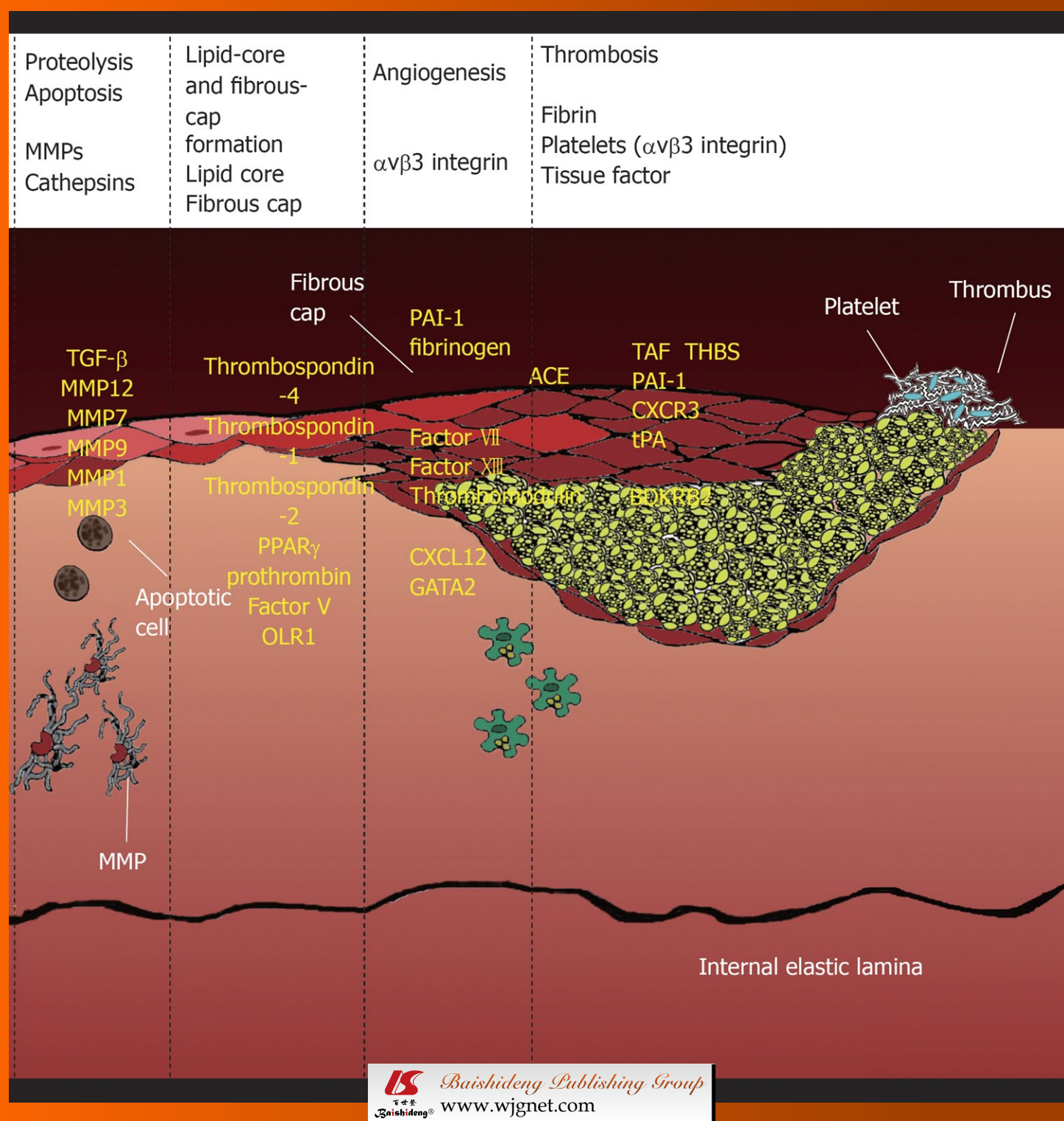
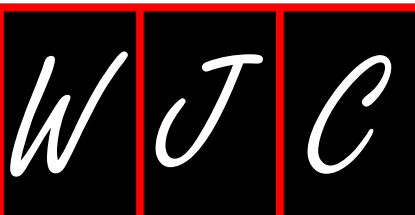


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Acute and recurring pericarditis: More colchicine, less corticosteroids

Paul Farand, Francis Bonenfant, Emilie P Belley-Côté, Nicholas Tzouannis

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INTRODUCTION

Pericarditis is a frequently encountered clinical entity. Studies have shown that pericarditis accounts for 5% of the final diagnoses among patients consulting in the emergency department for non-anginal chest pain^[1]. Few studies have addressed the treatment of acute and recurring pericarditis and, until recently, recommendations were based on a few small studies. Moreover, one of the most complete publications on the subject, the “Guidelines on the Diagnosis and Management of Pericardial Diseases” from the European Society of Cardiology^[2], is based predominantly on expert opinion. However, in the past decade, several clinical studies have been published on pericarditis, mainly from Italian and Israeli researchers. A recent publication in *Circulation*^[3] and a publication from our group^[4] summarized these studies. It is important to note that some of these studies reinforce the current recommendations to use corticosteroids only in cases of treatment failure or intolerance to other treatments and support the use of colchicine, even for a first episode.

This article focuses on the treatment of pericarditis after a brief exploration of its pathophysiology, diagnosis and prognosis. Other pericardial pathologies such as constrictive pericarditis, chronic pericardial effusion and tamponade will not be discussed.

PATHOPHYSIOLOGY

The pericardial space is bordered by two layers: the fi-

Abstract

Acute and recurring pericarditis are frequently encountered clinical entities. Given that severe complications such as tamponade and constrictive pericarditis occur rarely, the majority of patients suffering from acute pericarditis will have a benign clinical course. However, pericarditis recurrence, with its painful symptoms, is frequent. In effect, recent studies have demonstrated a beneficial role of colchicine in preventing recurrence, while also suggesting an increase in recurrences with the use of corticosteroids, the traditional first-line agent.

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Key words: Pericarditis; Colchicine; Corticosteroids; Recurring pericarditis; Pericardium

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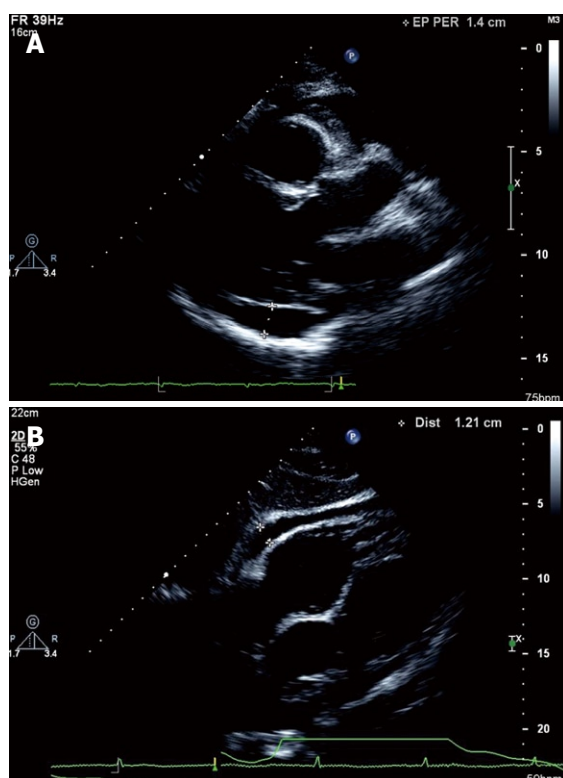


Figure 1 Transthoracic echocardiography. A: Parasternal long axis view showing a moderate pericardial effusion without compression of the cardiac cavities; B: Subcostal view showing a mild pericardial effusion without compression of the cardiac cavities.

brous pericardium and the visceral pericardium. The latter is in close contact with the epicardial fat. In normal conditions, a small amount of liquid (25-30 mL) is present in the pericardial space which prevents friction between the two layers of the pericardium and, as a result, between the beating heart and adjacent structures^[2] (Figure 1).

The most frequent pericardial disease is acute pericarditis. Most cases are idiopathic and assumed to result from viral infection. Other frequent, non-infectious etiologies are autoimmune disease, neoplasia and iatrogenic (i.e. post-cardiac procedures). Pericarditis due to tuberculosis is mainly seen in developing countries^[5]. After years of decreasing prevalence, the frequency of tuberculous pericarditis has been increasing in the context of the HIV epidemic.

Inflammation of the pericardial layers leads to increasing exudation of fluid while decreasing the normal pericardial drainage by the lymphatic ducts. Thus, inflammation often leads to pericardial effusion. Although usually small, if sufficient, this volume of liquid can impinge on the filling of the right cardiac chambers, culminating with tamponade. Pericardial effusion occurring in a context of acute pericarditis is distinguished from chronic effusion by the fact that the effusion regresses when the inflammation resolves and the pericardium resumes its normal functions. On the other hand, chronic inflammation can lead to fibrosis and rigidity of the pericardium, as seen in constrictive pericarditis.

PROGNOSIS

Tamponade and constrictive pericarditis are the most serious complications of acute pericarditis. Tamponade can be life-threatening: the accumulated fluid causes compression of the cardiac chambers, preventing their filling. Prompt treatment is vital and consists in removal of the pericardial effusion, usually *via* pericardiocentesis. In contrast, in constrictive pericarditis, compression of the cardiac cavities results from a stiffened pericardium. Constrictive pericarditis presents with right heart failure and low cardiac output. The definitive treatment of this pathology is surgical removal of the pericardium, a high risk procedure. Fortunately, both of these complications rarely arise following acute or recurring pericarditis such as demonstrated in a review of several clinical studies on pericarditis. This review reports that 3 % of patients with recurring pericarditis evolved towards tamponade and only 1 out of 296 patients developed constrictive pericarditis^[6].

Several studies have tried to identify patients at high risk for the development of complications from an episode of acute pericarditis. Their goal was to determine the patients who would benefit from hospitalization for surveillance. High-risk features included: a pericardial effusion more than 20 mm, risk factors for hemorrhagic pericardial effusion (anticoagulation, neoplasia, accidental or iatrogenic thoracic trauma), temperature above 38°C, myopericarditis, pulsus paradoxus, evidence of systemic inflammation, subacute evolution (in contrast to acute) and, finally, patients with treatment failure^[7]. However, it is important to note that the majority of patients present none of these characteristics. Besides identifying the patients with a higher risk of complications, these characteristics can also help identifying those for whom a precise etiology is more likely to be discovered and who would benefit from a more extensive investigation.

A significant proportion of patients with acute pericarditis also present a certain degree of myocarditis, possibly because they share several common etiologies, in particular viral infections. Detection of these patients relies on an elevation of cardiac biomarkers (troponins, CK-MB) and a new left ventricular dysfunction as usually demonstrated by echocardiography^[8]. Besides providing hemodynamic support, if needed, guideline recommended therapy for non-ischemic left ventricular dysfunction is advised (β -blockers and angiotensin-converting enzyme inhibitors). Moreover, because of their possible harmful effects on myocarditis, non-steroidal anti-inflammatory drugs (NSAIDs) should be used with caution in perimyocarditis. It is recommended to use a lower dosage and to favour the use of acetylsalicylic acid. However, some causes of myocarditis require specific treatments, as described in a recent New England Journal of Medicine article^[9].

The main concern in the evolution of acute pericarditis lies in the high rate of recurrences, which occur, on average, in 24 % of patients, according to the biggest case-series available^[10]. This rate is approximately twice as frequent in recurring pericarditis.

Table 1 Suggested pharmacological treatments of acute and recurring pericarditis

Drugs	Discharge dose (adults)	Tapering (wait until symptom free and normal CRP)	Monitoring/follow-up (in addition to follow-up for the clinical condition)
Acetylsalicylic acid (preferred for patients with known atherosclerosis)	650 mg <i>po qid</i> for 1-2 wk (2-4 wk when recurring)	Taper the dose by 30 % every 1-2 wk then stop	-Use gastric protection
Ibuprofen	600 mg <i>po tid</i> for 1-2 wk (2-4 wk when recurring)	Taper the dose by 30 % every 1-2 wk then stop	-Use gastric protection
Indomethacin	50 mg <i>po tid</i> for 1-2 wk (2-4 wk when recurring)	Taper the dose by 30 % every 1-2 wk then stop	-Use gastric protection
Colchicine	0.5 mg (or 0.6 mg) <i>po bid</i> for 3 mo (6 mo when recurring) Use 0.5 mg (or 0.6 mg) <i>po</i> daily in patients intolerant to higher doses, over 70 yr old or less than 70 kg	-	-Adjust for renal function -AST ALT CK, creatinine initially, then at 1 mo
Prednisone	0.2-0.5 mg/kg <i>po</i> daily for 2 wk (2-4 wk when recurring)	-Taper the dose by 10% every 1-2 wk -Taper slowly, especially when it comes to 15 mg/d, where decreases could be as low as 1.0 mg/d every 6 wk	-Osteoporosis prophylaxis

Modified from the tables in the last recommendations^[3,5]. CRP: C-reactive protein.

DIAGNOSIS

Two out of four criteria are required to diagnose acute pericarditis. They include the presence of (1) a characteristic chest pain; (2) diffuse ST elevations; (3) a pericardial rub; and (4) a pericardial effusion^[11].

If suspected, the basic investigation, after history and physical examination, should include the following: an electrocardiogram, a chest X-ray, a transthoracic echocardiogram, cardiac biomarkers (troponins and creatine kinase), markers of inflammation [C-reactive protein (CRP) and sedimentation rate] as well as a complete blood count and a basic renal function profile. A more extensive workup is usually not required because the majority of pericarditis cases, seen in the emergency department in developed countries, are idiopathic. If a specific etiology is suspected, such as neoplasia, systemic disease, or tuberculosis, appropriate investigations should be performed^[3].

TREATMENT

Pericardial diseases are one of the few areas in cardiovascular medicine without multiple practice guidelines. The European Society of Cardiology is the only major organisation that has established guidelines concerning pericardial diseases (2004). Moreover, these guidelines are mainly based on expert opinion because, until recently, few randomized controlled trials were available. In the past few years, several trials explored the subject, particularly regarding the role of colchicine and corticosteroids as treatments for acute and recurring pericarditis. This section will discuss these treatments in further detail.

NSAIDs

Although there is no large study on the use of anti-inflammatory drugs for the treatment of acute and recurring pericarditis, they remain the cornerstone of treat-

ment. Clinical experience shows that in order to prevent treatment failure, both the dosage of NSAIDs and the duration of treatment must be adequate^[2]. Recommended dosages and tapering are presented in Table 1. Tapering of NSAIDs should not be attempted before complete resolution of symptoms. Also, the follow-up of inflammatory markers, such as CRP, should guide the tapering. It is recommended to wait for their normalization before every decrease in dosage^[3].

Corticosteroids

Corticosteroids are often used in acute and recurring pericarditis because of their ability to quickly induce a positive clinical response. However, recent recommendations limit their role because of the lack of clinical evidence showing medium and long-term benefit. Indeed, the use of corticosteroid therapy for recurring pericarditis is only supported by a retrospective study of 12 patients treated with high doses of prednisone^[12].

Recent data identified the use of corticosteroids as being a factor favoring the recurrence of pericarditis. High vs low dosage of corticosteroids in the treatment of recurring pericarditis was evaluated in a retrospective study of 100 patients^[13]. The high dose group not only experienced more side effects, mainly osteoporosis, but also had more recurrences of pericarditis and hospitalizations. This increase in recurrences was also observed in two retrospective observational studies^[11,14].

These findings are reflected in the European recommendations which limit the use of corticosteroids to refractory and recurring pericarditis or in cases of intolerance, contraindication or failure of the usual treatments (NSAIDs and colchicine)^[15]. Patients receiving steroids for more than 3 mo should receive osteoporosis prophylaxis: calcium, vitamin D and bisphosphonates^[16]. The proposed corticosteroid doses are inferred from the experience acquired in the treatment of serositis such as in lupus and

other systemic inflammatory diseases. They are presented in Table 1. As with NSAIDs, corticosteroid tapering should begin only after the resolution of symptoms and normalization of the inflammatory markers.

Colchicine

The action of colchicine is mainly obtained through modulation of the cellular microtubule formation. Colchicine is mainly used in the treatment of gout and familial Mediterranean fever^[17]. During the 1990s, the analysis of several small series of patients raised a potential benefit of colchicine in the prevention of pericarditis recurrences. However, it was only in 2005, with the publication of the CORE^[18] and COPE^[19] trials that colchicine was clearly demonstrated to be effective in the treatment of pericarditis.

The COPE trial was randomized although not blinded. One hundred and twenty patients were approached during their first episode of pericarditis and randomly assigned to an anti-inflammatory treatment alone or in combination with colchicine. The recurrences of pericarditis at 18 mo were 11% in the colchicine-treated group *vs* 32% for the group not receiving colchicine. Only 5 patients stopped colchicine because of diarrhea, the most frequent side effect of this medication.

Similarly, the CORE trial was randomized but not blinded. It recruited patients suffering from a first recurrence of pericarditis. Eighty-eight patients were randomized to an anti-inflammatory treatment alone or in association with colchicine. In the colchicine group, recurrence was at 24% *vs* 51% for the group not receiving colchicine.

Three randomized controlled trials, in progress since 2007, are exploring the use of colchicine in the treatment of acute^[20] and recurring^[21] pericarditis. Their results should allow clinicians to clarify the possible benefits of colchicine in the treatment of pericarditis. While waiting for these results, colchicine should be preferred over corticosteroids in the context of more robust data and a more favourable side effect profile associated with its use.

Other treatments

In refractory cases, investigations should focus on trying to identify a secondary cause of pericarditis, where specific treatments could be available, or a cause that could explain treatment failure.

Literature recommendations for patients with recurring pericarditis that is refractory to conventional treatments are sparse. First-line therapy consists in combining NSAIDs, colchicine and corticosteroids^[3]. If this triple therapy fails, a trial of immunosuppressive agents can be attempted and proved successful in small trials. Azathioprine and methotrexate are the most often reported and less toxic of such agents^[22]. It is important to note that, according to expert opinions, patients suffering from pericarditis should not participate in competitive sports for a period of 3 mo^[23].

CONCLUSION

Acute and recurrent pericarditis are frequently encoun-

tered clinical entities. Since severe complications such as tamponade and constrictive pericarditis occur in rare cases, the majority of patients suffering from acute pericarditis will have a benign clinical course. However, recurrence of pericarditis with its painful symptoms is much more frequent. On this matter, recent studies have demonstrated a benefit of colchicine in reducing the risk of recurrence of pericarditis, while also suggesting an increase in recurrence with the use of corticosteroids, the traditional first-line agent.

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Inflammation and reactive oxygen species in cardiovascular disease

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Abstract

Reactive oxygen species (ROS) have long been proposed to be mediators of experimental cardiovascular pathology. There is also a wealth of data indicating that ROS are involved in clinical cardiovascular pathology. However, multiple clinical studies have shown little benefit from anti-oxidant treatments, whereas nearly all experimental studies have shown a marked effect of anti-oxidant therapy. One reason for this discrepancy is that ROS are produced through multiple different mechanisms of which some are clinically beneficial; thus, in a defined experimental system where predominately pathological ROS are generated does not mimic a clinical setting where there are likely to be multiple ROS generating systems producing beneficial and pathological ROS. Simple inhibition of ROS would not be expected to have

the same result in these two situations; ergo, it is important to understand the molecular mechanism underlying the production of ROS so that clinical treatments can be tailored to target the pathological production of ROS. One such example of this in cardiovascular biology is tissue specific inflammation-mediated ROS generation. This and the following series of articles discuss the current understanding of the role of ROS in cardiovascular disease, specifically focusing on the molecular mechanisms of ROS generation and the actions of ROS within the cardiovascular system. Although there are still many areas with regard to the effects of ROS in the cardiovascular system that are not completely understood, there is a wealth of data suggesting that blocking pathological ROS production is likely to have beneficial clinical effects compared to traditional anti-oxidants.

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Key words: Anti-oxidants; Inflammation; Oxidants; Pathology; Reactive oxygen species

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The World Health Organization in 2004 estimated that 17.1 million people died due to a cardiovascular event. This represents 29% of all deaths and is the greatest single cause of death worldwide. Reactive oxygen species (ROS) play a critical role in the pathogenesis of cardiovascular disease^[1,2] and clinically related disorders such as obesity^[3]

and metabolic syndrome^[4]. ROS inhibition has thus been proposed as a potential therapy for cardiovascular disease. Animal models confirm that inhibition of ROS generation, specifically from NAD(P)H oxidase, ameliorates and even prevents cardiovascular disease^[5]; however, clinical studies indicate that antioxidants have, at best, a marginal effect in reducing cardiovascular disease^[6,7]. One postulate for this disconnect is that clinically viable antioxidants merely scavenge all ROS indiscriminately, which will block pathological ROS production along with physiologically important ROS^[8]. For example, hydrogen peroxide (H₂O₂) is a vasodilator and, in the proper concentration, is beneficial^[9,10]. Since H₂O₂ is produced by superoxide dismutase, the proper concentration and location of superoxide (O₂⁻), the most studied ROS, also has beneficial cardiovascular effects; thus, complete scavenging of O₂⁻ eliminates these benefits. Rather, the mediators of pathological ROS generation should be identified and targeted for the treatment of cardiovascular disease.

Recently, inflammation has been linked, both experimentally and clinically, to cardiovascular disease^[11]. A key hallmark of inflammation is the generation of ROS, which can be due to immune cells [dendritic cells (DCs), lymphocytes, and macrophages] or interleukins and other inflammatory cytokines, such as tumor necrosis factor (TNF)- α ^[12,13]. Inflammatory cytokines activate vascular production of ROS, specifically O₂⁻, primarily through activation of NAD(P)H oxidase. Vascular NAD(P)H oxidase is a multimeric protein complex consisting of five primary subunits: a Nox isoform, p22^{nox}, p40^{nox}, p47^{nox} or its homologue NoxO1, and p67^{nox} or its homologue NoxA1. Additionally, the Rho family small G protein Rac1 in its active (GTP-bound) state is required for activation of the complex^[14]. There are a number of Nox isoforms (Nox 1-5, DUOX1 and 2) all of which share some homology with the first identified Nox subunit, gp91^{nox}/Nox2^[15]. The Nox isoforms and p22^{nox} are membrane bound and although the catalytic core is comprised within the Nox subunit, it requires p22^{nox} to be active; similarly, the rest of the subunits are cytosolic and are involved in the regulation of the Nox subunit. The various components of NAD(P)H oxidase are differentially expressed and regulated, but all of the NAD(P)H oxidases generate O₂⁻, which is known to participate in growth, apoptosis, and migration of vascular smooth muscle cells, as well as in the modulation of endothelial function, including endothelium-dependent relaxation and expression of the proinflammatory phenotype and in the modification of the extracellular matrix^[16]. Additionally, O₂⁻ is also linked to hypertension, pathological states associated with uncontrolled growth, and inflammation leading to coronary artery disease^[16,17]. However, the various Nox isoforms do not generate ROS equally, and moreover, they occur at specific locations within a cell and are activated *via* unique mechanisms^[18]. Interestingly, recent data indicates that some Nox isoforms are localized within the endoplasmic reticulum (ER)^[19-21], providing a molecular link to ER stress and cell death seen in atherosclerosis^[22] and diabetes^[23]. Although NAD(P)H oxidases have been identified as major players in redox signaling in several cardiovas-

cular disorders, there are still many questions that remain regarding cell specific NAD(P)H oxidase localization and function. Recent and ongoing research tends to highlight the regulation^[24], localization^[25], and structure-function^[26] of this enzyme complex, but further studies are required to completely understand the mechanisms of action of the multiple potential NAD(P)H oxidase complexes in normal physiology and disease.

The following articles in this editorial address several topical aspects of the roles of inflammation and ROS in cardiovascular disease. The overview articles by Zhang *et al.*^[27] and Zuidema *et al.*^[28] consider the effects of inflammatory cytokines and general mechanisms including the roles of DCs, immune cells and the relevance of ROS signaling, which summarize our current understanding of inflammatory and immune mechanisms in ischemic heart disease. This editorial is followed by articles from Capobianco *et al.*^[29], Gao *et al.*^[30], Lee *et al.*^[31] and Zhang *et al.*^[32] addressing recently identified novel mechanisms for cardiovascular dysfunction in ischemic heart disease regarding the roles of stem cells, endothelium-derived hyperpolarizing factor, exercise training and adipokines as mediators in cardiovascular disease. Picchi *et al.*^[33] evaluate the roles of hyperglycemia, oxidative stress, polyol pathway, protein kinase C, advanced glycation end products, insulin resistance, peroxisome proliferator-activated receptor γ , inflammation, and diabetic cardiomyopathy as a “stem cell disease”. They also discuss the potential pathogenic importance of superoxide production by nitric oxide synthase (NOS), the enzyme that normally generates nitric oxide (NO) but can switch to ROS production when the NOS co-factor tetrahydrobiopterin is deficient—for example, in diabetic vasculopathy. Fay^[34] reviews the linkage between inflammation and thrombosis by focusing on the role of C-reactive protein (CRP); the identification of elevated inflammatory marker CRP as a transient independent risk factor for endothelial dysfunction may provide an important clue to link a systemic marker of inflammation to progression of atherosclerotic disease. The review by Pung *et al.*^[35] discusses aspects of redox signaling in the growth of coronary collateral circulation. Finally, DeMarco *et al.*^[36] discuss the contribution of oxidative stress to pulmonary arterial hypertension (PAH), and suggest that statins may be an attractive option for treatment of PAH and cor pulmonale because they may simultaneously prevent further tissue damage by decreasing oxidative stress and enhancing repair to injured sites in both the pulmonary vasculature and right ventricle. Our aim in this mini-symposium is to provide an up-to-date overview of inflammation, oxidative stress and redox signaling as they relate to clinical cardiovascular disease.

Inflammation and ROS have increasingly been recognized to play an important role in cardiovascular disease and related pathologies manifested by obesity and diabetes. Available evidence suggests that low-grade inflammation is accompanied by a decreased bioavailability of endogenous NO, and, as summarized in the accompanying reviews, inflammation plays a larger, more complex, role in cardiovascular disease. Thus, randomized longitudinal studies are now needed to investigate whether or not vari-

ous anti-inflammatory treatment strategies (such as anti-TNF- α treatment) improve cardiovascular function and decrease the unacceptably high cardiovascular mortality rate. However, many of the mechanisms being scrutinized here need further elucidation. Our understanding of inflammation and ROS, especially with respect to structure-function and signal transduction relationships as well as their pathophysiological role in cardiovascular dysfunction, is still in its infancy. A potentially important and relatively new direction is the concept that inflammatory cells contribute to ischemic heart disease. Future studies are needed to understand the interaction of inflammation, immune cells and ROS with cardiovascular disease and how this might be interrupted to provide therapeutic benefit. We believe that further investigations in this exciting field will facilitate the development and/or delivery of selective anti-inflammatory agents (antioxidants) to specifically inhibit the pathological generation of O_2^- . These compounds and delivery systems are expected to provide for better management of cardiovascular diseases.

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Endothelial progenitor cells as factors in neovascularization and endothelial repair

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Abstract

Endothelial progenitor cells (EPCs) are a heterogeneous population of cells that are provided by the bone marrow and other adult tissue in both animals and humans. They express both hematopoietic and endothelial surface markers, which challenge the classic dogma that the presumed differentiation of cells into angioblasts and subsequent endothelial and vascular differentiation occurred exclusively in embryonic development. This breakthrough stimulated research to understand the mechanism(s) underlying their physiologic function to allow development of new therapeutic options. One

focus has been on their ability to form new vessels in injured tissues, and another has been on their ability to repair endothelial damage and restore both monolayer integrity and endothelial function in denuded vessels. Moreover, measures of their density have been shown to be a better predictor of cardiovascular events, both in healthy and coronary artery disease populations than the classical tools used in the clinic to evaluate the risk stratification. In the present paper we review the effects of EPCs on revascularization and endothelial repair in animal models and human studies, in an attempt to better understand their function, which may lead to potential advancement in clinical management.

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Key words: Atherosclerosis; Bone marrow; Endothelial dysfunction; Endothelial progenitor cells; Neovascularization; Stem cells

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INTRODUCTION

Stem cells are primal cells found in all multicellular organisms. The biologic hallmark of stem cells is their ability to

renew through mitotic cell division and differentiate into a different specialized cell types. The three broad categories of mammalian stem cells are (1) embryonic stem cells derived from blastocysts; (2) intermediate stem cells isolated from fetal tissue and extra-embryonic membranes; and (3) adult progenitor cells found in adult tissues. Stem cells can be cultured *in vitro* and transformed into specialized cells, potentially offering treatment for a variety of diseases which were previously considered incurable. Other types include manually-manipulated stem cells such as human induced pluripotent stem cells^[1,2], nuclear transfer stem cells^[3] and pluripotent adult unipotent germline stem cells^[4].

We begin by clarifying our terminology in order to better address this topic. The term progenitor cell is used in cell and developmental biology to refer to an immature or undifferentiated cell, typically found in post-natal animals. While progenitor cells share many common features with stem cells, these two terms are often incorrectly used as synonymous. Stem cells have unlimited self-renewal ability, while the self-renewal ability of progenitor cells is limited. Another differentiating feature is that stem cells are *pluripotent* (can differentiate into cells derived from any of the three germ layers) while adult progenitor cells are *unipotent* (can produce only one cell type, but have the property of self-renewal, which distinguishes them from non-stem cells) or *multipotent* (can produce only cells of a closely related family of cells, e.g. hematopoietic stem cells differentiate into red blood cells, white blood cells, platelets, *etc.*). Embryonic stem cells can differentiate into all of the specialized embryonic tissues that form the organism. In adults, progenitor cells act as a repair system for the body, replenishing specialized cells as and when needed.

The focus of this review is endothelial progenitor cells (EPCs), a population coming from mobilization and differentiation of precursors present in the bone marrow (BM) or other tissues such as fat, adventitia and skeletal muscles. EPCs have the ability to elicit neovascularization in response to ischemia, and to repair injured or damaged endothelium.

EPCS

The close regional and functional development of peripheral blood and vascular wall cells from the angioblast during embryonic development suggested the existence of a common origin, the hemangioblast. However, differentiation of these mesodermal cells to angioblasts and subsequent endothelial differentiation was believed to exclusively occur in embryonic development. This belief was first challenged by Asahara *et al.*^[5] in 1997, who isolated an angioblast from peripheral blood of adult humans, which differentiated *in vitro* into endothelial cells and contributed to *in vivo* neoangiogenesis in response to tissue ischemia. These cells were named EPCs^[6,7]. They express various surface markers, hematopoietic CD34 surface marker and endothelial phenotype marker vascular endothelial growth factor receptor 2 (VEGFR2). Further observations also

reported the existence of “circulating BM-derived EPCs” in adults, a subset of the CD34 blood-derived cell population, which was shown to differentiate into the endothelial lineage and express endothelial marker proteins. Because CD34 was not exclusively expressed on hematopoietic stem cells, further studies used a more immature stem cell marker CD133 and demonstrated that purified CD133 cells can differentiate to endothelial cells *in vitro*^[8]. CD133+/CD34- EPCs have a higher vascular regeneration potential compared to CD133+/CD34+ EPCs^[9]. Peripheral blood-derived EPCs can also form ‘late-outgrowth colony-forming unit endothelial cells, and this ability characterizes the “true” EPCs *in vitro*, being a marker of their clonogenic potential^[10]. Thus, CD133/VEGFR2-positive cells more likely reflect immature EPCs, whereas CD34/VEGFR2-positive cells represent circulating endothelial cells both derived from EPC differentiation and/or shed from the vessel endothelium into the blood^[11]. Importantly, EPCs have been shown to differentiate *in vitro* into vascular smooth muscle cells^[12].

We speculate that heterogeneity in cell markers may reflect different developmental stages of EPCs during the maturational process from the BM residual cell to the mature vascular wall cell. In addition to the BM (myelomonocytic)-derived cells, spleen-derived mononuclear cells, cord blood derived mononuclear cells^[13], fat tissue derived stem cells^[14], adventitial stem cells^[15] and skeletal muscle progenitor cells^[13] contribute to the pool of progenies of the endothelial cell lineage. Therefore, EPCs are a heterogeneous population of cells from BM or other adult tissue that share a common phenotype which when properly stimulated *in vivo* and *in vitro*, give rise to endothelial cells.

EPCS AND NEOVASCULARIZATION

The finding that BM-derived cells can mobilize to sites of ischemia and express endothelial marker proteins have been demonstrated in animal models and in humans. This suggests that isolated EPCs may be used in therapeutic vasculogenesis as a way to rescue tissue from critical ischemia. Infusion of various distinct cell types, either isolated from the BM or *ex vivo* cultivation, was shown to augment capillary density and neovascularization in ischemic tissue. In animal models of myocardial infarction, injection of *ex vivo* expanded EPCs or stem and progenitor cells significantly improved blood flow, improved cardiac function and reduced left ventricular scarring^[16,17]. Similarly infusion of *ex vivo* expanded EPCs derived from peripheral blood mononuclear cells in athymic nude mice or rats improved neovascularization in hind limb ischemia models^[18-21].

Initial human trials indicate that BM-derived or circulating blood-derived progenitor cells are useful for therapeutically improving blood supply to ischemic tissue. Autologous implantation of BM mononuclear cells in patients with ischemic limbs significantly augmented ankle-brachial index and reduced rest pain. In addition, transplantation of *ex vivo* expanded EPCs significantly im-

proved coronary flow reserve and left ventricular function in patients with acute myocardial infarction^[22,23]. The role of EPCs in neovascularization remains to be elucidated. Basal incorporation of EPCs in non-ischemic tissues is very low^[24] but the incorporation of EPCs in ischemia-injured tissues shows contradictory results. Data showing a wide range of EPC incorporation rates have been published, but other studies detected BM-derived cells only adjacent to the vessel, and they did not express endothelial markers^[25-29]. This heterogeneity may be due to differences among models of ischemia that may significantly influence the incorporation rate. A homogenous finding among these studies was that the incorporation rate in an ischemic injured tissues model was quite low, or at least not enough to explain the observed effective increase in the neovascularization process. The challenge is to explain how such a low number of endothelial stem cells can improve neovascularization. One possible explanation is that the efficiency of neovascularization may combine the incorporation of EPCs in newly formed vessels and the release of proangiogenic factors in a paracrine manner.

Various studies have been conducted to evaluate the extent of neovascularization after infusion of EPCs or monocyte/macrophages lines. EPCs additionally incorporated into the newly formed vessel structures show endothelial marker protein expression *in vivo*^[30,31]. Infusion of macrophages (which are known to release growth factors but are not incorporated into vessel-like structures), induces only a slight increase in neovascularization after ischemia, much less than the one induced by injection of EPCs. These studies indicate that the capacity of EPCs to physically contribute to vessel-like structures leads to their potent capacity to improve neovascularization^[18,32,33]. Human studies performed to demonstrate the usefulness of EPCs gave mixed results. The TOPCARE-AMI^[34] trial showed the safety and feasibility of intracoronary infusion of EPCs (either BM-derived cells or circulating progenitor cells) in patients successfully revascularized by stent implantation post-acute myocardial infarction. EPCs and BM mononuclear cells have been clinically evaluated for their benefits in limb ischemia^[35-37], acute myocardial infarction, and dilated cardiomyopathy^[38-42]. These cell types showed modest cardiovascular benefits with 2%-8% improvement in left ventricular ejection fraction^[38] compared to significant improvement in limb ischemia. Many questions remain unanswered, and further studies are needed to elucidate the contribution of physical incorporation, paracrine effects and possible effects on vessel remodeling and facilitation of vessel branching to obtain EPC-mediated improvement of neovascularization.

EPCS AND ENDOTHELIAL REPAIR

Previously, the regeneration of the injured endothelium was believed to come only from the migration and proliferation of neighboring endothelial cells. However, accumulating evidence shows that additional mechanisms may exist, and may be mediated by the circulating pool of

EPCs. Rafii *et al.*^[11] showed that a subset of CD34-positive cells (hematopoietic marker) have the capacity to differentiate into endothelial cells *in vitro* in the presence of basic fibroblast growth factor, insulin-like growth factor-1, and VEGF. These differentiated endothelial cells stained for von Willebrand factor (vWF), and incorporate acetylated low-density lipoprotein (LDL), therefore showing both hematopoietic (CD34) and endothelial phenotype (LDL and vWF). This also suggests the existence of a BM-derived precursor endothelial cell. To demonstrate this phenomenon *in vivo*, the authors further used a canine BM transplantation model, in which the BM cells from the donor and recipient were genetically distinct. After BM transplantation, a Dacron graft was implanted in the descending thoracic aorta, and they found that only donor alleles were detected in DNA from cells on the Dacron graft, indicating that re-endothelialization was mediated by circulating EPC derived from donor BM^[43].

Xu *et al.*^[44] demonstrated that endothelial repair of a vein grafted into an artery is mediated by a circulating pool of endothelial cells provided by the recipient. They used TIE2-LacZ mice, which are transgenic mice with a promoter (TIE2) placed before an intron fragment containing the enhancer for LacZ gene. This combination allows LacZ gene expression (β -galactosidase) specifically on vascular endothelial cells. Thus TIE2-LacZ endothelial cells can be recognized by β -galactosidase staining. When they grafted the TIE2-LacZ vena cava into the carotid artery of wild-type mice, endothelial cells of freshly harvested vena cava from TIE2-LacZ mice showed β -galactosidase staining, whereas the intensity of blue color of β -gal cells in vein grafts was decreased 1 d after surgery, and almost disappeared 3 d after the implantation. Hence, these results confirmed that the endothelial cells of vein segments implanted in an artery are totally destroyed by the acute exposure to mechanical stress due to increased blood pressure. When they grafted the vena cava from wild-type mice into the TIE2-LacZ carotid artery, no β -galactosidase staining was observed on the surface of the freshly-harvested vena cava from the wild-type mouse, which in contrast appeared 24 h after grafting into the carotid artery of a TIE2-LacZ mouse and increased in number to reach a monolayer at 4 wk after the graft. These results showed that the endothelial cells on the grafted vein were coming from the recipient mice and not from the donor. To show that those cells were coming from the BM of the recipient, they further created chimeric mice by transplanting the BM from TIE2/LacZ mice to wild-type animals that were previously irradiated: these chimeric mice expressed β -galactosidase activity only on endothelial cells that were actually provided by its BM. The investigators then grafted a vena cava coming from the wild-type into the carotid artery of the chimeric mice, and 3 d after transplantation, β -galactosidase activity was detected on the vein. Since the chimeric mice expressed β -galactosidase activity only on endothelial cells provided by the BM, these β -galactosidase-positive cells could only come from their BM^[44-46]. However, in a model of transplant arteriosclerosis, BM-

derived cells appeared to contribute only to a minor extent to endothelial regeneration by circulating cells^[47]. These data indicated that there might be at least two distinct populations of circulating cells that principally are capable of contributing to re-endothelialization, namely mobilized cells from the BM and non-BM derived cells. The latter may arise from circulating progenitor cells released by non-BM sources (e.g. tissue resident stem cells) or represent vessel wall-derived endothelial cells.

We emphasize here that the source of transfused endothelial cells after *in vitro* expansion is critical for interpreting the results. The observation that EPCs directly influence lesion formation and progression comes from experimental models using progenitor cell transfusion. The systemic application of healthy wild-type EPCs in atherosclerotic apolipoprotein E-knockout mice has been shown to improve endothelial function and to inhibit atherosclerotic lesion progression independent of high serum cholesterol levels^[48]. However, these beneficial effects were not observed in a study conducted by George *et al*^[49] in which aortic sinus lesion size was significantly increased in mice receiving EPCs compared with controls. Mice receiving EPCs showed plaques with larger lipid cores, thinner fibrous caps, and a higher number of infiltrating CD3 cells, suggesting an effect on plaque stability. An important aspect of this study was that intravenously transfused spleen-derived cells were administered without splenectomy of the recipient animals, and the tendency of spleen-derived cells to migrate back to the organ of origin may have affected the results.

Fujiyama *et al*^[50] showed that infusion of EPCs leads to regeneration of a functionally active endothelium confirmed by release of nitric oxide (NO). They also noted a significant reduction in neointima formation. Similarly, Griese *et al*^[51] showed that infused peripheral blood monocyte-derived EPCs deposit on bioprosthetic grafts and balloon-injured carotid arteries, with significantly reduced neointima deposition. These studies indicate that administration of EPCs not only facilitates re-endothelialization but also helps with recovery of endothelial function, while inhibiting neointima deposition.

MOBILIZATION, CHEMOTAXIS, ADHESION, TRANSMIGRATION AND DIFFERENTIATION

The mobilization of stem cells in the BM is determined by the local microenvironment, the so-called “stem cell niche,” which consists of fibroblasts, osteoblasts, and endothelial cells^[52]. Basically, mobilizing cytokines [VEGF, stromal-derived factor (SDF)-1] hamper the interactions between stem cells and stromal cells, which finally allow stem cells to leave the BM *via* trans-endothelial migration. Thereby, activation of proteinases such as elastase, cathepsin G, and matrix metalloproteinase (MMP) cleave adhesive bonds on stromal cells, which interact with integrins on hematopoietic stem cells. MMP-9 was addition-

ally shown to cleave the membrane-bound Kit ligand and induce the release of soluble KitL (also known as stem cell factor). Physiologically, ischemia is believed to be the predominant signal to induce mobilization of EPCs from the BM. Ischemia is thus believed to upregulate VEGF or SDF-1, which in turn are released into the circulation and induce mobilization of progenitor cells from the BM *via* a MMP-9 dependent mechanism. Several mediators increase the number of circulating EPCs in the blood of both humans and animal models. Granulocyte-colony stimulating factor, a cytokine that is typically used for mobilization of CD34 cells, has been shown to increase the levels of circulating EPCs. A related cytokine, the granulocyte monocyte-colony stimulating factor, also increases EPC levels^[53].

In a clinical environment, the effects of VEGF and erythropoietin (EPO) on EPC mobilization have been evaluated, and both demonstrated an augmentation of EPC levels in humans^[40,41,54,55]. Moreover, the correlation between EPO serum levels and the number of CD34 or CD133 hematopoietic stem cells in the BM of patients with ischemic coronary artery disease (CAD) further supports an important role of endogenous EPO levels as a physiologic determinant of EPC mobilization. Some athero-protective drugs can also positively modulate the number of circulating EPCs. Statins increase the number and the functional activity of EPCs *in vitro*, in mice, and in patients with stable CAD. This increase in EPC numbers was associated with increased BM-derived cells after balloon injury and accelerated endothelial regeneration^[56]. Other factors that augment the circulating EPCs are estrogen^[57] and exercise^[58,59].

The molecular signaling pathways have not, as yet, been identified. However, several studies indicate that the activation of the PI3K/Akt pathway may play an important role in the statin-induced increase in EPC levels^[60]. Likewise, EPO^[61], VEGF^[62], estrogen^[63] and exercise (shear stress)^[64] are also well known to augment the PI3K/Akt-pathway. Thus, these factors may share some common signaling pathways. Recent data shows that endothelial NO synthase is essential for mobilization of BM-derived stem and progenitor cells^[65], and we speculate that these stimuli may increase progenitor cell mobilization by PI3K/Akt-dependent activation of the NO synthase within the BM stromal cells.

Factors that drive the EPCs to the site of endothelial injury (chemotaxis) may be the same that normally stimulate engraftment of hematopoietic cells to the BM, such as SDF-1 or sphingosine-1-phosphate. SDF-1 has been proven to stimulate recruitment of progenitor cells to the ischemic tissue. SDF-1 protein levels increase during the first days after induction of myocardial infarction^[66]. Integrins are known to mediate the adhesion of various cells including hematopoietic stem cells and leukocytes to extracellular matrix proteins and to endothelial cells^[67,68]. Integrins capable of mediating cell-cell interactions are the $\beta 2$ -integrins and $\alpha 4\beta 1$ -integrin. Various cell types including endothelial cells and hematopoietic cells express

β 1-integrins, whereas β 2-integrins are found preferentially on hematopoietic cells^[69]. Because adhesion to endothelial cells and transmigration events are involved in the *in vivo* homing of stem cells to tissues with active angiogenesis, integrins such as the β 2-integrins and the α 4 β 1-integrin may be involved in the homing of progenitor cells to ischemic tissues. However, the data regarding physiologic mobilization, chemotaxis and differentiation of EPCs at the site of endothelial injury is limited.

EPCS AND CARDIOVASCULAR RISK FACTORS, RISK STRATIFICATION AND PROGNOSTIC VALUE

Endothelial cell number and function is a valuable surrogate biologic marker for vascular function and cumulative cardiovascular risk and a strong predictor of the risk of cardiovascular events^[70-73]. Hill *et al.*^[74] started from Ross's classic paradigm stating that endothelial cell injury is the stimulus for the development of atherosclerotic plaque. This model argues that seemingly disparate risk factors act on a final common pathway that culminates in endothelial-cell injury, including both direct endothelial damage and endothelial dysfunction. They speculated that indicators of cumulative risk, such as the Framingham score, or function, such as brachial reactivity, represent useful composite measures of overall vascular status. In 45 healthy adult subjects, with different associations of cardiovascular risk but with no symptoms of atherosclerosis or active organ ischemia, the correlation between EPC count in the peripheral blood, and both Framingham risk score and brachial vascular reactivity was evaluated. The number of colony-forming units was negatively correlated with Framingham risk score and positively correlated with brachial reactivity. Interestingly, when the subjects were divided according to the number of EPCs circulating into "high" and "low", activity of EPCs was a stronger predictor of flow-mediated brachial reactivity than the presence or absence of conventional cardiovascular risk factors. Tepper *et al.*^[75] isolated EPCs from human type II diabetics and age-matched control subjects and found that the proliferation of diabetic EPCs relative to control subjects was significantly decreased and inversely correlated with patient levels of glycosylated hemoglobin. Diabetic EPCs had normal adhesion to fibronectin, collagen, and quiescent endothelial cells but a decreased adherence to human umbilical vein endothelial cells (HUVEC) activated by tumor necrosis factor (TNF)- α . These authors conclude that type II diabetes may alter EPC biology in processes critical for new blood vessel growth and, furthermore, EPC monitoring may identify a population at high risk for morbidity and mortality after vascular occlusive events.

In addition to what was shown in the healthy population, in patients with CAD the number of cardiovascular risk factors negatively correlated with progenitor cell counts. Vasa *et al.*^[76] determined the number and functional activity of EPCs in 45 patients with CAD and in 15

healthy volunteers. The number of isolated and circulating EPCs was significantly reduced in patients with CAD. To determine the influence of atherosclerotic risk factors, a risk factor score including age, sex, hypertension, diabetes, smoking, positive family history of CAD, and LDL cholesterol levels was used. The number of risk factors was significantly correlated with a reduction of EPC levels. Analysis of the individual risk factors demonstrated that smokers and patients with a family history of CAD had significantly reduced levels of EPCs. EPCs isolated from patients with CAD also revealed an impaired migratory response, which was inversely correlated with the number of risk factors. They concluded that patients with CAD show reduced levels and functional impairment of EPCs, which correlated with risk factors for CAD. Another potentially attractive marker for risk stratification in patients with atherosclerotic disease seems to be the so-called "endothelial cell-derived microparticles (EMP)": endothelial cell damage mediated by chemical or mechanical injury leads to endothelial cell apoptosis, which is associated with conformational changes of the cell's plasma membrane leading to the release of membrane microparticles, in which antigens derived from their mother cell and can be quantified *in vivo* by flow cytometry^[77]. Elevated EMP levels have been described in all conditions of severe endothelial cell damage (e.g. thrombotic thrombocytopenic purpura^[78], diabetes^[79], arterial hypertension^[80], acute coronary syndromes^[81], and myocardial infarction^[82]) and microparticles themselves have been shown to elicit direct effects on endothelium-dependent vasorelaxation *in vitro*. Microparticles derived from patients with acute coronary syndromes or preeclampsia directly impaired endothelial function in rat aortic rings or myometrial arteries^[83], and in humans, increased apoptotic microparticle counts positively correlated with the impairment of coronary endothelial function^[84]. In a study investigating coronary endothelial function in 50 patients with CAD, multivariate analysis revealed that increased apoptotic microparticle counts predict severe endothelial dysfunction independent of classical risk factors such as hypertension, hypercholesterolemia, smoking, diabetes, age, and gender^[84]. In the context of human atherogenesis, it may be pivotal to evaluate the current status of regeneration and endothelial cell apoptosis in each individual. EPC and EMP may be valuable biomarkers in patients with atherosclerotic disease.

Werner *et al.*^[85] performed a clinical study to evaluate the prognostic value of circulating EPCs and their potentially vasculoprotective role. The number of EPCs was measured in 519 patients with angiographically documented CAD and correlated with cardiovascular outcomes. Primary end points included cardiovascular mortality, the occurrence of a first major cardiovascular event (myocardial infarction, hospitalization, revascularization, and cardiovascular death), revascularization, hospitalization, and all-cause mortality after 1 year. The cumulative event-free survival increased stepwise across tertiles of baseline EPC levels for cardiovascular mortality, first major cardiovascular event, revascularization, and hospitalization. After adjustment for

vascular risk factors, drug therapy, and concomitant disease, increased EPC levels were independently associated with a lower risk of cardiovascular death, first major cardiovascular event, revascularization, and hospitalization. Primary and secondary prevention trials suggest that statins possess favorable effects on atherosclerosis development and progression and that these effects are independent of cholesterol reduction. Statins can also improve vascular perfusion by causing several positive side effects such as reduction of both hypertrophy and proliferation of smooth muscle cells^[86-89], an increase in NO synthesis^[90] and a decrease in production of adhesion molecules^[91-93]. In a model of carotid balloon-injury, Walter *et al.*^[94] investigated whether statin therapy may also accelerate re-endothelialization after carotid balloon injury: they treated male Sprague-Dawley rats with simvastatin and found that this treatment accelerated re-endothelialization of the balloon-injured arterial segments and resulted in a dose-dependent significant reduction in neointimal thickening when compared with saline-injected controls. They further tried to elucidate the mechanism, and investigated the contribution of BM-derived EPCs by BM transplantation from Tie2/lacZ mice to background mice or athymic nude rats. As described earlier TIE2-LacZ endothelial cells can be recognized by β -galactosidase staining. β -galactosidase staining of mouse carotid artery specimens revealed a significant increase in the number of β -galactosidase-positive cells per mm² appearing on the carotid artery luminal surface of treated rats. In addition, statins increased circulating rat EPCs and induced adhesiveness of cultured human EPCs by upregulation of the integrin subunits $\alpha 5$, $\beta 1$, $\alpha(v)$, and $\beta 5$ of human EPCs. These findings showed physiological evidence that EPC mobilization represents a functionally relevant consequence of statin therapy^[94]. Furthermore, Werner *et al.*^[95] investigated vascular lesion formation in mice after transplantation of BM transfected by means of retrovirus with enhanced green fluorescent protein; they induced carotid artery injury, resulting in neointimal formation. Fluorescence microscopy and immunohistological analysis revealed that BM-derived progenitor cells were involved in re-endothelialization of the vascular lesions. Treatment with rosuvastatin enhanced the circulating pool of EPCs, propagated the advent of BM-derived endothelial cells in the injured vessel wall, and, thereby, accelerated re-endothelialization and significantly decreased neointimal formation. These results also show that statin treatment promotes BM-dependent re-endothelialization and diminishes vascular lesion development. Estrogens increase EPC numbers in mice and humans, which contributes to repair mechanisms of the vascular wall^[96]. Also physical activity, which is known to reduce cardiovascular morbidity and mortality by mainly unknown mechanisms, increases the number and function of EPCs in rodents and healthy humans^[58].

INFLAMMATION, REACTIVE OXYGEN SPECIES AND STEM CELLS

Mesenchymal stem cells (MSCs) are a heterogeneous subset of stromal stem cells that can be isolated from many

adult tissues. MSCs are multipotent stem cells that can differentiate into a variety of cell types. Cell types that MSCs differentiate into *in vitro* or *in vivo* include osteoblasts, chondrocytes, myocytes, adipocytes, endothelium, and, as described recently, β -pancreatic islets cells^[97]. Interestingly, some studies disputed the differentiation potential of adult BM-derived stem cells^[98-100]. MSCs can interact with cells of both the innate and adaptive immune systems, leading to the modulation of several effectors functions. Once MSCs are administered *in vivo* they may induce peripheral tolerance and migrate to the injured tissues, where they help damaged cells survive by inhibiting the release of pro-inflammatory cytokines^[101]. The key role of myeloid dendritic cells (DCs) is to present antigen to naive T cells following DC maturation, induced by cytokines. As the DCs are maturing, they acquire expression of co-stimulatory molecules and upregulate expression of MHC class I and class II molecules together with other cell-surface markers^[101-103]. MSCs have been shown to inhibit the maturation of monocytes, cord blood and CD34+ hematopoietic progenitor cells into DCs *in vitro*^[101]. The final outcome of the immunomodulatory activity of MSCs is likely to be significantly influenced by the micro environmental cues encountered following *in vivo* administration. Micro environmental cues encountered following *in vivo* administration influences the immunomodulatory activity of MSCs including their effect on target cells, as exemplified by the opposite outcomes that can arise from the interaction of MSCs with DCs and natural killer cells in the presence of high or low concentrations of interferon- γ ^[101]. Stem and progenitor cells are critical for organogenesis during the fetal stage of development^[104]. Recently the existence of somatic stem cells has been reported in adult organs^[104]. Somatic stem cells and progenitor cells are thought to sense and repair damaged tissues and organs^[104]. Reactive oxygen species accelerate the senescence of stem and progenitor cells^[104].

Clinical studies suggest that TNF levels in the serum are correlated negatively with the CD34+ stem cells and EPCs circulating in the peripheral blood in patients with congestive heart failure. This is thought to be related to the myelosuppressive effect of circulating TNF^[55]. In a murine congestive heart failure model, elevated serum TNF levels and reduced BM progenitor cells have been reported^[55]. An *in vitro* study indicated a causal relationship between TNF and suppression of hematopoietic stem cell growth, and that TNF directly inhibited stem cell factor-stimulated proliferation of CD34+ hematopoietic progenitor cells^[103]. Human CD34+ myeloid leukemic cells and BM progenitor cells (CD34+CD38-) demonstrated similar results^[103]. Similar results were also found in human CD34+ myeloid leukemic cells and primitive human BM progenitor cells (CD34+CD38-)^[103]. Interestingly, the inhibitory effects of TNF in these studies were consistently mediated by TNFR- I, but not TNFR- II. To the contrary, the TNFR- II signaling pathway shows a protective profile on stem cell function. Thus, distinct effects of TNF are mediated by different subtypes of TNF receptors in stem

cells while the overall effect might be dependent on the expression level and ratio of these two receptors. Apart from the direct effect, TNF is able to indirectly influence the fate of stem cells. TNF markedly stimulates production of granulocyte macrophage-colony stimulating factor, a strong mobilizer of stem cells from the BM^[105]. Activation of the TNF/Fas pathway in lymphocytes in the BM may play a pathogenic role in suppressing hematopoiesis^[103]. EPC adhesion to HUVEC, a process mediated by upregulation of E-selectin, was significantly increased by TNF pre-treatment of HUVEC in the peripheral circulation. Interestingly, EPC adhesion to HUVECs was not induced when pretreatment was carried out for EPCs instead of HUVECs^[103]. TNF also has effects on stem cell differentiation: administration of TNF switched the differentiation of these cells from granulocytes to almost complete production of macrophages when mouse Lin-Sac⁺ hematopoietic progenitor cells were cultured with stem cell factor and IL-7^[103]. In summary, TNF plays an important role in regulating stem cell-mediated vascular repair and remodeling. However, the overall effect of TNF on stem cell mobilization, proliferation and function is complicated, depending on the subtypes of the TNF receptors, the presence of other cytokines as well as other cells. Although stem cell-based treatments are effective in myocardial infarction, the vascular protective effects of stem cells in ischemia-reperfusion injury in coronary microcirculation have not been studied. Further studies will improve our understanding of the mechanisms and remediation of ischemia-reperfusion injury.

CONCLUSION

Stem and progenitor cells possess the ability to self-regenerate and differentiate into many cell types, and inflammation is involved in most cardiovascular diseases. An understanding of the communication and interaction between TNF and stem cells is important^[103]. The mechanism underlying this function remains unclear because the number of endothelial cells incorporated in ischemic tissue is too low to create a new vessel just by incorporating themselves into it; we speculate they may act through two different mechanisms, which may consist of both physical incorporation and paracrine stimulation of another “*in loco*” population to stimulate their differentiation into vessel cells. This aspect needs further study. The molecular mechanisms for effective mobilization of stem cells are, however, poorly understood. We speculate that the functional properties of EPCs in cardiovascular disease are impaired and that regeneration by endogenous cells without further mobilization of cells is diminished or absent in the presence of cardiovascular disorders or risk factors. Consistently, impaired mobilization of EPCs has been associated with older age, the presence of cardiovascular risk factors, and the presence of atherosclerotic disease. The presence of cardiovascular risk factors may interrupt the delicate equilibrium between endothelial damage and repair, leading to manifestation of endothelial dysfunction and ath-

erosclerosis. The fact that physiological mechanisms of EPC mobilization, homing adhesion and differentiation is poorly understood adds to the challenge of unraveling this complex problem. Further studies are needed to elucidate the complexities of stem cell mobilization, homing and differentiation to identify mechanisms and develop therapies suitable for clinical application.

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Corruption of coronary collateral growth in metabolic syndrome: Role of oxidative stress

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especially the metabolic syndrome, that negatively affect collateral growth through the corruption of redox signaling processes.

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Abstract

The myocardium adapts to ischemic insults in a variety of ways. One adaptation is the phenomenon of acute preconditioning, which can greatly ameliorate ischemic damage. However, this effect wanes within a few hours and does not confer chronic protection. A more chronic adaptation is the so-called second window of preconditioning, which enables protection for a few days. The most potent adaptation invoked by the myocardium to minimize the effects of ischemia is the growth of blood vessels in the heart, angiogenesis and arteriogenesis (collateral growth), which prevent the development of ischemia by enabling flow to a jeopardized region of the heart. This brief review examines the mechanisms underlying angiogenesis and arteriogenesis in the heart. The concept of a redox window, which is an optimal redox state for vascular growth, is discussed along with signaling mechanisms invoked by reactive oxygen species that are stimulated during ischemia-reperfusion. Finally, the review discusses of some of the pathologies,

INTRODUCTION

Recently, there has been a rapid increase in the incidence of metabolic syndrome, a term used to describe a condition characterized by abdominal obesity, hyperglycemia, insulin resistance and hyperinsulinemia, to near epidemic levels. People with metabolic syndrome are particularly at increased risk for ischemic heart disease (IHD) and approximately 30% to 40% of these patients show little to no coronary collateral growth. Importantly, patients with well-developed coronary collaterals have a better prognosis in recovering from a myocardial infarction than those with poorly developed collaterals^[1]. Because collateral growth is a chronic event, patients without collaterals that have an acute coronary occlusion have a poor prognosis because the wavefront of necrosis proceeds faster (minutes to hours) than vascular growth (days

Table 1 Differences in the underlying mechanisms of induction, as well as mediator and growth factor involvement in angiogenesis and arteriogenesis^[8,9]

	Angiogenesis	Arteriogenesis
Inducer	Ischemia	Shear stress, inflammation, ischemia
Promoter	Hypoxia inducible factor (TACGTGCT)	Hypoxia inducible factor (TACGTGCT) Shear stress responsive element (GAGACC)
Substrate	Pre-existing capillaries	Pre-existing arterioles
Cell type	Endothelial cells	Endothelial and smooth muscle cells; likely fibroblast
Result	Increase capillaries density	Remodeling of arteries into collateral arterioles
Duration	Days	Days to weeks
Growth factors	VEGF, bFGF, PDGF, PIGF, MCP-1, MMPs, GM-CSF, <i>etc.</i>	VEGF, bFGF, PDGF, PIGF, MCP-1, MMPs, GM-CSF, <i>etc.</i>

VEGF: Vascular endothelial growth factor; bFGF: Basic fibroblast growth factor; PDGF: Platelet-derived growth factor; PIGF: Placenta growth factor; MCP-1: Monocyte chemoattractant protein-1; MMPs: Matrix metalloproteinases; GM-CSF: Granulocyte-macrophage colony-stimulating factor.

to months). Coronary collaterals carry insufficient flow to completely prevent infarction in most cases, although their presence is known to limit the damage and reduce infarct size^[2]. Thus, the growth of coronary collaterals has earned the name “mother nature’s by-pass”. The complex mechanisms mediating the enlargement and/or development of new blood vessels in the heart are not well-understood. In this review, we discuss redox-sensitive mechanisms that lead to coronary collateral growth and how redox-dependent signaling should be considered in therapies designed to stimulate the growth of blood vessels in the heart, particularly in patients with metabolic syndrome.

MECHANISMS OF CORONARY COLLATERAL FORMATION IN THE HEART

Coronary collateral growth is the enlargement of arterial-arterial connections in the heart. It is a chronic coronary adaptation to myocardial ischemia that helps to restore the coronary flow and prevent or minimize myocardial ischemic injury^[3]. Under physiological conditions, collateral vessels are very small and thus resistance to net blood flow is high^[4]. However, collaterals can greatly expand their calibers and serve as conduits offering little resistance to blood flow if challenged with appropriate stimuli^[5]. The stimuli that trigger this physiologic remodeling in an outward direction, rather than pathologic remodeling in which cell proliferation is involved in the development of a neointima and atherosclerotic plaque formation, remain unknown^[4].

Vascular growth is usually categorized as angiogenesis (the tightly regulated sprouting of new capillaries from pre-existing ones) or vasculogenesis (the *in situ* development of vessels from angioblasts, which is normally confined to the embryonic phase of development)^[6]. Arteriogenesis, formerly regarded as a variant of angiogenesis, is a relatively new term that was introduced to distinguish it from other mechanisms of vascular growth; i.e. angiogenesis and vasculogenesis^[7-9]. Arteriogenesis describes the formation of mature arteries from pre-existent interconnecting arterioles after an arterial occlusion. According to Cai *et al.*^[10], the fundamental difference between the two

types of vascular growth is that arteriogenesis occurs in a normoxic environment; whereas angiogenesis depends on tissue hypoxia/ischemia that leads to the activation of the transcription factor hypoxia-inducible factor-1 α (HIF-1 α). However, these generalizations are far too simplistic because, in the heart, arteriogenesis or collateral growth is initiated by ischemia/tissue hypoxia. Several years ago, Chilian *et al.*^[11] attempted to resolve the contributions of shear stress from ischemia in the coronary circulation by distally embolizing the microcirculation of the heart with microspheres (thus producing ischemia, but without pressure gradients across upstream collaterals). Under these conditions, initiation of collateral growth was observed, but the magnitude of collateral growth was not nearly as robust as with other models. Importantly, Toyota *et al.*^[3] further demonstrated that neutralizing antibodies to vascular endothelial growth factor (VEGF) prevented coronary collateral growth. Because VEGF has an HIF responsive element in the promoter, such an observation is consistent with the early initiation of collateral growth being regulated by ischemia (tissue hypoxia). As collaterals develop, tissue hypoxia is ameliorated because the collaterals enable the delivery of oxygenated blood. Thus, at least in the heart, ischemia can be thought of as an initiating factor for collateral development, but shear stress is likely a factor that contributes to remodeling during the continuation of this process as the tissue hypoxia is abated^[4].

Whether the growth and enlargement of coronary collaterals is due to the enlargement of pre-existent vessels, *de novo* arteriogenesis, or both, remains a controversy. In our opinion, we think that repetitive occlusions in the heart may give rise to a mixed arteriogenic/angiogenic adaptation due to the close proximity of the stenosing vessel and the downstream region at risk. Indeed, we previously found an increase in capillary density in a canine model of collateral growth induced by episodic ischemia^[12]. However, what is not resolved is whether these capillaries can arterialize and contribute to the formation of the collateral network. Obviously, the underlying mechanisms of this “natural process” of coronary collateral growth/arteriogenesis are a complex orchestration of the expression of numerous growth factors and signaling cascades that have not been well elucidated, as illustrated in Table 1 and Figure 1. Figure 1 summarizes how both

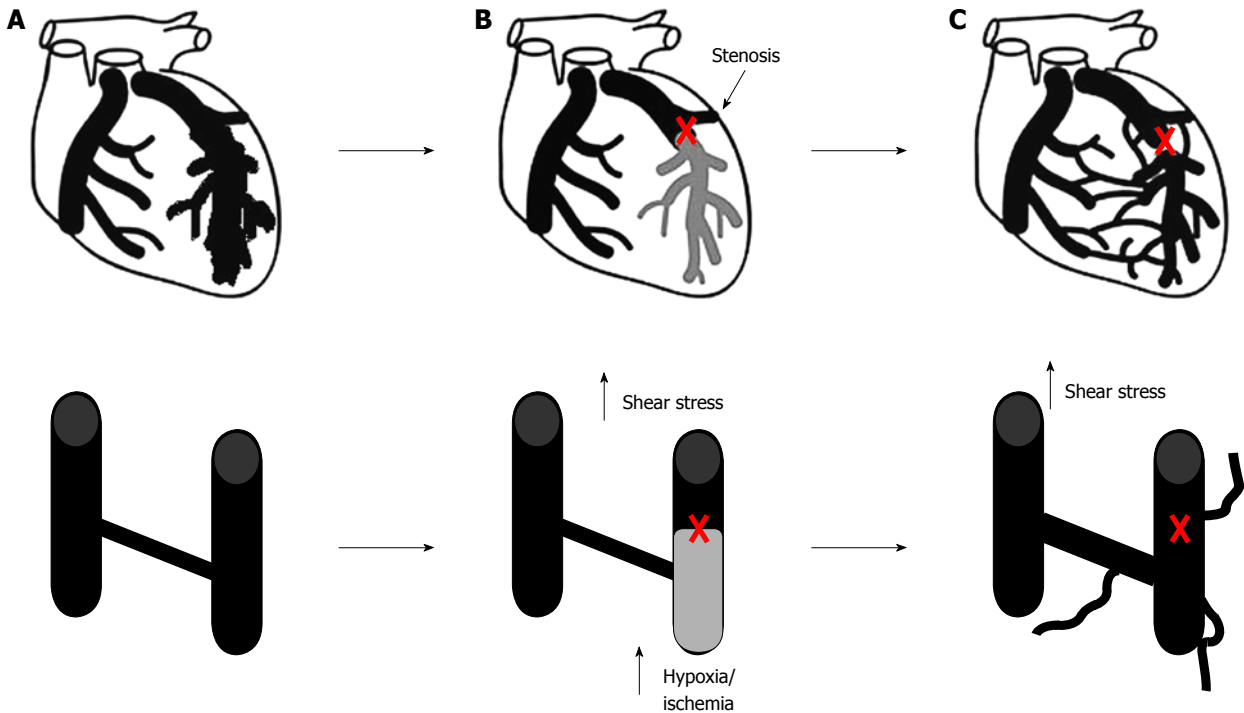


Figure 1 Compensatory mechanism of coronary collateral growth/arteriogenesis. A: A non-functioning collateral between parallel circuits in the absence of flow obstruction; B: Following proximal occlusion, there is a pressure drop across the pre-existing collateral stimulated by shear stress-driven redirection of flow; C: A complex intracellular signaling cascade involving various growth factors act in a coordinated manner to facilitate migration and proliferation of endothelial and smooth muscle cells (likely fibroblasts), leading to enlargement/ formation of arteries, arterioles and capillaries.

tissue hypoxia/ischemia and shear stress can contribute to coronary growth. In this figure, an acute occlusion produces ischemia and increases shear stress across collaterals. As the collaterals grow, ischemia is absolved but shear stress may still be elevated compared to the normal state. Table 1 summarizes key aspects, regulators and components of growth of a collateral vessel (arteriogenesis) and growth of new vessels (angiogenesis). This table shows that the two processes overlap in various categories.

ISCHEMIA-REPERFUSION INJURY

In the heart, ischemia or ischemia-reperfusion (IR) is the initiating stimulus for collateral growth and angiogenesis. IR injury in the myocardium is a biphasic process, in which exposure of the myocardium to prolonged reduction in blood flow (ischemic phase) produces a variety of events including hypoxia, which initiates cell injury or even death in the affected region of the heart. This is then followed by further injury commencing upon reestablishment of blood flow (reperfusion phase), which further cellular destruction, including stunning and death^[3,13]. Massive amounts of reactive oxygen species (ROS) released during reperfusion have been shown to be the major cause of death of myocardial tissue that was still alive before the onset of reperfusion. Treatment with superoxide dismutase-1 (SOD-1), catalase (cat) and nitric oxide synthase (NOS) inhibitors at the onset of reperfusion have been shown to be cardioprotective^[3,14]. The caveat to IR is that

if the period of occlusion is brief, then instead of inducing cell death, adaptive processes of cardio-protection and collateral growth are initiated.

OXIDATIVE STRESS

ROS are a family of molecules, including molecular oxygen and its derivatives, produced in all aerobic cells. Many ROS possess unpaired electrons and thus are free radicals. These include superoxide anion (O_2^-), hydroxyl radical (HO^\bullet), nitric oxide (NO^\bullet) and lipid radicals. Other ROS, such as hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), and hypochlorous acid ($HOCl$), are not free radicals *per se*, but have potent oxidizing effects that contribute to oxidative stress^[15]. As stated previously, there is a burst in the production of ROS during the reperfusion phase of IR, which has been implicated in the regulation of intracellular signaling pathways and biological functions of the cells^[14,16]. Superoxide is rapidly converted to the more stable H_2O_2 by the actions of superoxide dismutase 1 and 2 (SOD-1 and -2). Catalase (cat) then converts H_2O_2 into water and O_2 . Peroxynitrite is formed by the reaction of O_2^- and NO , and has been implicated in the disruption of intracellular signaling by nitration of tyrosine residues^[17-19]. However, if there is an imbalance between pro-oxidant generation and anti-oxidant defenses, oxidative stress may ensue. Oxidative stress can result in oxidation of biological macromolecules, such as DNA, protein, carbohydrates and lipids. Oxidative modification of lipids, tyrosine residues, nucleotides, and a shift in the ratio of

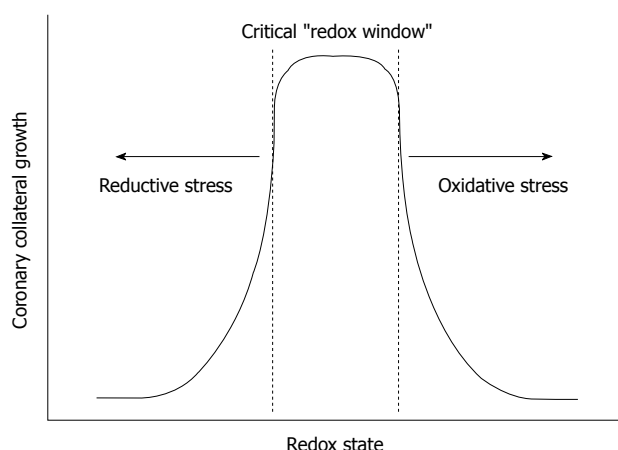


Figure 2 “Redox-window” hypothesis on coronary collateral growth. Existence of a critical “redox-window” is required; either reductive or oxidative stress will corrupt collateral growth and redox-dependent growth-factor signaling. The figure illustrates the principal measure in our system—collateral growth—as a function of the redox state in cells. The summary of this concept is best described as, in situations where there is reductive stress or oxidative stress, collateral growth is corrupted because of either too many neutral or oxidized thiol groups.

reduced to oxidized thiols are all standard measurements to evaluate oxidative stress. However, when does the shift to an oxidative state become a stress and therefore is outside the bounds of the norm? This question is difficult to answer, but in fact is the most important, and we pose it in a different venue, as shown in Figure 2 where we illustrate a “redox window”. In Figure 2, the outcome of redox-dependent signaling is coronary collateral growth. However, we emphasize that there are many surrogates for the effects of oxidative or reductive stresses. In our model, the final measure of redox signaling is coronary collateral growth and, accordingly, we plotted this variable as the outcome for redox signaling in the heart. In some instances oxidative stress should be confined to a situation where the normal metabolism or biology of a cell is altered by excess production of oxidants or inadequate antioxidant defenses. Alternatively, reductive stress occurs with insufficient production of ROS, excessive amounts of free radical scavengers or enhanced levels of NADPH or NADH (because of inadequate oxidation of these substrates). Although oxidative stress is generally perceived as being more important than reductive stress, the latter is known to corrupt growth factor signaling^[20] and also induce cardiomyopathy^[21]. The likely mechanism of reductive stress relates to the incomplete oxidation of protein thiols on cysteine residues that, when oxidized, produce the negatively charged sulfenic acid. If too many (oxidative stress) or too few (reductive stress) thiol groups are oxidized into sulfenic acid, the activity of the enzyme is affected *via* conformational changes in tertiary structure that is determined by either neutrality or negatively charged thiols.

Despite many potential enzymatic sources of ROS, only xanthine oxidase, NADH/NADPH oxidase and NO synthase, have been studied extensively in the cardiovas-

cular system^[15]. In recent years, more studies have revealed several important mechanistic details of mitochondrial ROS production in the heart. Mitochondria, which encompass 40% to 50% of a cardiac myocyte volume, are densely packed with various protein electron carriers (mitochondrial complexes) that, instead of transferring electrons for the production of energy, have electrons inadvertently leak from the complexes and reduce oxygen to form the superoxide anion. Superoxide anion then serves as a ROS progenitor to induce a positive feedback loop (“vicious cycle”) wherein ROS-mediated oxidative damage to cells favors further elevated ROS production^[22]. Many cell types have been proposed to contribute to the enzymatic production of ROS during IR, namely infiltrating neutrophils, cardiac myocytes and endothelial cells^[23]. However, potential roles for both cardiac and vascular fibroblasts and vascular smooth muscle cells have also been reported^[23].

REDOX-DEPENDENT SIGNALING IN CORONARY COLLATERAL GROWTH

Studies have shown that ROS modulate cellular function *via* intricate mechanisms. Ambient production of O_2^- and H_2O_2 , maintained by basal activity of pre-assembled NADPH oxidases or mitochondrial respiration, is necessary for the growth, proliferation and migration of vascular cells. Under pathological conditions, activation of vascular NADPH oxidase, xanthine oxidase, uncoupled from eNOS and even mitochondrial dysfunction, lead to detrimental consequences. The functions of some ROS, particularly H_2O_2 , are often viewed in a dichotomous manner as an important physiological mediators in certain concentrations (e.g. endogenous vasodilator^[24,25]) but harmful in large amounts (e.g. anti-microbial and apoptotic^[15]).

The likely actions of H_2O_2 are mediated *via* its effects on cellular thiols, in which oxidation of a free thiol by H_2O_2 produces sulfenic acid. The conferrence of a negative charge *via* oxidation of a free thiol to sulfenic acid performs a similar action as when a protein is phosphorylated by a kinase; the introduction of the negatively charged phosphate induces a change in the tertiary structure of the protein causing an alteration in function; e.g. activation, docking and inhibition. However, one critical issue related to ROS signaling is that a little is “good”, but a lot seems to be “bad”. Perhaps there are critical thiol residues that are normally subjected to oxidation by ROS, but if there are too many ROS, then additional thiols may be converted into sulfenic acids, which could result in improper tertiary changes in structure. An observation supporting this contention is that different thiols show varying sensitivity to oxidative modification^[26] and, therefore, it is not unreasonable to assume that oxidative stress can induce a very different effect on protein structure than physiological levels of ROS.

Rocic *et al.*^[20] demonstrated that too many or too few

ROS have a negative effect on endothelial tube formation *in vitro*, which may be a shared common mechanism with angiogenesis and collateral growth. These investigators showed that there was robust tube formation induced by VEGF when endothelial cells were seeded on two-dimensional Matrigel. However, tube formation was inhibited when the cultures were treated with either diphenylene iodonium (DPI) to block O_2^- formation or diethyldithiocarbamic acid (DETC) to inhibit SODs^[20]. The former shifted the redox state to a more reductive condition and the latter to a more oxidative environment. To further establish the physiological relevance of the impact of the critical “redox window” in mediating the angiogenesis process, the study was extended to an *in vivo* coronary collateral growth model. Healthy, lean rats were subjected to a 10-d protocol of brief episodic ischemia, a sham group with the surgical procedure but without repetitive ischemia, and two experimental groups in which O_2^- production was decreased by administration of DPI or increased by DETC, respectively. The desired redox state induced by drug treatment was monitored and confirmed using X-band electron paramagnetic resonance spectroscopy. Compared to the sham group, the experimental group produced an increase in O_2^- , which was blocked by DPI and augmented by DETC. Robust growth of coronary circulation (ratio of flow to the collateral relative to normal zones) was observed as opposed to abrogated growth by either too little or too much O_2^- ^[20]. This *in vivo* study again emphasized the existence and importance of redox-dependent signaling in coronary collateral growth.

OXIDATIVE STRESS IN METABOLIC SYNDROME AND CORONARY COLLATERAL GROWTH

Metabolic syndrome is associated with overproduction of ROS, leading to the concept that the amelioration of risk factors of metabolic syndrome, including insulin resistance, elevated blood pressure, elevated lipid levels, inflammation and endothelial dysfunction, may reduce oxidative damage and curtail the progression of IHD^[27]. As mentioned, enzymatic sources of ROS production under pathological conditions, including activation of vascular NADPH oxidase, xanthine oxidase and uncoupling of eNOS, are well characterized in cardiovascular diseases. In our opinion, cardiac mitochondrial dysfunction is the major cause and/or effect of mitochondrial ROS generation, leading to a vicious cycle of “ROS-induced, ROS-released” in the diabetic myocardium. Supporting this argument are many observations showing alterations in mitochondrial structure and function in the metabolic syndrome^[28-33], with some of these manifestations ameliorated by scavenging ROS in mitochondria.

Mitochondria are the principal source of high energy phosphate (ATP) production. Tissues that have high energy requirements, such as the heart, have a higher density of mitochondria and are reliant on mitochondrial aerobic

metabolism to produce energy to maintain contractile function. In addition to energy production, mitochondria continuously produce ROS as a by-product of electron transfer. This is because the transfer of e^- through the mitochondrial electron transport chain is not 100% efficient, and a small percentage of e^- leak out and react with O_2 to produce O_2^- . Although the heart is able to oxidize a broad variety of substrates for ATP production, the normal heart generates ATP mainly from the mitochondrial oxidation of fatty acid (60% to 70% of ATP generated) and to a lesser extent from glucose, lactate and other substrates (30% to 40%). In contrast, hearts of diabetic and obese animals use relatively more fatty acids to generate ATP, while glucose oxidation rates are decreased in isolated working heart perfusions of *db/db* and *ob/ob* mice, as shown by Andreyev *et al.*^[22] and Buchanan *et al.*^[34]. Similarly, increased fatty acid oxidation has also been observed in Zucker Diabetic Fatty rats (ZDF)^[35]. The resulting increase in reducing equivalent delivery to the respiratory chain may increase chances of e^- leakage, leading to mitochondrial oxidative stress. In addition, studies have shown that fatty acids are less efficient fuel when compared to glucose in terms of the yield of ATP per oxygen atom consumed. Substrate switched from 100% palmitate to 100% glucose would increase the ATP yield per oxygen atom by 12% to 14%^[36]. Thus increased fatty acid utilization in the diabetic myocardium may be energetically detrimental because of the higher cost to produce ATP to keep up with increased cardiac work. Importantly, reduction in ATP would also prevent a phenotypic switch of endothelial and smooth muscle cells (likely fibroblasts) from quiescent to proliferating and migrating phenotypes, which is essential for angiogenesis and collateral growth.

To understand whether amelioration of oxidative stress would confer a positive effect on VEGF gene therapy, we studied coronary collateral growth in Zucker Obese Fatty rats (ZOF). ZOF rats are a rat model of human metabolic syndrome because these rats share many of the same afflictions including obesity, insulin resistance, hyperlipidemia, hyperinsulinemia and hyperphagia. The ZOF rats also demonstrated endothelial dysfunction and oxidative stress^[37]. We first observed that coronary collateral growth was markedly compromised in response to episodic ischemia in the obese rats^[38]. VEGF gene therapy was administered *via* transfected smooth muscle cells that were introduced into the coronary circulation. There was no significant improvement in coronary blood flow to the ischemic zone. However, correction of oxidative stress with ecSOD (SOD-3), using the same smooth muscle-based gene delivery system as for VEGF, partially restored coronary collateral development^[38]. Importantly, this observation was also confirmed in a different model of MS; i.e. JCR rats^[39]. These results argue that amelioration of oxidative stress in diabetic/pre-diabetic myocardium will restore growth-factor redox-dependent signaling and thus enable the VEGF gene therapy to stimulate collateral growth. Clearly, redox-dependent signaling plays a critical role in collateral growth in the heart, and corruption of

this signaling by either reductive or oxidative stress can have negative influences and actions of growth factors on collateral growth.

CONCLUSION

The metabolic syndrome compromises vascular adaptations to ischemia^[40-42] resulting in impaired coronary collateral growth. Central to this inadequate adjustment, are impairments in endothelial function produced by oxidative stress, which also corrupts the signal transduction of growth factors. These issues represent a major challenge for clinical application of any therapeutic strategy, because the presence of oxidative stress prevents the actions of growth factors administered as gene therapy or recombinant protein. The correction of oxidative stress to restore redox-dependent signaling is imperative to enable realization of therapies designed to stimulate collateral growth.

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Role of genomics in cardiovascular medicine

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INTRODUCTION

Cardiovascular disease is considered the primary cause of death in developed countries and is becoming a major cause of death in the developing world. As with all fields of medicine, much attention must be paid to classification of the disease and, consequently, to its genetic characterization. The field of cardiovascular medicine involves a broad spectrum of abnormalities that are characterized by a host of clinical and etiological features that range from simple congenital diseases related to metabolic defects to complex diseases that manifest in adulthood.

In congenital heart disease, chromosomal aberrations or mutations in genes regulating cardiac development are usually the cause of the disease^[1-25]. The environment can have a small "teratogenic" effect in that some substances (e.g. early exposure to angiotensin converting-enzyme inhibitors, alcohol abuse and *Rubella virus*) alter the function of certain genes during embryogenesis^[1].

A rough categorization of the remaining adulthood cardiovascular diseases is ischemic and non-ischemic. Non-ischemic cardiomyopathies are usually associated

Abstract

As all branches of science grow and new experimental techniques become readily accessible, our knowledge of medicine is likely to increase exponentially in the coming years. Recently developed technologies have revolutionized our analytical capacities, leading to vast knowledge of many genes or genomic regions involved in the pathogenesis of congenital heart diseases, which are often associated with other genetic syndromes, coronary artery disease and non-ischemic cardiomyopathies and channelopathies. The knowledge-base of the genesis of cardiovascular diseases is likely going to be further revolutionized in this new era of genomic medicine. Here, we review the advances that have been made over the last several years in this field and discuss different genetic mechanisms that have been shown to underlie a variety of cardiovascular diseases.

with heart failure and sudden cardiac death (SCD) in the young, and ischemic cardiomyopathies are usually related to atherosclerosis and its sequelae, which include stroke and myocardial infarction (MI) in older populations^[26,27].

SCD in the young is believed to be mainly due to non-ischemic cardiomyopathy (often involving structures of the heart or tissues) and channelopathies (in which ion channels are malfunctioning and cause conduction defects). In these cases, a distinction between primary and secondary cardiomyopathy must be made. In fact, these diseases can also be the consequence of other clinical phenotypes, such as hypertension and peri-myocarditis. Furthermore, they can also be related to excessive physical activity and illicit drug and alcohol abuse. In all, these cases are defined as “secondary”^[27]. When there is no clear etiology, or there is familial recurrence, they are defined as “primary.”

Several mutations in different genes have been identified and functionally defined in both sporadic and familial forms of these diseases, and different models of inheritance; e.g. Mendelian and non-Mendelian, have been observed^[28-37]. Despite the low frequency of functional mutations that lead to these phenotypes and inheritance models that can be applied to such variations, geneticists are reluctant to define any of them as “simple.” Due to environmental influence and the modulation of common single nucleotide polymorphisms (SNPs) on the effect of disease causing mutations^[38,39], most of these diseases should be considered as “complex.” When over the age of 35-40 years, cardiac death is usually related to ischemic cardiomyopathy, which is secondary to the atherosclerotic process that leads to occlusion of the coronary artery resulting in acute myocardial ischemia or infarction. Except for very rare and clear cases of Mendelian mutations that cause premature coronary artery disease (CAD), atherosclerosis is a complex phenotype, in which environmental factors play a major role interacting with one another and with the biological background of each individual in determining the insurgence of the disease. The biological background of each individual can be considered unique, as one or more of the risk factors interplay with environmental factors. At least seven processes are involved in atherogenesis, leading to approximately 6 lesion stages as classified by the American Heart Association^[40]. In the early stages, mainly endothelial dysfunction, endothelial cell activation and inflammation are involved, whereas proteolysis and apoptosis are essential in the formation of the lipid core and fibrous cap of each lesion. Finally, platelet aggregation, angiogenesis and thrombosis are major players in the last stages of the disease that involve plaque growth and rupture^[40] (Figure 1). In each of these stages, hundreds of genes and proteins are believed to be involved. Nonetheless, genetic variants can have a major and diverse role in different stages of the disease, and risk variants in each stage may have no effect or even a protective role in other stages. The “mutation theory of atherosclerosis,” which underlines the similarity between atherosclerotic and carcinogenic processes, is currently under study. This theory remains to be defined,

but there is already significant evidence, in the form of microsatellite instability and loss of heterozygosity in smooth muscle cells of human plaques, that supports the hypothesis of genetic aberrations in atherogenesis^[41,42]. Recent studies have also correlated chromosome telomere length and coronary heart disease (CHD), suggesting that, in atherosclerosis, as in other complex phenotypes such as type 2 diabetes and cancer, telomere length probably contributes as a primary abnormality^[43].

CHD and MI are characterized by a high level of genetic and clinical heterogeneity. As mentioned earlier, the study of such diseases is complicated by the considerable impact of the environment on disease development, by the multiplicity of pathways involved in the response to environmental stress in different phases of disease evolution, and the multiplicity of clinical sub-phenotypes, such as hypertension and hypercholesterolemia.

ROLE OF GENETICS IN THE PATHOGENESIS OF CARDIOVASCULAR DISEASE

In 1964, Detweiler *et al.*^[44] investigated whether heart diseases, such as atherosclerosis, non-ischemic cardiomyopathy, congenital malformations, arrhythmias, conduction disturbances, congestive heart failure and hypertension, could be genetically determined in different species and breeds within species. Since then, much effort has gone into developing knowledge of the genetics of cardiovascular diseases. We briefly report the information that has accumulated over the last 4 decades. A schematic summary of cardiovascular defects and involved genes, which is far from being exhaustive, is shown in Table 1.

Congenital heart disease

Although biases related to recruitment methods must be considered, early studies on dogs comparing mongrels and purebreds, summarized in Detweiler's review, suggested a correlation between consanguinity and congenital heart disease^[44].

Most types of congenital heart disease are usually associated with other syndromes, and are caused by chromosomal aneuploidies or mutations usually located in genes that have been implicated in cardiac development. The heritability of congenital heart disease depends on the disease and on the underlying genetic cause.

These defects can be caused by errors in meiosis (and the predisposition to such errors can be due to external variables, such as teratogens), by the heritability of a parental chromosomal translocation, or may be due to *de novo* mutations^[1-25]. Since there is complexity in the classification of such diseases and their association with diverse genetic phenotypes, a rough scheme of the involved genes is presented in Table 1.

Non-ischemic cardiomyopathy

As mentioned previously, only the primary forms of these

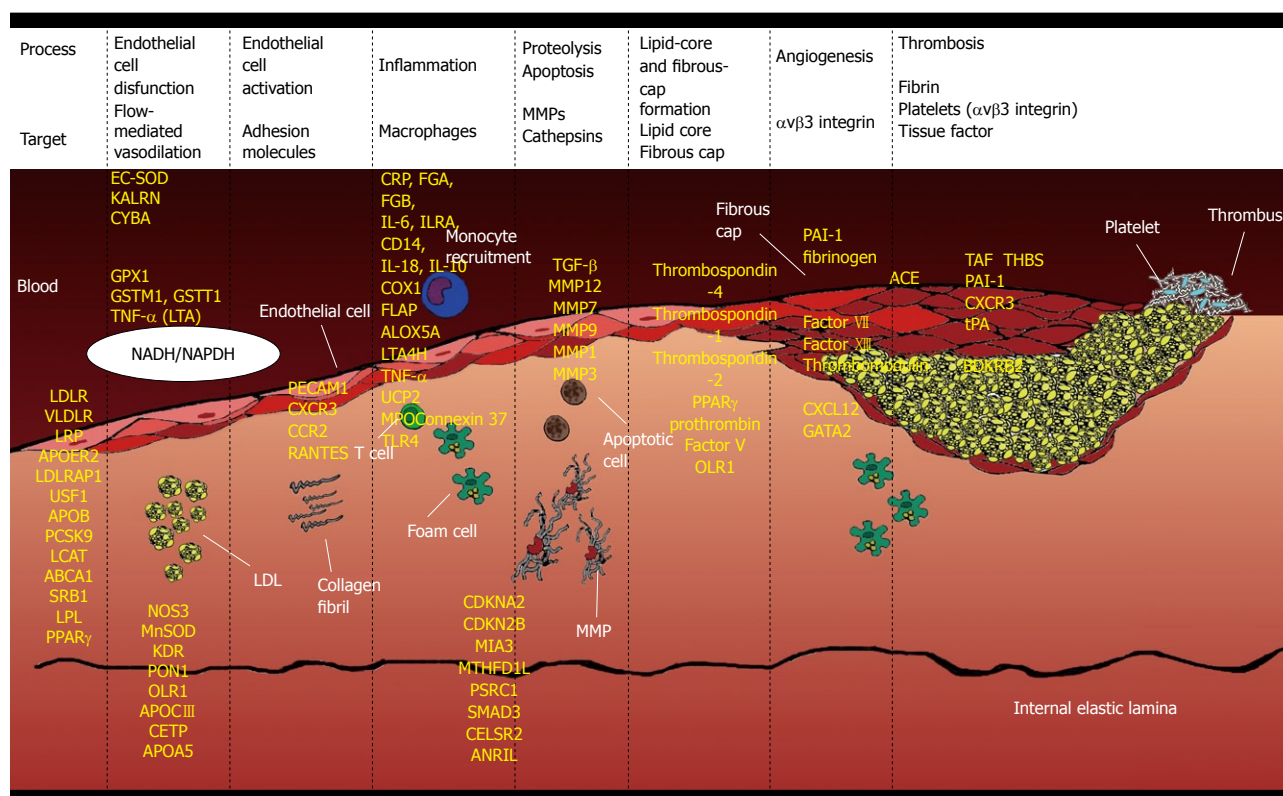


Figure 1 Clinical, biological and hypothesized genetic complexity of atherosclerosis as a model of complex disease. Modified from Watkins *et al.*^[40], 2006. MMPs: Matrix metalloproteinases; IL: Interleukin; LDL: Low density lipoprotein; TNF: Tumor necrosis factor; TGF: Transforming growth factor; PPAR: Peroxisome proliferator-activated receptor.

diseases, which are not the consequence of other phenotypes, such as hypertension, myocarditis and environmental factors like drug consumption or physical activity, can be ascribed to genetic factors. Familial cardiomyopathy and ion channelopathies are often described as single gene disorders. However, even in these disorders there are modifier genes that have a significant influence on phenotype, which may not be detected by conventional genetic techniques such as linkage analysis. Nonetheless, there is some suggestive evidence that arrhythmias, such as atrial fibrillation occurring in association with structural heart disease, are more prevalent in individuals with a certain genetic predisposition.

Quantification of the influence of genetics in these pathologies is quite difficult due to the complexity of aetiologies. To date, adult onset hypertrophic cardiomyopathy, which is the most common cardiomyopathy, is considered a genetically linked condition caused by inheritance or new mutations in genes that encode sarcomeric proteins. These include the cardiac β -myosin heavy chain (*MYH7*, 14q11.2), the cardiac myosin-binding protein C (*MYBPC3*, 11p11.2), cardiac troponin T (*TNNT2*, 1q32.1), cardiac troponin I (*TNNI3*, 19q13.42), essential myosin light chain (*MYL3*, 3p21.31), regulatory myosin light chain (*MYL2*, 12q24.11), α -tropomyosin (*TPM1*, 15q22.2), cardiac actin (*ACTC*, 15q14), and titin (*TTN*, 15q31.2). Mutations in the $\gamma 2$ regulatory subunit of AMP-activated protein kinase (*PRKAG2*, 7q36.1) result in early-onset left ventricular hypertrophy with arrhythmias and, more rarely, fatal infantile cardiac glycogenesis. Mutations in the

gene encoding lysosome-associated membrane protein 2 (*LAMP2*, Xq24) cause massive left ventricular hypertrophy in male subjects in whom systemic manifestations (phenotype known as Danon's disease) may also develop. In 2008, Morita *et al.*^[28] sequenced 9 genes known to cause adult onset disease in 84 children with idiopathic cardiac hypertrophy diagnosed at an early age (under 15 years). The authors concluded that cardiac hypertrophy in children and adults has a common genetic basis; the cause of half of the presumed sporadic cases and of nearly two-thirds of familial cases of childhood-onset hypertrophy was mutations predominantly in *MYH7* and *MYBPC3*^[28].

Other mutations in structural protein or ion channel subunit coding genes have been identified as underlying factors for other forms of cardiopathy, channelopathies (Brugada, LQT, SQT) and atrial fibrillation (Table 1).

Although intense efforts have been made to qualify and quantify the role of genetics in victims of SCD, it is still not possible to explain the role of mutations, modifier polymorphisms and environmental factors in a vast majority of cases^[26-39].

Ischemic cardiopathy

Over the past 4-5 decades, information on the role of environmental and genetic factors predisposing to atherosclerosis and to its clinical sub-phenotypes has accumulated. The classical environmental risk factors are well established and are mainly associated with lifestyle (diet and smoking) and family history of early CAD^[45,46].

With the exception of disease causing mutations that

Table 1 Summary of defects affecting the cardiovascular system and list of involved genes

	Phenotype	Involved genes	Associated diseases
Congenital heart disease			
Cyanotic heart disease	Transposition of the great arteries	NKX2-5, THRAP2	DiGeorge syndrome, alagille syndrome
	Tetralogy of fallot	NKX2-5, NOTCH1, TBX1, JAG1, NOTCH2	
	Tricuspid atresia	NKX2-5	Alagille syndrome
	Pulmonary atresia	PTPN11, JAG1, NOTCH2	
	Ebstein's anomaly of the tricuspid valve	NKX2-5	DiGeorge syndrome
	Double outlet right ventricle	NKX2-5, THRAP2	
	Persistent truncus arteriosus	TBX1	
	Anomalous pulmonary venous connection		
Left-sided obstruction defects	Hypoplastic left heart syndrome	NOTCH1	DiGeorge syndrome
	Mitral stenosis		
	Aortic stenosis	NOTCH1, PTPN11	
	Aortic coarctation	NOTCH1, PTPN11	
Septation defects	Interrupted aortic arch	TBX1	HOS
	Atrial septation defects	NKX2-5, GATA4, TBX20, MYH6, TBX5	
	Ventricular septal defects	NKX2-5, GATA4, TBX20, TBX1, TBX5	Noonan syndrome
	Atrioventricular septal defects	PTPN11, KRAS, SOS1, RAF1, CRELD1	
Other congenital heart defects	Bicuspid aortic valve	NOTCH1	Char syndrome
	Patent ductus arteriosus	TFAP2B	
Non ischemic cardiopathies			
Structural defects	CMH	MYH7, TNNT2, TPM1, MYBPC3, PRKAG2, TNNI3, MYL3, TTN, MYL2, ACTC1, CSRP3, LAMP2	CMH1, CMH2, CMH3, CMH4, CMH5, CMH6, CMH7, CMH8, CMH9, CMH10, CMH11, CMH12, Danon disease
	Dilated cardiomyopathy	ACTC, DES, SGCD, MYH7, TNNT2, TPM1, TTN, VCL, MYBPC, MLP, ACTN2, PLN, ZASP, MYH6, ABCC, TNNC1, TCAP, EYA4, LMNA, SCN5A, DMD, TAZ, TNNI3	Laminopathies, hypertension, ischemic disease
	Arrhythmogenic right ventricular dysplasia/cardiomyopathy	JUP, DSP, PKP2, DSG2, DSC2, RYR2, TGFB3	Naxos disease, Carvajal disease
Channelopathies	Long QT syndrome	SCN5A, SCN4B, KCNQ1, KCNH2, KNE1, KNE2, KCNJ2, ANK2, CAV3	Romano-Ward syndrome, Jervell Lange-Nielsen syndrome, Andersen-Tawil syndrome, Timothy syndrome
	Brugada syndrome	SCN5A, SCN1B, GPD1L, CACNA1C, CACNB2b	
	Sindrome di Lev-Lenègre	SCN5A	
	Short QT syndrome	KCNH2, KCNQ1, KCNJ2	
	Sindrome di Wolff-Parkinson-White	AMPK	
	Tachicardia ventricolare	ADRB1, ADRB2, ADRB3	
	Tachicardia ventricolare polimorfica catecolaminergica	RYR2, CASQ2	
	Atrial fibrillation	KCNQ1, KCNE2, KCNJ2, KCNH2	
Ischemic cardiopathy			
Coronary artery disease, myocardial infarction	Mendelian inheritance	LDLR, APOB, ABCG5, ABCG8, APOA1, ABCA1, CBS	Familial hypercholesterolemia
	Complex disease	9p21, SH2B3, MRP56-SLC5A3-KCNE, PHACTR1, CELSR2-PSRC1-SORT, CXCL12, MIA3, PCSK9	

CMH: Hypertrophic cardiomyopathy; HOS: Holt-Oram syndrome.

lead to premature CAD, genetic factors leading to atherosclerosis are often addressed as polymorphisms, which are variants that show high frequencies in the general population and participate in individual susceptibility to develop the disease. Mendelian forms of CAD are caused by mutations in genes involved in sterol metabolism, HDL concentration regulation, cholesterol efflux in macrophages and homocysteine concentration regulation. These include the low density lipoprotein receptor (*LDLR*, 19p13.2), the apolipoprotein B and A1 (*APOB*, 2p24.1 and *APOA1*,

11q23.3), members 5 and 8 of the subfamily G of the ATP binding cassette (*ABCG5* and *ABCG8*, 2p21), member 1 of the subfamily of the ATP binding cassette (*ABCA1*, 9q31.1) and the cystathionine-beta-synthase (*CBS*, 21q22.3) genes. Identification of the *LDLR* gene in the pathogenesis of familial hypercholesterolemia advanced knowledge on the cholesterol metabolism pathway as a major player in atherogenesis^[40]. Since this discovery, many studies, in particular large scale genome-wide association studies, identified several common variants in genes encoding for

proteins involved in cholesterol metabolism, inflammation and immunity that are associated with atherogenesis. In particular, an association between CAD and a region on chromosome 9 (9p21) was first identified in 2005^[47]. This result was replicated in another 25 different studies. A recent meta-analysis of 16 of these 25 studies has confirmed a statistically significant association between 9p21 polymorphisms and CAD^[48]. Nevertheless, this chromosomal region is devoid of protein-coding genes and a clear functional interpretation is still lacking. However, it is known that this region neighbours *CDKN2A/B* (encoding cyclin-dependent kinase inhibitors involved in cell cycle) genes. Recently, Visel *et al.*^[49] observed that deletion of the orthologous 70 kb non-coding region on *Mus musculus* chromosome 4 affects cardiac expression of the neighbouring genes, as well as proliferation properties of cells in the vessel wall. As a consequence, Chr4^{Δ70/Δ70} mice showed rapid weight gain and increased mortality during the developmental phase as well as in adulthood. Upon necropsy, 45% of these animals were found to have neoplasms of various types suggesting that this region could have a pivotal role in the regulation of cell proliferation and senescence^[49]. This region is also associated with other phenotypes, such as sporadic amyotrophic lateral sclerosis, cutaneous nevi development, and intracranial aneurism^[50-52].

The analysis of phenotypes, such as CAD or MI, presents two main obstacles: (1) the complexity of phenotypes (e.g. differences between early and late age onset MI, ST elevation MI and non-ST elevation MI) that can lead to non-replications^[53]; and (2) corrections that must be applied when analyzing multiple variants^[54], which can lead to false negatives. It is possible that, in the years to come, with the refinement of samples and the development of new methods, data unravelling the complexity of CAD will be easier to obtain. To give an idea of how quickly information on complex diseases increases, 5 new loci associated with CAD were identified in 2009 alone. Gudbjartsson *et al.*^[55] found genome-wide significance for a non-synonymous SNP on *SH2B3* gene (at 12q24) in association with inflammation in endothelial cells, elevated eosinophil count, and acute MI in six populations^[55]. The Myocardial Infarction Genetics Consortium, studying a sample of early onset acute MI, identified three new variants: 21q22 near *MRPS6-SLC5A3-KCNE* (encoding genes for mitochondrial ribosomal protein 28s, a sodium and myo-inositol transporter in response to hypertonic stress, and a potassium channel involved in the pathogenesis of arrhythmias), 6p24 in *PHACTR1* (encoding for an inhibitor of protein phosphatase 1 involved in serine and threonine dephosphorylation crucial for cell growth and differentiation), and 2q33 in *WDR1*, a member of the Pes1-Bop1 complex (required for ribosome biogenesis and, once again, crucial for cell proliferation). This consortium also replicated genome-wide significance for 6 previously identified variants (9p21, 1p13 near *CELSR2-PSRC1-SORT1*, 10q11 near *CXCL12*, 1q41 in *MLA3*, 19p13 near *LDLR* and 1p32 near *PCSK9*)^[56]. Erdmann *et al.*^[57] identified a new susceptibility locus on 3q22.3

(*MRAS*, a RAS related protein encoding gene involved with cell growth and differentiation.).

Although much effort has been spent on identifying and interpreting the involvement of different genetic variants in the pathogenesis of atherosclerosis, we can consider the problem far from being solved.

COMMON DISEASES AND VARIANT HYPOTHESES

Mutations that have a deleterious effect are usually associated with disease and, hence, often remain rare, with the result that the related disease is also rare. Variants conferring an advantage are often the basis for evolutionary change and tend to rise rapidly to high frequency, a phenomenon known as genetic hitchhiking^[58]. On the other hand, a polymorphism is defined as a frequent variant that is often neutral. Although the majority of neutral mutants are lost by chance, a minority of them eventually become fixed in the population^[59]. There are two hypotheses on the genetic basis of common diseases. On the one hand, the common disease common variant (CDCV) hypothesis postulates that genetic variants have low penetrance but high frequency in the population and contribute to the genetic background of common diseases; and on the other hand, the common disease rare variant (CDRV) hypothesis proposes that rare variants with strong penetrance provide this attribute to common diseases. On the basis of the second hypothesis, new generation sequencing methods are being tested to identify the rare variations that have escaped in genome-wide association studies^[60]. Over the last few years, these studies have identified a number of SNPs in the genome, resulting in recognition of about 150 common variants in robust association with over 30 common phenotypes. Given the very low penetrance and number of studies that have analyzed such variants, it is difficult to give an accurate predictive value for a complex disease state such as CAD, diabetes or hypertension.

UTILITY AND LIMITATIONS OF TESTING GENETIC VARIATIONS

Despite the discussions and concerns that have been described elsewhere^[60-68], there is an immense need to validate and provide a qualification process for genomic biomarkers before use in clinical practice, from a practical point of view.

Further, it is important to interpret the results of genetic testing using a set of parameters that includes family history and a scoring system for the range of clinical manifestations associated with the disease. Genome-wide association studies have so far identified only a small fraction of the heritability of CAD, so the ability to make meaningful predictions is still quite limited. Nonetheless, direct-to-consumer marketing of genetic risk prediction for CAD is attracting early adopters.

To date, the only genomic biomarkers that have been

Table 2 List of genetic markers that have been approved by the US Food and Drug Administration and by the European Medicines Agency (source: <http://www.fda.gov>)

Biomarker	Representative label	Drug
HLA-B*5701 allele presence	Patients who carry the HLA-B*5701 allele are at high risk for experiencing a hypersensitivity reaction to abacavir	Abacavir
Her2/neu over-expression	Over-expression of Her2/neu necessary for selection of patients appropriate for drug therapy (breast cancer)	Trastuzumab (Herceptin®)
EGFR expression with alternate context	Epidermal growth factor receptor presence or absence (colorectal cancer)	Cetuximab (Erbix®)
UGT1A1 variants	UGT1A1 mutation patients, exposure to drug and hence their susceptibility to toxicity (colon-rectum cancer)	Irinotecan (Camptosar®)
TPMT variants	Increased risk of myelotoxicity associated to thiopurine methyltransferase deficiency or lower activity	Azathioprine (Imuran®)
Protein C deficiencies (hereditary or acquired)	Hereditary or acquired deficiencies of protein C or its cofactor protein S	Warfarin (Coumandin®)
C-KIT expression	Gastrointestinal stromal tumour c-kit expression	Imatinib mesylate (Glivec®)
CYP2C19 variants	CYP2C19 variants (poor metabolizers PM and extensive metabolizers EM) with genetic defect leads to change in drug exposure	Voriconazole (Vfend®)
CYP2C9 variants	CYP2C9 variants PM and EM genotypes and drug exposure	Celecoxib (Celebrex®)
CYP2D6 variants	CYP2D6 variants PM and EM genotypes and drug exposure	Atomoxetine (Strattera®)
CYP2D6 with alternate context	CYP2D6 PM and EM variants and drug exposure and risk	Fluoxetine HCl (Prozac®)
DPD deficiency	Severe toxicity (stomatitis, diarrhoea, neutropenia and neurotoxicity) associated to deficiency of dihydropyrimidine dehydrogenase	Capecitabine (Xeloda®)
EGFR expression	Epidermal growth factor receptor presence or absence (NSCLC, pancreas cancer)	Erlotinib (Tarceva®)
EGFR expression with alternate context	Epidermal growth factor receptor presence or absence (squamous cell carcinoma of head and neck)	Cetuximab (Erbix®)
G6PD deficiency	G6PD deficiency and risk for haemolysis	Rasburicase (Elitek®)
G6PD deficiency with alternate context	G6PD deficiency (or NADH methemoglobin reductase deficiency) and risk for haemolytic reactions	Primaquine (Primaquine®)
KRAS mutation	Retrospective subset analyses of metastatic colorectal cancer trials have not shown a treatment benefit for Vectibix in patients whose tumors had KRAS mutations in codon 12 or 13. Use of Vectibix is not recommended for the treatment of colorectal cancer with these mutations	Panitumumab (Cetuximab®)
NAT variants	N-Acetyltransferase slow and fast acetylators and toxicity	Rifampin isoniazid (Rifater® and pyrazinamide)
Philadelphia chromosome deficiency	Philadelphia (Ph1) chromosome presence and efficacy-Busulfan is less effective in patients with CML lacking the Philadelphia chromosome	
UCD efficiency disorders	Valproate therapy and urea cycle disorders interaction	Valproic acid (Depakene®)
VKORC1 variants	Polymorphisms of vitamin K epoxide reductase complex subunit identify warfarin-sensitive patients who require a lower dose of the drug	Warfarin (Coumandin®)
PML/RAR α gene expression (retinoic acid receptor responders and non-responders)	PML/RAR (α) fusion gene presence	Tretinoin (Avita®, Renova®, Retin-A®)

approved and are recommended by the European Medicines Agency (EMA) and by the Food and Drug Administration (FDA) are localized in the ambit of pharmacogenomics (e.g. *VKORC1/CYP2C9* genotype for warfarin dosing in coagulation defects and HLA-B*5701 for the prevention of adverse reactions in antiretroviral therapy in HIV infected patients^[68]; Table 2 from <http://www.fda.gov>).

LEVELS OF BIOLOGICAL VARIATION

DNA is a very stable molecule that is easy to extract and is less prone to degradation compared to RNA. DNA is, hence, easier to study. However, one must consider somatic cell mutations, tissue-specific epigenetic effects such as DNA methylation, histone modification and micro-RNA expression, which can significantly and constantly change expression in the cell. Such changes can alter the activities of the cell and cannot be neglected when studying the biology of a complex disease^[69]. Figure 2 shows a

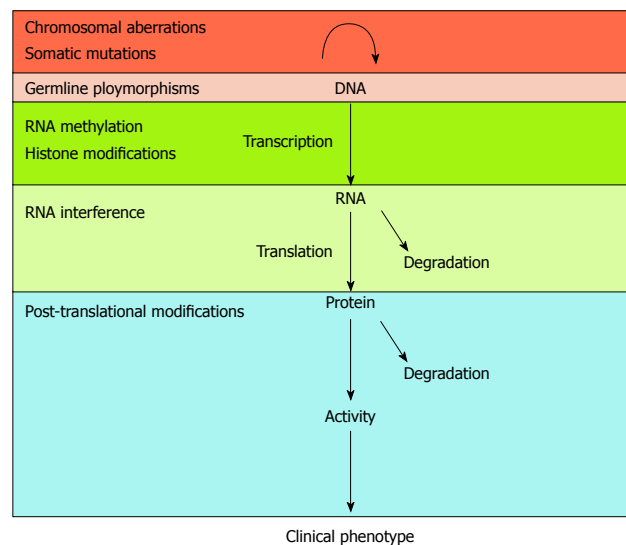


Figure 2 Biological variation at different levels. Modified from Brockmüller *et al.*^[69], 2008.

simplified scheme of these variations. As an example, in the cardiovascular field, we have already mentioned the “mutation theory of atherosclerosis”, which underlines the similarity between atherosclerotic and carcinogenic processes^[41,42]. Furthermore, different microRNAs have been found to be involved in different phases of ischemic heart disease^[70]. These levels of variation are much harder to analyze, and are not constant during the individual's lifetime as DNA variations, but are important in the pathology of all diseases, including cardiovascular diseases. The study of epigenetics, transcriptomics and proteomics is, therefore, another important issue in all disease studies and needs to be well integrated with studies of genome variability. These discussions and integration of these issues is beyond the scope of this mini-review.

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Ergonovine stress echocardiography: Recent experience and safety in our centre

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Abstract

AIM: To study recent experience and safety of ergonovine stress echocardiography in our centre.

METHODS: In this study we collected the clinical variables of patients referred since 2002 for ergonovine stress echocardiography, in addition to indications, the results of this test, complications, blood pressure and heart rate values during the test and the number and results of tests requested before this technique.

RESULTS: We performed 40 tests in 38 patients, 2 tests were carried out to verify therapy efficacy. The prevalence of classic cardiovascular risk factors was low and the most frequent indication was chest pain (57.5%). Coronary angiography was performed in 32 patients, and showed normal coronary arteries in 27 patients and non-significant stenosis in 5 cases. In 16 patients, coronary angiography was carried out after a positive or inconclusive ischemia test. Another 6 patients had a normal stress test (5 exercise electrocardiography

tests and 1 nuclear imaging test). Of the 40 ergonovine stress echocardiography tests, 6 were positive (4 in the right coronary artery territory and 2 in the circumflex coronary artery territory), all of them by echocardiographic criteria, and by electrocardiographic criteria in only 3 (50%). The presence of non-significant coronary artery stenosis was more frequent in patients with positive ergonovine stress echocardiography (50% *vs* 6%, $P = 0.038$), and were related to ischemic territory. During the maximum stress stage, there was a higher systolic (130.26 ± 19.17 mmHg *vs* 136.58 ± 27.27 mmHg, 95% CI: -12.77 to 0.14 mmHg, $P = 0.055$) and diastolic blood pressure (77.89 ± 13.49 mmHg *vs* 83.95 ± 15.73 mmHg, 95% CI: -10.41 to -1.69 mmHg, $P = 0.008$) than at the baseline stage, and the same was registered with heart rate (73 ± 10.96 beats/min *vs* 79.79 ± 11.72 beats/min, 95% CI: -9.46 to -4.11 beats/min, $P < 0.01$). Nevertheless, there were only 2 hypertensive reactions during the last stage, which did not force a premature end to the test, without sustained tachy or bradyarrhythmias, and the technique was well tolerated in 58% of cases. A unique complication (2.5%) of this test was a prolonged vasospasm with a slight increase in necrosis biomarkers, however, this was without reperfusion.

CONCLUSION: Ergonovine stress echocardiography can be performed with safety, is well tolerated in the majority of cases, and is useful for determining the ischemia mechanism in selected cases.

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Key words: Coronary angiography; Ergonovine; Myocardial ischaemia; Stress echocardiography; Vasospasm

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INTRODUCTION

Coronary vasospasm may be the cause of effort angina, myocardial infarction, syncope and sudden death^[1-3]. When there is no documentation of the initial clinical picture, the unique tests available for diagnosis orientation are the vasospasm provocation techniques.

There are several substances that can be used to cause a coronary vasospasm. Methylergometrine (a synthetic medicine chemically related to ergonovine) and acetylcholine are the most commonly used; another substances which can provoke vasospasm are histamine, dopamine and serotonin^[4,5].

There is a significant demand for non-invasive tests in Cardiology for the assessment of ischemia due to fixed coronary stenosis. The situation in vasospasm provocation techniques is different, because their safety have been questioned^[6]. However, there are a number of studies which have demonstrated that ergonovine stress echocardiography is a safe tool for the diagnosis of vasospasm, after confirming the absence of inducible ischemia or significant coronary stenosis by coronary angiography, and has many advantages compared with invasive provocation tests. These techniques have relevancy, especially, in the differential diagnosis of resting chest pain, which is suspected to be related to the vasospastic origin, in which coronary angiography or coronary computed tomography do not show significant stenosis^[7-17].

In this study, we assessed the experience related to ergonovine stress echocardiography over the last few years in our centre, collected the clinical variables of patients referred to our laboratory and the test results.

MATERIALS AND METHODS

Study group

We retrospectively collected the results of ergonovine stress echocardiography carried out in our centre since 2002. The tests were performed with the administration protocol of an intravenous bolus of ergonovine (methylergometrine) every 5 min across 3 stages (initial dose of 0.05 mg, middle dose of 0.10 mg and later dose of 0.20 mg), with blood pressure measurement and 12-lead electrocardiography at each stage and continuous echocardiography and electrocardiography (1-lead) monitoring. The criteria for finishing the test were to have reached the highest dose of ergonovine, the detection of electrocardiographic changes (ST segment deviation of 1 mm or more in at least 2 contiguous leads) or wall motion abnormalities (transient worsening of myocardial function

in at least 2 contiguous segments) that led to suspicion of ischemia, significant arrhythmias, symptomatic hypertensive or hypotensive response (systolic blood pressure > 200 or < 90 mmHg, respectively), or symptoms which forced a premature end to the test. At the end of each study, an intravenous bolus of diltiazem (0.125 mg/kg) was administered to all patients. The criteria for considering a test as abnormal were the detection of electrocardiographic or wall motion abnormalities that suggested ischemia, with or without associated symptoms.

Collected variables

We included the following patient baseline characteristics: age, gender, current or ex-smoker, high blood pressure, diabetes mellitus, hypercholesterolemia, chronic kidney disease, and antecedents of myocardial infarction, typical and variant angina, and surgical or percutaneous coronary revascularization), interruption of treatment that could modify the test accuracy, number and results of prior tests (coronary angiography, dobutamine or dipyridamole stress echocardiography, nuclear perfusion imaging and exercise tests) and indication, time between the studied event and ergonovine stress echocardiography, and its results (positive tests and criteria for positivity, affected territory, symptoms, dose of ergonovine administered, complications and blood pressure and heart rate at baseline and during the maximum stress stage). Informed consent was obtained from all patients.

Statistical analysis

Data are shown as average \pm SD or as number and percentage. Continuous variables were analyzed with the *t* test for paired samples. Quantitative variables were analyzed with the chi-square or Fisher test. We considered a difference as statistically significant when the *P* value was < 0.05.

RESULTS

There were 40 tests carried out in 38 patients, 2 tests were performed to verify the efficacy of therapy with calcium channel blockers. Baseline characteristics and test indications are shown in Table 1 and Figure 1, respectively. The criteria for requesting these tests were based on the clinical suspicion of coronary vasospasm as the reason for the clinical picture studied, after rejecting the possibility of inducible ischemia or significant coronary artery stenosis. Before ergonovine stress echocardiography, all patients underwent an ischemia test or coronary angiography. Coronary angiography was performed in 32 patients, and showed normal coronary arteries in 27 patients and non-significant stenosis in 5 cases, all confirmed by flow coronary reserve measurement. The percentage of stenosis in patients with non-significant coronary artery disease was 35% in 2 cases and 30%, 28% and 25% in the other 3 patients, which was performed by quantitative assessment. In 16 cases, coronary angiography was carried out after a positive or inconclusive ischemia test. Another 6 patients

Table 1 Baseline characteristics of patients referred for ergonovine stress echocardiography (*n* = 38) *n* (%)

Age (yr, mean \pm SD)	55 \pm 12.11
Masculine gender	24 (63)
Active smokers	14 (37)
Ex-smokers	4 (10.5)
Alcohol consumption > 2 drinks/d	6 (16)
Arterial hypertension	17 (48)
Diabetes mellitus	5 (13)
Hypercholesterolemia	15 (39)
Chronic kidney disease	2 (5)
Antecedents of myocardial infarction	1 (2.6)
Antecedents of typical angina	2 (5)
Antecedents of variant angina	3 (8)
Antecedents of percutaneous coronary revascularization	2 (5)
Antecedents of surgical coronary revascularization	1 (2.6)

Chronic kidney disease was defined as a creatinine clearance of < 60 mL/min.

Table 2 Results of ergonovine stress echocardiography (*n* = 40) *n* (%)

Positive tests	6 (15)
Transient segment function worsening	6 (15)
Dynamic ischemia in electrocardiogram	3 (7.5)
Chest pain during the test	6 (15)
Hypertensive reaction	2 (5)
Sustained tachy or bradyarrhythmias	0 (0)
Complications	1 (2.5)
Mild adverse reactions	16 (40)

had a normal stress test (5 exercise electrocardiography tests and 1 nuclear imaging test). Among the group with an abnormal ergonovine stress echocardiography, 3 patients (50%) had a non-significant stenosis in the vessel related to ischemia during the test, while among the group with an initial negative ergonovine stress echocardiography only 6% had a non-significant stenosis (Figure 2); this difference was statistically significant ($P = 0.038$). With regard to the baseline characteristics of the patients and the clinical picture studied, there were no significant differences between patients with a positive and negative ergonovine stress echocardiography. A baseline echocardiography was performed in all patients, and was normal in 28 (74%). Findings included 1 case (2.6%) of stress cardiomyopathy in relation to transient apical dyskinesia, 1 case of mild rheumatic mitro-aortic valvulopathy (mild aortic stenosis with mild mitral stenosis), 1 case of non-obstructive septal hypertrophic cardiomyopathy and 1 case of non-ischemic dilated cardiomyopathy, due to cardiac magnetic resonance imaging that did not show late gadolinium enhancement and suggested ischemic necrosis.

The ergonovine stress echocardiography results are shown in Table 2. Among the 6 abnormal cases, 4 were related to the right coronary artery territory while 2 were related to the circumflex coronary artery territory, without multivessel spasm. Among the positive tests, all patients presented with wall motion abnormalities and 5 (83%) had chest pain, whereas only 3 (50%) presented with significant ST segment changes, that consisted of ST segment

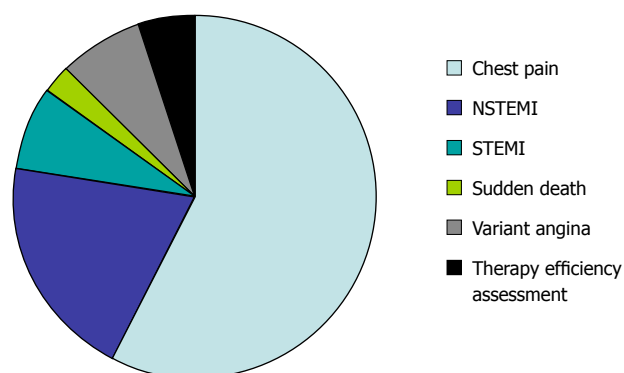


Figure 1 Indications for ergonovine stress echocardiography (*n* = 40). In all cases with myocardial infarction and referred for ergonovine stress echocardiography, coronary angiography did not show significant stenosis, and a vasospasm provocation technique was performed to orientate the study of its aetiology. Ergonovine echocardiography for studying chest pain was performed when there was a suspicion of coronary vasospasm due to clinical characteristics. We defined variant angina when there was documentation of transient ST-segment elevation in patients with chest pain with normal blood levels of necrosis biomarkers. NSTEMI: Non ST elevation myocardial infarction; STEMI: ST elevation myocardial infarction.

elevation of at least 1 mm in 2 contiguous leads in all cases (2 in inferior leads and 1 in lateral leads). On the other hand, 1 patient had chest pain with the highest ergonovine dose with neither worsening in regional myocardial function nor significant electrocardiographic changes (only a diffuse decrease in T wave voltage), that was attributed to a dose-dependent diffuse coronary vasoconstriction.

In 16 tests (40%) the patients developed an adverse effect: headache (10 cases), nausea (4 cases) and anxiety (2 cases). There was a hypertensive response in 2 patients (highest systolic blood pressure 200 mmHg in both patients) in the last stress stage, which did not force a premature end to the test. A statistical trend was found where higher systolic blood pressure values were observed during the test (95% CI: -12.77 to 0.14, $P = 0.055$) and significant differences were noted in relation to higher diastolic blood pressure values (95% CI: -10.41 to -1.69, $P = 0.008$) and heart rate (95% CI: -9.46 to -4.11, $P < 0.01$). Blood pressure and heart rate values are shown in Figure 3. A unique and serious complication was a prolonged vasospasm in the lateral segments with 0.15 mg of ergonovine, which led to a mild increase in necrosis biomarkers (highest troponin I = 10.2 ng/mL), but without wall motion abnormalities in the control echocardiography performed 1 mo later. This patient had presented with a non ST elevation myocardial infarction, and coronary angiography showed a luminal stenosis of 30% in the first diagonal branch. The other 5 positive cases improved rapidly with intravenous nitrates.

The time between the studied event and the ergonovine stress echocardiography was, in cases with elevation of necrosis biomarkers, 56 ± 11 d, and 3 ± 1 d for the remaining cases. In all patients, treatments that could modify the test results were withdrawn at least 48 h before the ergonovine stress echocardiography, with the exception of 2 cases in which the aim was to show treatment efficacy

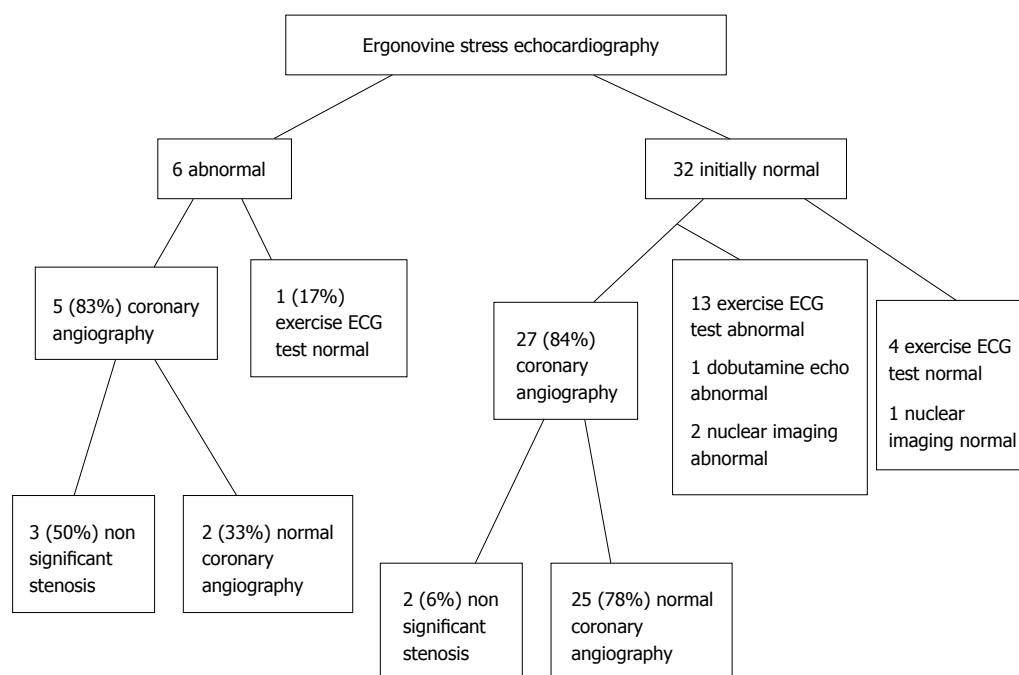


Figure 2 Tests performed before ergonovine stress echocardiography depending on the results of the stress test. ECG: Electrocardiography.

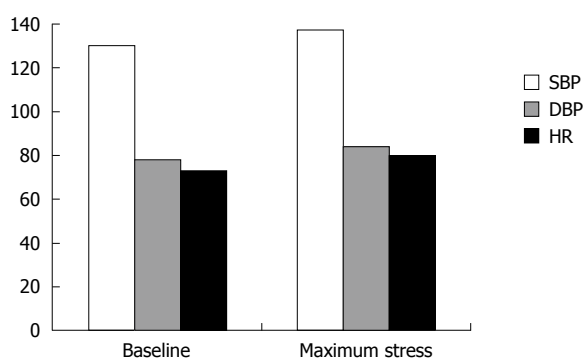


Figure 3 Blood pressure and heart rate at baseline and during the maximum stress stage of ergonovine stress echocardiography. DBP: Diastolic blood pressure (mmHg); HR: Heart rate (beats/min); SBP: Systolic blood pressure (mmHg).

and who had a prior abnormal stress test. The total dose of ergonovine administered was 0.15 mg in 2 studies (5%) that were abnormal, and 0.35 mg in the other cases (average dose 0.34 ± 0.04 mg).

DISCUSSION

Among patients referred for ergonovine stress echocardiography, the prevalence of classic cardiovascular risk factors was low and the most frequent indication was chest pain. This technique allowed us to orientate the clinical picture diagnosis to coronary vasospasm in 15% of tests. Although there was a mild increase in blood pressure and heart rate, this technique was well tolerated in the majority of patients. The test was safe, resulting in the unique complication a prolonged vasospasm with a slight increase in necrosis biomarkers, but without wall motion

abnormalities on control echocardiography. The presence of non-significant coronary stenosis was more frequent among the group of patients who had a positive ergonovine stress echocardiography, and these stenoses were related to the ischemic territory.

The induction of coronary vasospasm in patients who are suspected to have variant angina can occur in as many as 31% of Caucasian patients, and appears to be more common among east Asian patients^[18]. Vasospasm may be related to a hypersensitivity to vasoconstrictor stimulus, with an imbalance between relaxing factors, such as nitric oxide, and constrictor factors released by the endothelium. Oxidative stress, endothelial dysfunction, high endothelin-1 activity and regional sympathetic denervation demonstrated by nuclear imaging may play an important part in the pathogenesis of coronary vasospasm^[12,13,19-21].

Test positivity would support the diagnosis of coronary vasospasm, contributing to a complete differential diagnosis of chest pain, while if the test is normal, suspicion of vasospasm has to be based on treatment response and clinical picture. Vasospasm may be confirmed with Holter electrocardiography in cases with frequent symptoms, if it shows typical ischemic electrocardiographic changes^[18,20].

Ergonovine stress echocardiography has demonstrated excellent accuracy in orientating the diagnosis of coronary vasospasm, compared with the invasive provocation test, with a sensitivity of 93%, specificity of 96%, positive predictive value of 96% and negative predictive value of 86%^[12,22]. The fast recognition of wall motion abnormalities, information that is usually unavailable during invasive techniques, occur before electrocardiographic changes and onset of symptoms^[23,24], and allow us to prevent complications related to ischemic waterfall. On the other hand, ergonovine stress echocardiography avoids

the use of contrast and radiation associated with coronary angiography. One problem with this test may be the lack of a central venous approach for temporary pacing or an intracoronary catheter for intracoronary medicine infusion. However, with regard to its safety, several studies have reported a very low rate of complications, similar to dobutamine or dipyridamole, when appropriate patient selection is carried out by excluding those with significant stenosis and rapidly administering the antidote^[9-13].

The most frequently affected territory is normally the one related to the left anterior descending coronary artery, followed by the right coronary artery^[9,10]. In our study the latter territory was the most common; this situation may be explained by the smaller sample size compared with another studies.

Consistent with the literature, an important number of positive cases did not have electrocardiographic changes; this finding does not support the performance of techniques with only electrocardiographic monitoring^[12,13,22].

Among the patients with positive tests, 5 (83%) developed chest pain, while only 1 patient with a negative test had chest pain with the highest ergonovine dose, but without worsening of myocardial wall motion or significant electrocardiographic changes. Chest pain is not a variable systematically registered in other studies because it is not a criterion for a positive response^[3,12,14,15,20,22]. In one study in which 52 patients with acute coronary syndrome and normal or near-normal coronary angiograms were enrolled^[13], chest pain was registered in 19 cases (76%) with positive ergonovine stress echocardiography and in 4 patients (15%) in the group with a negative test. These data were similar to the data found in the present study.

With regard to prognosis, patients with a positive test seemed to have more frequent events and a lower survival during follow-up, although these patients continued with the treatment. This was more evident in smokers or if there had been a multivessel spasm^[11].

In this study, we found a higher rate of non-significant stenosis on coronary angiography in patients with a positive ergonovine stress echocardiography compared with patients who had a negative test. Thus, we expect that with the extension of non-invasive coronary angiography^[7,8,25] and the uncertain meaning of non-significant coronary artery stenosis, the request for ergonovine stress echocardiography will increase in the near future. On the other hand, we are aware of not having performed coronary angiography in all patients, and the real rate of cases with non-significant stenosis and a negative ergonovine stress echocardiography may be higher. Thus, these findings have to be interpreted with caution.

There are other possible uses for ergonovine stress echocardiography. This technique may be useful for determining the cause of acute heart failure without structural or coronary heart disease, due to the fact that it has been described as severe mitral regurgitation related to spasm of a posterior branch, which can be manifested during an ergonovine stress test^[26]. This technique could have additional value in the differential diagnosis between stress

cardiomyopathy and coronary vasospasm, as several studies have demonstrated that this test is negative in stress cardiomyopathy. Nevertheless, the diagnosis of this pathology remains complex especially in patients with transient apical dyskinesia and normal coronary angiography^[27,28].

Our data suggest that ergonovine stress echocardiography can be performed with safety in the majority of patients, usually with good tolerance.

Thus, based on our experience, we consider that ergonovine stress echocardiography is a safe and useful technique for orientating the ischemia mechanism if patients are appropriately selected on the basis of either previous normal coronary angiography, normal exercise electrocardiography or pharmacological stress test, contributing to a more complete differential diagnosis of chest pain.

COMMENTS

Background

The diagnosis of coronary vasospasm is often complicated. Frequently, there is no documentation of the initial clinical picture, and the unique tests available for diagnosis orientation in these situations are the vasospasm provocation techniques. A positive result in these tests supports the diagnosis of coronary vasospasm, contributing to a more complete differential diagnosis of chest pain.

Research frontiers

Ergonovine stress echocardiography has demonstrated excellent accuracy for the diagnosis of coronary vasospasm, compared with the invasive provocation tests, and has many advantages compared with these tests. With regard to prognosis, patients with a positive test seemed to have more frequent events and a lower survival during follow-up, and this was more evident in smokers or if there had been a multivessel spasm.

Innovations and breakthroughs

The safety of vasospasm provocation techniques has been questioned. However, there are a number of studies that have demonstrated that ergonovine stress echocardiography is a safe tool for vasospasm diagnosis, with a very low rate of complications if patients are appropriately selected on the basis of either previous normal coronary angiography or normal exercise electrocardiography or pharmacological stress test. Several studies have reported a very low rate of complications, similar to dobutamine or dipyridamole stress echocardiography, when appropriate patient selection is carried out, and rapidly administering the antidote.

Applications

In this study we have registered a higher rate of non-significant stenosis on coronary angiography in patients with positive ergonovine stress echocardiography compared with patients who had a negative test, and these stenoses were related to the ischemic territory. We expect that with the extension of non-invasive coronary angiography and the uncertain meaning of non-significant coronary artery stenosis, the request for ergonovine stress echocardiography will increase in the near future. Based on our experience, ergonovine stress echocardiography can be performed with safety in the majority of patients, usually with good tolerance, and we consider it a safe and useful technique for orientating the ischemia mechanism if patients are appropriately selected.

Terminology

Coronary vasospasm: a sudden constriction of a heart blood vessel, causing a reduction in local blood flow that can provoke a myocardial infarction. Coronary angiography: Examination of the heart blood vessels using x-rays following the injection of a radiopaque substance. Echocardiography: examination of the heart using ultrasound techniques. Ergonovine: an alkaloid derived from ergot that induces muscular contraction. It can be used for assessing the predisposition to a coronary vasospasm. Myocardial infarction: destruction of an area of heart muscle as the result of occlusion of a coronary artery.

Peer review

This is a well-written paper dealing with the topic of ergonovine stress echocardiography. The authors studied 40 patients with this method and report their

experience. All the patients had either normal or non-significant coronary angiography or normal stress tests before ergonovine stress echocardiography. The test was positive in 6 patients. No complications were registered.

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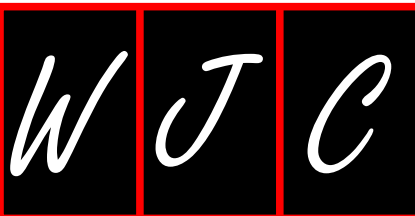
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Meetings

Events Calendar 2010

January 12-13
Riyadh, Saudi Arabia
1st International Cardiovascular Pharmacotherapy Conference

January 17-21
Hollywood, United States
22nd Annual International Symposium on Endovascular Therapy

January 20-23
Sao Paulo, Brazil
World Cardiology, Metabolism and Thrombosis Congress

January 21-24
Phoenix, United States
13th Society for Cardiovascular Magnetic Resonance Annual Scientific Sessions

January 28-30
Brussels, Belgium
29th Belgian Society of Cardiology Annual Scientific Meeting

January 28-31
Nashville, United States
31st Annual Meeting of The American Academy of Cardiovascular Perfusion

February 3-6
Snowbird, United States
35th Annual Cardiovascular Conference at Snowbird

February 4-5
Leuven, Belgium
Leuven Symposium on Myocardial Velocity and Deformation Imaging

February 6-9
St. Petersburg, United States
10th Annual International Symposium on Congenital Heart Disease

February 8-10
Tel Aviv, Israel
10th International Dead Sea Symposium on Cardiac Arrhythmias and Device Therapy

February 11-12
London, United Kingdom
2nd National Chronic Heart Failure and Hypertension

February 18-21
Istanbul, Turkey
The 2nd World Congress on Controversies in Cardiovascular Disease (C-Care)

February 22-25
Maui, United States
Arrhythmias & the Heart Symposium

February 22-26
Cancun, Mexico
15th Annual Cardiology at Cancun-Advances in Clinical Cardiology and Multi-Modality Imaging

February 25-28
Valencia, Spain
First International Meeting on Cardiac Problems in Pregnancy

February 26-28
Hong Kong, China
International Congress of Cardiology

February 28-March 4
Scottsdale, United States
International Congress XXIII on Endovascular Interventions

February 28-March 5
Keystone, United States
Keystone Symposia: Cardiovascular Development and Repair (X2)

March 3-5
Kish Island, Iran
Islamic Republic of 4th Middle East Cardiovascular Congress

March 4-7
Newport Beach, United States
30th Annual CREF: Cardiothoracic Surgery Symposium

March 7-12
Snowmass Village, United States
Interventional Cardiology 2010: 25th Annual International Symposium

March 14-16
Atlanta, United States
American College of Cardiology 59th Annual Scientific Session

March 18-20
Rome, Italy
VIII Congress of the Italian Society of Cardiovascular Prevention

March 18-20
Prague, Czech Republic
XI International Forum for the Evaluation of Cardiovascular Care

March 24-25
Jeddah, Saudi Arabia
12th KFAFH Cardiovascular Conference: A balanced approach to treatment of cardiovascular diseases

April 8-11
Guangzhou, China
The 12th South China International Congress of Cardiology

April 14-15
Tel Aviv, Israel
The 57th Annual Congress of the Israel Heart Society in Association with The Israel Society of Cardiothoracic Surgery

April 15-18
Izmir, Turkey
59th European Society for Cardiovascular Surgery International Congress

May 5-7
Prague, Czech Republic
EuroPrevent 2010-Cardiovascular Prevention: a Lifelong Challenge

May 8-9
St. Paul, United States
Controversies in Cardiovascular Disease: Practical Approaches to Complex Problems: Medical and Surgical

May 12-16
Marrakesh, Morocco
7th Metabolic Syndrome, type II Diabetes and Atherosclerosis Congress

May 17-20
Whistler, Canada
6th IAS-Sponsored HDL Workshop on High Density Lipoproteins

May 21-22
Sydney, Australia
3rd Cardiovascular CT, Concord Conference 2010

May 29-June 1
Berlin, Germany
Heart Failure Congress 2010

June 1-4
Seoul, Korea, Republic of
9th Asian-Pacific Congress of Cardiovascular & Interventional Radiology (APCCVIR 2010)

June 16-19
Beijing, China
World Congress of Cardiology Scientific Sessions

June 17-19
Port El Kantaoui, Tunisia
The 7th Tunisian and Europeans Days of Cardiology Practice

July 1-3
Singapore, Singapore
6th Asian Interventional Cardiovascular Therapeutics Congress

July 16-19
Berlin, Germany
Frontiers in CardioVascular Biology 2010-1st Meeting of the CBCS of the ESC

July 24-27
Vancouver, Canada
15th World Congress on Heart Disease, Annual Scientific Sessions 2010

August 13-15
Krabi, Thailand
East Meets West Cardiology 2010

September 16-18
Athens, Greece
5th International Meeting of the Onassis Cardiac Surgery Center

September 25-29
Belo Horizonte, Brazil
65th Brazilian Congress of Cardiology

September 30-October 2
Berlin, Germany
5th International Symposium on Integrated Biomarkers in Cardiovascular Diseases

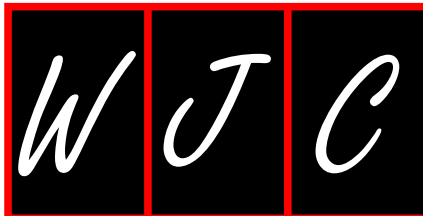
October 10-13
Rochester, United States
26th Annual Echocardiography in Pediatric and Adult Congenital Heart Disease Symposium

October 16-19
Copenhagen, Denmark
Acute Cardiac Care 2010

October 20-23
Boston, United States
2010 Cardiometabolic Health Congress

November 25-26
London, United Kingdom
13th British Society for Heart Failure Annual Meeting

December 9-11
Lisbon, Portugal
Heart, Vessels & Diabetes-The European Conference



Instructions to authors

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The columns in the issues of *WJC* will include: (1) Editorial: To introduce and comment on major advances and developments in the field; (2) Frontier: To review representative achievements, comment on the state of current research, and propose directions for future research; (3) Topic Highlight: This column consists of three formats, including (A) 10 invited review articles on a hot topic, (B) a commentary on common issues of this hot topic, and (C) a commentary on the 10 individual articles; (4) Observation: To update the development of old and new questions, highlight unsolved problems, and provide strategies on how to solve the questions; (5) Guidelines for Basic Research: To provide guidelines for basic research; (6) Guidelines for Clinical Practice: To provide guidelines for clinical diagnosis and treatment; (7) Review: To review systematically progress and unresolved problems in the field, comment on the state of current research, and make suggestions for future work; (8) Original Articles: To report innovative and original findings in cardiology; (9) Brief Articles: To briefly report the novel and innovative findings in cardiology; (10) Case Report: To report a rare or typical case; (11) Letters to the Editor: To discuss and make reply to the contributions published in *WJC*, or to introduce and comment on a controversial issue of general interest; (12) Book Reviews: To introduce and comment on quality monographs of cardiology; and (13) Guidelines: To introduce consensus and guidelines reached by international and national academic authorities worldwide on the research in cardiology.

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Acknowledgments

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- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarhoea. *Shijie Huaren Xiaobua Zazhi* 1999; **7**: 285-287

In press

- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID: 2516377 DOI: 10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI: 10.1097/01.ju.0000067940.76090.73]

No author given

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI: 10.1136/bmj.325.7357.184]

Volume with supplement

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI: 10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; **(401)**: 230-238 [PMID: 12151900 DOI: 10.1097/00003086-200208000-00026]

No volume or issue

- 9 Outreach: Bringing HIV-positive individuals into care. *HRS-A Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

- 12 **Breedlove GK**, Schorffheide AM. Adolescent pregnancy. 2nd ed. Wicczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

- 15 Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/eid/index.htm>

Patent (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean \pm SD or mean \pm SE.

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Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as *v* (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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