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Going beyond iron studies for iron deficiency anaemia: new cellular biomarkers for diagnosis

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Abstract

The full blood count is one of the simplest and most ordered blood investigations in daily practice. Its use and that of other classic markers for iron deficiency have been well established. Recently, there has been increased attention to the potential for fluorescence flow cytometry as an enhancement to the classic blood count. This paper explores the potential of new cellular biomarkers using this technology to enhance our diagnosis of iron deficiency anaemia (IDA) and differentiate between sepsis and systemic inflammation. For IDA, parameters such as the reticulocyte haemoglobin equivalent (RET-He) and the difference between reticulocyte and erythrocyte haemoglobin equivalent (DELTA-He) are exciting additions to enhance the speed and accuracy of its diagnosis. RET-He, which is defined as the haemoglobin content in reticulocytes, offers a more immediate reflection of iron availability for erythropoiesis compared to traditional markers such Hb concentration and mean corpuscular volume. The integration of advanced technologies, such as fluorescence flow cytometry, into routine blood counts can significantly improve diagnostic precision, allowing for a more nuanced understanding of the haematopoietic system and immune response. In critically ill patients,

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these new parameters can help to differentiate between various inflammatory responses and infections, providing valuable insights into the activation status of neutrophils and other immune cells. Parameters such as immature granulocytes and neutrophil reactivity intensity have shown promise in discriminating between systemic inflammatory response syndrome and sepsis. The introduction of these markers holds the potential for quicker and more cost-effective assessments for sepsis. The addition of fluorescence flow cytometry parameters to our armament of investigations for blood counts could enhance our abilities to practice precision medicine.

Keywords: cytometry, anaemia, sepsis, reticulocyte, immune response

Introduction

The continuous flow of blood through the body ensures that the tissues and organs receive the vital supplies required to survive, and waste products are removed. The various cells within the blood stream function as carriers, as defenders and perform repairs when required. As such, analysis of the blood cells would show great insight in the overall health of the body.

The full blood count is one of the simplest and most ordered blood investigations in daily practice. It allows the clinician to review the key components of blood, namely the oxygen carrying capacity through measurement of haemoglobin (Hb), the leucocyte counts, and the platelet counts. This provides a quantitative overview of the haematopoietic system and allows the clinician to infer various conclusions with regards to oxygen carrying capacity and the body's immune response. In this review, we explore the potential of the new parameters using fluorescence flow cytometry enhancing diagnostic performance in iron deficiency anaemia (IDA) and sepsis.

New parameters from the blood count reflect the immune response

The COVID pandemic highlighted the devastation that a systemic inflammatory disease can have on our patients as well as on the healthcare system. The various biomarkers used to measure the inflammatory response were wrought with a lack of specificity and sensitivity to predict disease progression accurately.[1] There is no single biomarker that can accurately differentiate between a bacterial, viral

or systemic non-infectious reaction (SIRS). The introduction of fluorescence flow cytometry in modern blood count analysers may offer a useful tool in our armament to detect and differentiate between infection and inflammation.[2]

Advanced inflammatory parameters from the routine blood count can provide important initial information within minutes at the start of the diagnostic process. They provide quantitative and qualitative information about the status of the patient's immune system, which can allow conclusions to be drawn about the type and severity of the infection. The fluorescence flow cytometry of modern blood count analysers makes it possible to analyse the leukocyte populations in greater depth and provides an insight into the status of the immune response. The activity of neutrophil granulocytes and lymphocytes plays a major role in bacterial and viral infections, and reacts at a very early stage of the immune response.

Both systemic-inflammatory (non-infectious) and infectious inflammations result in an immune system response in which different cells show more or less activation depending on the trigger. The innate immune system is a first non-specific line of defence against pathogens. It is divided into 2 areas: an early, cell-based immune response, characterised by an increase in activated T lymphocytes and NK cells, and a humoral immune response, characterised by activated B lymphocytes (plasma cells). Its main function is to identify and remove foreign substances by specialised leukocytes in order to activate the adaptive (acquired) immune system through presenting the antigen of the pathogen for recognition. In the first phase of the innate immune system, the number and activity of activated neutrophils increase (NEUT-RI, NEUT-GI*),* activated monocytes (RE-Mono), and immature granulocytes (IG) are typically observed. In addition, more reactive lymphocytes (RE-LYMP) and T-cell-independent activated plasma cells (AS-LYMP) can be observed. The combination of the RE-LYMP and AS-LYMP parameters provides additional information on the cellular activation of the innate and adaptive immune system.

Measurement technology

Fluorescence flow cytometry enables the identification and quantification of cell populations based on their fluorescence parameters and light-scattering properties.

Detergents are used to increase permeability of cell membranes and to enhance specific fluorescence marker to bind to intracellular ribonucleic acids (RNA). Given that activated but also immature cells (lymphoid, granulocytic, and monocytic)

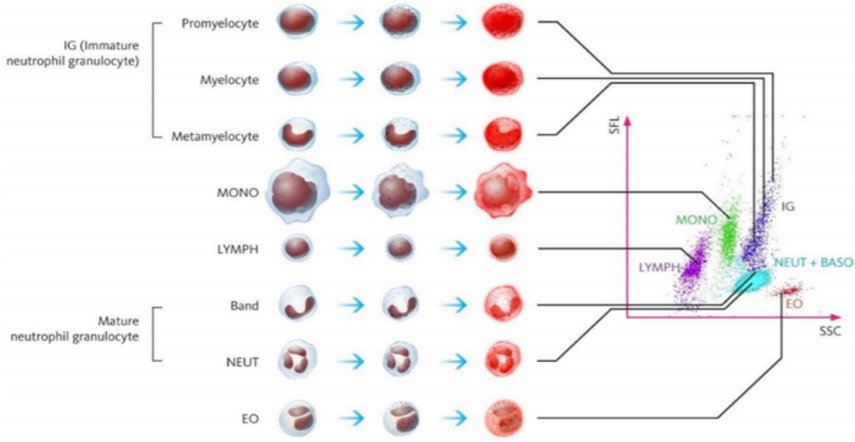

Fig. 1. WDF scattergram: differentiation of leukocytes. Adapted from Sysmex Systems, Kobe, Japan.[3]

have an altered lipid membrane composition, the cell permeability for the lysis reagent used on the haematology system is higher compared to non-activated or mature cells, and cellular nucleic acids can be stained more intensively with a fluorescent marker. Each cell is detected by a laser beam in a sheath current method.

The 3 signal intensities measured are:

- Forward scattered light (FSC): indicates the size of the cells.
- Side scattered light (SSC): indicates nuclear shape and surface structure.
- Side fluorescence light (SFL): indicates the labelling of nucleic acids and determination of activity intensity.

These measurements are used in all Sysmex XN systems (Sysmex Systems, Kobe, Japan) for counting and differentiating leucocyte populations, nucleated erythrocyte precursors, reticulocytes, and the flow cytometric measurement of thrombocytes. The measurements are reported as a white blood cell differential fluorescence (WDF) scattergram (Fig. 1).

Clinical benefits of the extended blood count parameters

According to the current state of research, the clinical benefit of these extended blood count parameters lies in the major fields of anaemia diagnostics and infection diagnostics. These 2 areas are closely linked from a diagnostic point of view. During inflammation, the acute phase reaction results in fever, leucocytosis, and production of acute phase proteins (APP) such as hepcidin. Hepcidin, a key regulator of iron homeostasis in the body, is an APP expressed in the liver that decreases iron availability to inhibit bacterial DNA synthesis, and enzymatic function. Its expression leads to iron-restricted erythropoiesis. At the same time, commonly used parameters to diagnose anaemia are compromised. Ferritin, being an APP itself, is falsely increased, whereas transferrin decreases.[4]

Table 1. Overview of the extended blood count parameters of the XN series with their respective immunological interpretation, units, and reference intervals.

Cell population and/or properties	Immunological interpretation	Parameter	Unit	Reference interval
Total number of reactive lymphocytes	Increased in innate and cell-based adaptive immune response	RE-LYMP# RE-LYMP%[I]	Cells/L %	0–0.5 x 109/L 0–5 %
Antibody-forming lymphocytes[II]	Increased in innate and humoral adaptive immune response	AS-LYMP# AS-LYMP%[I]	Cells/L %	0 cells/L 0%
Granularity of neutrophils	Increased in early innate immune response	NEUT-GI	SI	142.8–159.3 SI
Reactivity of the neutrophils	Increased in early innate immune response	NEUT-RI	FI	39.8–51.0 FI
Immature granulocytes	Indicates the severity of an infection	IG# IG%	Cells/L %	0–0.06 x 109/L 0–0.6 %
Delta haemoglobin equivalent	Detects a systemic bacterial infection at a very early stage	DELTA-He	pg	1.7–4.4 pg
Reticulocyte haemoglobin equivalent	Current availability of iron for the reticulocytes	RET-He	pg	29.7–35.4 pg

#: count; %: percentage; SI: scatter intensity; FI: fluorescence intensity; pg: picogram
[I]As a percentage of all white blood cells.
[II]If antibody-forming lymphocytes (AS-LYMP) are present, these are also recorded in the number of all reactive lymphocytes (RE-LYMP).
Adapted from Pekelharing *et al.* 2010.[2]

This is where the parameters of reticulocyte haemoglobin equivalent (RET-He) and the delta haemoglobin equivalent (DELTA-He) help, as they are not masked by the acute phase reactions. Table 1 summarises the extended blood count parameters from fluorescence spectrometry.

Anaemia diagnostics with extended parameters from the blood count

Anaemia is one of the most common diagnoses in anaesthesiology and intensive care medicine. Anaemia describes the lack of erythrocytes or Hb and can, under certain circumstances, lead to an undersupply of oxygen to vital organs. According to the World Health Organization (WHO), anaemia is defined by values of Hb < 12 g/dl in women and Hb < 13 g/dl in men.[5]

Approximately 30% of all surgical patients are already anaemic before an operation. A low Hb value is often regarded as an indication for a transfusion with red blood cell (RBC) concentrates instead of considering clinical options to improve oxygen supply. RBC transfusions are associated with a multitude of risks and side effects. These include allergic reactions as well as haemolytic and non-haemolytic transfusion reactions. In addition, it should not be forgotten that a blood transfusion can also be described as a "transplantation of the liquid organ blood", during which millions of foreign cells are introduced into the recipient's body. Such an intervention not only interferes with the patient's immune system but can also put additional strain on the patient's recovery. The immune modulation that this may trigger could be associated with an increased nosocomial infection rate and is currently the subject of clinical investigations.[6]

Since 2011, the WHO has been calling for the introduction of a medical concept to increase patient safety by strengthening the body's own blood reserves in everyday medical practice, aptly named patient blood management.[7] The concept is based on 3 pillars: early diagnosis and treatment of any anaemia that may be present, minimisation of blood loss, and rational use of blood reserves. Preoperative anaemia is associated with a longer length of hospital stay, higher risk of infection, higher risk of kidney damage, and mortality. These patients also often require more blood transfusions. According to one study, 11–48% of surgical patients suffer from anaemia at the time of surgery and are therefore at high risk of blood transfusion, which is not much lower even in patients with latent anaemia.[8] Even patients who stay in intensive care for a longer period after surgery and initially have a normal Hb value often develop iron deficiency or anaemia during their stay due to, *e.g.*, daily blood sampling.

Determination of Hb is the standard for the diagnosis of anaemia.[9] However, Hb only reveals an iron deficiency if the iron stores have already been depleted and/or iron has been insufficiently available for haematopoiesis for a long time, as can be the case in patients with chronic inflammation, for example. This parameter is not able to recognise acute or latent iron deficiency at an early stage, as it depends on the lifespan of erythrocytes.

RET-He and DELTA-He

RET-He indicates the Hb content of the reticulocytes. It is a useful parameter for the diagnosis and therapy control of iron deficiency anaemia.[10] The lifespan of circulating erythrocytes is approximately 120 days. Therefore, iron deficiency states and changes in the iron supply to erythropoiesis are detected relatively late using Hb concentration, mean corpuscular erythrocyte volume, mean corpuscular haemoglobin content, or the proportion of hypochromic erythrocytes. Reticulocytes, the precursor cells of mature erythrocytes, are formed in the bone marrow and washed out into the bloodstream. In the peripheral blood, the reticulocyte usually develops into a mature erythrocyte within 2 days. Determination of the reticulocyte count therefore provides very prompt quantitative information about erythropoiesis in the bone marrow. Determining the Hb content of the reticulocytes provides information about the current iron supply and thus enables a qualitative assessment of the cells so that changes in the iron status can be identified earlier than by determining the Hb content of the mature erythrocytes.

The reference range for RET-He is 28–35 pg. Iron deficiency is present at less than 28 pg. In a consensus document on the treatment of postoperative anaemia by Munoz *et al.*, RET-He is described as a marker equivalent to transferrin saturation and ferritin for the detection of iron deficiency, but unaffected by the acute phase reaction.[8] A reticulocyte (RET) scattergram is shown in Figure 2.

DELTA-He is the calculated difference between haemoglobinisation of reticulocytes (RET-He) and the Hb concentration of mature erythrocytes. In normal physiological conditions, DELTA-He is a positive value between 1.7 and 4.4 pg, as reticulocytes show a slightly higher Hb equivalent than mature cells.[11] In an acute phase reaction, the RET-He decreases very quickly because the increased release of hepcidin by interleukin-6 (IL-6) immediately leads to iron deficiency in the bone marrow. DELTA-He reflects this shift in haemoglobinisation between mature and immature erythrocytes within a few hours.

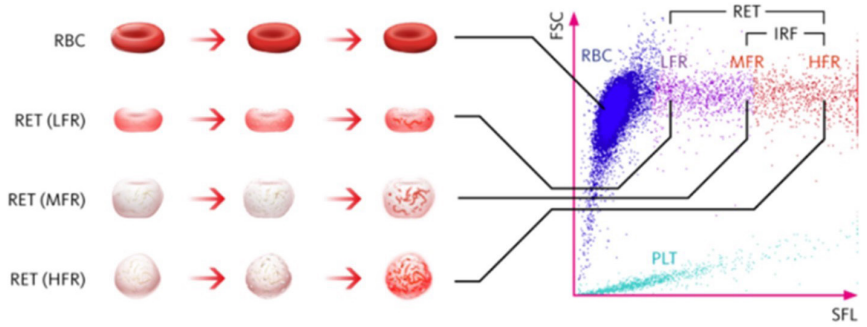

Fig. 2. RET scattergram: differentiation of erythrocytes. Adapted from Sysmex Systems, Kobe, Japan.[3]

In their review, Hönemann *et al.*, recommend RET-He as a routine preoperative parameter to identify patients with latent iron deficiency at low cost.[12] In terms of perioperative prehabilitation and the concept of "enhanced recovery after surgery" (ERAS), patients with iron deficiency could be treated proactively at an early stage to prevent complications, and avoid prolonged hospitalisation.

The determination of RET-He and DELTA-He in the blood count is already available in many hospitals and can replace the classic parameters of ferritin, transferrin, and iron as they are more sensitive, specific, and faster.

Infection diagnostics with extended parameters from the blood count: support in sepsis diagnostics

Sepsis is defined as a systemic, dysregulated reaction of an organism to an infection leading to a life-threatening condition.[13] Positive pathogen detection, *e.g.*, with a positive blood culture, increases the likelihood of an infectious cause of acute illness as well as the diagnosis of sepsis. However, laboratory diagnosis of infection takes 2 days at the earliest, potentially leading to a delay in diagnosis. Conventional parameters such as procalcitonin are not recommended to guide antimicrobial initiation. Cellular markers, such as leucocytes and thrombocytes, are far too unspecific for recognising sepsis.

Neutrophil reactivity intensity

Neutrophil granulocytes play a major role in inflammation. Activated neutrophils secrete a variety of proinflammatory cytokines to attract further immune cells and lysozyme to kill the pathogens. The neutrophil reactivity intensity (NEUT-RI) parameter shows the reactivity intensity of the neutrophils and represents their metabolic activity. Elevated NEUT-RI values reflect the activation of RNA biosynthesis in the neutrophils. This activation of neutrophils is the first event of the immune response to bacterial infections after the onset of infection.[14] Healthy patients show a reference interval of 39.8–51.0 fluorescence units (FI).[14] A NEUT-RI greater than 51.6 FI indicates a bacterial infection. [11] The differentiation between healthy and septic patients is reported with an area under the curve (AUC) of 0.909, sensitivity of 71.3%, and specificity of 96.8%. In a publication by Urrechaga *et al.*, NEUT-RI values over 54 FI can distinguish patients with non-systemic infections from septic patients (AUC 0.825, sensitivity 83.5%, specificity 68%).[15] Stiel *et al.* showed that the NEUT-RI parameter had a high sensitivity and specificity in the diagnosis of disseminated intravascular coagulopathy in patients with septic shock.[16]

Immature granulocytes

The presence of immature granulocytes (IG) is indicative of an increased consumption of neutrophil granulocytes in the periphery and points to a bacterial infection. This does not apply to patients undergoing chemotherapy or glucocorticoid therapy, nor to patients with underlying haematological disease or pregnant women. In healthy individuals, IG are almost never found in the blood. Ayres *et al.* describe that IG > 0.3% for the discrimination of SIRS and sepsis according to blood culture has an AUC of 0.75, sensitivity of 75.7%, and specificity of 45.5%. An IG value greater than 2% already shows a specificity of 90% with a sensitivity of 69.8%.[17] Nierhaus *et al.* state that IG was superior to C-reactive protein (CRP), lipopolysaccharide binding protein, and IL-6 in the discrimination of SIRS against sepsis in the first 48 hours in the intensive care unit.[18] In order to provide rapid and meaningful support in sepsis diagnostics with parameters from the blood count, it is possible to obtain an indication of the exclusion of sepsis or the very high probability of the presence of sepsis with various parameter combinations. With the help of the NEUT-RI, IG, and DELTA-He parameters, these indications can be generated for the treating physicians via so-called rule sets in a work area management system (Fig. 3).

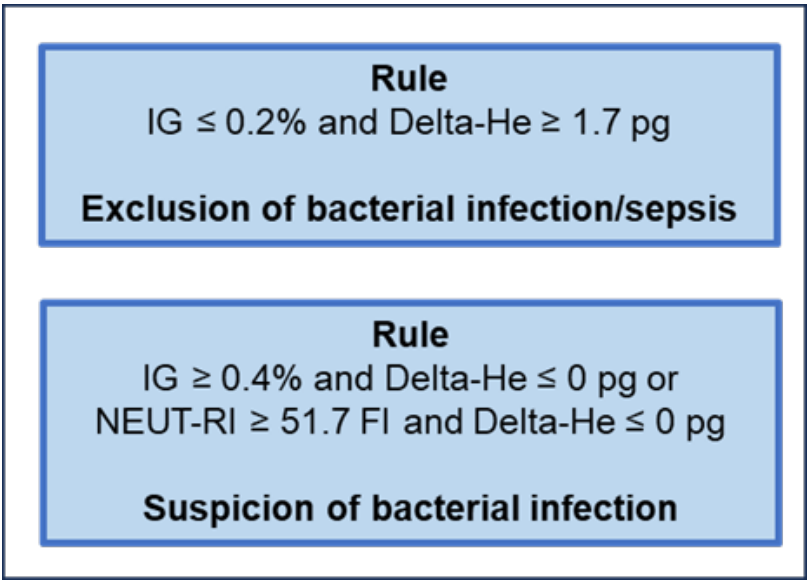

Fig. 3. General guide to using the new parameters to exclude or suspect a bacterial infection.

Differentiation of bacterial or viral infections using RE-LYMP/AS-LYMP

The RE-LYMP parameter is used to record activated T lymphocytes and B lymphocytes. AS-LYMP is the quantification of highly fluorescent lymphocytes, such as antibody-producing B lymphocytes and plasma cells. In contrast to IG, RE-LYMP, and AS-LYMP are mainly elevated in viral infections.

Support in assessing the course of disease in COVID-19 patients

In a multicentre study of 12 European hospitals with 1000 hospitalised COVID-19-positive patients, Linssen *et al.* described the development of a prognostic score to identify critically ill patients with a SARS-CoV-2 infection at an early stage and evaluate the need for critical care.[19] The score included 10 parameters from the blood count of the Sysmex XN series predicting requirement of critical care within the next 14 days, as well as recovery after 3 days of admission. With an AUC of 0.875, the score performed better than any other individual parameter including

the neutrophil/lymphocyte ratio at a value of > 3 at recognising a critical situation in patients. It correctly discriminated 70.5% of COVID-19 patients into critical and non-critical disease progression just 3 days after hospitalisation, and on day 6 of hospitalisation, 93% of patients were correctly identified as critically ill and requiring intensive care. The COVID-19 Prognostic Score enables early detection of critical disease progression, assessment of the need for intensive medical care, and allocation of resources.

Intensive Care Infection Score

Weimann *et al.* published the Intensive Care Infection Score (ICIS), a diagnostic score for the early detection of bacterial infections in postoperative intensive care patients.[20] The score combines 5 parameters from the blood count, some of which are immediately elevated (NEUT-RI) while others increase with the progression of infection (NEUT, IG, DELTA-He, AS-LYMP). With an AUC of 0.852, a sensitivity of 82.93%, and a specificity of 75.11%, the ICIS outperformed conventional sepsis markers such as CRP (AUC 0.77, sensitivity 82.93%, specificity 54.4%) or PCT (AUC 0.696, sensitivity 73.17%, specificity 58.56%). ICIS can be determined within few minutes and facilitates diagnosis and monitoring of infection. ICIS is currently available at research level and can be used for study purposes on request if the system requirements are met.

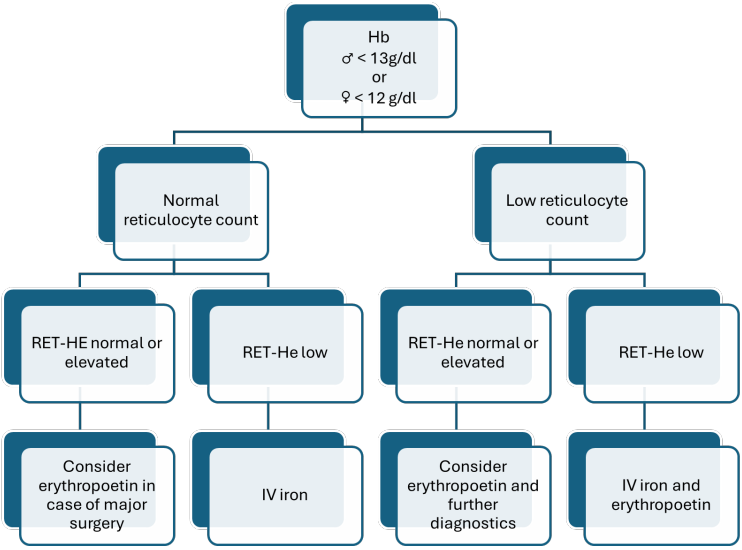

Fig. 4. Algorithm to assess and treat preoperative anaemia (own representation).

Routine parameters in preoperative anaemia management

Especially at the time of elective surgery, it is extremely important that patients are optimally prepared considering potential blood loss and decrease of Hb during and after surgery. Patients with a diagnosed iron deficiency and additional inflammation are at high risk to prolonged stay in hospital. According to Froessler *et al.*, the length of hospital stay could be reduced by 3 days in patients with preoperative anaemia if they were treated with intravenous iron.[21] Anaemia should be treated preoperatively using Hb, RET-He, and reticulocyte count, as shown in (Fig. 4).

Summary

The interpretation of the new extended blood count parameters described in this review makes it possible to obtain additional information for diagnosis and treatment decisions cost-effectively and, above all, quickly. These diagnostic parameters help to diagnose and treat patients with inflammatory diseases, to differentiate between viral or bacterial sources of infection, to initiate antimicrobic medication, and to monitor therapy as they provide additional information regarding immune response activation and quantitative assessment of the activation status of neutrophils (NEUT-RI, NEUT-GI), IG, and activated lymphocytes (RE-LYMP, AS-LYMP). Further clinical prospective investigations are essential to improve clinical interpretation.

In addition, the RET-He and DELTA-He parameters help to assess available iron and to diagnose iron deficiency in the acute phase. RET-He is an early indicator of response to iron therapy and/or erythropoiesis-stimulating agents allowing for evaluation of therapy success within 2 to 4 days. Furthermore, DELTA-He appears to be an early indicator of infections and helps to discriminate between IDA and anaemia in inflammation.

Many laboratories in Malaysia have the capabilities to run these tests, but they are currently not requested as part of the standard blood count. The possibility of enhancing our diagnosing capabilities for anaemia and sepsis makes these new markers an exciting possibility for advancing perioperative and intensive care.

Declarations

Ethics approval and consent to participate

This review article did not require ethics approval or informed consent.

Competing interests

Christian Honemann, MD, PhD, received honoraria for talks and travel expenses from Draeger Medical (Germany), CSL Vifor Pharma (Germany), and Sysmex Europe (Netherlands). Marie-Luise Ruebsam, MD, has received one author honorarium from Sysmex Europe and one from Draeger Medical. Kevin Ng Wei Shan reports no competing interests.

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