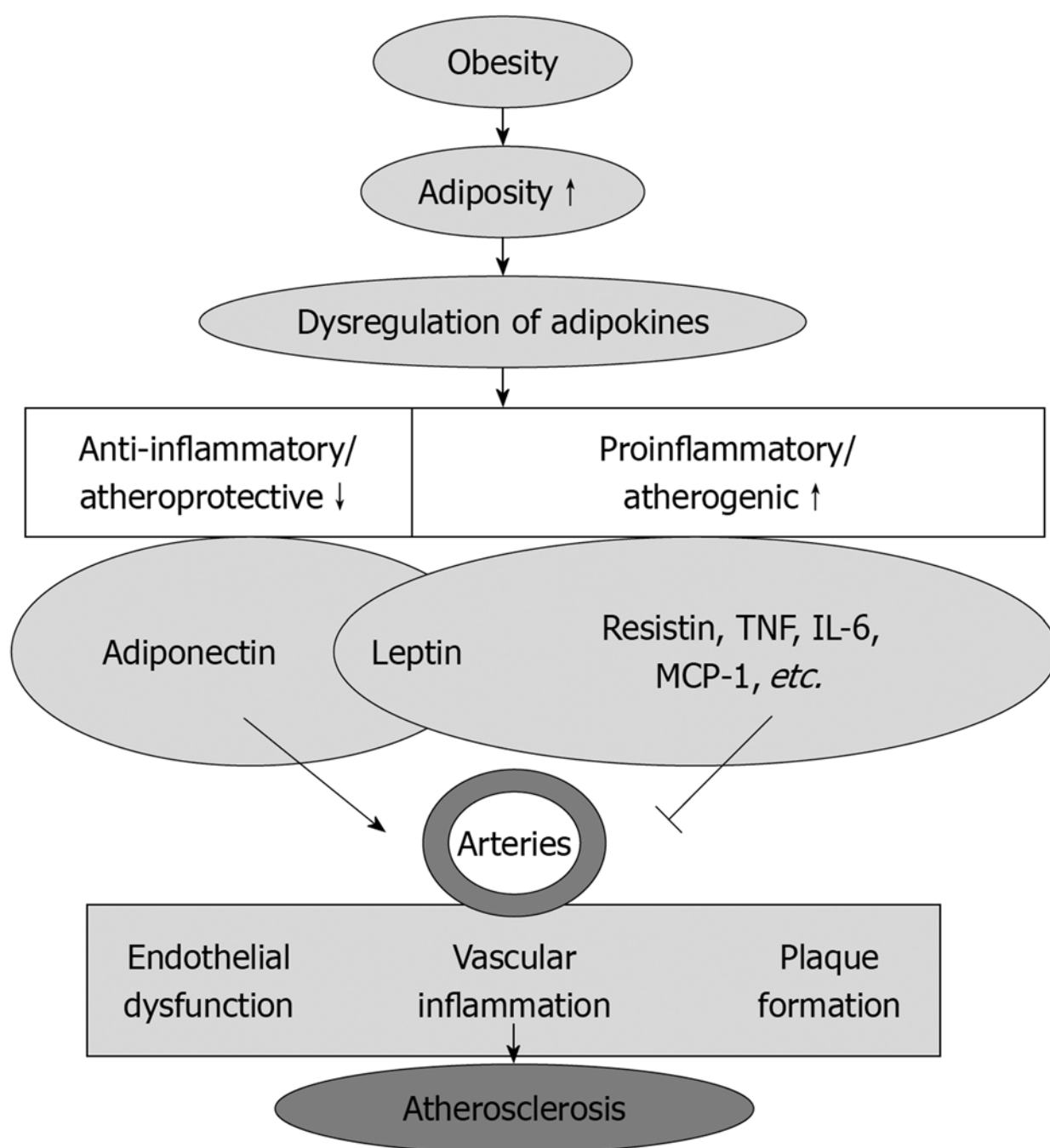


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Cuihua Zhang, MD, PhD, FAHA, Associate Professor, Series Editor

## Role of dendritic cells in cardiovascular diseases

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Author contributions: Zhang Y wrote the review article; Zhang C edited the manuscript and mentored Zhang Y.

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development of new approaches to treat many cardiovascular diseases, including atherosclerosis, cardiac IRI and transplantation.

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**Key words:** Dendritic cells; Atherosclerosis; Ischemia reperfusion; Transplantation

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### Abstract

Dendritic cells (DCs) are potent antigen-presenting cells that bridge innate and adaptive immune responses. Recent work has elucidated the DC life cycle, including several important stages such as maturation, migration and homeostasis, as well as DC classification and subsets/locations, which provided etiological insights on the role of DCs in disease processes. DCs have a close relationship to endothelial cells and they interact with each other to maintain immunity. DCs are deposited in the atherosclerotic plaque and contribute to the pathogenesis of atherosclerosis. In addition, the necrotic cardiac cells induced by ischemia activate DCs by Toll-like receptors, which initiate innate and adaptive immune responses to renal, hepatic and cardiac ischemia reperfusion injury (IRI). Furthermore, DCs are involved in the acute/chronic rejection of solid organ transplantation and mediate transplant tolerance as well. Advancing our knowledge of the biology of DCs will aid

### INTRODUCTION

Dendritic cells (DCs) were initially identified as potent antigen-presenting cells that play a key role in induction of the innate immune response<sup>[1]</sup>. Further investigation revealed DCs were also a link between the innate and adaptive immune systems<sup>[2,3]</sup>. DCs that were activated *via* endogenous or exogenous antigens progressed from immature DCs to mature DCs, which initiated the release of cytokines that activated the T cell/B cell immune responses<sup>[2,4-6]</sup>. DCs have now been confirmed to be involved in many different disease conditions, such as rheumatoid arthritis, pulmonary allergic disease, rhinitis and other autoimmune diseases<sup>[7-9]</sup>. DCs have also been implicated in various cardiac pathologies, such as atherosclerosis<sup>[10]</sup>, endothelial dysfunction<sup>[11]</sup>, ischemia reperfusion injury (IRI)<sup>[12]</sup> and heart transplantation<sup>[11]</sup>. The purpose of this review is to summarize the biology and function of DCs and draw attention to their role in cardiovascular diseases.

## BIOLOGY OF DCs

Steinman and Cohen originally described the role of DCs in the initiation of immune responses in 1973<sup>[13]</sup> and many workers since then have further elucidated the classification, origin, life cycle and functions of DCs.

DCs have been classified into two categories: plasmacytoid DCs (pDCs) and conventional DCs (cDCs)<sup>[2,14-16]</sup>. cDCs are sub-divided into two major populations, including non-lymphoid tissue migratory and lymphoid tissue-resident DCs<sup>[4]</sup>. pDCs and cDCs differ in origin, phenotype, localization and immunological function. pDCs, which are named for morphological similarity to plasma cells, have expression markers, including CD123 (IL-3R) and BDCA-2<sup>[17]</sup>. They recognize oligodeoxynucleotides *via* Toll-like receptors (TLR) 7 and TLR 9, whose functions are to produce cytokines of interferon (IFN)- $\alpha$ <sup>[18]</sup>. In contrast, cDCs have expression markers of CD1c, CD11c, CD33 and CD209 *via* TLR2, TLR4 and TLR7. cDCs usually produce different cytokines, such as tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-12 and IL-23<sup>[19,20]</sup>. Locational differences also exist. pDCs circulate in the blood and produce IFN- $\alpha$  when they are activated by an exogenous virus. Lymphoid tissue-resident cDCs homeostatically reside in all lymphoid organs, while non-lymphoid tissue migratory cDCs are located in different tissues, such as skin, lung, heart, kidney, liver or intestine<sup>[4]</sup>. Those non-lymphoid tissue migratory cDCs are mobile sentinels that reside in the blood. They become “inflammatory” DCs during inflammation or infection and migrate to lymphoid organs where they accumulate to stimulate T cells<sup>[21]</sup>.

The origin of DCs still remains controversial. The initial concept was that all DCs were derived from a hemopoietic stem cell (CD34<sup>+</sup> common myeloid precursor, CMP) in bone marrow<sup>[1]</sup>. Later studies demonstrated some DCs may originate from common lymphoid precursors (CLP)<sup>[22]</sup>. Recent evidence supports the idea that the FLT3 expressing hemopoietic precursors, as a growth factor for hemopoietic progenitors, are the precursors of both CLP and CMP pathways to produce cDCs and pDCs in the steady state<sup>[23-25]</sup>, while granulocyte-macrophage colony-stimulating factor, a primary growth factor for DCs, will increase the number of monocyte-derived DCs during inflammation and infection<sup>[26]</sup>. León *et al*<sup>[27]</sup> suggested that monocyte-derived DCs, which form at an infection site, control the induction of protective T helper 1 response. In contrast to the traditional concept that DCs are end-stage and nondividing cells, Liu *et al*<sup>[28]</sup> reported that there are two processes that account for DC homeostasis in lymphoid organs: (1) most DCs arise *via* a constant replenishment by DC precursors that replace dying nondividing DCs; and (2) about 5% of resident DCs replicate *in situ* for a limited number of divisions before dying.

Accumulated evidence indicates that DCs have several important checkpoints in their life cycles, including antigen-capture, maturation, migration, antigen-presentation and homeostasis<sup>[1,4,6]</sup>. In most tissues, other than secondary lymphoid tissues (lymph node and spleen), immature

DCs monitor exogenous and endogenous antigens to fulfill their sentinel/surveillance functions. In the steady-state, without an overt presence of stimuli, those immature DCs induce and maintain peripheral self-tolerance<sup>[29]</sup>. In the inflammatory state, where there is infection from bacteria/virus or intrinsic damage induced by hypoxia or ischemia, the immature DCs will capture antigens by various mechanisms, including phagocytosis, macropinocytosis, or receptor-mediated antigen uptake (C-type lectin receptors, scavenger receptors and complementary receptors), which in turn initiates DC maturation and migration<sup>[2]</sup>. Several factors have been proposed to induce and regulate DC maturation, including pathogen-related molecules (LPS, bacterial DNA or double-strand RNA), pro-inflammatory signals (TNF- $\alpha$ , IL-1, IL-6, IL-10, transforming growth factor- $\beta$  and prostaglandins) and T cell-derived signals (CD40L)<sup>[2]</sup>. These factors induce activation of pattern recognition receptors (PRRs), such as TLRs and CD1 receptors, both of which are bacteria and virus antigen presenting receptors<sup>[6]</sup>. TLRs also combine with endogenous ligands to induce an immune response after ischemia, hypoxia or tissue damage<sup>[30,31]</sup>. DC maturation is accompanied by the loss of endocytic phagocytic receptors, morphological changes of DCs, up-regulation of co-stimulatory molecules like CD40, CD58, CD80 and CD86, and MHC-II molecules/production of pro-inflammatory cytokines (TNF- $\alpha$  and IL-12)<sup>[2,4]</sup>. Commensurate with the activation of TLRs and CD1-mediated maturation, immature DCs will down-regulate DC's endocytic capacity and migrate from local tissues to secondary lymphoid organs. The anatomical routes for DC trafficking were reported to include afferent lymphatics or high endothelial venules<sup>[19]</sup>. Migratory mature DCs and lymphoid tissue-resident cDCs reach the T-cell zone in the lymph nodes and spleen, which in turn presents antigens to naïve T cells to initiate an adaptive immune response<sup>[20]</sup>. T cell activation consists of several effectors, such as helper T cell, regulatory T cells and cytotoxic T cells, which in turn secrete cytokines to activate other cells (macrophages, NK cells and eosinophils), or to lyse the infected cells<sup>[2]</sup>. DCs are eliminated by apoptosis after their interaction with T cells<sup>[2,6]</sup>.

DCs play a critical role by bridging the innate and adaptive immune responses<sup>[5,32]</sup>. The innate immune response is the first line of defense against foreign pathogens and tissue injuries or malignancies. DCs, as an important component of the innate immune response, scan and detect the environmental signs of foreign infection or tissue damage. DCs then become activated, which is followed by their migration to lymph nodes and maturation *via* TLR activation. TLRs are type I transmembrane protein receptors and are expressed on the surface of DCs<sup>[33]</sup>. There were 11 human and 13 mouse TLRs cloned after the first TLR was reported in 1997<sup>[34]</sup>. In the innate immune response, TLR activation initiates acute inflammatory responses by releasing inflammatory cytokines/chemokines to recruit neutrophils and the activation of macrophages, leading to the killing of pathogens directly<sup>[35]</sup>. On the other hand, TLR activation was found to ini-



tiate, maintain, modulate and terminate the innate host defenses by either producing pro-inflammatory cytokines or stimulating DC maturation to initiate T-cell expansion and activate antigen-specific adaptive immune responses<sup>[36]</sup>. In addition, TLRs promote the expression of co-stimulatory molecules (CD80 and CD86) to maintain the activation of adaptive immunity. DC activation *via* TLRs serves as a major link between innate and adaptive immunity<sup>[37]</sup>. Accumulated evidence has demonstrated that the engagement of DCs and TLRs defends against skin/pulmonary infection and autoimmune diseases<sup>[7,9]</sup> and also plays a key role in cardiovascular diseases, such as atherosclerosis, myocardial IRI and cardiac transplantation<sup>[10-12]</sup>.

## DCs AND ATHEROSCLEROSIS

The mechanisms of atherosclerosis have evolved to include inflammation as one of its important causes. Atherosclerosis can be characterized as a disease arising from the immune response<sup>[38,39]</sup>. Vascular inflammation has been linked to innate and adaptive immunity that includes DCs playing an important role in the pathogenesis of atherosclerosis<sup>[10]</sup>, as well as arising through traditional pathways, such as those involving macrophages and monocytes.

DCs reside in healthy arteries in small amounts, as well as in other organs such as kidney, lung or intestines. DCs have been reported to occur in the adventitia and intima of large arteries, including aorta, coronary and carotid arteries<sup>[40]</sup>. In contrast, DCs were not present in normal veins and they only occurred in diseased veins after vessel injury<sup>[41]</sup>. The vascular resident DCs are “immature DCs”, which are continuously and efficiently monitoring exogenous and internal antigens in the steady physiological state. DCs are reported to be key elements of vascular-associated lymphoid tissues, which function as sentinels to screen for potential harmful antigens that arise in vascular tissues<sup>[42,43]</sup>.

Direct evidence for a relationship between DCs and atherosclerosis came in 1995 when Bobryshev and his group reported that DCs accumulated in atherosclerotic lesions<sup>[44]</sup>. They went on to demonstrate a greater amount of DCs clustered in the intima of atherosclerosis-prone areas than in the atherosclerosis-resistant areas of the non-diseased aorta<sup>[45]</sup>. Thus, DCs occurred in healthy arteries and pre-atherosclerosis stage arteries, and in atherosclerotic lesions<sup>[46]</sup>. Subsequent work further implicated DCs in atherosclerosis<sup>[47,48]</sup>. Both local vascular DCs and blood DCs *via* inflamed neo-vessels were shown to contribute to the formation of atherosclerotic lesions<sup>[46,47]</sup>. Kawahara *et al*<sup>[49]</sup> confirmed that the expression of vascular DCs was observed in human atherosclerotic carotid plaques, which may be strongly associated with the occurrence of ischemic stroke; they found a close relationship between the mean signal intensity of DCs in plaques and the symptoms of patients with significant carotid plaques as well. Mechanisms that govern how vascular DCs contribute to the pathogenesis of atherosclerosis have been proposed. Vascular DCs activated by different antigens (viral, bacterial or auto-antigens) are followed by activation of T

cells and natural killer cells and initiate an inflammatory response in the arterial wall<sup>[10]</sup>. Bacci *et al*<sup>[50]</sup> demonstrated that smooth muscle cells, DCs and mast cells are sources of TNF- $\alpha$  and nitric oxide in human carotid artery atherosclerosis.

Vascular DCs not only contribute to the formation of atherosclerosis, but also play a crucial role in plaque destabilization. Plaque destabilization is an important risk factor for acute myocardial infarction and acute stroke after coronary or carotid artery plaque rupture. Clinical cardiology has confirmed that more than 50% of coronary artery plaques rupture in the plaque shoulder and the markedly increased number of DCs (more than 90%) were located in the plaque shoulder - the plaque-prone area<sup>[51]</sup>. Yilmaz *et al*<sup>[52]</sup> analyzed the frequency of different immune cells in atherosclerotic carotid plaque and demonstrated that immune cells were strongly associated with neovascularization. They concluded that enhanced recruitment of immune cells through neovessels into the upstream shoulder might contribute to plaque destabilization. In addition, Niessner *et al*<sup>[53]</sup> recently reported that human coronary and carotid plaques contain myeloid DCs (mDCs) in close cell-cell contact with T cells and mDCs are highly activated to produce the T-cell-attracting chemokines CCL19 and CCL21, which results in plaque destabilization. The evidence of co-accumulation of DCs and natural killer T cells within rupture-prone regions in human atherosclerotic plaques from Bobryshev's group supports the view that DCs shape the functional activity of natural killer cells to destabilize plaque<sup>[54]</sup>. Furthermore, Erbel *et al*<sup>[55]</sup> demonstrated that activated and fully mature DCs are represented in the inflammatory infiltrate that characterizes unstable carotid and coronary atheroma. More recently, Yilmaz *et al*<sup>[56]</sup> investigated the different levels of DC precursors and demonstrated a significantly lower level of circulating DC precursors in stable coronary artery disease (CAD) patients compared to healthy individuals, which is an independent predictor of the presence of stable CAD.

Inflammation as a mechanism of atherosclerosis and the involvement of DCs in the process of atherosclerosis have inspired possible therapeutic interventions for atherosclerosis by changing the DC profile. Statin was found to inhibit the maturation and antigen-presenting function of DCs, which may show a beneficial effect in atherosclerosis<sup>[57]</sup>. Vaccine therapy may also prevent atherosclerosis<sup>[58]</sup>.

## INTERACTION BETWEEN DCs AND ENDOTHELIAL CELLS

DCs have a close anatomical and functional relationship with endothelial cells (ECs). Immature DCs patrol in the blood in the steady state. Under inflammatory conditions, DCs will capture antigens and migrate from peripheral tissue to nearby lymph nodes to present antigens. When DCs are exiting from the bloodstream, they (either from blood or from tissue) will need to tether to the ECs, during which some proteins, such as E-selectins or P-selectins, may be involved. Robert *et al*<sup>[59]</sup> have demonstrated that

blood DCs constitutively interact with normal murine skin endothelium *in vivo* via selectins. In contrast, ECs play a crucial role in the inflammatory response. Their activation significantly promotes vascular permeability, edema and leukocyte recruitment, including DC activation. EC and DC cell lineages are closely related and they exert a reciprocal effect on their differentiation. Fernandez Pujol *et al.*<sup>[60]</sup> provided evidence of a phenotypic overlap between monocyte-derived DCs and microvascular endothelium and confirmed that DCs derived from peripheral monocytes express endothelial markers.

Increasing evidence has supported the idea that the adhesion and migration of DCs are affected by EC activation. Weis *et al.*<sup>[61]</sup> found the endothelial determinants of DC adhesion and migration and concluded that adhesion and migration of DCs are increased by endothelial activation and prevented by the augmentation of endothelial NO synthase activity. Inflamed lymphatic endothelium was also found to be able to suppress DC maturation and function *via* the MAC-1/ICAM-1-dependent mechanism<sup>[62]</sup>. Angelot *et al.*<sup>[63]</sup> reported that EC-derived microparticles induce pDC maturation and the production of inflammatory cytokines. More recently, Zhu *et al.*<sup>[64]</sup> suggested that homocysteine increased vascular oxidative stress and decreased NO release, which enhanced DC adhesion to and transmigration across the endothelium, indicating the importance of DC-EC interaction.

DC-EC cross-talk plays a pivotal role in angiogenesis<sup>[65]</sup>. There are several mechanisms responsible for this cross-talk. DCs were found to have the ability to transdifferentiate into endothelial-like cells to contribute to vasculogenesis<sup>[65]</sup>. DCs can produce and release pro- and anti-angiogenic mediators, including the potent angiogenic growth factor vascular endothelial growth factor-A, which directly acts on the endothelium by combining its signaling receptors on the EC surface. Furthermore, DCs, upon activation, can release cytokines and chemokines, which increase or decrease the responsiveness of ECs. IFN- $\alpha$ -producing pDCs can inhibit EC motility and promote the production of anti-angiogenic chemokines<sup>[66]</sup>. Therefore, a better understanding of DC-EC cross-talk in the pathophysiological process of angiogenesis will aid in the discovery of important mechanisms for inflammatory diseases.

## DCs AND IRI

CAD is a major problem in the USA and worldwide; early reperfusion of occluded coronary artery by thrombolytic therapy or percutaneous coronary interventions is the standard treatment. IRI, however, has become an important problem since it limits the full potential benefit of reperfusion therapy<sup>[67]</sup>. IRI was reported to involve several mechanisms, including oxygen paradox, calcium paradox, pH paradox, immune response and inflammation. Based on classic models, acute ischemia leads to endothelial activation and production of oxygen free radicals, which

promotes the secretion of inflammatory cytokines/chemokines and activity of adherent molecules<sup>[68]</sup>. Such changes recruit effector cells into the post-ischemic tissues. Reperfusion then further increases endothelial permeability and cell activation, which exacerbates the inflammatory reaction.

Recently, increasing evidence has demonstrated that both the innate immune response and the adaptive immune response mediated by T cell activation contribute to IRI<sup>[69,70]</sup>. DCs, which bridge innate and adaptive immune responses, are an important component in the pathogenesis of IRI<sup>[69]</sup>. Ischemia leads to tissue damage, which releases endogenous ligands, such as heat shock proteins, matrix components and products of necrotic cells. These ligands will combine with a family of PRRs, such as TLRs, which activate the first line of defense in the innate immune system<sup>[30]</sup>. TLRs were reported to be expressed in many cells, including antigen presenting cells, such as DCs. As discussed above in the biology of DCs, immature DCs will migrate to secondary lymphoid tissues (spleen and lymph nodes) to stimulate naïve T lymphocytes and trigger naïve T cell response.

The role of DCs in the hepatic and renal IRI have been extensively studied. DCs were found to be present in healthy liver and kidney. The number of DCs in the liver and kidney was significantly increased during ischemia and reperfusion<sup>[71,72]</sup>. Wu *et al.*<sup>[73]</sup> demonstrated that renal IRI resulted in DC infiltration of the outer medulla of the kidney after 2 d of reperfusion. Resident liver DCs migrated from the sinusoidal lumen to the hepatic lymph *via* the space of Disse to become mature DCs<sup>[74]</sup>. Loi *et al.*<sup>[75]</sup> showed that liver IRI itself induces DC maturation, migration and preferential production of inhibitory cytokines in a mouse IRI model. Resident DCs were reported to be the predominant secretory source of TNF- $\alpha$  in early renal ischemia-reperfusion injury and *in vivo* depletion of DCs from the kidney substantially attenuated TNF- $\alpha$  secretion following IRI<sup>[76]</sup>. Furthermore, TLR signaling activation in the pathogenesis of liver and renal IRI were found in several recent studies<sup>[77,78]</sup>.

DCs exist in the heart and cardiac DCs are closely associated with the endocardial blood vessels and connective tissue. Cardiac DCs were reported to be aligned parallel to cardiac myocytes with their processes interdigitating between the myocytes<sup>[79]</sup>. Cardiac DCs are fewer in number but higher in density than in other organs, including liver, pancreas and kidney<sup>[80]</sup>. Zhang *et al.*<sup>[81]</sup> reported the accumulation of DCs in the “border zones” of infarct sites at 7 d post-infarct in a rat myocardial infarction model. Compared to the numerous published studies for liver and renal IRI, there is a lack of experiments or studies regarding the role of DCs in the pathogenesis of cardiac IRI. However, the role of TLR on IRI has been extensively studied. TLR2 and TLR4 activations were confirmed to be related to IRI by different groups of researchers, showing the reduction of infarct size and inflammation in TLR4 deficient mice after 1 h of ischemia and 24 h of reperfusion<sup>[82]</sup>. Sakata *et al.*<sup>[83]</sup> recently demonstrated that

TLR deficient murine hearts showed better cardiac function after myocardial infarction than the wild-type. More studies are warranted to explore the role of DCs on cardiac ischemia-reperfusion injury.

## DCs AND CARDIAC TRANSPLANTATION

Cardiac transplantation has proven to be an effective treatment for advanced heart failure patients, although the shortage of heart donors limits clinical application. An evolving understanding of the multiple pathways involved in immune activation has resulted in many advances in immunology for transplantation medicine<sup>[84]</sup>. The adaptive immune responses mediated with T cell/B cell activation have been emphasized for the regulation of transplant rejection and the advance of immunosuppressive agents has improved long-term survival after transplantation. The traditional infectious-non-self model indicates that the activation of PRRs identifies pathogen-associated molecular patterns to produce significant cytokine release by the engagement of TLR, which in turn activates the adaptive immune response. Recently, the innate immune response after transplantation has attracted much attention. Based on the new “Danger Model”, the damaged or necrotic self-tissue, rather than foreign tissue, is what will activate innate immune responses *via* increased TLR reactivity, leading to increased cytokine release<sup>[31]</sup>. DCs, as an important bridge between innate and adaptive immune responses, therefore play a significant role in the field of transplantation medicine.

DCs are potent antigen presenting cells involved in direct, indirect and semi-direct pathways of alloantigen recognition by the host immune system<sup>[84-86]</sup>. First, for the direct pathway, donor-derived DC and monocytes/macrophages that originally exist in the donor heart will leave the graft after transplantation and migrate to the recipient's secondary lymph system (including lymph node and spleen). They present donor antigen to recipient T cells directly to induce an adaptive immune response. This direct pathway is responsible for the acute rejection. Larsen *et al.*<sup>[87]</sup> demonstrated that donor resident DCs migrate from cardiac allografts into the host spleen during the first days after transplantation. In contrast, for the indirect pathway, the recipient's DCs migrate into the graft after heart transplantation, where they pick up and process the donor antigen, to activate the recipient's adaptive immune response after their presentation to the recipient's T cells. The indirect pathway may actually be the cause of chronic rejection. Kofler *et al.*<sup>[11]</sup> reported that DCs frequently infiltrate the cardiac allograft with a peak during the first post-operative year and confirmed the graft-infiltrating DCs and coronary endothelial dysfunction after human heart transplantation. Finally, for the semi-direct pathway, recipient T cells recognize donor MHC molecules, which are transferred from donor cells to the surface of recipient cells intact<sup>[88]</sup>. Loverre *et al.*<sup>[89]</sup> also reported that myeloid and pDCs were significantly increased, with few mature DCs during delayed graft function. In addition, DC binding

and transmigration on allogenic ECs exposed to calcineurin inhibitors were concentration-dependently increased, indicating that long-term immunosuppression mediates enhanced invasion of DCs to the donor organ and aggravates chronic rejection<sup>[90]</sup>. Furthermore, DCs have been used as tools for controlling allograft rejection in organ transplantation<sup>[91,92]</sup>. Shlomchik *et al.*<sup>[93]</sup> provided evidence of prevention of graft versus host disease by inactivation of host antigen-presenting cells.

DCs not only induce an immune response and participate in organ rejection, but also mediate transplant tolerance<sup>[84,94]</sup>. Hawiger *et al.*<sup>[95]</sup> first reported that, in the absence of additional stimuli, DCs induce transient antigen-specific T cell activation followed by T cell deletion and unresponsiveness. Subsequently, Bonifaz *et al.*<sup>[96]</sup> demonstrated that small amounts of injected antigen, targeted to DCs by the DEC-205 adsorptive pathway, are able to induce solid peripheral CD8<sup>+</sup> T cell tolerance, indicating a constitutive tolerance role for DCs in peripheral lymphoid organs in the steady state. It is commonly accepted that, in the steady state, CD8<sup>+</sup> and CD8 $\alpha$ <sup>+</sup> DCs remain quiescent after capturing and processing exogenous antigen, which in turn express low levels of co-stimulatory molecules and then induces deficient activation of naïve T cells, T cell apoptosis or anergy and the generation of regulatory T cells. Huang *et al.*<sup>[97]</sup> found that a distinct DC subset constitutively endocytoses and transports apoptotic cells to T cell areas, suggesting a role for those DCs in inducing and maintaining peripheral self-tolerance. In addition, Lambolez *et al.*<sup>[98]</sup> showed that self-antigen presentation exclusively by peripheral DCs resulted in very efficient deletion of the majority of antigen-specific T cells with the remaining cells left in an anergic state. Finally, evidence has accumulated that deliberately generated tolerogenic DCs might be a useful tool for the induction of donor-specific tolerance to prevent rejection after solid organ transplantation. Jiga *et al.*<sup>[99]</sup> found that mitomycin treatment converts rat DCs into tolerogenic cells, whose mechanism is mediated by decreased ICAM-1, CD80 and CD86.

In summary, DCs are a bridge connecting the innate immune response and the adaptive immune response. DCs play an essential role in the pathogenesis of many cardiovascular diseases, including atherosclerosis, cardiac IRI and cardiac transplantation. DCs also interact with other cells, such as endothelial, natural killer and T cells, to affect immunogenic responses in the body. DCs have great potential for use in the treatment/prevention of cardiovascular disease.

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## Linking inflammation and thrombosis: Role of C-reactive protein

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### Abstract

C-reactive protein (CRP) is a biomarker of inflammation. Increased plasma levels of CRP are associated with an increased risk of myocardial infarction. However, the correlation between plasma CRP concentration and atherosclerotic plaque burden is poor. Based on these observations, it has been hypothesized that CRP increases the risk of myocardial infarction by promoting thrombosis. This article reviews available data that link enhanced CRP expression to increased risk of thrombosis, with a focus on the effects of CRP on hemostasis, platelet function, and fibrinolysis. Overall, the available data support the hypothesis that CRP is an important mechanistic link between inflammation and thrombosis.

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**Key words:** C-reactive protein; Thrombosis; Inflamma-

### INTRODUCTION

Inflammation is defined as a localized protective reaction of tissue to irritation, injury, or infection, which is characterized by pain, redness, swelling, and loss of function. Inflammation plays a central role in the pathogenesis of atherosclerosis<sup>[1]</sup>. C-reactive protein (CRP) is an acute phase reactant plasma protein that is present in plasma of healthy humans and whose plasma concentration increases significantly during acute and chronic inflammation<sup>[2]</sup>. Several studies have demonstrated that plasma CRP concentration is independently associated with the incidence of atherothrombotic events in humans, most notably myocardial infarction<sup>[3,4]</sup>. However, whether CRP plays a causal role in atherosclerosis and its complications, or is simply an important clinical marker of inflammation and cardiovascular risk, continues to be debated<sup>[5]</sup>. Plasma CRP levels are only weakly associated with the extent of atherosclerosis in humans<sup>[6]</sup>. The Dallas Heart Study, which measured coronary artery calcification and aortic

plaque size in > 2000 individuals, concluded that CRP is a poor predictor of atherosclerotic burden<sup>[7]</sup>. These results are consistent with recent experiments in which CRP-transgenic mice were crossed to atherosclerosis-prone, hyperlipidemic mice to test directly the hypothesis that enhanced CRP expression drives atherosclerosis formation. Although an initial report was positive<sup>[8]</sup>, two subsequent studies have found no apparent effect of CRP on atherosclerotic plaque development<sup>[9,10]</sup>, and another study has found that CRP retards atherogenesis in mice<sup>[11]</sup>. However, transgenic mice that express human CRP demonstrate accelerated thrombosis after arterial injury compared to non-transgenic control mice<sup>[12]</sup>, and administration of highly purified preparations of CRP to humans activates the blood coagulation system<sup>[13]</sup>. These observations support the hypothesis that CRP increases the risk of ischemic vascular events, such as myocardial infarction, not by promoting atherosclerotic plaque size, but rather by activating the blood coagulation system and increasing the risk of thrombosis. The regulatory systems that control hemostasis and thrombosis, although functioning in a highly coordinated manner, can be subdivided into three major components, namely: (1) blood platelets; (2) blood coagulation proteins present in plasma and the vascular wall; and (3) the fibrinolytic system. In this article, the scientific evidence that links CRP to the regulation of each of these systems is reviewed. As a whole, the available data support the hypothesis that CRP is an important mechanistic link between inflammation and thrombosis.

## STRUCTURE AND BIOLOGIC ROLE OF CRP

CRP belongs to the pentraxin family of plasma proteins<sup>[2]</sup>. Native CRP consists of five identical subunits, each composed of 206 amino acids with a molecular weight of 23 000, which bind non-covalently to form a symmetrically shaped, pentameric molecule with a molecular weight of 118 000. Pentameric CRP can be dissociated into monomers *in vitro*<sup>[14]</sup> and *in vivo*<sup>[15]</sup>, with pentameric and monomeric forms exerting significantly different biological effects<sup>[16-18]</sup>. CRP binds to phosphocholine residues in bacterial cell membranes, thereby playing an important role in the innate immune response by facilitating the recognition and clearance of bacteria<sup>[19-21]</sup>. CRP also binds phosphocholine residues in apoptotic eukaryotic cells and to several mammalian proteins<sup>[22]</sup>. Aggregated or ligand-bound CRP activates the complement cascade, which suggests an additional mechanism by which CRP participates in host defense<sup>[23]</sup>. CRP is synthesized predominantly in the liver, where its production is controlled by several cytokines<sup>[2]</sup>.

## CRP ACTIVATES VASCULAR CELLS

CRP is detectable in the walls of diseased blood vessels, including atherosclerotic plaque<sup>[24,25]</sup>. Although vascular

wall CRP can be deposited from blood<sup>[26]</sup>, CRP mRNA is detectable in the arterial wall<sup>[25]</sup>, which indicates that CRP is also produced locally, particularly within atherosclerotic plaque, in which one study has found higher CRP mRNA concentrations than those in liver tissue<sup>[27]</sup>. Macrophages within plaques produce CRP<sup>[27]</sup>. CRP mRNA and protein are present in vascular smooth muscle cells (VSMCs) within human atherosclerotic plaques, which indicates that VSMCs synthesize CRP *in vivo*<sup>[24,27]</sup>. Inflammatory cytokines induce CRP expression by cultured human coronary artery VSMCs<sup>[28]</sup>. Exposure of cultured vascular endothelial cells to CRP inhibits nitric oxide synthase expression and upregulates expression of interleukin-8, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1<sup>[29,30]</sup>. In addition to phosphocholine residues, CRP binds to protein receptors that are present on plasma membranes of eukaryotic cells. CRP binds to Fcγ receptor I (FcγR I CD64), a member of the IgG Fc receptor family, which is expressed on macrophages<sup>[31,32]</sup>. FcγR II a (CD32), which is expressed by macrophages and platelets and plays an important role in the pathophysiology of immune-mediated thrombocytopenia, binds CRP to activate intracellular signaling pathways<sup>[33-35]</sup>. One study has suggested that CRP binds to vascular endothelial cells *via* FcγR I and FcγR II a<sup>[30]</sup>. However, some studies have concluded that CRP does not bind directly to FcγR II a, and that the observed interactions of CRP with cells that express FcγR II a might have been due to binding of the intact Fc region of anti-CRP antibodies to FcγR II a<sup>[36]</sup>.

## EFFECT OF CRP ON BLOOD PLATELETS

Platelets express CRP receptors FcγR III (CD16) and FcγR II a<sup>[37]</sup>. Several studies have found that CRP inhibits platelet aggregation induced by a variety of agonists, including thrombin, platelet aggregating factor (PAF), and immunoglobulin<sup>[38-40]</sup>. CRP appears to inhibit PAF-induced platelet aggregation by binding to the phosphocholine moiety of PAF<sup>[41]</sup>. However, CRP induces platelet adhesion to endothelial cells and monocytes<sup>[42,43]</sup>; interactions that promote thrombosis. The conformation of CRP (i.e. monomeric *vs* pentameric) might play a major role in controlling platelet aggregation. Pentameric CRP binds FcγR II a on platelets, which inhibits binding of platelets to neutrophils<sup>[37,44]</sup>. Conversely, monomeric CRP binds to FcγR III, which promotes platelet capture of neutrophils<sup>[37]</sup>. Activated platelets convert pentameric CRP to the monomeric form<sup>[44,45]</sup>. Hence, CRP-platelet crosstalk is bidirectional; i.e. CRP regulates platelet activation, whereas activation of platelets regulates the conformational status and biological function of CRP. Conversion of pentameric CRP to monomeric CRP by activated platelets leads to activation of monocytes, which potentially provides a mechanism to link platelet activation to monocyte activation and invasion into the vascular wall.

## REGULATION OF BLOOD COAGULATION FACTORS BY CRP

Tissue factor (TF) appears to be an important mechanistic link between inflammation, CRP, and thrombosis. TF is a 44000 molecular weight membrane-bound glycoprotein that plays a key role in initiating thrombosis after vascular injury by binding factor VIIa<sup>[46]</sup>. The TF-VIIa complex activates factor X and factor IX, thereby initiating proteolytic cascades that result in thrombin formation and blood clotting. TF is synthesized in the adventitia of normal blood vessels<sup>[47]</sup>, where it functions to maintain hemostasis after vascular trauma. TF is not detectable in the intima of normal arteries, but is abundant in the lipid-rich cores of atherosclerotic plaques<sup>[46,48]</sup>. CRP stimulates TF expression by blood monocytes *in vitro*<sup>[49]</sup>, and it has been proposed that the monocyte is an important target cell of CRP that mediates its prothrombotic effects. CRP also induces TF expression by VSMCs, both *in vitro* and *in vivo*<sup>[50,51]</sup>, which provides a mechanism by which CRP can promote fibrin formation after endothelium-denuding vascular injury.

## EFFECT OF CRP ON THE FIBRINOLYTIC SYSTEM

Plasminogen is converted to plasmin, the enzyme that degrades fibrin clots, by tissue-type plasminogen activator (t-PA). Plasminogen activator inhibitor-1 (PAI-1) is the main physiological inhibitor of t-PA and urinary-type PA. PAI-1 is present in plasma, platelets, endothelial cells, VSMCs, and extracellular matrix. CRP inhibits release of t-PA and stimulates release of PAI-1 from vascular endothelial cells<sup>[52,53]</sup>. Therefore, CRP can alter the fibrinolytic balance of endothelial cells so as to promote intravascular fibrin formation.

## CONCLUSION

In summary, CRP appears to play an important role in regulating the function of blood platelets, the extrinsic blood coagulation cascade, and the fibrinolytic system. *In vivo*, CRP enhances the thrombotic response to vascular injury. Inflammation upregulates CRP expression; hence, CRP appears to be an important mechanistic link between inflammation and thrombosis. Activation of the blood clotting system - specifically, activation of platelets - regulates CRP structure and biological function. Therefore, the CRP-dependent crosstalk between inflammation and thrombosis is bidirectional. Further studies are necessary to define more precisely the pro-thrombotic functions of CRP. In addition, more research is warranted to determine the impact on thrombosis of pharmacological inhibition of CRP expression level and function, which can be achieved with statins and compounds that specifically target CRP<sup>[54,55]</sup>.

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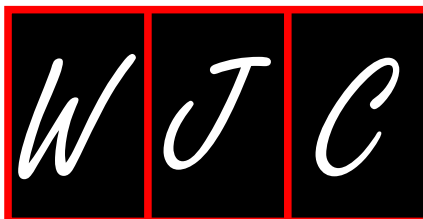


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## Emerging role of adipokines as mediators in atherosclerosis

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### Abstract

Atherosclerotic cardiovascular disease is a major health problem around the world. Obesity is a primary risk factor for atherosclerosis and is associated with increased morbidity and mortality of cardiovascular diseases. However, the precise molecular pathways underlying this close association remain poorly understood. Adipokines are cytokines, chemokines and hormones secreted by adipose tissue that couple the regulation of lipid accumulation, inflammation, and atherogenesis, and therefore serve to link obesity with cardiovascular disorders. Obesity-related disorders including metabolic syndrome, diabetes, atherosclerosis, hypertension, and coronary artery disease are associated with dysregulated adipokine(s) expression. Recent studies demonstrate the proinflammatory effects as well as atherogenic properties of adipokines. Adipokines also participate in the regulation of endothelial function, which is an early event in atherosclerosis. By contrast, adiponectin, an adipocyte-derived hormone, exerts anti-inflammatory, anti-atherogenic and vascular protective effects. Fur-

thermore, there is an interactive association among adipokines, by which adipokines reciprocally regulate each other's expression. Understanding this interplay may reveal plausible mechanisms for treating atherosclerosis and coronary heart disease by modulating adipokine(s) expression. In this review, we discuss insights into the role and the therapeutic potential of adipokines as mediators of atherosclerosis.

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**Key words:** Obesity; Inflammation; Adipokines; Endothelial function; Atherosclerosis

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### INTRODUCTION

Obesity is becoming pandemic<sup>[1]</sup>. Obesity and overweight conditions pose a major risk for a number of comorbidities including atherosclerotic diseases. The increasing prevalence of obesity, and recognition of the role of abdominal adiposity, has again focused attention on the relationship of obesity to atherosclerosis and coronary heart diseases<sup>[2]</sup>.

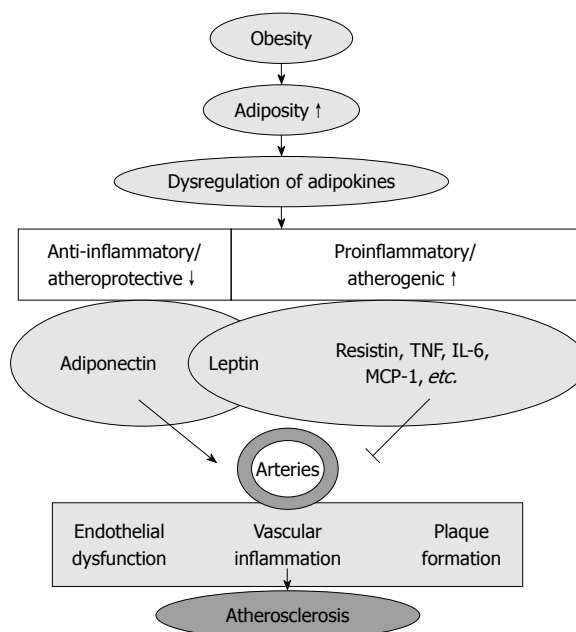
Although the association of obesity with atherosclerotic diseases has been widely reported<sup>[3]</sup>, mechanisms that describe how excess fat causes impairment of vascular function and atherosclerosis formation have not yet been fully elucidated. Recent advances in obesity research

strongly indicate that adipose tissue is an active endocrine organ that secretes multiple bioactive factors categorized as adipokines<sup>[4,5]</sup>. The adipokines include a large number of cytokines [e.g. tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-6], chemokines [e.g. IL-8 and monocyte chemoattractant protein (MCP)-1] and hormones (e.g. leptin, resistin and adiponectin)<sup>[6-8]</sup>. This review focuses on the contribution of excess adipose tissue to the major underlying cause of cardiovascular death, atherosclerosis and the evidence regarding potential mechanisms by which excess adipose tissue and the dysregulated adipokine expression profile could adversely affect the vessel wall.

## OBESITY AND ATHEROSCLEROSIS

Atherosclerosis is characterized by the progressive deposition of fatty substances, cholesterol, *etc.* (called plaques) in the intima and contributes to many cardiovascular diseases. Plaques can rupture and lead to formation of a blood clot, which can cause fatal complications, such as heart attack or stroke. The classical perception of atherosclerosis merely as a cholesterol storage disease has been replaced by the notion that inflammatory processes regulate all stages of atherosclerosis<sup>[9]</sup>. Obesity is an independent risk factor for the development of coronary artery atherosclerosis<sup>[3]</sup>. The fact that the prevalence of obesity is increasing among youth as well as adults, highlights the necessity for closely examining the association of obesity with atherosclerosis and the prevention of obesity<sup>[2]</sup>. Abdominal adiposity in particular is a major factor associated with accelerated progression of atherosclerosis, which emphasizes the need to develop strategies to avoid abdominal obesity and prevent atherosclerotic diseases<sup>[10]</sup>.

There are a number of mechanisms by which obesity can adversely affect the vasculature and thereby increase cardiovascular mortality. Obesity has various consequences known to accelerate atherosclerosis, including hypertension, diabetes, and dyslipidemia<sup>[11]</sup>. Adipokines released by adipose tissue can target distant organs, such as liver, skeletal muscle and hypothalamus. These adipokines impact the atherogenic environment of the vessel wall through metabolic changes, such as hyperlipidemia, hyperglycemia and insulin resistance. Moreover, systemic inflammation attributed to proinflammatory adipokines produced by inflamed adipose tissue serve as an important factor contributing to the adverse effects of adiposity on the vasculature<sup>[12]</sup>. Visceral adipose tissue, with its favored access to the portal circulation, could be particularly important in this pathway. In addition to the systemic effects, perivascular adipose tissue exhibiting a proinflammatory phenotype may exert paracrine effects and promote inflammatory cell infiltration into the vascular wall, thereby exerting local effects<sup>[13]</sup>. In addition to proinflammatory effects and atherogenic properties, the most abundant adipocyte protein, adiponectin, has potent anti-inflammatory as well as anti-atherogenic effects<sup>[14]</sup>. Those adipose-derived factors influence gene expression and cell function in endothelial cells, arterial smooth muscle cells, and monocytes/macro-



**Figure 1** Increased adiposity (obesity) is associated with dysregulated adipokine production, which is characterized by decrease in anti-inflammatory/atheroprotective adipokines (adiponectin) and increase in proinflammatory/atherogenic adipokines [resistin, tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-6, macrophage chemoattractant protein (MCP)-1, *etc.*]. Those adipokines participate in the regulation of endothelial function, vascular inflammation and plaque formation, which contribute to the inception and progression of atherosclerosis.

phages, which represent the major cell types of the artery wall and are major components for defending vessel wall homeostasis.

Therefore, adipokines may provide a link between adipose tissue lipid accumulation and atherosclerotic plaque formation, as well as the crosstalk between perivascular adipose and blood vessels in the regulation of atherosclerotic disease<sup>[13]</sup> (Figure 1).

## THE PROINFLAMMATORY CAPACITY OF ADIPOSE TISSUE

Obesity is associated with a chronic low-grade inflammatory condition in adipose tissue<sup>[15]</sup>. During obesity, adipose tissue is infiltrated by macrophages and displays secretion of proinflammatory cytokines<sup>[16,17]</sup>. Very recently, T cells have also been detected in adipose tissue with increased infiltration in obesity<sup>[18-20]</sup>, suggesting the potential role of adaptive immunity in obesity-related inflammation. Meanwhile, chemokines expressed in obese adipose tissue likely mediate the recruitment of these cells<sup>[21]</sup>, supporting the view of the existence of feedback regulation in perpetuating inflammatory status. Although it remains unclear which inflammatory cell types play predominant roles in the regulation of adipose inflammation, it is widely accepted that adipose inflammation, especially visceral adipose inflammation, increases vascular risk of disease due to secretion of adipokines by cellular constituents of the adipose tissue<sup>[22]</sup>. Atherosclerotic mice exhibit increased

inflammation in periadventitial and visceral adipose tissue<sup>[23]</sup>. The transplanted fat depots revealed chronically increased macrophage infiltration with characteristics identical to those observed in fat harvested from obese animals. Interestingly, by transplanting epididymal fat depots into atherosclerosis-prone apolipoprotein E knockout (ApoE KO) mice, plasma levels of leptin, resistin, and MCP-1 were increased. Mice transplanted with visceral fat developed significantly more atherosclerosis compared with sham-operated animals. The above results further support the idea that adipose inflammation contributes to atherosclerosis formation<sup>[24]</sup>.

The cellular source of adipokines released by adipose are examined in several studies. Leptin and adiponectin are predominantly produced by adipocytes in adipose and are released to the blood as hormones<sup>[25]</sup>. Over 90% of the adipokines released by adipose tissue, except for adiponectin and leptin, could be attributed to nonfat cells<sup>[25]</sup>. The location of adipose depots also affects the production and release of adipokines. Visceral adipose tissue released greater amounts of vascular endothelial growth factor, IL-6, and plasminogen activator inhibitor 1 compared with abdominal subcutaneous adipose tissue<sup>[25]</sup>.

Therefore, the proinflammatory properties may serve as the molecular basis for linking obesity with cardiovascular disorders. Knowledge of how alterations in the endocrine function of adipose tissue occur may help to identify mechanisms underlying the high cardiovascular risk associated with obesity<sup>[26]</sup>.

## ROLE OF ADIPOKINES IN ENDOTHELIAL DYSFUNCTION DURING ATHEROSCLEROSIS

Endothelial dysfunction is a key early event in the development of atherosclerosis<sup>[27,28]</sup>, which can be detected before structural changes to the vessel wall are apparent using angiography or ultrasound. Atherosclerotic endothelial dysfunction, particularly in the early disease stages, is primarily due to dysregulation of endothelial nitric oxide synthase (eNOS) enzymatic activity and inactivation of nitric oxide (NO) through oxidative stress<sup>[29]</sup>. Reduced bioavailability of NO is involved in the initiation, progression and complications of atherosclerosis<sup>[30,31]</sup>. NO opposes the effects of endothelium-derived vasoconstrictors and inhibits oxidation of low-density lipoprotein (LDL)<sup>[30]</sup>. Thus, reduced NO bioavailability leads to endothelial dysfunction<sup>[30]</sup>.

Recent studies by our and other groups suggest adipokines play a role in regulating atherosclerotic endothelial function by mediating NO production and oxidative stress. Adiponectin induces eNOS activation and NO production in endothelial cells<sup>[32]</sup>. Adiponectin reduces reactive oxygen species (ROS) production as well as improving endothelial function in aortas of ApoE KO mice<sup>[33]</sup>. Adiponectin deficiency increases leukocyte-endothelium interactions *via* upregulation of endothelial cell adhesion molecules *in vivo*. The protective role of globular adipo-

nectin in inhibiting leukocyte-endothelium adhesion was abolished by the blockade of eNOS with N(omega)-nitro-L-arginine methyl ester<sup>[34]</sup>. TNF- $\alpha$  (TNF- $\alpha$  plays an important role in both atherogenesis and vascular dysfunction. Inhibition of TNF- $\alpha$  reduces atherosclerosis<sup>[35]</sup> and improves endothelial function in ApoE KO mice (unpublished data). Aortic ROS formation and nuclear factor- $\kappa$ B (NF- $\kappa$ B) expression were higher in aortas of ApoE KO mice compared with control mice<sup>[33]</sup>. Genetic deletion of TNF- $\alpha$  reduced aortic superoxide production and improved NO availability<sup>[36]</sup>. Thus, vascular inflammation and oxidative stress may contribute to TNF- $\alpha$ -induced endothelial dysfunction.

Thus, maintaining endothelial health is important in preventing atherosclerotic diseases. The role of adipokines in atherosclerotic endothelial dysfunction warrants further attention.

## ROLE OF ADIPOKINES IN ATHEROGENESIS

Some adipokines are atherogenic while others have atheroprotective effects.

Adiponectin exerts atheroprotective effects. In *in vitro* studies, adiponectin inhibited TNF- $\alpha$ -induced increase in endothelial expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selection<sup>[37]</sup>. Adiponectin also suppresses vascular smooth muscle cell proliferation<sup>[38]</sup>, as well as macrophage to foam cell transformation<sup>[39]</sup>. Adiponectin reduces lipid accumulation in macrophage foam cells<sup>[40]</sup> and prevents atherosclerosis by increasing cholesterol efflux from macrophages<sup>[41]</sup>. *In vivo* studies further demonstrated that apolipoprotein E/adiponectin double-deficient mice had increased plasma IP-10 levels, accelerated T-lymphocyte accumulation in atheromata, and augmented atherogenesis compared with ApoE single-deficient mice. Indeed, adiponectin inhibits the production of CXC Receptor 3 chemokine ligands in macrophages and reduces T-lymphocyte recruitment in atherogenesis<sup>[42]</sup>. Adiponectin prevents adventitial fibroblasts from proliferating, transforming to myofibroblasts, and migrating to the intima, thus worsening atherosclerosis<sup>[43]</sup>. Macrophage adiponectin transgenic mice exhibited reduced macrophage foam cell formation in the arterial wall when these transgenic mice were crossed with a LDL receptor knockout (*ldlr*<sup>-/-</sup>) mouse model and were fed a high-fat diet<sup>[44]</sup>. A previous study suggested that an adenovirus-mediated increase in plasma adiponectin significantly suppressed the progression of atherosclerotic lesions in aortic sinus by 30% in ApoE KO mice<sup>[45]</sup>. Globular adiponectin also showed effects on amelioration of atherosclerosis, which was associated with decreased expression of class A scavenger receptor and TNF<sup>[46]</sup>. Thus, adiponectin modulates multiple pathways to impact the development of atherosclerosis.

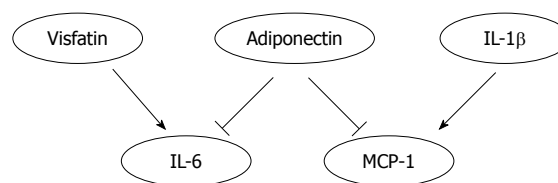
There is some discrepancy regarding the role of leptin in atherogenesis. One study suggested that leptin treatment of ApoE KO mice did not affect lesion size and surface area occupied by atherosclerotic lesions, but did



increase lesion calcification and the expression of the osteoblast-specific markers, osteocalcin and osteopontin. Thus, leptin may increase cardiovascular risk by promoting osteogenic differentiation and vascular calcification<sup>[47]</sup>. Another study showed that recombinant leptin treatment resulted in an increase in atherosclerosis (lesion surface coverage) and a shortened time to occlusive thrombosis after vascular injury, which promotes atherosclerosis and thrombosis in ApoE KO mice<sup>[48]</sup>. Leptin deficient hyperlipidemic mice (ob/ob; ApoE KO mice) developed significantly less atherosclerosis than ApoE KO mice, when fed an atherogenic diet for 16 wk from 8 wk of age. Histological analysis revealed that most of the atherosclerotic lesions in leptin deficient ApoE KO mice remained as fatty streaks, while those in ApoE KO mice were mainly fibrous plaques<sup>[49]</sup>. Leptin-deficiency (ob/ob) in LDL receptor deficient mice induces an unexpected 2.2- to 6-fold reduction in atherosclerotic lesion development<sup>[50]</sup>. The above findings support the notion that leptin accelerates atherosclerosis. However, one previous study reported that leptin deficiency in atherosclerosis-susceptible LDL receptor knockout or ApoE knockout background resulted in the development of larger atherosclerotic lesions<sup>[51]</sup>, suggesting a protective role for leptin in atherosclerosis. These results identify a critical role for the leptin/leptin receptor pathway in the modulation of atherogenesis, and further studies are needed before drawing conclusions. Resistin is a cardiovascular and atherosclerotic risk factor<sup>[52]</sup>. Resistin is a 12.5 kDa protein originally found to be secreted by mouse adipocytes<sup>[53]</sup>. Whereas in rodents the adipocyte is the major source of resistin, in humans resistin is mainly expressed in macrophages<sup>[54]</sup>. Resistin protein is present in both murine and human atherosclerotic lesions. ApoE KO mice had significantly higher resistin mRNA and protein levels in their aortas, and elevated serum resistin levels. Incubation of murine aortic endothelial cells with recombinant resistin increased MCP-1 and soluble VCAM-1 protein levels in the conditioned medium, suggesting a possible mechanism where resistin contributes to atherosclerotic diseases<sup>[55]</sup>.

TNF- $\alpha$  also plays an important role in atherosclerosis<sup>[56]</sup>. TNF- $\alpha$  has been detected in atherosclerotic lesions throughout all stages of human atherosclerosis<sup>[57,58]</sup>, and was found to be associated with atherosclerosis in mouse models<sup>[35,59]</sup>. Mice deficient in both ApoE and TNF- $\alpha$  showed less advanced atherosclerosis than ApoE KO mice<sup>[60]</sup>. mRNA levels of pro-atherosclerotic factors, i.e. IL-1 $\beta$ , interferon- $\gamma$ , ICAM-1, VCAM-1, MCP-1, GM-CSF and NF- $\kappa$ B (p65) were significantly downregulated in ApoE and TNF- $\alpha$  double-knockout mice<sup>[60]</sup>. Lectin-like oxidized LDL receptor-1 (LOX-1) is an important mediator of atherogenesis. TNF- $\alpha$ -induced increase in LOX-1 expression was demonstrated in various cell types, including endothelial cells, macrophages, vascular smooth muscle cells, *etc.*<sup>[61-63]</sup>.

IL-6 contributes to both atherosclerotic plaque development and plaque destabilization *via* a variety of mechanisms<sup>[64]</sup>, which involve the release of other pro-inflammatory cytokines and prothrombotic mediators,



**Figure 2 An heuristic diagram positing the interplay of adipokines.** Visfatin treatment increased the level of interleukin (IL)-6 in circulation. Adiponectin reduced the production of IL-6 and macrophage chemoattractant protein (MCP)-1 by inflamed adipocytes. IL-1 $\beta$  increased the expression of MCP-1 in aortic smooth muscle cells. Thus, the interplay among adipokines may significantly affect the pathogenesis of atherogenesis.

oxidation of lipoproteins by phospholipases, stimulation of acute phase protein secretion, and the activation of matrix metalloproteinases<sup>[64]</sup>.

MCP-1 plays a key role in monocyte/macrophage infiltration to the sub-endothelial space of the blood vessel wall, which is a crucial initial step in atherosclerosis<sup>[65]</sup>. Anti-MCP-1 gene therapy inhibits vascular smooth muscle cell proliferation<sup>[66]</sup>, suggesting it has atheroprotective potential.

In summary, adiponectin is anti-atherogenic, but resistin, TNF- $\alpha$ , IL-6 and MCP-1, *etc.* exert atherogenic effects through profound mechanisms. The role of leptin in atherogenesis remains to be controversial. The role of various adipokines in the pathogenesis of atherosclerosis and the therapeutic potential of modulating adipokine expression warrants further investigation (Figure 1).

## ADIPOKINES INTERPLAY

Reciprocal regulation may occur among adipokines. Transgenic mice that specifically express the gene coding for human adiponectin in mouse macrophages exhibit enhanced whole-body glucose tolerance and insulin sensitivity with reduced MCP-1 and TNF- $\alpha$  levels both in macrophages and serum<sup>[44]</sup>. Visfatin (pre-B cell colony-enhancing factor) has recently been identified as a new adipocytokine affecting insulin resistance<sup>[67]</sup>. Visfatin treatment increased the level of circulating IL-6 without affecting that of TNF<sup>[68]</sup>. In addition to systemic effects, adipokines showed interactive regulation in various cell types. Both full length and globular adiponectin attenuated IL-6 and MCP-1 production from inflamed adipocytes<sup>[69]</sup>. IL-1 $\beta$  promotes the expression of MCP-1 in human aortic smooth muscle cells *via* the NF- $\kappa$ B signaling pathway<sup>[70]</sup>. Thus, the interplay among adipokines may be an important contributor in the pathogenesis of atherogenesis (Figure 2).

## FUTURE PERSPECTIVES

A strong correlation is observed between obesity and atherosclerosis. However, the existence and nature of a communication/linkage between adipose tissue-derived factors and pathogenesis of atherosclerosis warrants further investigation. Future studies may better elucidate the



mechanisms of this communication by delving into these aspects: (1) Direct and specific evidence is needed to determine if/how adipose communicates with vasculature through adipose proinflammatory cells and/or various adipose-derived cytokines, hormones and lipid signals; (2) The role of adipose, as an endocrine organ, results in altered levels of systemic regulators of chronic inflammation and energy metabolism. Therefore, the metabolic parameters associated with adipose dysfunction may indirectly affect the vascular tissues. Thus, studies are needed to examine the local pathogenic effects of perivascular fat vs. the systemic effects of obesity-induced metabolic changes in the pathophysiology of atherosclerosis; (3) Investigate using animal models relevant to atherosclerosis in human beings; and (4) If obesity is rescued, can that rescue atherosclerosis?

Of particular importance is the need to identify novel adipokines that play crucial roles in atherogenesis or exert atheroprotective effects and to improve the translation of the exciting scientific results to patients.

## CONCLUSION

Obesity, especially visceral obesity, is associated with dyslipidemia, impaired glucose metabolism, and hypertension, all of which exacerbate atherosclerosis. One plausible mechanism involves adipokines, produced by adipose tissue in obesity that can directly impact the atherogenic environment of the vessel wall by regulating gene expression and function in endothelial, arterial smooth muscle, and macrophage cells, *etc.* Thus, this review explores the connection between adipose and atherosclerosis with particular emphasis on the role of adipokines as mediators in the development of atherosclerosis. We suggest that the proinflammatory capacity of adipose tissue provides new insights in the pathophysiology of atherosclerosis.

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## Coronary microvascular dysfunction in diabetes mellitus: A review

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els, where lesions of small coronary arteries have been described. These concepts are epitomized in the classic microvascular complications of diabetes, i.e. blindness, kidney failure and distal dry gangrene. Most importantly, accumulating data indicate that insights gained from the link between inflammation and diabetes can yield predictive and prognostic information of considerable clinical utility. This review summarizes the evidence for the predisposing factors and the mechanisms involved in diabetes, and assesses the current state of knowledge regarding the triggers for inflammation in this disease. We evaluate the roles of hyperglycemia, oxidative stress, polyol pathway, protein kinase C, advanced glycation end products, insulin resistance, peroxisome proliferator-activated receptor- $\gamma$ , inflammation, and diabetic cardiomyopathy as a "stem cell disease". Furthermore, we discuss the mechanisms responsible for impaired coronary arteriole function. Finally, we consider how new insights in diabetes may provide innovative therapeutic strategies.

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**Key words:** Coronary artery; Diabetes; Endothelial dysfunction; Hyperglycemia; Inflammation; Insulin; Microcirculation; Nitric oxide; Oxidative stress

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### Abstract

The exploration of coronary microcirculatory dysfunction in diabetes has accelerated in recent years. Cardiac function is compromised in diabetes. Diabetic patients manifest accelerated atherosclerosis in coronary arteries. These data are confirmed in diabetic animal mod-

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## INTRODUCTION

### **Diabetes and coronary artery disease**

Cardiovascular diseases are significantly increased in patients with metabolic syndrome and type 2 diabetes. In particular, coronary artery disease (CAD) causes much of the serious morbidity and mortality in patients with diabetes, who have a 2- to 4-fold increase in the risk of CAD<sup>[1]</sup>. In one population-based study<sup>[2]</sup>, the 7-year incidence of first myocardial infarction or death was 20% for diabetic patients, but only 3.5% for non-diabetic patients. The aging of the population and an increasing prevalence of obesity and sedentary life habits in the United States contribute to an increasing prevalence of diabetes.

### **Metabolic and vascular dysfunction**

Factors such as chronic hyperglycemia, lipid abnormalities, inflammation, oxidative stress, endothelium dysfunction, increased thrombosis and decreased fibrinolysis are likely to promote cardiovascular events in patients with metabolic syndrome and type 2 diabetes<sup>[3]</sup>. Clinical and experimental studies have demonstrated that cardiac function is compromised in type 2 diabetes, suggesting that alterations in myocardial metabolism in the diabetic state are responsible for this impairment<sup>[4]</sup>. Also, changes in coronary vessel function can lead to a mismatch of myocardial supply and demand, thereby provoking ischemic episodes in the diabetic heart.

### **Functional evidence precedes morphological changes**

Vascular lesions of small coronary arteries have been described in diabetic patients and experimental animals. Characteristic morphological features include thickening of the arterial wall<sup>[5]</sup> and capillary basement membrane, periodic acid-Schiff positive deposits in the vessel wall of small arteries<sup>[6]</sup>, microaneurysms, perivascular and interstitial fibrosis, and fibrosis in the wall of small coronary arteries<sup>[7]</sup>. However, previous studies<sup>[8,9]</sup> in small arteries and arterioles of diabetic subjects have demonstrated that before the appearance of morphological changes, a vasomotor dysfunction develops in microvessels, affecting both smooth muscle- and endothelium-mediated regulatory mechanisms. The first studies on abnormal nitric oxide (NO) production were performed in rats and then confirmed in diabetic humans. The relaxation of coronary arteries in response to pharmacological stimuli was reduced or suppressed in diabetic rats<sup>[10]</sup>. In humans, the vasodilation of coronary arteries was also altered after pharmacological [acetylcholine (ACh)] or mechanical (cold test) stimuli, but these abnormalities of large vessels were not associated with angiographic lesions, and were independent of other cardiovascular risk factors<sup>[11]</sup>, suggesting impaired endothelial function without any anatomical lesions. Bagi *et al*<sup>[12]</sup> have described the effects of diabetes mellitus on coronary arterioles. In type 2 diabetic mice, agonist- and flow-induced dilation of coronary arterioles was reduced. Furthermore, Miura *et al*<sup>[13]</sup> confirmed the impairment of coronary microvascular function in human

coronary arterioles isolated from patients affected by type 1 or type 2 diabetes mellitus: coronary arterioles showed a reduced vasodilation to hypoxia due to a decreased activity of ATP-sensitive potassium channels. The mechanisms underlying type 2 diabetes-induced impaired vasodilation and scientific consensus will be evaluated in this review.

## ETIOLOGY OF CORONARY MICROVASCULAR DYSFUNCTION IN DIABETES

### **Role of hyperglycemia**

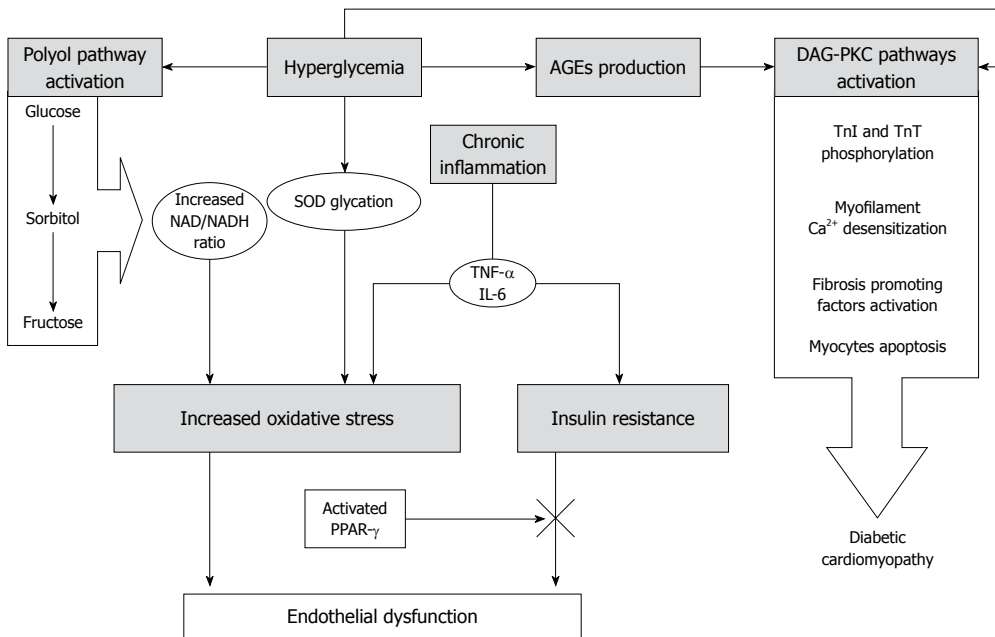
Hyperglycemia suppresses flow-mediated endothelial dependent vasodilation, and impairs endothelial-dependent vasodilation in diabetic and healthy human subjects<sup>[14-17]</sup>. Hyperglycemia is clearly recognized as the primary culprit in the pathogenesis of diabetic complications, inducing repeated acute changes in intracellular metabolism (activation of the polyol pathway, activation of diacylglycerol (DAG)-protein kinase C (PKC), increased oxidative stress, endothelial cell glycocalyx perturbation<sup>[18]</sup>), as well as cumulative long-term changes in the structure and function of macromolecules through formation of advanced glycation end products (AGEs). Coronary microvascular endothelial cells exposed to hyperglycemia exhibit increased oxidative stress that may arise from enhanced pro-oxidant enzyme activity and diminished generation of antioxidant glutathione<sup>[19]</sup> (Figure 1).

Blood glucose control is not significantly different between type 1 and type 2 diabetic patients, suggesting<sup>[20]</sup> that other factors are involved. A longer period of undetected blood glucose abnormalities, lipid alterations or decreased insulin sensitivity could be involved in type 2 diabetes. Furthermore, the initial ACh-induced endothelium-dependent dysfunction can be improved by the normalization of blood glucose control in type 1 diabetes mellitus<sup>[21]</sup>, whereas such remediation has never been observed in type 2 diabetes mellitus. Consequently, hyperglycemia is probably not the sole mechanism by which diabetes mellitus induces vascular endothelial dysfunction.

### **Role of insulin resistance**

**Role of insulin and insulin resistance in endothelium-dependent vasodilation:** Endothelial cells express insulin receptors. Insulin is known to elicit NO-dependent vasodilation in human skeletal muscle<sup>[22]</sup>; it may stimulate basal NO production directly by itself or indirectly by a second messenger. *In vitro* evidence indicates that insulin activates the L-arginine-NO vasodilator pathway in men<sup>[23]</sup> and stimulates the release of endothelium-derived relaxing factors (NO and PGI<sub>2</sub>). The L-arginine availability for endothelial NO synthase (NOS) could be reduced by either diminished L-arginine recycling or increased L-arginine metabolism. The study by Hein *et al*<sup>[24]</sup> suggests that one mechanism by which ischemia/reperfusion (I/R) inhibits NO-mediated arteriolar dilation is through increased arginase activity, which limits the availability of L-arginine





**Figure 1 Etiology of mechanisms involved in coronary vascular dysfunction in diabetes.** Increased oxidative stress is the unifying element common to all pathways through which the various mechanisms described interact to cause endothelial dysfunction and cardiomyopathy in diabetes. Hyperglycemia acts directly through the glycation of the scavenger enzyme superoxide dismutase (SOD) and other antioxidant enzymes, and indirectly through the cross activation of advanced glycation end product (AGE)/advanced glycation end product receptor (RAGE) interaction and of the polyol pathway. The latter raises the NADH/NAD<sup>+</sup> ratio, modifying the redox state of the cells and leading to the production of superoxide anions. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) has been shown to affect intracellular insulin signaling, promoting insulin resistance and consequently impairing the insulin-mediated endothelial function in coronary arterioles. The insulin-resistance mediated endothelial dysfunction can be restored by the activation of peroxisome proliferator-activated receptor- $\gamma$  by thiazolidinediones and rosiglitazone. Then, if we consider diabetes as a chronic, subclinical inflammatory disease, it acts through TNF- $\alpha$  and Interleukin (IL)-6, increasing the cellular oxidative stress. AGE/RAGE interaction and hyperglycemia can in turn activate the diacylglycerol (DAG)-protein kinase C (PKC) pathway, thus promoting fibrosis, myofilament desensitization, and apoptosis, and finally leads to diabetic cardiomyopathy. TnI: Troponin I; TnT: Troponin T.

to NOS for NO production. ACh-induced vasodilation is correlated with insulin sensitivity in healthy subjects, suggesting that insulin plays an important role in the early processes of endothelial dysfunction<sup>[25]</sup>. Insulin resistance precedes the development of type 2 diabetes mellitus and is associated with increased plasma concentrations of endothelin and von Willebrand factor (vWF) in obese subjects, even in the absence of diabetes mellitus<sup>[26]</sup>. Insulin vascular action may be blunted in insulin resistant states, such as obesity<sup>[26]</sup>, hypertension, impaired glucose tolerance and type 2 diabetes mellitus<sup>[27]</sup>. Wang *et al.*<sup>[28]</sup> showed that, in cultured endothelial cells, free fatty acid, which is increased in metabolic syndrome, induces insulin resistance, inhibits eNOS activation and consequently causes endothelial dysfunction. Avogaro *et al.*<sup>[29]</sup> confirmed this hypothesis in uncomplicated type 2 diabetic patients by linking cellular glucose disposal defects with insulin resistance.

**Mechanisms of insulin resistance-mediated impaired vasodilation:** The mechanisms by which hyperinsulinemia acts on endothelial function have been studied by Rask-Madsen *et al.*<sup>[30]</sup>, showing the role of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in insulin-stimulated endothelial function in humans. They found that the forearm blood flow response to ACh was inhibited by TNF- $\alpha$  and the inhibitory effect of TNF- $\alpha$  was larger during co-infusion of insulin. The results demonstrate that TNF- $\alpha$  plays a

pivotal role in insulin mediated endothelial dysfunction. TNF- $\alpha$  affects intracellular insulin signaling in fat, skeletal muscle and other insulin sensitive tissues by inhibiting kinase activity in the proximal part of the insulin signaling pathway<sup>[31,32]</sup>. A similar signaling pathway in vascular endothelium results in production of NO<sup>[33]</sup>, which is necessary for insulin-stimulated vasodilation<sup>[34]</sup>. In a rat model of obesity-associated insulin resistance, vascular insulin signaling is compromised<sup>[35]</sup>. In cultured endothelial cells, 10 min of exposure to TNF- $\alpha$  inhibits insulin signaling and NO production.

#### Role of peroxisome proliferator-activated receptor- $\gamma$

**Peroxisome proliferator-activated receptor- $\gamma$  and the amelioration of insulin resistance:** Peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ) is a ligand-activated transcription factor belonging to the nuclear receptor family<sup>[36]</sup>. PPAR- $\gamma$  is a regulator of lipid and glucose metabolism and is the target of insulin-sensitizing drugs such as thiazolidinediones, which are frequently used to treat metabolic complications associated with type 2 diabetes mellitus<sup>[37]</sup>. Long-term activation of PPAR- $\gamma$  by thiazolidinediones in type 2 diabetic subjects reduces plasma levels of insulin and glucose with the consequent attenuation of vascular dysfunction<sup>[38]</sup> (Figure 1). PPAR- $\gamma$  is also expressed in vascular tissues, specifically in vascular smooth muscle cells<sup>[39]</sup> and endothelial cells<sup>[40]</sup>. Activators of PPAR- $\gamma$ , *via* a mechanism that is unrelated to lipid and carbohydrate

metabolism, may also protect vascular function in diabetes mellitus<sup>[41]</sup>. Furthermore, PPAR- $\gamma$  has been demonstrated to increase the release of NO in porcine pulmonary artery and human umbilical vein endothelial cells in culture<sup>[42]</sup>.

PPAR- $\gamma$  antagonists also provided encouraging results in *in-vivo* human studies. Thiazolidinediones improved coronary vasomotor abnormalities in Mexican Americans with insulin resistance<sup>[43]</sup>. The addition of pioglitazone to conventional lipid-lowering therapy in non-diabetic but insulin-resistant patients affected by familial combined hyperlipidemia significantly enhanced not only myocardial glucose uptake, but also myocardial blood flow measured by positron emission tomography<sup>[44]</sup>.

#### PPAR- $\gamma$ activation improves endothelial dysfunction:

Both experimental and clinical evidence has revealed the role of PPAR- $\gamma$  in diabetic endothelial dysfunction. Bagi *et al*<sup>[45]</sup> have shown that short-term treatment of type 2 diabetic mice with the PPAR- $\gamma$  activator, rosiglitazone, augments NO-mediated, flow-dependent dilation of coronary arterioles, despite the presence of hyperglycemia and hyperinsulinemia. These changes are associated with a reduction in vascular NAD(P)H oxidase activity and enhancement of vascular catalase activity. The study demonstrates functionally important antioxidant activity of the PPAR- $\gamma$  ligand. In fact, rosiglitazone, by activating vascular PPAR- $\gamma$ , prevents the impairment of NO mediation of coronary arteriolar dilations. The underlying mechanism is most likely the enhancement of NO bioavailability *via* a reduction in the level of vascular reactive oxygen species (ROS). In the clinical environment, Esposito *et al*<sup>[46]</sup> showed that treatment for over one year with rosiglitazone reduces circulating inflammatory markers and improves endothelial function in patients with metabolic syndrome. These observations show that rosiglitazone increases the number and migratory activity of cultured endothelial progenitor cells in type 2 diabetic patients<sup>[47]</sup>.

#### Role of inflammation

**Insulin resistance and diabetes mellitus in inflammatory disorders:** Inflammation is a condition that underscores many cardiovascular pathologies including endothelial dysfunction, insulin resistance and, consequently metabolic syndrome and diabetes mellitus. The inflammatory biomarkers, C reactive protein (CRP), interleukin-6 (IL-6) and TNF- $\alpha$  are potentially informative given their involvement in biologically plausible mechanisms of insulin resistance. Among these cytokines, TNF- $\alpha$  is likely to play a pivotal role because it is one of the key inflammatory mediators expressed during a variety of inflammatory conditions<sup>[30]</sup> and is capable of initiating the expression of an entire spectrum of inflammatory cytokines ranging from interleukins to interferons.

Several studies have shown the potential application of these cytokines in the prediction of diabetes risk. Yudkin *et al*<sup>[48]</sup> showed that CRP, IL-6 and TNF- $\alpha$  are elevated in the insulin resistance syndrome, from quantitative measures of insulin resistance. Hak *et al*<sup>[49]</sup> found similar

relationships among healthy middle-aged women. In the insulin resistance and atherosclerosis study<sup>[50]</sup>, among 1008 non-diabetic subjects with no prior history of CAD, CRP levels were independent of insulin sensitivity as measured by a frequently sampled intravenous glucose tolerance test. They also found that higher geometric mean CRP levels were linearly related to an increase in a number of components of the metabolic syndrome. These observations suggest that an enhanced acute phase response is associated with insulin resistance and may presage the development of type 2 diabetes.

These epidemiological findings are strengthened by experimental studies that demonstrate the hyperglycemic effects of several proinflammatory cytokines including IL-6 and TNF- $\alpha$ , both of which are partly derived from adipose tissues. In rodent models of glucose homeostasis, IL-6 modifies glucose-stimulated insulin release from isolated pancreatic  $\beta$  cells and diminishes insulin-stimulated glycogen synthesis by hepatocytes in culture<sup>[51,52]</sup>. In humans, the exogenous administration of recombinant IL-6 induces dose-dependent hyperglycemia and concordant elevations in circulating levels of glucagons<sup>[53]</sup>. TNF- $\alpha$  may induce insulin resistance through a variety of mechanisms, including direct inhibitory effects on the glucose transporter protein GLUT4, the insulin receptor, and insulin receptor substrates<sup>[54]</sup>. TNF- $\alpha$  has been shown to alter the intracellular insulin signaling in fat, skeletal muscle, and other insulin-responsive tissues by inhibiting kinase activity in the proximal part of the insulin-signaling pathway<sup>[31,32]</sup>. A similar signaling pathway in vascular endothelium results in the production of NO, which is necessary for insulin-stimulated vasodilation<sup>[33,34]</sup>. Jiang *et al*<sup>[35]</sup> demonstrated in a comparable rat model that vascular signaling is compromised. In cultured endothelial cells, 10 min of exposure to TNF- $\alpha$  inhibits insulin signaling and NO production.

The role of TNF- $\alpha$  in coronary microvascular dysfunction was first identified in the setting of ischemia reperfusion-injury by using a murine genetic model (TNF- $\alpha$  overexpression mice, TNF<sup>+/+/+</sup>)<sup>[55]</sup>; recent studies have shown that TNF- $\alpha$  is also likely to be involved in coronary microvascular dysfunction occurring in the setting of both pre-diabetic metabolic syndrome and overt diabetes. Picchi *et al*<sup>[56]</sup> recently demonstrated in an animal model of metabolic syndrome (i.e. Zucker fatty rats) that impairment in coronary endothelial function is caused by TNF- $\alpha$  overexpression; we demonstrated that endothelial dysfunction occurring in obesity is the result of the effects of the inflammatory cytokine TNF- $\alpha$  and subsequent production of superoxide (O<sub>2</sub><sup>-</sup>). We also used genetic models of obesity and type 2 diabetes (Lepr<sup>db</sup> mouse), heterozygote lean controls (m Lepr<sup>db</sup>), and Lepr<sup>db</sup> mice null for TNF- $\alpha$  (db<sup>TNF-/-</sup>/db<sup>TNF-/-</sup>)<sup>[57]</sup>. Our results revealed that endothelial function is normal in TNF-deficient diabetic mice and that TNF- $\alpha$  overexpression impairs endothelium-dependent vasodilation which can be restored toward normal by administration of TNF- $\alpha$  antibodies. The mechanism by which TNF- $\alpha$  affects endothelial

function is through increased superoxide production by NAD(P)H oxidases, which in turn leads to a reduced NO bioactivity by direct scavenging. Moreover, we observed that AGE receptor (RAGE) for these products seem to amplify TNF- $\alpha$  expression in diabetes; thus, TNF- $\alpha$  and the AGE/RAGE signaling pathway play pivotal roles in endothelial dysfunction in type 2 diabetes<sup>[58]</sup>.

In order to document the extent of endothelial dysfunction at the different stages of type 2 diabetes, we also studied type 2 diabetic (db/db) mice aged 12, 18 and 24 wk<sup>[59]</sup>. We demonstrated that TNF- $\alpha$  is the key factor in producing O<sub>2</sub><sup>•-</sup> and induces endothelial dysfunction in db/db (a model of obesity and type 2 diabetes) and Db/db (lean control) mice as they age. Furthermore, we found that in younger mice (12-18 wk) the mechanism by which TNF- $\alpha$  affects endothelial function is through an increased O<sub>2</sub><sup>•-</sup> production by NAD(P)H oxidases which in turn leads to reduced NO bioactivity; on the contrary, in older mice (18-24 wk), TNF- $\alpha$  impairs endothelial function through an increased O<sub>2</sub><sup>•-</sup> production by mitochondria, which in turn leads to a reduced NO bioactivity by direct scavenging. Further support for the role of TNF- $\alpha$  in endothelial dysfunction comes from a recent study where aorta rings from diabetic mice instead of coronary arterioles were used: polyphenol (resveratrol) is capable of exerting a protective effect against vascular oxidative stress by inhibiting the activation of vascular NAD(P)H oxidase, which leads to a downregulation of eNOS phosphorylation induced by TNF- $\alpha$ <sup>[60]</sup>. In conclusion, these recent findings contribute to understanding the link between inflammation, insulin resistance and coronary microvascular dysfunction and highlight the key role of TNF- $\alpha$  as a common denominator in these pathologies.

**Should anti-inflammatory treatment be considered among diabetes therapies?** Despite significant evidence, prospective data for evaluation of the relationship between chronic subclinical inflammation and the incidence of type 2 diabetes are sparse. In the Atherosclerosis Risk Communities Study, markers of inflammation (such as white blood cell count, fibrinogen, and low serum albumin)<sup>[61]</sup> and inflammation-associated hemostasis variables (such as factor VIII and vWF)<sup>[62]</sup> were associated with the risk of type 2 diabetes. However, these relationships were largely abolished after adjustment for obesity. Pradhan *et al.*<sup>[63]</sup> showed the association between elevated levels of CRP and IL-6 and the risk of type 2 diabetes in otherwise healthy middle-aged women. Among participants of the Women's Health Study followed for 4 years, through age-matched analyses on obesity control, family history of diabetes, and other clinical risk factors, the authors found that elevated CRP levels are associated with a 4-fold increase in risk for presenting with diabetes. Recent reports on reduction in the incidence of type 2 diabetes accompanying pharmacological interventions for coronary heart disease prevention offer further support for links between inflammation, diabetogenesis, and atherosclerosis. In two large intervention trials of angiotensin-converting enzyme

(ACE) inhibitors for the prevention of cardiovascular disease, treatment assignment to captopril and ramipril<sup>[64]</sup> was associated with a statistically significant reduction in the incidence of type 2 diabetes. Similarly, the use of pravastatin in the primary prevention of coronary heart disease was also associated with a 30% reduction in the risk of type 2 diabetes<sup>[65]</sup>. One compelling hypothesis that may account for these effects is mitigation of subclinical inflammation. Angiotensin II induces IL-6 expression from both macrophages and smooth muscle cells, and is co-localized with IL-6 in human atheroma<sup>[66]</sup>. Furthermore, long-term ACE-inhibition lowers CRP levels among individuals with CAD<sup>[67]</sup>. In addition, statin therapy in general appears to lower CRP levels and exhibits beneficial adjunctive effects on the restoration of endothelial function<sup>[68]</sup>.

### Role of oxidative stress

Oxidative stress is defined as an increase in the steady-state levels of ROS and may occur as a result of increased free radical generation and/or decreased antioxidant defense mechanisms. This seems to be the common final pathway that leads to endothelial dysfunction in diabetes mellitus (Figure 1). There are multiple intracellular sources for the formation of oxygen free radicals [e.g. mitochondria, xanthine oxidase, NAD(P)H oxidase *etc.*]. Our results<sup>[57]</sup> suggest that the primary proximate route to radical production in type 2 diabetes is through NAD(P)H oxidase activation by TNF- $\alpha$ .

### Mechanisms that decrease antioxidant mechanisms:

Hyperglycemia promotes glycation and inactivation of antioxidant proteins such as Cu/Zn superoxide dismutase (SOD), leading to inactivation and reduction in antioxidant defense for these proteins<sup>[69]</sup>. Experimental studies in streptozotocin-induced diabetic rats have shown decreased concentrations of antioxidants such as vitamin E, SOD and catalase<sup>[70]</sup>. For example, the consumption of NAD(P)H leads to decreased glutathione activity, which is efficient for capturing free radicals<sup>[71]</sup>. Experimentally, when the activities of SOD (which captures O<sub>2</sub><sup>•-</sup>) and catalase (H<sub>2</sub>O<sub>2</sub> inhibitor) were maintained, endothelial function was not altered even in hyperglycemic patients.

### Mechanisms of increased generation of oxygen free radicals:

Many experimental studies suggest that increased superoxide production accounts for a significant proportion of the NO deficit in diabetic vessels. Potential sources of vascular superoxide production include NAD(P)H-dependent oxidases<sup>[72,73]</sup>, xanthine oxidase<sup>[74]</sup>, lipoxygenase, mitochondrial oxidase and NOS<sup>[75]</sup>. Hyperglycemia increases oxidative stress through ROS overproduction at the mitochondrial transport chain level; Piconi *et al.*<sup>[76]</sup> proposed that the mitochondrial oxidative activity may be a therapeutic target in diabetes. However, NAD(P)H oxidase appears to be the principal source of superoxide production in several animal models of vascular disease, including diabetes<sup>[77]</sup>. An increase in TNF- $\alpha$  expression induces activation of NAD(P)H oxidase and



production of ROS, leading to endothelial dysfunction in type 2 diabetes<sup>[57]</sup>.

Guzik *et al.*<sup>[78]</sup> have described the mechanisms of increased superoxide production in human diabetes mellitus. First they found that basal superoxide release is significantly elevated in vessels from patients with diabetes. They demonstrated that endothelium is a net contributor to total vascular superoxide production. In fact, in arteries of non-diabetic patients, endothelium removal resulted in a significant increase in superoxide release, which suggests that in these vessels the net contribution of the endothelium is to reduce vascular superoxide release by production of NO. In marked contrast, endothelium removal in artery segments from diabetic patients significantly reduces superoxide release, suggesting a key role of the endothelium in superoxide production. Similarly, NOS inhibition in diabetic vessels decreases superoxide release, suggesting that the net effect of NOS activity in these vessels is superoxide production rather than NO production. Furthermore, they found that sepiapterin (a BH<sub>4</sub> precursor) significantly reduces vascular superoxide production in vessels from patients with diabetes. Therefore, NAD(P)H oxidase seems to be the most important source of superoxide production in diabetes mellitus and the superoxide anion is likely to reduce NO bioactivity by direct scavenging. Nevertheless, in diabetic vessels, the endothelium is a significant net source of superoxide because of a profound loss of normal eNOS function, characterized by a transition from NO production to superoxide production. In fact, peroxynitrite, generated from NO and superoxide, directly oxidizes BH<sub>4</sub> to BH<sub>2</sub> (dihydrobiopterin), a biopterin that does not support eNOS enzymatic activity<sup>[79]</sup>. Indeed, some data suggest that competition between BH<sub>2</sub> and BH<sub>4</sub> for eNOS binding may increase eNOS uncoupling. Therefore, upregulation of vascular superoxide production by NAD(P)H oxidases may in turn lead to eNOS uncoupling through oxidation of BH<sub>4</sub>. This reduces NO production and further increases endothelial superoxide production. Bagi *et al.*<sup>[80]</sup> confirmed these results: they found that *in vitro* administration of the NAD(P)H oxidase inhibitor apocynin restores flow-induced coronary arteriolar dilation in type 2 diabetes mice, suggesting that NAD(P)H oxidase is likely to be the main source of the enhanced superoxide production in coronary microvessels. A recent study supports the role of NAD(P)H oxidase<sup>[81]</sup>: the authors demonstrated that endothelial dysfunction in atherosclerosis is mediated, at least in part, *via* the interaction of oxidizing low density lipoproteins (Ox-LDL) with its receptor, LOX-1, which in turn stimulates endothelial generation of superoxide by activation of NAD(P)H oxidase.

However, different sources of superoxide anions have been described in recent reports<sup>[82,83]</sup>, suggesting that an enhanced level of mitochondrial superoxide, likely produced during the enhanced rate of glucose metabolism, might be responsible for all high glucose related processes. Bagi *et al.*<sup>[43]</sup> reported that in carotid arteries, either the presence of 2-DG (a competitive inhibitor of glycolysis) or the presence of TTFA (inhibitor of the mitochondrial complex II) significantly reduces hyperglycemia-induced

superoxide production. Correspondingly, in skeletal muscle arterioles, the presence of 2-DG during hyperglycemia prevents the reduction of flow-induced dilation. Moreover, the presence of TTFA substantially moderates the hyperglycemia-induced reduction in flow-induced dilation of arterioles. These results indicate that during hyperglycemia a higher rate of glucose metabolism (glycolysis and mitochondrial utilization) is likely to elicit enhanced production of superoxide in mitochondria of skeletal muscle arterioles.

### Role of the polyol pathway

High blood glucose levels increase activity in the polyol pathway. In the polyol pathway, glucose is reduced to sorbitol by aldose reductase, leading to depletion in cellular stores of NAD(P)H<sup>[84]</sup>. Reduced NAD(P)H is required for the functioning of many endothelial enzymes, including NOS and cytochrome P450, as well as for the antioxidant activity of glutathione reductase. Sorbitol is then oxidized to fructose by sorbitol dehydrogenase. This reaction uses NAD<sup>+</sup> and raises the NADH/NAD<sup>+</sup> ratio, which modifies the redox state of the cells and results in the production of superoxide anions (Figure 1). Alternatively, a high polyol pathway flux consumes large amounts of ATP and may thus provide the energy supply required for endothelial-derived relaxing factor production<sup>[85]</sup>.

### Role of PKC

**PKC and its physiological role:** PKC is a family of serine/threonine kinases that consists of at least 12 members<sup>[86]</sup> and can be classified into three groups: conventional PKC, novel PKC, and typical PKC. PKC can be activated through multiple pathways in response to a wide array of stimuli, including cytokines, mechanical shears, stresses, hormones, and even glucose. With exposure to hyperglycemia in diabetes, accumulation of the glycolytic intermediate, glycerol-3-phosphate, stimulates the *de novo* synthesis of DAG, which in turn activates specific isoforms of PKC. In addition, chronic hyperglycemia can also increase the production of AGEs and generate ROS, which have been shown to activate the DAG-PKC pathway<sup>[87]</sup> (Figure 1).

Hyperglycemia increases circulating cytokines, growth factors, and hormones such as endothelin-1 and angiotensin II; these secreted cytokines can also activate PKCs by binding to their cell surface receptors<sup>[88,89]</sup>. Diabetes is also associated with severe dyslipidemia, in which increased levels of circulating free fatty acids have been reported to activate PKC either directly or through *de novo* synthesis of DAG in endothelial cells<sup>[90,91]</sup>.

**PKC isoforms and diabetic cardiomyopathy:** In the myocardium, several isoforms of PKC are activated to the membrane fraction of the heart by hyperglycemia: PKC-β II and -δ are the major isoforms to be activated by chronic hyperglycemia. Targeted overexpression of the β II isoform in transgenic mice results in a cardiac phenotype reminiscent of that seen in diabetic cardiomyopathy, characterized by early diastolic dysfunction, small vessel disease, myocardial hypertrophy and loss of cardiac con-

tractility and cardiomyocytes<sup>[92,93]</sup>. This is probably due to PKC- $\beta$  II-mediated phosphorylation of troponin I, which may decrease myofilament  $\text{Ca}^{2+}$  responsiveness<sup>[94]</sup>. This observation is consistent with a previous finding that PKC activation can induce phosphorylation of troponin I and T and downregulation of calcium-stimulated ATPase in actomyosin, with subsequent inhibition of cardiac sarcoplasmic reticulum  $\text{Ca}^{2+}$  accumulation which in turn reduces cardiac contractility<sup>[95]</sup>. Overexpression of PKC- $\beta$  II resulted in extensive cardiac fibrosis, probably due to upregulation of the expression of fibrosis-promoting factors such as transforming growth factor- $\beta$  I and connective tissue growth factor<sup>[93]</sup>. These factors can further result in the transcription and deposition of extracellular matrix components such as collagens and fibronectins. This phenomenon is consistent with the observation that collagen and fibronectin deposition is increased in myocardial tissues from diabetic humans and animals<sup>[96]</sup>. Loss of cardiomyocytes has been blamed, in part, for the ventricular dysfunction in diabetic hearts and is probably caused by hyperglycemia-induced cardiomyocyte apoptosis<sup>[97]</sup>. Activation of PKC- $\delta$  is known to induce cellular apoptosis in many cell types including neutrophils, keratinocytes, neuronal cells, fibroblasts, transformed cells and cardiomyocytes<sup>[98]</sup>.

### PKC targeted therapies for diabetic cardiomyopathy:

Because pathologic activation of the DAG-PKC pathway has been shown to play key roles in the onset and progression of cardiovascular complications, efforts have been focused on developing effective approaches to regulate PKC activities, and therefore, to reverse or even prevent these lethal complications. Several PKC inhibitors have been developed, but few of them demonstrated PKC isoform specificities, with the exception of a PKC- $\beta$  II-selective inhibitor (LY333531) that has been demonstrated to ameliorate diabetes-induced PKC- $\beta$  II activation and its related vascular abnormalities in cell culture, animal models and clinical trials<sup>[99]</sup>. Specific receptors for activated C kinases (RACKs) regulate PKC translocation and confer PKC isoform-selective-interactions. Peptides have been designed according to the protein sequence of RACK and have been shown to inhibit the translocation of specific isoforms of PKC<sup>[100]</sup>. By a similar strategy, peptide activators of specific PKC isoforms have been developed from a pseudo RACK sequence<sup>[101]</sup>. These peptide inhibitors and activators have been used successfully to evaluate cardiac functions both *in vitro* and *in vivo*<sup>[102,103]</sup>.

### Role of AGEs

Glucose is known to bind to free amino groups in proteins or to lipids. Through a series of oxidative and non-oxidative reactions, AGEs are formed irreversibly and accumulate in tissues over time. Although AGE formation occurs during the normal aging process, it is markedly accelerated during diabetes, as a consequence of an increase in substrates such as glucose and in the prevailing oxidant stress in diabetes<sup>[104]</sup>. AGEs promote atherogenesis by Ox-LDL and by causing changes in the intimal collagen. A major contribution of AGEs to atherogenesis, however,

emerges from important studies that have led to the isolation of a RAGE on the cell surface. RAGE acts as a signal transduction receptor and also binds with non-AGE-related proinflammatory molecules such as S100/calgranulins and amphotericins<sup>[105]</sup>. The overlapping accumulation and expression of RAGE and its ligands at sites of tissue lesions sustain RAGE-mediated cellular activation and the induction of multiple signaling pathways.

The endothelium is exposed to AGEs localized on circulating proteins or cells (for example diabetic red blood cells), as well as to AGEs present in the underlying subendothelial matrix. Receptors for AGEs have been found on the endothelial cell surface, and mediate both the uptake and transcytosis of AGEs, as well as the internal signal transduction. AGE-RAGE interaction causes alteration of the barrier function that has been documented by an increased permeability of endothelial cells incubated with AGEs and increased transit of macromolecules through the endothelial monolayer. The increase in permeability is accompanied by alterations in the physical integrity of the endothelium, as shown by the destruction of organized actin structures and alterations in cellular morphology<sup>[106]</sup>. Binding of AGEs to endothelial RAGE also results in the depletion of antioxidant defense mechanisms (e.g. glutathione, vitamin C)<sup>[107]</sup> and the generation of ROS<sup>[108]</sup>, leading to increased oxidative stress (Figure 1). As a consequence, NF- $\kappa$ B activation occurs and thus promotes the expression of NF- $\kappa$ B regulated genes, including procoagulant tissue factor and adhesion molecules such as E-selectin, intercellular adhesion molecule-1 and vascular adhesion molecule-1<sup>[109,110]</sup>. In addition, interaction of RAGEs leads to an increase in thrombomodulin and also activates the receptors for the cytokines, IL-1, TNF- $\alpha$  and growth factors, causing the migration and proliferation of smooth muscle cells.

AGEs linked to the vascular matrix can chemically interfere with the bioavailability of NO. AGEs, when added to NO *in vitro*, block NO activity in a concentration-dependent manner. Studies using animal models with experimentally-induced diabetes demonstrated that an alteration of endothelium-dependent dilation occurs quickly within 2 mo from diabetes induction<sup>[111]</sup>. Presumably, the inactivation of NO occurs through a direct reaction of the NO radical with other free radicals that are formed during the reactions of advanced glycation. Interestingly, AGEs impair endothelial NOS activity in rabbit aorta and femoral artery<sup>[112]</sup>. In human saphenous vein endothelial cells, PCR upregulates RAGE expression in a dose and time-dependent manner, enhancing the binding ability of RAGE with its endogenous ligands<sup>[113]</sup>. In parallel, AGEs induce the expression of the potent vasoconstrictor, endothelin-1, changing endothelial function towards vasoconstriction<sup>[114]</sup>. Zhang *et al.*<sup>[115]</sup> have reported that AGEs induce phosphorylation of 2 signal transduction kinases (ERK1/2 and JNK) and produce inflammatory responses in adventitial cells of porcine coronary arteries. Antioxidants and inhibitors of NAD(P)H oxidase may attenuate this inflammatory response. Moreover, the authors hypothesized that AGE signaling inhibition may produce a



vascular protection: soluble RAGE, which prevents AGE-mediated signaling, has been shown to inhibit vascular inflammation and lesion formation in diabetic apolipoprotein E-deficient mice<sup>[116,117]</sup>.

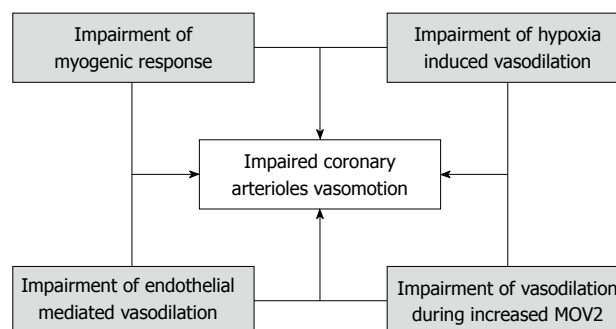
### Diabetic cardiomyopathy as a “stem cell” disease

Diabetic cardiomyopathy is described as the structural and functional changes caused by death of cardiac cells, with chronic loss of myocytes and vascular cells, which lead to a decrease in muscle mass, chamber dilation, impaired ventricular function, and finally, to symptoms of heart failure. Recent and accumulating evidence supports the concept that the heart possesses a store of multipotent progenitor cells (stem cells), which have the ability to differentiate into myocytes, endothelial cells and smooth muscle cells, both *in vivo* and *in vitro*<sup>[118-120]</sup>. Based on these observations, it has been postulated that under diabetic conditions, an imbalance between myocytes death and their regeneration may occur, with defects in both growth and survival of the store of multipotent cells in the heart that can be mediated by at least two underlying different mechanisms: the enzymatic o-glycosylation of proteins and oxidative stress-mediated stem cell damage. Ventricular acute hyperglycemia can promote apoptosis of ventricular myocytes *via* enzymatic glycosylation and the activation of the transcription factor p53 and effector responses involving the local renin-angiotensin system<sup>[97]</sup>. Moreover, in the same study, angiotensin II secretion and its receptor binding can promote the generation of ROS and initiate an oxidative-stress mediated cell death. This demonstrates that hyperglycemia can act through both direct and indirect mechanisms. Previously, the targeted deletion of the p66<sup>shc</sup> gene was shown to decrease ROS and improve cell resistance to oxidative damage, prolonging their life<sup>[121]</sup>. Based on this observation, Rota *et al.*<sup>[122]</sup> used an insulin dependent-streptozotocin induced diabetes model in wild type and p66<sup>shc</sup> Knockout mice, in order to test the hypothesis that oxidative stress in diabetes can alter the store of multipotent progenitor cells homeostasis, producing aging, loss of growth and death of stem cells, leading to diabetic cardiomyopathy. They found that p66<sup>shc</sup> deletion was followed by a reduction in oxidative stress, in cardiac stem cell senescence and that it favors the activation of cell growth mechanisms. However, the most important finding was that it prevented the increase in cardiac size and shape, diastolic and systolic dysfunction, which are the main features of the diabetic-failing heart. If we can consider diabetic cardiomyopathy as a “cardiac stem cell compartment disease”, it will open new previously unexpected therapeutic options in the near future.

## MECHANISMS RESPONSIBLE FOR IMPAIRED CORONARY ARTERIOLAR VASOMOTION

### Myogenic response

Microvessels respond to an increase or a decrease in transmural pressure by constriction and dilation, respectively.



**Figure 2 Possible mechanisms involved in vasomotion of impaired coronary arterioles in diabetes.** Several mechanisms have been postulated to be responsible for impaired vasomotion in coronary artery disease. There is strong evidence accumulating in favor of each impairment category as causal. We hypothesize that they interact in as yet unspecified ways rather than operate through separate pathways to cause diabetes. Major challenges in this field include better understanding each of these mechanisms, but the greatest opportunity for seminal breakthroughs may reside in reconciling our understanding among these mechanisms and their roles in diabetes.

Active myogenic responses are present in both subendo-cardial and subepicardial arterioles: Kuo *et al.*<sup>[123]</sup> also demonstrated that subepicardial arterioles exhibited greater vasodilatory responses at low pressure and augmented constriction at higher pressures. Changes in local regulatory mechanisms, intrinsic to the vascular wall, such as pressure sensitive myogenic response, have been proposed to contribute to the decreased dilator capacity of skeletal vessels in type 2 diabetes mellitus; because coronary vascular resistance is influenced by myogenic reactivity, enhanced myogenic tone could adversely affect vasodilator function of arterioles. In type 2 diabetic db/db mice, Lagaud *et al.*<sup>[124]</sup> found that in mesenteric arterioles, there is enhanced pressure-induced myogenic tone due to the upregulation and activation of smooth muscle PKC. In obese Zucker rats, Frisbee *et al.*<sup>[125]</sup> also reported enhanced myogenic tone in skeletal muscle arterioles. In contrast, Bagi *et al.*<sup>[12]</sup> found that no significant difference between active and passive diameters of coronary arterioles of db/db and control mice developed at 80-mmHg intraluminal pressure. Moreover, the myogenic tone of arterioles in response to stepwise increases in intraluminal pressure from 20 to 120 mmHg was also not significantly different in the two groups, indicating that in db/db mice, enhanced myogenic constriction is unlikely to be responsible for the decreased vasodilation of coronary arterioles (Figure 2).

### Endothelium-dependent NO mediated-dilation

One of the primary *in vivo* physiological stimuli for local regulation of arteriolar diameter is the presence of intraluminal blood flow. Increases in intraluminal flow elicit endothelium-dependent vasodilation *via* the release of vasodilator substances, such as NO<sup>[126]</sup>. *In vivo* flow-mediated dilation in skeletal muscle microvessels was significantly reduced in type 2 diabetic obese Zucker rats compared with controls<sup>[125]</sup>. Lagaud *et al.*<sup>[124]</sup> and Pannirselvam *et al.*<sup>[127]</sup> previously demonstrated that in mesenteric arteries of db/db mice, dilations in response to ACh were reduced,

suggesting impaired endothelium-dependent NO-mediated dilation. Interestingly, they found unaltered dilation in response to the NO donor, sodium nitroprusside (SNP); therefore, they speculated that the reduced NO-mediated dilation might be due to a decreased synthesis of NO caused by reduced availability of the eNOS substrate, L-arginine, or reduced levels of tetrahydrobiopterin (BH<sub>4</sub>). These results have been confirmed by Bagi *et al*<sup>[45]</sup>, who studied the effects of acute hyperglycemia on skeletal muscle arterioles of healthy rats. They found that transient elevation of glucose concentrations resulted in the reduction of NO-mediation of flow-induced dilation; acute hyperglycemia was likely to elicit enhanced production of superoxide which reduced the bioavailability of NO and the level of the NOS cofactor, BH<sub>4</sub>, thereby eliciting a reduction in flow-induced arteriolar dilation.

Little information is available in the literature regarding the effect of diabetes mellitus on endothelial function in coronary arterioles. Ammar *et al*<sup>[128]</sup> demonstrated that, in an *in vivo* beating heart, dilation of epicardial coronary arterioles to the endothelium-dependent vasodilator, ACh, was impaired in diabetic animals, while responses to adenosine and SNP were intact. Topical application of SOD and catalase restored ACh vasodilatory responses suggesting a pivotal role of ROS in destroying NO. However, which ROS was responsible for endothelial dysfunction was unclear. Bagi *et al*<sup>[45]</sup> showed that in coronary arterioles isolated from diabetic mice, NO mediation of flow- and agonist-induced dilation was reduced. Nevertheless, the dilation in response to the NO donor, NONOate, was also decreased suggesting that an alteration in NO synthesis, due to reduced levels of L-arginine or BH<sub>4</sub>, is unlikely to be the main cause of the decreased dilation. In fact, the authors found enhanced vascular production of superoxide anions which is likely to interfere with the mediation by NO of flow- and agonist-induced dilation. The reduced dilations to flow, ACh, and NONOate could be reversed by administration of SOD to the organ chamber, suggesting that in coronary arterioles of db/db mice, an enhanced level oxidative stress is present in both the endothelial and smooth muscle layers of microvessels. Interestingly, Zhang *et al*<sup>[129]</sup> showed that even if endothelial dysfunction is a main characteristic of I/R models, the underlying mechanisms may be different. They found that the process is partially mediated by increased arginase activity that leads to reduced availability of L-arginine, and partially mediated by the downregulation of endothelial NOS production. They also obtained the same results in hypertension-mediated endothelial dysfunction<sup>[129]</sup>. Bagi *et al*<sup>[45]</sup> showed that NAD(P)H is likely to be the main source of the enhanced superoxide production in coronary microvessels. In fact, *in vivo* administration of the NAD(P)H inhibitor, apocynin, restores flow-induced coronary arteriolar dilation in mice with type 2 diabetes. They also demonstrated that short-term treatment of type 2 diabetic mice with the PPAR- $\gamma$  activator, rosiglitazone, augments NO-mediated flow-dependent dilations of coronary arterioles by reducing vascular superoxide

production *via* a favorable alteration of oxidant/antioxidant enzyme activities (Figure 2).

### Hypoxia-induced vasodilation

Hypoxia is known to induce potent endothelium-dependent vasodilation. Several animal studies have reported that ATP-sensitive potassium channels (K<sub>ATP</sub>) play a pivotal role in mediating such a vasodilation in conduit and resistance arteries<sup>[130]</sup>. It is not well established how hypoxia elicits vasodilation, but interestingly, Quayle *et al*<sup>[131]</sup> demonstrated that anoxia, but not hypoxia, is enough to activate the K<sub>ATP</sub> current in rat femoral artery, suggesting that part of the relaxant effect of hypoxia may be mediated by changes in intracellular [Ca<sup>2+</sup>] through modulation of calcium channel activity. However, Miura *et al*<sup>[13]</sup> described the vasodilation to hypoxia and the role of K<sub>ATP</sub> in human coronary arteries from patients with diabetes. They confirmed that the mechanism of hypoxia-induced vasodilation involves opening of K<sub>ATP</sub> and they found that vasodilation to both hypoxia and K<sub>ATP</sub> stimulation is impaired in both type 1 and type 2 diabetes mellitus in the human coronary microcirculation. The exact mechanism of impaired vasodilation is not completely understood. The impairment seems to be specific to the K<sub>ATP</sub> mechanism, because no reduction is observed in vascular smooth muscle cell relaxation followed by a decrease in intracellular Ca<sup>2+</sup> concentration either by cGMP production attributable to NO (SNP) or by membrane hyperpolarization through Ca<sup>2+</sup>-activated K<sup>+</sup> channel activation. Therefore, the reduced hypoxia-induced dilation in coronary arterioles could be responsible for the impaired myocardial perfusion in patients affected by diabetes mellitus by hindering vasodilator responses during ischemia (Figure 2).

### Vasodilation during increased metabolic demand

Coronary arterioles are responsible for the close coupling of coronary blood flow and myocardial oxygen consumption (MVO<sub>2</sub>)<sup>[132]</sup>, although the exact coupling mechanism remains unclear. Coronary blood flow may increase five- to six-fold from baseline<sup>[133]</sup>. The primary means by which oxygen delivery to the myocardium may be increased during higher demand is *via* coronary dilation. Microvascular dilation during raised MVO<sub>2</sub> is heterogeneous according to the vessel size<sup>[132]</sup>, with the greatest magnitude of dilation being inversely related to baseline coronary diameter. Adenosine has received most attention as a potential mediator of metabolic vasodilation, but its role has been seriously questioned. Jones *et al*<sup>[134]</sup> reported that NO participates in coronary microvascular dilation during increases in metabolic demand with rapid atrial pacing. Furthermore, Embrey *et al*<sup>[135]</sup> demonstrated that coronary microvascular dilation during increase in MVO<sub>2</sub> by a dobutamine and pacing control is virtually abolished by NOS inhibition. In contrast, several authors reported that glibenclamide prevents the increase in coronary blood flow associated with increase in MVO<sub>2</sub>, demonstrating a possible role for K<sub>ATP</sub> channels in mediating faster coronary blood flow during larger oxygen consumption<sup>[136]</sup>.

Ammar *et al*<sup>[137]</sup> demonstrated that coronary blood flow during increase in MVO<sub>2</sub> is impaired in hyperglycemic dogs. Coronary arteriolar dilation to ACh is impaired, while dilatory response remains unaltered during administration of the NO-donor, SNP, demonstrating that impaired dilation during hyperglycemia is selective for endothelium-mediated dilation. The authors also reported that both endothelium-derived contracting factors, such as thromboxane A and prostaglandin H<sub>2</sub>, and free radicals are likely to cause impaired metabolic dilation during hyperglycemia, because reversal of either mechanism permits normal vasodilation to raise the metabolic demand (Figure 2).

The work by Xu *et al*<sup>[138]</sup> on the role of LOX-1 in atherosclerosis provides direct evidence that endothelial dysfunction in atherosclerosis is mediated, at least in part, *via* the interaction of Ox-LDL with its receptor, LOX-1, which in turn stimulates endothelial generation of superoxide radicals by activation of NAD(P)H oxidase. The results of this study contribute to the development of novel adjunctive therapies using anti-Ox-LDL and/or anti-LOX-1 antibodies or soluble receptors to prevent endothelial dysfunction following atherosclerosis. This work is an example of how basic research can provide important insight into the underlying mechanisms of ischemic heart disease, obesity, type 2 diabetes and atherosclerosis and provide valuable guidance on therapeutic design.

## CONCLUSION

Our understanding of type 2 diabetes has begun to recognize many factors involved in etiology, pathophysiology and clinical and microvascular manifestations of this common disease. The mechanisms involved in the etiology of microvascular complications have been described separately, but the signaling pathways always interact to amplify one another and induce endothelial dysfunction in diabetes. However, hyperglycemia is known to be the primary culprit in the pathogenesis of diabetic microvascular complications. This induces acute changes in cellular metabolism such as glycation and consequent inactivation of protein involved in the control of microvascular function. Hyperglycemia also activates and being activated by several other mechanisms, particularly the generation of AGEs, polyol, activation of DAG PKC pathways, and chronic or subclinical inflammation. The common final pathway is, however, the increase in oxidative stress, which seems to play a pivotal role in diabetic endothelial dysfunction and cardiomyopathy (Figure 1). Among the mechanisms responsible for impaired vasomotion, endothelial dysfunction is most likely to play the primary role (Figure 2). Knowledge gained from these studies will help further understand the increased cardiovascular risk and development of chronic vascular disease in type 2 diabetes. Furthermore, the quest to identify proximal stimuli for diabetes may provide a solution to the specific problem and new approaches for aiding development of therapeutic strategies. Future studies will gauge their utility as guides to monitor therapy.

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## Mediastinitis in pediatric cardiac surgery: Prevention, diagnosis and treatment

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### Abstract

In spite of advances in the management of mediastinitis following sternotomy, mediastinitis is still associated with significant morbidity. The prognosis is much better in pediatric surgery compared to adult surgery, but the prolonged hospital stays with intravenous therapy and frequent required dressing changes that occur with several therapeutic approaches are poorly tolerated. Prevention includes nasal decontamination, skin preparation, antibioprophylaxis and air filtration in the operating theater. The expertise of the surgical team is an additional factor that is difficult to assess precisely. Diagnosis is often very simple, being made on the basis of a septic state with wound modification, while retrosternal puncture and CT scan are rarely useful. Treatment of mediastinitis following sternotomy is always a combination of surgical debridement and antibiotic therapy. Continued use of numerous surgical techniques demonstrates that there is no consensus and the best treatment has yet to be determined. However, we suggest that a primary sternal closure is the best surgical option for pediatric patients. We propose a simple technique with high-vacuum Redon's catheter drainage that allows early mobilization and short term antibiotherapy, which thus decreases physiological and psychological trauma for patients and families. We have demonstrated the ef-

ficiency of this technique, which is also cost-effective by decreasing intensive care and hospital stay durations, in a large group of patients.

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**Key words:** Pediatric cardiac surgery; Mediastinal infection; Staphylococcus post cardiac surgery infection; Mediastinitis drainage; Prevention of mediastinitis; Treatment of mediastinitis

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### INTRODUCTION

Mediastinitis is a complication of pediatric cardiac surgery, with an incidence of 0.2% to 5%<sup>[1-3]</sup>. It is a retrosternal wound infection frequently associated with a macroscopically sternal osteomyelitis. The prognosis of mediastinitis is better for pediatric patients than for adult patients but this complication is still serious, particularly in the rare instances where mediastinitis is associated with endocarditis. Mediastinitis is uncomfortable for patients, is poorly accepted by parents, leads to a prolonged hospital stay and, thus, leads to an increase in health care costs.

There is considerable lack of consensus on the prevention, diagnosis and optimal treatment of mediastinitis. The goal of this manuscript is to describe the different suggestions that have been offered: (1) to prevent medi-

astinitis; (2) to confirm the diagnosis of mediastinitis; and (3) to treat poststernotomy deep wound infection. We will especially focus on an original and simple closed chest technique that we described in 1989<sup>[4]</sup> and reevaluated in 2007<sup>[5]</sup>.

## PREVENTION OF MEDIASTINITIS

Mediastinitis is a post-operative infection that is mainly due to intra-operative contamination. The origin of the germ is the patient, the surgical team or the operating room air<sup>[6-8]</sup>. Among the factors involved in germ pathogenesis in mediastinitis are three highly relevant factors: (1) the quality of the pre- and intra-operative patient disinfection; (2) the expertise of the surgical team; and (3) the quality of the air in the operating theater. Airborne particles with germs can reach the wound or enter into the bypass circuit through the suckers used during the cardiopulmonary bypass.

### *Quality of the pre- and intra-operative patient disinfection*

Nasal carriers of staphylococcus aureus are at increased risk for post-operative mediastinitis<sup>[9,10]</sup>, but there is no consensus about the efficiency of mupirocin prophylaxis in decreasing post-operative wound infection. Some works have demonstrated a significant reduction in surgical-site infection in patients treated with mupirocin<sup>[11-13]</sup>, while others failed to show any difference between patients treated with mupirocin nasal ointment and patients treated with placebo<sup>[14,15]</sup>. One of the most recent studies on mupirocin pre-operative nasal decontamination concluded that decolonization of nasal and extra nasal sites decreased the risk of surgical site infection in adults. However, this work did not analyze mupirocin alone; the treated group received nasal mupirocin twice daily for 5 d and also had a total-body wash with chlorhexidine soap every day, while the control group had only placebo<sup>[16]</sup>.

### *Some facts about mediastinitis prevention are noteworthy*

Before mupirocin ointment nasal therapy, nasal carriage must be identified either by microbiological culture techniques or with real-time polymerase chain reaction (which allows detection of staphylococcus within a few hours instead of days when using a classical microbiological approach). The treatment is prescribed for 5 d (delay consistent with the manufacturer's recommendations) so that emergency patients and neonates, who are usually considered at higher risk for mediastinitis, are not included in many studies. Staphylococcus resistance to mupirocin is rare and mupirocin is a safe and inexpensive product, however, the emergence of mupirocin high-level resistance has been described in many countries<sup>[17]</sup>. Prolonged or widespread use of mupirocin is likely to be a significant predisposing factor for acquired resistance<sup>[14-17]</sup>. In many studies, lack of information regarding skin disinfection and the type of antibiotic prophylaxis that was used limits

the pertinence of the results. As a final remark, nasal decontamination with mupirocin has been tested for over 20 years without clearly demonstrating an advantage.

There is less debate about skin preparation. Skin disinfection is considered as a major factor in the reduction of post-operative infection. There are two major products used as antiseptic solutions: povidone-iodine and chlorhexidine. Because of the permeable nature of the skin in small infants, significant iodine absorption is possible and likely to occur in pre-term infants. However, even in neonates, TSH levels may be significantly affected by povidone-iodine and transient thyroid dysfunction may result from topical exposure to iodine-containing antiseptic solutions<sup>[18-20]</sup>. This is known as the acute Wolff-Chaikoff effect, described in 1948, and is the reason why iodine containing solution should be avoided in neonates. Furthermore, chlorhexidine-based antiseptic solution is often considered as the best therapy for skin disinfection<sup>[21,22]</sup>. Compared to iodine solution, it has a more prolonged action and no known sensitivity<sup>[23]</sup>. Intra-operative skin disinfection is of the utmost importance, however, it seems that showers or baths with skin disinfectant before surgery are not required<sup>[24,25]</sup>.

The rationale for systematic antibioprophyllaxis in cardiac surgery is questionable; however, it is accepted worldwide and classically performed with a second generation cephalosporin, in the absence of a patient sensitivity. The vast majority of mediastinitis cases are due to staphylococcus and the incidence of resistance to methicillin (varying from one country to another and from one surgical unit to another, with an increase in incidence for US hospitals from 35.9% in 1992 to 64.4% in 2003<sup>[26]</sup>). Antibiotic prophylaxis induces a change in staphylococcal flora. There is some relation between the efficiency in eradication of carriage of staphylococcus aureus and the emergence of resistant strains of coagulase-negative staphylococci<sup>[27]</sup>. Staphylococci cultured from the skin of cardiac surgery patients are more resistant after surgery than before surgery and, furthermore, staphylococci causing post-operative infections have the same antimicrobial resistant phenotypes as do colonizing isolates<sup>[28]</sup>. This supports a modification of patient skin flora induced by antibiotics and is most unlikely due to in-hospital acquired germs. Pefloxacin was compared to cefamandole in perioperative cardiac surgery prophylaxis. Eradication of germs was better achieved with pefloxacin, however, while pefloxacin and oxacillin resistant strains were 0% before prophylaxis in the perianal area, 70% of the patients had resistant strains after pefloxacin prophylaxis. Such an emergence is not seen with cefamandole prophylaxis<sup>[29]</sup>. Antimicrobial agents given as prophylaxis may select resistant organisms. In patients who do not receive antibiotic prophylaxis, the staphylococcal flora remains unaffected. In patients receiving cephalosporin prophylaxis, 61% of the sites colonized with a low-level of methicillin-resistant strains before surgery were colonized with high levels of methicillin-resistant staphylococci on the third post-operative day<sup>[30]</sup>. In this study, the authors also demonstrated that the plasmid profile patterns were identical between



pre- and post-operative methicillin-resistant staphylococci. This is in accordance with previous works suggesting that the resistant pathogen is an alteration of patient skin flora rather than a contamination from in-hospital flora. The benefit of a prophylactic antibiotic must be balanced with the possible selection of resistant strains that are likely to make up a nosocomial reservoir for new patients and for the hospital staff.

## THE EXPERTISE OF THE SURGICAL TEAM

Obviously many risk factors of mediastinitis could be decreased with a high-quality level of care. Immunomodulation and an increase in gut mucosal permeability, induced by cardiopulmonary bypass and increased by hypothermia or deep hypothermic circulatory arrest, are also considered as predisposing factors<sup>[31,32]</sup>. They can be minimized with a more physiological cardiopulmonary bypass and warm surgery. In pediatric patients younger than 1-year-old, length of surgery, redo for bleeding, post-operative open-chest, ECMO and blood transfusion are also classical risk factors for deep wound infection<sup>[33-35]</sup>.

Optimal thoracic blood drainage is important in the prevention of post-operative infection. Stagnant blood in the mediastinum is a perfect growth medium for micro-organisms, which are protected from immunologic host defense. A hematoma collected in the supra-sternal space is clearly, in some patients, the origin of an abscess that may diffuse in the retrosternal space.

In an adult retrospective study of 18532 patients who underwent on-pump coronary artery bypass grafting, blood transfusion was considered as the major preventable risk factor of post-operative mediastinitis<sup>[36]</sup>. The risk associated with blood transfusion also exists after off-pump coronary artery bypass<sup>[37]</sup>. It is noteworthy that the association of red blood cell transfusion with infection is dose-dependent<sup>[38]</sup>. In neonates and young infants, blood-free surgery is unrealistic but efforts have to be taken to decrease blood use. Blood conservation with a miniaturized bypass circuit, vacuum-assisted venous drainage and microplegia is effective<sup>[39-41]</sup> and, thus, is likely to decrease the risk of pediatric post-operative mediastinitis.

Skin closure with cyanoacrylate glue, initially used for treatment of sternal instability, was also described as a protective factor against deep wound infection<sup>[42]</sup>.

Finally, the "human factor" is probably the most difficult to assess and the most difficult to control but not the least important. An optimal protocol is the first step of an efficient prophylaxis and its practical application is crucial, which depends on the motivation and quality of the medical staff.

## THE QUALITY OF THE AIR IN THE OPERATING THEATER

One particularity of open heart surgery is the need for cardiopulmonary bypass. In this technique, the sucker

system drains blood into the cardiectomy from the mediastinum or from cardiac cavities to the venous reservoir of the bypass circuit. In the sucker system tubing, room air is mixed with blood and thus potentially contaminated air enters into the bypass circuit. There is some evidence of transmission of fungal infections through contaminated air-handling systems<sup>[43,44]</sup>, and air filtration as well as radiation were considered efficient ways to protect from mediastinitis<sup>[45-47]</sup>. Gram negative bacilli may also be transmitted from the environmental flora<sup>[48]</sup> but contamination with coagulase negative staphylococci is probably only due to patient or surgical team flora<sup>[49]</sup>.

## DIAGNOSIS

The delay between surgery and diagnosis of mediastinitis varies from a few days to a few weeks. When a patient does not receive any antibiotic therapy, mediastinitis is usually an early complication and simple to diagnose. Babies are often grouchy and tired, and fever is constant. The incision is erythematous and painful, and wound dehiscence and purulent drainage from the incision are frequent as well as sternal instability. Biological signs of infection are also constant (i.e. leukocytosis and C reactive protein elevation) and blood cultures are often positive. Bacterial examination of the purulent drainage confirms the presence of altered leukocytes and germs. Antibiotic treatment based on empirical probability should be immediately initiated, followed within a few hours by surgical drainage, which may confirm the retrosternal infection.

The diagnosis may be more difficult when the patient has received antibiotic therapy. Epicardial pacing wire cultures are not satisfactory for the diagnosis of mediastinitis<sup>[50]</sup>, while bacteriological samples from the sternal or retrosternal puncture are considered safe and powerful<sup>[51]</sup>. CT scans have been considered of great value to localize infected tissues<sup>[52]</sup>, however, there is also some doubt about the validity of a CT scan in performing an early diagnosis<sup>[53,54]</sup>. Furthermore, erroneous diagnoses due to Surgicel packing<sup>[55]</sup>, to Surgicel body foreign reaction<sup>[56]</sup> or to iodine accumulation following irrigation<sup>[57]</sup> have been reported.

## TREATMENT

Treatment of mediastinitis is based on surgery and antibiotic therapy, but there is still controversy about the best strategy to help speed-up wound healing.

There are at least some consensual points: (1) Surgical revision with careful debridement of the infected areas, removal of foreign material and curettage of sternal edges until normal bleeding are essential; (2) Samples of purulent fluids must be collected to confirm the diagnosis and to perform germ identification and antibiotic susceptibility testing; and (3) Intravenous antibiotics, based on empiric probability, need to be injected before surgery and modified according to germ susceptibility.

Following surgery, several therapeutic approaches have been successively described. Open dressing or closed irrigation are two conventional treatments.

Open dressing has disadvantages, namely, thoracic instability requiring mechanical ventilation and prolonged immobilization, increasing risk of muscular weakening and patient discomfort. Multiple open dressing changes require heavy sedation and are time consuming for the medical staff. Cytotoxicity of classical antiseptics has been demonstrated<sup>[58]</sup> and even lethal iodine toxicity following povidone-iodine irrigation in a 34-mo-old patient has been described<sup>[59]</sup>. To overcome this problem, topical treatment with granulated sugar was also proposed and was considered by several authors as a simple, efficient and inexpensive alternative to irrigation during open chest management<sup>[60,61]</sup>. However, the numerous drawbacks of open chest management have stimulated the emergence of new therapies, such as primary closed sternum with continuous irrigation and drainage. This approach was described in 1963 and had theoretical advantages<sup>[62,63]</sup>. Mechanical ventilation was not required and the overall length of treatment was shorter than with the open chest technique. However, the results were disappointing and far from the expected progress, with a high rate of failure<sup>[64,65]</sup> and mortality. Cardiac tamponade induced by imbalance between irrigation and drainage, or even cardiac rupture, were reported<sup>[66,67]</sup>. A combination of two techniques, primary open chest management followed by delayed closed chest irrigation, was also proposed but was also less than satisfactory. However, irrigation is still used and is considered a cost-effective therapy<sup>[68]</sup>.

Procedures using reconstructive plastic surgery with vascularized soft tissue flaps were described in 1980. The pectoral muscle flap was proposed for patients who fail conventional closed irrigation techniques. The obliteration of all of the dead space with well-vascularized tissue is probably the major positive aspect of this approach<sup>[69]</sup>. By eliminating any residual cavity and by filling all of the space with healthy tissue, from an infectious point of view, this treatment represents real progress. The mediastinal cavity is anfractuous, thus failure of irrigation and drainage is probably due to incomplete irrigation and drainage with persistence of one or several septic residual cavities. Unfortunately, drawbacks of plastic procedures are also numerous. Morbidity associated with the muscle flap technique includes pain, weakness, hernia and an esthetic prejudice, which is of great significance in pediatric patients and more specifically in young girls<sup>[70,71]</sup>. Chest wall instability, bleeding, recurrence of infection were noticed and omental transfer was proposed as an alternative to the pectoral flap<sup>[72]</sup>. On the other hand, the omental flap has been credited to alter respiratory function by decreasing the percent vital capacity and oxygen consumption at the anaerobic threshold<sup>[73]</sup>.

When compared to closed mediastinal irrigation, the benefit of muscle flap reconstruction is not obvious<sup>[70]</sup>. Plastic procedures may have short-term and long-term results equivalent to irrigation, but the length of stay in intensive care is longer for patients treated with muscle flap closure<sup>[74]</sup>.

One of the latest developments in mediastinitis sur-

gical treatment is the introduction of vacuum-assisted closure therapy. The application of negative pressure has several advantages: (1) Wound drainage is enhanced by negative pressure; (2) The negative pressure avoids any residual mediastinal cavity so that the mediastinum is fill with healthy tissue; (3) The negative pressure helps approximate the wound edges and favors stabilization of the chest; and (4) Some benefits in local microcirculation have been described.

This newly emerging technique was used with success in small groups of pediatric patients<sup>[75-78]</sup>. The mechanism by which the vacuum technique improves wound healing is still unclear. A reduction in germ burden was suspected but not demonstrated<sup>[79]</sup>.

Vacuum-assisted closure was also used following continuous irrigation, when irrigation was ineffective or in patients with low cardiac output syndrome after a successful treatment of their hemodynamic instability<sup>[80]</sup>.

The benefit obtained with vacuum-assisted wound closure over classical management is unquestionably accepted by the medical community. However, there are constraints for the patient: the vacuum-assisted closure system must be changed every 2 or 3 d, positive results are seen after a long time and confinement in bed is necessary for days or weeks; following vacuum device therapy, a delayed closure is necessary and, thus, the intensive care stay is prolonged. Cardiac ruptures were described in adults during topical negative pressure<sup>[81,82]</sup> and the best negative pressure in pediatric patients is still to be determined. There is also a real concern about the risk of hemodynamic instability during negative pressure application in patients with Fontan- or Rastelli-type procedures; experimental data remains conflicting, and the precise location of the foam placement is important<sup>[83]</sup>. Magnetic resonance imaging has confirmed a reduction in cardiac output and stroke volume after initiation of vacuum therapy at the levels currently used in clinical human applications. This hemodynamic effect can be minimized by interposition of paraffin gauze dressing over the heart during application of negative pressure<sup>[84]</sup>.

More interesting is a recent approach with an early primary closure over a single chest tube that serves as a routine mediastinal drain (usually removed in 1 d or 2 d). This simple technique is more comfortable for the patient and achieves a high rate of success<sup>[85]</sup>. However, in 3 of 42 patients, this approach failed and they all required reoperation for continuing sepsis with suspicion of ongoing mediastinitis. Furthermore, the medical therapy is a lengthy course of intravenous antibiotics. The 6-wk intravenous antibiotic treatment has a significant disadvantage, especially when a central venous line is necessary (because of small-sized patients or the poor peripheral venous tolerance to antibiotics).

In 1989, we described a simple closed technique with primary closure and high-vacuum drainage<sup>[4]</sup>. The technique was progressively modified to decrease the length of hospital stay and to decrease patient physical and psychological trauma<sup>[5]</sup>. The surgical technique is classic with debridement of all infected or necrotic tissue and sternal



**Figure 1 Redon's catheter.** 1: Multiperforated polyvinyl tubing; 2: Luer-lock connector (with clamp above and under the connector) allowing safe disconnection of the plastic bottle; 3: Control of bottle vacuum (collapsible cap, when inflated the cap indicate the loss of vacuum).

edge revision until normal bleeding. Drainage is achieved through high-vacuum Redon's catheters connected to lightweight plastic bottles. The catheter is 2.7 mm in diameter with multiperforated polyvinyl tubing, and the plastic graduated bottle is manufactured with a negative pressure of 90 kPa and has a vacuum indicator that inflates in case of loss of negative pressure (Drainobag® Lock 150, Braun, Melsungen, Germany, Figure 1). The antibiotic therapy is composed of a synergistic association of two molecules. We usually have a bacteriological monitoring of the effluent fluids every day. Sterility of the effluent fluids is obtained in 4 to 5 d for staphylococcus aureus or epidermitis, however methicillin sensitive or resistance they might be. Sterility is delayed 8 d for gram-negative bacilli. When the effluent is sterile, the catheter is progressively withdrawn (2 cm every day). This technique avoids any recurrence of sepsis. In the rare cases of unusual delay in mediastinal sterilization, antibiotic dosage in the blood and in the effluent fluid allows an assessment of antibiotic diffusion, and increased antibiotic dosage or prescription of a third antibiotic is always efficient in achieving sterility. In the group of 64 patients studied, 40 cases had isolated mediastinitis (nine in neonates, 20 in infants and 11 in children), seven patients had mediastinitis associated with endocarditis who needed a longer antibiotic therapy course, and 17 had mediastinitis associated with organ failure. The mortality rate was 4%; one patient had pneumococcal endocarditis and mediastinitis following a pulmonary atresia with a ventricular septal defect cure. One patient had a redo operation for coronaroplasty following an arterial switch operation with mediastinitis and acute respiratory distress syndrome, and the third patient had an arterial switch and Senning procedure. The three patients died from associated complications after sterilization of the mediastinal effluent and withdrawal of Redon's catheters.

The major advantages of this technique are: (1) The primary sternal closure allows for a short mechanical ventilation time and short intensive care stay; (2) The lightweight plastic bottles allow patient mobilization; they are in fact less inconvenient than the intravenous perfusion needed for antibiotics therapy; (3) The high-vacuum is well-tolerated. This drainage is used in pediatric or adult cardiac surgery (instead of conventional underwater-seal drains) without hemodynamic drawback<sup>[86]</sup> (except for Norwood stage 1) and without damage to surrounding tissue; (4) The high-vacuum avoids any residual cavity and helps filling all of the mediastinum with healthy well-vascularized tissue; (5) Duration of antibiotic therapy is short: 11 d in cases without organ failure and 15 d in patients with organ failure; namely, respiratory failure, renal failure or ECMO; (6) A short antibiotic course is probably the best way to avoid fungal superinfection and to prevent catheter-related complications; (7) The efficiency of the short antibiotic course is due to the efficiency of the mediastinal drainage; (8) The overall infection eradication rate is 100% on a group of 64 patients treated during a 10-year period<sup>[5]</sup>; (9) There is no need for sophisticated or expensive material and the technique is not time consuming for the medical staff; and (10) As a result of these advantages, the technique is cost effective.

This therapy was adopted by several centers in Europe<sup>[87-90]</sup> and *via* humanitarian activities in Asia, South America and Africa.

The literature about mediastinitis is prolific for adult cardiac surgery, but there is much less data for pediatric cardiac surgery. Pediatric patients are very different from adult patients; many factors make the prognosis of mediastinitis worse including diabetes, chronic lung disease, and unilateral or bilateral use of internal mammary artery for coronary artery bypass. All of the different techniques were first used in adults and subsequently applied in pediatric cases. The lack of consensus about surgical management is illustrated by the numerous strategies proposed. None of the techniques are totally satisfactory, but we must keep in mind the differences between the pediatric and adult populations. We have demonstrated, on a large group of patients, that primary closure, allowing quick mobilization with or without minimal discomfort, is a valid alternative to all other techniques. This technique is, to our knowledge, the shortest, simplest and most efficient method for mediastinitis that has been published.

## CONCLUSION

Prevention of mediastinitis is still a difficult challenge. The prognosis of mediastinitis is very different in adult compared to pediatric patients, with mortality being very rarely related to mediastinal infection in pediatric cardiac surgery. A primary sternal closure is the technique of choice in pediatric patients. Short-term therapy is the main goal to decrease the physical and psychological trauma induced by this complication.



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## Walking with Gianluca Di Bella during the development of clinical cardiac imaging

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### Abstract

Cardiac magnetic resonance imaging (MRI) for the diagnosis and management of many cardiac diseases has been established in clinical practice. It provides anatomic and functional information and is the most precise technique for quantification of ventricular volume, function and mass. Among cardiac MRI sequences used in clinical practice, delayed contrast enhancement is an accurate and reliable method used in the diagnosis of ischemic and nonischemic cardiomyopathies. In addition, new technology applied in echocardiographic imaging has permitted quantification of myocardial deformations with 2-dimensional strain imaging (longitudinal, circumferential and radial strain). Cardiac MRI and echocardiography therefore both play a crucial role in the diagnosis and management of cardiovascular disease. Dr. Di Bella and colleagues have defined the roles of cardiac MRI and echocardiography in many clinical and experimental settings.

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**Key words:** Cardiac magnetic resonance imaging; Strain echocardiography; Myocarditis; Myocardial infarction; Cardiomyopathy; Heart failure

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### INTRODUCTION AND EDUCATIONAL EXPERIENCE

Dr. Gianluca Di Bella (Figure 1) is a Professor of Cardiology and researcher in the Faculty of Medicine and Surgery at the University of Messina, Italy. He received his first class degree in the Faculty of Medicine and Surgery at the University of Messina in 2001. He was certified as a specialist in cardiovascular disease at the University of Messina in 2005. He pursued graduate work on the application of cardiac magnetic resonance in the magnetic resonance imaging (MRI) laboratory at the Clinical Physiology Institute (CNR), Pisa, Italy under Dr. Lombardi M in 2004-2005. He received training in the application of cardiac magnetic

resonance and computed tomography in the Radiological Department of the University Hospital of Leuven (Belgium) under Prof. Dr. Bogaert J in 2007. He received PhD qualification in “methodologies and techniques of cardiovascular imaging” at the University of Messina, Italy in 2009. His research has been supported by research grant and fellowship awards from the Italian Society of Cardiology since 2004. As an independent investigator, he received the young researcher award from the Italian Society of Cardiology in 2006 and 2008. He is a reviewer for international journals and plays a key role in the Italian Society of Cardiology (e.g. vice-chairman of the cardiac MRI working group) and Italian Society of Echocardiography (e.g. member of the task force on integrated cardiac imaging).

## RESEARCH FOCUS AND STRATEGIES

Over recent years, Dr. Di Bella's research group has investigated cardiovascular diseases using cardiac MRI and strain echocardiography. An integrated approach with MRI and echocardiography provides an excellent and useful tool that permits identification of the pathophysiology of cardiovascular diseases. Dr. Di Bella's research has permitted identification of the relationship between myocardial damage and deformation in acute myocarditis<sup>[1-6]</sup>. Furthermore, Dr. Di Bella's research has included the study of clinical signs of heart failure, systolic function and cardiomyopathies<sup>[7-17]</sup>.

Another focus of Dr. Di Bella's research has been on cardiac imaging in patients with myocardial infarction<sup>[18-32]</sup>, heart valve disease<sup>[33,34]</sup>, atrial septal defect<sup>[35]</sup> and congenital anomalies<sup>[36-38]</sup>. He has authored papers on the correlation between percutaneous transluminal coronary angioplasty (PTCA) time and acute myocardial damage during ST elevation myocardial infarction<sup>[18]</sup>, the relation between Q waves and scar tissue<sup>[20]</sup>, the evidence that obese patients have less scar tissue than patients without obesity (obesity paradox)<sup>[22]</sup> and the correlation between scar tissue and non-sustained ventricular tachycardia<sup>[23]</sup>. Recently, he identified early MRI signs in patients with cardiac amyloidosis<sup>[39]</sup>.

## RESEARCH ACHIEVEMENTS

The following highlight Dr. Di Bella's contributions in the field of cardiac imaging, diagnosis and pathophysiology of cardiac diseases.

### **Role of cardiac MRI and echocardiography in the diagnosis and pathophysiology of acute myocarditis**

Di Bella *et al*<sup>[1]</sup> demonstrated the role of cardiac MRI in the diagnosis of focal myocarditis, even with the lack of wall motion abnormalities. Furthermore, Di Bella *et al*<sup>[2,3,6]</sup> identified the role of endocardium and epicardium in left ventricular deformation. They showed that both longitudinal and circumferential myocardial deformations were impaired in patients with acute myocarditis with preserved wall motion and evidence of subepicardial damage<sup>[3]</sup>. Particularly, longitudinal strain was reduced in all myocardial

walls independently from the presence or absence of subepicardial damage, although segments with subepicardial damage had greater impairment of longitudinal strain than those without damage. This suggests that subepicardium contributes together with subendocardium to longitudinal strain.

### **New advances of echocardiography and cardiac MRI in cardiac function**

Cardiac function and its pathophysiology were studied by Dr. Di Bella and colleagues in different clinical and experimental settings. Di Bella *et al*<sup>[7]</sup> studied cardiac function during hypoxemia induced by prolonged breath holding (in air) in healthy diving athletes. Results showed that, during breath holding, the stroke volume and cardiac output increased due to recruitment of left ventricle preload reserve, which counterbalanced the reduction in systolic function.

Others studies investigated the role of symptoms and signs in identifying left ventricular dilatation and/or systolic dysfunction<sup>[7,8]</sup>. Recently, Di Bella *et al*<sup>[10]</sup> showed that strain echocardiography allows an accurate, rapid, easy and reliable semiautomatic quantification of the left ventricular ejection fraction.

### **Scar tissue and myocardial infarction: new observations using cardiac MRI**

Dr. Di Bella and colleagues published studies on the role of scar tissue in patients with acute and chronic myocardial infarction. The most interesting results have demonstrated the following: (1) the impact of no reflow and scar tissue in patients with ST elevation myocardial infarction and the relation with PTCA time<sup>[18]</sup>; (2) the capability of cardiac MRI in simultaneous visualization of myocardial scar, no-reflow phenomenon and ventricular and atrial thrombi<sup>[19]</sup>; (3) the relationship between regional function and scar tissue<sup>[20]</sup>; (4) the predictive role of Q waves to identify localization and extension of scar tissue<sup>[21]</sup>; (5) the relationship between body mass index and scar tissue<sup>[22]</sup>; and finally; and (6) the combined role of regional function and scar tissue in the genesis of non-sustained ventricular tachycardia<sup>[23]</sup>. In this latter study, entitled “Different substrates of non-sustained ventricular tachycardia in post-infarction patients with and without left ventricular dilatation,” Dr Di Bella showed that necrotic and viable myocardium coexistence within the same wall segments predicts occurrence of non-sustained ventricular tachycardia in patients without left ventricular dilatation, whereas left ventricular mass and end-systolic volume are predictors of non-sustained ventricular tachycardia in those with left ventricular dilatation.

### **Early identification of cardiac amyloidosis by cardiac MRI**

The diagnosis of early stage cardiac amyloidosis is very difficult, however, studies have shown that cardiac MRI is a helpful tool for identification of amyloidotic deposition in patients with heart failure due to amyloidosis. Di Bella *et al*<sup>[39]</sup> showed that cardiac MRI is a suitable technique to identify cardiac amyloidosis in asymptomatic patients.



In this paper, Di Bella and colleagues showed an unusual localization of myocardial damage (hyperenhancement) in mid-basal segments of inferior and inferolateral walls. Furthermore, they observed an enhancement of the atria and/or tricuspid valve and/or right ventricle in all patients affected by cardiac deposition of amyloidosis.

### Outcome in patients with suspected arrhythmogenic right ventricular dysplasia

Aquaro *et al*<sup>[40]</sup> studied the role of right ventricular (RV) abnormalities detected by cardiac MRI to predict adverse outcome in patients with suspected arrhythmogenic right ventricular dysplasia (ARVD). They showed that RV abnormalities are also associated with worse outcomes in patients without a definite diagnosis of ARVD.

## CONCLUSION

Cardiac imaging has greatly modified the diagnostic process with noninvasive, rapid and accurate diagnosis of cardiac diseases. Echocardiography is the first step of cardiac imaging while an appropriate use of cardiac MRI is mandatory for identification of the substrate of many diseases. Overall, Dr. Di Bella's research has contributed to a better understanding of the role of strain echocardiography and cardiac MRI in clinical practice.

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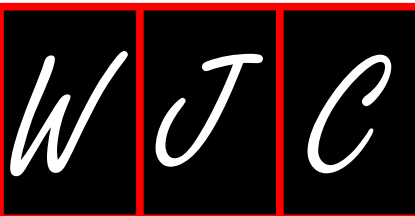
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## Meetings

### 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

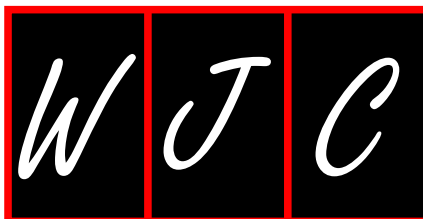
October 16-19  
Copenhagen, Denmark  
Acute Cardiac Care 2010

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

### GENERAL INFORMATION

*World Journal of Cardiology* (*World J Cardiol*, *WJC*, online ISSN 1949-8462, DOI: 10.4330) is a monthly peer-reviewed, online, open-access (OA), journal supported by an editorial board consisting of 352 experts in cardiology from 41 countries.

The biggest advantage of the OA model is that it provides free, full-text articles in PDF and other formats for experts and the public without registration, which eliminates the obstacle that traditional journals possess and usually delays the speed of the propagation and communication of scientific research results. The open access model has been proven to be a true approach that may achieve the ultimate goal of the journals, i.e. the maximization of the value to the readers, authors and society.

#### Maximization of personal benefits

The role of academic journals is to exhibit the scientific levels of a country, a university, a center, a department, and even a scientist, and build an important bridge for communication between scientists and the public. As we all know, the significance of the publication of scientific articles lies not only in disseminating and communicating innovative scientific achievements and academic views, as well as promoting the application of scientific achievements, but also in formally recognizing the "priority" and "copyright" of innovative achievements published, as well as evaluating research performance and academic levels. So, to realize these desired attributes of *WJC* and create a well-recognized journal, the following four types of personal benefits should be maximized. The maximization of personal benefits refers to the pursuit of the maximum personal benefits in a well-considered optimal manner without violation of the laws, ethical rules and the benefits of others. (1) Maximization of the benefits of editorial board members: The primary task of editorial board members is to give a peer review of an unpublished scientific article via online office system to evaluate its innovativeness, scientific and practical values and determine whether it should be published or not. During peer review, editorial board members can also obtain cutting-edge information in that field at first hand. As leaders in their field, they have priority to be invited to write articles and publish commentary articles. We will put peer reviewers' names and affiliations along with the article they reviewed in the journal to acknowledge their contribution; (2) Maximization of the benefits of authors: Since *WJC* is an open-access journal, readers around the world can immediately download and read, free of charge, high-quality, peer-reviewed articles from *WJC* official website, thereby realizing the goals and significance of the communication between authors and peers as well as public reading; (3) Maximization of the benefits of readers: Readers can read or use, free of charge, high-quality peer-reviewed articles without any limits, and cite the arguments, viewpoints, concepts, theories, methods, results, conclusion or facts and data of pertinent literature so as to validate the innovativeness, scientific and practical values of their own research achievements, thus ensuring that their articles have novel arguments or viewpoints, solid evidence and correct conclusion; and (4) Maximization of the benefits of employees: It is an iron law that a first-class journal is unable to exist without first-class editors, and only first-class editors can create a first-class academic journal. We insist on strengthening our team cultivation and construction so that every employee, in an open, fair and transparent environment, could contribute their wisdom to edit and publish high-quality ar-

ticles, thereby realizing the maximization of the personal benefits of editorial board members, authors and readers, and yielding the greatest social and economic benefits.

#### Aims and scope

The major task of *WJC* is to rapidly report the most recent developments in the research by the cardiologists. *WJC* accepts papers on the following aspects related to cardiology: arrhythmias, heart failure, vascular disease, stroke, hypertension, prevention and epidemiology, dyslipidemia and metabolic disorders, cardiac imaging, paediatrics, nursing, and health promotion. We also encourage papers that cover all other areas of cardiology as well as basic research.

#### Columns

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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

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- 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

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- 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]

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- 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]

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- 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]

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- 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. Wiczorek 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

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