ISSN 2222-0682 (online)

World Journal of *Methodology*

World J Methodol 2018 June 28; 8(1): 1-8



Published by Baishideng Publishing Group Inc

World Journal of Methodology

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NAME OF JOURNAL
World Journal of Methodology

ISSN
ISSN 2222-0682 (online)

LAUNCH DATE
September 26, 2011

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Baishideng Publishing Group Inc
7901 Stoneridge Drive, Suite 501, Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
Fax: +1-925-2238243
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Suite 501, Pleasanton, CA 94588, USA
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PUBLICATION DATE
June 28, 2018

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World J Methodol 2018 June 28; 8(1): 1-8

DOI: 10.5662/wjm.v8.i1.1

ISSN 2222-0682 (online)

MINIREVIEWS

Noninvasive hemodynamic monitoring of septic shock in children

Emad Mohamed Fathi, Hassib Narchi, Fares Chedid

Emad Mohamed Fathi, Department of Critical Care, Al Jalila Children's Specialty Hospital, Dubai 7662, United Arab Emirates

Hassib Narchi, Department of Pediatrics, College of Medicine and Health Sciences, United Arab Emirates University, Al Ain 17666, United Arab Emirates

Fares Chedid, Neonatal Intensive Care Unit, Oasis Hospital, Al Ain 1016, United Arab Emirates

ORCID numbers: Emad Mohamed Fathi (0000-0003-2899-4740); Hassib Narchi (0000-0001-6258-2657); Fares Chedid (0000-0001-6606-3141).

Author contributions: All authors contributed equally to this paper.

Conflict-of-interest statement: No potential conflicts of interest. No financial support.

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Manuscript source: Unsolicited manuscript

Correspondence to: Dr. Emad Mohamed Fathi, MSc, MRCPCH, EPIC, Department of Critical Care, Al Jalila Children's Specialty Hospital, Al Jaddaf, Dubai 7662, United Arab Emirates. efmoustafa@ajch.ae
Telephone: +971-50-3127281

Received: March 3, 2018
Peer-review started: March 6, 2018
First decision: April 4, 2018
Revised: April 10, 2018
Accepted: May 11, 2018
Article in press: May 11, 2018
Published online: June 28, 2018

Abstract

Septic shock in children is associated with high mortality and morbidity. Its management is time-sensitive and must be aggressive and target oriented. The use of clinical assessment alone to differentiate between cold and warm shock and to select the appropriate inotropic and vasoactive medications is fraught with errors. Semi-quantitative and quantitative assessment of the preload, contractility and afterload using non-invasive tools has been suggested, in conjunction with clinical and laboratory assessment, to direct shock management and select between vasopressors, vasodilators and inotropes or a combination of these drugs. This review aims to describe non-invasive tools to assess the hemodynamic status in septic shock including echocardiography, trans-thoracic/trans-esophageal Doppler and electrical cardiometry. As septic shock is a dynamic condition that changes markedly overtime, frequent or continuous measurement of the cardiac output (CO), systemic vascular resistance (SVR) and other hemodynamic parameters using the above-mentioned tools is essential to personalize the treatment and adapt it over time. The different combinations of blood pressure, CO and SVR serve as a pathophysiological framework to manage fluid therapy and titrate inotropic and vasoactive drugs. Near infrared spectroscopy is introduced as a non-invasive method to measure end organ perfusion and assess the response to treatment.

Key words: Hemodynamics; Monitoring; Septic shock; Pediatric; Trans-esophageal Doppler; Echocardiography; Cardiometry; Near infrared spectroscopy; Trans-thoracic Doppler

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Core tip: We have reviewed noninvasive tools, such as echocardiography, Doppler and electrical cardiometry used to evaluate the hemodynamic status and response

to treatment of children with septic shock. As septic shock is a dynamic condition that changes markedly overtime, we suggest a practical approach to guide patients' management by assessing cardiac output, preload, and afterload using these methods. Near infrared spectroscopy is described as a method to assess end organ perfusion.

Fathi EM, Narchi H, Chedid F. Noninvasive hemodynamic monitoring of septic shock in children. *World J Methodol* 2018; 8(1): 1-8 Available from: URL: http://www.wjgnet.com/2222-0682/full/v8/i1/1.htm DOI: http://dx.doi.org/10.5662/wjm.v8.i1.1

INTRODUCTION

Sepsis is a systemic illness following invasion of the body by an infectious agent, to which the body reacts by releasing a storm of inflammatory mediators including cytokines, interleukins and many others, constituting the systemic inflammatory response syndrome (SIRS). Unfortunately, these mediators also depress the myocardium and the vascular tone, increase vascular permeability resulting in fluid leakage and hypovolemia. These changes affect body organs particularly the kidneys, brain, liver, lungs and coagulation system, leading to inadequate tissue and organ perfusion with insufficient supply of oxygen and nutrients to them and defective removal of metabolic wastage. If not early corrected, it will lead to intense cellular damage with multi-organ system failure and ultimately death.

Septic shock develops when sepsis affects the cardiovascular system resulting in different types of shock states, depending on the underlying pathophysiology. On one hand, sepsis can cause cardiac depression with (CO) which is the product of heart rate (HR) and stroke volume (SV). As blood pressure is the product of CO × (SVR), this low CO state caused by shock triggers a reactive increase in the SVR to maintain an adequate BP, to keep the vital organs well perfused at the expense of non-vital organs such as the skin. This results the extremities becoming cold, hence the name of "cold shock". This high SVR will lead to increase in afterload with further decrease in CO leading to a narrow pulse pressure. Cold shock is more commonly seen in infants and children than in adults. While the latter can double their HR to compensate for a falling CO, children have a much narrower margin to increase their HR and rely instead on intense peripheral vasoconstriction to maintain an adequate BP. The alternate mechanism for shock, more often seen in adults, is vasodilation and decreased SVR. Intense vasodilation will make the skin warmer, hence the term "warm shock". The body tries to increase the falling CO by increasing the cardiac contractility and HR, leading to bounding pulses and a hyperdynamic state.

These shock states either cold or warm could happen with much overlap and variability overtime thus, the classification of shock either pure "cold" or "warm" has been shown to be prone to errors. Normal BP in a patient with septic shock is not necessarily assuring as it does not always equate adequate vital organ perfusion. Although tissue and cellular impairment may cause altered consciousness level, tachypnea, tachy- or bradycardia, hypo- or hyper-thermia, decreased urine output, with increased blood lactate and metabolic acidosis, physical examination and laboratory testing alone are not sufficient for the proper management of septic shock[1].

Traditional classification of shock state includes: (1) hypovolemic; (2) cardiogenic; (3) distributive; and (4) obstructive shock. In septic shock, all these states could happen at different degrees at the same time due to variable effects on preload, afterload and contractility. Hypovolemia results from a combination of relatively decreased blood volume caused by vasodilation, increased insensible water loss and increased capillary permeability to the interstitial and third spaces fluid pooling. A depression of the myocardial function by bacterial toxins and inflammatory cytokines may also occur. Septic shock can also present as distributive shock due to peripheral vasodilation or as obstructive shock from diffuse small end-vessel thrombosis. Therefore, adjunctive objective hemodynamic parameters are needed to assess the cardiovascular condition.

Once shock is recognized, the goal of therapy is to improve tissue oxygen delivery (DO_2), which is the product of CO multiplied by arterial O_2 content (CaO_2), *i.e.*, $DO_2 = CO \times CaO_2$, by improving SV and therefore CO, perfusion pressure and O_2 supply. The left ventricular SV depends on the preload, contractility, afterload and HR. Successful therapy should target a normal HR and BP (adjusted for age), capillary refill time of $\leqslant$ 2 seconds, adequate peripheral pulses, normal temperature of extremities, normal consciousness level, urine output of more than 1 mL/kg per hour, and a central venous oxygen saturation ($ScVO_2$) $\geqslant$ 70%[2]. These targets must be achieved within a short "golden" time frame to avoid multi-organ failure and death[3]. The response to therapy should be frequently monitored, in addition to the essential clinical and laboratory assessment, by objective tools for prompt time-directed management. Invasive tools -mainly through pulmonary artery catheter (PAC) - are more accurate than the noninvasive ones; however, they are time consuming, with special technical difficulties and complications in the small sized infants and children. These obstacles favor the use of noninvasive tools, which should be bedside and user-independent with fast response time to the ongoing hemodynamic changes. They are crucial to guide selection and titration of the most appropriate therapy, including fluid boluses and cardiovascular med-

Table 1 Measurement of hemodynamic parameters using different non-invasive tools

Hemodynamic parameter	Echo	Trans-thoracic Trans-esophageal Doppler	Electrical cardiometry	NIRS
Preload (fluid responsiveness)				
IVCDI	+	-	-	-
CI before and after fluid bolus	+	+	+	-
Afterload				
SVRI	+	+	+	-
Contractility				
CI	+	+	+	-
End-organ perfusion				
rSO_2	-	-	-	+

IVCDI: Inferior vena cava distensibility index; CI: Cardiac index; SVRI: Systemic vascular resistance index; rSO_2: Regional tissue oxygen saturation; NIRS: Near infra-red spectroscopy.

ications, and to monitor its effectiveness.

CHALLENGES IN THE MANAGEMENT OF SEPTIC SHOCK

Left ventricular SV (and therefore CO) is improved by optimizing the preload, contractility and afterload. However, as up to 50% of patients are fluid refractory[4], their management must be directed towards optimizing contractility and afterload. The pediatric surviving sepsis campaign (SSC) 2012 guidelines recommend classifying patients with fluid refractory shock as cold or warm based on clinical assessment, and to proceed accordingly by administering dopamine/epinephrine to cold shock, or norepinephrine to warm shock respectively. However, such subjective classification of shock as warm or cold was found to be inaccurate, with up to 66% of shock diagnosed as being cold shock by experienced clinicians were found to have vasodilation by invasive measurement[5]. It is therefore recommended to institute instead a therapy guided by the hemodynamic monitoring, as detailed in the section titled "Suggested therapy guided by hemodynamic monitoring". Septic shock also has various hemodynamic profiles, with children presenting with different combination of changes in CO and SVR that rapidly change over time[6,7]. Mortality was observed to be high in patients with septic shock with persisting low CO after fluid resuscitation[7,8]. Mortality has also been shown to be more frequently related to patients with low CO rather than with low SVR[9].

The management of septic shock is further complicated by the fact that the medications used, including inotropes, vasopressors and vasodilators, act differently on various receptors at different doses. Selecting the appropriate vasoactive medications and monitoring their effect for titration requires frequent measurements of the CO and SVR in addition to subjective clinical assessment. Relying exclusively on the clinical assessment by feeling the temperature of the patient extremities, capillary refill time or blood pressure, which is not a good marker for end organ perfusion, is prone to error. Prompt and accurate assessment of preload, contractility, afterload and tissue perfusion, preferably non-invasively, is therefore essential. The available non-invasive tools and the hemodynamic parameters that they measure are summarized in (Table 1).

Assessment of preload

The pediatric SSC 2012 recommends initial management of septic shock with aggressive fluid therapy, which may exceed 60 mL/kg in the first hour until either improvement of perfusion occurs, or the appearance of rales or hepatomegaly[2]. In 2017, The American College of Critical Care Medicine endorsed the SSC recommendations but advised to assess patients for rales and hepatomegaly before initiating fluid boluses[10].

Fluid bolus therapy is essential for improving SV and hence cardiac output (CO = SV × HR) until a certain limit according to the Frank-Starling curve (Figures 1 and 2). At the steep limb of the curve, the SV increases in response to fluid boluses (fluid responsiveness), but no further benefit is observed when extra fluid is given at the flat limb of the curve. Furthermore, these extra fluids become hazardous, as they will accumulate in various body tissues including the lungs and the liver manifesting clinically as audible rales and palpable hepatomegaly respectively. These changes are detected clinically only after the patient had already received extra fluids and lost a valuable time in the critical initial window for the management of shock. These extra fluids will also extend the patient time on mechanical ventilation, PICU length of stay and increase the risk of morbidity and mortality[4]. Moreover, it was found that as much as 50% of patients presented with septic shock will not be fluid responsive from early on due to initial myocardial depression and changed sensitivity to adrenergic hormones[4,11]. Because of all these factors, there is a definite need for bedside non-invasive tools to assess fluid responsiveness and prevent overload and its hazards.

Inferior vena cava distensibility index: Distensibility index is a bedside noninvasive parameter for the evaluation of preload volume status and fluid responsiveness, measured by ultrasound. Inferior vena

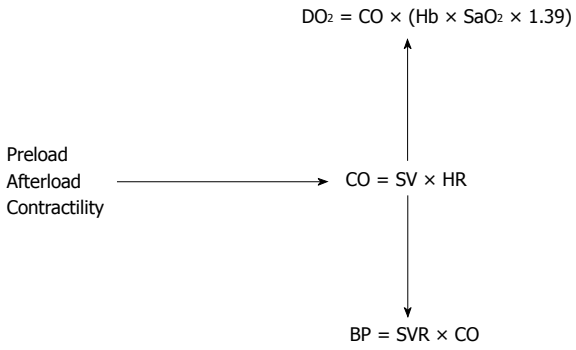


Figure 1 Interplay of hemodynamic parameters. The interplay among the preload, the afterload and the cardiac contractility determines the CO. Cardiac output is essential to maintain a good blood pressure and tissue oxygen delivery. DO_2: Systemic oxygen delivery; SaO_2: Arterial oxygen saturation; Hb: Hemoglobin level; CO: Cardiac output; SV: Stroke volume; HR: Heart rate; BP: Blood pressure; SVR: Systemic vascular resistance.

cava (IVC) has no valves and is distensible; its size is easily changeable by changes in blood volume and intrathoracic pressure. IVC Distensibility index is the result of maximum IVC diameter during inspiration minus minimum IVC diameter during expiration divided by the minimal IVC diameter during expiration [IVC distensibility = ($maxIVC_{insp}$ – $minIVC_{exp}$)/$minIVC_{exp}$]. IVC distensibility index has a great value for the assessment of fluid bolus responsiveness in septic shock patients; values more than 18% indicate fluid responsiveness with 90% sensitivity and 90% specificity[12]. However, there are many pre-requisites that must be met to guarantee precise values. Patients must be relaxed and on mechanical ventilation in order to control intrathoracic pressure. They should not be on a trigger mode to avoid spontaneous efforts, which may cause unpredictable changes in the tidal volume and intrathoracic pressure and should be receiving low positive end-expiratory pressure (PEEP) as high PEEP may decrease the delta change in IVC diameter. Patients should not also have any medical condition associated with, or complicated by, increased intra-abdominal pressure[13]. It is also imperative that the attending intensivist possesses the required ultrasonography skills and experience to perform the measurement.

Functional echocardiography, Doppler and cardiometry: They are used for serial measurements of CO and SV to decide the initial resuscitative fluid bolus therapy and assess the response to repeated volume administrations before reaching the flat limb of the Starling Curve. An increase of SV (and so CO) by 10%-15% indicates fluid responsiveness in septic shock[14].

Assessment of CO

Cardiac output is the result of the overall effect of preload, afterload and cardiac contractility. Thermodilution, through a pulmonary artery catheter (PAC), is the gold standard method for measurement of CO, but it is invasive, time consuming and impractical in infants and small sized children due to technical difficulties and risk of complications. Various minimally invasive and non-invasive methods have been developed for a bedside measurement of hemodynamic parameters including Doppler based approaches, electrical cardiometry and bio-reactance.

As in children CO depends on their size, it is imperative to quantify the cardiac index (CI) instead, to remove the influence of different body sizes and to provide a reference number suitable for children of all sizes. Cardiac index L/(min.m^2) is the product of CO divided by the body surface area (CI = CO/BSA). In patients without septic shock, a CI of more than 2 L/(min.m^2) is considered adequate[15]. However, in those with septic shock, it is recommended to aim a CI of 3.3 - 6 L/(min.m^2)[7,8] to compensate for the impaired SVR in order to maintain a good perfusion pressure for best outcome. In 2017, the American College of Critical Care Medicine has endorsed the SSC 2012 recommendation, in the septic shock management algorithm, to measure the CI in fluid-resistant and catecholamine-resistant shock late at the end of that algorithm[10]. This sequence of targeting the CI, after the patient had already received fluid boluses and cardiovascular shock medications, might be intended for settings with no intensive care facilities. In advanced PICU settings, CI monitoring should be considered early on for proper decision on fluid boluses and further choice of cardiovascular shock medications.

Two-dimensional transthoracic and transesophageal echocardiography: Unlike transesophageal two-dimensional (2-D) echocardiography, transthoracic 2-D echocardiography is noninvasive and both methods reliably measure the SV and therefore the CI and systemic vascular resistance index (SVRI). SV is considered as the volume of a virtual cylinder of blood pushed out of the heart with each beat through its left ventricular outflow tract (LVOT). The base of this cylinder is the LVOT area and its height is the VTI (velocity time integral), which is the velocity of blood in systole moving through the LVOT (Figure 3). Accurate measurement of the SV by this method is time consuming, needs a qualified operator and requires manual calculations. Hence, 2D-echocardiography is an impractical method to use for frequent, serial hemodynamic assessment of critically ill children.

Transesophageal and transthoracic Doppler: The SV is calculated using the same principle as in 2-D echocardiography; the blood velocity is measured in the descending aorta in case of transesophageal Doppler devices and in the ascending aorta or pulmonary artery in case of transthoracic Doppler. The cross-sectional area of the corresponding vessel is calculated by a built-in nomogram based on the patient's age, gender, weight and height that are fed to the machine[16]. Both machines have an inbuilt algorithm that provides cal-

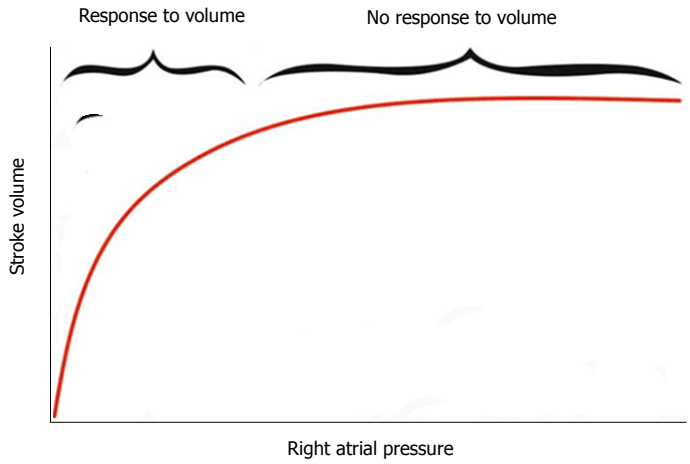


Figure 2 Frank-Starling curve depicting the relation between preload (right atrial pressure) and stroke volume. At the the steep limb of the curve, there is improvement of stroke volume in response to fluid boluses while there is no more response to the fluid boluses at the flat limb of the curve.

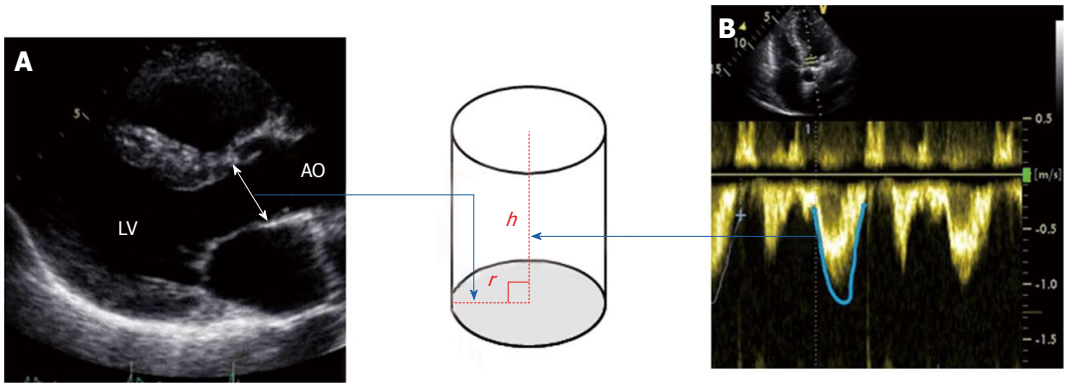


Figure 3 Stroke volume is considered as the volume of a virtual cylinder of blood ejected each beat out of the heart at its left ventricular outflow tract. The base of this cylinder is the LVOT area (area = $\pi.r^2$) π = 3.14, *r* is the radius and its height (*h*) is the velocity time integral (VTI), which is the velocity of blood in systole moving through the LVOT. So, SV will be the net result of π (LVOT diameter/2)^2 × LVOT VTI. The diameter of the LVOT is measured using a parasternal long axis view during systole (A) and the VTI is measured with continuous wave Doppler (B). LVOT: Left ventricular outflow tract.

culations for hemodynamic parameters like CI, SVRI, stroke volume variability and corrected flow time (FTc). The last two parameters are particularly useful in assessing and monitoring preload or ventricular filling. A minimal training is required to adequately place the probe in order to display the best Doppler signal seen on the screen and to hear it through the machine. Doppler based methods are not as accurate as echocardiography, but they are easy to use and they provide trends of the measured values that are valuable in the management of shock. The transesophageal Doppler allows a continuous measurement of the CO in the descending aorta but does not consider the flow in the aortic arch arteries, which represents 30% of the CO. Despite these limitations, its use was found to decrease complication rates and hospital stay in perioperative outcome studies[17]. Although transthoracic Doppler does not offer continuous monitoring of the hemodynamic parameters, the machine is portable, bedside and easy to use allowing frequent monitoring in case of shock. Validation of transthoracic Doppler in children against the gold standard PAC thermodilution method has shown conflicting results. A study in 24 children undergoing cardiac catheterization found that transthoracic Doppler CO measurements were not reliable (Bias -0.13 and precision 1.34 L/min)[18], while another similar study on 31 children demonstrated reliable CO measurements (Bias 0.1 L/min and 95% limits of agreement of -0.6 to +0.9 L/min)[19].

Electrical cardiometry: Electrical cardiometry is a recent non-invasive continuous CO monitoring tool. Its idea is based on its precedent electrical impedance. Four electrodes are attached to the left neck and left lower chest sides. A low magnitude (2 mA), high frequency (30-100 KHz) alternating electrical current (AC) of constant amplitude is applied through the outer electrodes and the resulting voltage is received by the other inner electrodes. The ratio of the detected voltage to the applied current defines bio-impedance.

The theory behind it is that during systole, red cells flow in a parallel manner allowing the electrical current

to flow easily thus increasing "electrical velocity" and decreasing impedance. During diastole, red blood cells are arranged randomly, hence impeding the electrical current (increased impedance) and decreasing electrical velocimetry[20]. The changes of impedance overtime are integrated in a complex algorithm that allows to measure CO along with essential hemodynamic parameters including preload (Thoracic Fluid Index), afterload (SVR) and CO, in addition to many other hemodynamic parameters that are beyond the scope of this manuscript. Electrical cardiometry is FDA approved and validated for use in neonates[21], children[22] and adults[23]. A metanalysis in 2016, including 20 studies and 624 patients comparing the CO measurement accuracy by using electrical cardiometry with other noninvasive technologies in pediatrics, demonstrated that electrical cardiometry was the device that offered the most correct measurements. However, there was a high heterogeneity among the individual studies[24]. Furthermore, the accuracy of cardiometry may be affected by severe tachycardia or bradycardia, aortic regurgitation[25], chest wall edema, and high frequency ventilation[26].

Bioreactance: Bioreactance is a modification of thoracic bioimpedance. It needs attachment of two dual electrodes on both sides of the chest and so, tracks the phase of the electrical currents traversing the chest. The underlying scientific phenomenon is that the higher the cardiac SV, the more significant these phase shifts become. Kupersztych-Hagege *et al*[27] (2013) have shown that bioreactance is not reliable for estimating CO and it could not predict fluid responsiveness in critically ill patients.

Assessment of afterload

Afterload is assessed by measuring the SVR. The resistance (R) in any circuit is the gradient of pressure (ΔP) divided by the flow (R = ΔP/Flow). SVR is the gradient between the mean arterial pressure (MAP) at the exit of the left ventricle and the pressure at the entrance of the right atrium (CVP) divided by the flow, which is the CO; [SVR = (MAP – CVP)/CO]. Including body surface area in reporting SVRI[(MAP – CVP)/CI], makes its value independent of body size. SVRI is further multiplied by 80 to convert the results from Woods unit to dyn·s·cm^{-5} unit. There are now available reference ranges of CI and SVRI according to patient age. Cardiac index and SVRI include the SV in their calculations (Figure 1), making SV the corner stone for these measurements. Echocardiography and Doppler based methods, as described earlier, are used to measure SV and calculate both CI and SVRI.

Although there is still no clear evidence that monitoring CI and SVRI improves the outcome of children in septic shock[14], it makes physiological sense and provides intensivists objective parameter to adequately select and guide cardiovascular shock therapy.

Assessment of end organ perfusion

The CO, BP and SVR cannot inform about the end organ perfusion because the CO is not evenly distributed among all organs due to the inhomogeneous vascular resistance across the body. The effect of the commonly used vasoactive medications is not always predictable and may vary at different doses. Concurrent $PaCO_2$, PaO_2 and hemoglobin level may affect cerebral oxygen delivery independent of the CO and SVR. Medical conditions affecting oxygen consumption like fever, irritability and thyroid hyper function may also increase oxygen extraction and shift the balance towards oxygen and metabolites deficit.

Near infrared spectroscopy: This method allows continuous measurement of the regional tissue saturation and oxygen extraction. It works non-invasively by placing a simple electrode on the forehead, kidneys, abdomen or muscles depending on the target organ. The electrode contains an emitter of infrared light at specific wavelengths and a receptor measuring the regional O_2 saturation based on the amount of light absorbed by the hemoglobin. The method is comparable to arterial SpO_2 monitoring. However, while the measurements of arterial SpO_2 are performed about 2200 times/min, allowing the machine to capture SpO_2 during the pulsations, near infrared spectroscopy (NIRS) emits an infrared light continuously, thus providing a measurement of O_2 saturation mainly at the venous side of the capillaries (70%). Therefore, NIRS is primarily a measurement of venous saturation and should be considered as a non-invasive alternative of $ScVO_2$. The method has been validated extensively and its use was shown to improve outcomes[28]. New generations of NIRS are being developed to enable the assessment of cerebral autoregulation and to choose the appropriate target blood pressure within the autoregulation zone.

SUGGESTED THERAPY GUIDED BY HEMODYNAMIC MONITORING

Considering that BP = CO × SVR, measuring BP, CI and SVRI is recommended as it helps to select the most appropriate therapy based on the different combinations of these parameters (Table 2). For example, a patient with a high SVRI, low CI and normal BP is better treated with inodilators such as milrinone or levosimendan to increase cardiac contractility and decrease afterload. Frequent assessments are required until the target clinical and laboratory hemodynamic parameters are reached, and CI and SVRI normalized. On the other hand, if SVRI, CI and BP are all low, norepinephrine is a first choice as it increases the SVRI, to be then followed by epinephrine for its beta 1 effect

Table 2 Suggested therapy in response to measured hemodynamic parameters in children with septic shock

Hemodynamic parameters	Pathophysiological mechanism	Treatment
High SVRI, low CI and normal BP	Increased afterload Decreased contractility	Inodilators (milrinone, levosimendan)
Low SVRI, low CI and low BP	Decreased afterload	Vasopressors (norepinephrine, epinephrine)
	Decreased contractility	Dobutamine if CI remains low
Low SVRI, high CI and low BP	Decreased afterload Good contractility	Vasopressors (norepinephrine or low dose vasopressin)
		Dobutamine or low dose epinephrine if CI decreased

SVRI: Systemic vascular resistance index; CI: Cardiac index; BP: Blood pressure.

or by dobutamine if the CI remains low. Frequent assessments are always required until the hemodynamic targets are reached. In cases of low SVRI, high CI and low BP fluid boluses are administered first, followed by norepinephrine, then by low dose vasopressin if needed. As these potent vasoconstrictors can decrease CO, inotropes like dobutamine or low dose epinephrine can be added to improve cardiac contractility[29]. The whole situation needs close, frequent monitoring by clinical and laboratory assessment, in addition to these parameters. They could also guide the titration and the choice of these cardiovascular medications.

CONCLUSION

Early hemodynamic management in the first golden hour of presentation of septic shock is associated with better outcome[30]. Non-invasive bedside assessment of the preload, contractility and afterload provides a rational guide to the fluid therapy and selection of appropriate cardiovascular medications. Following the trend of hemodynamic parameters instead of individual readings allows for personalized management and proper titration of therapy over time. Proper training and a good understanding of the used techniques and their limitations is needed. With a paucity of existing data analyzing the effect of non-invasive bedside hemodynamic monitoring on mortality in children with septic shock, adequately designed and powered outcome studies are urgently needed.

ACKNOWLEDGMENTS

Dr. Samah Al Asrawi for providing the echocardiography picture which illustrates the measurement of the stroke volume.

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P- Reviewer: Al-Biltagi M, Losano G **S- Editor**: Ji FF **L- Editor**: A
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ISSN 2222-0682 (online)

World Journal of *Methodology*

World J Methodol 2018 October 12; 8(2): 9-16



Published by Baishideng Publishing Group Inc

World Journal of Methodology

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INDEXING/ABSTRACTING

World Journal of Methodology is now abstracted and indexed in PubMed, PubMed Central, China National Knowledge Infrastructure (CNKI), and Superstar Journals Database.

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NAME OF JOURNAL
World Journal of Methodology

ISSN
ISSN 2222-0682 (online)

LAUNCH DATE
September 26, 2011

EDITOR-IN-CHIEF
Yicheng Ni, MD, PhD, Professor, Department of Radiology, University Hospitals, KU Leuven, Herestraat 49, B-3000, Leuven, Belgium

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PUBLICATION DATE
October 12, 2018

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World J Methodol 2018 October 12; 8(2): 9-16

DOI: 10.5662/wjm.v8.i2.9

ISSN 2222-0682 (online)

MINIREVIEWS

Towards a better understanding of anesthesia emergence mechanisms: Research and clinical implications

Marco Cascella, Sabrina Bimonte, Maria Rosaria Muzio

Marco Cascella, Sabrina Bimonte, Division of Anesthesia and Pain Management, Department of Supportive Care, Istituto Nazionale Tumori "Fondazione G. Pascale" - IRCSS, Naples 80131, Italy

Maria Rosaria Muzio, Division of Infantile Neuropsychiatry, UOMI-Maternal and Infant Health, ASL NA3 SUD Torre del Greco, Naples 80059, Italy.

ORCID number: Marco Cascella (0000-0002-5236-3132); Sabrina Bimonte (0000-0002-5408-9675); Maria Rosaria Muzio (0000-0002-8172-2325).

Author contributions: Cascella M and Muzio MR contributed equally to this work, generated the table and wrote the manuscript; Bimonte S contributed to the writing of the manuscript.

Conflict-of-interest statement: All the authors declare no conflict of interest.

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Manuscript source: Invited manuscript

Correspondence to: Marco Cascella, MD, Academic Fellow, Professor, Division of Anesthesia and Pain Management, Department of Supportive Care, Istituto Nazionale Tumori "Fondazione G. Pascale" - IRCSS, Via Mariano Semmola, Naples 80100, Italy. m.cascella@istitutotumori.na.it
Telephone: +39-81-5903586
Fax: +39-81-5903778

Received: June 29, 2018
Peer-review started: June 30, 2018
First decision: July 19, 2018
Revised: August 1, 2018
Accepted: August 26, 2018
Article in press: August 26, 2018
Published online: October 12, 2018

Abstract

Emergence from anesthesia (AE) is the ending stage of anesthesia featuring the transition from unconsciousness to complete wakefulness and recovery of consciousness (RoC). A wide range of undesirable complications, including coughing, respiratory/cardiovascular events, and mental status changes such as emergence delirium, and delayed RoC, may occur during this critical phase. In general anesthesia processes, induction and AE represent a neurobiological example of "hysteresis". Indeed, AE mechanisms should not be simply considered as reverse events of those occurring in the induction phase. Anesthesia-induced loss of consciousness (LoC) and AE until RoC are quite distinct phenomena with, in part, a distinct neurobiology. Althoughanaesthetics produce LoC mostly by affecting cortical connectivity, arousal processes at the end of anesthesia are triggered by structures deep in the brain, rather than being induced within the neocortex. This work aimed to provide an overview on AE processes research, in terms of mechanisms, and EEG findings. Because most of the research in this field concerns preclinical investigations, translational suggestions and research perspectives are proposed. However, little is known about the relationship between AE neurobiology, and potential complications occurring during the emergence, and after the RoC. Thus, another scope of this review is to underline why a better understanding of AE mechanisms could have significant clinical implications, such as improving the patients' quality of recovery, and avoiding early and late postoperative complications.

Key words: Delirium; Anesthesia; Isoflurane; Propofol; Consciousness; Awareness; Electroencephalography

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Core tip: Emergence from general anesthesia is not simply the reverse process of induction. The exhaustive knowledge of its complex neurobiological mechanisms is mandatory for avoiding or limiting a large number of anesthesia complications including altered mental status, and emergency awareness. Moreover, in a fascinating translational perspective, the study on this topic could provide new insights into the processes involved in cortical arousal, offering significant data to the research on brain arousal. On the other hand, research on the sleep-wake regulatory network, and on alterations in arousal processes could provide interesting suggestions for the general anesthesia research.

Cascella M, Bimonte S, Muzio MR. Towards a better understanding of anesthesia emergence mechanisms: Research and clinical implications. *World J Methodol* 2018; 8(2): 9-16 Available from: URL: http://www.wjgnet.com/2222-0682/full/v8/i2/9.htm DOI: http://dx.doi.org/10.5662/wjm.v8.i2.9

INTRODUCTION

Emergence from anesthesia (AE) is the ending stage of anesthesia featuring the transition from unconsciousness to complete wakefulness and recovery of consciousness (RoC)[1]. Although smooth and safe emergence is a primary target of anesthesia, during this critical phase a wide range of undesirable complications may occur. These AE complications include coughing, which may induce an increase in intracranial and intraocular pressures, respiratory events (*e.g.,* laryngospasm) resulting in oxygenation problems, hypertension, and tachycardia as well as mental status changes such as emergence delirium (ED)[2], and delayed RoC (*i.e.*, hypoactive emergence)[3].

From a neurobiological perspective, AE and RoC processes should not be simply considered as reverse events occurring in the induction of anesthesia. In mathematical terms, this non-linear system between induction and AE mechanisms represents a fascinating neurobiological example of "hysteresis"; thus, anesthesia can be ideally compared as a travel with a forward way (induction) which differs from that of the return (emergence). Recently, several research groups have demonstrated that the anesthetics-induced transition from wakeful state to loss of consciousness (LoC), and vice versa the RoC phase are subjected to the control of distinct neural circuits[4,5].

The aim of this work is to provide a comprehensive review of the literature, for assessing the state of the art in research on AE processes. Because most of the research in the field concerns preclinical investigations, translational suggestions and research perspectives are proposed. However, little is known about the relationship between AE neurobiology and AE potential complications. Thus, another scope of this review is to underline why a better understanding of AE mechanisms could have significant clinical implications, including an improvement of the patients' quality of recovery, and avoiding early and late postoperative complications.

NEUROBIOLOGY OF EMERGENCE

Mechanisms of emergence

Explanation of these mechanisms presupposes the knowledge of the action mechanisms of anesthetics, namely the neural correlates of anesthetics-induced unconsciousness. Although it does not represent the primary goal of this work, here we will try to underline the most recent pieces of evidence on the topic[6-9]. This extremely complex matter can be simplified by assuming that anesthetics interfere with cortical and subcortical signals, inducing, in turn, changes in the functional/effective connectivity across brain regions. During general anesthesia, alterations in functional and effective connectivity from different brain regions (*e.g.*, from frontal to parietal regions) have been widely demonstrated[9]. For instance, inhalation anesthetic agents impair frontal-posterior interactions by interfering with the gamma (20-60 Hz) oscillations which have a key role in arousal and maintenance of consciousness[10]. High-density electroencephalography (hd-EEG) demonstrated that propofol-induced LoC is characterized by an increase in frontal delta power as the result of cortical propagation of processes starting from subcortical regions (*e.g.*, lateral sulci and cingulate gyrus)[11]. In turn, these slow-delta oscillations propagate asynchronously across the cortex, inducing a functional disruption of the connectivity between distinct cortical areas. Moreover, by using a combination of positron emission tomography and functional magnetic resonance imaging (PET/MRI), Akeju *et al*[12] demonstrated that the main effect of dexmedetomidine-induced LoC was the impairment of the thalamo-cortical functional connectivity.

Connectivity changes within distinct brain regions lead to different depths of anesthesia (DoA). Thus, loss of communication between the thalamus and the cortex is responsible for the beginning of LoC. Subsequently, changes in the cortico-cortical functional connectivity, and in the functioning of several brainstem nuclei (*e.g.*, the ventrolateral preoptic nucleus) as well as in the connectivity between these structures and cerebral cortex are responsible for completing the induction process and, in turn, for maintaining the surgical anesthesia status. During the AE period, mechanisms responsible for LoC and anesthesia maintenance are gradually reversed, whereas other specific awakening mechanisms are activated. These mechanisms encompass several ascending arousal brain pathways

responsible for the activation and promotion of the emergence until the RoC. Among the arousal-promoting brain regions involved in the active AE processes, the thalamus has a key role[13]. Alkire *et al*[14] proved in rats that midline intrathalamic microinfusion of nicotine reversed sevoflurane-induced loss of righting reflex (LORR), an indicative sign of unconsciousness in rodents. Other preclinical investigations, focusing on voltage-gated potassium channels, were performed to assess the role of thalamic central medial nucleus for AE induction[15,16]. However, the thalamic systems are not the sole pathways involved in active AE. Investigations on the dopaminergic (DA) projections from the substantia nigra (SN) and ventral tegmental area (VTA) of the midbrain to the pedunculopontine, thalamus, dorsal raphe, locus ceruleus (LC), and laterodorsal tegmental areas, basal forebrain (BF), and lateral hypothalamus)[17,18] suggested the existence of a mesencephalic arousal pathway. It has been shown in animals that the intravenous administration of methylphenidate, or dextroamphetamine which increases the dopaminergic and adrenergic neurotransmission through the reuptake inhibition or the use of a D1 receptor agonist (chloro-APB) restores LORR and increases theta oscillations (decreasing delta- and alfa-power) during inhaled[19,20] or endovenous anesthesia[21]. Furthermore, Taylor *et al*[22] obtained the same results through a selective stimulation of the VTA dopaminergic neurons, whereas the administration of a D1 antagonist (SCH-23390) attenuated the arousal response. Thus, they called this active transition from the anesthetized state to the awake state "reanimation from general anesthesia"[22].

The hypothalamus is another brain structure involved in the AE mechanisms. The orexinergic neurons are localized in a hypothalamic area around the fiber bundle of fornix. This orexin system (OS) plays a key role in induction of sleep-to-wake transitions, and maintenance of wakefulness[23]. A series of studies proved that it facilitated AE in both intravenous[24], and inhaled general anesthesia[25]. More recently, Zhang *et al*[26] demonstrated in rats that isoflurane depressed the excitability of orexinergic neurons. Although both orexins (*i.e*., orexin-A, OXA, and orexin-B, OXB) promoted emergence, OXA played a more significant role (through orexin receptor-1).

Functionally, the OS is related to the locus coeruleus norepinephrine system (LC NE), and the posterior hypothalamic histaminergic tuberomammillary nuclei (TMN HA)[27] which are well-known wake-promoting cell groups of the sleep-wake regulatory network[28], and implicated in the action mechanisms of inhaled anesthesia[29]. In particular, LCNE is functionally connected to the posterior cingulate cortex (PCC), thalamus, and basal ganglia forming the LCNE arousal system, which has been suggested to have an important role in the AE[30]. Orexinergic projections to the hippocampus, and basal ganglia have been also demonstrated[31].

In this complex scenario, there is a functional connection between OS, BF cholinergic structures (*i.e*., medial septum, vertical limbs of the diagonal band of Broca, nucleus basalis of Meynert, and substantial innominate), and the brainstem ascending reticular arousal system (ARAS). Indeed, the BF has diffuse projections to all parts of the neocortex, basolateral amygdala, and hippocampus, whereas the ARAS has cholinergic cortical projections, and connection with the thalamus, hypothalamus, and the BF region, which in turn modulate the OS (feedback mechanism). The OS contributes to arousal processing by increasing cortical activity due to excitatory projections to wake-promoting cell groups in the posterior hypothalamus, BF, and brainstem. On the other hand, orexin neurons are controlled by positive and negative feedback mechanisms mainly mediated by the hypothalamus and other areas (*e.g.*, perifornical area)[32] and more details on orexin pathways could be found in literature[33]. Taken together, these data suggest that arousal processes at the end of anesthesia are triggered by structures deep in the brain, rather than being induced within the neocortex.

Electrical activity during recovery from anesthesia

The brain's response to anesthetics recorded with scalp electroencephalogram (EEG) represents the cortical synaptic activity of both excitatory and inhibitory post-synaptic potentials from cortical or thalamic neurons[34]. Apart from this non-invasive EEG modality used in human studies, other approaches such as the electrocorticogram (ECoG, EEG measured directly from the cortical surface), stereoelectroencephalography (SEEG), an EEG performed via depth probes, are used in specific clinical settings (*e.g*., SEEG in epilepsy) or for experimental investigations in animals. Moreover, neurophysiological changes in the brain under general anesthesia are often studied through a combination of EEG (including hd-EEG methods) with brain activity measures such as functional near-infrared spectroscopy (fNIRS)[35], and neuroimaging modalities, (*e.g*., functional magnetic resonance imaging, fMRI) [36], or by combining EEG with electrodiagnostic methods, including electromyography and evoked potentials (EP)[37].

Studies on EEG activity during anesthesia induced a significant impetus to research aimed at elucidating the dynamics of anesthesia. Again, technological advances, and mathematical approaches, allowed to apply several brain monitoring devices which are commonly used in clinical practice. However, explanation of features and clinical utility of DoA monitoring systems is not the scope of this review[38].

Anesthesia-related electrical activity consists of a wide range of EEG patterns, mainly depending on the anesthesia phase (induction, maintenance and emergence), the DoA status, and the type of anesthetics used. Before induction, the awake subject with eyes closed shows a prominent alpha activity (10 Hz) which

is maximal over parieto-occipital scalp locations. After inducing anesthesia, EEG pattern shows an increase in beta activity (13-25 Hz) until the LoC[39], whereas during the maintenance phase, different EEG patterns are observed, depending on the DoA level. During a light anesthesia, a decrease in EEG beta band (13-30 Hz) and an increase in both EEG alpha (8-12 Hz) and delta activities (0-4 Hz) may occur. As the DoA state becomes deeper, beta activity decreases, and there is an increase in delta and in alpha frequency band oscillations, with the latter being anteriorly located ("alpha anteriorization")[40]. A further DoA status features an EEG pattern comprising flat periods interspersed with periods of alpha and beta activity. This characteristic EEG pattern, is known as *burst suppression*. It can be also recognized in deeper coma status due to various conditions including cerebral anoxia, cancers, drug intoxications, encephalopathies, or hypothermia[41]. The anesthesia-induced burst suppression seems to be associated with a state of cortical hyperexcitability generated by decreased inhibition[42].

As the anesthesia state deepens, EEG shows a progressive stretching between the alpha activities. The amplitudes of the alpha and beta activities progressively decrease, and in turn, the EEG assumes isoelectric form. In this context, the deepest DoA status has been reached[43]. As to the correlation between EEG findings and different anesthetic agents, previous studies showed that this general scheme is particularly applicable for halogenated inhalational anesthetics, and propofol whereas, in contrast, opioids and ketamine usually induce less marked EEG changes. Furthermore, etomidate and barbiturates lead to a rapid shift toward the high voltage delta and theta frequencies[44].

Classically, during emergence it is possible to observe a loss of delta activity, combined with a progressive decrease in frontal alpha power and increased higher frequencies[45]. Moreover, ECoG studies showed that specific findings (*i.e.*, slow oscillation in large-scale functional networks) are maintained during the LoC and RoC phases[46]. However, the canonical EEG sequence during AE can undergo changes. In a fascinating clinical study, Chander *et al*[47] described different AE patterns. At the beginning of AE, they recognized a pattern characterized by high power of alpha and beta bands (in 95% patients) and termed it as "Slow-Wave Anesthesia" (SWA). The minority of patients had an EEG with a very low spindle and delta power (called "Non Slow-Wave Anesthesia", NSWA). Interestingly, they also found that EEG patterns between start of emergence and RoC vary, and described four trajectories between the beginning of AE and the RoC. More recently, Liang *et al*[48] classified emergence EEG patterns in sevoflurane anesthesia. Using an integrated approach obtained by a multivariate statistical model, they identified four types of emergence EEG patterns. Interestingly, some of these emergence modalities were age-related and could be associated with postoperative mental changes.

It can be assumed that the occurrence of different EEG patterns at the emergence reflects the different degrees of influence of brainstem activity on cortical re-connectivity, through the thalamus mediation[13]. This evidence could prove that AE modulation can be mostly obtained by controlling brainstem activity (*e.g.*, by opioids). Although we adopted a general descriptive scheme (*i.e.,* anesthesia-induced EEG changes at the emergence), it is important to underline that because of the distinct mechanisms of action, different anesthetics may induce different types of EEG dynamics, also in the AE phase. Recent studies are increasingly characterizing these profiles[49], especially with regard to dexmedetomidine[50].

CLINICAL IMPLICATIONS

A better understanding of AE mechanisms has significant clinical implications, such as improving the quality of recovery of patients following surgery. It has been proved that the EEG modality at the emergence affected the residual level of sedation and post-operative pain[47]. Thus, in a hypothetical scenario, it could be possible to modulate the path of emergence, choosing the one which correlates with the desired target. The possibility to increase the predictability of the time of emergence may help prevent delayed emergence - defined as the failure to regain consciousness 30-60 min after general anesthesia[51] - and other more frequent AE complications, such as ED, and respiratory complications. This is a significant issue as delayed emergence was associated with a longer postoperative hospital stay[52], whereas ED in children may lead to physical harm in the children and distress in patients, parents and staff. Moreover, although usually self-limiting, it can last up to 48 h, and children who manifested ED are more likely to suffer from new-onset postoperative maladaptive behavioral changes[53]. Again, ED in adults can lead to serious complications, such as self-extubation, accidental removal of catheters and injury[54].

Although about 20% of accidental awareness with recall during general anesthesia (AAWR) occur at the AE, and 90% of these cases are potentially preventable (*e.g.*, through the use of neuromuscular monitoring)[55], in very rare AAWR cases there is no readily identifiable cause[56]. A more precise AE management can help avoid these unexpected awakening events, which are often associated with severe psychological consequences, such as post-traumatic stress disorder (PTSD).

TRANSLATIONAL DATA AND RESEARCH PERSPECTIVES

Positive results from preclinical and clinical studies on this topic should encourage additional research (Table 1). For instance, clinical investigations should translate preclinical findings to evaluate possible interventions for inducing active AE, and in turn for

Table 1 Suggestions for additional research on emergence from anesthesia

Type of study	Topic(s)
Multicenter RCTs	Effects of drugs on AE time, features, and postoperative complications including mental status changes
Multicenter RCTs	Effects of antinociceptive interventions (*e.g.*, neuraxial anesthesia) on accelerating emergence and improving patient outcomes
Animal research (molecular/behavioral research)	Effects of AE modulation on molecular targets of neuroinflammation
Animal research (behavioral research)	Effects of AE modulation on early postoperative behavioral changes
Animal research (molecular/imaging/behavioral research)	Anesthetics mechanisms; Linkage between brain areas involved in cognitive functioning and AE features
Animal research/in humans	Neurophysiological changes under general anesthesia (*e.g.*, by combining EEG approaches with electrodiagnostic methods, including EMG and EPs, or with brain activity measures such as fNIRS, and neuroimaging modalities like fMRI)
In vitro/*In vivo* (*e.g.*, mutant analysis in Drosophila)	Anesthetic mechanisms (*e.g.*, genes encoding for second-messengers, memory formation substrates, ion channels, synaptic proteins)

RCT: Randomized controlled trial; AE: Anesthesia emergence; EMG: Electromyography; EPs: Evoked potentials; fNIRS: Functional near-infrared; fMRI: Functional magnetic resonance imaging.

preventing AE complications. For this purpose, thanks to its actions consisting of arousal promotion, and breathing enhancement, the dopamine uptake inhibitor methylphenidate was the first drug to be tested in humans. Researchers from the Ohio State University assessed, in adult patients, whether methylphenidate (given orally 20 mg, 2 h before induction) decreased the emergence time from isoflurane general anesthesia, and gave rise to a fast cognitive improvement with efficient pain control and post-operative nausea and vomiting (PONV) prevention (NCT02327195). To date, the recruitment status of this prospective, randomized, double-blind, placebo-controlled trial (RCT) is indicated as completed (n = 54) and we are waiting for the publication of the results. Probably, this RCT will encourage further research with a multicenter involvement and a greater sample. Another RCT in adult patients scheduled for pancreatic surgery is on-going at Massachusetts General Hospital (NCT02051452). Apart from the AE time effect, the investigators focused on safety and tolerability of methylphenidate in this clinical setting, and the impact on postoperative delirium (PD) and post-operative cognitive function (POCD). The study is expected to be completed in December 2018. We hope that results from this RCT will offer clinical data to better define the correlation between AE and postoperative mental status changes. Data from preclinical research suggested that other interesting molecules should be tested for evaluating their effects on emergence features and postoperative cognitive outcomes. Zhang *et al*[57] demonstrated that amantadine, a dopamine agonist used to treat Parkinson's disease and parkinsonism syndromes, attenuated post-operative learning and memory decline *via* inhibition of neuroinflammation in rodents. Interestingly, this study may suggest that interventions focusing on AE modulation may interfere with microglial activation and the cascade of neuroinflammation, implicated in POD/POCD pathogenesis[58].

Clinical investigations should be carried out to better clarify results, and discrepancies of preclinical and clinical studies. Although OXA has been proved to be involved in arousal from general anesthesia in rodents[24,26], and the Kushikata[59]'s studies showed that plasma OXA significantly increased at AE from both propofol and sevoflurane anesthesia, Wang *et al*[60] proved that higher plasma OXA concentrations were not associated with a reduction in AE time in elderly patients. However, these patients showed a higher level of plasma OXA compared to young patients[60], suggesting an age-dependent difference in the orexin-induced anesthesia arousal regulation. Probably, the lower density of orexin receptors in elderly can offer a potential explanation to the evidence that the elderly require a longer AE time[61] despite a higher orexinergic activation.

In addition, we believe that further preclinical research may be necessary to evaluate correlations between AE mechanisms and postoperative cognitive complications. More detailed investigations in rodents should investigate the effect of AE modulation on early postoperative behavioral changes. In a translational perspective, indeed, a paramount aim should be to demonstrate whether any potential intervention on active AE processes can effectively induce an improvement in cognition, rather than just reducing the AE times.

Again, studies on the pathophysiology of PD, and POCD offer interesting prospects for further research. Alterations in the prefrontal cortex, and in the dopaminergic projection to the LC are implicated in the genesis of PD and POCD. Moreover, the orexinergic system is connected through the functional mediation of the TMN HA to the hippocampus, neostriatum, nucleus accumbens, and amygdala, which represent key regions involved in the pathogenesis of PD, and POCD[62].

Further research is also warranted to better explain the mechanisms which induce AE activation.

Certainly, many aspects of the anesthetics are still to be elucidated. For instance, it has been demonstrated that the OS could be another possible target for isoflurane[26], whereas the role of serotonergic neurones in dorsal raphe nucleus implicated in the mechanisms of general anesthesia[63] on the orexinergic signal should be an interesting field of research for investigating the linkage between AE modalities and RoC features, such as pain and mood. Effects of specific antinociceptive interventions (*e.g.*, neuraxial anesthesia) as potential mechanisms interfering with emergence processes and clinical consequences should be addressed in order to prove specific experimental findings such as the brainstem involvement in arousal dynamics. AE translational approaches could promote a feedback between different neuroscience fields of study. Thus, general anesthesia research could offer significant information to the research on mechanisms controlling arousal processes involved in physiological and pathological phenomena, such as sleep and coma[43].

CONCLUSION

Neurobiologically, the ending stage of anesthesia is not simply the reverse process of induction. Recent findings demonstrated that induction and emergence are partly subjected to the control of different neural pathways. The exhaustive knowledge of these mechanisms may help prevent a large percentage of anesthesia complications, including altered mental status, and AAWR phenomena. Consequently, a better understanding of AE neurobiology could open a new era in anesthesia aiming to design new and safer anesthetic strategies. Moreover, in a fascinating translational perspective, the study on this topic could offer new insights into the complex mechanisms involved in cortical arousal, and provide significant data to the research on brain arousal processes and relative alterations. On the other hand, research on the sleep-wake regulatory network, and on alteration in arousal, and cognitive processes, could provide interesting suggestions for the general anesthesia research.

ACKNOWLEDGMENTS

The authors are deeply grateful to Mr Nagoth Joseph Amruthraj, Senior Researcher - Clinical, Experimental and Medical Sciences, Chair of Nephrology, Department of Cardio-Vascular Medicine, University of Study of Campania "Luigi Vanvitelli", Caserta, Italy 81100 - for his valuable pro bono help in revising the manuscript in order to improve and polish language.

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P- Reviewer: Bugaj AM, Ciccone MM, Maric I, Neri V, Tomizawa M
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ISSN 2222-0682 (online)

World Journal of *Methodology*

World J Methodol 2018 November 29; 8(3): 17-50



Published by Baishideng Publishing Group Inc

World Journal of Methodology

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World Journal of Methodology is now abstracted and indexed in PubMed, PubMed Central, China National Knowledge Infrastructure (CNKI), and Superstar Journals Database.

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NAME OF JOURNAL
World Journal of Methodology

ISSN
ISSN 2222-0682 (online)

LAUNCH DATE
September 26, 2011

FREQUENCY
Continuous

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Jin-Lei Wang, Director
World Journal of Methodology
Baishideng Publishing Group Inc
7901 Stoneridge Drive, Suite 501, Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
Fax: +1-925-2238243
E-mail: editorialoffice@wjgnet.com
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PUBLISHER
Baishideng Publishing Group Inc
7901 Stoneridge Drive,
Suite 501, Pleasanton, CA 94588, USA
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Fax: +1-925-2238243
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PUBLICATION DATE
November 29, 2018

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World J Methodol 2018 November 29; 8(3): 17-36

DOI: 10.5662/wjm.v8.i3.17

ISSN 2222-0682 (online)

EDITORIAL

Precision medicine allergy immunoassay methods for assessing immunoglobulin E sensitization to aeroallergen molecules

Florin-Dan Popescu, Mariana Vieru

Florin-Dan Popescu, Mariana Vieru, Department of Allergology, "Carol Davila" University of Medicine and Pharmacy, Bucharest 022441, Romania

Florin-Dan Popescu, Mariana Vieru, Department of Allergology and Clinical Immunology, "Nicolae Malaxa" Clinical Hospital, Bucharest 022441, Romania

ORCID number: Florin-Dan Popescu (0000-0001-6316-3155); Mariana Vieru (0000-0001-7396-5688).

Author contributions: Popescu FD and Vieru M conceived the editorial and drafted the manuscript; both authors approved the final version of the article.

Conflict-of-interest statement: The authors have no conflict of interest to declare.

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Corresponding author to: Florin-Dan Popescu, MD, PhD, Associate Professor, Chief Doctor, Department of Allergology and Clinical Immunology, "Nicolae Malaxa" Clinical Hospital, Sos Vergului 12, Sector 2, Bucharest 022441, Romania. florindanpopescu@ymail.com
Telephone: +40-212-555405
Fax: + 40-212-555275

Received: July 9, 2018
Peer-review started: July 9, 2018
First decision: August 2, 2018
Revised: August 9, 2018
Accepted: October 9, 2018
Article in press: October 9, 2018
Published online: November 29, 2018

Abstract

Molecular-based allergy diagnosis for the *in vitro* assessment of a patient immunoglobulin E (IgE) sensitization profile at the molecular level uses allergen molecules (also referred to as allergen components), which may be well-defined, highly purified, natural allergen components or recombinant allergens. Modern immunoassay methods used for the detection of specific IgE against aeroallergen components are either singleplex (such as the fluorescence enzyme immunoassay with capsulated cellulose polymer solid-phase coupled allergens, the enzyme-enhanced chemiluminescence immunoassay and the reversed enzyme allergosorbent test, with liquid-phase allergens), multiparameter (such as the line blot immunoassay for defined partial allergen diagnostics with allergen components coating membrane strips) or multiplex (such as the microarray-based immunoassay on immuno solid-phase allergen chip, and the two new multiplex nanotechnology-based immunoassays: the patient-friendly allergen nano-bead array, and the macroarray nanotechnology-based immunoassay used as a molecular allergy explorer). The precision medicine diagnostic work-up may be organized as an integrated "U-shape" approach, with a "top-down" approach (from symptoms to molecules) and a "bottom-up" approach (from molecules to clinical implications), as needed in selected patients. The comprehensive and accurate IgE sensitization molecular profiling, with identification of the relevant allergens, is indicated within the framework of a detailed patient's clinical history to distinguish genuine IgE sensitization from sensitization due to cross-reactivity (especially in polysensitized

patients), to assess unclear symptoms and unsatisfactory response to treatment, to reveal unexpected sensitizations, and to improve assessment of severity and risk aspects in some patients. Practical approaches, such as anamnesis molecular thinking, laboratory molecular thinking and postmolecular anamnesis, are sometimes applied. The component-resolved diagnosis of the specific IgE repertoire has a key impact on optimal decisions making for prophylactic and specific immunotherapeutic strategies tailored for the individual patient.

Key words: Singleplex; Multiplex; Immunoglobulin E sensitization; Aeroallergens; Immunoassays

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Core tip: Allergic respiratory diseases affect many people of all ages worldwide, showing increased prevalence, severity and complexity. New generation immunoassays using allergenic molecules represent a great precision medicine approach in research and clinical practice, allowing *in vitro* assessment of the immunoglobulin E (IgE) sensitization pattern at the molecular level, with favorable impact on allergy diagnosis and treatment, especially in selected patients with multiple aeroallergen sensitizations. The choice for a specific IgE immunoassay (singleplex, multiparameter or multiplex) for the allergenic extracts and molecular specificities, and the correct interpretation of the results, require optimal knowledge of the tests' methodologies and characteristics, and good clinical judgments.

Popescu FD, Vieru M. Precision medicine allergy immunoassay methods for assessing immunoglobulin E sensitization to aeroallergen molecules. *World J Methodol* 2018; 8(3): 17-36 Available from: URL: http://www.wjgnet.com/2222-0682/full/v8/i3/17.htm DOI: http://dx.doi.org/10.5662/wjm.v8.i3.17

INTRODUCTION

The precision medicine concept is both appealing and challenging[1] for chronic allergic airway diseases, including allergic rhinitis and asthma, which are a major and growing global health problem. In this regard, it is worth mentioning that chronic respiratory and allergic diseases affect over one billion people of all ages worldwide, with increasing prevalence and severity. Precision medicine represents a novel, modern approach to the management of some of these patients, embracing as key features: Personalized care based on molecular, immunologic and functional endotyping, considering predictive and preventive aspects, with participation of the patient in the decision-making process. Implementation of precision medicine into clinical practice may help to combat allergies and chronic airways diseases. Significant healthcare system changes are required to achieve that[1-5].

Assessing immunoglobulin E (IgE) sensitization to aeroallergens, in combination with detailed clinical history of the patients, represents the cornerstone for diagnosis of allergic airway diseases[5]. IgE sensitization and allergy, although very often correlated, are not always fully comparable. A positive IgE test result (IgE sensitization) is likely to correspond to a clinical reaction, but this cannot be considered universally valid because there are cases in which the clinical significance of some protein-IgE recognitions is not clear. Specific IgE, even in the absence of allergy, could be a risk factor for future clinical reactions or the memory of a previous allergic status[6,7]. International guidelines still indicate that clinical history and skin prick testing (SPT) are the first-level starting procedures of every allergy diagnosis ("top-down" approach). Specific IgE immunoassays with whole allergen extracts are considered a second-level diagnostic, and molecular allergy diagnosis a third-level one. Some authors suggest that a "bottom-up" diagnostic approach with wide IgE profiling based on allergen micro- or macroarray-based immunodiagnostics may also have advantages[8,9].

The methods usually applied in clinical practice to assess IgE-mediated sensitization to aeroallergens are skin prick tests and/or specific IgE immunoassays with allergen extracts. Skin tests represent the first diagnostic method in patients with a suggestive clinical history of allergic rhinitis/rhinoconjunctivitis and/or asthma[10]. SPT is a reliable method to diagnose IgE-mediated allergic disease in such patients[11]. There are European standards and North American practice parameters for a SPT panel and selection of key aeroallergens. Such extracts of plant, fungal and animal origin are used for the *in vivo* allergy assessment of patients with allergic rhinitis and asthma[5,11-13]. Intradermal skin tests are not useful for allergy diagnosis with aeroallergen extracts[10]. There are circumstances in which the *in vivo* and *in vitro* tests have their distinct advantages and limitations in the assessment process. In general, there is a good concordance between a positive skin test result and a positive blood test result for the most potent aeroallergens from house dust mites, cat and dog epithelia, and pollen of trees, grasses and weeds. Comparing evaluations of the two test methods mentioned, skin tests seem to be more sensitive (lower false-negative rate), while serum allergen-specific IgE immunoassays seem to be more specific (lower false-positive rate)[14,15].

The skin prick tests and specific IgE immunoassays confirm sensitization to a specific aeroallergen; however, the clinical relevance must be interpreted based on medical history and clinical symptoms. Positive results to skin tests or specific IgE assays do not mean that an allergen is causing symptoms, and the relevance of allergen exposure and its relation with symptoms must be confirmed by the patient's history[16]. Allergen provocation tests (such as local allergen challenge tests

or controlled exposure in allergen challenge chambers) can reproducibly confirm the clinical significance of a sensitized allergen, but may be difficult to perform and present limitations. Nasal and ocular challenges may be helpful as diagnostic tools for selected patients in clinical settings, and especially for research purposes[11,17,18]. Bronchial allergen challenge using an aerosol provocation system nebulizer or segmental allergen challenge using bronchoscopy are used only in research[19,20]. Few studies have assessed the basophil activation test (BAT) to determine the allergenicity of individual aeroallergens. Some researchers have concluded that BAT is not sensitive enough to be used for the routine diagnosis of individual pollen allergy, and they believe this may be due to a non-specific IgE cross-linking in the performance of BAT using CD63 expression[21]. In contrast, others have considered that BAT using CD203c expression is a reliable method in the diagnosis of pollen allergy[22]. It must be mentioned here that up-regulation of CD63 (lysosomal-associated membrane glycoprotein-3) is representative of anaphylactic degranulation, being expressed on the surface of degranulated basophils, while up-regulation of CD203c (glycosylated type Ⅱ transmembrane molecule constitutively expressed in low levels on basophil surface) may be associated with piecemeal degranulation[23].

IgE is the least abundant human antibody, with approximately half being found as free IgE in the intravascular compartment and the other half being bound to IgE receptors of a various cells, especially mast cells and basophils, *via* the high-affinity IgE receptor (FcεRI). Although free serum IgE has a short half-life of approximately 2 d, FcεRI-bound IgE persists for about 2 mo. While serum IgE immunoassay determination directly measures free IgE, SPT and BAT yield indirect information on mast cell- and basophil-bound IgE. Although all these methods offer qualitative diagnostic information, there are quantitative variations between the results, particularly due to different allergen sources and other methodological considerations[24]. The need for consistent quality is essential for immunology laboratories undertaking specific IgE antibody assays. External quality assessment is essential for approval by accreditation organizations[25].

Notwithstanding that the introduction of highly-purified natural and recombinant allergen molecular components represents an important improvement in the diagnosis of IgE sensitization to aeroallergens, the allergy skin testing cannot be completely replaced by molecular diagnosis in the near future. Besides costs and availability aspects, molecular allergy testing can be ordered by any physician; thus, patient selection and interpretation of results might not always be optimal. No allergy immunotherapy trial has yet shown efficacy in patients selected solely on the basis of molecular diagnosis. Moreover, molecular allergen treatment has still not been introduced in clinical practice[26]. Diagnostic molecular approaches are, however, currently revolutionizing the assessment of allergic patients[27]. Molecular allergen immunotherapy approaches have the potential to improve the treatment of allergic diseases and may be used as allergen-specific forms of secondary and eventually primary prevention for allergy[28].

Although it has been shown that it would be possible to use molecular allergen components instead of allergen extracts for skin prick or intradermal testing and for topical mucosal provocations such as nasal challenge, these methods are not available for routine clinical applications. Their use is important, however, in the development of new hypoallergenic allergen immunotherapies[29-31]. The number of published studies using *in vivo* testing with recombinant allergens has declined substantially over the past years, due to implementation of regulations prohibiting the approval of clinical studies with non-Good Manufacturing Practice produced recombinant allergens[29]. Instead, over the recent years, significant technological developments allowed the use of such allergenic components in the *in vitro* measurement of allergen-specific IgE[32]. Thus, molecular technology has changed the way that clinical laboratories diagnose IgE sensitization to allergens in respiratory allergies.

Precision medicine is a structural model aimed at customizing healthcare, with medical products/decisions tailored to the individual patient at a highly detailed level. Precision medicine allergy immunoassays support the molecular-based allergy diagnosis. They also allow the accurate definition of the IgE sensitization profile of the patient (*i.e.,* the patient's IgE repertoire). Molecular-based allergy diagnosis, also known as "component resolved diagnostics", is a patient IgE sensitization *in vitro* diagnostic approach at the molecular level using allergenic molecules, also referred to as "allergen components". Two types of molecular allergen components are used in current immunoassays[33,34]: (1) Well-defined highly purified natural allergens (isolated and purified from natural allergen sources); and (2) Recombinant allergens (produced by recombinant DNA technology). The successful sequencing of the first allergen-encoding DNA kickstarted the era of molecular allergy diagnostics 30 years ago[35,36].

Allergen molecules or allergen components are highly defined, and purified proteins from a given allergen source. These molecular allergens in their native or recombinant forms are typically homogeneous, and have comprehensive quality control. By contrast, allergen extracts are crude, heterogenous, unfractionated mixtures of many allergenic and nonallergenic proteins, polysaccharides and lipids obtained by extraction from an allergen source. Even if they are less expensive, due to easier preparation, such natural extracts are difficult to standardize by detailing their composition and allergenic potency. Moreover, the protein mixture complexity is a factor for low

Table 1 Classification of immunoassays used for the detection of specific immunoglobulin E against allergen components[9,40,41]

Type of immunoassay	Description
Singleplex	Detect specific IgE against a single allergen component
Multiparameter	Detect specific IgE against a few allergen components at once (usually - 10)
Multiplex	Detect simultaneously specific IgE against many different allergen components (> 100)

IgE: Immunoglobulin E.

specificity, while endogenous degradation is a risk for low sensitivity[9].

Regarding the preparation of allergen components, natural allergens are purified from different allergen sources (such as pollen grains or house dust mites) by chromatographic techniques. Recombinant allergens are mainly produced in prokaryotic expression systems (*Escherichia coli*), with several exceptions produced in eukaryotic systems (the yeast *Pichia pastoris*). Only few allergen molecules are produced under Good Manufacturing Practice conditions and are considered biologic reference preparations, due to their comprehensive characterization by physicochemical and immunological methods. Detailed physicochemical characterization includes protein identification and amino acid sequencing by mass spectrometry-based methods as well as quantification determined by amino acid analysis using reversed-phase high-performance liquid chromatography. Homogeneity is assessed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis silver-stained, isoelectric focusing Coomassie-stained and immunoblotting experiments, while folding and denaturation analysis by far-ultraviolet circular dichroism and Fourier-transform infrared spectroscopy. High-performance size-exclusion chromatography and dynamic light scattering are used for aggregation behavior and stability in solution. Biological activity is assessed *in vitro* by BAT, with up-regulation of CD203c, and by enzyme-linked immunosorbent assay (ELISA) inhibition for batch-to-batch consistency regarding allergenic potency[37-39].

Immunoassays used for the detection of specific IgE to allergen components are either singleplex, multiparameter or multiplex assays (Table 1), according to the number of allergen extracts and molecular components used[9,40,41].

SINGLEPLEX IMMUNOASSAYS FOR SPECIFIC IgE TO ALLERGEN COMPONENTS

Molecular-based singleplex specific IgE immunoassays refer to laboratory methods in which one analyte is measured per analysis. They are designed to detect and measure circulating IgE antibodies that can bind to one specific allergen or molecular component[9,42]. The basic chemistry of such *in vitro* IgE assays has remained essentially unchanged since their introduction more than 40 years ago. After serum specific IgE binding to solid- or liquid-phase allergen, bound IgE antibody is detected with a labeled anti-IgE reagent, with these reactions' incubation periods being separated by buffer washes[43,44].

Historically, the first generation solid-phase isotopic immunoassay is the radioallergosorbent test (RAST), with allergens covalently coupled to a filter paper disc allergosorbent (solid-phase with low surface area), radioiodinated polyclonal anti-human IgE used as signal detection antibody, two overnight incubations needed, and bound radioactivity quantified in a gamma counter[24,44]. This first *in vitro* qualitative test for specific IgE-antibodies introduced in 1973 was used as a manual method for detection of serum specific IgE against various allergen extracts, but not against molecular components. It has been replaced by nonisotopic, more sensitive immunoassays, which are in use currently; thus, the term RAST should be abandoned when referring to these *in vitro* IgE testing methods[45,46]. Automation of new generation, US Food and Drug Administration (FDA)-cleared, clinically used immunoassays (such as ImmunoCAP® and Immulite® systems, which now hold the largest share of the global market) have optimized precision, reproducibility, and linearity to a performance standard of less than 15% coefficients of variation. These new generation immunoassays must report comparable analytical sensitivity and calibration schemes traceable to the World Health Organization (WHO) IgE international standard. Interassay differences may exist, and have been attributed particularly to the differences in composition of the allergen extract-based reagents used and details of the calibration systems[44].

Modern singleplex immunoassays used to determine allergen-specific IgE antibodies comprise the following components[24]: (1) Allergen-containing reagent: Solid-phase allergosorbent or liquid-phase labeled allergen; (2) Reaction compartment: Plastic capsule reserve with cellulose polymer, plastic reaction tube with dispensed assay-specific polystyrene bead, plastic microtiter plate with wells; (3) Human serum with specific IgE antibodies and negative serum controls; (4) Anti-IgE detection reagent: Monoclonal antibody specific to the constant Fc fragment of human IgE; and (5) Calibration and data processing systems.

The total calibration curve used in most immunoassay systems nowadays is linked to the WHO IgE standard and reported in arbitrary units, kU$_A$/L kilo mass units of allergen-specific antibody per unit volume of

sample, where the "$_A$" stands for allergen-specific. These are distinguished from internationally standardized units IU/mL or kU/L for total IgE measurement (1 kU$_A$/L = 0.994 kU/L), introduced to express the level of IgE in peripheral blood to alleviate the inconvenience in expressing the very low levels of serum IgE. The reference curve calibrated to the official WHO standard for total IgE is generated following each immunoassay run according to manufacturers' specifications. The measurement signals obtained for allergen-specific IgE are converted into corresponding units (kU$_A$/L) with the help of total IgE reference curve as heterologous calibration[24].

Performance specifications for immunoassays are established for several characteristics, including the reportable range. Accuracy means trueness assessed by comparison-of-methods studies, while precision refers to the standard deviation or coefficient of variation (CV) estimated by replication studies. Analytical specificity is the ability to detect IgE, not antibodies of other classes, *via* interference studies, while analytical sensitivity, the limit-of-detection studies. Allergen molecules allow improved analytical specificity (selectivity) by binding a partial amount of the specific IgE repertoire. Analytical sensitivity is often improved (the lower Limit of Quantitation, commonly referred to as LoQ) when using allergen molecules, particularly if these are under-represented in the natural extracts or even entirely absent due to their instability. LoQ itself is the lowest concentration of specific IgE antibodies that can be reliably detected within a predefined precision. LoQ may be equivalent to or higher than the Limit of Detection (LoD), which refers to the weakest signal or lowest concentration of specific IgE antibodies reliably determined from the test, calculated using the Limit of Blank (the signal of a serum sample without allergen-specific IgE)[9,24,47,48].

Specific IgE immunoassays report results in a class system (classes 0-6) based on the amount of detected serum specific IgE. The higher the allergen-specific IgE level, the greater the likelihood of a patient to suffer from allergic symptoms caused by the exposure to the sensitizing allergen. These randomly assigned classes have evolved over time, to semiquantitatively and broadly categorize serum IgE concentrations. However, this class system has become obsolete with the quantitative reporting of specific IgE using kU$_A$/L[15,24]. The lower detection threshold for specific IgE determination was formerly 0.35 kU$_A$/L. Presence of specific IgE against a particular allergen above this level is deemed positive for that allergen, and a positive test (a level $\geqslant$ 0.35 kU$_A$/L) for aeroallergens generally correlates well with the clinical expression[49,50]. The sensitivity of new generation specific IgE immunoassays is now higher due to more sensitive calibration and improved resolution of low IgE values, being able to provide values below 0.35 kU$_A$/L, until down to 0.1 kU$_A$/L. This range is particularly informative and relevant when total IgE is extremely low (< 20 kU/L). Thus, the ratio of specific IgE to total IgE (referred to as antibody-specific activity) is particularly important in this case. The upper detection limit is 100 kU$_A$/L for most specific IgE immunoassays. Serum samples with higher specific IgE should be measured in diluted forms (1:10) in order to determine the actual value by multiplying with 10. Specific IgE/total IgE ratio is also important in samples with very high total IgE levels (> 1000 kU/L)[24,51]. Complete concordance between specific IgE immunoassays and SPT cannot be expected. An *in vitro* assay measures circulating allergen-specific IgE antibodies, whereas skin testing assesses cutaneous mast cell reactivity based on assumed cell-bound specific IgE[52].

The currently available singleplex immunoassays aim to determine serum specific IgE to molecular allergen components using either solid-phase coupled allergens (*i.e.,* fluorescence enzyme immunoassay) or liquid-phase allergens *(i.e.,* chemiluminescence immunoassay and reversed enzyme allergosorbent test). Advantages of singleplex assays for allergen-specific IgE testing with allergenic molecules/components, when compared with multiplex technology, include increased assay analytical sensitivity (lower LoQ) and greater sensitivity at low specific IgE levels. In addition, they have similar units for total IgE and allergen-specific IgE, due to heterologous calibration allowing calculation of allergen-specific IgE/total IgE-ratio, as well as more established quality control measures[9,33].

The fluorescence enzyme immunoassay (FEIA) with capsulated cellulose polymer solid-phase (Immuno CAP®) coupled allergens is currently used to measure specific IgE antibodies to many allergen extracts (> 650) and 105 individual molecular allergens, in serum or plasma. Introduced as a second generation immunoassay in 1989, this ImmunoCAP technology has advantages regarding sensitivity and efficiency. Later generations of ImmunoCAP® specific IgE Phadia ™ instruments (Thermo Fisher Scientific Inc., Phadia AB, Uppsala, Sweden) with full automation, using the same test principle, brought further improvements in precision and reproducibility, more rapid procedure (100 min), higher capacity and continuous random access ability availability. Based on sandwich fluoro-enzyme immunoassay method, ImmunoCAP FEIA offers the opportunity to assess the patient's allergic sensitization profile not only for natural extracts but also at molecular level. It has several steps[42,44,53-55]: (1) Specific IgE binding to solid-phase step: Native purified or recombinant allergen component covalently coupled to a flexible solid-phase, with a large surface area, a highly branched, hydrophilic cellulose CNBr-activated polymer/sponge encased in a capsule or capsulated carrier polymer (ImmunoCAP with 1-2 μg allergen), reacts with the specific IgE from the patient plasma/serum sample; (2) Conjugate/labeled anti-

IgE detection antibody step: After washing away unbound antibodies, β-galactosidase-labeled anti-IgE mouse monoclonal antibody is added to form the so-called antigen-antibody immune complex; and (3) Fluorescent signal step: After the unbound enzyme-anti-IgE is washed away, 4-methlyumbelliferyl-β-galactoside is used as a fluorogenic substrate, incubated with the bound complex to produce the fluorescent 4-methylumbelliferone. After stopping the reaction, the fluorescence measurement of the eluate is performed with a fluorocounter, and there is a correlation between fluorescence and the allergen-bound IgE established from a standard curve of concentration points.

The ImmunoCAP FEIA quantitative method delivers accurate results, as studies using mouse-human chimeric IgE antibodies to allergens have revealed[56,57]. ImmunoCAP immunoassay needs only 40 μL serum or plasma per test, and the intra-assay precision is as good as standard clinical chemistry assays. This is important and should be emphasized considering the extremely low serum concentrations of IgE antibodies (μg/L). Moreover, each native allergen contains many protein components that may provoke an IgE antibody response, and there are possible interferences with immunoglobulins from other classes[58]. Low CV (10%) translates into fewer replicates, avoiding unnecessary reruns and assay delays. LoQ for ImmunoCAP specific IgE is 0.1 kU$_A$/L. The detection limits are 0.10-100 kU$_A$/L[44,55]. In the ImmunoCAP system, 1 kU$_A$/L specific IgE represents 0.994 kU/L total IgE, and is equal to 2.4 ng/mL specific IgE. Despite this good conversion from kU$_A$/L to ng/mL, it must be stressed that interlaboratory CVs were observed for both units of measurement. The conversion ratios have not been established with other immunoassay systems. Results from different specific IgE systems are not always comparable to each other even if they are provided in same units[15,59]. Despite methodological differences, results obtained with ImmunoCAP solid-phase immunoassay and Immulite liquid-phase allergens immunoassay for specific IgE against molecular allergens are similar[60], but such results are not interchangeable by means of mathematical conversion[57].

The ImmunoCAP specific IgE classes are defined using six calibrators: 0, 0.35, 0.7, 3.5, 17.5 and 100 kU$_A$/L (Class 0: from 0 to < 0.35 kU$_A$/L; Class 1: from 0.35 to < 0.7 kU$_A$/L; Class 2: from 0.70 to < 3.5 kU$_A$/L; Class 3: from 3.50 to < 17.5 kU$_A$/L; Class 4: from 17.5 to < 50 kU$_A$/L; Class 5: from 50 to < 100 kU$_A$/L; and Class 6: from ⩾ 100 kU$_A$/L)[55].

The enzyme-enhanced chemiluminescence immunoassay with liquid-phase allergens is another advanced singleplex detection method that exploits liquid-phase kinetics in a bead format (3gAllergy™ Immulite® 2000 and Immulite® 2000 XPi immunoassay; Siemens Healthcare Diagnostics Inc., Erlangen, Germany). It is considered as a third generation assay to measure serum specific IgE antibodies against various allergen extracts and 21 individual molecular allergens. This new generation automated liquid-phase immunoassay was introduced in 2003. The use of fluid-phase allergens allows rapid binding kinetics between IgE and the allergenic protein conformations, and a time-to-first-result of only 65 min, while enzyme-enhanced chemiluminescence is used for optimal accuracy[43,55]. This automated quantitative chemiluminescent method can also be used in molecular allergy diagnostics. It has the following steps[42,53,61]: (1) Specific IgE binding in liquid-phase step: Native purified allergen component covalently bound to soluble biotinylated polylysine polymer in a fluid phase binds to streptavidin-coated polystyrene bead (as solid-phase) in the reaction tube (through a streptavidin-biotin interaction) and reacts with specific IgE from the patient's serum sample (during the incubation of streptavidin-coated bead, biotinylated liquid allergen, and patient sample); (2) Conjugate/labeled anti-IgE detection antibody step: After spin washing, alkaline phosphatase enzyme-labeled anti-human IgE monoclonal murine antibody is added to form the so-called antigen-antibody immune complex; (3) Chemiluminescent signal step: After the bead is washed again (efficient washing with spinning at high speed of the tube on its vertical axis), adamantyl 1,2-dioxetane aryl phosphate is added as chemiluminescent substrate. In the luminogenic reaction, the action of bound alkaline phosphatase on this stable substrate creates an unstable adamantly dioxetane anion, with its rapidly and spontaneously breakdown emitting a photon of light; and (4) Chemiluminescence measurement: Performed by a photon-counting photomultiplier tube/luminometer, and there is a correlation between the chemiluminescent signal and the allergen-bound IgE established from a standard curve of concentration points.

Defining features of such a third generation immunoassay include a true zero calibrator with a detection limit of 0.1 kU$_A$/L and functional sensitivity of 0.2 kU/L. The detection limits are 0.10-100 kU$_A$/L, and the sample volume is 50 μL. A high diagnostic accuracy of the specific IgE to allergen components measurement with this Immulite® 2000 system and a high agreement with ImmunoCAP® platforms were revealed[61]. An important difference between these methods is the source and quality of the allergenic extracts used[53]. Results of *in vitro* assessments for IgE sensitization using both new generation ImmunoCAP® and Immulite® systems show substantial correlation with respect to serum specific IgE detection for common aeroallergens. However, the results are not interchangeable. Although these two singleplex FDA-cleared assays have the same basic reaction sequence for IgE detection and report the results using the same units, methodological differences are important regarding allergen binding methods, signal detection methods (amplified chemiluminescence used in Immulite *vs* fluorescence in ImmunoCAP) and

test running time (reduced from 100 min to 65 min).

The Immulite standard classification system uses eight calibrators (Class 0: from 0 to < 0.35 kU$_A$/L, but with the possibility to detect values from 0.1 to 0.35 kU$_A$/L; Class 1: from 0.35 to < 0.7 kU$_A$/L; Class 2: from 0.70 to < 3.5 kU$_A$/L; Class 3: from 3.50 to < 17.5 kU$_A$/L; Class 4: from 17.5 to < 52.5 kU$_A$/L; Class 5: from 52.5 to < 100 kU$_A$/L; and Class 6: from ⩾ 100 kU$_A$/L)[55].

The reversed enzyme allergosorbent test (REAST) with liquid-phase allergens (Allerg-O-Liq™; Dr. Fooke-Achterrath Laboratorien GmbH, Neuss, Germany) is a reliable singleplex immunoassay using microwells, based on a sandwich ELISA, for the quantitative determination of specific IgE antibodies against about 500 allergen extracts and 50 individual highly-purified native and recombinant allergen components, in serum or plasma. Determination of specific and total IgE is possible in the same test run, with high sensitivity and specificity. Total of the incubation times are 3 h for the manual procedures. Fifty microliters of undiluted calibrators, controls and patient samples are needed, to be pipetted into wells. Fully automated microplate procedure for REAST is possible. It has low variations between different instruments and between manipulators. REAST Allerg-O-Liq has several steps[24,62-64]: (1) IgE binding to solid-phase step: All serum IgE antibodies are bound by immobilized anti-human IgE antibodies coating the microwells (microtiter plates); (2) Fluid-phase allergen binging step: After washing away unbound antibodies, biotinylated allergen is incubated in the microwells; (3) Detection conjugate step: After another washing procedure, the added horseradish peroxidase (commonly referred to as HRP)-conjugate forms a complex consisting of specific IgE/bound allergen/HRP-conjugate; (4) Chromogenic substrate step: After further well washing, the substrate 3,3', 5,5'-tetramethylbenzidine (commonly known as TMB) is added for colorimetric detection, resulting in the development of a blue color, and after stopping the enzymatic reaction with acid, the color changes to yellow; and (5) Optical density of the colored product is measured by spectrophotometry at 450 nm (reference wave length of 620 nm), with the specific IgE concentration of the patient sample being proportional to the optical density.

Calibrators with defined concentrations of IgE are assayed simultaneously with the patient samples to generate a calibration curve. IgE concentrations are calculated from this curve. This REAST immunoassay detects specific IgE concentrations between 0.35 IU/mL and 100 IU/mL. Specimens with higher specific IgE concentrations should be diluted and retested to determine the exact content. A level of specific IgE < 0.35 IU/mL is rated as class 0, ⩾ 0.35 to < 0.7 IU/mL as class 1, ⩾ 0.7 to < 3.5 IU/mL as class 2, ⩾ 3.5 to < 17.5 IU/mL as class 3, ⩾ 17.5 to < 50 IU/mL as class 4, ⩾ 50 to < 100 IU/mL as class 5, and ⩾ 100 IU/mL as class 6. Test with a level of specific IgE ⩾ 0.35 IU/mL is defined as positive. Although no single method has been officially designated as the gold standard for specific IgE detection, the worldwide spread ImmunoCAP® assay is commonly used for comparisons. The agreement between this FEIA and the Allerg-O-Liq method is good to excellent[62,64,65].

The chemiluminescence reverse sandwich immunoassay with liquid-phase allergens performed on the Advia Centaur® analyzer (Bayer HealthCare Diagnostics Division, Tarrytown, New York, United States) was also used as a fully automated, quantitative specific IgE *in vitro* test, using a calibration method based on a recombinant reference allergen. Monoclonal mouse anti-human IgE antibody covalently bound to paramagnetic particles in the solid-phase captures the sample specific IgE. Bound specific IgE reacts with liquid biotinylated allergen, which is detected as chemiluminescence using acridinium ester-labeled streptavidin. Specific IgE was considered positive at ⩾ 0.35 kU$_A$/L. This immunoassay for determination of specific IgE to allergen components performed with good reproducibility. It also correlated well with the ImmunoCAP® system. A good agreement and correlation for some allergen components with the ImmunoCAP Immuno Solid-phase Allergen Chip (ISAC) microarray was revealed[66-68].

MULTIPARAMETER IMMUNOASSAYS FOR SPECIFIC IGE TO ALLERGEN COMPONENTS

A multiallergen screen is not considered a true multiplex immunoassay despite the fact that it detects IgE antibodies to more individual allergens with a single serum addition[9]. Several immunoassays for the *in vitro* assessment of IgE sensitization to allergen molecules are considered multiparameter tests because they detect specific IgE against few allergen components at once, usually about 10 (2-11 recombinant or native molecules), along with specific IgE against several natural aeroallergen extracts.

The multiparameter line blot immunoassay for defined partial allergen diagnostics with purified, biochemically-characterized allergen components coating membrane strips in thin parallel lines as line blots (Euroline™; EUROIMMUN AG, Lübeck, Germany) is used to measure simultaneously (on one test strip) specific IgE antibodies against few allergen extracts and several individual molecular allergens. This *in vitro* diagnosis with defined, in part recombinant partial allergens involves one panel with two pollen extracts plus eight pollen components, and another with one mold and five pollen extracts plus one mold and ten pollen components). Such a component-resolved multiparameter assay, based on immunoblot technology, uses defined proteins as single purified allergen components for IgE antibody detection along with whole

raw allergen extracts. It has several steps[40,41,69,70]: (1) Specific IgE binding to solid-phase step: Recombinant or native purified allergen component coupled to blot strips as thin parallel lines at defined positions on the moistened solid-phase binds to specific IgE patient serum/plasma sample; (2) Conjugate/labeled anti-IgE detection antibody step: After washing away unbound antibodies, alkaline phosphatase enzyme-labeled mouse anti-human IgE monoclonal antibody is added to form the so-called antigen-antibody immune complex; and (3) Chromogenic substrate step: bound antibodies are stained with nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) for colorimetric detection; scanning is performed on the completely dried membrane strip line using a computer-linked flatbed scanner.

The Euroline immunoassay is a semiquantitative method. The incubation protocol includes an undiluted serum sample (0.4 mL) 60-min incubation, a 60-min incubation with conjugate and a 10-min incubation with substrate, all performed in a blot strip incubation channel on a rocking shaker and separated by washing procedures. The measurement range is given in the enzyme-allergo-sorbent test (EAST) system, in classes from 0-6, the detection limits being 0.35–100 kU_A/L. With respect to the concentration grades, the EAST is similar to the previously mentioned ImmunoCAP specific IgE classes: Class 1 (very low antibody titer, frequently no clinical symptoms where sensitization is present); Class 2 (low antibody titer, existing sensitization, frequently with clinical symptoms in the upper range of class); Class 3 (significant antibody titer, clinical symptoms usually present); Class 4 (high antibody titer, almost always with clinical symptoms); and Classes 5 and 6 (very high antibody titers). Introduced in clinical practice as a reliable and costly efficient *in vitro* specific IgE test in the molecular diagnosis of tree and grass pollen allergy, EAST classes of Euroline blot assay have acceptable correlation with ImmunoCAP[41,69,70].

A similar manual or automated multiparameter technology-based enzyme immunoassay is performed with allergen extracts and components coated separately in lines on a carrier membrane fixed in the well of a cassette (Polycheck®; Biocheck GmbH, Münster, Germany). There are two panels containing recombinant allergens, one with 20 allergen extracts plus 2 recombinant pollen allergen components, and another with 2 aeroallergen extracts with 4 allergen components. Only 200 μL of patient sera is needed. Anti-IgE antibody is the monoclonal murine antibody labeled with ligand, the enzyme-labeled anti-ligand is a ligand conjugated to alkaline phosphatase, and the substrate solution is also NBT/BCIP. Duration of the immunoassay in manual procedure is 2.5 h. The data of specific IgE serum levels presented in categorical (class) form range from class 0-6 according to the calibration curve[71-73].

MULTIPLEX IMMUNOASSAYS FOR SPECIFIC IgE TO ALLERGEN COMPONENTS

Multiplex specific IgE immunoassays allow the characterization of IgE sensitization repertoire against a broad array of preselected allergens (more than 100 allergens from various allergen sources), independently of the clinical history[9,36].

The most important advantages of multiplex assays for allergen-specific IgE testing are the provision of an extended panel of specific and cross-reactive allergen molecules (widest allergen spectrum for *in vitro* allergen testing) for a broad IgE sensitization profiling, especially suited for patients with complex sensitization pattern or symptoms. Critical benefits include distinguishing genuine sensitization from sensitization due to cross-reactivity, assessing biomarkers for allergy phenotypes and allergen immunotherapy, and software interpretation of results. Other advantages over singleplex assays include conservation of sample volume, optimized costs due to fewer required reagents, and increased speed of analysis of the specific IgE repertoire[9,33,36,74].

Multiplex allergen microarray technology has been applied recently to the field of *in vitro* allergy diagnosis, being considered an extremely useful advanced diagnostic approach[75]. The first report of an allergen chip-based microarray technology was published in 2002[76], and the chemistry of this immunoassay (called the ISAC™) was patterned. Ten years of rapid development, resulting in this first generation multiplex allergy test, increased the number of allergenic molecules from 74 to 112. Additional multiplex/oligoplex IgE immunoassays were used in the clinical setting or in research development strategies. Moreover, after the first generation of microarray-based tests, in 2016 and 2017 two new macroarray nanotechnology-based immunodiagnostics tools were launched. They captured the interest of allergists due to their direct impact on the management of patients with allergies in the context of a precision medicine approach[7,32,75].

Multiplex microarray-based immunoassays

A manual allergen microarray-based immunoassay on polymer-coated slide as solid-phase, the Immuno CAP® ISAC™ (Thermo Fisher Scientific Inc., Phadia AB, Uppsala Sweden), is an European Conformity-marked (CE-marked), miniaturized multiplex *in vitro* molecular diagnostic test. It enables simultaneous measurement of specific IgE antibodies against a fixed selection of 112 recombinant or purified native allergen components, derived from over 51 allergen sources (2011 version), from a 30 μL of serum, plasma or capillary blood. Because the ISAC panel holds more than 100 allergen components and no allergen extract, it is a good mul-

tiplex tool for a detailed exclusively molecular IgE profile assessment of patients[36,77]. ISAC is a multiplex immunoassay with two reaction steps[32,54,78,79]: (1) Specific IgE binding to solid-phase step: IgE antibodies from the patient sample bind to immobilized multiple allergen components spotted in triplicate onto a pre-activated amine reactive polymer-coated glass slide as solid-phase (100 pg allergen are immobilized on a single spot of the chip, spot size being 200 μm; each glass slide contains four microarrays giving results for four samples per slide); (2) Conjugate/labeled anti-IgE antibody binding step: After the nonspecific antibodies are washed away, fluorophore-labeled anti-human IgE monoclonal antibodies are added to promote the IgE-allergen complex formation; and (3) Fluorescence measuring and image processing: Performed after unbound labeled antibodies are removed by washing, using a confocal laser microarray scanner and microarray image analysis software.

ISAC test results are analyzed with microarray image analysis software and reported in arbitrary ISAC standardized units for IgE (ISU-E). The operating range is 0.3-100 ISU-E. This range is about the same as a concentration range of 0.3-100 kU$_A$/L (1 kU$_A$/L is equal to 2.4 ng/mL). The calibration curve is adjusted to approximately match the units in the ImmunoCAP singleplex method (kU$_A$/L). ISU-E measurements are assigned to one of four categories on a semiquantitative scale, ranging from undetectable or very low (< 0.3 ISU-E) to low (≥ 0.3 to < 1 ISU-E), moderate to high (≥ 1 to < 15 ISU-E) and very high (≥ 15 ISU-E). This miniaturized platform provides a relatively rapid and efficient means of assessing IgE sensitizations to a broad panel of allergen components. The immunoassay is performed in 4 h[80,81]. In addition to the integrated Xplain® software (Thermo Fisher Scientific Inc., Phadia AB), the AllerGenius® expert system (ARMIA, Genova, Italy) was developed to support the interpretation of allergy tests obtained with the ISAC microarray technology[82]. The average CV for all allergens tested in intra- and interassay comparisons is below 20%. The LoD of 0.3 ISU-E is assumed for all allergens tested. High concentrations of total IgE have no effect on test performance. The kinetics of ISAC 112 ensure that high-affinity specific IgE is preferentially bound, whereas the kinetics generated by the large excess of allergen in the ImmunoCAP singleplex assay allow binding of low-affinity specific IgE. The overall assay sensitivity (LoD and LoQ) of ISAC 112 is to be considered lower than that of the ImmunoCAP singleplex method. Although there are different epitope exposure on the solid-phase of the assay, due to immobilization on the polymer coating of the glass ISAC chips and covalent binding of allergens to the cellulose matrix in the ImmunoCAP® system, a good to very good correlation of the ISU-E values with the ImmunoCAP-derived values (kU$_A$/L) is noted for the majority of aeroallergens[36]. Furthermore, the ISAC microarray platform has revealed comparable results to the traditional singleplex method ImmunoCAP and SPT[83].

The Mechanisms for the Development of Allergies (MeDALL) allergen chip is a version of the ISAC platform developed for the European Union-funded project MeDALL. It is a research tool containing an expanded repertoire of 170 allergen molecules used for the sensitive detection of allergen-specific IgE against natural purified and recombinant allergen components. For each allergen protein, 50–200 fg, corresponding to 1–5 attomol, is spotted in triplicates onto the MeDALL chip, as compared to ImmunoCAP, in which one spot of the microarray contains 10000000 times less protein, influencing the microarray saturation levels[32,84].

Additional multiplex/oligoplex IgE immunoassays applied in the clinical setting or in development

An automated allergen microarray-based immunoassay on Microtest chip (Microtest Diagnostics, Worthing, United Kingdom) is a CE-marked, miniaturized *in vitro* diagnostic test to measure specific antibodies to allergen extracts and allergen components at the same time. The allergens used in the Microtest microarray can be customized. In contrast to the ISAC, it uses 100 μL of serum and may employ 19 allergen extracts and 16 allergenic molecules covering a total of 26 allergen specificities, according to published data, representing a simplified version of a proof-of-concept assay that used 95 allergen extracts and 8 recombinant proteins on aldehyde-activated glass microscope slides[9,85]. Allergen molecules are immobilized covalently in triplicate or more onto a precoated slide as solid-phase in the form of a matrix with more than 150 spots, to which bind the specific IgE antibodies from serum or plasma. Each slide contains one matrix microarray. Up to five microarrays can be assayed at the same time on the platform. A HRP-conjugated antibody detects the IgE-allergen complex, and a detection solution is used to develop the fluorescent signal assessed by a fluorimeter. The procedure is reported to take about 4 h. Results are reported in specific IgE classes (Class 0: < 0.35 kU$_A$/L; Class 1: 0.35-1 kU$_A$/L; Class 2: 1.01-15 kU$_A$/L; Class 3: > 15 kU$_A$/L). The Microtest allergen panel is much simpler than the ISAC panel but appears to give comparable results for specific IgE against some prevalent aeroallergens[81,83].

A different fluorescence covalent microbead immunosorbent assay was assessed as a Luminex bead-based suspension array technology test. It allowed the simultaneous quantitative detection of serum total and allergen-specific IgE against six natural purified and recombinant aeroallergen molecules. Multianalyte profiling fluorescent bead microspheres coupled with allergen molecules were used to bind antibodies from human serum and, once bound, the IgE antibody was subsequently detected with a biotinylated anti-human IgE and streptavidin-conjugated phycoerythrin,

fluorescence being measured by a multiplex microplate reader. The analytes were measured with very good interassay reproducibility, from a total serum volume of less than 20 μL and in 6 h. Total and allergen-specific IgE levels correlated with enzyme-linked and fluorescent enzyme immunoassay results[32,86].

A point-of-care fully automated fluorescence-based sandwich nanofluidic immunoassay is available in a disposable capsule containing nanofluidic biosensors with immobilized allergens for *in vitro* diagnostics (IVD Capsule Aeroallergens; Abionic SA, Biopôle, Switzerland). It is used for the quantitative determination of total IgE and specific IgE to five key aeroallergen components using the abioSCOPE® clinical analyzer (Abionic SA). A 50 μL capillary blood sample with specific IgE mixed with a solution composed of fluorescently labeled anti-IgE antibody to form a complex is drawn through a capsule by capillary action. Using diffusion phenomena, molecules interact together in the biosensors and form molecular complexes in case of specificity. IgE-anti-IgE antibody complex is bound by capsule allergens immobilized within the read-out area. The surface of each biosensor contains specific immobilized allergens (either from natural sources or purified allergens). The capsule with aeroallergens is placed into a disc mounting plate, which is then inserted into the abioSCOPE. These fluorescent immobilized complexes are optically measured by this reading unit, which contains a miniaturized fluorescent microscope. The measured fluorescence signal is reported in kU$_A$/L, according to the six classes: < 0.7 kU$_A$/L (absent, low or undetectable level of allergen-specific IgE); 0.7-3.4 kU$_A$/L (moderate level of allergen-specific IgE); 3.5-17.5 kU$_A$/L (high level of allergen-specific IgE); 17.6-50 kU$_A$/L (very high level of allergen-specific IgE); 51-100 kU$_A$/L (ultra-high level of allergen-specific IgE); > 100 kU$_A$/L (extremely high level of allergen-specific IgE). A good agreement was noted between the allergen-specific IgE values measured in ImmunoCAP Phadia 250 and in the abioSCOPE. Nanofluidic-based biosensor containing a nanochannel that accelerates molecular interactions reduces the incubation duration and the immunoassay time to a matter of minutes[36,87,88]. Such a new disrupter nanotechnology-based diagnostic approach provides healthcare professionals with tools that help them to make a rapid point-of-care diagnosis.

New generation multiplex nanotechnology-based immunoassays

Protein arrays constitute a powerful tool for multiplexed protein analysis. Multiplex tests allow to detect specific IgE to many different preparations at once, assessing a patient's IgE sensitization profile and allowing for tailoring of decisions for interventions[89]. The terms "microarray" and "macroarray" may be used to differentiate between spot size and the number of spots on the support. The ISAC microarray single spot size of the chip is 200 μm[78]. The term macroarray is usually used for the larger spot array. Multiplex macroarray- and nanotechnology-based immunoassays make available for the allergist an unprecedented quantity of data, which is very useful to explore polysensitized patients and to disclose unknown sensitizations. They offer, nowadays, the widest possible knowledge of the patient's IgE sensitization profile[75,89,90].

The patient-friendly allergen nano-bead array (FABER®; MacroArray Diagnostics, Vienna, Austria, in collaboration with Centri Associati di Allergologia Molecolare-CAAM, Rome, Lazio, Italy, and its partners) is a new advanced multiplexed nanotechnology-based *in vitro* immunoassay for specific IgE measurement, having 122 molecular allergens and 122 allergenic extracts (FABER 244), coupled to chemically activated nanoparticles. The large majority of aeroallergens are represented. The inclusion of allergenic extracts is strategic to confirm or complement results obtained with the single allergenic molecules. Allergenic preparations (either produced in-house or obtained from commercial providers) are individually coupled to nano-beads by means of optimized protocols in order to achieve maximum test performance and provide high diagnostic accuracy for each spotted allergenic item. Once coupled, they are arrayed to a solid-phase matrix to form a one-step comprehensive array-based testing approach using 100 μL of patient serum or plasma. FABER results expressed in arbitrary units (FIU) are considered negative ($\leqslant$ 0.01 FIU/mL), doubtful (> 0.01 to < 0.30 FIU/mL) or positive ($\geqslant$ 0.30 FIU/mL). Test interpretation is supported by a center digital reporting system (an online dynamic visualization system). It provides real-time information and easier understanding of the test results, as a patient-friendly, multilanguage tool accessible from personal computers and mobile devices. The test is exclusively available from CAAM partner laboratory (Sermolab S.r.l., Italy), so the patients have to wait to get the results. FABER IgE measurements perform very well with most allergens, but improving the quality of some extracts will lead to better performances. FABER, ImmunoCAP® and Immulite® systems-having different reference standards-do not completely overlap each other[7,89, 91,92].

The macroarray nanotechnology-based immunoassay used as a molecular allergy explorer (ALEX®; MacroArray Diagnostics) is the latest launched *in vitro* multiplex tool for precision medicine in allergy diagnosis. It is based on a state-of-the-art proprietary nano-bead technology. This new array contains 282 allergen reagents (157 allergenic extracts and 125 molecular components), with a large majority of aeroallergen families and cross-reactive food allergens being represented. This *in vitro* allergy explorer is the first *in vitro* multiplex allergy test allowing simultaneous measurement of total IgE and specific IgE against a plethora of allergen extracts and molecular allergens. The combination of second- and third-level assays in the same immunoassay allows to define the presence of IgE sensitization, whether it is

genuine or cross-reactive, and saves time and costs, particularly in polysensitized patients and/or with pollen-food syndromes[75,93].

The ALEX® *in vitro* allergy test core technology is based on a two-phased manufacturing process and it represents a multiplex ELISA-based test with proven immunoassay chemistry and detection methods, as discussed below[75]. Initially, allergens are coupled to activated nanoparticles, for coupling individual and combinatorial optimization. Each allergen is attached reflecting its biochemical properties and specific requirements for stability, thereby preserving the full epitope complexity. The nanoparticles multiply the surface of the solid-phase presenting the allergen during the immunoassay, enabling highly sensitive detection. In the next step, the allergen-bearing nanoparticles are deposited onto a solid-phase matrix, forming a macroscopic array of individual assay parameters. The different allergens and components, spotted onto a nitrocellulose membrane as immunosorbent in a cartridge chip, are incubated with 0.5 mL of a 1:5 dilution of serum under agitation, the serum diluent containing a Cross-reactive Carbohydrate Determinants (CCDs) inhibitor. After incubation for 2 h, the chips are extensively washed. A pretitered dilution of anti-human IgE labeled with alkaline phosphatase is added and incubated for 30 min. Following another washing cycle, the enzyme substrate is added, and after a few minutes, the reaction is complete. After the membranes are dried, the quantification of this colorimetric enzyme assay is performed with an easy-to-use and affordable image explorer. The image acquisition and analysis of a single test takes only a few seconds. The assay time is 3.5 h, and tests per run are up to 50 per operator, with manual processing.

The ALEX® immunoassay protocol integrates a powerful CCDs inhibitor during serum incubation, as previously mentioned. This reduces the interpretative burden for physicians of CCD-positive patients and increases the specificity of the test results. CCDs refer to a group of related glycans produced by invertebrates and plants but not by vertebrates. Induction of IgE antibodies against CCDs is thought to be driven in humans by pollen exposure and/or insect stings[9]. Most natural allergen preparations originating from plants or insects contain CCDs, but CCDs do not behave as allergens *in vivo* and are clinically insignificant[94]. IgE antibodies directed against CCDs cross-react with all proteins containing these CCDs epitopes. Therefore, they are an important cause of cross-reactivity for *in vitro* specific IgE assays regarding CCD-containing allergens from pollen, plant foods and insect venoms if a CCDs inhibitor is not used. A notable 22% of serum samples from patients with suspected sensitizations to pollen, foods or insect venoms were detected to have anti-CCD IgE antibodies, the incidence reaching 35% in the teenage group[95]. A similar overall prevalence of 23% of positive IgE to CCD was recorded in a previous study in subjects with suspected allergic respiratory disease. The prevalence varied when subsets of non-allergic (5%), non-pollen allergic (10%), and pollen allergic (31%) subjects were considered, and further increased in subsets with multiple pollen sensitization (71%)[96]. Because many patients have anti-CCD IgE antibodies, there are a significant number of positive specific IgE results without the use of a CCDs inhibitor. The presence of such anti-CCD IgE could be misleading for the *in vitro* reactivity in the case of extract-based testing or when using many CCD-containing natural purified glycoproteins from pollen grains[74,97,98]. Recombinant proteins produced in *Escherichia coli* bacteria are not affected by CCD recognition, because of the lack of the posttranslational glycosylation of proteins[99]. nAna c 2 (MUXF3) is a purified *N*-glycan from *Ananas comosus* bromelain (nAna c 2) able to detect IgE against *N*-glycans in most pollen sources, anti-CCD IgE being a biomarker of reactivity to carbohydrate moieties of glycoproteins[74,97,98].

The ALEX measuring range for specific IgE is 0.3-50 kU$_A$/L (quantitative) and for total IgE is 1-2500 kU/L (semiquantitative). The sample requirement is 100 μL serum or plasma. The results are expressed as Class 0 (< 0.3 kU$_A$/L), Class 1 (0.3-1 kU$_A$/L), Class 2 (1-5 kU$_A$/L), Class 3 (5-15 kUA/L), and Class 4 (> 15 kU$_A$/L). ALEX is commercially available, having attained CE certification, which assures that the quality of the assay, regarding LoD, precision and repeatability as well as specificity and linearity, is in line with *in vitro* diagnostic features. There is no significant interference from high total IgE, hemoglobin, bilirubin or triglycerides. A flexible Raptor analysis software (specifically designed for ALEX®) allows to analyze tailormade allergen panels, as considered fit for clinical needs (multiplex on-demand)[75].

PRECISION MEDICINE IMMUNOASAYS FOR ASSESSING IgE SENSITIZATION TO AEROALLERGENS

Molecular allergy diagnosis work-up may be organized as an integrated "U-shape" approach, with a classical "top-down" approach (from symptoms to molecules, using extract-based skin prick tests and/or IgE singleplex assays) combined with a "bottom-up" approach (from molecules to clinical implications, using multiplex assays), as needed in selected patients. This is done in order to explain various allergic phenotypes or diseases, by exploring in detail the individual serum IgE profile or repertoire[9,33].

The selection of IgE immunoassays, allergenic extract and molecular specificities, and the interpretation of the results assessing *in vitro* IgE sensitization to aeroallergens require knowledge of test principle, methods and characteristics, and clinical judgments based on carefully collected history and physical exa-

Table 2 Allergen molecules of house dust and storage mites origin[1] used in singleplex and multiplex immunoassays[9,74,102,103]

Allergen molecule	Biological function, comments, CR
House dust mites (*Dermatophagoides pteronyssinus, Dermatophagoides farinae, Euroglyphus maynei, Blomia tropicalis*)	
nDer p 1	Cysteine-protease, cleavage of regulatory IgE synthesis CD23, CD25
rDer f 1	Midgut (colon, intercolon, postcolon) and mite fecal pellets
	Group 1 major mite allergen, CR with nDer f 1
rDer p 2	Functional homologue of adaptor MD-2, TLR4 coreceptor
nDer p 2	Midgut (ventriculus, paired caeca), male reproductive tract, fecal pellets
rEur m 2	Group 2 major mite allergen, NPC2 family, CR Der p 2, Der f 2, homologue Lep d 2
rDer p 4	Group 4 major mite allergen
rDer p 5	Group 5 major mite allergen, homologue rBlo t 5
rDer p 7	LPS-binding protein, stimulation TLR2, group 7 mite allergen
rDer p 9	Serine protease, group 9 mite allergen
rDer p 10	Invertebrate panallergen tropomyosin muscular protein from mite locomotory muscles
	Group 10 CR mite allergen, invertebrate panallergen
	CR rAni s 3, nBla g 7, rPer a 7, rPen a 1, Pen m 1, Lit v 1, Hel as 1, Ven g 1, Uro du 1
	Involved in house dust mites-crustaceans-mollusks syndrome
rDer p 11	Paramyosin, major allergen
rDer p 14	Apolipophorin-like allergen, lipid transport particles
rDer p 15	Chitinase-like allergens
rDer p 18	
nDer p 20	Arginine kinase, CR shrimp *Penaeus monodon* Pen m 2
rDer p 21	Group 21 mite allergen, gut and fecal particles
rDer p 23	Peritrophin-like protein (8 kDa), group 23 mite major allergen
	Peritrophic lining of mite gut, fecal pellets
Storage mites (*Glycyphagus domesticus, etc.*)	
rGly d 2	Group 2 mite allergens, NPC2 protein
	CR Lep d 2, homologue Der p 2 (limited CR)

[1]n: Native purified; r: Recombinant; Per a: Cockroach *periplaneta americana* allergen molecule; Mite *Dermatophagoides pteronyssinus* (Der p), *Dermatophagoides farinae* (Der f), *Glycyphagus domesticus* (Gly d), *Lepidoglyphus destructor* (Lep d), *Blomia tropicalis* (Blo t) allergen molecules; Nematode *Anisakis simplex* (Ani s) allergen molecule; Shrimp *Penaeus aztecus* (Pen a), *Penaeus monodon* (Pen m), *Litopenaeus vannamei* (Lit v 1), clam *Venus gallina* (Ven g), squid *Uroteuthis duvauceli* (Uro du) and snail *Helix aspersa* (Hel as) allergen molecules; CD: Cluster of Differentiation; CR: Cross-reactivity; LPS: Lipopolysaccharide; NPC2: Niemann-Pick type C2 protein; TLR: Toll-like receptor.

mination[100]. Although some multiplex assays, such as ISAC 112, may be correlated to singleplex assays, such as ImmunoCAP, from the point of view of the results for important corresponding molecular allergens, the tests' results are not interchangeable because of the different applied technologies. Due to the underlying different methodological backgrounds, it is not surprising that differences appear between different immunoassays. They may be caused by differences in method sensitivity, the use of native or recombinant allergens and the representation of the sensitizing molecule in the testing procedure[101].

Specific and cross-reactive aeroallergen components of animal (*e.g.,* house dust mites, cockroaches, mammalian pets), fungal (molds) and plant (pollen) origin used in allergy immunoassays are either well-defined highly purified natural or recombinant molecules. They are utilized in singleplex and multiplex immunoassays for the detection of IgE sensitization (Tables 2-7). Such precision medicine immunoassays used for *in vitro* assessment of the IgE sensitization to aeroallergen components[9,33,109-111] are: (1) Indicated within the framework of a patient's detailed clinical history, because IgE sensitization to a given allergen does not necessarily imply clinical significance and comprehensive case history alone may overlook relevant aeroallergens, especially in multisensitized patients, but also in the case of unclear symptoms and/or sensitization patterns or for assessing unsatisfactory response to treatment. Some history-related information may indicate certain underlying allergenic molecules in the process of anamnesis molecular thinking (*e.g.,* apple oral allergy syndrome with symptoms of rhino-conjunctivitis during birch pollen season suggests Bet v 1 involvement); (2) Essential for comprehensive and accurate IgE sensitization profiling with identification of the clinically relevant allergens, especially when using the multiplex approach, distinguishing genuine IgE sensitization from sensitization due to cross-reactivity in polysensitized patients, and revealing unexpected sensitizations or helping rule out potential triggers by delivering IgE results for a broad spectrum of aeroallergens. Some data correlations may be referred as laboratory molecular thinking (*e.g.,* high levels of specific IgE to all pollen species without plant food allergic reactions may be related to sensitization to polcalcins) or postmolecular anamnesis (*e.g.,* mite-shrimp syndrome related to the presence of a sensitization to Der p 10 tropomyosin); (3) Particularly useful for IgE sensitization profiling in several groups/situations, including small children with limited skin area, elderly with less reliable skin tests, all settings of

Table 3 Allergen molecules of domestic insect pest origin[1] used in singleplex and multiplex immunoassays[9,74,104]

Allergen molecule	Biological function, comments, CR
Cockroaches (*Blattella germanica, Periplaneta americana*)	
rBla g 1	Midgut microvilli protein-homolog CR Per a 1 (group 1), secreted in the digestive system and excreted in fecal particles, concentration in feces of adult females higher vs adult males and nymphs
rBla g 2	Unusual inactive aspartic protease with strong allergenic properties Digestive organs (esophagus, gut and proventriculus) and fecal particles
rBla g 4	Insect calycin lipocalin, calcium binding protein, binds tyramine/octopamine with role in reproduction of insects
rBla g 5	GST Involved in detoxification of toxic compounds role in insecticide resistance
rPer a 7	Invertebrate tropomyosin, muscle contraction protein, invertebrate panallergen CR Bla g 7, locomotor insect body parts

[1]n: Native purified; r: Recombinant; Cockroaches *Blattella germanica* (Bla g) and *Periplaneta americana* (Per a) allergen molecules; CR: Cross-reactivity; GST: Glutathione S-transferase.

Table 4 Allergen molecules of mammalian pet/pest origin[1] used in singleplex and multiplex immunoassays[9,74,105]

Allergen molecule	Biological function, comments, CR
Mammalian pets (*Felis domesticus, Canis familiaris*)	
rFel d 1	Secretoglobin, major cat allergen, species-specific, sebaceous glands (testosterone influenced, production higher in males) Dispersed by dander (regardless of race) Sublingual salivary glands (saliva), lacrimal and anal glands
rFel d 2	Feline serum albumin in cat serum, dander, saliva, minor allergen CR nBos d 6 BSA CR Sus s 1 serum albumin: pork-cat syndrome involvement
rFel d 4	Feline lipocalin in cat serum, dander, saliva CR moderate risk with lipocalins rEqu c 1 and Can f 6
rCan f 1	Non-CR lipocalin from sublingual salivary glands (saliva) and dander Major allergen not influenced by hair length or hormonal status
rCan f 2	Non-CR lipocalin, major allergen in dander and saliva Together with rCan f 1 and rCan f 5 are species-specific markers of sensitization
nCan f 3	Canine serum albumin, found in serum, dander, saliva High CR with other serum albumins: rFel d 2, nEqu c 3
rCan f 4	Non-CR lipocalin major allergens
rCan f 6	(both Can f 4 and Can f 6 are dog lipocalins)
rCan f 5	Dog prostatic kallikrein, an arginine esterase, major allergen in male urine, dander
House mouse (*Mus musculus*)	
nMus m 1	Lipocalin-odorant binding rodent family of MUPs

[1]n: Native purified; r: Recombinant; Cat *Felis domesticus* (Fel d) and dog *Canis familiaris* (Can f) allergen molecules; Horse *Equus caballus* (Equ c), cow *Bos domesticus* (Bos d) and domestic pig *Sus scrofa* (Sus s) allergen molecules; BSA: Bovine serum albumin; CR: Cross-reactivity; MUPs: Major urinary proteins.

inflamed or atopic skin or when medications interfering with SPT cannot be discontinued; (4) Equipped with the ability to improve assessment of severity of reactions associated with allergens. Multisensitization to several different allergen components from a single allergen source may increase symptom severity. Combination of several sensitizing aeroallergen exposures at a given time - the allergen load-is related to disease severity, pushing the patient over the symptom-onset threshold, particularly during viral infections. Moreover, molecular allergy diagnosis may reveal potential risk for food-related reactions; and (5) Posing a key impact on the optimal decision making for prophylactic measures and specific immunotherapy. The detailed assessment of the molecular pattern of IgE sensitization to aeroallergens may improve interventions to reduce allergen exposure or for allergen avoidance, may decrease the need for provocation testing and be useful to guide prescription of allergen immunotherapy, with a better selection of patients and immunotherapy products, potential prediction the efficacy and, in some cases, prediction of the adverse reactions risk.

CONCLUSION

Precision medicine is increasingly recognized as the way forward for optimizing patient care[112]. Introduction of the new generation immunodiagnostics, with well-defined purified natural and recombinant allergens,

Table 5 Allergen molecules of fungal origin[1] used in singleplex and multiplex immunoassays[9,74,106-108]

Allergen molecule	Biological function, comments, CR
Ascomycetes fungi (*Alternaria alternata, Cladosporium herbarum, Aspergillus fumigatus*)	
rAlt a 1	Fungal beta-barrel protein, detected in spores before germination
	Involvement in *Alternaria*-spinach syndrome
rAlt a 6	Fungal enolase, panallergen CR Cla h 6, Asp f 22, Pen c 22
rCla h 8	Fungal mannitol dehydrogenase, major allergen, CR Alt a 8
rAsp f 1	Fungal ribotoxin, specific major allergen, member of the mitogillin family
	Not present in spores, but produced after germination and growth
	Genuine exposure and IgE sensitization to A. fumigatus germinated in the respiratory tract
rAsp f 2	Species-specific allergen component, with high frequency of sensitization among patients affected by ABPA
rAsp f 3	Fungal peroxisomal protein, CR Pen c 3
rAsp f 4	Fungal protein, highly specific allergen
	With high frequency of sensitization among ABPA patients
rAsp f 6	Fungal Mn-SOD, CR Alt a 14, Mala s 11

[1]n: Native purified; r: Recombinant; Fungi *Alternaria alternata* (Alt a), *Cladosporium herbarum* (Cla h), *Aspergillus fumigatus* (Asp f), *Penicillium citrinum* (Pen c), *Malassezia sympodialis* (Mala s) allergen molecules; ABPA: Allergic bronchopulmonary aspergillosis; CR: Cross-reactivity; Mn-SOD: Mn superoxide dismutase.

Table 6 Main species-specific allergen molecules of pollen origin[1] used in singleplex and multiplex immunoassays[9,74,93]

Plant source	Allergenic molecule	Biological function, comments
Specific allergen components of tree pollen origin		
(birch *Betula verrucosa*, olive tree *Olea europaea*, plane tree *Platanus acerifolia*, cypress *Cupressus arizonica*, *etc.*)		
Betulaceae family	rBet v 1	PR-10 protein, heat sensitive
		CR with Bet v 1-like proteins Cor a 1, Mal d 1
		Involved in birch-hazelnuts-Rosaceae fruit syndrome
Oleaceae family	rOle e 1	Trypsin inhibitor
Platanaceae family	rPla a 1	Invertase inhibitor
	nPla a 2	Polygalacturonase
Cupressaceae family	nCup a 1	Pectate lyase
Specific allergen components of grass pollen origin		
(Timothy grass: *Phleum pratense*, Bermuda grass: *Cynodon dactylon*, *etc.*)		
Poaceae family	rPhl p 1	Beta-expansin
Pooideae subfamily	rPhl p 5	Ribonuclease;
		other allergen molecules: rPhl p 2, rPhl p 6
Chloridoideae subfamily		
nCyn d 1		
Beta-expansin		
Specific allergen components of weed pollen origin		
(mugwort *Artemisia vulgaris*, ragweed *Ambrosia artemisiifolia*, *Pellitory Parietaria judaica*, plantain *Plantago lanceolata*, saltwort *Salsola kali*, goosefoot *Chenopodium album*, *etc.*)		
Asteraceae family	nArt v 1	Defensin-like protein
	nAmb a 1	Pectat lyase, CR Art v 6
Urticaceae family	rPar j 2	Lipid transfer protein
Plantaginaceae family	rPla l 1	Trypsin inhibitor
		Ole e 1-like protein
Amaranthaceae/Chenopodiaceae family	nSal k 1	Pectin methylesterase
	rChe a 1	Trypsin inhibitor
		Ole e 1-like protein

[1]n: Native purified; r: Recombinant; Fruit hazelnut *Corylus avellana* (Cor a) and apple *Malus domestica* (Mal d) allergen molecules; Pollen *Betula verrucosa* (Bet v), *Olea europaea* (Ole e), *Platanus acerifolia* (Pla a), *Cupressus arizonica* (Cup a), *Phleum pratense* (Phl p), *Cynodon dactylon* (Cyn d), *Artemisia vulgaris* (Art v), *Ambrosia artemisiifolia* (Amb a), *Parietaria judaica* (Par j), *Plantago lanceolata* (Pla l), *Salsola kali* (Sal k), *Chenopodium album* (Che a) allergen molecules; PR: Pathogenesis-related plant proteins; CR: Cross-reactivity.

in the field of allergy research and clinical practice represents a great precision medicine approach. Modern IgE immunoassays allow for accurate and affordable patient detailed IgE sensitization profiling and identification of the clinically relevant allergens, with potential favorable impact on the allergy diagnosis and treatment, especially in selected patients, including refined useful information regarding clinically relevant allergens, allergenic risks, early interventions, effective and optimized management. Collectively, this will lead to improvements in patient health, quality of life and overall costs.

Table 7 Cross-reactive allergen components of pollen origin[1] used in singleplex and multiplex immunoassays[9,74]

Allergen molecule	Biological function, comments, CR
Polcalcins	
rPhl p 7	Calcium-binding protein, polcalcin biomarker Minor allergen CR with other polcalcins contained in pollen grains: grasses: *Pooideae* and non-*Pooideae*, *e.g.*, Bermuda grass (Cyn d 7) trees: *e.g.*, birch (Bet v 3), alder (Aln g 4), olive (Ole e 3), juniper (Jun o 4) weeds: *e.g.*, mugwort (Art v 5), short ragweed (Amb a 10)
rBet v 4	Polcalcin, which contains only two calcium-binding domains (*vs* Bet v 3, which contains three calcium-binding domains) Polcalcin biomarker
Non-specific lipid transfer proteins	
nArt v 3	PR-14 protein, CR with peach Pru p 3 from, hazelnut Cor a 8 Involved in mugwort-peach association, *Asteraceae*-hazelnut association
nOle e 7	PR-14 protein, CR with peach Pru p 3, pear Pyr c 3, melon Cuc m LTP, kiwifruit Act d 10, Involved in olive pollen-fruit syndrome
rPla a 3	PR-14 protein, CR with hazelnut Cor a 8, banana Mus a 3, celery Api g 2 Involved in *Platanus* pollen-fruit/vegetables association
Profilins	
rPhl p 12	Plant panallergen actin-binding protein (cytoskeleton dynamics) Profilin biomarker with great sequence identity with other profilins CR with profilins from: grass pollen: *Pooideae* and non-*Pooideae*, *e.g.*, Bermuda grass (Cyn d 12) tree pollen: *i.e.*, birch (Bet v 2), olive (Ole e 2), date palm (Pho d 2) weed pollen: *i.e.*, ragweed (Amb a 8) and mugwort (Art v 4); sunflower (Hel a 2) CR with profilins in latex (Hev b 8) and exotic fruits, involved in pollen-latex-fruit syndrome: profilins from ananas (Ana c 1), banana (Mus xp 1), kiwi (Act d 9) and olive pollen (Ole e 2) CR with profilins from various plant foods: profilin Art v 4 CR with Dau c 4, Api g 4, involvement in celery-mugwort-spice syndrome profilin Amb a 8 CR with Cuc m 2, Mus xp 1, involved in ragweed-melon-banana association
rBet v 2	Cross-reactive biomarker, important for the profilin group CR profilins in Apiaceae (Dau c 4, Api g 4) Involvement in birch-Apiaceae vegetables association

[1]n: Native purified; r: Recombinant; Fruit hazelnut *Corylus avellana* (Cor a), *Prunus persica* (Pru p), *Pyrus communis* (Pyr c), *Cucumis melo* (Cuc m), *Actinidia deliciosa* (Act d), Musa acuminata (Mus a), *Ananas comosus* (Ana c) allergen molecules; Latex *Hevea brasiliensis* (Hev b) allergen molecule; Pollen *Betula verrucosa* (Bet v), *Alnus glutinosa* (Aln g), *Olea europaea* (Ole e), *Platanus acerifolia* (Pla a), *Juniperus oxycedrus* (Jun o), *Phoenix dactylifera* (Pho d), *Phleum pratense* (Phl p), *Cynodon dactylon* (Cyn d), *Artemisia vulgaris* (Art v), *Ambrosia artemisiifolia* (Amb a), *Helianthus annuus* (Hel a) allergen molecules; Vegetables *Apium graveolans* (Api g), *Daucus carota* (Dau c) allergen molecules; CR: Cross-reactivity; PR: Pathogenesis-related plant proteins.

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P- Reviewer: Amrousy DI, Ciccone MM, Neri V **S- Editor**: Dou Y
L- Editor: A **E- Editor**: Wu YXJ

World Journal of Methodology

Submit a Manuscript: http://www.f6publishing.com

World J Methodol 2018 November 29; 8(3): 37-39

DOI: 10.5662/wjm.v8.i3.37

ISSN 2222-0682 (online)

EDITORIAL

Can extracorporeal shock-wave therapy be used for the management of lateral elbow tendinopathy?

Dimitrios Stasinopoulos

Dimitrios Stasinopoulos, Department Health Sciences, School of Sciences, European University Cyprus, Nicosia 1516, Cyprus

ORCID number: Dimitrios Stasinopoulos (0000-0002-7725-8742).

Author contributions: Stasinopoulos D solely contributed to this manuscript.

Conflict-of-interest statement: None conflict of interest.

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Manuscript source: Invited manuscript

Corresponding author to: Dimitrios Stasinopoulos, PhD, Associate Professor, Director of Cyprus Musculoskeletal and Sports Trauma research centre (CYMUSTREC), Department Health Sciences, School of Sciences, European University Cyprus, 6, Diogenes Str. Engomi, Nicosia 1516, Cyprus. d.stassinopoulos@euc.ac.cy
Telephone: +357-22-713044

Received: July 30, 2018
Peer-review started: July 30, 2018
First decision: August 20, 2018
Revised: September 7, 2018
Accepted: October 10, 2018
Article in press: October 10, 2018
Published online: November 29, 2018

Abstract

Lateral elbow tendinopathy (LET) is one of the two most common tendinopathies of the upper limb. The most effective treatment in the management of LET is the exercise program. Clinicians combine exercise program with other physiotherapy, electrotherapeutic and no, techniques. Extracorporeal shock wave therapy (ESWT) is one of the most common recommended electrotherapeutic modalities for the management of LET. Further research is needed to find out the optimal treatment protocol of ESWT in the management of LET

Key words: Lateral elbow tendinopathy; Extracorporeal shock wave therapy; Exercise program; Electrotherapy; Tennis elbow; Lateral epicondylitis; Physiotherapy; Calcific tendinopathy

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Core tip: Extracorporeal shock wave therapy (ESWT) is one of the most common recommended electrotherapeutic modalities for the management of lateral elbow tendinopathy (LET). ESWT does not use as a substitute for exercise but as a supplement to exercise program. More research is needed to find out the optimal treatment protocol of ESWT in the management of LET.

Stasinopoulos D. Can extracorporeal shock-wave therapy be used for the management of lateral elbow tendinopathy? *World J Methodol* 2018; 8(3): 37-39 Available from: URL: http://www.wjgnet.com/2222-0682/full/v8/i3/37.htm DOI: http://dx.doi.org/10.5662/wjm.v8.i3.37

Lateral elbow tendinopathy (LET) commonly referred as lateral epicondylitis or tennis elbow is a common sports/musculoskeletal injury. LET is usually defined as a syndrome of pain in the area of the lateral epicondyle which may be degenerative rather than inflammatory. The main complaints of patients with LET are de-

creased function and pain both of which may affect daily activities. Pain and function can be assessed by a variety of outcomes like gripping activities, palpation on the facet of the lateral epicondyle and specific clinical tests like middle finger extension and mill's test. Moreover, the Patient - Rated tennis elbow evaluation questionnaire provides a quick, standardized and easy quantitative description of functional disability and pain in LET patients. Physiotherapy is usually proposed for the treatment of LET. Many physiotherapy maneuvers, electrotherapeutic and non-electrotherapeutic modalities, has been recommended for the rehabilitation of LET. These treatments have different theoretical mechanisms of action, but all have the same aim, to improve function and reduce pain. Thus, there is need for more research in order to find out the most effective treatment technique in LET patients since this variety of treatment modalities suggests that the most proper treatment approach is not known.

The most common physiotherapy treatment for LET is a supervised or in clinic exercise programme. One program consisted of isometric exercises of extensor carpi radialis brevis (ECRB), the most common site injury of LET, as well as isotonic (concentric and eccentric mainly) and static stretching exercises of ECRB is usually recommended[1]. The exercise program should include exercises not only for ECRB strengthening but also for supinator strengthening[2]. In addition, rotator cuff and scapular muscles strengthening is also needed[3]. Finally, therapists should use techniques to improve the proprioception since LET patients have also reduced proprioception[4].

Electrotherapeutic modalities, have also been recommended in the management of LET. Extracorporeal shock wave therapy (ESWT) is one of the most common recommended electrotherapeutic modalities for the management of LET. The available literatures on the use of ESWT therapy for LET have revealed contradictory results. ESWT does not use as a substitute for exercise but as a supplement to exercise program. The mechanism of this method is not yet completely understood, but many mechanisms have been described in clarifying its effects, including direct stimulation of healing, neovascularization, direct suppressive effects on nociceptors and a hyper stimulation mechanism that would block the gate control mechanism. It seems that it can increase the number of neovessels at the normal tendon-bone junction, through the release of growth factors and some other active substances[5]. Therefore, ESWT does not reverse the pathology of LET (disorganized collagen, an increased presence of fibroblasts, the absence of prostaglandins and inflammatory cells) but it can improve the symptoms of LET. The question that arises is whether ESWT can be used for any kind of LET, *i.e.*, acute, chronic and calcification.

ESWT is not applied in acute LET when the inflammatory signs are active. It is recommended in the management of chronic LET. However, the term chronic LET is not clear in the literature. The term chronic LET is ranged in the literature from 4 wk to 6 mo after the first onset. A patient with 4 wk of LET does not have the same symptoms with a patient with 6 mo of LET, so the ESWT is applied in a different way in a patient with four weeks of LET and in a patient with 6 mo of LET. There is not a standard protocol in the management of chronic LET using the ESWT. There is not a standard protocol in the management of chronic LET because the medical society does not define the term chronic LET. If the medical society defines the term chronic LET, a standard recommended protocol of ESWT in the management of chronic LET will be applied.

ESWT is usually recommended when the symptoms persist for more than 6 mo or all the other types of conservative therapy fail. However, there is lack of strong evidence in the literature to support the above recommendations. Why someone recommend the use of ESWT 6 mo after the first onset? Can it be used earlier than 6 mo after the first onset? I think the above will be solved when the medical society defines the term chronic LET. Moreover, the exercise program is the most effective conservative treatment approach in the management of LET, as mentioned above. The ESWT is recommended when the exercise program fails or other conservative techniques fail. It is known that all the other types of conservative therapy are less effective than the exercise program in the management of LET. In addition, ESWT does not use as a substitute for exercise but as a supplement to exercise program, as mentioned previously.

Although there are conflicting results on the effectiveness of ESWT in the management of chronic (need definition as mentioned previously) LET, it cannot be ruled out from research, as it is a dose-response modality and the optimal treatment dose (ESWT parameters such as focused or radial ESWT, anaesthesia or not, energy flex density, frequency, number of sessions, and impulses) has obviously not yet been discovered in order to be used in rehabilitation protocols[6]. It is worth to mention one more time that ESWT does not use as a substitute for exercise but as a supplement to exercise program. The optimal protocol can be formulated taking information from RCTs, reviews (systematic and narrative) and experts opinion.

The ESWT is usually recommended in the management of calcific tendinopathies (CT). The majority of research on ESWT for CT that has been carried out involved calcific shoulder tendinopathy (CST). One of the mechanisms of the therapeutic effect of ESWT for the treatment of CST is destruction of calcifications[7]. There is a lack of *in vitro* studies to explain how the increasing pressure produced by ESWT causes fragmentation and cavitation effects inside amorphous calcifications, leading to disorganization and disintegration of the deposits[8]. Alternatively, disintegration of calcifications in shoulder tendinopathy after ESWT has been shown in *in vivo* studies[8]. High-energy ESWT under anaesthesia effectively treats

(strong and moderate evidence) CST in the short, mid, and long terms[9]. Focus on the calcific deposit is more effective (moderate evidence) than focus on the tuberculum majus[9]. However, further studies are needed to standardize ESWT parameters (energy flex density, number of sessions, and impulses) to be used in rehabilitation protocols[10]. It is believed that ESWT is an effective treatment approach for LET with calcification using the same, under investigation, parameters for CST. However, more research is needed to support this belief.

ESWT is applied only in the area of pain, in the case of LET in the ECRB. I wonder if it can be applied in a different way, like scanning the whole area for example muscles of the forearm or parallel to the elbow joint line. Perhaps, the combination of the above two ways of application, scanning and painful (sensitive) point, leads to a better clinical result. More research is needed to support the above recommendation.

Finally, the aim of this editorial is two-fold, which suggests the following: First, future studies are needed to standardize ESWT parameters in the management of LET (chronic and calcific), and secondly, well-conducted trials are needed to find out the effectiveness of ESWT in the treatment of any kind of LET. A cost-effectiveness analysis should be incorporated into the analysis of the effectiveness of such a treatment approach in future trials.

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P- Reviewer: El Amrousy D, Wan TTH **S- Editor**: Ji FF
L- Editor: A **E- Editor**: Wu YXJ

World Journal of Methodology

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World J Methodol 2018 November 29; 8(3): 40-43

DOI: 10.5662/wjm.v8.i3.40

ISSN 2222-0682 (online)

EDITORIAL

Microembolic signal detection by transcranial Doppler: Old method with a new indication

Sombat Muengtaweepongsa, Charturong Tantibundhit

Sombat Muengtaweepongsa, Charturong Tantibundhit, Department of Internal Medicine, Faculty of Medicine, Thammasat University, Pathum Thani 12120, Thailand

ORCID number: Sombat Muengtaweepongsa (0000-0003-3715-4428); Charturong Tantibundhit (0000-0002-3889-7314).

Author contributions: Muengtaweepongsa S contributed to conception and design of the work, data collection, drafting and critical revision of the article, and gave final approval; Tantibundhit C contributed to data collection and drafting of the article.

Conflict-of-interest statement: The authors declare they have no conflicts of interest.

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Corresponding author to: Sombat Muengtaweepongsa, MD, MRCP, MSc, Associate Professor, Department of Internal Medicine, Faculty of Medicine, Thammasat University, Pathum Thani 12120, Thailand. musombat@staff.tu.ac.th
Telephone: +66-86-9994208
Fax: +66-29-269793

Received: August 4, 2018
Peer-review started: August 5, 2018
First decision: August 24, 2018
Revised: September 30, 2018
Accepted: October 17, 2018
Article in press: October 18, 2018
Published online: November 29, 2018

Abstract

Transcranial Doppler (TCD) is useful for investigation of intracranial arterial blood flow and can be used to detect a real-time embolic signal. Unfortunately, artefacts can mimic the embolic signal, complicating interpretation and necessitating expert-level opinion to distinguish the two. Resolving this situation is critical to achieve improved accuracy and utility of TCD for patients with disrupted intracranial arterial blood flow, such as stroke victims. A common type of stroke encountered in the clinic is cryptogenic stroke (or stroke with undetermined etiology), and patent foramen ovale (PFO) has been associated with the condition. An early clinical trial of PFO closure effect on secondary stroke prevention failed to demonstrate any benefit for the therapy, and research into the PFO therapy generally diminished. However, the recent publication of large randomized control trials with demonstrated benefit of PFO closure for recurrent stroke prevention has rekindled the interest in PFO in patients with cryptogenic stroke. To confirm that emboli across the PFO can reach the brain, TCD should be applied to detect the air embolic signal after injection of agitated saline bubbles at the antecubital vein. In addition, the automated embolic signal detection method should further facilitate use of TCD for air embolic signal detection after the agitated saline bubbles injection in patients with cryptogenic stroke and PFO.

Key words: Cryptogenic stroke; Patent foramen ovale; Transcranial Doppler; Recurrent stroke; Patent foramen ovale closure; Brain ischemia; Real-time emboli

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Core tip: Patent foramen ovale (PFO) is an emerging etiology of cryptogenic stroke, and PFO closure therapy has been shown to reduce the rate of recurrent stroke.

Detection of the air embolic signal by transcranial Doppler (TCD) after injection of agitated saline bubbles at the antecubital vein will help to confirm the importance of PFO as the cause of a concurrent stroke. In addition, the automated embolic signal detection method should further facilitate use of TCD for air embolic signal detection after the agitated saline bubbles injection in patients with cryptogenic stroke and PFO.

Muengtaweepongsa S, Tantibundhit C. Microembolic signal detection by transcranial Doppler: Old method with a new indication. *World J Methodol* 2018; 8(3): 40-43 Available from: URL: http://www.wjgnet.com/2222-0682/full/v8/i3/40.htm DOI: http://dx.doi.org/10.5662/wjm.v8.i3.40

INTRODUCTION

Transcranial Doppler (TCD) is a noninvasive method for evaluating blood flow velocities in intracranial arteries. The TCD instrument is also used to detect emboli in real time, as they emerge in the main intracranial circulation. In general, an embolic signal representing an embolus has some characteristics that are distinctive from the signal that represents normal blood flow. Specifically, the embolic signal is classified as high intensity transient signals (commonly referred to as "HITS") lying on top of the Doppler signal, deflected through an angle of 180 degrees by red blood cells. However, artefacts, caused by a variety of situations (*e.g.,* probe motion, patient movement, and sound waves from the patient speaking) are sometimes detected as HITS. Distinguishing embolic signals from artefacts requires an expert-level evaluation of the morphology of the signals, resulting in a subjective finding[1,2]. The multigated method was developed to improve the objective differentiation between embolic signals and artefacts. This technique samples signals from different depths of a similar vessel to demonstrate the motion of the following embolus from proximal to distal (Figure 1); in contrast, an artefact shows no movement property but appears in all depths simultaneously (Figure 2)[3].

CRYPTOGENIC STROKE AND PATENT FORAMEN OVALE

Cryptogenic stroke is described as a cerebral infarct of unclear or undetermined etiology, according to the Trial of Org 10172 in Acute Stroke Treatment stroke-subtype categorization system (TOAST). The source of cryptogenic stroke remains unclear mainly because the episode itself is temporary or reversible, and the available forms of clinical investigation do not address all possible etiologies. It is also important to consider that there may be additional etiologies that have yet to be recognized[4]. The finding that more than one-third of reported cerebral infarcts are categorized as cryptogenic etiology highlights the need for technologies and procedures to better investigate them[5].

Patent foramen ovale (PFO) is a possible etiology of cryptogenic stroke, especially in young adults. Prevalence of PFO is considerable, with estimates as high as 25% for the overall population. Moreover, it has been reported that around 50% of cryptogenic stroke patients of age less than 55 have a PFO[6]. The consideration of PFO as an etiology of consecutive cryptogenic stroke has been controversial, however, since early clinical trials of PFO closure therapy did not show any benefit for prevention of recurrent stroke[7-9]. Moreover, due to this reported lack of benefit, a routine practice of investigating cryptogenic stroke patients for PFO was not included among the recommendations in standard guidelines[10].

Three recent large randomized control trials (*i.e.*, RESPECT[11], REDUCE[12] and CLOSURE[13]) demonstrated benefit of PFO closure for secondary stroke prevention in selected cases of patients with cryptogenic stroke[11-13]. Of the three, the RESPECT study had the longest follow-up time, at 6 years[11]. The REDUCE study showed the benefit of PFO closure therapy over antiplatelet therapy, at 3 years after treatment[12]. The CLOSURE study included high-risk PFO cases with an atrial septal aneurysm or large interatrial shunt[13]. Data on device-related atrial fibrillation was reported in the REDUCE[12,13] and CLOSURE[12,13] studies. Furthermore, other recent studies carried out as meta-analyses also confirmed the benefit of PFO closure for secondary stroke prevention[14,15]. Considering these studies and their findings collectively, the next step would be carrying out systematic investigation of the potential for routinely seeking PFO in patients with cryptogenic stroke, particularly since the PFO itself holds promise as a target of therapy.

TCD FOR PATIENTS WITH CRYPTOGENIC STROKE AND PFO

Echocardiography plays a major role in diagnosis of PFO. In some cases, the PFO is detectable with color flow Doppler imaging in the echocardiogram. In most cases, the agitated saline bubbles test is mandatory for the diagnosis of PFO. In this procedure, a bolus of agitated saline is injected into an antecubital vein, after which air bubbles appear in the right atrium. A positive PFO finding is indicated when the air bubbles appear in the left atrium within three cardiac cycles of their initial appearance in the right atrium. The mechanism underlying this finding is the Valsalva maneuver, which increases right atrial pressure and facilitates right-to-left shunting[16].

Use of TCD for detection of the air embolic signal after injection of agitated saline bubbles into an antecubital vein is an alternative procedure to confirm right-to-left shunting. Detection of an air embolic signal

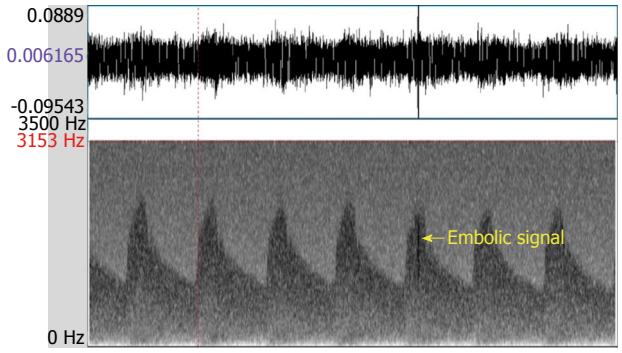


Figure 1 Embolic signal. The NICOLET Pioneer transcranial Doppler 4040 Doppler Waveform Analyzer was used.

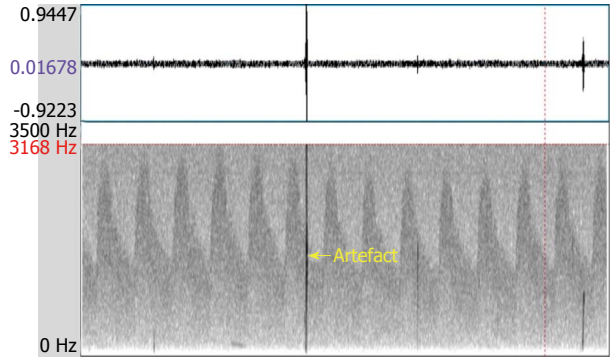


Figure 2 Artefact. The NICOLET Pioneer transcranial Doppler 4040 Doppler Waveform Analyzer was used.

in intracranial arteries should affirm that an embolus from the heart is able to reach the brain and cause the ischemic lesions. With the new indication for PFO closure, the use of TCD for air embolic signal detection with agitated saline bubbles test will be increased. The number of air embolic signals may be related to the size of the PFO, and such would help to strengthen the interpretation of clinical significance for the shunting.

Furthermore, the automated embolic signal detection method should improve the differentiation between artefacts and real emboli, and allow for counting the number of emboli[17,18]. The sensitivity and specificity of the automated system for differentiation between real emboli and artefacts were demonstrated to be as high as those of experts' opinions[17,18]. With this automated method, TCD for air embolic signal detection with agitated saline bubbles test should be more useful in patients with cryptogenic stroke with PFO. Moreover, the automated method may extend use of TCD for embolic signal detection in other indications, such as emboli detection during invasive cardiac or great vessels procedure and microembolic monitoring during the first 48 h after onset of stroke[19,20].

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P- Reviewer: Ciccone MM, Sharma P, Vieyra JP, Weng CF
S- Editor: Ji FF **L- Editor**: A **E- Editor**: Wu YXJ



World Journal of Methodology

Submit a Manuscript: http://www.f6publishing.com

World J Methodol 2018 November 29; 8(3): 44-50

DOI: 10.5662/wjm.v8.i3.44

ISSN 2222-0682 (online)

ORIGINAL ARTICLE

Retrospective Study

Assessment of quality control system by sigma metrics and quality goal index ratio: A roadmap towards preparation for NABL

Monica Verma, Kiran Dahiya, Veena Singh Ghalaut, Vasudha Dhupper

Monica Verma, Kiran Dahiya, Veena Singh Ghalaut, Vasudha Dhupper, Department of Biochemistry, Pt. B.D. Sharma, University of Health Sciences, Rohtak 124001, Haryana, India

ORCID number: Monica Verma (0000-0002-8070-7123); Kiran Dahiya (0000-0002-7734-5387); Veena Singh Ghalaut (0000-0002-1716-4277); Vasudha Dhupper (0000-0003-4759-8997).

Author contributions: Verma M designed the research, interpreted the results, and drafted the manuscript; Dahiya K and Ghalaut VS helped in statistical analysis, critical revision, and final approval of the article; Ghalaut VS and Dhupper V helped in making critical revisions and final approval of the article.

Conflict-of-interest statement: None exists.

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Manuscript source: Unsolicited manuscript

Corresponding author to: Monica Verma, DNB, MBBS, MD, Assistant Professor, Doctor, Department of Biochemistry, Pt. B.D. Sharma, University of Health Sciences, House No. 315, 3rd Floor, Model Town, Rohtak 124001, Haryana, India. monisoni26@gmail.com
Telephone: +91-98-13334543

Received: July 31, 2018
Peer-review started: July 31, 2018
First decision: August 20, 2018
Revised: September 4, 2018
Accepted: October 17, 2018

Article in press: October 17, 2018
Published online: November 29, 2018

Abstract

AIM

To study sigma metrics and quality goal index ratio (QGI).

METHODS

The retrospective study was conducted at the Clinical Biochemistry Laboratory, PGIMS, Rohtak, which recently became a National Accreditation Board for Testing and Calibration of Laboratories accredited lab as per the International Organization for Standardization 15189:2012 and provides service to a > 1700-bed tertiary care hospital. Data of 16 analytes was extracted over a period of one year from January 2017 to December 2017 for calculation of precision, accuracy, sigma metrics, total error, and QGI.

RESULTS

The average coefficient of variation ranged from 2.12% (albumin) to 5.42% (creatinine) for level 2 internal quality control and 2% (albumin) to 3.62% (high density lipoprotein-cholesterol) for level 3 internal quality control. Average coefficient of variation of all the parameters was below 5%, reflecting very good precision. The sigma metrics for level 2 indicated that 11 (68.5%) of the 16 parameters fall short of meeting Six Sigma quality performance. Of these, five failed to meet minimum sigma quality performance with metrics less than 3, and another six just met minimal acceptable performance with sigma metrics between 3 and 6. For level 3, the data collected indicated eight (50%) of the parameters did not achieve Six Sigma quality performance, out of

which three had metrics less than 3, and five had metrics between 3 and 6. QGI ratio indicated that the main problem was inaccuracy in the case of total cholesterol, aspartate transaminase, and alanine transaminase (QGI > 1.2), imprecision in the case of urea (QGI < 0.8), and both imprecision and inaccuracy for glucose.

CONCLUSION

On the basis of sigma metrics and QGI, it may be concluded that the Clinical Biochemistry Laboratory, PGIMS, Rohtak was able to achieve satisfactory results with world class performance for many analytes one year preceding the accreditation by the National Accreditation Board for Testing and Calibration of Laboratories. Aspartate transaminase and alanine transaminase required strict external quality assurance scheme monitoring and modification in quality control procedure as their QGI ratio showed inaccuracy.

Key words: Sigma; Quality goal index; Bias; Imprecision; Inaccuracy; Coefficient of variation

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Core tip: As the majority of tests take place in the biochemistry laboratory, it plays a major role in patient care. Therefore, it is necessary to follow a proper quality management system to provide accurate and precise reports to patients. National Accreditation Board for Testing and Calibration of Laboratories accreditation is an important benchmark for "A" grade quality. Sigma metrics is also a well-known self-assessment tool to guide quality control strategy design. On the basis of sigma metrics and quality goal index ratio, it may be concluded that the Clinical Biochemistry Laboratory, PGIMS, Rohtak was able to achieve satisfactory results with world class performance for many analytes one year preceding accreditation by the National Accreditation Board for Testing and Calibration of Laboratories.

Verma M, Dahiya K, Ghalaut VS, Dhupper V. Assessment of quality control system by sigma metrics and quality goal index ratio: A roadmap towards preparation for NABL. *World J Methodol* 2018; 8(3): 44-50 Available from: URL: http://www.wjgnet.com/2222-0682/full/v8/i3/44.htm DOI: http://dx.doi.org/10.5662/wjm.v8.i3.44

INTRODUCTION

Over 60% of tests are carried out in a clinical biochemistry laboratory. Therefore, it plays a major role in diagnosing and managing diseases. It is imperative to follow a proper quality management system (QMS) to provide accurate and reliable reports in an agreed upon time frame[1]. The Clinical Biochemistry Laboratory, PGIMS, Rohtak, (CBL, PGIMS, Rohtak) is a National Accreditation Board for Testing and Calibration of Laboratories (NABL) accredited lab as per the International Organization for Standardization (ISO) 15189:2012. It has become the first laboratory in the government sector attached to a postgraduate institute to be accredited by NABL in the whole North India region.

In a CBL, total testing process consists mainly of three stages: pre-analytical phase, analytical phase, and post-analytical phase. QMS includes strict compliance at all phases as error can occur at any step. In the mid-1980s, a revolution came in QMS that reduced the cost of products, decreased variability in processing, and eliminated defects. This evolution was Six Sigma methodology that was developed by a Motorola engineer named Bill Smith[2]. Sigma metrics is an important tool to evaluate the errors in quality control of a laboratory system. Sigma is a metric that quantifies the performance of a process at a rate of defects-per-million[3]. The sigma value indicates how often errors are likely to occur. The higher the sigma value, the lower the chance of false test results by the laboratory. It can easily quantify the exact number of errors by combining bias, precision, and total allowable error (TEa). A sigma level < 3 is an indication of a poor performance procedure, whilst a good performance is indicated by a sigma level > 3. Sigma level of 6 or greater indicates world-class performance[2].

To calculate precision and bias, internal quality control (IQC) and external quality assurance scheme (EQAS) are being carried out in our laboratory. IQC is run daily as per NABL guidelines and is interpreted by Levy Jennings' charts and Westgard's rules. The samples to be analyzed are run only when the IQC results are within control limits. EQAS sample is run monthly and is interpreted by Z score or Standard Deviation Index (SDI). Z-score is a calculated value that tells us how many standard deviations (SDs) a control result has shifted from the mean value, which is expected for that material[2]. Quality goal index (QGI) is a newer parameter to represent the relative extent to which both bias and precision meet their respective quality goals.

The CBL, PGIMS, Rohtak is a large laboratory catering to 500 outpatient samples and 300 inpatient samples per day. The laboratory has regularly run IQC and EQAS for several years. To obtain the NABL accreditation by our large, government laboratory was a daunting task. However, once a system was established, the task became achievable and motivated us to share our experience regarding EQAS data from the year preceding the NABL accreditation.

The aim of the present study is to measure the sigma metrics and QGI for individual parameters in the scope for NABL accreditation.

MATERIALS AND METHODS

The retrospective study was conducted at the CBL, PGIMS, Rohtak, which provides laboratory service to a > 1700-bed tertiary care hospital. Data was extracted

over a period of one year from January 2017 to December 2017. A total of 16 analytes were included in the study which were: glucose, urea, creatinine, total bilirubin, total protein, albumin, calcium, phosphorus, uric acid, total cholesterol, triglyceride, high density lipoprotein-cholesterol (HDL-cholesterol), aspartate transaminase (AST), Alanine Transaminase (ALT), alkaline phosphatase (ALP), and amylase. All parameters were run along with IQC and EQAS. IQC data was analyzed for imprecision and EQAS data for inaccuracy. The parameters were done on a Randox Suzuka autoanalyzer by using Randox kits obtained from the manufacturer following the standard operating procedures at CBL.

As per laboratory policy, two levels of controls (level 2: normal and level 3: pathological, Randox Laboratories Limited) were run twice daily along with monthly EQAS lyophilized sample obtained from Christian Medical College, Vellore throughout the study period. The laboratory follows the Westgard's rule to accept and reject the run. 1_{3s}, 2_{2s}, R_{4s}, 4_{1s}, and 10x were considered a rejection, and 1_{2s} as a warning rule for each respective run. Mean, SD, and coefficient of variance (CV) were calculated for each month for both levels. The laboratory receives EQAS sample in three batches of four samples every year. The sample was reconstituted and analyzed the same day. All the EQAS samples were handled as routine patient samples and were analyzed by the senior lab technician on duty without his knowledge. Reports were uploaded before the 20th of every month. On the 4th of the next month SDI was checked. SDI within 0 ± 2 was considered acceptable. Bias was also noted.

Sigma metrics

Mean of the CV of both levels and bias was calculated and used for estimating sigma metrics by the following formula:

Sigma = (TEa – Bias)/CV[4]

Where, TEa is total allowable error, and bias and CV are the indicators of systematic and random errors, respectively. The minimum acceptable performance of process was a 3 sigma level.

QGI

QGI represents the relative extent to which both bias and precision meet their respective quality goals. It was calculated using the following formula:

QGI = Bias/1.5 CV

QGI represents the reason behind lower sigma value *i.e*., imprecision, inaccuracy, or both. For analytes which fall short of Six Sigma quality, a QGI score of < 0.8 indicates imprecision, QGI > 1.2 indicates inaccuracy, and QGI score 0.8-1.2 indicates both imprecision and inaccuracy[4].

Coefficient of variation

The coefficient of variation (CV) is the SD expressed as a percentage and is a measure of the variability of an assay[5].

CV = (SD/Mean) × (100)

Bias

Bias is the systematic difference between the expected results obtained by the laboratory test method and the results that would be obtained from an accepted reference method[6].

TEa

TEas were followed as per the Clinical Laboratory Improvement Amendments (CLIA) guidelines[1]. Total error (TE) of parameters was also calculated by the following formula[7]:

TE = Bias + 1.65CV

RESULTS

Tables 1 and 2 summarize the CV % of level 2 and level 3 IQC, respectively for 16 biochemical parameters from January 2017 to December 2017 along with their average values. The average CV ranged from 2.12% (albumin) to 5.42% (creatinine) for level 2 IQC and 2% (albumin) to 3.62% (HDL-cholesterol) for level 3 IQC. Average CV of all the parameters is below 5% reflecting very good precision. Table 3 summarizes the bias % obtained from EQAS from CMC Vellore for 16 parameters and their average for the same duration. Table 4 summarizes the average CV %, average bias %, TEa (CLIA), calculated TE, and sigma metrics of the 16 parameters.

The sigma metrics for level 2 indicated that 11 (68.5%) of the 16 parameters fell short of meeting Six Sigma quality performance. Of these, five failed to meet minimum sigma quality performance with metrics less than three and another six just met minimal acceptable performance with sigma metrics between three and six. For level 3, the data collected indicated that eight (50%) of the parameters did not achieve Six Sigma quality performance, out of which three had metrics less than 3 and five had metrics between 3 and 6. Calculated TEa of all the parameters were less than specified TEa (CLIA) except for AST and ALT. Table 5 summarizes the results of sigma metrics of various parameters. Table 6 summarizes the QGI ratio of analytes with lower sigma values (< 3). QGI ratio indicated that out of five and three parameters of level 2 and level 3, which failed to meet Six Sigma quality performances, the main problem was inaccuracy in the case of total cholesterol, AST, and ALT (QGI > 1.2), imprecision in the case of urea (QGI < 0.8), and both imprecision and inaccuracy for glucose.

DISCUSSION

Currently, it is a requirement to constantly verify the pre-analytical, analytical, and post-analytical processes

Table 1 The CV % of 16 parameters of level 2 internal quality control for a period of one year (Jan-Dec, 2017) and their average

Parameter	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Average
Glucose	2.04	1.81	2.12	1.98	2.99	2.07	4.74	2.96	3.07	4.01	2.94	2.17	2.74
Urea	3.13	4.27	3.87	5.84	4.08	5.30	4.43	4.08	3.89	3.70	3.61	4.39	4.21
Creatinine	7.02	8.30	8.02	7.15	4.47	3.38	6.28	4.13	3.45	4.02	4.26	4.66	5.42
Total bilirubin	2.88	3.89	3.27	2.93	2.52	4.60	4.75	4.62	2.99	2.54	4.07	4.86	3.66
Total protein	1.95	2.18	2.67	2.94	3.81	2.99	3.03	3.00	3.83	3.99	2.34	3.27	3.00
Albumin	0.99	2.55	2.04	0.96	3.40	2.18	1.86	1.86	2.27	1.94	3.00	2.47	2.12
Calcium	2.09	2.90	2.76	2.70	2.75	2.38	2.57	2.55	2.06	3.24	2.74	1.85	2.54
Phosphorus	5.48	6.83	6.70	4.47	5.40	6.72	4.52	3.58	3.87	3.68	4.22	3.54	4.91
Uric acid	3.63	4.17	2.73	3.59	3.64	3.81	2.80	3.21	3.36	2.53	2.66	2.6	3.22
Total cholesterol	2.26	1.86	3.42	2.17	2.53	4.16	3.64	2.62	2.38	2.87	3.00	3.49	2.86
Triglyceride	3.74	4.95	5.07	5.30	7.17	5.07	5.36	3.96	3.62	4.23	5.13	3.29	4.74
HDL cholesterol	5.08	3.43	5.68	1.74	4.56	2.85	4.57	3.89	3.14	2.90	2.94	3.56	3.69
AST	5.74	6.18	6.79	4.22	4.59	4.97	5.72	4.31	3.49	3.40	4.38	3.56	4.77
ALT	5.82	6.10	4.80	5.09	5.25	5.39	5.56	5.64	1.99	3.05	4.90	3.46	4.75
ALP	2.98	3.59	2.81	2.56	2.51	2.87	3.02	3.77	2.26	5.97	4.17	2.84	3.27
Amylase	4.03	5.70	4.71	4.24	4.00	3.14	5.47	4.72	4.10	3.30	3.07	3.87	4.19

CV: Coefficient of variation; IQC: Internal quality control; HDL: High-density lipoprotein; AST: Aspartate transaminase; ALT: Alanine transaminase; ALP: Alkaline phosphatase.

Table 2 The CV % of 16 parameters of level 3 internal quality control for a period of one year (Jan-Dec, 2017) and their average

Parameter	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Average
Glucose	1.20	1.31	1.59	1.50	1.96	1.65	2.80	2.05	4.32	2.45	1.76	1.90	2.04
Urea	2.60	2.91	3.50	4.40	3.23	3.84	3.41	3.36	3.65	4.02	3.28	2.60	3.40
Creatinine	3.34	3.49	5.29	4.77	2.75	1.97	3.10	3.03	2.98	2.95	2.92	2.16	3.22
Total bilirubin	2.50	2.35	2.52	2.05	2.35	3.34	2.86	3.99	2.12	3.91	2.66	2.71	2.78
Total protein	2.36	2.41	1.96	2.36	3.84	2.90	2.75	4.24	3.50	3.82	3.25	3.34	3.06
Albumin	2.25	1.95	3.29	1.73	1.69	1.84	1.99	1.69	1.86	1.74	1.96	2.02	2.00
Calcium	1.37	2.95	2.57	2.54	2.00	2.21	2.92	2.93	2.14	2.22	2.34	2.2	2.36
Phosphorus	3.50	4.85	4.77	4.78	3.85	4.02	2.22	1.88	1.62	2.78	2.41	2.61	3.27
Uric acid	2.90	3.03	2.33	2.12	2.66	2.42	3.56	2.51	2.54	1.97	3.11	2.23	2.61
Total cholesterol	2.09	1.30	2.86	2.35	2.61	4.61	2.30	3.91	2.03	2.57	2.6	2.79	2.66
Triglyceride	2.50	2.26	3.61	2.85	3.18	4.22	3.80	2.70	1.98	2.72	3.49	2.83	3.01
HDL cholesterol	3.03	3.72	4.19	1.95	5.17	4.54	5.22	2.91	1.97	4.35	4.1	2.35	3.62
AST	2.12	3.40	2.65	2.75	3.45	2.55	3.48	3.76	3.95	2.92	3.62	2.70	3.11
ALT	3.19	2.81	3.09	2.89	4.45	2.66	4.22	4.32	2.89	2.67	2.56	3.11	3.23
ALP	2.93	2.61	2.67	2.74	2.59	2.53	2.53	2.3	2.30	4.08	2.82	2.90	2.75
Amylase	3.10	3.51	3.57	3.47	3.30	2.84	5.30	2.25	3.03	3.42	2.73	3.33	3.32

CV: Coefficient of variation; IQC: Internal quality control; HDL: High density lipoprotein; AST: Aspartate transaminase; ALT: Alanine transaminase; ALP: Alkaline phosphatase.

of the laboratory by internal or external audit. Sigma metrics is an important self-assessment tool to guide QC strategy design. It helps to improve the process quality by removing defects. We analyzed 16 parameters for sigma metrics over one year (January-December, 2017). Similar studies have been conducted by Singh *et al*[8], Adiga *et al*[2], Iqbal *et al*[3] and Nanda *et al*[9], but none of these assessed the cause of low sigma, *i.e*., either imprecision, inaccuracy, or both. Only a single study in literature could be found that carried out both sigma metrics and QGI[10].

The Six Sigma model is similar to Total Quality Management, which follows a "Plan, Do, Check, Act" cycle. The basic scientific model in Six Sigma metrics is "Define, Measure, Analyze, Improve and Control." The Six Sigma model has the extra step of control, which is important in modern quality management. This step helps in preventing the recurrence of defects, *i.e*., if an error is detected, it has to be solved it and prevented from affecting the process again. With this step, errors are effectively decreased until a desirable degree of quality is obtained[11]. The same is to be followed for the parameters with lower sigma values to attain desirable performance level, as continual improvement is necessary as per ISO standards for good laboratory practices.

In this study, four parameters (albumin, uric acid, HDL-cholesterol, and ALP) showed a sigma of > 6 for both level 2 and level 3 of IQC showing excellent performance, while creatinine, total bilirubin and amylase showed > 6 for level 3 IQC only. Total cholesterol, AST, and ALT were short of sigma metrics with a value < 3 for both level 2 and level 3. Glucose and urea showed < 3 sigma for level 2 only. Nanda *et al*[9] and Kumar

Table 3 The bias % obtained from external quality assurance scheme from CMC Vellore for 16 parameters for a period of one year (Jan-Dec, 2017) and their average

Parameter	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Average
Glucose	10.8	-9.7	6.1	1.2	1.2	6	3.8	3.2	0.9	7.5	8.5	4.5	3.66
Urea	-3.8	-7.2	-2.5	-2.2	2.2	-12.2	-1.8	-5.4	4	-10.1	-1.6	5.3	-2.94
Creatinine	-8.3	-13.6	-18.8	-7.7	-10.9	-9.1	-9.9	14.3	-7.5	-14	0	-10.7	-8.01
Total bilirubin	0	-20	-4.8	5.4	5.9	-5.9	-4	-10	3.3	-3.8	6.2	10.9	-1.4
Total protein	-4.2	-14	-2	-6	-2	-9.6	-5.8	-8.3	-6.2	-10	-8.2	-4.1	-6.7
Albumin	-3.3	-16.1	-6.5	-9.4	-12.9	-15.6	-6.3	-10	-10	-6.5	0	-3.3	-8.32
Calcium	1.2	-0.8	4.1	-4.3	3.1	-1.6	2.2	3.4	1.1	2.1	15.7	12.6	3.23
Phosphorus	-12.8	-37.5	-20.6	-15.8	-12.5	-9.3	-25.9	-4.4	-20	1.9	-18.8	7.3	-14
Uric acid	-14.5	-20.4	-16.2	-11.6	-22.9	-3.3	-4.8	-26.7	-5.7	-7.1	-8.3	-5.5	-12.2
Total cholesterol	-0.6	-6.4	1.8	2	-1.9	-3.5	3.3	6	26.9	8.9	15.8	8.7	5.08
Triglyceride	9.1	-1.3	4	5.9	-2.8	6.5	12.2	1.5	15.5	17.5	11.5	9.4	7.41
HDL cholesterol	-12.1	-23.6	-19.4	-14.7	-18	-27.7	-13	-15	-17.4	-18	-15.3	-14.6	-17.4
AST	20	2.4	20.7	7.6	0.8	5.8	13.7	17.8	20.1	11	14.8	24.3	13.2
ALT	33.5	12.3	22	-3.6	6.7	-4.3	13.2	-11.9	18.3	7.6	29	29.6	12.7
ALP	-5.6	-5	4.3	-8.3	-6	-18.2	-6.1	-4.5	1.5	6.6	7.3	3.2	-2.56
Amylase	12.4	-0.7	17.9	4.8	8.2	4.8	15.1	15.3	17.6	5.9	-1.6	16.4	9.67

CV: Coefficient of variation; EQAS: External quality assurance scheme; HDL: High-density lipoprotein; AST: Aspartate transaminase; ALT: Alanine transaminase; ALP: Alkaline phosphatase.

Table 4 Sigma metrics (Level 1 and 2) and quality goal index ratio (Level 1 and 2) of 16 parameters calculated from coefficient of variation (Level 1 and 2), total allowable error (Clinical Laboratory Improvement Act), and bias %, for a period of one year (Jan-Dec, 2017)

Parameter	CV%		Bias %	TEa (CLIA)	TE (calculated)		Sigma	
	Level 2	Level 3			Level 2	Level 3	Level 2	Level 3
Glucose	2.74	2.04	3.66	10	8.18	7.03	2.31	3.11
Urea	4.21	3.4	-2.94	9	4.01	2.67	2.84	3.51
Creatinine	5.42	3.22	-8.01	15	0.93	-2.7	4.25	7.15
Total bilirubin	3.66	2.78	-1.4	20	4.64	3.19	5.85	7.7
Total protein	3	3.06	-6.7	10	-1.75	-1.65	5.57	5.46
Albumin	2.12	2	-8.32	10	-4.82	-5.02	8.64	9.16
Calcium	2.54	2.36	3.23	11	7.42	7.12	3.06	3.29
Phosphorus	4.91	3.27	-14	10	-5.9	-8.6	4.89	7.34
Uric acid	3.22	2.61	-12.2	17	-6.89	-7.89	9.07	11.19
Total cholesterol	2.86	2.66	5.08	10	9.8	9.47	1.72	1.85
Triglyceride	4.74	3.01	7.41	25	15.23	12.38	3.71	5.84
HDL cholesterol	3.69	3.62	-17.4	30	-11.31	-11.43	12.85	13.09
AST	4.77	3.11	13.2	20	21.07	18.33	1.43	2.19
ALT	4.75	3.23	12.7	20	20.54	18.03	1.54	2.26
ALP	3.27	2.75	-2.56	30	2.84	1.98	9.96	11.84
Amylase	4.19	3.32	9.67	30	16.58	15.15	4.85	6.12

CV: Coefficient of variation; TE: Total error; CLIA: Clinical Laboratory Improvement Act; HDL: High-density lipoprotein; AST: Aspartate transaminase; ALT: Alanine transaminase; ALP: Alkaline phosphatase.

Table 5 Sigma metrics of various parameters

Sigma metrics	Level 2	Level 3
< 3	Glucose, urea, total cholesterol, AST, ALT	Total cholesterol, AST, ALT
3-6	Creatinine, total bilirubin, total protein, calcium, phosphorus, triglyceride	Glucose, urea, total protein, calcium, triglyceride
> 6	Albumin, uric acid, HDL-cholesterol, ALP	Creatinine, total bilirubin, albumin, uric acid, HDL-cholesterol, ALP, amylase

HDL: High-density lipoprotein; AST: Aspartate transaminase; ALT: Alanine transaminase; ALP: Alkaline phosphatase.

et al[10] reported four parameters with < 3 sigma metrics. The lowest value for sigma was found for total cholesterol (1.72) at level 2 and the highest value for HDL-cholesterol (13.09) at level 3. For parameters showing lower sigma values, root cause analysis is done. Strict monitoring as well as increased frequency

Table 6 Quality goal index ratio of analytes performed low for sigma for accuracy and precision problem

Analytes	Qc levels	Bias%	CV%	Sigma	QGI	Problem
Glucose	Level 2	3.66	2.74	2.31	0.89	Imprecision and inaccuracy
Urea	Level 2	-2.94	4.21	2.84	0.47	Imprecision
TC	Level 2	5.08	2.86	1.72	1.18	Inaccuracy
	Level 3	5.08	2.66	1.85	1.27	Inaccuracy
AST	Level 2	13.2	4.77	1.43	1.84	Inaccuracy
	Level 3	13.2	3.11	2.19	2.83	Inaccuracy
ALT	Level 2	12.7	4.75	1.54	1.78	Inaccuracy
	Level 3	12.7	3.23	2.26	2.62	Inaccuracy

QGI: Quality goal index ratio; AST: Aspartate transaminase; ALT: Alanine transaminase; TC: Total cholesterol.

of IQC run is required. QGI ratio for parameters with sigma < 3 depicted inaccuracy in the case of TC, AST, and ALT (QGI > 1.2), imprecision in the case of blood urea (QGI < 0.8), and imprecision and inaccuracy in the case of glucose. There are certain limitations in the sigma metrics system because we have observed no problems in the CV % and bias % of glucose (level 2), urea (level 2), and TC (level 2 and level 3), but sigma is showing a lesser value. In the case of AST and ALT, the calculated TE is higher compared to the allowable error as per CLIA, which is reflected in the QGI and sigma metrics. In our opinion, if TE of an analyte is within allowable error limits specific for that analyte, bias % and CV % might be more reliable than sigma metrics. However, this claim needs to be supported by further studies.

On the basis of sigma metrics and QGI, it may be concluded that the CBL, PGIMS, Rohtak was able to achieve a quality of results that allowed a NABL accreditation to the laboratory as per ISO standard 15189:2012. AST and ALT required strict EQAS monitoring and modification in quality control procedure as their QGI ratio showed inaccuracy. Although sigma metrics is a well-known industrial standard, it might not be applied universally for all the analytes.

ARTICLE HIGHLIGHTS

Research background

Accreditation is a formal recognition from a third party body, which demonstrates the competence and capability to carry out a certain task it is claiming to do.

Research motivation

Over 60% of tests are carried out under clinical biochemistry section; hence it plays a critical role in diagnosing and managing diseases. It is imperative to follow a proper quality management system by the laboratory so as to provide accurate and reliable reports in an agreed upon time frame.

Research objectives

Assessment of the analytical phase of quality control system by sigma metrics and quality goal index ratio (QGI).

Research methods

This retrospective study was conducted at the Clinical Biochemistry Laboratory, PGIMS, Rohtak, which recently became a National Accreditation Board for Testing and Calibration of Laboratories (NABL) accredited lab per the International Organization for Standardization (ISO) 15189:2012 and provides service to a > 1700-bed tertiary care hospital. Data of 16 analytes were extracted over a period of one year from January 2017 to December 2017 for calculation of precision, accuracy, sigma metrics, total error, and QGI.

Research results

The average coefficient of variation of all the parameters was below 5%, reflecting precision. The sigma metrics for level 2 indicated that five of the sixteen parameters fell short of meeting minimal Six Sigma quality performance. For level 3, the data collected indicated three of the parameters do not achieve minimal Six Sigma quality performance. QGI ratio indicated that the main problems were inaccuracy in the case of total cholesterol, aspartate transaminase, and alanine transaminase (QGI > 1.2), imprecision in the case of urea (QGI < 0.8), and imprecision and inaccuracy for glucose.

Research conclusions

On the basis of sigma metrics and QGI, it may be concluded that the Clinical Biochemistry Laboratory, PGIMS, Rohtak was able to achieve satisfactory results with world class performance for many analytes one year preceding the NABL accreditation as per ISO standard 15189:2012.

Research perspectives

Although sigma metrics is a well-known industrial standard, it might not be applied universally for all the analytes.

ACKNOWLEDGEMENTS

The authors acknowledge the laboratory technical staff for their cooperation and assistance.

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P- Reviewer: Banerjee D, Tomizawa M, Wan TTH **S- Editor**: Ji FF
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ISSN 2222-0682 (online)

World Journal of *Methodology*

World J Methodol 2018 December 14; 8(4): 51-58



Published by Baishideng Publishing Group Inc

World Journal of Methodology

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INDEXING/ABSTRACTING

World Journal of Methodology is now abstracted and indexed in PubMed, PubMed Central, China National Knowledge Infrastructure (CNKI), and Superstar Journals Database.

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NAME OF JOURNAL
World Journal of Methodology

ISSN
ISSN 2222-0682 (online)

LAUNCH DATE
September 26, 2011

FREQUENCY
Continuous

EDITORS-IN-CHIEF
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EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/2222-0682/editorialboard.htm

EDITORIAL OFFICE
Jin-Lei Wang, Director

PUBLICATION DATE
December 14, 2018

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World Journal of Methodology

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World J Methodol 2018 December 14; 8(4): 51-58

DOI: 10.5662/wjm.v8.i4.51

ISSN 2222-0682 (online)

MINIREVIEWS

Radiosurgical techniques for the treatment of brain neoplasms: A short review

Tomaz Velnar, Roman Bosnjak

ORCID number: Tomaz Velnar (0000-0002-6283-4348); Roman Bosnjak (0000-0001-7565-0989).

Author contributions: Velnar T and Bosnjak R contributed equally to this work; Velnar T designed the research; Bosnjak R analyzed the data; Velnar T and Bosnjak R wrote the paper.

Conflict-of-interest statement: The authors declare that they have no conflicts of interest.

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Manuscript source: Invited manuscript

Correspondence to: Tomaz Velnar, MD, PhD, Department of Neurosurgery, University Medical Centre Ljubljana, Zaloska 7, Ljubljana 1000, Slovenia.
tvelnar@hotmail.com
Telephone: +386-1-5223250
Fax: +386-1-5222218

Received: September 28, 2018
Peer-review started: September 28, 2018

Tomaz Velnar, Roman Bosnjak, Department of Neurosurgery, University Medical Centre Ljubljana, Ljubljana 1000, Slovenia

Tomaz Velnar, AMEU-ECM, Maribor 2000, Slovenia

Abstract

Radiotherapy has long been used as an adjunct to neurosurgery for the treatment of malignant and benign intracranial tumors and other intracranial lesions. Intracranial tumors can be irradiated in three different ways: I) fractional radiotherapy, II) stereotactic radiotherapy and III) stereotactic radiosurgery. The third is most often by means of a gamma knife or a specially designed linear accelerator. Additionally, radiosurgery is increasingly used in combination with systemic therapy to treat metastases.

Key words: Neurosurgical techniques; Radiotherapy; Stereotaxy; Gamma knife; Linear accelerator

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Core tip: Intracranial tumors can be irradiated in three different ways: with fractional radiotherapy, stereotactic radiotherapy, and stereotactic radiosurgery. Additionally, radiosurgery is increasingly used in combination with systemic therapy to treat metastases.

Velnar T, Bosnjak R. Radiosurgical techniques for the treatment of brain neoplasms: A short review. *World J Methodol* 2018; 8(4): 51-58
URL: https://www.wjgnet.com/2222-0682/full/v8/i4/51.htm
DOI: https://dx.doi.org/10.5662/wjm.v8.i4.51

INTRODUCTION

Radiotherapy has long been used to treat malignant and benign intracranial neoplasms. Its use permits greater control of tumor growth and leads to increased patient survival[1-3]. Recently, a number of new neurosurgical therapies have been developed, such as stereotactic surgery for deep-seated lesions, neuronavigation,

First decision: October 29, 2018
Revised: November 6, 2018
Accepted: November 16, 2018
Article in press: November 16, 2018
Published online: December 14, 2018

minimally invasive endoport surgery, and navigated endoscopic surgery of small parenchymal lesions[2,4-6]. These allow a more effective and targeted treatment of lesions (removal or biopsy) whilst causing less damage to the surrounding tissue (Figure 1). In modern neuro-oncological radiotherapy, the same working principle was transferred from stereotactic neurosurgery into radiotherapy, with the aim being the treatment of surgically inaccessible lesions with a beam from a radioactive source or with the aid of electron acceleration[2]. A variety of radiosurgical techniques have recently emerged.

At the start of the 20th century, neurosurgeon Harvey Cushing carried out over 2000 brain tumor operations; amongst these, there were 800 glioma procedures. During some operations, he placed radioactive radium needles or wires, which he called radium "bombs". These were designed to irradiate the tumor and hence improve patient survival[7]. The treatment, known as brachytherapy, is a form of radiotherapy where a radioactive source is placed directly into a tumor by means of a neurosurgical operation[8].

In some centers, this technique is still being used for treating brain metastases and primary brain tumors such as glioblastomas, oligodendrogliomas, astrocytomas, meningiomas, and craniopharyngiomas[9-12]. Although the results initially seemed positive, the use of brachytherapy did not ultimately increase survival times. The most harmful complication reported in connection to brachytherapy was the radiation-induced late tissue necrosis[8-13].

RADIOSURGICAL TECHNIQUES FOR THE TREATMENT OF INTRACRANIAL TUMORS

With the development of radiotherapy and surgical techniques, the tendency has been to use radiotherapy either as a primary or a secondary method to treating brain tumors. For the successful treatment of these tumors, a sufficient dose of ionizing radiation, which is aimed at the target volume, ensures the tumor tissue is destroyed, while conserving healthy tissue. This method of radiotherapy is possible with high-dose targeted radiation[14]. Three methods exist for the irradiation of intracranial tumors: (1) fractional radiotherapy, (2) stereotactic radiotherapy, and (3) stereotactic radiosurgery.

In fractional radiotherapy, many fractions of radiation are given to treat a lesion. Stereotaxy is the precise three-dimensional localization of a target within a space. In stereotactic radiotherapy, the radiation beams are stereotactically (very precisely) targeted onto a tumor in a single dose or fractionally, relying on detailed imaging and computerized three-dimensional treatment planning. Stereotactic radiosurgery is a highly accurate type of radiation therapy, which uses high-powered X-rays that are stereotactically focused onto a lesion. In contrast to other forms of radiation therapy, which are more likely to affect the nearby healthy tissue, stereotactic radiosurgery more specifically targets the abnormal area. Stereotactic radiosurgery is related to stereotactic neurosurgery and radiotherapy. All three methods are stereotactic methods, as they use the exact positional localization of a target and patient positioning to consistently and accurately direct radiation to the same area[14,15]. They differ by the number of fractions applied to form the total dose received by the patient, as well as the method of application[15]. In fractional and stereotactic radiotherapy, a number of fractions are given to reach the total dose, with stereotactic radiotherapy differing by stereotactically using a beam aimed at the target. In stereotactic radiosurgery, the whole dose is stereotactically applied to the target in one fraction[14].

The type of treatment will depend upon the size, location and number of lesions, the radiation dose needed to destroy the tumor mass, the volume of healthy tissue that will receive the dose, important surrounding structures, and the tolerance of the healthy tissue to the dose of radiation, and hence, its ability to regenerate. Dividing radiation into fractions allows healthy cells to repair any damage to the genetic material between the individual fractional treatments of low dose radiation at a faster rate than cancerous cells[14-17].

Swedish neurosurgeon Lars Leksell, the pioneer of stereotactic radiosurgery, who collaborated in the development of the gamma knife, defined radiosurgery as a method that directs a single high-dose ionizing radiation onto an intracranial target, precisely decided by computed tomography (CT) or magnetic resonance imaging (MRI). A stereotactic frame or a polyester mesh mask, to fix and reference the head, is used to stereotactically direct multiple beams to a target and apply a therapeutic dose without incision[14-17].

Imaging has an important role in radiotherapy treatment. Firstly, proper imaging is

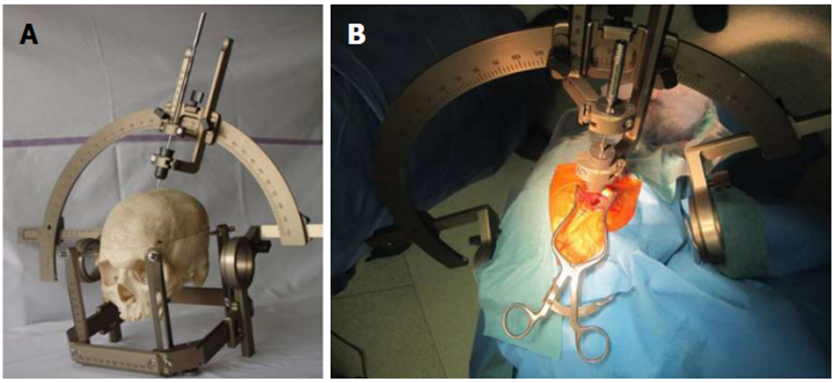


Figure 1 An illustration of the stereotactic biopsy procedure. A: A stereotactic frame (Leksell, Sweden) is mounted on the skull, and a probe is inserted through a previously drilled burr hole into the brain parenchyma, the tip of the probe reaching the lesion of interest; B: A stereotactic procedure in surgical practice with the tip inserted.

very important during the entirety of radiosurgery treatment, not only at the beginning for pre-treatment planning but then again during the treatment itself and during the follow-up[8,13]. Before deciding the treatment options with the patients, modern management of brain tumors includes comprehensive staging and surveillance with both CT and MRI to identify the intracranial metastases as early as possible[17,18]. Thereafter, prompt management with radiosurgery may commence, with the purpose to prevent the need for surgical resection, development of neurologic symptoms, and worsening of the clinical condition. Both CT and MRI are often needed for planning the stereotactic treatment. Additionally, pre-treatment position imaging verification is strongly advised to avoid possible mistreatments during the course of stereotactic radiosurgery, although the stereotactic system itself is extremely accurate[13,17,19]. In the follow-up period, imaging is important to monitor the effects of the stereotactic treatment. As brain metastases may at first increase in size after the stereotactic radiosurgery, it is very difficult to distinguish post-radiation changes from a possible tumor relapse. In these situations, imaging with multiparametric MRI techniques is very useful. In the initial identification of radiation-related changes, it enables assessment of metabolic and physiological characteristics of the tissue and possible tumor recurrence, and thus, may be useful for monitoring treatment changes in intracranial neoplasms[17-19].

Secondly, proper radiotherapy treatment cannot be planned without accurate CT and MR images. The planning itself is performed on a computer, which combines and develops CT or MR images. At this point, an oncologist and a medical physicist or a radiographer select the target to be irradiated and calculate the dose that the tissue will receive. The target is defined by means of a coordinate system so that each target is defined with coordinates relating to the X-, Y-, and Z-axes. Before therapeutic irradiation, a computer performs a simulation during which the apparatus moves into the position for target irradiation[14-16]. The stereotactic frame is used while CT images are taken to plan the coordinates for the target, therefore playing a role as an external reference for the coordinate system. This method of irradiation allows the target to receive the maximal dose, with the dose received by neighboring tissue dropping significantly. The healthy tissue receives a negligible dose of radiation, allowing for decreased morbidity and better quality of life. With the maximum limit of the diameter of the target being around 3 cm, this method is particularly appropriate for the use in small brain metastases and primary intracranial lesions[15,16].

The two most used units in radiosurgery are the linear accelerator and gamma knife. The characteristics of both are: (1) application of a single high dose of radiation; (2) stereotactic localization of the lesion with CT imaging; (3) the use of computerized dose calculation, which can precisely define the amount of radiation that the central nervous system receives; (4) a steep dose-gradient, with the target receiving the highest dose and the dose steeply falling in the area surrounding it, so that these tissues receive a negligible or manageable dose; and (5) an accurate application of radiation, both spatially and in terms of dose. Technical development has allowed more accurate planning and irradiation of intracranial lesions[1,18,19].

GAMMA KNIFE

The gamma knife was first developed in 1968. The modern gamma knife is equipped

with 201 sources of the radioactive isotope Cobalt-60 built into a special "hood" (Figure 2). The gamma knife allows for the application of a narrow beam of rays, accurately directed at a predetermined region in the brain where the lesion is located (Figure 3). Thus, the dose-gradient on the periphery of the target volume is very steep so that the target receives a high dose of radiation with the surrounding tissue preserved[2,20]. The treatment is planned using a stereotactically determined image base with a computer program. The treatment is usually completed in 1 d and one session. The gamma knife is suitable for the treatment of deep and surgically inaccessible intracranial lesions with low mortality and with few side effects from radiation. It is also suitable for the management of lesions that are insensitive to conventional radiotherapy. The gamma knife is also used to treat functional disorders, pain syndromes (such as trigeminal neuralgia), arteriovenous malformations, benign and malignant brain tumors and their remnants after surgery. Effective treatment is predominantly in the area of arteriovenous malformations and tumors (meningiomas, acoustic neuromas, pituitary adenomas, and brain metastases)[1,2,20].

In comparison with micro-neurosurgery, the neurological deficits in patients treated with radiosurgical techniques tend to be smaller, although technical limits and the risk of radiation damage to the brain do exist. Even though the gamma knife is a minimally invasive method for the treatment of a multitude of intracranial diseases, it is not without risk. In some patients, complications such as syncopal episodes, anxiety, and acute vessel complications can develop[21,22]. Late complications include headaches, strong facial pain (*e.g.*, post trigeminal neuralgia treatment), newly developed motor dysfunction (*e.g.*, ataxia caused by edema, paresis of the facial and oculomotor nerves, which can also occur a long time after the procedure), cases of hydrocephalus, and late-onset epileptic seizures. The most common indications for gamma knife treatment are: metastases (33% of patients), vestibular Schwannomas (17%), meningiomas (16%), neuropathic pain from trigeminal neuralgia (14%), arteriovenous malformations (8%), gliomas (5%) and the post-operative remnants of pituitary adenomas (2% of patients)[22-24].

Radiosurgery is also indicated for the treatment of some cases of epilepsy and movement disorders, as damage to a precisely defined point in the deep brain nuclei from electromagnetic irradiation may improve a patient's symptoms. however, this treatment has fallen out of favor more recently, as neuromodulatory techniques are being used more frequently. Here, the cerebral nuclei or cortex is stimulated or inhibited by implanting an electrode to improve neural pathway function. The benefit of this technique is that it is reversible and causes less tissue damage[21].

LINEAR ACCELERATOR

Linear accelerators were first used in the 1970s and are the most commonly used devices for conventional radiotherapy and radiosurgery today. They work by focusing a directed and shaped beam of X-rays onto a stereotactically determined target (Figure 4). The main direction of entry of the beam of X-rays from the linear accelerator (gantry) rotates around the patient to form a crisscross of beams, which all intersect at the target (Figure 5). The bed can rotate about the horizontal axis to allow irradiation from multiple trajectories to intersect at a certain point, the isocenter of the target[1,25]. As is also the case with the gamma knife, radiation beams that intersect on the target provide a large dose of ionizing radiation while minimizing the dose that the surrounding brain tissue receives. Linear accelerators use a special device called a collimator to shape and target the rays. In addition to primary and secondary collimators, rays can be further shaped with circular divergent tertiary collimators[25].

The modifications of linear accelerators include the gantry, which synchronously rotates the robotic table carrying the patient with the entry of the beam to the target, the system for stereotactic localization of the lesions, and improved collimators. With the wider use of linear accelerators, it is now possible to treat larger targets. Circular collimators were first developed for targets smaller than 3 cm in diameter and for irregularly shaped targets. In larger targets, the irradiated volume includes an unacceptably large volume of normal tissue. This is why collimators were developed. They allow radiosurgery with the dynamic form of the field of exposure, and hence, the irradiation of irregular and concave-shaped lesions. They are designed so that they are supplemented with a special multileaf collimator system, which allows exact coverage of the field of irradiation and comprises a multitude of micro-leaves that allow for irradiation of even bigger (diameter 3 cm to 4 cm) and geometrically more complex lesions. Recently, there has been a resurgence of interest in arc-based intensity modulated radiotherapy through the use of 'conventional' multileaf collimator systems that can treat large tumor volumes in a single or very few passes of

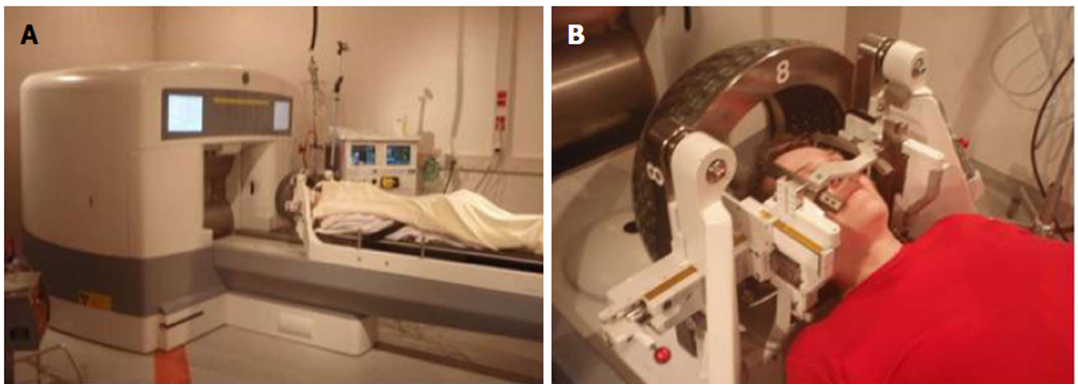


Figure 2 Positioning of the patient. A: Gamma knife and the patient position before the treatment procedure; B: Sources of radioactive cobalt are hidden in the in the "helmet" of the gamma knife, which is located over the patient's head.

the gantry[1,14,25,26].

The benefits of the linear accelerator over the gamma knife are the ability of more accurate shaping of the field of irradiation, a wider range of settings for the size of the collimator, a more homogeneous field of irradiation for large lesions, a higher energy of irradiation, the possibility to irradiate other parts of the body, the use of modern computer programs, and lower costs. Linear accelerators are also widely used for stereotactically focused fraction radiotherapy and for radiosurgical procedures. It is also possible to radiosurgically treat extracranial lesions[26].

CYBERKNIFE

The latest versions of linear accelerators are in the form of a robotic knife known as a Cyberknife[21]. The Cyberknife is a robotic radiosurgical system that is computer controlled with the help of CT or MRI. The system functions because of the interaction between the automatic guide, the imaging system, and the linear accelerator[21,22].

The guidance system includes a unit for X-ray imaging with the unit for the application of radiation being provided by a linear accelerator located on a robotic arm. There is a specific system for the guidance of the robotic arm during irradiation. Therefore, the robotic knife does not require a rigid attachment onto the stereotactic frame, as is the case with a linear accelerator[22]. Therefore, the position of the patient during the treatment is more comfortable in comparison to the gamma knife or linear accelerator. Treatment times typically range from 30 to 90 min, depending on the type of tumor. The treatment is usually concluded in one session. Occasionally, the patient can receive up to five sessions of radiotherapy. Besides intracranial tumors, the robotic knife can also be used to irradiate other organs, such as lungs, liver, prostate, kidneys, and pancreas. Another benefit of the Cyberknife is that tumors that move during respiration can also be treated[21-24].

CONCLUSION

Alongside neurosurgery, radiosurgery is a desirable secondary treatment option for many intracranial lesions. As a primary treatment, it is most suitable in cases of deep neurosurgically inaccessible or multiple lesions, where conventional forms of radiotherapy are not appropriate due to the greater risk of radiation tissue damage[25-29]. Examples include arteriovenous malformations, vestibular Schwannomas, meningiomas, certain primary brain tumors, and brain metastases. The number of metastases that can be treated with radiosurgery is up to five or a total diameter of 3.5 cm[30-32]. The uses of radiosurgery in treating epilepsy, functional motor, and behavioral disorders are limited to a small number of centers globally. The radiosurgical treatment of lesions requires a source of high energy rays and a method of accurately applying the radiation, conforming to the target volume and preserving the surrounding tissue[27,28].

Radiosurgical techniques can be used as an independent form of treatment, in case the volume of the lesion is not too large, or as a part of combined treatment with surgical removal or intravascular techniques. A combination of high-resolution diagnostic imaging, powerful computers, robotic systems, irradiation techniques, and

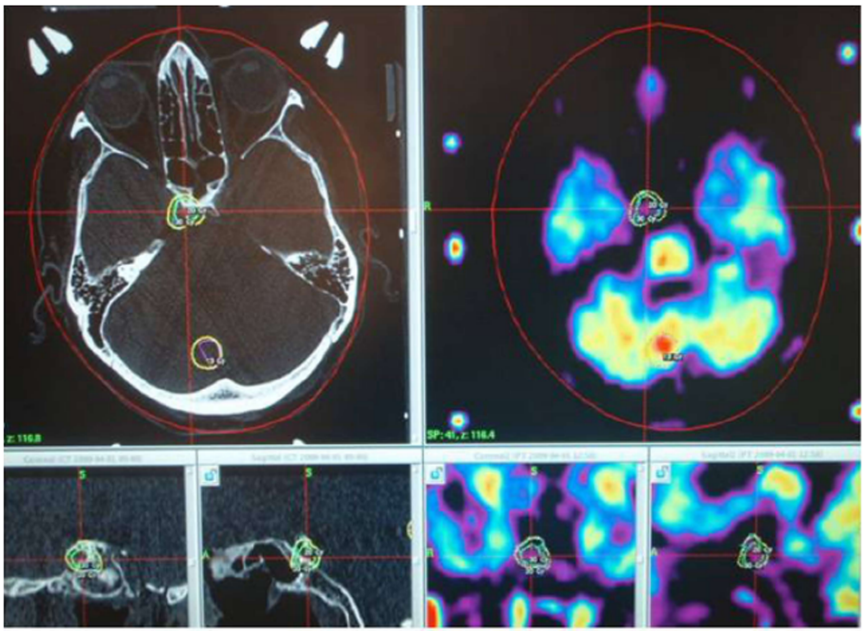


Figure 3 An example of computer planning for irradiation procedure of a residual pituitary adenoma, which was not completely removed from the parasellar space during a transnasal approach. In the upper row are the axial views of CT and MRI-PET images with the tumor location. In the lower row from left to right: the frontal and sagittal views of CT and MRI-PET images. CT: Computed tomography; MRI-PET: Magnetic resonance imaging - positron emission tomography.

new achievements in radio-biological research has enabled more successful treatment[30,31]. In addition to treating intracranial lesions, radiosurgery is effectively used in combination with systemic therapy in metastatic cancer treatment and as a local ablative therapy with stereotactic irradiation of metastases in the lungs, liver, and spine[30-34].

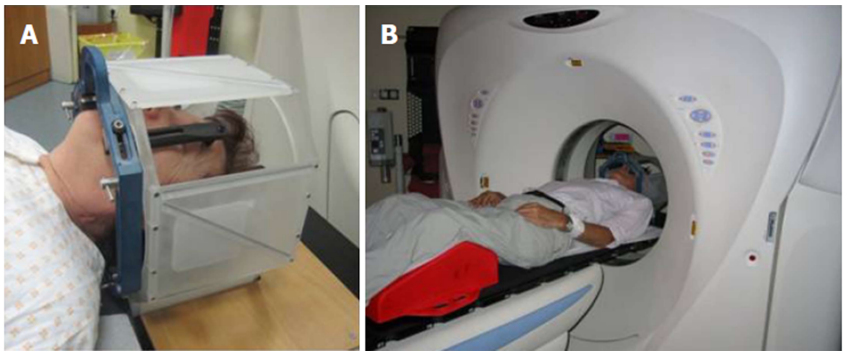


Figure 4 The stereotactic frame. A: The stereotactic frame (BrainLab, Germany) with the localizer cube attached, mounted on the patient's head with four screws that fix the frame onto the skull bone; B: The patient is brought into the CT machine prior to the combined CT imaging with the frame. CT: Computed tomography.

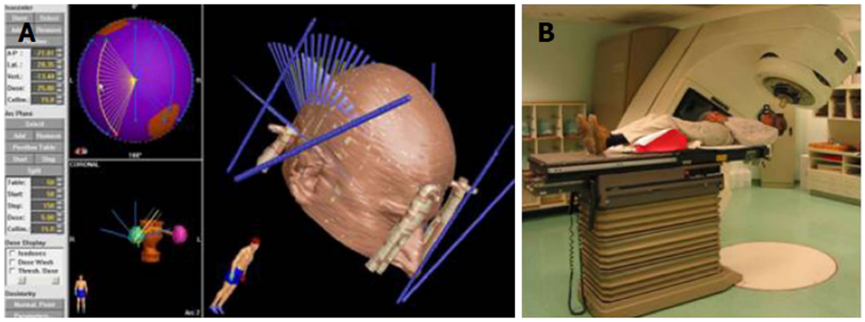


Figure 5 Irradiation with a linear accelerator. A: A computer irradiation plan showing a localizer cube attached to the stereotactic frame (thick blue lines) (right image) and the direction of irradiation of the intracranial lesion with X-rays (thin blue lines). Small images on the left show the location of two intracranial lesions that are going to be irradiated; B: The patient is resting on the table with the head fixed in the stereotactic frame. The bed and gantry rotate about various axes, and this allows for irradiation by means of multiple trajectories intersecting at a certain point.

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