−0.33	−5.05	8.219	−16.67–11.70
	Advia 2120i	0.95	**1.10**	−0.27	10.50	9.693	−0.78–29.63
	XE-5000	0.96	**0.97**	**1.31**	−1.32	9.834	−14.00–22.22
	XN-2000	0.97	1.00	0.00	−3.04	7.840	−25.00–7.89
RET, %	Sapphire	0.87	**1.19**	**0.05**	0.41	0.470	−0.10–1.20
	DxH 800	0.82	1.00	0.00	0.02	0.458	−0.60–1.30
	Advia 2120i	0.75	**0.86**	**−0.14**	−0.41	0.511	−1.80–0.30
	XE-5000	0.95	1.00	0.00	−0.04	0.152	−0.40–0.20
	XN-2000	0.91	1.00	0.00	0.07	0.196	−0.30–0.50
NRBC, %	Sapphire	0.57			−0.04	1.759	−0.80–1.00
	DxH 800	0.46			−0.09	1.752	−1.10–0.60
	Advia 2120i	0.47			0.27	5.636	−1.70–3.70
	XE-5000	0.85			0.24	1.768	0.00–1.40
	XN-2000	0.84			0.09	0.692	0.00–0.50
		Regression to microscopy					Differences to microscopy
NRBC, %	Sapphire	0.54			−0.05	3.845	−2.00–1.10
	DxH 800	0.56			−0.23	3.481	−2.00–0.80
	Advia 2120i	0.37			0.26	6.062	−2.00–4.30
	XE-5000	0.63			0.20	3.297	−1.00–1.20
	XN-2000	0.66			0.03	2.701	−1.20–0.20
			Regression to CD61				Differences to CD61
PLT, $10^3/\mu L$	Sapphire	0.92	1.04	0.21	7.72	22.95	−35.48–94.03
	DxH 800	0.91	**0.91**	**3.05**	23.84	47.24	−20.12–160.00

Bereitgestellt von | Deutsche Vereinte Gesellschaft für Klinische Chemie und Laboratoriumsmedizin e.V. (DGKL)
Angemeldet
Heruntergeladen am | 10.03.20 16:27

Table 2 (continued)

System	τ_b	b	a	Mean	SD	95% limits of agreement
		Regression to CD61				Differences to CD61
Advia 2120i	0.93	**1.09**	**3.97**	42.07	43.09	−1.15–173.33
XE-5000	0.90	1.01	**1.98**	19.75	33.60	−29.03–122.22
XN-2000	0.96	0.97	0.59	2.24	17.86	−25.93–56.67

τ_b, Kendall's τ_b; b, slope (numbers in bold are significantly different from 1); a, intercept (numbers in bold are significantly different from 0); SD, standard deviation.

Supplemental Material, Figure 1, that accompanies the article http://www.degruyter.com/view/j/cclm.2015.53.issue-7/cclm-2014-0945/cclm-2014-0945.xml?format=INT). MCH and MCV revealed correlation coefficients $\tau_b \geq 0.84$. Systematic differences between the various analyzers were seen for WBC counts, which were slightly higher with Advia 2120i (slope 1.05), the latter also revealing the highest variability (95% limits of agreement −0.17–1.70) (Table 2, Figure 1A). Furthermore, as compared to other instruments, Advia 2120i revealed consistently higher MCV values (slope 1.08) (Table 2, Figure 1E). PLT measurements performed with Cell-Dyn Sapphire and Advia 2120i were significantly higher (slopes 1.07 and 1.10, respectively) and significantly lower for DxH 800 and XE-5000 (slopes 0.94 and 0.97, respectively) (Table 2, Figure 1G). Comparable results were found focussing on PLT counts $<50\times10^3/\mu L$ (data not shown). Comparison of anti-CD61 based PLT analysis with automated PLT analysis of different instruments was performed using 30 additional EDTA blood samples, of which 22 revealed CD61 based counts below $50\times10^3/\mu L$. Method comparison revealed τ_b values ≥0.90, but also confirmed a maximum deviation to higher values using Advia 2120i (slope 1.09) (Table 2). The highest concordance to CD61 based PLT measurement could be shown for XN-2000 using PLF-F technology.

Reticulocyte counts determined with different instruments revealed correlation coefficients in the range between 0.75 and 0.95 (Table 2, Supplemental Figure 1H). Values were comparable between DxH 800, XE-5000 and XN-2000. The greatest systematic difference was found between Cell-Dyn Sapphire showing significantly higher values (slope 1.19) and Advia 2120i showing significantly lower values (slope 0.86) (Table 2, Figure 1H).

Inter-instrument comparison of NRBC counts and comparison to microscopy (n=349)

Inter-instrument comparison of NRBC counts resulted in a wide range of values for τ_b, with 0.57 for Cell-Dyn Sapphire, 0.46 for DxH 800, 0.47 for Advia 2120i, 0.85 for XE-5000 and 0.84 for XN-2000 (Table 2, Figure 2C). The highest variability in NRBC analysis (SD 5.64%) was found for Advia 2120i, with 95% limits of agreement between –1.70 and 3.70, (Table 2, Figure 2A). A rather low concordance was also observed between automated NRBC analysis performed with different analyzers and manual counts, with correlations of 0.54 for Cell-Dyn Sapphire, 0.56 for DxH 800, 0.37 for Advia 2120i, 0.63 for XE-5000 and 0.66 for XN-2000 (Table 2, Figure 2D). The greatest difference between automated and manual NRBC counts could be shown for Advia 2120i, with 95% limits of agreement between –2.0 and 4.30 (Table 2, Figure 2B). Sensitivities for the detection of NRBCs ≥1% in manual count (n=30) were 43.3% for Cell-Dyn Sapphire, 46.7% for DxH 800, 40% for Advia 2120i, 60% for XE-5000, and 53.3% for XN-2000. Respective specificities ranged from 95% to 99%.

Inter-instrument comparison of WBC differential and comparison to microscopy (n=292)

As blasts and variant lymphocytes are not included in WBC differentials of hematology analyzers, comparisons were performed in a set of 292 out of 349 samples, after excluding samples containing blasts and variant lymphocytes in blood smears.

Neutrophil counts

Inter-instrument correlation of automated neutrophil counts revealed τ_b values ≥0.96 for all instruments (Table 3, Supplemental Figure 2A). The lowest variability in inter-instrument comparison (SD 0.15) with 95% limits of agreement between −0.30 and 0.20 was found for XN-2000 (Table 3, Figure 3A). Comparison of automated and manual neutrophil counts showed a somewhat lower correlation with τ_b values ≥0.93 (Table 3, Supplemental Figure 2B). For two samples, a difference

Bereitgestellt von | Deutsche Vereinte Gesellschaft für Klinische Chemie und Laboratoriumsmedizin e.V. (DGKL)
Angemeldet
Heruntergeladen am | 10.03.20 16:27

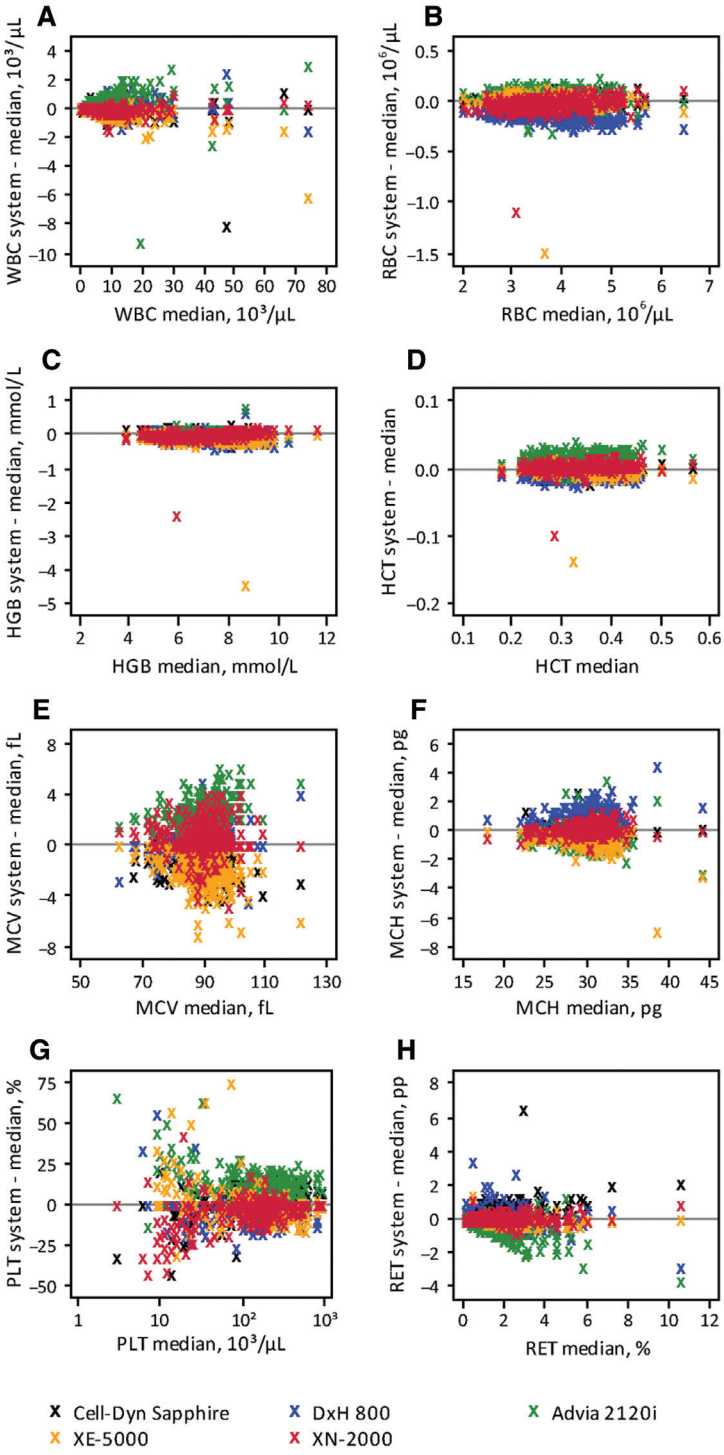


Figure 1 Difference plots for blood count parameters and reticulocyte counts.
Inter-instrument comparisons of blood and reticulocyte counts were determined in 349 routine samples. Differences between single measurements and the median of all five analyzers were plotted against the median of all analyzers. (A) WBC count, (B) RBC count, (C) hemoglobin concentration, (D) hematocrit, (E) MCV, (F) MCH, (G) PLT, (H) reticulocyte count.

of more than 5×10^3/µL between automated and microscopic neutrophil counts was observed for all instruments, due to misclassification of smudge cells or IGs to neutrophils.

Lymphocyte counts

Inter-instrument correlation of automated lymphocyte counts showed τ_b values ≥0.94 (Table 3, Supplemental

Bereitgestellt von | Deutsche Vereinte Gesellschaft für Klinische Chemie und Laboratoriumsmedizin e.V. (DGKL)
Angemeldet
Heruntergeladen am | 10.03.20 16:27

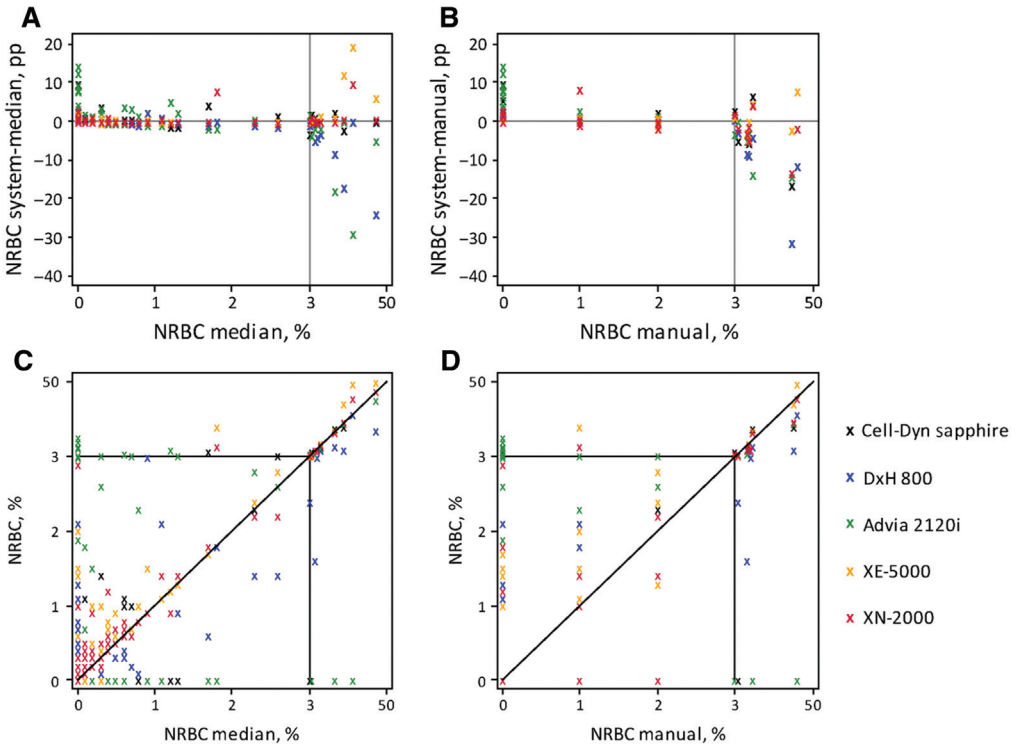


Figure 2 Inter-instrument comparison of NRBC counts and comparison to microscopy.
Inter-instrument comparison and comparison between automated and manual NRBC counts were performed in 349 routine samples. (A) Differences between single measurements of each analyzer and the median of all five analyzers plotted against the median of all analyzers. (B) Differences between automated and manual NRBC counts plotted against manual NRBC counts. (C) Single NRBC measurements of each analyzer compared to median of all five analyzers. (D) Single NRBC measurements of each analyzer compared to single manual counts. The line in the scatter plot represents identity. Axes were scaled to include all data except one data point of 94% NRBCs measured by Advia 2120i and results of one sample with manual NRBC count of 54% and automated counts between 81% and 100%.

Figure 2C), comparison with microscopy revealed τ_b values ≥0.80 (Table 3, Supplemental Figure 2D).

Monocyte counts

Inter-instrument correlation of automated monocyte counts revealed τ_b values between 0.85 (Advia 2120i) and 0.95 (DxH 800 and XN-2000), with Advia 2120i tending to lower values (slope 0.80) (Table 3, Figure 3E, Supplemental Figure 2E). Comparing automated and manual monocyte counts, correlation coefficients in the range of 0.66 (Advia 2120i)–0.72 (XN-2000) were found, with Advia 2120i revealing the lowest mean of differences to manual values (mean 0.06) (Table 3, Figure 3F, Supplemental Figure 2F).

Eosinophil counts

Inter-instrument correlation of automated eosinophil counts showed τ_b values between 0.90 (Cell-Dyn Sapphire) and 0.97 (XE-5000, XN-2000) (Table 3, Supplemental Figure 2G), comparison with microscopy revealed τ_b values in the range of 0.76–0.79 (Table 3, Supplemental Figure 2H).

Basophil counts

A poor inter-instrument correlation for automated basophil counts with τ_b values ranging from 0.37 to 0.82, and between automated basophil counts and microscopic examination with τ_b values ranging from 0.17 to 0.35 was observed (Table 3).

Inter-instrument comparison of flagging quality in unselected samples (n=349)

For inter-instrument comparison of flagging quality, results of microscopic evaluation were compared to flagging alerts of different analyzers. Defining a >0% cut-off

Bereitgestellt von | Deutsche Vereinte Gesellschaft für Klinische Chemie und Laboratoriumsmedizin e.V. (DGKL)
Angemeldet
Heruntergeladen am | 10.03.20 16:27

Table 3 Inter-instrument comparison of differential blood count and comparison to manual count (n=292 out of 349 samples, excluding samples with blasts and atypical lymphocytes).

	System	τ_b	b	a	Mean	SD	95% limits of agreement
			Regression to median				Differences to median
NEU, $10^3/\mu L$	Sapphire	0.98	1.00	0.00	−0.04	0.158	−0.40–0.20
	DxH 800	0.98	1.00	0.00	0.06	0.205	−0.20–0.60
	Advia 2120i	0.96	**1.08**	0.02	0.45	0.400	−0.10–1.40
	XE-5000	0.98	**0.98**	**−0.04**	−0.17	0.191	−0.60–0.10
	XN-2000	0.99	1.00	0.00	−0.03	0.147	−0.30–0.20
LYM, $10^3/\mu L$	Sapphire	0.96	1.00	0.00	0.05	0.123	−0.10–0.30
	DxH 800	0.97	1.00	0.00	−0.04	0.085	−0.20–0.10
	Advia 2120i	0.94	1.00	**0.10**	0.14	0.154	0.00–0.40
	XE-5000	0.97	1.00	0.00	−0.01	0.081	−0.20–0.20
	XN-2000	0.97	1.00	0.00	−0.05	0.077	−0.20–0.00
MON, $10^3/\mu L$	Sapphire	0.94	1.00	0.00	−0.01	0.078	−0.20–0.10
	DxH 800	0.95	1.00	0.00	0.00	0.064	−0.10–0.10
	Advia 2120i	0.85	**0.80**	0.02	−0.12	0.157	−0.50–0.10
	XE-5000	0.94	1.00	0.00	0.01	0.077	−0.10–0.20
	XN-2000	0.95	1.00	0.00	0.03	0.061	−0.10–0.20
EOS, $10^3/\mu L$	Sapphire	0.90	1.00	0.00	0.02	0.060	−0.10–0.10
	DxH 800	0.96	1.00	0.00	−0.01	0.029	−0.10–0.00
	Advia 2120i	0.93	1.00	0.00	0.02	0.042	−0.00–0.10
	XE-5000	0.97	1.00	0.00	0.00	0.031	−0.10–0.00
	XN-2000	0.97	1.00	0.00	−0.01	0.028	−0.10–0.00
BAS, $10^3/\mu L$	Sapphire	0.37	1.00	0.00	0.00	0.057	−0.10–0.10
	DxH 800	0.62	1.00	0.00	0.01	0.048	−0.10–0.10
	Advia 2120i	0.82	1.00	0.00	0.01	0.033	0.00–0.10
	XE-5000	0.67	1.00	0.00	−0.01	0.036	−0.10–0.00
	XN-2000	0.80	1.00	0.00	0.01	0.038	0.00–0.10
			Regression to microscopy				Differences to microscopy
NEU, $10^3/\mu L$	Sapphire	0.94	1.00	−0.05	−0.03	0.606	−0.90–0.90
	DxH 800	0.94	1.00	0.00	0.07	0.714	−0.90–1.30
	Advia 2120i	0.93	**1.05**	0.04	0.46	0.760	−0.30–2.10
	XE-5000	0.94	**0.98**	−0.05	−0.18	0.506	−1.00–0.60
	XN-2000	0.93	1.00	−0.10	−0.02	0.633	−0.90–1.00
LYM, $10^3/\mu L$	Sapphire	0.82	1.00	**0.20**	0.19	0.301	−0.30–0.90
	DxH 800	0.84	1.00	**0.10**	0.09	0.275	−0.40–0.70
	Advia 2120i	0.80	1.05	**0.19**	0.28	0.327	−0.30–1.10
	XE-5000	0.83	1.00	**0.10**	0.13	0.281	−0.40–0.80
	XN-2000	0.83	1.00	**0.10**	0.09	0.285	−0.40–0.80
MON, $10^3/\mu L$	Sapphire	0.70	1.14	**0.09**	0.17	0.215	−0.20–0.60
	DxH 800	0.70	1.14	**0.10**	0.18	0.237	−0.20–0.70
	Advia 2120i	0.66	1.00	**0.10**	0.06	0.205	−0.40–0.50
	XE-5000	0.69	1.17	**0.08**	0.19	0.235	−0.10–0.70
	XN-2000	0.72	1.14	**0.13**	0.21	0.238	−0.10–0.70
EOS, $10^3/\mu L$	Sapphire	0.76	1.00	0.00	0.04	0.109	−0.10–0.20
	DxH 800	0.78	1.00	0.00	0.02	0.096	−0.20–0.20
	Advia 2120i	0.76	1.00	0.00	0.04	0.099	−0.10–0.20
	XE-5000	0.78	1.00	0.00	0.02	0.094	−0.20–0.20
	XN-2000	0.79	1.00	0.00	0.02	0.094	−0.20–0.20
BAS, $10^3/\mu L$	Sapphire	0.17	1.00	0.00	0.01	0.065	−0.10–0.10
	DxH 800	0.26	1.00	0.00	0.01	0.065	−0.10–0.10
	Advia 2120i	0.34	1.00	0.00	0.01	0.062	−0.10–0.10
	XE-5000	0.35	1.00	0.00	−0.01	0.049	−0.10–0.10
	XN-2000	0.30	1.00	0.00	0.01	0.066	−0.10–0.10

τ_b, Kendall's τ_b; b, slope (numbers in bold are significantly different from 1); a, intercept (numbers in bold are significantly different from 0); SD, standard deviation.

Bereitgestellt von | Deutsche Vereinte Gesellschaft für Klinische Chemie und Laboratoriumsmedizin e.V. (DGKL)
Angemeldet
Heruntergeladen am | 10.03.20 16:27

for presence of blasts in blood smear, cut-offs ≥1% plasma cells, ≥5% atypical lymphocytes or ≥2% abnormal lymphocytes for presence of variant lymphocytes, cut-offs ≥2% metamyelocytes or ≥1% myelocytes/promyelocytes for presence of IG and ≥8% bands for presence of left shift, microscopy identified 34 samples with blasts, 25 samples with variant lymphocytes, 90 samples with IGs and 76 samples with left shift out of 349 unselected samples. Seven samples revealed PLT clumps in microscopy. A complete overview of samples with pathological findings in microscopy, the number of true-positive and false-positive flagging alerts in automated analysis with different instruments and respective sensitivities and specificities are shown in Table 4.

Blast flagging

All except one out of 34 blast positive samples could be identified with a blast flag by XN-2000, whereas 8–12 blast positive samples could not be correctly identified by other instruments (Table 4). The one not identified sample by XN-2000 and 4, 7, 3 and 6 not identified samples by Cell-Dyn Sapphire, DxH 800, Advia 2120i and XE-5000, respectively, revealed WBC counts $<2\times10^3/\mu L$. Two out of eight samples showing blasts in microscopy but without blast flagging in Cell-Dyn Sapphire revealed a variant lymphocyte flag, four an IG flag. Five out of nine samples without correct blast flagging in DxH 800 revealed a variant lymphocyte flag, two an IG flag. Six out of 12 blast containing samples were flagged to be positive for variant lymphocytes in Advia 2120i, five to be positive for IGs. Two out of 12 samples showing blasts in microscopy but without blast flagging in XE-5000 revealed a variant lymphocyte flag, five an IG flag. Two samples with blasts in microscopy were not even flagged by Cell-Dyn Sapphire and DxH 800, respectively, one sample by Advia 2120i and XN-2000, respectively, and four samples by XE-5000.

A blast flagging without verification of blasts in microscopy was found in 21 samples for Cell-Dyn Sapphire, in 15 samples for DxH 800, in 12 samples for Advia 2120i, in six samples for XE-5000 and in 14 samples for XN-2000. Flagging sensitivity was highest for XN-2000 (97%) and markedly lower for all other instruments (Cell-Dyn Sapphire, 76%; DxH 800, 74%; Advia 2120i and XE-5000, 65%). Setting the cut-off from >0 to ≥5% presence of blasts in blood smear, slightly better sensitivities could be shown for different instruments (Cell-Dyn Sapphire, 90%; DxH 800, 84%; Advia 2120i, 74%; XE-5000, 79%; XN-2000, 100%). Flagging specificity was comparable between instruments (93% to 98%).

Variant lymphocyte flagging

Samples described as positive for variant lymphocytes in microscopy (n=25) clearly revealed activation or atypical characteristics. Fourteen, 16 or 18 variant lymphocyte positive samples were correctly identified by Cell-Dyn Sapphire, DxH 800 or Advia 2120i. XE-5000 and XN-2000 each correctly identified 20 positive samples (Table 4). Two out of 11 samples showing variant lymphocytes in microscopy but without variant lymphocyte flagging in Cell-Dyn Sapphire revealed a blast flag, two an IG flag. Two out of nine samples that were not correctly flagged by DxH 800 for variant lymphocytes revealed a blast flag. One out of seven samples without correct variant lymphocyte flagging was classified as IG positive by Advia 2120i. One or four out of five samples showing variant lymphocytes in blood smear but without variant lymphocyte flagging in XE-5000 or XN-2000 were flagged to be positive for blasts, three samples were flagged to be positive for IGs by XE-5000. Seven or six samples with variant lymphocytes in microscopy were not even flagged by Cell-Dyn Sapphire, DxH 800 or Advia 2120i, and one sample by XE-5000 and XN-2000, respectively.

A variant lymphocyte flag without verification in microscopy was found in 18 samples for Cell-Dyn Sapphire and DxH 800, in 40 samples for Advia 2120i, in 17 samples for XE-5000 and in 14 samples for XN-2000. Flagging sensitivity was highest for XE-5000 and XN-2000 (80%) and markedly lower for other instruments (Cell-Dyn Sapphire, 56%; DxH 800, 64%; Advia 2120i, 72%). Setting the cut-off to ≥5% presence of variant lymphocytes in blood smear resulted in clearly better sensitivities (Cell Dyn Sapphire, DxH 800, XN-2000, 78%, respectively; Advia 2120i, 89%; XE-5000, 100%). Except Advia 2120i (88%), flagging specificity was comparable between instruments (94% to 95%).

Combining the clinically most relevant flags for blasts and variant lymphocytes resulted in sensitivities from 74% (Cell-Dyn Sapphire) to 96% (XN-2000), and in specificities from 94% to 96% for different instruments (Table 4).

IG and left shift flagging

Out of 90 samples revealing IGs (promyelocytes, myelocytes or metamyelocytes) in microscopy, 49 samples were correctly flagged by Cell-Dyn Sapphire, 60 samples by DxH 800, 35 samples by Advia 2120i, 72 samples by XE-5000 and 82 samples by XN-2000 (Table 4). Numbers of false-positive IG flags were 24 for Cell-Dyn Sapphire, 16 for DxH 800, 11 for Advia 2120i, 21 for XE-5000 and

Bereitgestellt von | Deutsche Vereinte Gesellschaft für Klinische Chemie und Laboratoriumsmedizin e.V. (DGKL)
Angemeldet
Heruntergeladen am | 10.03.20 16:27

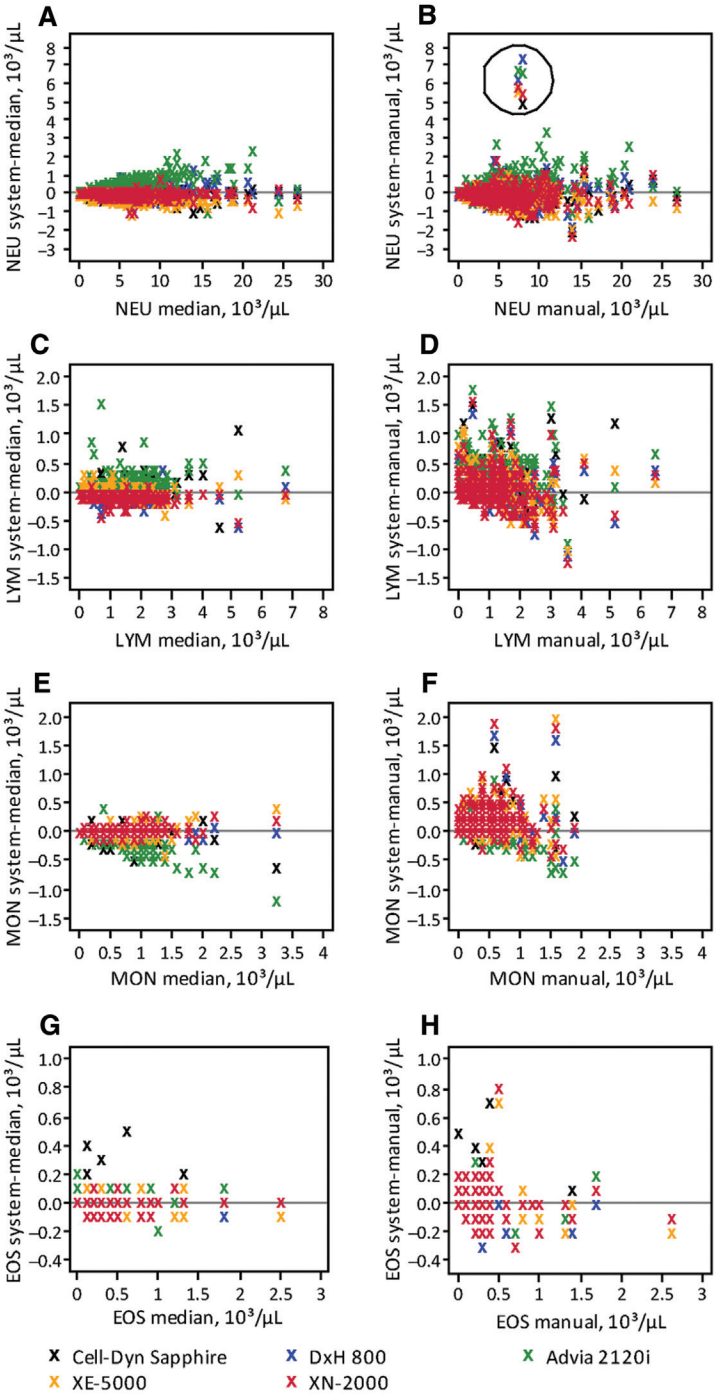


Figure 3 Difference plots for differential blood counts.
Automated and manual differential blood counts were compared in samples without blasts or variant lymphocytes in blood smear (n=292). A, C, E, and G show differences between automated counts and the median of all five analyzers plotted against the median. B, D, F, and H show differences between automated and manual counts plotted against manual count. (A, B) neutrophil count, (C, D) lymphocyte count, (E, F) monocyte count, (G, H) eosinophil count. Samples with a difference of more than 5×10^3/µL between automated and microscopic neutrophil counts are circled.

35 for XN-2000. Out of 76 samples revealing left shift in microscopy, 39 were correctly flagged by Cell-Dyn Sapphire, 64 by DxH 800, 39 by Advia 2120i, 38 by XE-5000 and 36 by XN-2000. As compared to microscopy, a false-positive left shift flag occurred in 13 samples for Cell-Dyn Sapphire, in 27 samples for DxH 800, in 14 samples for

Bereitgestellt von | Deutsche Vereinte Gesellschaft für Klinische Chemie und Laboratoriumsmedizin e.V. (DGKL)
Angemeldet
Heruntergeladen am | 10.03.20 16:27

Table 4 Inter-instrument comparison of pathological flaggings in 349 samples taken randomly out of routine analysis.

Instrument flagging	Pathological samples in microscopy, n	Instrument	True positives, n	Sensitivity 95% CI, %	False positives, n	Specificity 95% CI, %
Blasts	34	Sapphire	26	76 (59–89)	21	93 (90–96)
		DxH 800	25	74 (56–87)	15	95 (92–97)
		Advia 2120i	22	65 (46–80)	12	97 (94–98)
		XE-5000	22	65 (46–80)	6	98 (96–99)
		XN-2000	33	97 (85–100)	14	96 (93–98)
Variant lymphocytes	25	Sapphire	14	56 (35–76)	18	94 (91–97)
		DxH 800	16	64 (43–82)	18	94 (91–97)
		Advia 2120i	18	72 (51–88)	40	88 (84–91)
		XE-5000	20	80 (59–93)	17	95 (92–97)
		XN-2000	20	80 (59–93)	14	95 (93–98)
Immature granulocytes	90	Sapphire	49	54 (44–64)	24	91 (87–94)
		DxH 800	60	67 (56–76)	16	94 (90–96)
		Advia 2120i	35	39 (29–50)	11	96 (93–98)
		XE-5000	72	80 (70–88)	21	92 (88–95)
		XN-2000	82	91 (83–96)	35	86 (82–90)
Left shift	76	Sapphire	39	51 (40–63)	13	95 (92–97)
		DxH 800	64	84 (74–92)	27	90 (86–93)
		Advia 2120i	39	51 (40–63)	14	95 (92–97)
		XE-5000	38	50 (38–62)	1	99 (98–100)
		XN-2000	36	47 (36–59)	7	97 (95–99)
Platelet clumps	7	Sapphire	4	57 (18–90)	8	98 (96–99)
		DxH 800	6	86 (42–100)	7	98 (96–99)
		Advia 2120i	4	57 (18–90)	6	98 (96–99)
		XE-5000	4	57 (18–90)	8	98 (96–99)
		XN-2000	4	57 (18–90)	4	99 (97–100)
Blasts and/or variant lymphocytes	57	Sapphire	42	74 (60–84)	16	95 (91–97)
		DxH 800	46	81 (68–90)	15	95 (92–97)
		Advia 2120i	44	77 (64–87)	18	94 (90–96)
		XE-5000	43	75 (62–86)	11	96 (93–98)
		XN-2000	55	96 (88–100)	18	94 (90–96)
Blasts and/or variant lymphocytes and/or immature granulocytes	103	Sapphire	70	68 (58–77)	29	88 (84–92)
		DxH 800	80	78 (68–85)	29	88 (84–92)
		Advia 2120i	66	64 (54–73)	26	89 (85–93)
		XE-5000	88	85 (77–92)	30	88 (83–92)
		XN-2000	101	98 (93–100)	54	78 (72–83)

CI, confidence interval; n, number.

Advia 2120i, in one sample for XE-5000 and in seven samples for XN-2000. The highest flagging sensitivity for the presence of IGs could be shown for XN-2000 (91%), Advia 2120i revealed the lowest (39%). Flagging sensitivity and specificity for left shift were comparable among different instruments (47%–51%; 95%–99%), except DxH 800 revealing a sensitivity of 84% and a specificity of 90% (Table 4).

PLT clump flagging

PLT clumping was identified by microscopy in seven samples. Out of these, six were correctly flagged by DxH 800, and four samples were correctly flagged by the other instruments (Table 4). Four out of seven samples with PLT clumps in microscopy revealed automated PLT counts in the range of 30–100×10^3/µL and manually corrected PLT counts considerably above 100×10^3/µL, and were therefore of potential clinical relevance. Each of these four samples was correctly flagged by DxH 800, three were correctly identified by Cell-Dyn Sapphire, and only two by Advia 2120i, Sysmex XE-5000 and XN-2000, respectively. A PLT clump flag without evidence for PLT clumping in microscopy occurred in eight samples for Cell-Dyn Sapphire, in seven samples for DxH 800, in six samples for Advia 2120i, in eight samples for XE-5000 and in four samples for XN-2000.

Bereitgestellt von | Deutsche Vereinte Gesellschaft für Klinische Chemie und Laboratoriumsmedizin e.V. (DGKL)
Angemeldet
Heruntergeladen am | 10.03.20 16:27

Inter-instrument comparison of false-positive flags in samples without pathological criteria in microscopy (n=198)

In 198 out of 349 randomly selected samples, microscopy did not reveal pathological criteria. In this selection of samples, numbers of false-positive flags for blasts and variant lymphocytes did not differ significantly between the different instruments, but were significantly higher for IGs with the XN-2000 (n=18) compared to all other instruments (χ^2-test, Table 5).

Inter-instrument comparison of required sample volume

Minimal volumes required for CBC analysis including WBC differential by different instruments using different tubes are presented in Supplemental Table 1. Using 2.7 mL Sarstedt tubes, Cell-Dyn Sapphire required the smallest volume (300 µL), while the other analyzers required a minimal volume of 500 µL. Using 1.2 mL Sarstedt tubes, XN-2000 required the smallest volume (200 µL), while Cell-Dyn Sapphire and DxH 800 required minimal volumes of 300 µL, Advia 2120i and XE-5000 of 500 µL. Using 1.0 mL Siemens EZEE-nest tubes or 1.5 mL Sarstedt micro tubes, Cell-Dyn Sapphire, Advia 2120i and XN-2000 required a minimal volume of 150 µL, respectively. DxH 800 required minimal volumes of 200 µL using both tubes, XE-5000 required a minimal volume of 150 µL or 300 µL, respectively.

Discussion

To the best of our knowledge, this is the most comprehensive side-by-side comparison, investigating the diagnostic performances of five current top of the range routine hematology analyzers in the setting of a large university hospital central laboratory. Samples were collected randomly from routine diagnostics (n=349), permitting unbiased testing of the five instruments. A good correlation for CBC parameters was found in inter-instrument comparison, however, slight systemic differences of instruments were observed (Table 2, Figure 1, Supplemental Figure 1). A consistently poorer inter-instrument concordance could be shown for reticulocyte counts, with Cell-Dyn Sapphire showing significantly higher and Advia 2120i showing significantly lower values (Table 2, Figure 1H, Supplemental Figure 1H). A rather low concordance for NRBC counts was observed between different instruments, the poorest concordance for automated NRBC counts with microscopy was found for Advia 2120i (Table 2, Figure 2). Without the use of a true reference method for most of the investigated parameters, interpretation of results of different analyzers is difficult. For inter-instrument comparison we therefore used the median of all five analyzers as a reference. Correlations of differential blood counts between instruments and with microscopy were less well, however, clear differences between the various instruments were not noted.

A major expectation of the clinical laboratory from any automated hematology analyzer is to decrease review-rates, requiring the highest possible flagging specificity and sensitivity. In our analysis of 349 randomly selected routine samples, XN-2000 outperformed the other instruments in blast flagging, while specificity was comparable between instruments (Tables 4 and 5).

It is difficult to compare our findings with previous published work, because different analyzers were used in each paper. The closest publication might be by Hotton et al. [3], where three of the instruments, the Cell-Dyn Sapphire, DxH 800 and XN-2000, also tested in our study, were used. In agreement with our work, these authors did not find relevant differences for blood count between the three analyzers, except slightly higher PLT counts in Cell-Dyn Sapphire. It must be noted that the Advia 2120i,

Table 5 Inter-instrument comparison of false-positive flags in 198 samples without pathological criteria in microscopy.

Instrument	Instrument flagging (n)					
	Blasts	Variant lymphocytes	Immature granulocytes	Left shift	PLT clumps	Other
Sapphire	1	0	6	6	2	3[a]
DxH 800	1	0	7	12	3	
Advia 2120i	0	2	5	11	3	
XE-5000	0	0	5	0	0	
XN-2000	1	1	18	4	2	2[b]

n, number; [a]unidentified fluorescence cells; [b]WBC abnormal distribution.

Bereitgestellt von | Deutsche Vereinte Gesellschaft für Klinische Chemie und Laboratoriumsmedizin e.V. (DGKL)
Angemeldet
Heruntergeladen am | 10.03.20 16:27

which showed a slightly lower concordance of WBCs, MCV and PLTs compared to other analyzers in our work, was not tested in that study. Unfortunately, there are no current side-by-side comparisons of Advia 2120i, except for NRBC analysis [14, 15] and flagging performance [16, 17], which are however, not informative with regard to CBC parameters. In our study, we were able to show a slightly lower concordance of PLTs for Advia 2120i systematically tending to higher counts compared to DxH 800, XE-5000 and XN-2000 and to a lesser extent compared to Cell-Dyn Sapphire (Table 2). Previous studies also observed higher PLT counts with Cell-Dyn Sapphire and Advia 120, the predecessor model of Advia 2120i, when compared with DxH 800, XE-2100 the predecessor model of XE-5000, and XN-2000 [1–3]. Considerably higher PLT counts were also confirmed in a recent publication comparing Advia 2120i with CD61 based PLT counts and an immunological reference method [18].

The application of PLT-F channel as first-line PLT testing in XN-2000 in our study may not reflect routine algorithms, because the impedance method is normally used as first-line diagnostics followed by fluorescence based testing in case of abnormal scattergram or low PLT count. However, based on the fact that impedance and optical PLT counts are measured with the same technology in Sysmex XE-5000 and XN-2000, we decided to define the PLT-F channel as first-line testing in XN-2000 in order to investigate a potential benefit of PLT-F technology in the present study. We could not find clear differences of PLT counts generated by PLT-F based technology as compared to XE-5000, but we were able to confirm recent studies showing a slightly better concordance of PLT-F based analysis as compared to CD61 based analysis (Table 2) [19].

Regarding differential blood count, we found an overall high inter-instrument correlation of neutrophil counts (Supplemental Figure 2A), lymphocyte counts (Supplemental Figure 2C) and eosinophil counts (Supplemental Figure 2G) and with microscopy (Supplemental Figure 2B, D, H), also observed in previous studies [1, 2]. In our study monocyte counts between different instruments revealed a considerably lower agreement with Advia 2120i showing the maximum deviation to consistently lower values (Table 3, Figure 3E,). Except Advia 2120i, automated differentials revealed higher percentages of monocytes in comparison with microscopy (Table 3, Figure 3F). Lower percentages of monocytes in automated analysis have already been described for Advia 120 and were suspected to be due to underestimation by the peroxidase staining [2, 20]. As in previous validation studies, we were able to show low correlations of basophil counts between automated and manual counts (Table 3) [1, 2]. However, largely due to statistical uncertainty because of missing samples with higher basophil counts, the informative value of this analysis is quite limited.

The presence of NRBCs in peripheral blood is an indicator for pathologic conditions and the number of NRBCs was shown to be associated with the prognosis in neonates and in critically ill patients [21, 22]. Furthermore, accurate identification and quantification of NRBCs is a prerequisite for adequate correction of WBC counts. Here, we found a very limited concordance of NRBC counts in inter-instrument comparison and in comparison with microscopy (Table 2, Figure 2). Using linear regression analysis, a recent publication showed a good correlation of NRBC analysis between Cell-Dyn Sapphire, DxH 800 and microscopy [1, 23], however, overall concordance was also limited. Unlike Tan et al., we calculated Kendall's τ_b, which in our view better reflects the distribution of NRBC values with the majority in a very low range. In comparison to Pearson's correlation, τ_b resulted in lower correlation coefficients. Other studies showed a clear inferiority of Advia 2120i compared to DxH 800 [15] and XE-5000 [14] in NRBC analysis. Taking all instruments evaluated in our study into consideration, the worst relationship to microscopy as reference method was also found for Advia 2120i, other instruments revealed comparable quality in NRBC analyses.

An essential role of modern routine hematology analyzers in clinical practice is to screen for samples containing pathological cell types and therefore requiring microscopic evaluation. For blast flagging as one of the clinically most relevant warning messages, a significant improvement of sensitivity was found for XN-2000 in comparison to other instruments (Table 4). These data confirm a recent study showing sensitivities to detect blasts of 89%, 72% and 100% for Cell-Dyn Sapphire, DxH 800 and XN-2000 in a limited population of 18 blast positive samples [3]. The higher sensitivity of blast flagging in XN instruments might be due to the newly introduced white cell differential (WDF) and white cell nucleated red blood (WNR) channel allowing optimized identification of blast cells [4]. Our data revealing limited sensitivity of XE-5000 in blast flagging is in line with Eilertsen et al. showing 11 false negative out of 37 blast positive samples [24]. In addition, these authors showed a high variability of blast flagging in different XE-5000 instruments. The limited sensitivity of blast flagging in different instruments other than the XN-2000 was not restricted to samples with leucopenia. Despite an association of the three flags for blasts, variant lymphocytes or IGs for detection of blasts, different instruments still missed blast positive samples. It must also be noted, that in contrast to the study by Hotton

Bereitgestellt von | Deutsche Vereinte Gesellschaft für Klinische Chemie und Laboratoriumsmedizin e.V. (DGKL)
Angemeldet
Heruntergeladen am | 10.03.20 16:27

et al. [3], the XN-2000 still overlooked blasts in one out of 34 samples, thereby not providing 100% certainty for detecting these cells.

The highest sensitivity for flagging of variant lymphocytes was found for XE-5000 and XN-2000, continuously decreasing sensitivities were found for Advia 2120i, DxH 800 and Cell-Dyn Sapphire (Table 4). In their inter-instrument comparison between Cell-Dyn Sapphire, DxH 800 and XN-2000, in accordance with our data, Hotton et al. showed the highest lymphocyte flagging sensitivity for the XN-2000, however, remarkably lower sensitivities were shown for other instruments [3].

IGs are increased in various clinical conditions [25]. As microscopic evaluation of patient samples for presence of IGs is time consuming, identification of the respective samples in automated blood count analysis gains increasing importance in clinical practice. Comparing IG flagging of different instruments with presence of IGs in blood smear analysis, we were able to show a superior sensitivity for XN-2000 (91%) compared to other instruments with sensitivities ranging from 80% for XE-5000 to 39% for Advia 2120i (Table 4). Our findings are consistent with Hotton et al. showing considerably higher IG flagging sensitivities for XN-2000 compared with Cell-Dyn Sapphire and DxH 800 and with Meintker et al. showing lowest sensitivity for Advia 120 as compared to Cell-Dyn Sapphire and DxH 800 [2, 3].

There is only limited information available about the quality of PLT clump flagging of different hematology analyzers, however, a high flagging quality would be of great interest as agglutination of PLTs is associated with false low PLT counts in automated analysis. With the exception of DxH 800, all of the analyzers had limitations in the discrimination of PLT clumps (Table 4). Our data are in accordance with Sandhaus et al. describing only very limited sensitivities for PLT clump flagging in LH 750, XE-2100 and Advia 120, as predecessor models of DxH 800, XE-5000 and Advia 2120i [26].

Beside high flagging sensitivity, low numbers of false-positive flagging alerts are a prerequisite to reduce unnecessary manual smear reviews. As already described in recent publications, flagging parameters most contributing to the number of false-positive samples were IGs followed by left shift and PLT clumping [1–3, 17]. Combining flagging alerts for presence of blasts, variant lymphocytes and IGs, XN-2000 revealed the lowest specificity (78%) among all investigated instruments primarily due to a relatively high number of false-positive IG warnings. In accordance with existing publications comparing Cell-Dyn Sapphire versus DxH 800 [1] and DxH 800 versus Advia 2120i [17], we were able to show similar specificities ranging from 94% to 96% for the two most clinically relevant warning alerts (presence of blasts and variant lymphocytes) in inter-instrument comparison (Table 4).

The required sample volume of different analyzers for CBC analysis and differential is of great interest especially for pediatric samples. Inter-instrument comparison revealed that the minimal volume required by different instruments depended on usage of different tubes (Supplemental Table 1).

In conclusion, side-by-side testing of five current top of the range hematology analyzers, revealed a good concordance for blood count parameters, correlations between instruments were less well for reticulocyte counts, NRBCs, and differentials. With respect to automated NRBC analysis Advia 2120i revealed main limitations compared to microscopic analysis. Regarding flagging quality between different instruments, the highest overall sensitivity for presence of blasts, variant lymphocytes and IGs was found for XN-2000. Variable analyzer quality has to be taken into consideration in defining laboratory algorithms in clinical practice.

Acknowledgments: We thank Abbott, Beckman Coulter, Siemens and Sysmex for their support of the study by providing their instruments for evaluation free of charge.
Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.
Financial support: None declared.
Employment or leadership: None declared.
Honorarium: None declared.
Competing interests: The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

References

1. Tan BT, Nava AJ, George TI. Evaluation of the Beckman Coulter UniCel DxH 800, Beckman Coulter LH 780, and Abbott Diagnostics Cell-Dyn Sapphire hematology analyzers on adult specimens in a tertiary care hospital. Am J Clin Pathol 2011;135:939–51.
2. Meintker L, Ringwald J, Rauh M, Krause SW. Comparison of automated differential blood cell counts from Abbott Sapphire, Siemens Advia 120, Beckman Coulter DxH 800, and Sysmex XE-2100 in normal and pathologic samples. Am J Clin Pathol 2013;139:641–50.
3. Hotton J, Broothaers J, Swaelens C, Cantinieaux B. Performance and abnormal cell flagging comparisons of three automated blood cell counters: Cell-Dyn Sapphire, DxH-800, and XN-2000. Am J Clin Pathol 2013;140:845–52.

Bereitgestellt von | Deutsche Vereinte Gesellschaft für Klinische Chemie und Laboratoriumsmedizin e.V. (DGKL)
Angemeldet
Heruntergeladen am | 10.03.20 16:27

4. Briggs C, Longair I, Kumar P, Singh D, Machin SJ. Performance evaluation of the Sysmex haematology XN modular system. J Clin Pathol 2012;65:1024–30.
5. ICSH guidelines for the evaluation of blood cell analysers including those used for differential leucocyte and reticulocyte counting. International Council for Standardization in Haematology, Writing Group: Briggs C, Culp N, Davis B, d`Onofrio G, Zini G, Machin SJ; the International Council for Standardization of Haematology. Int J Lab Hematol 2014;36:613–27.
6. Clinical and Laboratory Standards Institute. Reference leukocyte (WBC) differential count (proportional) and evaluation of instrumental methods; CLSI document H20-A2 Approved Standard, 2nd ed. Wayne, PA: CLSI, 2007.
7. International Society for Laboratory Hematology. Consensus rules: consensus guidelines-positive smear findings. Available from: http://www.islh.org/2010/index.php?page=consensus_smear. Accessed on December 22, 2014.
8. Müller R, Mellors I, Johannessen B, Aarsand AK, Kiefer P, Hardy J, et al. European multi-center evaluation of the Abbott Cell-Dyn sapphire hematology analyzer. Lab Hematol 2006;12:15–31.
9. Jean A, Boutet C, Lenormand B, Callat MP, Buchonnet G, Barbay V, et al. The new haematology analyzer DxH 800: an evaluation of the analytical performances and leucocyte flags, comparison with the LH 755. Int J Lab Hematol 2011;33:138–45.
10. Harris N, Jou JM, Devoto G, Lotz J, Pappas J, Wranovics D, et al. Performance evaluation of the ADVIA 2120 hematology analyzer: an international multicenter clinical trial. Lab Hematol 2005;11:62–70.
11. Passing H, Bablok W. A new biometrical procedure for testing the equality of measurements from two different analytical methods. Application of linear regression procedures for method comparison studies in clinical chemistry, part I. J Clin Chem Clin Biochem 1983;21:709–20.
12. Kendall MG. The treatment of ties in ranking problems. Biometrika 1945;33:239–51.
13. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet 1986;1:307–10.
14. Pipitone S, Pavesi F, Testa B, Bardi M, Perri GB, Gennari D, et al. Evaluation of automated nucleated red blood cells counting on Sysmex XE5000 and Siemens ADVIA 2120. Clin Chem Lab Med 2012;50:1857–9.
15. Kwon MJ, Nam MH, Kim SH, Lim CS, Lee CK, Cho Y, et al. Evaluation of the nucleated red blood cell count in neonates using the Beckman Coulter UniCel DxH 800 analyzer. Int J Lab Hematol 2011;33:620–8.
16. Barnes PW, Eby CS, Shimer G. Blast flagging with the UniCel DxH 800 Coulter Cellular Analysis System. Lab Hematol 2010;16:23–5.
17. Kim SJ, Kim Y, Shin S, Song J, Choi JR. Comparison study of the rates of manual peripheral blood smear review from 3 automated hematology analyzers, Unicel DxH 800, ADVIA 2120i, and XE 2100, using international consensus group guidelines. Arch Pathol Lab Med 2012;136:1408–13.
18. Trabuio E, Valverde S, Antico F, Manoni F, Gessoni G. Performance of automated platelet quantification using different analyzers in comparison with an immunological reference method in thrombocytopenic patients. Blood Transfus 2009;7:43–8.
19. Tanaka Y, Tanaka Y, Gondo K, Maruki Y, Kondo T, Asai S, et al. Performance evaluation of platelet counting by novel fluorescent dye staining in the XN-series automated hematology analyzers. J Clin Lab Anal 2014;28:341–8.
20. Grimaldi E, Carandente P, Scopacasa F, Romano MF, Pellegrino M, Bisogni R, et al. Evaluation of the monocyte counting by two automated haematology analyzers compared with flow cytometry. Clin Lab Haematol 2005;27:91–7.
21. Baschat AA, Gungor S, Kush ML, Berg C, Gembruch U, Harman CR. Nucleated red blood cell counts in the first week of life: a critical appraisal of relationships with perinatal outcome in preterm growth-restricted neonates. Am J Obstet Gynecol 2007;197:286.e1–8.
22. Stachon A, Segbers E, Holland-Letz T, Kempf R, Hering S, Krieg M. Nucleated red blood cells in the blood of medical intensive care patients indicate increased mortality risk: a prospective cohort study. Crit Care 2007;11:R62.
23. Tan BT, Nava AJ, George TI. Evaluation of the Beckman Coulter UniCel DxH 800 and Abbott Diagnostics Cell-Dyn Sapphire hematology analyzers on pediatric and neonatal specimens in a tertiary care hospital. Am J Clin Pathol 2011;135:929–38.
24. Eilertsen H, Vøllestad NK, Hagve TA. The usefulness of blast flags on the Sysmex XE-5000 is questionable. Am J Clin Pathol 2013;139:633–40.
25. Buttarello M, Plebani M. Automated blood cell counts: state of the art. Am J Clin Pathol 2008;130:104–16.
26. Sandhaus LM, Osei ES, Agrawal NN, Dillman CA, Meyerson HJ. Platelet counting by the coulter LH 750, sysmex XE 2100, and advia 120: a comparative analysis using the RBC/platelet ratio reference method. Am J Clin Pathol 2002;118:235–41.

Supplemental Material: The online version of this article (DOI: 10.1515/cclm-2014-0945) offers supplementary material, available to authorized users.

Bereitgestellt von | Deutsche Vereinte Gesellschaft für Klinische Chemie und Laboratoriumsmedizin e.V. (DGKL)
Angemeldet
Heruntergeladen am | 10.03.20 16:27

Sepsis-associated changes of the arachidonic acid metabolism and their diagnostic potential in septic patients*

Mathias Bruegel, MD; Ute Ludwig; Alisa Kleinhempel; Sirak Petros, MD; Linda Kortz, PhD; Uta Ceglarek, PhD; Lesca Miriam Holdt, MD, PhD; Joachim Thiery, MD; Georg Martin Fiedler, MD

Objectives: **Sepsis-associated changes of the arachidonic acid metabolism and the utility of arachidonic acid metabolites for the diagnosis of sepsis have been poorly investigated so far. Therefore, the primary objective of our study was to screen for differentially regulated arachidonic acid metabolites in septic patients using a lipopolysaccharide whole-blood model and to investigate their diagnostic potential.**

Design: **Prospective, observational, single-center, clinical study.**

Setting: **Intensive care unit at University Hospital Leipzig.**

Patients: **Thirty-five patients (first cohort 25 patients, second cohort 10 patients) meeting the criteria for severe sepsis or septic shock were enrolled. Eighteen healthy volunteers (first cohort 15 subjects, second cohort 3 subjects) were enrolled as controls.**

Interventions: **None.**

Measurements and Main Results: **Arachidonic acid and its metabolites were investigated in supernatants of nonactivated (baseline) and lipopolysaccharide-activated heparinized whole blood of healthy subjects (n = 15) and septic patients (n = 25) by solid phase extraction and subsequent liquid chromatography-tandem mass spectrometry. Arachidonic acid, arachidonic acid analogues, and the cyclooxygenase-associated metabolites prostaglandin E_2, 11-hydroxyeicosatetraenoic acid, and thromboxane B_2 were identified as differentiating metabolites between septic patients and healthy subjects. Some of these compounds, including arachidonic acid, its analogues, and the cyclooxygenase metabolites prostaglandin E_2 and thromboxane B_2 differed at baseline. The inducibility of arachidonic acid and the cyclooxygenase metabolites 11-hydroxyeicosatetraenoic and prostaglandin E_2 were reduced by 80% to 90% in septic patients. The degree of the inducibility was associated with severity of sepsis and clinical outcome. A reduced inducibility of *COX-2* but preserved inducibility of mPGES-1 on gene expression level were confirmed in an independent cohort of septic patients (n = 10) by quantitative reverse-transcription polymerase chain reaction compared to healthy controls (n = 3).**

Conclusions: **Arachidonic acid metabolism is markedly affected in patients with sepsis. Our data suggest that the analysis of arachidonic acid metabolites in an *in vitro* whole blood activation model may be a promising approach for risk estimation in septic patients that has to be further evaluated in subsequent large-scale clinical studies. (Crit Care Med 2012; 40:1478–1486)**

Key Words: arachidonic acid metabolites; eicosanoids; lipopolysaccharide; sepsis; whole blood

Eicosanoids are lipid-signaling molecules primarily generated from arachidonic acid (AA), which is released by phospholipase A_2 enzymes from membrane phospholipids. The subsequent enzymatic metabolization via cyclooxygenase (COX), cytochrome P450, and lipoxygenase pathways or via nonenzymatic peroxidation mediates the generation of a broad spectrum of eicosanoids (1). As proinflammatory molecules (prostaglandin [PG] H_2), chemoattractants (leukotriene B_4), platelet (PLT) aggregation factors, and contractors of smooth muscle cells (thromboxane A_2), modifiers of vascular permeability (leukotrienes) and potent vasodilators (PGE_2 and PGI_2), these cell-derived mediators are involved in the pathogenesis of inflammatory diseases and sepsis (2, 3). Based on these central pathophysiological effects and their superordinate modulatory function on inflammatory reactions, eicosanoids can be considered as biomarkers for sepsis (4, 5). However, sepsis-associated changes of the AA metabolism and its diagnostic potential have been poorly investigated so far because of the analytical difficulties as well as the chemical and biological complexity of eicosanoids, which are characterized by a very short half-life and a rapid degradation. Recently, different methods based on liquid chromatography combined with tandem mass spectrometry were developed for the analysis of AA metabolites in biological fluids, allowing specific, accurate, and simultaneous analysis of eicosanoid profiles as a whole-systems biology approach.

The aim of the present prospective, observational, single-center, clinical study was to investigate sepsis-associated changes of the AA metabolism and to screen for differentially regulated AA metabolites

***See also p. 1663.**

From the Institute of Laboratory Medicine (MB, UT, AK, LK, UC, MH, JT, GMF), Clinical Chemistry and Molecular Diagnostics, University Hospital Leipzig, Leipzig; Department of Internal Medicine (SP), Intensive Care Unit, University Hospital Leipzig, Leipzig; Leipzig Interdisciplinary Research Cluster of Genetic Factors (LK, UC, MH, JT), Clinical Phenotypes and Environment, Leipzig, Germany.

Present affiliation for Dr. Bruegel: Institute of Clinical Chemistry, Ludwig-Maximilians-University Munich, Munich, Germany.

Present affiliation for Dr. Fielder: University Institute of Clinical Chemistry, Inselspital, University Hospital Bern, Bern, Switzerland.

Drs. Bruegel and Ludwig contributed equally to this work.

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (http://journals.lww.com/ccmjournal).

The authors have not disclosed any potential conflicts of interest.

For information regarding this article, E-mail: Mathias.Bruegel@med.uni-muenchen.de

Copyright © 2012 by the Society of Critical Care Medicine and Lippincott Williams & Wilkins

DOI: 10.1097/CCM.0b013e3182416f05

using a lipopolysaccharide (LPS) *in vitro* whole-blood activation model with subsequent multiparametric liquid chromatography combined with tandem mass spectrometry analysis. Furthermore, we investigated the diagnostic potential of differentially released eicosanoids in sepsis with regard to disease severity and prognosis.

MATERIALS AND METHODS

Study Population. In total, 35 septic patients divided into two study cohorts were enrolled within 12 hrs of admission to the medical intensive care unit (ICU) at the University Hospital Leipzig, Germany. All patients met the criteria for severe sepsis or septic shock according to the International Sepsis Definitions Conference consensus criteria (6). They received early goal-directed therapy consisting of adequate volume administration, appropriate antibiotics, and optimal oxygen delivery. Patients of the first study cohort (n = 25) were used for identification and quantification of differentially released AA metabolites. They were further classified regarding their clinical course of disease for association studies. A favorable clinical course was defined as sepsis survival and transfer to a general ward within a period of 2 wks, whereas an unfavorable clinical course was defined as death within a period of 2 wks or need for prolonged ICU treatment without clinical improvement. Patients of the second study cohort (n = 10) were used for investigation of target genes of the AA metabolism and for analysis of metabolic activity and endotoxin plasma concentrations. Demographic and clinical data including the Simplified Acute Physiology Score II were documented for all patients. Healthy volunteers not using anti-inflammatory drugs or not suffering from infectious diseases within 14 days before whole-blood testing were enrolled as controls (n = 18). Fifteen and three healthy subjects were used as controls for the first and second patient study cohort. Their differential blood counts displayed normal cell counts and blood smears showed no signs of inflammatory activation or infection. The study meets the ethical standards of the Declaration of Helsinki. It has been approved by the Ethics Committee of the Medical Faculty of the University Leipzig, Germany.

Specimen Collection. Whole blood was collected into 7.5-mL lithium heparin tubes (S-Monovette-system; Sarstedt, Nümbrecht, Germany) through indwelling venous catheters in all patients. Blood collections were performed on admission to ICU and on day 3 of therapy. Whole blood from healthy subjects was collected by peripheral venous blood puncture using Sarstedt Safety-Multifly needles (800 mm), with subsequent collection into 7.5 mL lithium heparin tubes (S-Monovette-system; Sarstedt). After blood collection, whole blood samples were immediately used for activation experiments. Heparin plasma was used for analysis of LPS concentration after centrifugation of whole blood for 5 mins at 4000*g*. Plasma was kept frozen at –80°C until analysis.

LPS Whole Blood Testing. Fresh heparinized whole blood (1 mL) was transferred into cell culture plates (Tissue Culture Plate 6-well Flat Bottom Cell +; Sarstedt) and mixed with 500 mL RPMI 1640 medium (Biochrom, Berlin, Germany) containing 1% penicillin/streptomycin and spiked with or without LPS from *Escherichia coli* (Sigma-Aldrich, St. Louis, MO) at 1 mg/mL. Whole blood was incubated with or without LPS for 24 hrs at 37°C and 5% CO_2. For preincubation experiments, blood cells were incubated with LPS (300 pg/mL) for 12 hrs ahead of stimulation with LPS (1 mg/mL) for 24 hrs. One sample aliquot containing whole-blood medium mix without LPS was immediately processed without further incubation, serving as baseline. Samples were centrifuged at 4000*g* for 10 mins, supernatants were transferred into microtubes (1.5 mL polypropylene; Sarstedt) and stored at –80°C until liquid chromatography combined with tandem mass spectrometry or endotoxin analysis. Cellular components were used for RNA isolation and gene expression analysis.

Laboratory Diagnostics. A Sysmex XE-2100 hematology analyzer (Sysmex, Kobe, Japan) was used for automated analysis of differential blood count and procalcitonin was analyzed by the Brahms procalcitonin Kryptor assay (Brahms Diagnostica, Hennigsdorf, Germany). Analysis of endotoxin was performed using the Endosafe-PTS system (Charles River, Wilmington, MA) based on limulus amebocyte lysate kinetic chromogenic methodology according to the manufacturers' instructions. An endotoxin standard (Charles River) was used for recovery testing in plasma samples. Trypan blue or CellTiter-Blue Cell Viability Assay (Promega, Madison, WI) were used for analysis of cell viability in whole blood before and after activation with LPS according to the manufacturers' instructions.

Sample Preparation and Liquid Chromatography Combined With Tandem Mass Spectrometry Analysis. Solid-phase extraction was used for extraction and concentration of the eicosanoids from liquid samples; 100 mL of supernatants were mixed with 50 mL of the internal standard, consisting of isotope-labeled eicosanoids: TxB_2-d4, PGF_{2a}-d4, PGE_2-d4, leukotriene B_4-d4, 5-S-hydroxyeicosatetraenoic (HETE) acid-d8 (100 ng/mL each), and AA-d8 (1000 ng/mL) (Cayman Chemical, Ann Arbor, MI). After protein precipitation with 200 mL of a methanol/zinc sulfate heptahydrate (89 g/L; 4/1 v/v) solution and centrifugation at 10,000*g* for 5 mins, 300 mL of the supernatant were mixed with 100 mL of 10% acetic acid in water and 800 mL water. Solid-phase extraction was performed using Strata-x 33 mm polymeric sorbent 60 mg/3 mL tubes (Phenomenex, Torrance, CA) that were conditioned with 2 mL methanol and 2 mL water and centrifuged at 170*g* for 1 min; 1.2 mL of the diluted supernatant were transferred onto the solid-phase extraction cartridge and centrifuged at 270*g* for 1 min. After washing with 2 mL methanol/water (10/90; v/v), the samples were eluted with 1 mL of pure methanol, followed by vacuum concentration for 2.5 hrs at 35°C (SpeedVac Concentrator; Thermo Scientific, Waltham, MA). Samples were stored at –80°C until analysis. After thawing and reconstitution in 50 mL acetonitrile/water (50/50; v/v) +0.02% formic acid, samples (10 mL) were injected into the liquid chromatography combined with tandem mass spectrometry system. A triple quadrupole mass spectrometer (API 4000 QTrap; AB SCIEX, Foster City, CA) with an electrospray ionization source was used in negative ion mode. Multiple reaction monitoring of 55 mass transitions was performed for eicosanoid analysis. Analytes were quantified via their corresponding deuterated internal standards (7) (Supplemental Table 1 [Supplemental Digital Content 1, http://links.lww.com/CCM/A396] shows a list of eicosanoids and isotope-labeled internal standards with specific mass transitions and retention times).

Isolation of RNA and Quantitative Fluorogenic Reverse-Transcription Polymerase Chain Reaction. RNA from cellular components of whole blood was isolated using QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer´s instructions, including additional DNA digestion with RNase-Free DNase (Qiagen). RNA was reverse-transcribed using SuperScript-II enzyme and random hexamer primers (Invitrogen, Carlsbad, CA). Gene expression was determined by quantitative fluorogenic reverse-transcription polymerase chain reaction. Assays for *COX-2* and *mPGES-1* were established using gene-specific primers and probes spanning two exons to avoid amplification of genomic DNA (*COX-2*: 5′ primer 5′-CTT CAC GCA TCA GTT TTT CAA G-3′; 3′ primer 5′-TCA CCG TAA ATA TGA TTT AAG TCC AC-3′; probe 5′-FAM-ATA AGC GAG GGC CAG CTT TCA CCA ACG-TAMRA-3′; *mPGES1*: 5′ primer 5′-CTG GGA TGA CAG GCA TGA AT-3′; 3′ primer 5′-GAC TCA CAT GGG AGC CTT TT-3′; probe 5′-FAM-CAC TGT GCT CAG CCA CCA TCT GGA GTT-TAMRA-3′). Results of gene expression analysis were normalized to mg RNA.

Data Evaluation and Statistical Analysis. MarkerView 1.2 Software (AB Sciex, Foster City, CA) was used for biomarker discovery by principal component analysis. Analyst 1.5 Software (AB Sciex) was used for quantitative analysis of chromatographic peaks. Statistical analyzes were performed using the GraphPad Prism 4 statistical software (GraphPad Software, LA Jolla, CA). Normality of distribution was assessed using the Kolmogorov-Smirnov test. Groups of normally distributed samples were analyzed using the *t* test and non-normally distributed samples were analyzed using Mann-Whitney test. A $p < .05$ was considered as statistically significant.

Table 1. Characteristics at study population

	First Cohort				Second Cohort	
		Septic Patients				
	Healthy Subjects	Total	Survival to a General Ward at 2 wks	Prolonged Intensive Care Unit Care or Death at 2 wks	Healthy Subjects	Septic Patients
Subjects (n)	15	25	13	12	3	10
Age (y)	26a (23-32)[b]	63a (50-73)[b]	70a (53-76)[b]	61a (48-67)[b]	36a (29-37)[b]	71a (69-76)[b]
Gender						
Male (n)	7	17	7	10	2	8
Female (n)	8	8	6	2	1	2
Severe Sepsis (n)	—	15	10	5	—	1
Septic Shock (n)	—	10	3	7	—	9
White Blood Cell Count (×109/L)	5.2a (4.2-6.9)[b]	9.4a (5.7-17.2)[b]	12.0a (7.2-17.3)[b]	7.9a (5.2-19.6)[b]	5.7a (5.4-6.0)[b]	17.0a (12.3-21.0)[b]
Platelet count (×109/L)	214a (192-257)[b]	184a (88-284)[b]	245a (175-314)[b]	87a (34-167)[b]	287a (244-319)[b]	186a (104-218)[b]
Procalcitonin (μg/L)	—	14.9a (2.6-27.2)[b]	11.4a (2.0-20.1)[b]	20.1a (2.9-38.7)[b]	—	29.0a (15.0-66.0)[b]
Microbiology (n)	—				—	
Gram-negative bacteria		8				5
Gram-positive bacteria		6				3
Fungi		2				1
Viral		2				--
None		7				1
Site of Infection (n)	—				—	
Pulmonary		11				4
Urogenital		2				2
Abdominal		8				4
Endocarditis		2				—
Orthopedic		1				—
Unknown		1				—
Simplified Acute Physiology Score II Score	—	43a (38-52)[b]	45a (38-50)[b]	42a (32-52)[b]	—	58a (45-64)[b]

Results are presented as absolute numbers or [a]medians or [b]lower (25%) and upper (75%) quartiles

RESULTS

Characteristics of Study Population

Characteristics of the study population are shown in Table 1. Fifteen patients of the first study cohort and one patient of the second study cohort met the criteria of severe sepsis, ten patients of the first study cohort and nine patients of the second study cohort had septic shock. The first study cohort was further subdivided into patients with survival to a general ward at 2 wks (n = 13), and into patients with prolonged ICU care (n = 5) or death (n = 7) at 2 wks. The clinical course of disease was not associated with the classification of patients into severe sepsis or septic shock. Patients with septic shock showed increased procalcitonin concentrations (median, 31.7 mg/L) compared to patients with severe sepsis (median, 5.8 mg/L; $p < .05$), whereas white blood cell and PLT counts did not significantly differ between both groups (data not shown). Patients with an unfavorable clinical course showed reduced PLT counts compared to patients with a favorable clinical course ($p < .005$), whereas procalcitonin and white blood cell counts as well as the Simplified Acute Physiology Score II scores did not significantly differ between both groups. Causative agents of sepsis were Gram-negative bacteria in 13 patients, Gram-positive bacteria in nine patients, fungi in three patients, and viruses in two patients, whereas no agent could be identified in eight patients.

Differential Release of AA Metabolites in Healthy Subjects and Septic Patients

A principal component analysis was performed to identify differentially released AA metabolites after LPS activation of whole blood of 15 healthy controls and 15 septic patients. As shown in Figure 1*A*, analysis of AA metabolites allowed differentiation between healthy controls and septic patients after LPS whole-blood activation (score plot). Nine mass signals could be detected, which differed between the two groups (Fig. 1*B*). According to their mass transition and retention time (RT), these signals could be identified as AA and the COX-associated metabolites PGE_2, 11-HETE, and TXB_2. In addition, AA-analogues could be detected, which are characterized by the mass transition of the AA standard (AA-d8) but different RTs (Table 2). As shown in the loading plot, which indicates the individual contribution of these metabolites to the separation of both groups, PGE_2 had the highest impact on differentiation (Fig. 1*B*). Differentiating metabolites apart from 11-HETE already could be identified in a principal component analysis performed without LPS activation at baseline. Discrimination, however, was worse compared to principal component analysis after LPS activation (data not shown). A principal component analysis after LPS activation of whole blood of ten other septic patients confirmed differentiating mass signals (data not shown). Results of quantitative analysis of differentiating metabolites between healthy subjects and septic patients with and without (baseline) LPS whole-blood activation are shown in Figure 2. Septic patients

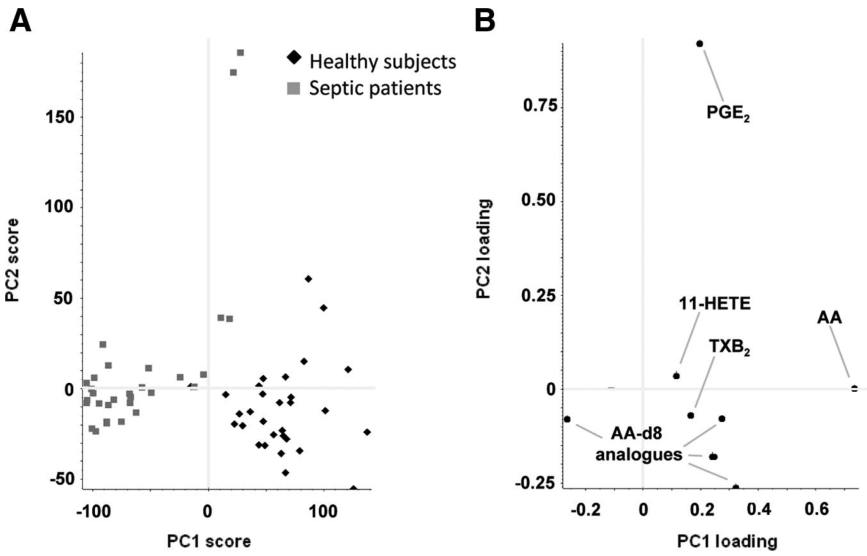


Figure 1. Principal component analysis of arachidonic acid (*AA*) metabolites in healthy subjects and septic patients. Data from 15 healthy controls and 15 septic patients after lipopolysaccharide whole-blood activation are shown. *A*, Duplicate values of all measurements are included in the score plot illustrating the differentiation between both groups. *B*, The loading plot shows identified metabolites and their individual contribution for the differentiation of both groups. The greater the distance of dots from the origin, the stronger their separating impact. *HETE*, hydroxyeicosatetraenoic; *PC*, principal component; *PG*, prostaglandin; *TX*, thromboxane.

Table 2. Mass transitions and retention times of identified metabolites

Parameter	Mass Transitions	Retention Time (min)
AA	303.2/259.0	12.2
AA-d8 analogue	311.3/267.2	2.9
AA-d8 analogue	311.3/267.2	6.8
AA-d8 analogue	311.3/267.2	9.5
AA-d8 analogue	311.3/267.2	10.3
AA-d8 analogue	311.3/267.2	10.9
11-hydroxyeicosatetraenoic	319.2/167.0	10.6
Prostaglandin E2	351.2/271.0	4.4
Thromboxane B2	369.2/169.0	3.4

AA, arachidonic acid.

Mass transitions and chromatographic retention times of differentially released metabolites are shown. Analogues are characterized by mass transitions of the isotope labeled AA standard (AA-d8: 311.3/267.2; 12.1 mins) but different retention times.

revealed increased concentrations of AA at baseline compared to healthy controls. A three-fold increase of AA after LPS activation could be detected in healthy subjects. No significant increase was found in septic patients, resulting in lower AA concentrations after LPS activation compared to healthy controls (Fig. 2*A*). The concentrations of AA-d8 analogues significantly differed between healthy subjects and septic patients already at baseline. AA-d8 analogues with RTs of 2.9 mins, 6.8 mins, 9.5 mins, and 10.3 mins showed up to ten-fold lower concentrations in septic patients (Fig. 2*B--E*). One analog characterized by RT of 10.9 mins could only be detected in septic patients (Fig. 2*F*). LPS activation induced a significant increase in the AA-d8 analog characterized by RT of 10.3 mins in septic patients ($p < .01$; Fig. 2*E*). Healthy subjects showed LPS-induced increase of AA-d8 analogues with RTs of 2.9 mins and 10.3 min ($p < .05$; Fig. 2*B*, 2*E*).

Because 11-HETE and PGE_2 are mainly generated in leukocytes (1, 8), concentrations of both metabolites were analyzed as absolute concentrations and normalized to the white blood cell count (Fig. 2*G–J*). 11-HETE could be detected in healthy subjects and septic patients within the limit of detection at baseline (Fig. 2*G*, 2*H*), whereas PGE_2 could only be detected in healthy subjects at baseline (Fig. 2*I*, 2*J*). LPS activation resulted in a significant increase in 11-HETE and PGE_2 both in septic patients and in controls; however, compared to septic patients, healthy subjects showed a two-fold higher increase in 11-HETE and a five-fold higher increase in PGE_2 with and without normalization to white blood cells (Fig. 2*G–J*).

Because the production of TXA_2 is mainly attributable to PLTs, the concentration of its stable metabolite TXB_2 was analyzed as absolute concentration and normalized to the PLT count (1) (Fig. 2*K*, 2*L*). Concentrations of TXB_2 at baseline were approximately eight-fold lower in septic patients compared to healthy controls, regardless of the normalization to PLTs. LPS activation only marginally affected the release of TXB_2 in healthy subjects but induced a significant increase in septic patients when considering absolute concentrations (Fig. 2*K*).

To exclude a potential effect of differences in cell viability between healthy subjects and septic patients on mediator release, cell viability was determined in whole blood from both groups. Trypan blue method and CellTiter-Blue cell viability assay did not reveal differences in cell viability in whole blood from septic patients compared to healthy controls. Also, no significant differences between cell viability before and after activation of whole blood with LPS for 24 hrs were observed (data not shown). To further exclude potential effects of endogenous LPS in septic patients on mediator release, endotoxin was determined in patients of the second study cohort, characterized by microbiologically described Gram-negative sepsis (n = 5). Endotoxin could not be detected in two out of five patients; the other three patients revealed plasma concentrations between 1.54 and 9.92 EU/mL (data not shown). Because effects of metabolite release were not associated with endotoxin levels in these experiments, endogenous endotoxin as a confounding variable was excluded.

Differential Release of AA Metabolites in Patients With Sepsis in Relation to Disease Severity and Clinical Outcome

To further investigate a potential association between the eicosanoid response and disease severity or clinical outcome, the release of AA metabolites was compared between patients with severe sepsis or septic shock (Fig. 3) and between patients with survival to a general

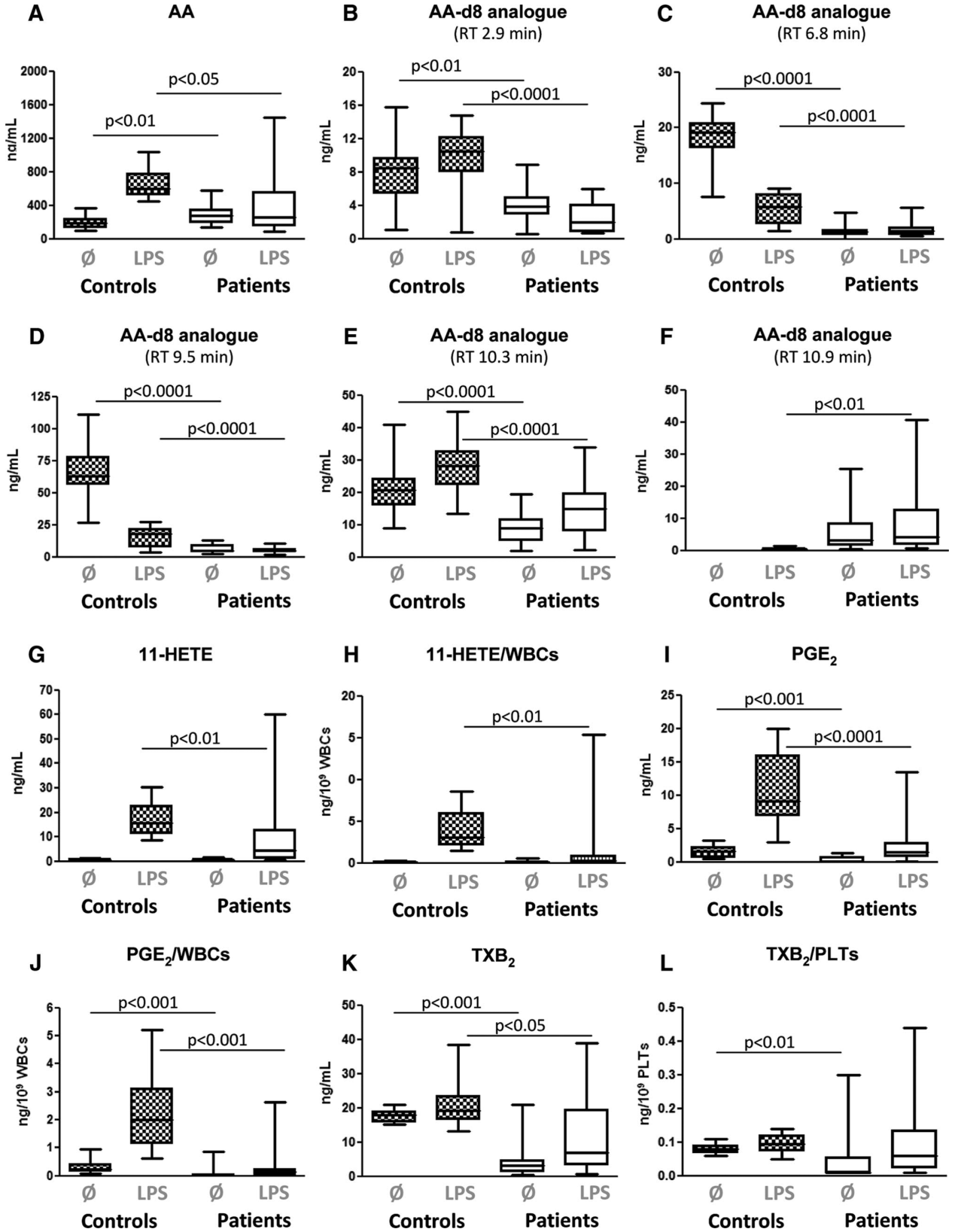


Figure 2. Quantitative analysis of arachidonic acid (*AA*) metabolites in healthy subjects and septic patients. AA metabolites that were differentially released in lipopolysaccharide (*LPS*)-activated or nonactivated whole blood of healthy subjects (n = 15, *dotted boxes*) and septic patients (n = 25, *plain boxes*) are shown (*A*, AA; *B*, AA-d8 analogue retention times [*RT*] 2.9 min; *C*, AA-d8 analogue RT 6.8 min; *D*, AA-d8 analogue RT 9.5 min; *E*, AA-d8 analogue RT 10.3 min; *F*, AA-d8 analogue 10.9 min; *G*, 11-hydroxyeicosatetraenoic [*HETE*]; *H*, 11-*HETE*/white blood cells [*WBCs*]; *I*, prostaglandin [*PG*] E_2; *J*, PGE2/WBCs; *K*, thromboxane [*TX*] B_2; *L*, TXB_2/platelets [*PLTs*]). AA and AA-d8 analogues are presented in absolute concentrations. 11-HETE, PGE_2, or TXB_2 concentrations and normalized to WBC or PLT. RT of AA-d8 analogues are given for identification. The *Ø* indicates medium control incubation of whole blood without LPS for 24 hrs; *LPS* indicates incubation of whole blood with LPS (1 mg/mL) for 24 hrs.

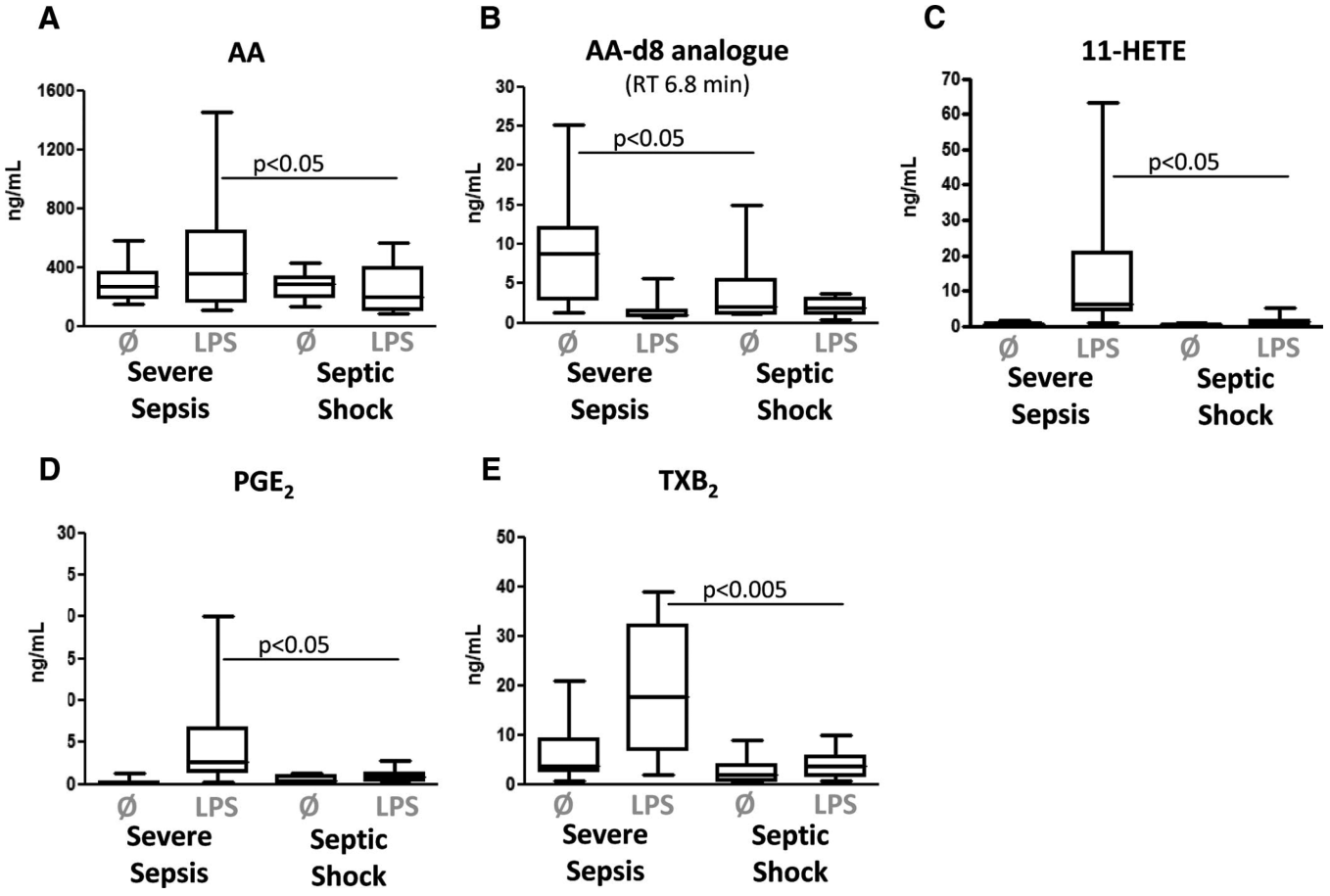


Figure 3. Quantitative analysis of arachidonic acid (*AA*) metabolites in patients with severe sepsis or septic shock. AA metabolites that were differentially released in lipopolysaccharide (*LPS*)-activated or nonactivated whole blood of septic patients with severe sepsis (n = 15) or septic shock (n = 10) are shown. (*A*, AA; *B*, AA-d8 analogue RT 6.8 min; *C*, 11-hydroxyeicosatetraenoic [*HETE*]; *D*, prostaglandin [*PG*] E_2; *E*, thromboxane [*TX*] B_2). AA and AA metabolites are presented in absolute concentrations. The *Ø* indicates medium control incubation of whole blood without LPS for 24 hrs; *LPS* indicates incubation of whole blood with LPS (1 mg/mL) for 24 hrs.

ward at 2 wks or with prolonged ICU care or death at 2 wks (Fig. 4).

Comparable concentrations of AA were found in patients with severe sepsis and septic shock at baseline. LPS activation in patients with severe sepsis resulted in a two-fold increase of AA, whereas no increase was observed in patients with septic shock (Fig. 3*A*). The concentration of the AA-d8 analog with RT of 6.8 mins was significantly lower in patients with septic shock at baseline ($p < .05$; Fig. 3*B*). Patients with septic shock further revealed a reduced release of 11-HETE at baseline when considering normalized concentrations (data not shown) and a significantly lower release (–90%) after LPS activation compared to patients with severe sepsis (Fig. 3*C*). Concentrations of PGE_2 did not significantly differ between the two groups at baseline; however, the LPS-induced release was approximately 80% lower in patients with septic shock (Fig. 3*D*). TXB_2 concentrations of patients with severe sepsis or septic shock did not significantly differ without LPS activation. Patients with septic shock showed no significant increase after LPS activation; in contrast, patients with severe sepsis revealed a three-fold increase of TXB_2 concentrations after activation with LPS (Fig. 3*E*).

Differentially released AA metabolites between patients with survival to a general ward at 2 wks or patients with prolonged ICU care or death at 2 weeks, namely 11-HETE, PGE_2, and TXB_2, are shown in Figure 4. Concentrations of 11-HETE, PGE_2, and TXB_2 did not differ between the two groups at the time of admission (day 0) and on day 3 without LPS activation. Furthermore, no increase or decrease in the release of 11-HETE, PGE_2, and TXB_2 was observed in the course of the disease (data not shown). LPS activation on day of admission induced a higher release of 11-HETE in patients with favorable clinical course; however, the difference between the two groups was not significant. On day 3, a significantly higher release of 11-HETE (two-fold) could be shown in patients with survival to a general ward compared to patients with prolonged ICU care or death (Fig. 4*A*). Furthermore, a nonsignificant trend toward higher 11-HETE concentrations after LPS activation could be shown for patients with survival to a general ward in the course of the disease. PGE_2 release on activation with LPS significantly increased on day 3 in these patients (five-fold), whereas no differences in LPS-induced PGE_2 release were observed between the two groups on the day of admission (Fig. 4*B*). TXB_2 concentrations after LPS activation significantly differed between the two groups on the day of admission ($p < .05$) and no significant differences could be shown on day 3. However, a nonsignificant trend toward higher TXB_2 concentrations was observed in patients with survival to a general ward in the course of the disease (Fig. 4*C*).

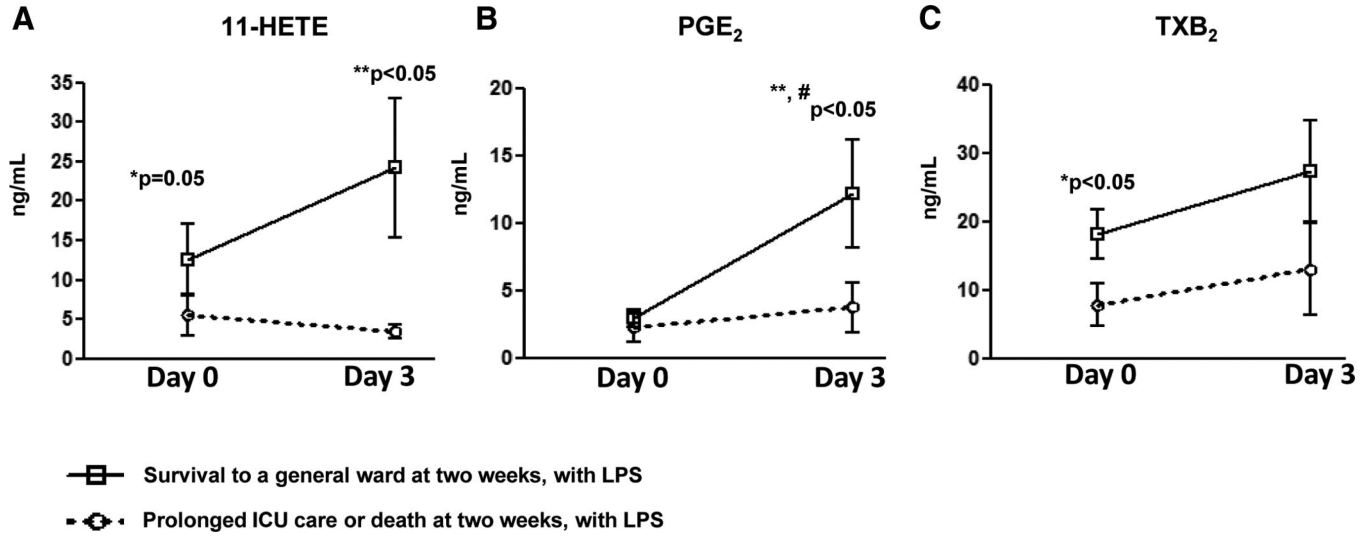


Figure 4. Quantitative analysis of arachidonic acid metabolites in septic patients with favorable or unfavourable course of disease. Arachidonic acid metabolites that were differentially released in lipopolysaccharide (*LPS*)-activated whole blood of septic patients with survival to a general ward at 2 wks (n = 13, *continuous line*) or prolonged intensive care unit (*ICU*) care or death at 2 wks (n = 12, *broken line*) are shown (mean ± SEM). (*A*, 11-hydroxyeicosatetraenoic [*HETE*]; *B*, prostaglandin [*PG*] E_2; *C*, thromboxan [*TX*] B_2). LPS activation was performed on day of admission (day 0) and on day 3 after admission. *Comparison between groups on day 0; **comparison between groups on day 3; #comparison within group, day 0 to day 3.

Differential Regulation of Target Genes of the AA Metabolism in Healthy Subjects and Septic Patients

To further elucidate the sepsis-associated effects on release of COX-dependent metabolites, comparative analyses of *COX-2* and *mPGES-1* as corresponding target genes were performed in a second independent study cohort consisting of ten septic patients and three healthy controls. *COX-2* gene expression of patients and healthy subjects did not significantly differ at baseline without LPS whole-blood activation. LPS activation of whole blood of septic patients resulted in a six-fold lower induction of *COX-2* mRNA expression compared to healthy controls ($p < .001$; Fig. 5*A*). A reduced LPS-induced release of the corresponding metabolite 11-HETE could be confirmed in septic patients of the second cohort (Fig. 5*B*). A nonsignificant trend to a higher gene expression of *mPGES-1* could be shown in septic patients without LPS activation. LPS activation induced a comparable increase of *mPGES-1* gene expression in healthy subjects and septic patients (Fig. 5*C*). In contrast to this finding, a reduced PGE_2 release in septic patients was found, confirming the results observed in the first study cohort (Fig. 5*D*).

Because endogenous LPS in septic patients might potentially induce a general hyporesponsiveness of LPS-dependent pathways, whole blood from healthy subjects (n = 3) was preincubated with LPS for 12 hrs at a final concentration of 300 pg/mL, corresponding with concentrations observed in septic patients (9). However, preincubation had no effect on mediator release of 11-HETE and gene expression of *COX-2* was still inducible to the same extent, suggesting that sepsis-associated changes of the *COX-2* pathway were not caused by endogenous endotoxin tolerance (data not shown).

DISCUSSION

Our study demonstrates that patients with sepsis were characterized by significant differences in their released AA metabolite concentrations compared to healthy subjects. LPS-activated whole blood showed a significant reduction of AA and the COX-associated AA metabolites 11-HETE, PGE_2, and TXB_2 in septic patients. Gene expression analysis of corresponding target genes confirmed a reduced inducibility of *COX-2* mRNA expression as a rate-limiting step in prostaglandin synthesis but revealed a preserved inducibility of downstream *mPGES-1* in septic patients. The reduced release of AA, 11-HETE, PGE_2, and TXB_2 was associated with increased disease severity or an unfavorable clinical course of disease.

The shown differences in AA metabolite patterns between healthy subjects and septic patients substantiate a pathophysiological role of these compounds in sepsis. AA is released by phospholipase A_2 from membrane phospholipids, followed by subsequent metabolization via specialized enzymes (1, 10). Our data showing a reduced LPS-induced release of AA in septic patients (Fig. 2) indicate a disturbance of the AA metabolism already in superordinate systems. One of the key regulatory enzymes of the AA metabolism is COX, which catalyzes the conversion of AA to PGH_2, the precursor for a large number of eicosanoids (11). The reduction in the release of COX-dependent metabolites (Fig. 2) and the reduced inducibility of *COX-2* gene expression in septic patients (Fig. 5) speak for a direct affection of the COX pathway in sepsis, presumably at the level of transcription. One could speculate about a COX-2 product-dependent feedback regulation of *COX-2* gene expression (12).

Sepsis is characterized by uncontrolled expression of the inflammatory cascade; however, there is often a shift toward an immunosuppressive state in the course of the disease (13). This might also be reflected by the reduced COX-associated eicosanoid response comparable to the frequently shown reduced release of proinflammatory cytokines after LPS whole-blood activation in septic patients (14). Former studies identified PGE_2, which is formed by COX-2 and the specific PGE synthase, as a central modulator balancing between the proinflam-

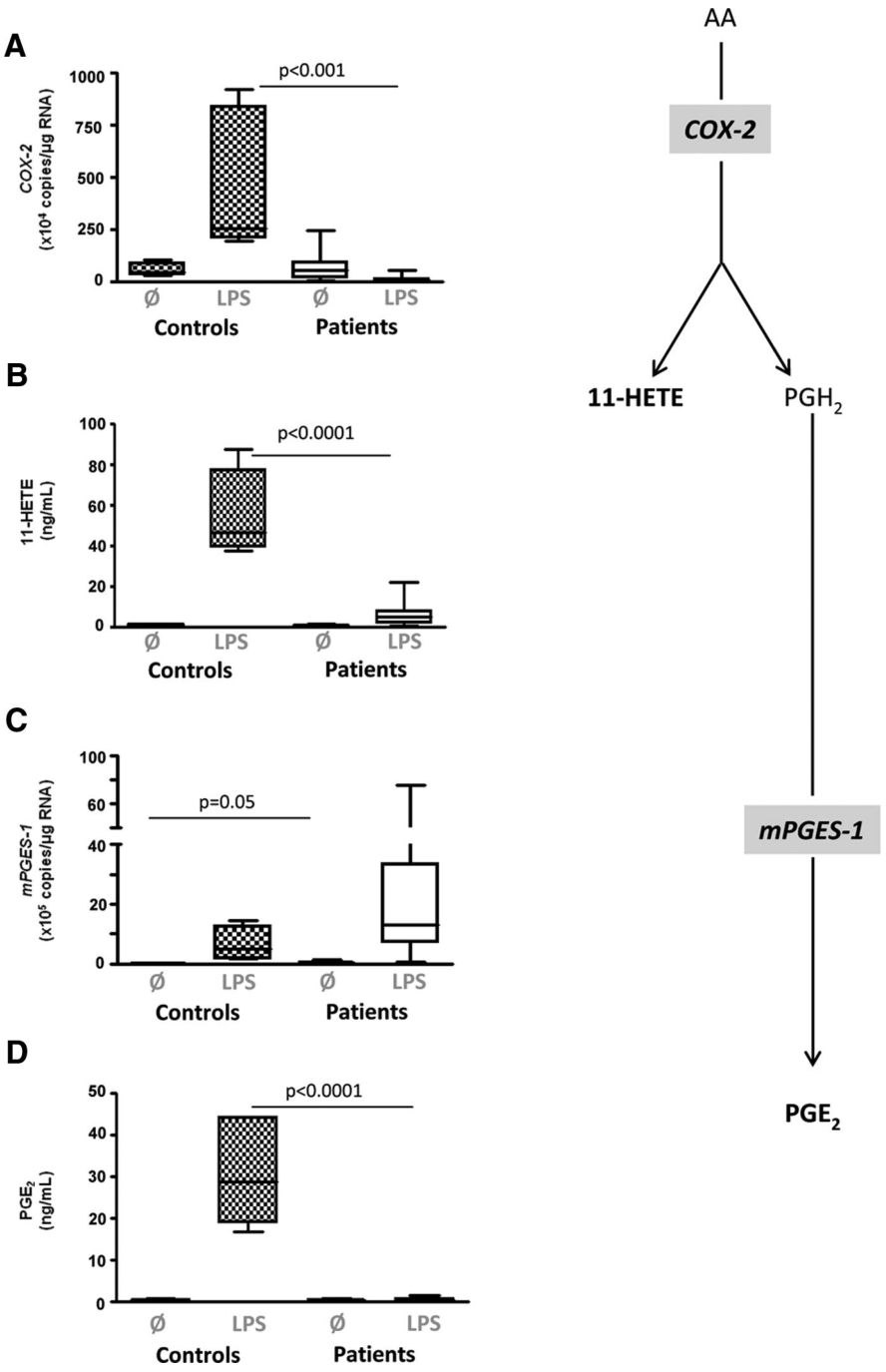


Figure 5. *COX-2* and *mPGES-1* gene expression and corresponding mediator release in healthy subjects and septic patients. Data from ten septic patients (*light squares*) and three healthy subjects (*dark squares*) are shown (*A*, COX_2 messenger RNA expression; *B*, 11-hydroxyeicosatetraenoic [*HETE*] release; *C*, mPGES-1 messenger RNA expression; *D*, PGE_2 release). Whole blood of patients and healthy subjects was incubated with or without lipopolysaccharide (*LPS*). Measurements were determined in duplicates. The investigated pathway is shown on the *right*, analyzed genes are highlighted in *gray*, and determined corresponding metabolites are given in *bold*. The *Ø* indicates medium control incubation of whole blood without LPS for 24 hrs; *LPS* indicates incubation of whole blood with LPS (1 mg/mL) for 24 hrs. *AA*, arachidonic acid; *PG*, prostaglandin.

matory and anti-inflammatory response (15, 16). The shown reduction of basal PGE_2 levels and of the LPS-induced PGE_2 release in septic patients (Fig. 2) may thus reflect a central pathogenic mechanism in sepsis leading to an imbalance in the inflammatory response. The preserved inducibility of *mPGES-1* in septic patients (Fig. 5) as the essential component for PGE_2 production could be interpreted as a compensatory mechanism for maintenance of homeostasis. The failure of subsequent increase of PGE_2 release is possibly based on our demonstrated downregulation of *COX-2* in sepsis (Fig. 5), because upregulation of both *COX-2* and *mPGES-1* were described to be necessary for stimulation of PGE_2 synthesis (17). A suspected positive modulating role of PGE_2 in sepsis is further supported by our data showing an increase of PGE_2 release in patients with a favorable clinical course of disease (Fig. 4).

The actual impact of HETEs in inflammation is poorly understood; however, because 11-HETE was shown to directly reflect COX activity on mediator level, the reduced release in whole blood of septic patients (Fig. 2) further confirms a direct affection of COX pathway in sepsis (8).

Our study further revealed a significant reduction of TXB_2 in septic patients compared to healthy controls with and without LPS activation (Fig. 2). TXB_2 is typically determined as the stable non-enzymatically degraded metabolite of TXA_2, which is formed mainly by COX-1 and the specific downstream enzyme TXA synthase (1). Earlier studies showed increased plasma levels of TXB_2 in septic patients (18, 19). This discrepancy might be explained by the determination of TXB_2 by immunoassays that are often hampered by cross-reactivity with other AA metabolites. The overall contribution of TXA_2 in sepsis is considered to be uniformly negative. However, our data tending to higher levels in patients with a favorable clinical course (Fig. 4) may be supported by a recent publication describing a protective effect of TXA_2 in sepsis preventing the development of vascular hyporesponsiveness (20).

The shown sepsis-associated changes of AA metabolism further implement a diagnostic and therapeutic impact. The differences of the eicosanoid response between patients with survival to a general ward at 2 wks or patients with prolonged ICU care or death at 2 wks and the shown recovery of the eicosanoid response after 3-day therapy in patients with a favorable clinical course (Fig. 4), together with the differences observed between patients with severe sepsis or septic shock (Fig. 3), may indicate a diagnostic potential of subsequent analyses of the eicosanoid response in disease or therapeutic monitoring. Furthermore, one could speculate that a decrease of AA metabolism in early stages of infectious disease may indicate an increased risk for sepsis development. Concentrations of distinct cytokines as

alternative markers of early inflammation were shown to predict mortality in septic patients (21). However, because AA metabolites are assumed to be superordinate to cytokines, analysis of AA metabolites may have a higher impact in sepsis diagnostics.

Another important finding of our study was the detection of AA-d8 analogues, which were significantly affected in patients with sepsis (Figs. 1 and 2). Even though we could not specify these compounds, they completely discriminated between healthy subjects and septic patients, indicating promising diagnostic potential.

Treatment of septic patients with COX inhibitors has been discussed as a potential therapeutic approach aiming to reduce the proinflammatory response. However, our data showing reduced inducibility of the COX pathway in sepsis (Figs. 2 and 5) may further explain the missing benefit of an inhibition of the COX or associated enzymes in septic patients as described in recent clinical studies (22–24). On the contrary, the recovery of the eicosanoid response in patients with better prognosis (Fig. 4) may even suggest a potential negative effect of a COX-inhibiting therapy.

The presented sepsis-associated effects on AA metabolism could be discussed as a general hyporesponsiveness to LPS attributable to endotoxin tolerance rather than a direct affection of related eicosanoid pathways (25). There are several points that disprove this hypothesis. First, sepsis-associated changes of AA metabolism could be demonstrated in all studied septic patients, whereas approximately only half of the study population experienced Gram-negative sepsis. Even in patients with Gram-negative sepsis, LPS was not always detectable or plasma concentrations showed high variability. Second, the differential regulation between *COX-2* and *mPGES-1* on gene expression level with preserved LPS-associated induction of *mPGES-1* (Fig. 5) argues against a general LPS hypo-responsiveness. Third, we could demonstrate in a cell culture model that preincubation of whole blood with LPS in concentrations corresponding to those of septic patients did not affect LPS whole-blood testing.

A considerable limitation of the present study is the missing inclusion of patients with early or developing sepsis, allowing a comparison of sensitivity of LPS-induced eicosanoid response in relation to conventional sepsis markers and of patients with otherwise critical illness, allowing an assessment of specificity of observed findings. Also, the selected time points for blood collection, directly after admission to ICU, potentially reflecting the highest disease activity, and after 3-day therapy, potentially indicating the further course of the disease, do not adequately allow assessment of the dynamic nature of sepsis. Subsequent clinical studies will have to address these limitations.

CONCLUSIONS

Using a systems biology approach, we were able to show that the AA metabolism is markedly affected in patients with sepsis. The identification of differentially released mediators and the association of the intensity of the eicosanoid response with disease severity as well as clinical outcome indicate a promising diagnostic potential. Further clinical studies may address the diagnostic and prognostic potential of eicosanoid analyses after LPS whole-blood activation in septic patients and a potential superiority to existing laboratory markers.

REFERENCES

1. Brock TG, Peters-Golden M: Activation and regulation of cellular eicosanoid biosynthesis. *Sci World J* 2007; 7:1273–1284
2. Tilley SL, Coffman TM, Koller BH: Mixed messages: Modulation of inflammation and immune responses by prostaglandins and thromboxanes. *J Clin Invest* 2001; 108:15–23
3. Bulger EM, Maier RV: Lipid mediators in the pathophysiology of critical illness. *Crit Care Med* 2000; 28:N27–N36
4. Levy BD, Clish CB, Schmidt B, et al: Lipid mediator class switching during acute inflammation: Signals in resolution. *Nat Immunol* 2001; 2:612–619
5. Peplow PV: Actions of cytokines in relation to arachidonic acid metabolism and eicosanoid production. *Prostaglandins Leukot Essent Fatty Acids* 1996; 54:303–317
6. Levy MM, Fink MP, Marshall JC, et al: 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference. *Intensive Care Med* 2003; 29:530–538
7. Kortz L, Geyer R, Ludwig U, et al: Simultaneous eicosanoid profiling and identification by liquid chromatography and hybrid triple quadrupole-linear ion trap mass spectrometry for metabolomic studies in human plasma. *J Lab Med* 2009; 3:341–348
8. Norris PC, Reichart D, Dumlao DS, et al: Specificity of eicosanoid production depends on the TLR-4-stimulated macrophage phenotype. *J Leukoc Biol* 2011; 90:563–574
9. Opal SM, Scannon PJ, Vincent JL, et al: Relationship between plasma levels of lipopolysaccharide (LPS) and LPS-binding protein in patients with severe sepsis and septic shock. *J Infect Dis* 1999; 180:1584–1589
10. Nevalainen TJ: Serum phospholipases A2 in inflammatory diseases. *Clin Chem* 1993; 39:2453–2459
11. Dubois RN, Abramson SB, Crofford L, et al: Cyclooxygenase in biology and disease. *FASEB J.* 1998; 12:1063-1073
12. Ishikawa T, Jain N, Herschman HR: Feedback regulation of Cyclooxygenase-2 transcription *ex vivo* and *in vivo*. *BBRC* 2009; 378:534–538
13. Hotchkiss RS, Karl IE: The pathophysiology and treatment of sepsis. *N Engl J Med* 2003; 348:138–150
14. Ertel W, Kremer JP, Kenney J, et al: Downregulation of proinflammatory cytokine release in whole blood from septic patients. *Blood* 1995; 85:1341–1347
15. Harris SG, Padilla J, Koumas L, et al: Prostaglandins as modulators of immunity. *Trends Immunol* 2002; 23:144–150
16. Xu XJ, Reichner JS, Mastrofrancesco B, et al: Prostaglandin E2 suppresses lipopolysaccharide-stimulated IFN-beta production. *J Immunol* 2008; 180:2125–2131
17. Cipollone F, Cicolini G, Bucci M: Cyclooxygenase and prostaglandin synthases in atherosclerosis: recent insights and future perspectives. *Pharmacol Ther* 2008; 118:161–180
18. Suchner U, Katz DP, Furst P, et al: Impact of sepsis, lung injury, and the role of lipid infusion on circulating prostacyclin and thromboxane A(2). *Intensive Care Med* 2002; 28:122–129
19. Reines HD, Halushka PV, Cook JA, et al: Plasma thromboxane concentrations are raised in patients dying with septic shock. *Lancet* 1982; 2:174–175
20. Yamada T, Fujino T, Yuhki K, et al: Thromboxane A2 regulates vascular tone via its inhibitory effect on the expression of inducible nitric oxide synthase. *Circulation* 2003; 108:2381–2386
21. Opal SM, DePalo VA: Anti-inflammatory cytokines. *Chest* 2000; 117:1162–1172
22. Celik I, Akbulut A, Kilic SS, et al: Effects of ibuprofen on the physiology and outcome of rabbit endotoxic shock. *BMC Infect Dis* 2002; 2:26
23. Bernard GR, Wheeler AP, Russell JA, et al: The effects of ibuprofen on the physiology and survival of patients with sepsis. The Ibuprofen in Sepsis Study Group. *N Engl J Med* 1997; 336:912-918
24. Shann F: Antipyretics in severe sepsis. *Lancet* 1995; 345:338
25. Biswas SK, Lopez-Collazo E: Endotoxin tolerance: new mechanisms, molecules and clinical significance. *Trends Immunol* 2009; 30:475-487