Research Toolbox for Peripheral Arterial Disease
— Minimally Invasive Assessment of the Vasculature and Skeletal Muscle —

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In 2010, more than 200 million people (3%) were afflicted with peripheral arterial disease (PAD). Because it is atherosclerotic in etiology, it is not surprising that PAD is a leading cause of cardiovascular morbidity. Cardiovascular disease (CVD) risk can be decreased if ambulatory physical function is improved. However, physical function is limited by a mismatch between oxygen supply and demand in the legs, which results in exertional pain, leg weakness, and balance problems. Therefore, a key factor for improving physical function, and decreasing CVD outcomes, is ensuring oxygen supply meets the oxygen demand. The purpose of this review is to highlight and evaluate practical and minimally invasive tools for assessing PAD etiology, with a specific focus on tools suited to studies focusing on improving physical function and CVD outcomes. Specifically, the macrovascular, microvascular, and skeletal muscle pathology of PAD is briefly outlined. Subsequently, the tools for assessing each of these components is discussed, including, where available, the evidence to contextualize these tools to PAD pathology as well as physical function and CVD outcomes. The goal of this review is to guide researchers to the appropriate tools with respect to their methodological design.

Key Words: Arterial stiffness; Circulating angiogenic cells; Endothelial function; Microvascular; Mitochondria

In 2010, 202 million people (3%) were afflicted with peripheral arterial disease (PAD) globally, a 23.5% increase from 2000. Moreover, the prevalence of PAD is rising among all age categories, beginning from 25–29 years. Besides the increased risk of cardiovascular disease (CVD), PAD is associated with decreased quality of life (QOL), and both are in large part attributable to impaired ambulatory physical function. Impairments to physical function include an inability to walk moderate distances and perform activities of daily living. Conversely, improvements to ambulatory physical function can decrease CVD risk and enhance QOL. The key to improving physical function is matching oxygen supply to increased oxygen demand. There has been growing interest in PAD and physical function. A PubMed search, performed 4 June 2018, using the Boolean operators (“peripheral arterial disease” OR “peripheral artery disease”) AND (“physical function” OR exercise OR “physical activity” or “activities of daily living”), returned 1,719 articles, which represents 12.5% of all PAD-focused articles (n=13,703). Moreover, the number of physical function-associated articles increased from 13 in the year 2000 to 158 in the year 2017. Considering the growth in literature, there is a clear need to summarize the tools available to researchers interested in improving physical function and CVD outcomes in PAD.

The purpose of the current review is to highlight practical and minimally invasive tools available to researchers interested in improving physical function and CVD outcomes in those with PAD. The goal of this review is not to summarize the literature on physical function and PAD pathology per se. Rather, the goal is to guide researchers to the appropriate tools with respect to their methodological design. Specifically, this review will briefly outline the macrovascular, microvascular and skeletal muscle pathology of PAD, and then contextualize the available research tools (Table, Figure) using available evidence.

PAD Pathology

In subjects with PAD, reduced physical function results from an impaired oxygen and nutrient supply (e.g., glucose/insulin) supply to the lower extremity musculature, following insufficient blood flow and perfusion. Inadequate oxygen and nutrient supply reduces oxidative phosphorylation of adenosine triphosphate (ATP) and places greater demand on anaerobic processes. Although the reduced oxygen supply is typically attributed to a progressive narrowing of the
conduit (macrovascular) arteries (Figure), a dysfunctional microvascular system likely further limits delivery of oxygen and nutrients to active skeletal muscle. Additionally, impaired skeletal muscle mitochondrial respiratory capacity may limit the ability to extract and utilize oxygen for aerobic metabolism.

Macrovascular Pathology
Blockage of one or more conduit arteries supplying the leg(s) leads to impaired delivery of oxygen to lower extremity muscle. Blockages typically occur at sites of geometric irregularities, such as the iliac artery branching to the femoral artery, or the femoral artery branching at the knee. These geometric abnormalities produce disturbed shear stress profiles, which then makes the site more prone to endothelial dysfunction, and subsequently to atherosclerosis. In the short- to medium-term, arterial stiffness, which is a dynamic property based on both vascular function and structure, will result. Longer term, endothelial dysfunction may lead to increased lipoprotein permeability, foam cell formation, T-cell activation, and smooth muscle migration into the arterial wall, which manifests as increased intima-media thickness (IMT). If these conditions persist, fatty streaks progress and plaques may become vulnerable to rupture. Ruptured plaque content may enter the systemic circulation, potentially leading to thrombotic vessel occlusion, including in the coronary (myocardial infarction) or cerebral (stroke) circulation.

Microvascular Pathology
Following the passage of oxygen and nutrients through the macrovascular system, the microvascular network must be recruited. The major function of the microvascular network is to provide an exchange surface area for adequate delivery of oxygen, nutrients, and hormones, as well as removal of metabolic waste. The microvasculature may limit vascular oxygen delivery if (1) capillary density is decreased or (2) there is poor capillary recruitment.

Impaired capillary recruitment may result from diminished nitric oxide (NO)-dependent vasodilation of the feed arteries. In turn, circulating angiogenic cells (CACs) may play a role in regulating NO-dependent vasodilation. First discovered in 1997, CACs are a heterogeneous mix of circulating monocytes, macrophages, and hematopoietic cells that respond to blood flow-induced shear stress. Importantly, these cells express endothelial NO synthase (eNOS) and produce NO, as well as contribute to angiogenesis and healthy function of the intimal layer of blood vessels.

Skeletal Muscle Pathology
Limited oxygen supply is not the only cause of decreased muscle functional capacity in PAD. Indeed, both in vivo and in vitro evidence demonstrate that even when abundant oxygen delivery is artificially permitted, there is reduced oxygen utilization in PAD skeletal muscle. Nevertheless, impaired oxygen supply is the first step in a cascade of events that likely lead to impaired muscle oxygen utilization and functional capacity. When subjects with PAD are at rest, the metabolic demands of lower extremity muscle are low, and oxygen delivery is sufficient. However, when metabolism reaches a critical threshold, such as during physical exertion, a mismatch between supply and demand results in muscle ischemia. This may lead to ischemia-reperfusion cycles with reactive oxygen species (ROS) production and subsequent mitochondrial damage, limiting the ability of skeletal muscle to utilize oxygen.

Following ROS damage, several changes can be observed...
Minimally Invasive Assessment for PAD

Assessing Macrovascular Health: Functional

Endothelial Function
Endothelial dysfunction is a pivotal, yet potentially reversible step that precedes and predicts overt CVD. This is because the vascular endothelium is responsible for governing several aspects of vascular homeostasis, including regulating lipoprotein permeability, platelet aggregation and vascular tone. Endothelial function is commonly assessed by measuring the vasodilatory response to chemical and/or physical endothelial-dependent stimuli such as acetylcholine or reactive hyperemia. Vasodilatory responses can be measured using strain-gauge venous occlusion forearm plethysmography, peripheral arterial tonometry, laser Doppler flowmetry, or flow-mediated dilation (FMD).

FMD Using commercial duplex Doppler ultrasound, coupled with dedicated image analysis software (e.g., FMD Studio, QUIPU; or Vascular Tools, Medical Imaging Applications), endothelial function is gauged by measuring the percent increase in brachial artery diameter above baseline following transiently increased shear stress during reactive hyperemia. Meta-analytic findings indicate that, after adjusting for confounding risk factors, a 1% increase in brachial artery FMD equates to a 13% (95% confidence interval (CI): 9–17%) reduction in the risk of future CV events.

In PAD muscle, some of which are likely to be compensatory mechanisms for decreased oxygen availability. Examples include changes in fiber type distribution, capillary networking or increased mitochondrial density. However, the findings are mixed and can be counterintuitive. For example, mitochondrial density increases with PAD severity. This unexpected finding may be explained by impaired mitophagy, which in turn would explain why mitochondrial density exhibits a U-shaped relationship with survival in PAD. Moreover, this finding suggests that mitochondrial respiratory capacity is a better indicator of the ability to meet oxidative ATP demand than total mitochondrial content in PAD.

Assessing Macrovascular Health: Functional

Microvascular Function

Circulating angiogenic cells

US - Reactive hyperemia
NIRS - blood flow
CEU - blood flow
Damage and Repair

Muscle Perfusion
NIRS - perfusion

Oxidative Function

Muscle

Mitochondria

Figure. Peripheral arterial disease pathology contextualized to available minimally invasive research tools. In order to improve physical function, and subsequently decrease cardiovascular disease risk, metabolically active skeletal muscle requires an adequate oxygen supply. Oxygen is transported through the conduit arteries to feed arterioles, which branch in to capillaries surrounding working skeletal muscle, from where oxygen can be extracted by the mitochondria and subsequently used for aerobic metabolism. Note: macrovascular function tools are numbered with respect to “utility of investigation vs. atherosclerotic change”. CACs, circulating angiogenic cells; CEU, contrast-enhanced ultrasound; eNOS, endothelial nitric oxide synthase; FMD, flow-mediated dilatation; IMT, intima-media thickness; NIRS, near infrared spectroscopy; NO, nitric oxide; PWV, pulse wave velocity; US, ultrasound.

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NO Bioavailability

NO produced by the vascular endothelium performs a myriad of antiatherogenic functions, and disrupted NO production has been implicated in the pathogenesis of CVD. Unfortunately, the rapid metabolism and short half-life of NO in the human circulation makes it impractical to measure directly in vivo. However, protected NO-derived species that are transported through the vasculature can release NO at tissues with a low pO2. A limited number of studies have also related FMD to functional performance, suggesting that FMD is associated with achievable walking distance and daily physical activity. Lastly, acute exercise has been demonstrated to reduce FMD in subjects with PAD, which is contrary to expectations.

Plasma NO2− is commonly measured using the Griess reaction or the superior ozone-based chemiluminescence method.
Assessing Macrovacular Health: Structural

Arterial Stiffness

Arterial stiffness is a general term that collectively describes the distensibility, compliance, and elastic modulus of the arterial vascular system. Generalized stiffening of the arterial vasculature has been observed in subjects with PAD, and it has been suggested that the stiffening of the large arteries contributes to impaired peripheral perfusion and reduced walking ability through both hemodynamic and structural mechanisms. Methodologies for assessing arterial stiffness fall into 3 broad groups; (1) local arterial stiffness as reflected by relating change in the area of an artery to distending blood pressure; (2) central arterial wave reflection assessed by augmentation index (AIx); and (3) regional arterial stiffness measured by pulse wave velocity (PWV). Carotid-femoral (aortic) PWV is considered the “gold-standard” and forms the focus of this section.

PWV

Carotid-femoral (aortic) PWV, the speed at which the forward pressure wave is transmitted between the carotid and femoral arteries, can be measured using dedicated equipment, including oscillographic and tonometric devices. Aortic PWV assessments confer excellent reliability (intra-class correlation coefficient [ICC]: 0.98), and commercial devices (e.g., SphygmoCor, AtCor Medical) automate PWV calculations and compare the outcome to known reference values.

A meta-analysis of 17 longitudinal studies (n=15,877, mean follow-up 7.7 years) reported that for a 1 m/s increase in aortic PWV, the risk of future CV events, CV death, and all-cause death increased by 14%, 15%, and 15%, respectively. However, the use of PWV in subjects with PAD has produced mixed findings. The only known prospective study that followed 177 symptomatic PAD subjects for 4.1 years reported that aortic PWV was not associated with CV or all-cause death. Paradoxically, aortic PWV has been associated with PAD pathology, including impaired microvascular function, as well as with physical function.

IMT

The intima-media complex comprises endothelial cells, connective tissue, and smooth muscle, and is the site of lipid deposition in plaque formation. Assessments of IMT are commonly made using brightness-mode ultrasound. Although IMT has been measured at peripheral sites, the common carotid arteries are typically measured and averaged to infer global atherosclerotic burden. When a standardized protocol is coupled with automated image analysis software (e.g., Carotid Studio, QUIPU; Vascular Tools, Medical Imaging Applications), carotid IMT assessments are highly objective and reliable (ICC: 0.90). Further, age-specific reference values are available.

Carotid IMT assessments are endorsed by the American Heart Association, because IMT is related to traditional CVD risk factors and atherosclerosis elsewhere in the arterial system, and demonstrates a consistent and gradual relation to risk of CV events. A meta-analysis that included 8 general population studies (n=37,197, mean follow-up 5.5 years) reported that for an absolute carotid IMT increase of 0.1 mm, the future risk of a myocardial infarction increased by 10–15%.

IMT has been assessed in PAD. A prospective study (10.3-year follow-up) reported that the incidence of PAD was predicted by a carotid IMT of <0.86 mm vs. <0.66 mm (relative risk: 1.95, 95% CI: 1.20–3.18) in 1,651 previously asymptomatic type 2 diabetic subjects. Two studies have also measured IMT at peripheral sites, with 1 prospective study (n=184 PAD, 4.5-year follow-up) reporting that brachial IMT had greater sensitivity and specificity for predicting death compared with brachial FMD. However, a small (n=20 PAD, n=10 controls) descriptive study reported that brachial IMT was not different between subjects with and without PAD (0.31 mm vs. 0.27 mm, P=0.05, respectively), but that the superficial femoral IMT was enlarged (1.4 mm vs. 0.8 mm, P<0.05).

As such, while there is sufficient evidence to recommend carotid IMT in terms of predicting coronary artery disease, further investigation is required to clarify the value of lower extremity IMT to PAD-based research.

Assessing Microvascular Health: Recruitment

Several techniques have been used to provide a global index of microvascular function, including venous occlusion plethysmography, and ultrasound-based assessment of reactive hyperemia. This section will focus on the latter technique.

Reactive Hyperemia

Using commercial duplex ultrasound, microvascular function can be assessed by monitoring the reactive hyperemia response to ischemia, induced by inflating a tourniquet to a suprasystolic pressure for 5 min. Reactive hyperemia is induced through dilation of the microvasculature, with the dilation being dependent on a variety of endothelial and other paracrine mediators, indicating that this is useful as a general vascular metric rather than being endothelial specific. The response can be measured as peak flow velocity, the velocity time integral, systolic/diastolic ratio, or vascular resistance response.

Several studies have examined reactive hyperemia in PAD subjects. The FATE study followed 1,578 middle-aged low-intermediate risk males for 7.2 years, and found that reactive hyperemia was an independent predictor of adverse CV outcomes. Additionally, a prospective study (median follow-up 309 days) that followed 267 PAD subjects reported mixed findings.
Assessing Microvascular Health: Perfusion and Blood Flow

Minimally invasive techniques to investigate human skeletal muscle microcirculation perfusion and blood flow in vivo include microdialysis, magnetic resonance imaging (MRI), near infrared spectroscopy (NIRS), and contrast-enhanced ultrasound (CEUS). Of these, NIRS- and CEUS-based methodologies are the most practical and will be the focus of this section.

NIRS

Modern NIRS devices can record real-time relative or absolute quantities of oxygenated and deoxygenated hemoglobin. Total hemoglobin, the sum of oxygenated and deoxygenated hemoglobin, is a proxy for tissue perfusion. Additionally, the rate of change in total hemoglobin following venous occlusion is used to determine skeletal muscle microvascular blood flow. Briefly, a tourniquet, placed upstream from the site of interest, is inflated to a subdiastolic pressure (e.g., 100 mmHg) to occlude venous outflow while minimally obstructing arterial inflow. Blood flow is then calculated by evaluating the linear increase in total hemoglobin during the first few seconds of the occlusion. Although this blood flow method agrees with traditional measurements using plethysmography and the Fick method, and has been reported to be reliable at rest (ICC: 0.83) and during exercise (ICC: 0.82–0.90), clinically relevant data are not available.

CEUS

CEUS combines traditional ultrasound imaging with intravenously infused contrast. The contrast agents are lipid-coated, perfluorocarbon gas-filled microbubbles. The microbubbles can pass through the circulation, but cannot pass through vessel walls into the extravascular compartments. As the microbubbles pass through a region of interest they are disrupted by high-energy ultrasound pulses, and the time course for refilling the microvascular bed is used to estimate microvascular perfusion and flow velocity.

A limited number of studies have used CEUs with PAD, one of which is the only known study to have investigated the between-day reliability of CEUS-estimated perfusion or blood flow. Time to peak intensity (TTP) and AUC were measured in the calf following 60 s of complete arterial occlusion in subjects with PAD, aged-matched controls, and a group of young, healthy controls. The reliability was poor for AUC (r=0.01–0.80), but acceptable for TTP (r=0.77–0.78). Further, TTP was longer for PAD (22 s) compared with young controls (8.9 s), but not compared with age-matched controls (19.3 s). Similarly, the TTP and blood flow response to exercise onset have been reported to be slower in PAD compared with healthy controls. Of particular interest, 1 study reported that the TTP under resting conditions was decreased (improved) 3–5 months following percutaneous transluminal angioplasty or bypass surgery.

Assessing Microvascular Health: Damage and Repair

CACs

CAC number and function can be assessed after isolating peripheral blood mononuclear cells from whole blood using cell surface markers and flow cytometry. The use of surface markers to identify CACs is an evolving field, but CD34 and VEGFR2 (KDR) are commonly used to identify CACs; additionally, subpopulations of CACs can be identified by CD133, CD31, and CD45. Angiogenic T cells (CD3+/CD31+) and monocytes (CD14+/CD31+) are also populations of interest, as these cells aid in vessel repair and growth.

CAC function is optimally assessed using highly purified populations derived from cell sorting. Immunomagnetic positive selection is an alternative CAC purification method that is simpler and potentially more cost-effective, albeit at the cost of purity and the ability to use multiple markers. Several distinct cultured populations are also available, including CACs (secreting proangiogenic growth factors), endothelial colony-forming cells (high proliferation rates, revascularization capability), and the colony-forming-unit Hill assay (mixed cell colony). The latter assay has been associated with CVD, but contains heterogeneous cell populations. Once isolated or cultured, functional outcomes may include changes in gene and protein expressions, cytokine production, intracellular ROS and NO production, and stimulated capillary formation.

Several studies have measured CACs in PAD subjects. Low CAC counts, in the form of endothelial progenitor cells, have been observed in PAD, and inversely correlated with the ankle-brachial index (ABI) and carotid IMT. Moreover, CAC apoptosis and oxidative stress levels are significantly higher in subjects with PAD. However, this has not been consistently observed, partly because of a lack of a standardized set of cell surface markers to identify these cells. Although PAD-specific evidence is limited, these cells may be important for studies targeting the delivery of oxygen and nutrients to PAD skeletal muscle.

Assessing Skeletal Muscle Health

The gold-standard noninvasive approach for assessing mitochondrial respiratory function is magnetic resonance spectroscopy (MRS)-based assessment of phosphocreatine following brief, rhythmic exercise. This technique provides a picture of mitochondrial respiration in situ. In situ, mitochondrial respiration is dependent on several physiological systems operating within a closed environment (i.e., microvascular perfusion of blood, tissue oxygen extraction, and terminal respiratory chain oxidative coupling). However, this technique does require access to costly equipment. A promising alternative to MRI is NIRS.

NIRS

Operationally, the NIRS-based procedure for measuring mitochondrial respiratory function is similar to that for MRS, in that first order Michaelis-Menten enzyme kinetics are assumed when monitoring ‘recovery’ following brief, rhythmic exercise. Unlike MRS, NIRS monitors the recovery of skeletal muscle oxygen consumption using a series of brief (5–10 s) arterial occlusions, separated by 20–60 s for reperfusion in PAD. Specifically, a NIRS
probes are placed distal to the arterial site and muscle oxygen consumption is calculated from the rate of the change in deoxygenated hemoglobin, assuming that, in the absence of blood flow, muscle deoxygenation occurs solely from oxygen consumption. NIRS-based assessments have been validated against MRS ($r=0.88–0.95$), have comparable reliability to MIRS MRS (ICC=0.93–0.95 vs. 0.90, respectively), and NIRS is relatively inexpensive, portable, and permits use across a greater range of movement and activity types. Currently, PAD-specific data are unavailable.

**Recommendations and Conclusions**

The goal of this review was not to summarize the literature on physical function and pathology per se. Rather, our goal was to guide researchers to the appropriate tools with respect to their methodological design and the limitations of each in assessing physiology. If the goal of said research is to focus on CVD outcomes, there will be a necessary trade-off between ease of use, time required to complete and analyze the test, and validity. For example, a limited number of prospective studies have used PWV, FMD, and IMT to examine CVD outcomes in PAD, and the findings have been mixed. However, of these measures PWV is the simplest to conduct, requires relatively minimal training, and aortic PWV has been associated with PAD pathology, including impaired microvascular function. 

If the research purpose is to investigate the influence of an intervention on a mechanistic pathway, the research tools will need to align with the hypothesized pathology. For example, if the purpose of a study is to chronically elevate antegrade stress in the lower extremities, to improve endothelial function and decrease IMT, endothelial function (FMD), and IMT can be measured using ultrasound, with the caveat that the validity of leg FMD in PAD subjects is questionable. Similarly, if the purpose of a study is to investigate whether an antioxidant cocktail can improve oxygen delivery and utilization, CEUS can be used to assess transient changes in microvascular recruitment, while NIRS can be used to track longitudinal changes in skeletal muscle blood flow and mitochondria respiratory function.

The research tools discussed in this review are by no means exhaustive, but do include those traditionally used in this area, together with emerging but promising tools. Further, we trust the discussion was sufficient to contextualize the value of these tools relative to PAD pathology, prevent selection of inappropriate tools, and provide direction for future research.

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**References**


