The Right Heart and Pulmonary Circulation (II)

Imaging Techniques and the Evaluation of the Right Heart and the Pulmonary Circulation

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Since the right side of the heart and the pulmonary circulation are regarded as secondary components of the circulatory system, their role in disease has traditionally not received the same attention as their counterparts in the systemic circulation. This was partly because precise noninvasive study of these structures was difficult. For many years, chest radiography and invasive angiography were the only techniques available for imaging the minor circulation. The development of transthoracic echocardiography and nuclear techniques has produced a significant leap forward for noninvasive imaging, particularly of the right ventricle. More recently, novel echocardiographic techniques, and advances in computed tomography and magnetic resonance imaging, in particular, have expanded our diagnostic armamentarium and provided new insights into the anatomy and function of the pulmonary circulation in both health and disease. This article contains a review of the current status of techniques for imaging the right side of the heart and the pulmonary circulation.

Key words: Right ventricle. Pulmonary circulation. Imaging

INTRODUCTION

The right heart chambers and pulmonary circulation have important roles in cardiovascular homeostasis both in normal and pathologic conditions. However, changes in their anatomy and function associated with disease have often been considered less relevant than those occurring on the left side, particularly in the clinical setting. One of the reasons for this disparity is the earlier and broader availability of noninvasive imaging modalities for accurate and reliable evaluation of left ventricular performance. For a long time, invasive right heart catheterization and contrast angiography have been the reference standards for the study of the anatomy of the right heart and pulmonary circulation, as well as the hemodynamic impact of different diseases. Over the past 3 decades there has been a progressive shift to a noninvasive approach, particularly because of improvements in echocardiography, computed

Técnicas de imagen en la evaluación del corazón derecho y la circulación pulmonar

Considerados componentes secundarios del sistema circulatorio, los papeles del corazón derecho y la circulación pulmonar en la enfermedad no recibieron la misma atención que sus análogos sistémicos por mucho tiempo. Esto se debió en parte a la dificultad de estudiar estas estructuras con precisión y de forma no invasiva. Durante muchos años la radiografía de tórax y la angiografía invasiva fueron las únicas modalidades de imagen disponibles para evaluar la circulación menor. El desarrollo de la ecocardiografía transtorácica y de técnicas nucleares constituyó un importante avance en imagen no invasiva, particularmente del ventrículo derecho. Más recientemente, nuevas técnicas ecocardiográficas, avances en tomografía computarizada y, particularmente, en resonancia magnética han aumentado nuestro armamento diagnóstico y proporcionan un mayor entendimiento de la anatomía y función de la circulación derecha tanto en la salud como en la enfermedad. En este artículo revisaremos el estado actual de las técnicas de imagen en el corazón derecho y la circulación pulmonar.

cardiac axis and lateral displacement of the RV outflow tract, which results in a more prominent "pulmonary artery" (PA) contour in the left cardiac border. A radiographic examination can potentially identify signs of underlying etiologies, such as lung parenchymal abnormalities or left heart disease.

Regarding the pulmonary circulation, enlargement of the central pulmonary arteries can be quantified by adding the horizontal distances of the first divisions of the right and left pulmonary arteries to the midline, divided by the maximum transverse diameter of the thorax. A ratio >0.38 is often indicative of elevated pulmonary pressures.

Diameters of the right and left descending PA diameters above 16 and 18 mm, respectively, on posteroanterior projections also indicate PH.

Whereas peripheral vessel density increases in systemic-to-pulmonary shunts ("shunt vascularity"), a typical finding of advanced PH is reduced peripheral vessel caliber and density ("pruning"). Post-stenotic dilatation of the main and left PA can be present in cases of pulmonary valve stenosis, whereas dilated PA and RV without shunt vascularity or pruning may indicate pulmonary regurgitation. In addition, signs of PH with normal lung parenchyma coupled with regional oligemia are suggestive of chronic thromboembolic PH (CTEPH).

CHEST RADIOGRAPHY

A chest roentgenogram is cheap and widely available, but provides only rough information regarding cardiopulmonary status. Although fairly sensitive, abnormalities in the plain chest x-ray often lack specificity. In the case of PH, radiographic signs tend to correlate poorly with disease severity and commonly occur late in its course. Right atrial enlargement is best noted as a prominently convex lower right heart border, as the posteroanterior projection (Figure 1A). Right ventricular (RV) dilatation is best detected on the lateral view when the cardiac silhouette occupies >40% of the lower retrosternal space (Figure 1B). In addition, enlargement of the right heart chambers leads to posterior rotation of the cardiac axis and lateral displacement of the RV outflow tract, which results in a more prominent "pulmonary artery" (PA) contour in the left cardiac border. A radiographic examination can potentially identify signs of underlying etiologies, such as lung parenchymal abnormalities or left heart disease.

ECHOCARDIOGRAPHY

Echocardiography is the most commonly used imaging modality for routine clinical evaluation of the RV since it is widely available, extremely safe and relatively inexpensive. The combination of 2-dimensional (2D), M-mode, and Doppler echocardiography allows for the simultaneous
evaluation of ventricular and valvular function. It provides invaluable hemodynamic information with flow characterization of both the left and right heart. However, it is not well suited for studying the pulmonary vasculature (except for the main PA). In addition, standard techniques are limited due to the complex RV geometry, retrosternal position, and the marked load dependence of RV function indices, so quantification is often only an estimation. Recent advances may be used for complementary information beyond standard 2D measurements (Table 1).

**Standard Echocardiographic Modalities**

*Evaluation of the RV*

Accurate evaluation of RV size, volume and, contractility requires a complete set of 2D standardized images. Typical changes in RV volume and/or pressure overload include right atrial and RV enlargement, with or without RV hypertrophy, and RV systolic dysfunction (Figure 2A). A value of end-diastolic free RV wall thickness above 5 mm (measured in the subcostal 4-chamber view) indicates hypertrophy and is strongly associated with chronically increased afterload.

RV volumes are difficult to quantify because of the complex RV geometry and the difficulty of tracing the markedly trabeculated endocardial surface. Hence, 2D methods that rely on Simpson's formula suffer from lack of standardization and tendency to underestimate volumes. Instead, visual estimation of the RV size relative to the left ventricle or measurements of transverse and longitudinal diameters are usually performed. An abnormal pattern of interventricular septal motion (leftwards displacement in systole or diastole when pressure or volume overload, respectively, are present) reflects RV hemodynamics. Left septal bowing leads to ventricular under-filling and reduced stroke volume, even in the presence of normal systolic function (Figure 2A). The left ventricular eccentricity index is calculated as the ratio of the antero-posterior to the septal-lateral short axis cavity dimension of the left ventricle. A value of 1 is considered normal, and an elevated diastolic eccentricity index has been associated with death or pulmonary transplant in idiopathic PH. In addition, the degree of septal curvature and its relation to the left ventricular free wall curvature can be used to estimate transseptal pressure gradients and RV systolic pressures.

Longitudinal displacement of the RV annular segment toward the apex (tricuspid annular peak systolic excursion [TAPSE]) is measured with M-mode in the apical 4-chamber view. A value of <1.8 cm indicates RV dysfunction and has been shown to predict survival in PH. This method shows a strong correlation with radionuclide angiography RV ejection fraction even though it correlated poorly with MRI. Although fast and simple, it is a one-dimensional approach, reflecting mostly regional (basal) RV systolic function. Assessment of RV outflow tract fractional shortening may add value to TAPSE. Right ventricular fractional area shortening is defined as the percentage of change in the RV chamber area in an apical 4-chamber view during the cardiac cycle, and seems to correlate best with MRI. The Tei index or RV myocardial performance index is a global assessment of both RV systolic and diastolic function. It is the ratio of the total RV isovolumetric time (isovolumetric contraction plus relaxation intervals) divided by the RV ejection time. The normal value is 0.28 (0.04), and an elevated index (≥0.83) is associated with increased cardiac mortality and lung transplantation in PH patients. The Tei index has shown significant correlation with RV ejection fraction by nuclear ventriculography and has been reported to be less affected by loading conditions or heart rate.

### Table 1. Echocardiographic Methods for the Assessment of the Right Ventricle and Pulmonary Circulation

<table>
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<th>Method</th>
<th>Description</th>
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<td>M-mode echocardiography</td>
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<td>Strain imaging</td>
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<td>Intracardiac echocardiography</td>
<td>Dp/dt indicates delta pressure/delta time; RV, right ventricle; TAPSE, tricuspid annular plane systolic excursion; TDI, tissue Doppler imaging.</td>
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Hemodynamic Assessment of the Pulmonary Circulation

Doppler echocardiography allows non-invasive estimation of PA pressures and vascular resistance. The tricuspid regurgitant jet is the most common method used in clinical practice to calculate the systolic PA pressure via the Bernoulli equation as $4v^2 + \text{right atrial pressure}$ (Figure 2B), where $v$ is the peak regurgitant velocity (m/s). Right atrial pressure is estimated from the inferior cava vein diameter and its respiratory changes. A diameter in long axis <1.5 cm with normal respiratory variation (~50%) corresponds to right atrial pressures <10 mm Hg.6

The peak velocity of the pulsed-Doppler envelope and the time to peak flow acceleration (acceleration time) in the main PA are reduced in PH. A shorter acceleration time (<100 ms) is suggestive of PH and a time <62 ms has been correlated with worsened survival in idiopathic PH.17 However, the acceleration time is dependent on heart rate and cardiac output and has to be interpreted with caution. Diastolic PA pressure can be determined also from $4v^2 + \text{right atrial pressure}$, with $v$ being in this case the velocity of the end-diastolic pulmonary regurgitant jet. Another contribution of Doppler echocardiography is the estimation of pulmonary vascular resistance, which is calculated as the ratio of tricuspid regurgitation velocity (m/s) to the velocity-time interval (cm) of the RV outflow. It has been validated in children with severe PH18 and has shown excellent correlation with invasive measurements.19 In addition, exercise echocardiography may reveal exercise-induced increments in systolic PA pressure, a finding that might represent early stages of pulmonary vascular disease.20

Emerging Echocardiographic Techniques

Three-Dimensional Echocardiography

In recent years, the introduction of new matrix transducers as well as advances in image acquisition and analysis have permitted real-time 3D echocardiography in the clinical setting.21 There are still limitations related to limited temporal resolution of real-time imaging or the need to average 4-7 cardiac cycles with full volume imaging, which may cause artifacts in cases of arrhythmia.

Direct visualization of the entire RV with 3D echocardiography is possible using the full volume mode acquisition. This capability is particularly attractive for the RV as it has the potential advantage to measure cardiac chambers without geometric assumptions. Both older22 and more recent real-time 3D techniques23,24 have been validated for RV volume quantification, information that can provide important prognostic information in various clinical scenarios such as congenital heart disease.25 The non-planar geometry of the tricuspid valve has been demonstrated, as well as how RV contributes to functional tricuspid regurgitation.26-27 Multiplane reconstruction analysis allows accurate evaluation of segmental RV geometry and function (Figure 3). In chronic PH secondary to left-sided heart disease, RV dilatation has been reported to occur mainly in segments closest to the outflow tract (basal and mid levels).28

Tissue Doppler Imaging (TDI)

Pulsed-wave Doppler at the tricuspid annulus can be used to measure low-frequency systolic and diastolic velocities that reflect longitudinal RV myocardial motion. Spectral TDI quantifies peak
Color TDI acquires color-coded images of the RV and represents the average velocities within a specific region of interest (Figure 4B). It allows simultaneous evaluation of the annular, basal, mid, and apical segments within the same cardiac cycle in a reproducible fashion. Color TDI improves spatial resolution of RV wall motion. However, it still remains largely a research tool because of angle and frame rate dependence, complex post processing, low temporal resolution and relative lack of experience.

Myocardial acceleration during isovolumetric contraction is a new parameter of global RV systolic function, calculated as the maximal isovolumetric myocardial velocity divided by time to peak velocity. It appears to be less load dependent than other methods. Regarding RV diastolic function, TDI may be a useful modality, combined with pulsed wave Doppler analysis of the tricuspid inflow, allowing the quantification of the E/A, E'/E' and E'/A' ratios. Isovolumetric relaxation time can be considered a simple method to estimate systolic PA pressure when corrected for heart rate, and has been shown to increase progressively in the presence of PH. However, results should be interpreted with caution when RV function is impaired.

velocities (Figure 4A). There are good correlations between TDI of the tricuspid annulus and RV ejection fraction quantified by radionuclide angiography. A value of S’ below 9.7 cm/s suggests abnormal RV contractility, and may be useful in the detection of early RV dysfunction. S’ is also lower in patients with idiopathic PH than in normal controls, and is inversely related to PA pressures and resistance. Myocardial acceleration during isovolumetric contraction is a new parameter of global RV systolic function, calculated as the maximal isovolumetric myocardial velocity divided by time to peak velocity. It appears to be less load dependent than other methods. Regarding RV diastolic function, TDI may be a useful modality, combined with pulsed wave Doppler analysis of the tricuspid inflow, allowing the quantification of the E/A, E'/E' and E'/A' ratios. Isovolumetric relaxation time can be considered a simple method to estimate systolic PA pressure when corrected for heart rate, and has been shown to increase progressively in the presence of PH. However, results should be interpreted with caution when RV function is impaired.

Figure 3. Real-time 3D echocardiogram (Full volume mode acquisition) of the right ventricle (RV) reformatted into coronal (A, right), sagittal (A, left), and multiple short-axis views showing segmental geometry of the RV (B, C: apex; D, E: mid; and F, G: basal segments).
Similarly, evaluation of RV dyssynchrony with TDI is still in early stages of development.

**Speckle Tracking**

Speckle tracking analyzes motion by tracking speckles in the myocardium with an algorithm that identifies speckle location on sequential frames (velocity vector imaging) and derives strain values. It is less dependent on 2D image quality, frame rate, and angle, and has the ability to measure RV strain in both long and short axis planes. Several studies have shown that it is a feasible and accurate method to assess global and regional RV function in normal volunteers and PH patients.38,39

**Intracardiac echocardiography**

An intravascular ultrasound catheter can provide 2D, color and pulsed Doppler views of intracardiac structures. Preliminary data on animal models demonstrates the feasibility and accuracy of this technique for quantifying RV volumes, and systolic function.40

**NUCLEAR IMAGING**

Advantages of nuclear techniques include the lack of geometric assumptions for count-based methods of RV ejection fraction quantification, high contrast-to-noise ratio and the possibility of simultaneously evaluating pulmonary ventilation/perfusion (V/Q scan). Main drawbacks include limited spatial resolution, relatively prolonged imaging times and the need for radioisotopes. For the evaluation of the RV, many centers now prefer other modalities such as echocardiography or MRI that do not involve ionizing radiation and provide superior resolution.

First-pass radionuclide ventriculography is based on the detection and quantification with a gamma camera of the transit of a tracer bolus through the RV. The preferred agent is Technetium-99m labelled diethylenetriamine pentaacetate (99mTc-DTPA), which has a fast renal elimination, resulting in less patient irradiation and allowing earlier repetition of the study if necessary.41 A quality bolus injection is crucial to provide enough RV counts, which will affect the accurate determination of RV ejection fraction. Normal values are 52% (6%) with a lower limit of 40%.42 Although limited by imaging in a single plane, it allows good separation of the RV and right atrium, correlates well with right heart catheterization and MRI (although with large limits of agreement) and is considered the nuclear method of choice for RV assessment.43,44

Functional indices can also be obtained during a longer period and from multiple views with equilibrium blood pool scanning (Figure 5), which is technically less demanding. Planar imaging is limited because of overlap of the RV cavity with adjacent structures.
INVASIVE ANGIOGRAPHY

Invasive angiography remains the gold standard for the evaluation of the pulmonary tree but has been largely replaced by noninvasive modalities. Both RV and pulmonary angiography are costly procedures and do not come without risk, with morbidity and mortality rates of 3.5%-6%, and 0.2%-0.5%, respectively. Complications may arise from arrhythmia, acute increase in pulmonary pressures related to acute volume overload or other mechanisms. They appear to be more common in patients with more severe PH and, particularly, more severe RV dysfunction.

RV cineangiograms obtained after the administration of contrast through a pigtail or similar catheter (Figure 7) can provide accurate estimations of RV volumes and function applying Simpson’s rule or simplified analytical approaches. These methods are nonetheless limited by the need for geometrical assumptions. Invasive pulmonary digital subtraction angiography (DSA) can be employed in the diagnosis of acute pulmonary embolism when results are inconclusive, although this is an infrequent indication. However, DSA is commonly performed in the setting of chronic PH after a positive result in the lung V/Q scan. Typical angiographic signs of CTEPH include: abrupt branch tapering, complete vessel occlusion, luminal irregularities caused by mural clot, pouch-like regions related to occlusive or sub-occlusive thrombi, and webs or bands that may cause branch narrowing and post-stenotic dilatation (Figure 8). It should be considered in patients with unexplained dyspnea and segmental or larger defects on ventilation-perfusion scanning.
especially if there is echocardiographic evidence of right atrial enlargement or RV dysfunction. The purpose of DSA is to confirm the diagnosis of CTEPH and to define the location of occlusive emboli, as successful thromboendarterectomy is more likely if thrombi involve the main, lobar, or proximal segmental arteries. DSA is still considered the procedure of choice for presurgical planning over alternative noninvasive modalities such as MRI and CT. Inconclusive results of DSA may lead to further preoperative invasive imaging with fiberoptic angioscopy.

**COMPUTED TOMOGRAPHY**

Contrast-enhanced CT angiography is the most commonly used technique for the evaluation of the pulmonary vasculature. It provides 3D datasets with excellent isotropic spatial resolution (submillimeter with current multidetector scanners) in very short scanning times. In addition, the lung parenchyma can be simultaneously evaluated and electrocardiographic gating allows for the study of cardiac function. On the other hand,
CT can reveal other findings suggestive of PH, regardless of the underlying etiology. The degree of enlargement of central pulmonary arteries correlates moderately with disease severity and a ratio between the main PA and the ascending aorta diameters >1 or a main PA diameter ≥29 mm are fairly specific markers of PH, although with limited sensitivity. An asymmetrical dilatation of the main arteries suggests CTEPH. In patients with an acute pulmonary embolism, a right-left maximal ventricular diameter ratio >0.9 in a reformatted 4-chamber view independently predicts 30-day mortality. Image acquisition with retrospective electrocardiographic gating has been validated as an accurate modality for the quantification of RV volumes and function in comparison with magnetic resonance (Figure 10), although at the expense of increased radiation dose. The feasibility of detecting RV dysfunction in patients with an acute pulmonary embolism has been reported; however, its prognostic significance or its application in chronic PH have not been tested systematically.

Simultaneous evaluation of the lung parenchyma may point to specific etiologies of PH, as many features of obstructive or interstitial lung disease can be readily identified. A mosaic pattern in combination with signs of PH may be caused...
by heterogeneous lung perfusion and is highly suggestive of CTEPH.\textsuperscript{71} Prior pulmonary infarcts (wedge-shaped consolidations in subpleural regions) also support the diagnosis of CTEPH.\textsuperscript{65} Other indirect signs of increased systemic venous pressure include enlargement of the superior and inferior vena cava, ascitis and pericardial and/or pleural effusions.

**MAGNETIC RESONANCE IMAGING**

MRI has evolved in the last decade as one of the most attractive imaging modalities for the study of both the right heart and the pulmonary circulation.\textsuperscript{72,73} It has a good balance of high spatial, temporal and contrast resolution, can obtain images in any desired orientation and has no “acoustic” window limitations. It is also safe, highly accurate and reproducible, and provides both anatomic and functional information. The main limitations are cost, less widespread availability and experience, and constraints related to the magnetic field. In addition, there has been concern on the potential association of gadolinium contrast agents with nephrogenic systemic fibrosis. In the specific context of PH, MRI has limited ability to quantify PA pressures.\textsuperscript{74}

**Evaluation of the RV**

MRI is currently considered the gold standard for the quantification of RV volumes and ejection fraction.\textsuperscript{31} The most commonly employed approach is to apply Simpson’s method to a stack of contiguous short-axis cine loops acquired from base to apex. This approach is highly accurate and does not rely on geometrical assumptions.\textsuperscript{73,75} The good interstudy reproducibility also indicates a role for MRI in the serial follow-up of patients, for example in the evaluation of the effect of therapies.\textsuperscript{76} The degree of RV dilatation, hypertrophy and systolic dysfunction is directly proportional to the severity of PH. As an example, an RV to left ventricular mass ratio >0.6 detects PH with a sensitivity of 84\% and a specificity of 71\% and has been reported to be more specific than Doppler echocardiography.\textsuperscript{77} Importantly, quantification of RV functional parameters appears to add prognostic information, as demonstrated in a study of patients with idiopathic PH in whom a RV end-diastolic volume index $\geq$84 ml/m$^2$ and a left ventricular end-diastolic volume index $\leq$40 ml/m$^2$ were independent predictors of 1-year mortality.\textsuperscript{78} More sophisticated analyses of RV performance can be obtained with simultaneous quantification of pressures with MRI-compatible catheters to derive RV volume/pressure loops.\textsuperscript{79} Myocardial tagging has been used to detect abnormal regional RV strain patterns in PH, although this technique has limited resolution and requires long post-processing times. These limitations can be overcome with recently developed strain encoded MRI.\textsuperscript{80} Alternatively, myocardial velocities can be quantified using phase-contrast imaging in a manner similar to DTI.\textsuperscript{81}

MRI can also be employed to quantify interventricular septal curvature (Figure 11), defined as the inverse of the radius. End-systolic septal curvature correlates strongly with PH severity and leftwards septal bowing has been associated with a systolic PA pressure $>67$ mm Hg.\textsuperscript{82} The comparison between the curvatures of the interventricular septum and of the left ventricular free wall can also be used to quantify systolic PA pressure accurately.\textsuperscript{83} Delayed contrast enhancement indicative of fibrosis can be noted in PH patients at the level of the septal insertion sites of the RV and often extending into the interventricular septum, a finding associated with paradoxical septal motion. The extent of fibrosis correlates directly with the degree of PH and is seen irrespective of PH etiology, probably representing increased mechanical stress at these points.\textsuperscript{84,85} It is currently unknown if this finding carries clinical implications.

**Evaluation of the Pulmonary Circulation**

High-resolution magnetic resonance angiography can be employed to detect pulmonary emboli or
depict typical features of chronic PH or CTEPH.\textsuperscript{86,87} Using this approach, preliminary reports suggest good diagnostic accuracy for the differentiation of idiopathic PH from CTEPH.\textsuperscript{87} In comparison with DSA and CT, MRI offers excellent depiction of proximal abnormalities but accuracy is lower for subsegmental vessels.\textsuperscript{49,86,87} In addition, time-resolved magnetic resonance angiography can be used to visualize the dynamic passage of the contrast through the lung vasculature (Figure 12). This technique can be employed to analyze lung perfusion in a manner that correlates well with nuclear scintigraphy.\textsuperscript{87,88} Preliminary data also demonstrates the feasibility of evaluating lung ventilation with MRI.\textsuperscript{89}

Figure 11. Short axis view obtained with magnetic resonance imaging in a patient with severe pulmonary hypertension. There is severe enlargement of the right ventricle which leads to septal bowing (arrow) and compression of the left ventricle.

Measurement of blood velocities with phase-contrast imaging is usually employed for the accurate quantification of systemic-to-pulmonary flow ratios in patients with congenital shunts.\textsuperscript{90} In addition, flow profiles in the cross-section of the main PA or its branches can be accurately characterized.\textsuperscript{91} Numerous parameters have shown correlation with the degree of elevation of PA pressures and/or resistance, although with inconsistent results.\textsuperscript{74} An average blood flow velocity <11.7 cm/s provides a sensitivity and specificity of 93\% and 82\%, respectively, for the detection of PH.\textsuperscript{92} More recently, 3D phase-contrast MRI revealed abnormal patterns in the main PA (end-systolic flow vortices and prolonged anterograde diastolic flows) that enabled differentiating patients with PH at rest, exercise-induced PH or no PH.\textsuperscript{93} In addition, phase-contrast or cine imaging can be employed to evaluate dynamic changes in arterial cross-section and therefore pulmonary artery stiffness.\textsuperscript{94} A change in PA cross-sectional area <16\% during the cardiac cycle was identified recently as a predictor of mortality in PH.\textsuperscript{95} Also, alterations in PA elasticity can be detected before overt PH occurs and these may be useful for early detection of abnormal circulatory physiology.\textsuperscript{96}

CONCLUSION

Several imaging modalities can be combined today for the evaluation of the right heart and the pulmonary circulation. Advances in nuclear techniques, echocardiography, CT and MRI have expanded our understanding of the crucial roles of the minor circulatory system in many pathologic states. Continuing technological advances, particularly in the field of MRI, promise to further improve our ability to detect early disease stages or evaluate the mechanisms of action and efficacy of novel therapeutic interventions. Advances in molecular imaging with any of these modalities will be of particular interest in the evaluation of multiple biological processes in vivo.
REFERENCES


