



# Future non-invasive imaging to detect vascular plaque instability and sub-clinical non-obstructive atherosclerosis

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## Abstract

Atherosclerosis underlies the major causes of death in the Western World. Our main goal is to detect early changes of atherosclerosis and to identify subjects at highest cardiovascular risk that may aid in the development of prevention approaches and better management that will decrease cardiovascular morbidity and mortality. The new methods that are of interest include the advanced vascular ultrasound methods, the infra red and near infra red imaging techniques, the EndoPat device that reflects peripheral arterial tone, the electron beam computed tomography, the magnetic resonance imaging, and the molecular imaging techniques. In this review we will focus on the future of advanced imaging techniques that are being developed to detect early (pre-clinical) development of atherosclerosis.

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## 1 Introduction

Atherosclerosis is the major cause of myocardial infarction, stroke, and other occlusive vascular diseases in the Western World.<sup>[1]</sup> Although there have been improvements in the manifestations of atherosclerosis, most of them are due to life style modifications such as, cigarette smoking reduction, better eating habits and knowledge, increased physical activity, and generally speaking an improvement in risk factor management.<sup>[2]</sup> Still, cardiovascular death is the leading cause of death, and more than 50% of the newly diagnosed cardiovascular patients did not have the traditional risk factors, there seem to be a “lag phase” between early undetected atherosclerotic vascular inflammatory processes and the eventual clinical syndromes and cardiovascular death.<sup>[3]</sup>

Endothelial dysfunction may be detected in childhood<sup>[4,5]</sup> with atherosclerotic plaques already detected in many adolescents and young adults.<sup>[6]</sup> It may suggest that early intervention and management of traditional risk factors may change the clinical outcome later on. Such functional events start with decreased local availability of endothelial derived

nitric oxide, enhancement of cell adhesion molecules expression on endothelial and surfaces of white blood cells, increased markers of inflammation, cytokines and growth factors, and oxidative stress and radicals that build the atherosclerotic plaque.<sup>[7-9]</sup> Structural changes in the vessel wall occur later, starting with vessel wall thickness and calcification that can be detected by ultra sound and computed tomography.<sup>[10-13]</sup>

## 2 Functional bio-assays for a non-invasive arterial endothelial function testing

Endothelial dysfunction is an early event in the development of atherosclerosis and in the progression to clinical syndromes of ischemia. The first description of an *in vivo* method to study endothelial function was published by Ludmer *et al.*<sup>[14]</sup> in 1986. However, this technique is an invasive procedure that is performed during coronary catheterization with the infusion of pharmacological agents.<sup>[15]</sup> Other disadvantages are the fact that it is not physiological, because using different pharmacological agents may change the natural physiology.<sup>[16-20]</sup> The next advance was described first in 1992, where an *in vivo* measurement of endothelium-dependent dilatation of peripheral arteries (as a surrogate for the coronary circulation) was made using a high-resolution external vascular ultrasound to measure changes in arterial diameter in response to reactive hyperemia. This response is achieved by inflating a pneumatic cuff distal to the imaging site to supra-systolic pressure for 5 min

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resulting in transient ischemia distally. After deflation, an increased flow causing a high shear stress enhances the activity of the enzyme endothelial nitric oxide synthase (e-NOS) to produce more nitric oxide (NO) from L-arginine. The method is based on measurements of the NO dependent flow mediated dilatation (FMD%) of the brachial artery after the ischemic trigger, with the exception that the diameter of the brachial artery will increase 10% and more in healthy subjects with an intact endothelium.<sup>[21]</sup> The response (FMD%) has been shown to correlate with the endothelial function measured invasively in the coronary arteries<sup>[22]</sup> and with the severity of coronary artery disease.<sup>[23]</sup>

### 2.1 Endothelial activation and atherosclerosis

The basic process involved in atherogenesis is the switch in signaling from an NO-mediated silencing of cellular processes to activation by redox signaling. Reactive oxygen species (ROS) lead to generation of hydrogen peroxide, which react with protein and alter their function.<sup>[24]</sup> e-NOS, which normally maintains the endothelium in a quiescent state can switch to generate the ROS part of the endothelial activation.<sup>[25]</sup> In certain situations, chronic ROS production may exceed the capacity of anti-oxidation, and thus contribute to vascular disease by inducing sustained endothelial activation. For example, ROS production is balanced by the mitochondria, however in obesity-related metabolic disorders or in diabetes mellitus, this balance of oxidative phosphorylation is impaired.<sup>[26]</sup> Other ROS sources include nicotine amide adenine dinucleotide phosphate oxidases and xanthine oxidase.<sup>[27,28]</sup> Inflammatory cytokines and growth factors can also initiate ROS signaling and the activation of white blood cells' activation and interaction with the endothelium.<sup>[29]</sup>

It is believed that there are two mechanisms of repair, namely adjacent endothelial cells and remote stem cells that are transferred to repair the damaged endothelium. In the presence of risk factors, loss of endothelial integrity will develop if there are no local repair cells nearby that can be recruited just for that mission.<sup>[30]</sup> Circulating endothelial progenitor stem cells (EPCs) are a "back-up" mechanism for maintenance and repair of the endothelium.<sup>[31]</sup> Mobilization of these cells is NO-dependent and this mechanism is impaired in patients with cardiovascular risk factors.<sup>[32]</sup> Factors that enhance endothelial function and NO-bioavailability, like statins and exercise, may do it through enhanced mobilization of EPCs from the bone marrow niches to the peripheral blood.<sup>[33-35]</sup>

### 2.2 The brachial artery method

An ideal test should be safe, non-invasive, reproducible,

repeatable, cheap, and standardized internationally. Endothelium-dependent vasomotion is the most widely used clinical tool to assess endothelial function. The basic mechanism of the test is a release of an NO molecule and other vasoactive compounds that dilate the brachial artery. The bioavailability of NO is reflects the vascular tone, but also other NO functions like platelet adhesiveness, cell adhesion, and cell proliferation. The test began with experiments in the 1980's conducted by Furchgott and Zawadzki demonstrating that an intact endothelium responds to acetylcholine by vasodilatation through NO release, but also causes vasoconstriction in subjects with endothelial dysfunction.<sup>[36]</sup>

To assess NO bioavailability, several clinical methods were developed, all of them had an invasive nature (pharmacological medication injections and invasive intra-arterial pressure measurements), until a non-invasive method was reported in 1992. This is the non-invasive ultrasound based test to assess arterial vascular function in the systemic circulation. The brachial artery diameter is measured before and after an increase in shear stress (induced by reactive hyperemia). This dilatation is called FMD and is expressed in percent of change, i.e., the ratio between the difference in the diameter and the baseline diameter (FMD%). A normal response is considered when a 10% change is observed. Technically, a sphygmomanometer cuff is placed on the forearm distal to the brachial artery and inflated to 50 mmHg above the systolic blood pressure for 5 min. After deflation, there is an increased shear stress in the artery, causing enhanced NO bioavailability and flow mediated dilatation of the brachial artery. Brachial artery FMD has been studied extensively and its advantages include the fact that it is a non-invasive, non-painful test, does not involve injection of any pharmacological agents, can be done bed side while the patient is relaxed and comfortable, and can be used for cardiovascular risk assessment but also for management success and can be used as a guide for further interventions. This test represents the gold standard for clinical research of conduit artery endothelial biology and enables epidemiological cardiovascular research in all groups, including children and old patients, mobile and bed ridden as well. There are, however, some limitations and practical challenges. For example, it has to be performed early in the morning because of a diurnal variation, while the patient is fasting, did not have a nitrite rich diet in the last 3 days, and without taking the vasodilator medications or drinking coffee on the morning of the exam. Other challenges include the need for highly trained operators, the expense of the equipment, the care required to minimize the effect of environmental or physiological influences, such as

exercise, eating, caffeine ingestion, and variation in temperature.<sup>[37]</sup>

### 2.3 Assessing endothelial vasodilator function with Endo-peripheral arterial tone (PAT)

The digital pulse volume signal is measured by the Endo-Pat device and reflects PAT. PAT was developed as part of a trend to develop noninvasive methods that would be widely applicable, reproducible, and informative relative to test endothelial function. Measurement of ultrasound based flow mediated dilatation in the brachial artery is also non-invasive, relatively repeatable and reproducible, reflects important biology, and the data can predict future clinical outcome. The test is also significantly correlated with the coronary circulation.<sup>[38]</sup> Still, this method is technically demanding, requires specific training, is very sensitive to factors that affect the vascular tone and function (viral disease, heavy meal, coffee) and has a circadian pattern.<sup>[39]</sup> Measuring digital reactive hyperemia PAT (RH-PAT) includes measuring arterial pulsatile volume at rest and during conditions of increased shear stress that result in a release of NO and other mediators that affect vascular tone and homeostasis. Systemic influences are controlled by simultaneous measurement of PAT without reactive hyperemia in the contra-lateral index fingertip. Endogenous NO-mediated vasoregulation is particularly prominent in the anastomoses of the arterial-venous connections of the fingertips,<sup>[40]</sup> and has been shown that 60% of the vascular response in that area is mediated by NO release.<sup>[41]</sup> One of the big dilemmas is the issue of reliability and the clinical/practical implications of an investigational tool, and the ability to use it repeatedly, easily and be cost-effective. Clinical studies, however, that investigated the relationship of digital pulse amplitude to clinical risk factors included small, selected samples. Impairment in the hyperemic response has been shown to correlate with coronary artery endothelial dysfunction,<sup>[42,43]</sup> and one large-scale study that included 1957 subjects (the Framingham Third Generation Cohort participants) observed an obesity-related decrease in PAT ratio (that was greater in men).<sup>[44]</sup> There was a clear gender effect – so that women had a higher digital pulse amplitude than men, and this effect was intensified with age, but was inversely related to multiple risk factors (diabetes mellitus, body mass index, higher cholesterol concentration and smoking).

Others have reported that a reduced PAT response predicted abnormal coronary endothelial function, with improvements in PAT hyperemic response after treatments that were beneficial for cardiovascular health.<sup>[45,46]</sup> On the other hand, there are still some unsolved issues that do not

add to the reliability of that test, for example, no significant relationship was found between hypertension and digital vasodilation,<sup>[47]</sup> or between systemic inflammation (CRP) and digital vascular function. Another study that evaluated RH-PAT among subjects with risk factors for atherosclerosis (all had a borderline ankle brachial index ABI between 0.90–1.00) did not show significant PAT values between subjects with different cardiovascular risk factors; however, patients with impaired fasting glucose had slightly lower PAT response than subjects without impaired fasting glucose.<sup>[48]</sup>

## 3 Imaging techniques for detecting early atherosclerosis

### 3.1 The near infra red imaging

Light in the near infra red (NIR) region between 700-900 nm can penetrate deep into living tissue, and may offer a unique opportunity to use near-infra red fluorescence (NIRF) imaging techniques to detect and visualize fluorescent probes *in vivo*. The vascular response to injury is a complex inflammatory response that triggers accumulation of macrophages and lymphocytes altogether with smooth muscle cell proliferation and the building of an atherosclerotic plaque, which is vulnerable and rupture-prone. Molecular imaging in atherosclerosis has evolved into clinical investigation and research with the ability to have an *in vivo* visualization of inflammation. The advent of high resolution optical imaging coupled with activated fluorescent probes led to the development of new intravascular strategies to improve biological imaging of human coronary atherosclerosis. Near infra red fluorescence molecular imaging utilizes excitation light with a defined band width (650–900 nm) as a source of photons that are delivered to an optical agent or fluorescent probe, which emits fluorescence in the NIR window that can be detected using an appropriate emission filter and a high sensitivity charge-coupled camera.<sup>[49]</sup> Epithelial and endothelial barriers may prevent penetration of the imaging dyes which is considered a limiting factor in this imaging approach. Small molecules have a faster blood clearance and with a higher target-to-background ratios.<sup>[50,51]</sup> Nanoparticles are now used to improve labeling of vascular cell adhesion molecules like vascular cell adhesion molecule 1 (VCAM-1), enabling a better resolution and detection by Magnetic Resonance Imaging (MRI). The nanoparticles were internalized into VCAM-1 expressing cells and achieved a 12-fold higher target to background ratio compared with fluorescently labeled antibodies against VCAM-1.<sup>[52,53]</sup> Until now, this approach has not been proven to be beneficial and effective as a non-invasive mo-

dality to detect pre-atherosclerotic vascular inflammation or endothelial function impairment *in vivo* in human subjects.

### 3.2 Preclinical diagnosis of atherosclerosis using Computed Tomography (CT) imaging

Cardiac computed tomography (calcium scoring and coronary CT angiography) has emerged as a reliable non-invasive tool for diagnosis of coronary artery disease and risk assessment/management. Coronary calcium scoring enables the detection and quantification of subclinical coronary disease, and has been shown to identify at risk patients with a greater predictive accuracy than conventional risk factor assessment.<sup>[54]</sup> No contrast is needed and the radiation doses are negligible (about 1 mSv, compared with an average annual radiation exposure of 3 mSv).<sup>[55]</sup> Coronary calcification increases with age, and reports are defined as an absolute score and relative to patient's age and gender of the patient. Recent studies have shown that a coronary calcium score provided additive prognostic information over and above the traditional risk assessment. The Multi-Ethnic Study of Atherosclerosis (MESA) trial of 6,809 patients demonstrated that calcium score was the most accurate predictor for long term cardiac events.<sup>[56-58]</sup> Coronary calcium score has been shown to be a better predictor of cardiac events compared to ultrasound assessment of the carotid intima-media thickness (CIMT) and to high sensitivity C reactive protein for risk assessment of intermediate-risk groups of patients for the prediction of future cardiovascular events.<sup>[59]</sup>

Plaque Burden Quantification\_by coronary computed tomographic angiography (CCTA) is limited by the spatial resolution of modern multi-detector CT (MDCT) systems (0.4-0.5 mm). By histology, vulnerable plaques are lipid rich with a necrotic core that measure around 2-17 m.<sup>[60]</sup> They are usually eccentric and the lipid pools can measure from 1 mm to 5 mm. A total of 1,059 patients that were evaluated for future cardiovascular risk had a CCTA for vessel remodeling and low attenuation plaques. Patients who had eccentric plaques with a lipid core had a 22% incidence of acute coronary syndromes in 3 years.<sup>[61]</sup> Other studies have shown by comparing images of plaques causing more than 50% stenosis using CCTA (with MDCT), cardiac catheterization or intravascular ultrasound (IVUS), that CCTA tended to underestimate the volumes of non-calcified and mixed plaques and overestimate the volumes of hard plaques.<sup>[62]</sup> One of the major disadvantages of CCTA is the ability to detect the borders between the lumen, plaque, and exterior wall. However, a recent study showed that "soft" plaques measured by automated 3-dimensional quantification CCTA had an excellent correlation with

IVUS.<sup>[63]</sup> Software can differentiate between different types of non-calcified "soft" plaques based on attenuation. The automated detection system correctly detected all patients with a > 50% stenosis in any vessel with a 100% sensitivity and 100% negative predictive value.<sup>[64]</sup> A recent meta-analysis included 17 studies found that for CT diagnosis of any plaque, respectively, the mean sensitivity and specificity were 92% and 93%; for calcified plaques 93% and 98%, and for diagnosis of non-calcified plaque (soft plaque) 88% and 92%.<sup>[65]</sup> A much higher sensitivity (95%) and specificity (94%) were achieved using CT scanners with more than 16 rows compared with older generation scanners (only 83% and 92%). The greatest disadvantage in CT imaging to detect coronary artery disease and plaques at risk is the lack of randomized studies that may allow earlier detection and may affect the patient's management and outcome. Recent research has demonstrated that patients came to the emergency room with chest pain and were evaluated by a CCTA, of those who had a negative examination, none died or suffered a myocardial infarction within 30 days. Comparing patients who underwent CCTA in the emergency room with patients who received the traditional management, the patients in the CCTA group had a higher discharge rate, a shorter length of stay, and a higher rate of detection of coronary artery disease.<sup>[66]</sup> With ongoing technical developments, CT may have a better potential to detect plaques at risk, and currently CT should be considered as a non-invasive alternative to IVUS for detecting coronary artery plaques.

### 3.3 Magnetic Resonance Imaging (MRI) of Atherosclerosis

Human studies have shown that MRI can identify the adventitial boundary of vessels walls with high accuracy.<sup>[67,68]</sup> A recent carotid artery study showed that errors in vessel wall volume measurement ranged between 4%-6% depending on contrast media methods that had been used. MRI can also detect the substructures of plaques. Using a new technique (three dimensional time-of-flight (TOF) bright-blood imaging technique), unstable fibrous caps were identified in atherosclerotic human carotid arteries *in vivo*.<sup>[69]</sup> Plaques could be characterized based on their appearance on MR images as being intact and thick, intact and thin, or ruptured. Comparing these plaques to histology, a sensitivity of 81% and a specificity of 90% were observed.<sup>[70]</sup> MRI findings of ruptured plaques were associated with recent transient ischemic attacks or stroke.<sup>[71]</sup> MRI is also able to detect plaque composition, and has detected tissue components including lipid rich necrotic core, hemorrhage, and calcification. Soft plaques could be identified with a sensitivity and specificity of 85% and 92%, respectively.<sup>[72]</sup> Con-

trast enhanced MRI (using gadolinium) improved differentiation of necrotic cores from fibrous tissue<sup>[73,74]</sup> and helped to differentiate between fibrous caps from lipid core. Contrast agents were able to demonstrate plaque inflammation, which was related to the amount of neovascularization. Recent reports have shown that MRI can study the morphology of coronary arteries, especially wall thickness, based on sophisticated imaging sequences with high spatial resolution, black-blood technique, and minimal motion interference.<sup>[75,76]</sup>

### 3.4 Molecular imaging – using the MRI platform

In order to detect atherosclerosis in the very early stages of development and to detect atherosclerosis in deeply located structures, we need to combine different techniques in order to get a better resolution at an earlier stage. Focal inflammation induces the expression of endothelial cell adhesion molecules, including vascular cell adhesion molecules-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and P-selectin – all of them participate in the recruitment and activation of growth factors and peptides that are involved with cell signaling and cell proliferation. These receptors can be activated and immunogenicity can be induced by the use of modified or “humanized” antibodies<sup>[77]</sup> and by using single chain antibodies.<sup>[78,79]</sup> Fluorescence techniques enable a better penetration and a higher resolution of detection of vascular inflammation and plaques that are at risk of rupture. Use of multiple Fluorochromes targeting different molecules opens a new window for new frontiers in imaging. In MRI the image is determined by the density and local environment of water protons. Contrast agents that are designed for MRI exert indirect effect by changing the local environment and the position of these protons. Gadolinium ions are used for contrast imaging, that are chelated into small molecules to attenuate toxicity. This contrast media shortens T1 and enables to get brighter images.<sup>[80]</sup> A higher sensitivity can be achieved by using super-paramagnetic iron oxides that induce proton dephasing (manifested as an intense negative contrast on T2-weighted images). Another advantage of the iron oxides is the ability to enhance the image detected (much greater, about 50 times, than the physical size of the particles).<sup>[81,82]</sup> Nanometer size range iron oxide particles (ultrasmall particles of iron oxide (USPIO)) are used for cellular imaging including for identifying plaque macrophages in humans.<sup>[83,84]</sup> These advanced techniques offers the ability to follow and detect migration of magnetically labeled intravenously administered bone marrow cells to injured arteries.<sup>[85]</sup> Based on that achievement there is now a new technique – “plaque specific imaging of atherosclerosis”. It allows evaluation of the efficacy of different therapies.<sup>[86]</sup>

The future of MRI for clinical application is now aimed in several key areas: (1) Plaque specific MRI of stem-progenitor cells for early detection of diffused atherosclerosis; and (2) Plaque specific gene therapy for atherosclerosis mediated by stem-progenitor cells.

### 3.5 Carotid Intima-media thickness

Carotid intima-media thickness (CIMT) is considered as a surrogate marker for atherosclerosis and can be used to detect an accelerated disease process and the presence of subclinical disease. The test is office based, and may be done by trained clinicians with simple training.<sup>[87]</sup> Increased intima-media thickness is associated with increased risk for MI,<sup>[88]</sup> stroke,<sup>[89]</sup> and cardiovascular death.<sup>[90]</sup> A positive finding of increased thickness was found to add significantly to risk prediction, above the usual Framingham risk factors.<sup>[91]</sup> Advantages of CIMT are that it is made with simple B mode ultrasound, is non-invasive, inexpensive, and can be repeatedly performed for evaluation of interventions effect. As such, it is used as a trial end point in large studies.<sup>[92]</sup>

Measurement of CIMT was accepted by the ACC/AHA guidelines committee as a reasonable for cardiovascular risk assessment in asymptomatic adults at intermediate risk (class IIa).<sup>[93]</sup>

## 4 Summary

The diffuse nature of atherosclerosis creates a unique challenge for early diagnosis and effective preventive treatment. We have described the physiological, functional bioassays for early detection (the Brachial Artery Method for endothelial function evaluation, and the HR-PAT technique), infra red approach (not clinically applicable yet), and the more advanced technologies of computed tomography and magnetic resonance imaging, with the new approach that combines fluorescent tagging of antibodies aimed to detect cell adhesion molecules (that helps to locate vascular inflammation) or using ultra-small particles of iron oxide to detect stem cells on their way to replace a denuded endothelium or to overcome an inflamed atherosclerotic artery. Future goals will be to detect atherosclerosis in the very first stages of its evolution and to follow its development or regression following interventions.

What is the best modality? Functional bioassays that may suggest (without direct imaging) that the patient is at risk, and enable the following of the condition of the patient and the success of an intervention, or alternatively a sophisticated advanced imaging system that will show directly the location of the dangerous plaque that has to be treated. Only future clinical studies will answer this question, and a com-

bination of these two approaches may lead to the “golden tract”.

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