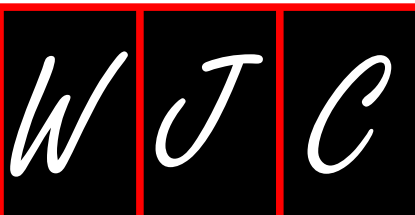


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- 583 Intimal pericytes as the second line of immune defence in atherosclerosis
Ivanova EA, Bobryshev YV, Orekhov AN

FRONTIER

- 594 Cost-effectiveness modelling of percutaneous coronary interventions in stable coronary artery disease
Beresniak A, Caruba T, Sabatier B, Juillière Y, Dubourg O, Danchin N

REVIEW

- 603 "Obesity paradox" in coronary artery disease
Akin I, Nienaber CA
- 609 Lipoprotein-associated phospholipase A2 prognostic role in atherosclerotic complications
Maiolino G, Bisogni V, Rossitto G, Rossi GP
- 621 Cardioprotection by remote ischemic conditioning: Mechanisms and clinical evidences
Aimo A, Borrelli C, Giannoni A, Pastormerlo LE, Barison A, Mirizzi G, Emdin M, Passino C
- 633 Cardiovascular disease in human immunodeficiency virus infected patients: A true or perceived risk?
Shahbaz S, Manicardi M, Guaraldi G, Raggi P

MINIREVIEWS

- 645 Concepts of hypoxic NO signaling in remote ischemic preconditioning
Totzeck M, Hendgen-Cotta U, Rassaf T
- 652 Potential of dietary nitrate in angiogenesis
Rammos C, Luedike P, Hendgen-Cotta U, Rassaf T
- 658 Is there a rationale for short cardioplegia re-dosing intervals?
Durandy YD
- 665 Role of platelet-rich plasma in ischemic heart disease: An update on the latest evidence
Spartalis E, Tomos P, Moris D, Athanasiou A, Markakis C, Spartalis MD, Troupis T, Dimitroulis D, Perrea D

ORIGINAL ARTICLE**Basic Study**

- 671 Enhanced caveolin-1 expression in smooth muscle cells: Possible prelude to neointima formation
Huang J, Wolk JH, Gewitz MH, Loyd JE, West J, Austin ED, Mathew R

Retrospective Cohort Study

- 685** Renal function assessment in atrial fibrillation: Usefulness of chronic kidney disease epidemiology collaboration vs re-expressed 4 variable modification of diet in renal disease

Abumuaileq RRY, Abu-Assi E, López-López A, Raposeiras-Roubin S, Rodríguez-Mañero M, Martínez-Sande L, García-Seara FJ, Fernandez-López XA, González-Juanatey JR

CASE REPORT

- 695** Percutaneous pulmonary valve implantation in a single artery branch: A preliminary experience

Chessa M, Butera G, Giugno L, Micheletti A, Negura DG, Carminati M

- 700** Intra-His bundle block in 2:1 atrioventricular block

Hong SP, Park YW, Lee YS

- 703** Late endocarditis of Amplatzer atrial septal occluder device in a child

Jha NK, Kiraly L, Murala JSK, Tamas C, Talo H, El Badaoui H, Tofeig M, Mendonca M, Sajwani S, Thomas MA, Al Doory SA, Khan MD

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Intimal pericytes as the second line of immune defence in atherosclerosis

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Abstract

Inflammation plays an essential role in the development of atherosclerosis. The initiation and growth of atherosclerotic plaques is accompanied by recruitment of inflammatory and precursor cells from the bloodstream and their differentiation towards pro-inflammatory phenotypes. This process is orchestrated by the production of a number of pro-inflammatory cytokines and chemokines. Human arterial intima consists of structurally distinct leaflets, with a proteoglycan-rich layer lying immediately below the endothelial lining. Recent studies reveal the important role of stellate pericyte-like cells (intimal pericytes) populating the proteoglycan-rich layer in the development of atherosclerosis. During the pathologic process, intimal pericytes may participate in the recruitment of inflammatory cells by producing signalling molecules and play a role in the antigen presentation. Intimal pericytes are also involved in lipid accumulation and the formation of foam cells. This review focuses on the role of pericyte-like cells in the development of atherosclerotic lesions.

Key words: Atherosclerosis; Arteries; Intima; Immune-inflammatory processes; Pericyte-like cells

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Core tip: Intimal stellate cells, expressing smooth muscle α -actin that co-express antigen 3G5 antigen (known to be specific for pericytes), reside in the intima of human large arteries. These cells have been defined as "pericytes-like satellite cells" or "intimal pericytes". Because of the peculiarities of the distribution of these cells and because of the current lack of our knowledge about the spectrum of the expression of other pericyte-associated markers in the arterial wall, it is reasonable to avoid at this time to identify smooth muscle α -actin(+)/3G5 antigen(+) stellate-shaped cells as true pericytes. The review highlights the importance of intimal pericytes in atherosclerosis.

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INTRODUCTION

Cardiovascular disorders (CVDs) are currently the main cause of mortality in developed countries, and atherosclerosis is the major contributor to the CVD risk^[1]. The disease affects large arteries causing lipid deposition in the arterial wall, local inflammatory process and the formation of plaques. Atherosclerotic plaques progression leads to luminal narrowing of blood vessels, which by itself can result in the ischemia of corresponding organs. However, most dangerous is the acute thrombus formation on the surface of a ruptured atherosclerotic plaque. Thrombosis can lead to rapid and life-threatening events, such as acute coronary syndrome and stroke^[2]. Early stages of atherosclerosis development are mostly clinically silent, and the first manifestations of the disease are often fatal. At the same time, subclinical atherosclerosis is a prevalent condition in the modern society, especially among the ageing population. It has been reported recently that up to 5% of women and 12% of men over the age of 80 suffer from asymptomatic atherosclerotic carotid artery stenosis^[3]. Other studies demonstrated the high rate of asymptomatic atherosclerosis incidence in young and middle-aged people, with up to 100% of apparently healthy individuals having atherosclerotic lesions at various stages of progression^[4,5]. Along with the well-known benefits of life style correction, the development of more specific therapies could help decreasing the risk of atherosclerosis progression and consequent CVD development. It is therefore important to improve our understanding of the pathological mechanisms that trigger atherosclerotic lesion development in the arterial wall.

The intimal barrier of human arterial wall consists of the endothelium and the subendothelial net-like tissue layer formed by pericyte-like cells that have recently

been characterized as true pericytes^[6,7]. Whereas the endothelium serves as the first line of defence against the development of atherosclerotic lesion, the pericyte-containing subendothelial layer can be regarded as the second defence line essential for normal functioning and protection of the arterial wall. Apart from pericytes, this layer contains other cell types, including smooth muscle cells, macrophages, lymphocytes, mast cells, dendritic cells, and other cell types. Pericyte-containing subendothelial layer is a characteristic component of both arterial and venous intima^[6]. Its role in the development of human pathology remained unknown until recently. However, the accumulating evidence demonstrated its importance in many severe vascular disorders, such as saphenous vein graft disease, thrombosis and atherosclerosis. Macrovascular pericytes are capable of rapid proliferation and participate in immune reactions. Moreover, these cells have been identified as a source of coagulation-inducing tissue factor^[7]. In this review we will summarize the current knowledge on the structure and function of the sub-endothelial leaflet of human macrovascular intima and its role in the pathogenesis of atherosclerosis.

STRUCTURE OF NORMAL AND ATHEROSCLEROTIC ARTERIAL INTIMA

Aortic intima is the part of the blood vessel wall located between the internal elastic lamina and the lumen with endothelial lining, and consists of two distinct layers (Figure 1)^[8,9]. Muscular-elastic layer represents the external part of the intima, and is separated from the internal intimal layer by the limiting membrane. This layer is formed by longitudinally oriented elongated cells and elastic fibres. The internal intimal layer, also known as proteoglycan-rich or connective tissue layer^[10,11], contains randomly-oriented connective tissue fibres and morphologically heterogeneous cell population^[12]. The intimal layers differ from each other by the composition of glycosaminoglycans and fibres. Muscular-elastic layer is rich in elastin fibres, and proteoglycan-rich layer - in collagen and reticulin^[13]. Moreover, collagen composition and structure are different between the two layers: longitudinally oriented collagens I and III are common in the muscular-elastic layer, while collagens IV and V are detected primarily in the proteoglycan layer, where they form the endothelial basal membrane.

The development of atherosclerotic lesion has been carefully studied by our group on human aorta samples from healthy subjects and atherosclerotic patients^[8-13]. Atherosclerotic process is tightly associated with thickening of the arterial intima. Careful analysis of the intima thickness along the arteries affected by atherosclerosis in comparison to normal tissues revealed that muscular-elastic layer remained unaltered in the fatty streak area and became only slightly thicker in the atherosclerotic plaque area^[8-13]. By contrast, the proteoglycan layer was 2-fold thicker in the fatty streak and 4-fold - in

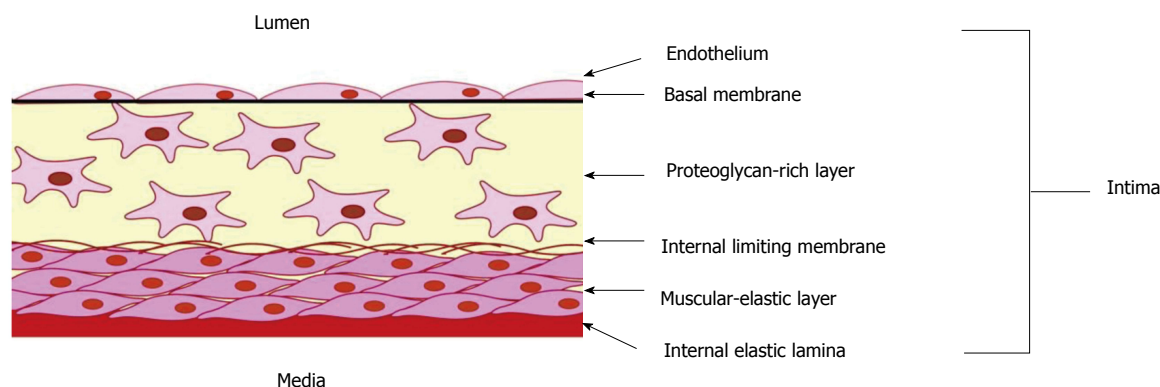


Figure 1 Schema showing the organization of the intima of the arterial wall. The proteoglycan-rich layer which contains a heterogeneous population of cells, including macrovascular pericytes, is located just below the endothelial monolayer. Intimal pericytes form a network of cells interconnected through gap junctions. The muscular-elastic layer, formed by elongated contractile smooth muscular cells, lies below the proteoglycan-rich layer.

the atherosclerotic lesion areas, and was the major contributor of the arterial stenosis. This thickened proteoglycan layer had increased collagen content. Moreover, longitudinal orientation of collagen fibres was altered, and lipid droplets were present between the interstitial collagen fibres^[8-13]. Atherosclerotic plaques were characterized by the accumulation of all collagen types, especially in the fibrous cap area. Collagens IV and V formed a thick layer in the endothelial basal membrane area and surrounded the subendothelial cells. At the same time, no prominent alterations of collagen structure and composition were present in the muscular-elastic layer of the intima^[8-13].

Changes of lipid content accompany the development of atherosclerotic plaques in the aortic intima. In the normal tissue, extracellular lipid droplets visualised by Oil Red O staining were located along the elastic membrane. In the fatty streaks, both extra- and intracellular droplets were present, mostly in the proximity of the lumen. In atherosclerotic plaques, lipid deposition could be observed in the proteoglycan layer, and total lipid content was 8-fold higher than in the unaffected tissue^[6]. Lipid content of muscular-elastic layer was increased to a lesser extent, being 4.4-fold higher than control. Cellular composition of the proteoglycan-rich layer of atherosclerotic intima was also altered in comparison with the healthy tissue^[6]. Total cell count assessed by alcohol-alkaline dissociation of the fixed tissue, was 1.5 and 2-fold higher in the fatty streak and atherosclerotic plaque respectively^[6].

Taken together, these observations clearly indicate that the subendothelial proteoglycan-rich layer of the arterial intima undergoes the most prominent alterations in course of the atherosclerotic plaque development, including thickening, lipid deposition and the accumulation of collagen fibres with disturbed orientation^[6,8-13].

CELLULAR COMPOSITION OF MACROVASCULAR INTIMA

Human macrovascular intima is made of a heterogeneous

cell population. Immediately below the endothelial lining and in close contact with it, there is a three-dimensional net formed by pericyte-like cells. Early studies have identified pericytes as mostly microvascular cells that play an important role in angiogenesis and vessel branching and are essential for maintaining the normal structure of the capillary wall and the endothelial barrier function, including the maintenance the blood-brain barrier^[14,15]. Pericytes were also demonstrated to contribute to embryonic development of the aorta^[16]. It has become evident, however, that pericyte-like cells are also present in the walls of large blood vessels^[17]. Apart from vasa vasorum microvessels^[18], pericyte-like stellate cells were found in the intima of large arteries^[6,7,19-21].

Accurate identification of pericytes, as well as their characterisation in *in vitro* studies, remains challenging, since these cells have flexible phenotype. The expression of numerous pericyte marker proteins is highly dynamic and depends on the surrounding milieu. Moreover, many marker proteins are shared between pericytes and vascular smooth muscle cells (VSMCs)^[14]. One of such markers is the α -smooth muscle actin (α SMA), which is normally expressed in VSMCs, but is also common in pericytes. However, its expression in the latter cell type can vary depending on the surrounding milieu^[22]. For instance, pericytes from non-contractile capillaries can be α SMA-negative^[23]. The expression of α SMA in pericytes can also be regulated by cell maturity^[23] or stimulation by endothelin-1^[24]. These considerations have to be taken into account while analyzing the cell population of macrovesicular intima. In the intima of large human arteries, the majority of resident cells were found to be α SMA-positive, especially in the muscular-elastic layer, where this population reached two thirds of the total amount of cells^[25]. In the proteoglycan layer, the percentage of α SMA-positive cells was much lower. These cells were different from typical VSMCs, were less densely packed and had a characteristic branched morphology, with long processes forming intracellular contacts. Moreover, some α SMA-positive intimal cells expressed marker proteins that were unusual for muscular cells,

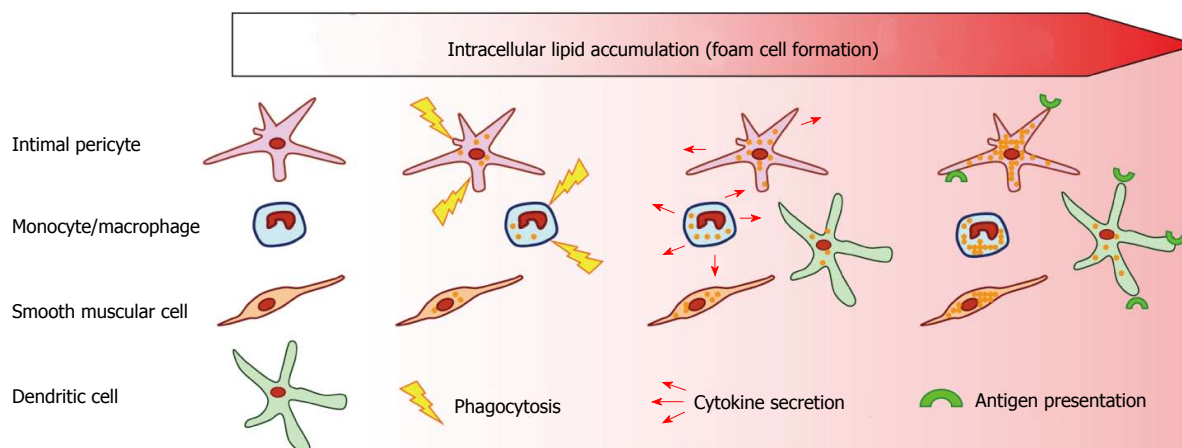


Figure 2 Scheme showing the roles of arterial wall cells in atherogenesis. Several types of arterial wall cells participate in lipid accumulation and foam cell formation. Macrophages and intimal pericytes accumulate lipids through phagocytosis and participate in the local inflammatory process secreting pro-inflammatory cytokines. Dendritic cells, together with macrophages and intimal pericytes express antigen-presenting complexes, further promoting the inflammatory process.

such as CD68, which is considered a macrophage-specific antigen^[26]. Interestingly, the proportion of α SMA+CD68+ cells increased in atherosclerotic lesions, as well as in primary cell culture exposed to atherogenic modified low-density lipoprotein (LDL). Apparently, the acquisition of the “macrophage”-specific marker reflects the engagement of cells into phagocytic activity^[27,28]. The large percentage of α SMA-negative cells in the arterial intima can partly be explained by the loss of contractile apparatus, since these cells acquire other functions than maintaining the vascular tone, such as metabolic homeostasis and nutrition or participation in the immune response in the blood vessel wall^[29].

Therefore, accurate identification of pericytes in the intimal layer should not be based on α SMA expression exclusively, and has to include the analysis of other pericyte markers expression and morphological data. Commonly used pericyte markers include platelet-derived growth factor receptor β , CD146, aminopeptidase A and N (CD13), endoglin, neuron-gial 2, non-muscle myosin, desmin, vimentin and nestin^[14,30]. However, most of these markers are shared with VSMCs and/or dependent on pericyte maturity or activation state, and therefore should be used in combination to avoid false-positive results. It has been demonstrated that some antigens expressed in resident intimal cells are not present in VSMCs. Antigen 3G5 is an O-sialoganglioside specific for microvascular pericytes^[31]. It was found in some intimal resident cells, but not in the tunica media^[26,32]. In humans, 3G5-positive pericytes form the net-like subendothelial tissue layer in normal human arterial intima and account for approximately one third of intimal cells (Figure 2)^[26]. Another pericyte antigen detected in the macrovascular intima is 2A7, also known as melanoma chondroitin sulphate proteoglycan^[26,33]. The expression of this antigen is typical for activated pericytes in the active angiogenesis phase. The expression of these pericyte marker antigens alters in atherogenesis. As

demonstrated on primary cultures of subendothelial cells exposed to atherogenic modified LDL, intracellular lipid deposition results in the reduction of 3G5-positive cell fraction, whereas 2A7-expressing fraction increases^[33].

Studies of the primary cell culture obtained from human macrovascular intima helped revealing the mechanisms of pericyte acquisition of characteristic stellate shape. Pericyte arborisation could be induced by increasing the intracellular cAMP concentration^[33]. Interestingly, cells originating from the proteoglycan layer were more susceptible of arborisation, with up to 100% of them being capable of acquiring the stellate shape, whereas in population originating from the muscular-elastic layer it could be induced in only 50% of cells. The arborisation was associated with re-distribution of connexin 43 (Cx43), which is responsible for gap junction formation. Stellate cells formed large Cx43-positive sites located at the ends of cellular processes, where intracellular contacts were formed. *In vivo*, pericytes form stable contacts with the endothelial cells, which could also be reproduced in co-culture of the two cell types^[7].

Apart from pericytes, other cell types populating the arterial intima have been described. These include cells of hematogenous lineage - macrophages^[34-36], lymphocytes^[37,38], mast cells^[39] and dendritic cells. Cells of hematogenous lineage are located exclusively in the subendothelial proteoglycan layer of intima and are enriched in atherosclerotic plaques, where they can account for up to 20% of total cell population.

ROLE OF PERICYTE-LIKE CELLS (INTIMAL PERICYTES) IN THE PATHOGENESIS OF ATHEROSCLEROSIS

According to current consensus, pericytes are pluripotent cells that can serve as progenitors for other cell types of

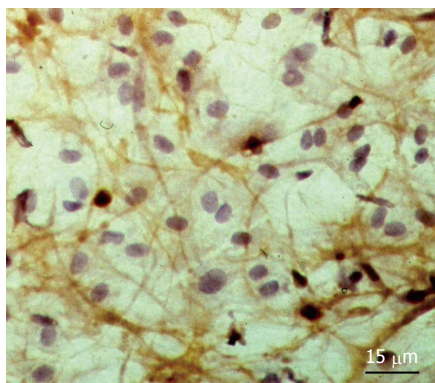


Figure 3 Cellular network formed by 3G5+ cells located under the luminal endothelium in undiseased intima. This cellular network was identified by means of the application of peroxidase-anti-peroxidase immunohistochemical analysis (brown colour of reaction product) in en face preparation of a tissue specimen of the human aorta. Counterstain with haematoxylin.

mesenchymal origin, including smooth muscle cells^[40], fibroblasts, osteoblasts^[41,42], chondrocytes^[40] and adipocytes^[43]. Pericytes are actively involved in various conditions associated with impaired microcirculation, such as diabetes, inflammation, wound healing and tumor growth. Converging evidence indicates pericyte implication in the pathogenesis of atherosclerosis. In presence of proinflammatory microenvironment and atherogenic modified LDL typical for early stages of atherosclerosis, intimal pericytes can accumulate lipids and become activated. The development of atherosclerotic plaque is associated with the increase of total cell count and the percentage of pericytes indicative of their involvement into the pathological process^[21]. In fatty streaks, these cells actively accumulate lipids, which leads to the increase of cell size, acquisition of irregular shape, loss of Cx43-mediated cell contacts^[44] and disturbance of the net-like subendothelial tissue^[45]. Pericytes may not only proliferate and store lipids contributing to the plaque growth^[46,47], but also promote thrombogenesis being a source of tissue factor^[17]. Figure 3 depicts the impact of different arterial cells, including intimal pericytes, in the atherosclerotic process.

Microvascular pericytes are important for atherosclerosis progression, as they participate in neovascularization of the atherosclerotic plaques^[48]. The recruitment of pericytes to growing neovessels could potentially be mediated by T-cadherin signalling. T-cadherin is an unusual member of the cadherin family, which is up-regulated in atherosclerosis^[49]. It might play a role during LDL-induced pericyte activation through ERK1/2-nuclear factor κ B signalling pathway^[50]. It has been demonstrated that pericytes could also be recruited to neovessels in atherosclerotic plaques through hepatocyte growth factor signalling triggering c-Met-PI3K/Akt pathway in pericytes^[51].

Participation in vascular wall remodelling and calcification is another possible contribution of pericytes to the pathological process in atherosclerosis. Activation of Wnt/ β -catenin signalling stimulates chondrogenic

differentiation of pericytes^[52], which can be enhanced by transforming growth factor- β ^[53]. The latter is abundantly produced by macrophages, foam cells and VSMCs in the atherosclerotic plaque^[54]. Intimal pericytes were shown to express vascular calcification-associated factor. Moreover, pro-inflammatory environment in the plaque may promote osteogenic differentiation of the resident vascular cells. These observations indicate the potential involvement of pericytes in ectopic vascular calcification associated with atherosclerosis^[55,56].

Taken together, the presented observations strongly support the hypothesis that macrovascular pericytes play an important role in all stages of the atherosclerotic lesion development, which makes them an attractive potential target for therapy development^[7,57]. Interestingly, pericytes may also take part in the innate immunity reactions, which will be discussed in the next section.

PERICYTE-LIKE CELLS IN INNATE IMMUNITY

The development of atherosclerotic lesion is tightly associated with local inflammatory process. At the initial stages of atherosclerosis, formation of focal intimal thickening is accompanied by the increase of intimal cell count^[58-60]. Such increase can be explained by the enhanced proliferation of intimal cells, recruitment of circulating hematogenous cells or a combination of these processes. Cell proliferation has been demonstrated to be important for atherosclerotic process^[61], although the differences in cell composition depending on different types of large arteries remained unknown. Detailed study of cell count and proliferation was performed on 29 post-mortem specimens of human carotid and coronary arteries with different stages of atherosclerotic plaque development: diffuse intimal thickening (grossly normal tissue), initial lesions, fatty streaks, lipofibrous plaques and fibrous plaques^[62]. Proliferating cells in S phase were identified by proliferating cell nuclear antigen (PCNA) staining. The authors report the increased total cell count in atherosclerotic lesions and more frequent macrophages and lymphocytes in comparison with normal tissue. Number of proliferating cells was also increased in atherosclerotic plaques as compared to normal intima. Maximal total cell count and maximal amount of proliferating cells were detected in lipofibrous plaques in both types of arteries. Interestingly, the proliferative index (determined as the percentage of PCNA-positive cells in the total subpopulation) hematogenous cells in atherosclerotic lesions was lower than in the unaffected intima. It is therefore likely that the increase of hematogenous cells in atherosclerotic intima occurs due to immune cell recruitment from the bloodstream and not from the local proliferation^[63-65]. By contrast, proliferative index of resident cells was higher in atherosclerotic lesions than in unaffected tissue, reaching its maximum in lipofibrous plaques^[62].

It is well documented that modified atherogenic LDL

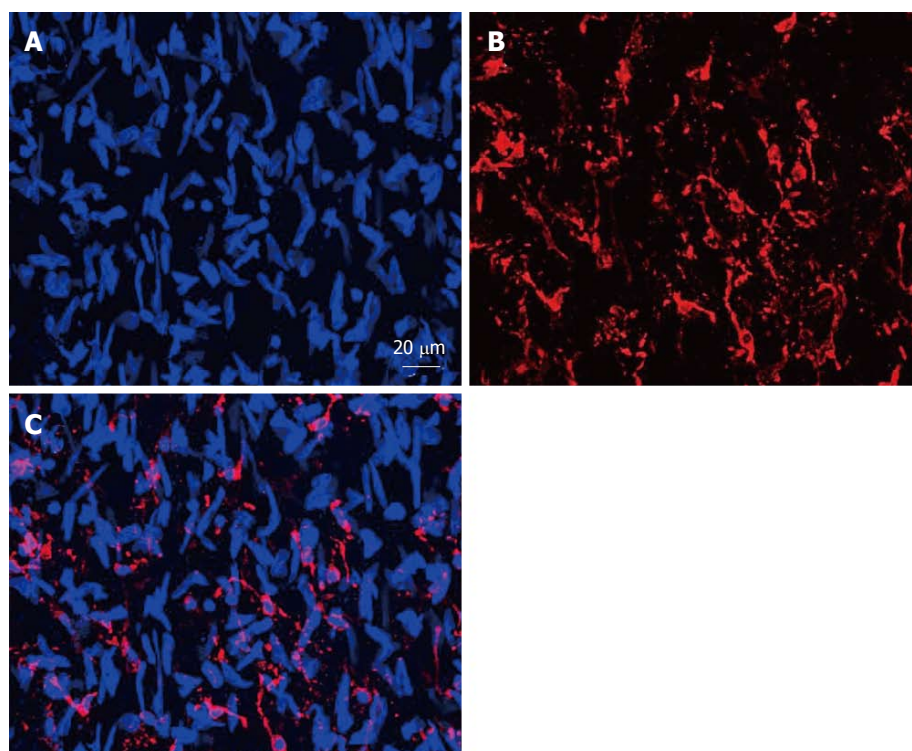


Figure 4 Immunofluorescent images representing the merge of 45 optic “confocal” sections of the intima obtained with interval of 1 μm between consecutive sections (A and B). A: Nuclei were visualized by staining with ethidium bromide; B: Distribution and networks formed by HLA-DR+ cells, detected with the use of anti-HLA-DR antibody conjugated with Alexa 633; C: A merge of the images shown in (A) and (B). Human aorta specimen. Reproduced from Bobryshev *et al*^[74], with permission from Elsevier. HLA: Human leukocyte antigen.

not only causes lipid deposition in the arterial intima, but also initiates pro-inflammatory conditions, stimulating both adaptive and innate immunity^[66,67]. During the atherosclerotic plaque formation, inflammatory cells are migrating into the lesion zone, where they release proinflammatory cytokines and chemokines, inducing proliferation of the resident intimal cells^[65,68]. This model may explain the observed divergence of inflammatory and resident cells proliferative index dynamics in atherosclerotic lesions. Study of immune-inflammatory processes in diffuse intimal thickening demonstrated the link between lipid deposition in the arterial wall and the immune-inflammatory cell content^[69]. Lipid deposition in the proteoglycan-rich layer of intima positively correlated with the expression of major histocompatibility complex class II (MHC II) molecule HLA-DR^[70] and the amount of immune-inflammatory cells in that area. These findings demonstrate that lipid accumulation at the early stages of atherosclerotic lesion development is accompanied by local immune activation. HLA-DR is a marker of antigen presenting cells (APCs)^[70,71] that include macrophages, B cells and dendritic cells. The latter were described as professional APCs, as they play the most prominent role in antigen presentation and initiation of the immune response. Apart from these cell types of hematogenous origin, some non-hematogenous cells can serve as APCs. For instance, epithelial cells of the thymus are involved in antigen presentation^[71]. Human endothelial cells are capable of T cell activation and basally express both MHCI and MHC II, although the expression is less prominent in larger arteries^[72]. Moreover, fibroblasts and endothelial cells can become activated to express MHC II under certain conditions both *in vitro* and *in vivo*^[70,73].

Immunofluorescent analysis of HLA-DR expression in the aortic intima revealed a large population of interconnected HLA-DR-positive cells that formed a net-like tissue (Figure 4)^[74]. In some segments of the intima up to 15% of total cell population was expressing HLA-DR^[74]. Morphologically, these cells could be identified as pericytes, with their typical net-forming intercellular contacts.

These results indicate that antigen presentation in the arterial wall can be performed by a variety of intimal cell types, including macrovascular pericytes. Importantly, microscopic study has revealed some HLA-DR-positive cells that also contained apo-B in the perinuclear space, possibly representing the early events of lipid accumulation in the intimal cells^[74]. It is therefore possible that pericytes play a double role in atherogenesis, participating both in lipid accumulation and in local immune reaction.

Immunohistochemistry demonstrated the expression of tumor necrosis factor (TNF)- α and CCL18 cytokines in both normal and atherosclerotic human aorta. These two cytokines were expressed by distinct populations of cells, with CCL18 mostly located in the subendothelial layer, and TNF- α - in deeper layers of the unaffected intima. The expression pattern was altered in atherosclerotic plaques, with both TNF- α and CCL18 upregulated compared to grossly normal arterial intima. Apart from macrophages, other cytokine-producing cells types were detected, including stellate pericyte-like cells.

The immune functions of pericytes remain an attractive topic for future investigation^[75]. Microvascular placental pericytes were demonstrated to interact with CD4⁺ T cells in co-culture. Pericytes stimulated the expression of CD25 and CD69 in resting allogenic CD4⁺

T cells, indicative of MHC recognition. This, however, did not lead to the induction of cytokine production and proliferation of T cells^[76]. The possibility that macrovascular pericytes have immune-modulating properties remains to be explored. The three-dimensional network of subendothelial cells participating in immune reactions may play important protective functions in the blood vessel wall. As demonstrated on the biopsy material from arteries, unaffected by atherosclerosis or with different stages of atherosclerotic lesion development, changes in this network occur early in the pathological process.

OTHER CELL TYPES PARTICIPATING IN IMMUNE REACTIONS IN THE SUBENDOTHELIAL INTIMA

Cells of hematogenous origin actively participate in immune reactions in the blood vessel wall. The development of atherosclerotic lesion is accompanied by infiltration and population of the lesion site by lymphocytes and macrophages. In human atherosclerotic lesions, the population of T cells consists mostly of the effector memory T cells. Substantial part of them are CD4⁺ T helper cells^[77]. CD8⁺ cytotoxic T lymphocytes are also present in atherosclerotic lesions. T cells are especially numerous in so called vulnerable plaques that are susceptible to rupture and thrombosis initiation^[78]. B cells have also been demonstrated to participate in atherogenesis^[79,80]. However, these cells are mostly found in the adventitia of the affected vessels, and not in the atherosclerotic plaques^[77,81].

Pro-inflammatory stimuli in blood vessels affected by atherosclerosis attract circulating monocytes that rapidly migrate to the atherosclerotic lesion site and differentiate where, predominantly to macrophages. This polarization is driven by stimulation of specific receptors, including C-C chemokine receptor type 2 and CX3C chemokine receptor 1^[82,83]. Both of these chemokines are implicated in the atherosclerotic plaque progression^[84]. Macrophages play a prominent role in the inflammatory process associated with the initiation and progression of atherosclerotic lesions. Macrophages are involved in the uptake of atherogenic modified LDL and form typical foam cells. Toll-like receptor (TLR) signalling in macrophages plays important role in lipid accumulation through regulation of scavenger receptor expression and suppression of cholesterol efflux^[85,86]. Macrophages also produce a number of inflammatory cytokines and contribute to vascular remodelling.

In atherosclerotic lesions, two distinct macrophage subpopulations can be found: M1 (classical) and M2 (alternative). Pro-inflammatory M1 phenotype can be induced in response to bacterial infection and pro-inflammatory cytokines, such as interferon (IFN)- γ and TNF- α ^[87]. They produce a spectrum of cytokines and chemokines stimulating the inflammatory res-

ponse, as well as nitric oxide and reactive oxygen species. M2 macrophages have anti-inflammatory properties, producing interleukin (IL)-10. In addition, M2 subpopulation of macrophages is heterogeneous: M2a macrophages are induced in response to IL-4 and IL-13 and participate in tissue remodelling, M2b cells are involved in immunoregulation, and M2c, induced by IL-10 and transforming growth factor- β , are involved in clearance of apoptotic cells^[88,89]. All three subtypes of M2 macrophages can be found in human atherosclerotic lesions. Human-specific M4 macrophages can be induced by platelet-derived chemokine CXCL4 and are characterized by resistance to excessive lipid uptake. This makes them susceptible to rapid transformation into foam cells and indicates their potential role in atherosclerotic lesion development^[90]. Specific populations of macrophages can be found in hemorrhagic atherosclerotic plaques. In humans, they represent M(Hb), HA-mac and Mhem subsets. HA-mac and M(Hb) macrophages are involved in hemoglobine clearance^[91]. All three subpopulations possess anti-inflammatory properties and can play a protective role in atherosclerosis.

Dendritic cells are also frequent in the subendothelial layer of intima, just below the liminal endothelium, where they contribute up to 10% of total cell population^[74,92,93]. In the subendothelial space, dendritic cells form cellular networks by means of their long interconnected processes^[74,92,93]. Dendritic cells play an important role in the immune response, producing a spectrum of cytokines and surface co-stimulatory molecules. They can be differentiated from monocytes at the periphery through stimulation with granulocyte-macrophage colony-stimulating factor (GM-CSF) and TLR4 ligands^[94]. These monocyte-derived dendritic cells are involved in antigen presentation and cross-presentation^[95]. It has been demonstrated that monocyte-derived DCs from patients with atherosclerosis had increased sensitivity to GM-CSF and IL-4 as compared to healthy subjects^[96]. Pro-inflammatory microenvironment of the atherosclerotic plaque accounts for preferential differentiation of monocytes and dendritic cell precursors towards pro-inflammatory dendritic cells that play an important role in the inflammatory process and stimulate differentiation of the naïve T cells to Th1 and Th17 phenotypes^[97]. Dendritic cells also produce pro-inflammatory IFN- α and a number of chemokines that induce the migration of inflammatory cells to the lesion site^[98]. In summary, pro-inflammatory dendritic cells play a prominent role in the development of atherosclerotic plaque. Populations of atheroprotective tolerogenic and anti-inflammatory dendritic cells could also be found in atherosclerotic plaques. In Apo-E-deficient mice, tolerogenic dendritic cells generated in response to immunogenic bacterial peptides were demonstrated to decrease inflammation and contribute to the plaque decrease through induction of IL-10-producing Tregs^[99]. It is possible that employing the

pathways of tolerogenic dendritic cell induction can be beneficial for treatment of atherosclerosis.

CONCLUSION

The subendothelial layer of human arterial intima contains a cellular network made of pluripotent pericytes that form multiple intracellular contacts through gap junctions. Together with the professional immune cells, such as macrophages and dendritic cells, pericytes may execute functions, typical for innate immune cell types: participate in LDL trapping by phagocytosis, produce pro- and anti-inflammatory cytokines and other factors and present antigens (Figure 2). Whereas the aortic endothelium plays a role as the first line of defence in atherosclerosis development, the subendothelial pericytes can be regarded as the second defence line and should be considered for the development therapeutic approaches for atherosclerosis treatment.

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Cost-effectiveness modelling of percutaneous coronary interventions in stable coronary artery disease

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Abstract

The objective of this study is to develop a cost-effectiveness model comparing drug eluting stents (DES) vs bare metal stent (BMS) in patients suffering of stable coronary artery disease. Using a 2-years time horizon, two simulation models have been developed: BMS first line strategy and DES first line strategy. Direct medical costs were estimated considering ambulatory and hospital costs. The effectiveness endpoint was defined as treatment success, which is the absence of major adverse cardiac events. Probabilistic sensitivity analyses were carried out using 10000 Monte-Carlo simulations. DES appeared slightly more efficacious over 2 years (60% of success) when compared to BMS (58% of success). Total costs over 2 years were estimated at 9303 € for the DES and at 8926 € for bare metal stent. Hence, corresponding mean cost-effectiveness ratios showed slightly lower costs ($P < 0.05$) per success for the BMS strategy (15520 €/success), as compared to the DES strategy (15588 €/success). Incremental cost-effectiveness ratio is 18850 € for one additional percent of success. The sequential strategy including BMS as the first option appears to be slightly less efficacious but more cost-effective compared to the strategy including DES as first option. Future modelling approaches should confirm these results as further comparative data in

stable coronary artery disease and long-term evidence become available.

Key words: Cost-effectiveness; Percutaneous coronary; Coronary artery disease; Drug eluting stent

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Core tip: The objective of this study is to develop a robust cost-effectiveness model comparing drug eluting stents (DES) *vs* bare metal stent (BMS) in patients suffering of stable coronary artery disease. DES appeared slightly more efficacious over 2 years (60% of success) when compared to BMS (58% of success). Mean cost-effectiveness ratios showed slightly lower costs per success for the BMS strategy (15520 €/success), as compared to the DES strategy (15588 €/success). The sequential strategy including BMS as the first option appears to be less efficacious but more cost-effective compared to the strategy including DES as first option.

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INTRODUCTION

Coronary heart disease is an important disorder in Western industrialized societies, with regard to both the epidemiologic and economic burden of illness^[1]. Stable angina (SA) is a clinical syndrome subset of the acute coronary artery disease (CAD), which is a major cause of emergency medical care in developed countries. The prognosis of SA is highly variable and depends on the initial treatment strategy which could be invasive (surgical procedure) or conservative (medical management). Angiographic and angioscopic studies suggest that CAD often results from the disruption of an atherosclerotic plaque and a subsequent cascade of pathological processes that decrease coronary blood flow. A modern therapeutic strategy consists of coronary interventions and the implantation of drug-eluting vascular stents. The idea that devices could be placed inside the arteries to maintain the blood flow came to a reality in 1986 when the first stents were successfully implanted in coronary arteries^[2,3]. The technology evolved rapidly even if the incidence of in-stent restenosis was between 20% and 30%^[4]. Then different generations of Drug Eluting Stents (DES) from heparin coated Palmaz-Schatz stents^[5] to chemotherapeutic releasing agent or copolymer coating have been proposed to lower the incidence of restenosis. Because of their high efficacy and good

safety profile, DES is reported to be used in 45% to 80% of all percutaneous coronary interventions^[6,7]. However, clinical evidence of medical devices is not really supported by robust randomized control clinical trials such as for pharmaceutical agents. Furthermore, cost-effectiveness of such strategies is rarely fully documented and based on numerous assumptions, making difficult the full evaluation of such strategies. Recent studies have continued to show improved procedural and clinical outcomes with DES both in the setting of acute coronary syndromes and stable coronary artery disease^[8]. A recent meta-analysis published by Palmerini *et al*^[9] analyzed twenty-two trials involving a total of 12453 patients and established that at one year DES were associated with lower rates of cardiac death or myocardial infarction and stent thrombosis than bare metal stents (BMS). Peterson *et al*^[10] studied the medical costs and outcomes of coronary stenting *vs* simple balloon angioplasty and estimated that the mean in-hospital cost for stent patients was \$3268 higher than for those receiving coronary angioplasty (\$14802 *vs* \$11534, $P < 0.001$). However, stent patients were less likely to be re-hospitalized (22% *vs* 34%, $P = 0.002$) or to undergo repeat revascularization (9% *vs* 26%, $P = 0.001$) than coronary angioplasty patients within six months of the procedure. A South Korean cost-minimisation model established that DES resulted in higher costs than Bare metal stent by 985 Euros per patient^[11]. However, it is possible that some selection bias influenced the results of such studies based on descriptive clinical data sources. A United States study published by Amin *et al*^[12] specifically focused on DES indications in current practices and concluded that the use of DES in the United States would vary widely among physicians, with only a modest correlation to patients' risk of restenosis. Thus less DES used among patients with low risk of restenosis would have the potential for significant cost savings for the United States health care system while minimally increasing restenosis events. As large controlled clinical trials are very difficult to implement in this indication, a modelling approach would allow to generate robust comparative results and assess the value for money^[13]. The objective of this study is to develop a cost-effectiveness model comparing DES *vs* BMS in patients suffering of stable coronary artery disease according to the French health system.

LITERATURE STUDY

Given the scarcity of head-to-head clinical trials, there is a need to use decision analytic models to assess and compare expected costs and effectiveness of percutaneous coronary interventions (PCI) strategies, using published evidence and cost estimates. Hence, evaluating the cost-effectiveness of a therapeutic sequence may identify the most clinically suitable population for a new strategy, and the most effective

Table 1 Charge table (i€)

Resource utilization	Mini	Mean	Max	Tariff
GP visit				22
Specialist visit (cardiologist)				23
Lab tests without markers of myocardial damage				21
Lab tests with markers of myocardial damage				39
ECG at rest				14
Stress test ECG				77
Stress test echocardiography				165
Myocardial scintigraphy				409
Coronary angiography				1954
Standard combination therapy (1 yr): aspirin + statin + ACE inhibitors	364		743	
GTN (1 yr)	39.4		73.2	
β-Blocker (1 yr)	52.9		210.9	
Calcium Inhibitors (1 yr)	87.5		311	
Hospitalization stay for PCI with BMS (1.6 stent)	4074		4573	
Hospitalization stay for PCI with DES (1.6 stent)	5284		5704	
Hospitalization stay for CABG surgery		14065		
Hospitalization stay for a clinical failure		2096		

DES: Drug eluting stents; BMS: Bare metal stent; PCI: Percutaneous coronary interventions; CABG: Coronary artery bypass graft; ACE: Angiotensin-converting-enzyme; ECG: Electrocardiogram.

and cost-effective treatment sequence. A model is a mathematic formula linking different variables to generate results relevant to a given environment based on local medical practices. A cost-effectiveness model is classically composed of a framework structure populated with costing and effectiveness data. Best modelling practices suggest that data populating a model should be based on relevant costs and existing published clinical data at the time of model development^[14]. The model assumptions should also be validated by expert clinicians according to their current medical practice in a given country. Specific to stable coronary artery disease management, results generated by such modelling approach provide unique information on the expected effectiveness, overall costs and cost-effectiveness of different PCI strategies to assist medical decision-making as well as resource allocation decisions.

Resource utilization

French direct medical costs were derived from a standard costing approach performed with a panel of three expert clinicians highly experienced in CAD management. Direct medical costs were estimated per 12 mo considering ambulatory costs (GP visits, cardiologist visit, laboratory tests, imaging, drugs) and hospital costs (percutaneous intervention, coronary artery bypass graft, coronary angiography alone, hospitalization due to complications), while initial diagnostic costs were not considered. Unit costs of interventions were derived from Diagnosis Related Groups list and from the national payer perspective for ambulatory

costs. Item costs were collected from minimum and maximum costs. Costs are reported in Euros (2012), and the charge table is reported in Table 1.

Resource utilizations were assessed from existing clinical guidelines. In the absence of guidelines, expert opinions were used from the clinician co-authors (YJ, OD and ND). Guidelines used were: "diagnosis and management of patients with stable ischemic heart disease" (American Heart Association 2012), "acts and services of long-term disease coronary heart disease" (Haute Autorité de Santé 2012) and "guidelines on the management of stable angina pectoris" (European Society of Cardiology 2006)^[15-17]. Treatment costs include a combination of drugs prescribed to every patient (low-dose aspirin, statin and angiotensin-converting enzyme inhibitor) and specific costs of the selected treatment. When the options are "PCI with BMS" or "PCI with DES", specific costs are associated with hospitalisation costs required for the angioplasty, the number and type of the implanted stents, and the costs of clopidogrel prescription to prevent intrastent thrombosis. Costs for the hospitalisation are determined using the French official hospital information system according to the specific diagnosis-related groups (DRGs) named "PCI without myocardial infarction". DRG costs include treatments and all examinations (invasive or non-invasive cardiac investigations, biological analyses, etc.). Total costs of stents are calculated according to the average number of stent implanted per patient: 1.6 per patient^[7]. The therapeutic option "drugs" includes the costs of basic drugs (see above) plus either β-blockers, calcium antagonists or long acting nitrates.

Costs of follow up after a clinical success include costs of ambulatory medicals visits, lab and medical imaging. The number of medical visits during follow-up has been estimated to 3 per year for GPs and 1 for specialist (cardiologist). Medical imaging includes one echocardiography and one myocardial scintigraphy. Lab tests (2 per year) include fasting lipid profile, including total cholesterol, low density lipoprotein (LDL), high density lipoprotein (HDL), and triglycerides, fasting glucose, full blood count including haemoglobin and white cell count, and serum creatinemia. Cost of follow up after a clinical failure are similar to costs after a clinical success plus one specific hospitalisation for management of the clinical failure. As no specific DRG exists for clinical failure, the following DRGs were considered: "myocardial infarction", "angina", "chest pain", "coronary atherosclerosis" and "arrhythmias and cardiac conduction disturbances". When a coronary angiography is not followed by an intervention, but by a drug prescription, the costs of the DRG "intravascular diagnostic procedure" have been used (mean costs of 5 related DRG).

Effectiveness

One relevant effectiveness endpoint aligned with stable coronary artery disease treatment goals has been used

Table 2 Effectiveness data of drug eluting stents model

	Minimum	Maximum	Ref.
Probability of success of a CABG surgery following a coronary angiography after a failure of a DES	90%	-	Sheiban <i>et al</i> ^[18]
Probability of success of pharmaceutical treatment after a failure of a DES	85%	90%	Sheiban <i>et al</i> ^[18]
Probability of success after a first DES	82.2%	97.5%	Simsek <i>et al</i> ^[19] Bakhai <i>et al</i> ^[20] Meredith <i>et al</i> ^[21] Morice <i>et al</i> ^[22] Toutouzas <i>et al</i> ^[23] Weisz <i>et al</i> ^[24] Serruys <i>et al</i> ^[25] Yan <i>et al</i> ^[26]
Probability of success after 1 yr of surveillance following a first DES	86.3%	98.7%	Simsek <i>et al</i> ^[19] Meredith <i>et al</i> ^[21] Park <i>et al</i> ^[27]
Probability of undergoing a second PCI following a coronary angiography after a failure of a DES	50%	60%	Malenka <i>et al</i> ^[28]
Probability of undergoing a surgery following a coronary angiography after a failure of a bare metal stent	30%	40%	Malenka <i>et al</i> ^[28]
Probability of undergoing a DES after a failure of a first DES	60%	70%	Sheiban <i>et al</i> ^[18]
Probability of success of a DES after a failure of a first DES	74.8%	90%	Steinberg <i>et al</i> ^[29] Ge <i>et al</i> ^[30] Lee <i>et al</i> ^[31]
Probability of success of a CBA after a failure of a first DES	60%	77%	Park <i>et al</i> ^[32]

DES: Drug eluting stents; PCI: Percutaneous coronary interventions; CABG: Coronary artery bypass graft; CBA: Coronary balloon angioplasty.

and expressed as treatment “success rate”. The success endpoint was defined as the absence of a MACE (major adverse cardiac events), that is to mean the absence of death or non fatal myocardial infarction or the need of a subsequent revascularization. Effectiveness estimates of PCI were derived directly from published clinical trials at the time of model development.

Assuming comparable patient populations, probabilities of patients in success at each simulated 12-mo time point have been estimated from an extensive literature review. If different values are presented in different publications, expert opinions were used to validate the use of a range between the minimum value and the maximum value observed in the literature. When no relevant values have been reported in the literature, a range of potential values is estimated based on the clinical experience of the expert panel. Then 10000 Monte-Carlo simulations were carried out to screen every possible value according to a uniform distribution shape. Effectiveness values are presented in Table 2 for the DES strategy and Table 3 for the BMS strategy.

Model structure

Using a 2-year time horizon, two PCI simulation models have been developed: BMS first line strategy (Figure 1A) and DES first line strategy (Figure 1B). The first step of the BMS model is the coronary angiography, followed by bare metal stent. After one year, patient could be in clinical situation of success or failure. In case of success, the surveillance without new treatment will occur during the second year. In case of failure at the end of the first year, a new coronary angiography will lead to either a PCI (either bare metal stent or DES), a Coronary Artery Bypass surgery (CABG), or

a pharmaceutical treatment. The first step of the DES model is the coronary angiography, followed by DES. In case of success, a simple surveillance is proposed as in the first model. In case of failure, a new coronary angiography will lead to either a PCI (new DES or balloon alone without any stent), a CABG, or a pharmaceutical treatment.

The models were specifically programmed in D-script language (Vanguard Studio 5.2). To manage uncertainty, and as per best practice in economic modeling, 10000 Monte-Carlo simulations generated mean values and standard deviations of the sub-model outputs: costs, effectiveness, and average cost-effectiveness over 2 years. Monte Carlo simulations consist of a class of computational algorithms that rely on repeated random sampling to compute their results. This approach, also called “probabilistic sensitivity analysis”, allows screening all possible values of a given parameter according to a defined distribution shape and to recalculate the results. For the purpose of this study, uniform distributions have been programmed between minimum-maximum ranges of values. Therefore, the models were able to construct distributions of results which are presented with their standard deviations (SD). Statistical tests (two groups mean tests with known variances deducted from cost-effectiveness SD) were performed to calculate potential significant differences between cost-effectiveness ratios of treatment strategies.

COST-EFFECTIVENESS

Using stable coronary artery disease management medical costs, cost of interventions and pharmaceutical therapies and published effectiveness data, the models

Table 3 Effectiveness data of bare metal stent model

	Minimum	Maximum	Ref.
Probability of success after a first bare metal stent	70.9%	86.4%	Simsek <i>et al</i> ^[19] Daemen <i>et al</i> ^[33] Bakhai <i>et al</i> ^[20] Morice <i>et al</i> ^[22] Weisz <i>et al</i> ^[24]
Probability of success after 1 yr of surveillance following a first bare metal stent	85%	96%	Simsek <i>et al</i> ^[19]
Probability of undergoing a second PCI following a coronary angiography after a failure of a bare metal stent	80%	-	Malenka <i>et al</i> ^[28]
Probability of undergoing a surgery following a coronary angiography after a failure of a bare metal stent	10%	15%	Malenka <i>et al</i> ^[28]
Probability of undergoing a DES after a failure of a bare metal stent	50%	-	Konstance <i>et al</i> ^[34]
Probability of success of a DES after a failure of a bare metal stent	78%	89%	Steinberg <i>et al</i> ^[29]
Probability of success of a second bare metal stent after a failure of a first bare metal stent	58%	67.3%	Singh <i>et al</i> ^[35]
Probability of success of a CABG surgery following a coronary angiography after a failure of a bare metal stent	90%	-	Malenka <i>et al</i> ^[28] Konstance <i>et al</i> ^[34]
Probability of success of pharmaceutical treatment after a failure of a bare metal stent	85%	90%	Sheiban <i>et al</i> ^[18]

DES: Drug eluting stents; PCI: Percutaneous coronary interventions; CABG: Coronary artery bypass graft.

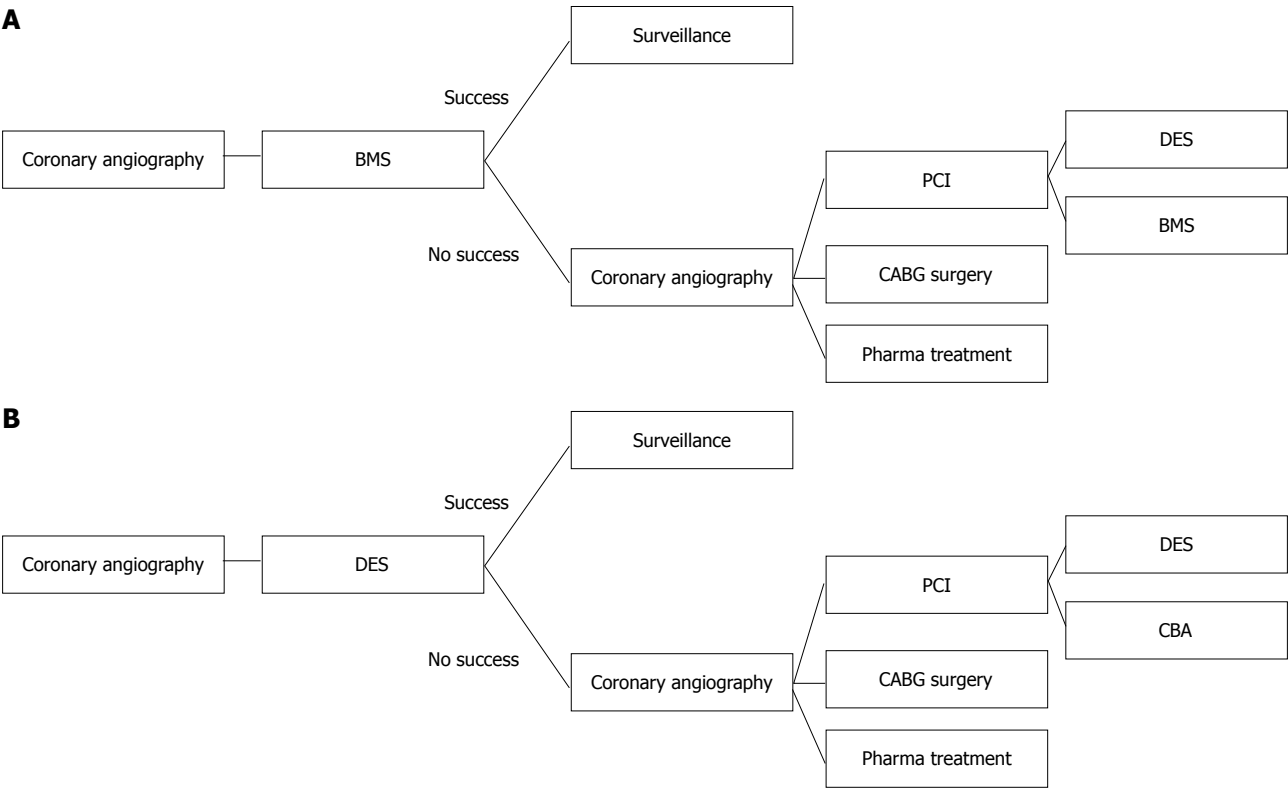


Figure 1 General architecture of the bare metal stent (A) and drug eluting stents (B) sequential model. DES: Drug eluting stents; BMS: Bare metal stent; PCI: Percutaneous coronary interventions; CABG: Coronary artery bypass graft; CBA: Coronary balloon angioplasty.

generated the overall treatment costs over 2 years, the probability of success, and the cost-effectiveness expressed in cost per success.

Strategy DES appeared slightly more efficacious (Figures 2 and 3) over 2 years (60% of success, SD 0.03) when compared to the strategy BMS (58% of success, SD 0.02). Total costs over 2 years were estimated at 9303 € (SD 3415) for the strategy DES and at 8926 € (SD 3778) for the strategy bare metal stent. Hence, corresponding mean cost-effectiveness ratios showed slightly lower costs per success for the bare metal stent

strategy (15520 €/success, SD 6634), as compared to the strategy DES (15588 €/success, SD 5787). Incremental Cost-Effectiveness Ratio is 18850 € for one additional percent of success.

DISCUSSION

The results of this cost-effectiveness model based on published clinical evidence suggest that in patients in stable coronary artery disease, the sequential strategy including bare metal stent as the first PCI option

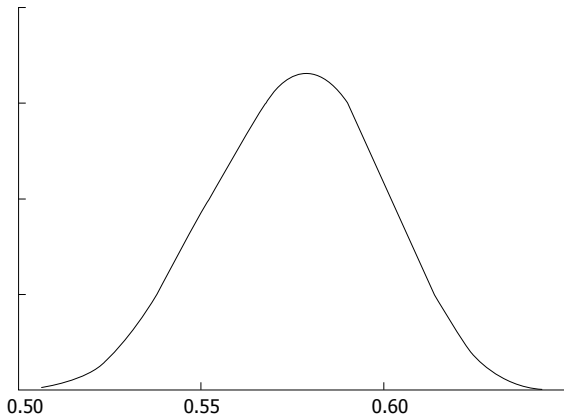


Figure 2 Cost distribution shape of the bare metal stent strategy (X axis: % success rate; Y axis: Occurrence probability).

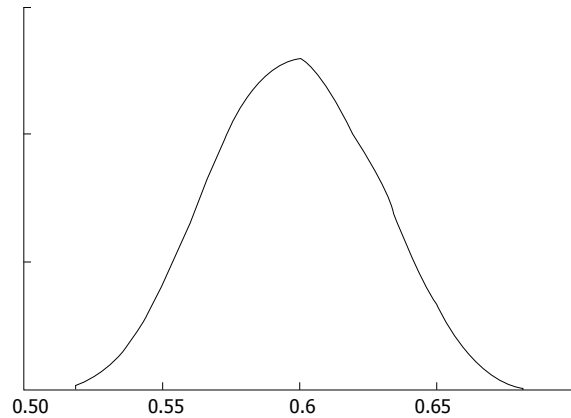


Figure 3 Cost distribution shape of the drug eluting stents strategy (X axis: % success rate; Y axis: Occurrence probability).

appears to be slightly less efficacious but more cost-effective compared to the strategy including DES as the first option. Two factors contribute to the better cost-effectiveness of the bare metal stent strategy. First, the results are driven by the efficacy reported in clinical trials. Secondly, the slightly higher success rates of the DES strategy do not offset the difference in costs between the two strategies. One of the most important issues in the creation of valid medico-economic models is the use of clinical effectiveness endpoints that are clinically meaningful and consistent across different settings. Selecting objective and consistent clinical outcomes allow defining clinical effectiveness of a given treatment more accurately and to compare across different treatment strategies for a specific patient population. Given that the goal of percutaneous coronary interventions is to reach a therapeutic success, this clinical endpoint appeared to be the most relevant effectiveness criteria for the purpose of conducting this cost-effectiveness analysis. Using a dichotomous approach of achieving success or no-success also appeared more clinically meaningful. Such cut-off points also avoid the need of using continuous scales with cardinal metrics (same origin and regular degrees) to compute effectiveness endpoints. Not only the proposed dichotomous approach success/no success is clinically meaningful, but it also requires fewer assumptions than using other outcomes,

The model assumes a 12-mo treatment period prior to allowing a potential switch to the next potential intervention in case of treatment failure. This assumption is based on the fact that most clinical trials report effectiveness data at 12 mo time points. These assumptions could be further discussed but they appeared to be consistent with medical practices in France, as validated with the expert panel. Furthermore, the time horizon of the model is limited to 2 years in order to reflect the data available at the time of model development. Hence, no long term effectiveness assumptions were made, as is it often the case in

published "lifetime" cost-effectiveness models in stable coronary artery disease. Wisløff *et al.*^[36] carried out a cost-effectiveness model comparing DES vs BMS and concluded that DES was more cost-effective over a life time horizon using life years saved as an effectiveness endpoint. However, this model is based on life-time horizon projections speculating well beyond clinical trials evidence and diluting costs over years.

Co-morbidity such as diabetes is a risk factor of re-stenose after stent implantation, particularly for BMS as confirmed by the meta-analysis of Bangalore *et al.*^[37] carried out on 42 controlled randomized clinical studies. However, no specific calculations have been carried out for patients suffering of co-morbidities such as diabetes. Data inputs come from studies where proportion of diabetes patients were similar than European population, as described in the Euro Heart Survey^[38].

This approach does not capture potential Quality of Life (QOL) improvement, as we would recommend that such evidence be considered separately, to its full merit. Furthermore, it is not the purpose of one clinical indicator to capture all the dimensions of life, so QOL dimensions should be collected separately using appropriate validated instruments. Many published "cost-utility" models (often presented under the label of "cost-effectiveness" models) consider the use of Quality Adjusted Life Years (QALY) as "effectiveness" criteria in order to take into account both the Quality of Life and the survival perspectives^[39]. Not only the QALY approach is not specifically recommended in France (and several other countries such as USA and Germany) for methodological issues, but such approach reveals to be inconsistent in Stable coronary artery disease^[40]. This is because the results are directly dependent on how the utility scores have been derived, explaining the possibility of data manipulation and why these studies often lead to divergent results^[41,42]. The advantage of cost-effectiveness models using clinical effectiveness outcomes (such as Success probabilities) from published clinical trials is that the effectiveness criteria are not further transformed into

utilities. Hence, classic cost-effectiveness assessments (cost/clinical outcome achieved or per medical event avoided) generate more transparent and consistent results. Also, this cost-effectiveness analysis does not use a societal perspective but the perspective of the public payer in France. In such case, the results do not take into account the reported favourable impact of PCI on indirect costs. As indirect costs related to CAD are substantial and are estimated to be 2-3 times as high as direct costs, the results of this economic evaluation are likely to be understated. Finally, a frequent concern about cost-effectiveness models is that most publications seem to support the product of the study sponsor, suggesting a potential publication bias such as for publications of clinical trials. As they are used to inform and optimize resource allocation decisions, cost-effectiveness models are country specific and should always define the assumptions and conditions underlying the results where a therapeutic strategy is found to be cost-effective^[14], which should also be in line with medical practices. Any model assuming very hypothetical clinical practices or theoretical outcomes should be considered with caution, as for any scientific studies.

CONCLUSION

This sequential cost-effectiveness model proposes a new approach to assess complex strategies based on clinical evidence based data and avoids any extrapolation over time, which could be subject to criticism. The model outcome expressed in costs per clinical success appears to be a clinically meaningful endpoint, allowing to compare various strategies. The sequential strategy including BMS as the first option appears to be slightly less efficacious but more cost-effective compared to the strategy including DES as first option in the frame of the French health system. Future modelling approaches should confirm these results as further comparative data in stable coronary artery disease and long-term evidence become available, but also to assess the value of innovative strategies such as biodegradable coronary stents.

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"Obesity paradox" in coronary artery disease

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Abstract

Obesity used to be among the more neglected public health problems, but has unfolded as a growing medical and socioeconomic burden of epidemic proportions. Morbid obesity is linked to traditional cardiovascular risk factors like, hypertension, hyperlipidemia and diabetes, and suspected to incur increased morbidity and mortality

in the Western and even third world populations. This patient cohort is also at greater risk to develop coronary artery disease. Recent population-based registries revealed that 43% and 24% of all cases of coronary revascularization were carried out in overweight and obese patients, respectively. However, despite evidence of a positive correlation between obesity and increased cardiovascular morbidity, some authors have described a better clinical outcome in overweight and obese patients, a phenomenon they coined "obesity paradoxon". Thus, there is an ongoing debate in light of conflicting data and the possibility of confounding bias causing misconception and challenging the "obesity paradox". In this review article we present the current evidence and thoroughly discuss the validity of the "obesity paradoxon" in a variety of clinical settings.

Key words: Coronary stent; Obesity paradox; Mortality; Body mass index

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Core tip: Obesity is one of the leading health problems within the last years and is associated with several cardiovascular risk factors resulting in increased prevalence of coronary artery disease as well as atherosclerotic disease in head vessels and peripheral artery system. Despite these positive correlations there are reports describing a protective effect in patients undergoing coronary revascularization. This review will enlight the potential causes and bias regarding this "obesity paradoxon" in several clinical setting and will present the latest data.

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INTRODUCTION

Epidemiologic data on obesity, traditionally defined

as a body mass index (BMI) $> 30 \text{ kg/m}^2$, range in the Western population with a prevalence of up to 36.5%^[1]. Prevalence in United States is up to 70% that is much higher than 40 years ago (25%)^[2]. Obesity and associated disorders like arterial hypertension, dyslipidemia, diabetes mellitus and sleep apnoea syndrome are linked to increased morbidity and mortality^[3,4]. Common recommendation is weight loss to modify cardiovascular risk as an attempt for primary and secondary prevention of cardiovascular disease in overweight and obese patients^[5-7]. Obesity is associated with increased atherosclerotic diseases, especially coronary artery disease by reduced insulin sensitivity, enhanced free fatty acid turnover, increased basal sympathetic tone, a hypercoagulable state, and finally by promotion of systemic inflammation^[8,9]. Population-based data revealed that 43% and 24% of all coronary revascularization were carried out in overweight and obese patients, respectively^[10]. Clinical outcome speculations should indicate that these patient cohorts would be associated with worse outcomes as compared to normal weight patients. Nevertheless, despite proven evidence of a causative association between morbid obesity and increased cardiovascular morbidity, previous studies have described the phenomenon of “obesity paradoxon”, reporting a protective effect of obesity with regards to postoperative morbidity and mortality in patients receiving a surgical or interventional revascularization^[11]. The observation of better clinical outcome is not only restricted to coronary revascularization but also in other clinical settings like acute myocardial infarction and heart failure^[12,13]. With this review article we would like to give an overview and summary on the evidence of an “obesity paradoxon” in coronary artery disease.

STABLE CORONARY ARTERY DISEASE

Correlation of BMI with clinical endpoints in the setting of interventional coronary revascularization was first reported in 1996 from a single-center experience in patients ($n = 3571$) receiving balloon angioplasty^[14]. In-hospital outcomes revealed higher rates of mortality (2.8% vs 0.9% vs 3.7%; $P < 0.001$) in normal weight and obese patients as compared to overweight patients. Similar differences were seen in need for blood transfusion (11.9% vs 7.4% vs 8.4%; $P = 0.003$) and rise in creatinine $> 1 \text{ mg/dL}$ (3.6% vs 1.8% vs 1.8%; $P = 0.018$); whereas rates for myocardial infarction were not different (3.5% vs 3.4% vs 4.7%; $P = \text{ns}$). A total of 3634 patients from the multicenter BARI registry undergoing elective revascularization [2108 by interventional procedure (PCI) and 1526 by surgery (CABG)] between 1988 and 1991 were evaluated for BMI at study entry^[15]. Body mass index (BMI) was associated with increased risk of a major in-hospital event just in the PCI arm. However, at five year follow-up there was a correlation between mortality and BMI only in the CABG arm. While these results from the

BARI trial suggested an inverse correlation of BMI with in-hospital outcome after PCI and no such difference with long-term follow-up, Gruberg *et al.*^[11] revealed an inverse correlation during 12-mo follow-up in 9633 patients that had been evaluated between 1994 and 1999 for mortality (10.6% vs 5.7% vs 4.9%; $P < 0.0001$); conversely rates of myocardial infarction (7.4% vs 7.0% vs 6.7%; $P = 0.66$) and target vessel revascularization (20.2% vs 22.0% vs 22.4%; $P = 0.16$) were not different.

Postprocedural clinical events like arterial hypotension, pulmonary congestion, impairment of renal function, as well as bleeding events, access site complications and mortality rates were more frequently seen in underweight patients than in overweight or obese ones. Different from previous all-comers trials, the Scottish Coronary Revascularisation Register included only patients ($n = 4880$) receiving elective PCI between 1997 and 2006 without a history of previous known coronary artery disease. During five years of follow up BMI between 27 and 30 kg/m^2 was correlated with lower all-cause mortality as compared to other weight groups. Introduction of a blanking time ($< 30 \text{ d}$) to exclude periprocedural events and an adjustment to different baseline data did not impact on outcomes of their study^[16]. These results could be confirmed in the APPROACH registry in 31021 patients treated medically ($n = 7801$), by PCI ($n = 7017$) or by CABG ($n = 15601$)^[17]. In the first group, mortality rates were lower in overweight and obese patients as compared to normal ones. Similar findings were found in both the CABG and PCI group. Interestingly, the use of bare-metal stents (BMS), or any metaanalysis of these single trials revealed an inverse relationship between BMI and clinical outcome after stenting^[18]. In contrast to the above-mentioned results from the balloon angioplasty and BMS era, some other studies from that time do not support the “obesity paradoxon” in patients with both, BMS or DES. Poston *et al.*^[19] revealed in 1631 patients that normal weight patients were older than the non-normal weight ones at the time of hospital admission^[18]. During one year follow-up mortality and risk for repeat procedures was not different between groups. In the TAXUS trials, 1307 patients were stratified according to BMI and use of stent type (BMS vs DES)^[20]; restenosis rates with use of BMS were higher in obese and overweight patients as compared to normal weight ones (29.2% vs 30.5% vs 9.3%, respectively; $P = 0.01$). Although rates for major cardiac events were also significantly different in favour of normal weight patients, clinical event rates were not different in patients receiving DES. These findings were underlined by the results of the German DES.DE registry^[21]. At 98 sites in Germany 5806 patients receiving DES in an all-comers design were included and followed over 12 mo. Similar to previous trials baseline comorbidity index was higher in obese patients as compared to overweight and normal weight patient and in-hospital events were similar in all three

Table 1 Overview of literature addressing the “obesity paradox” in patients suffering from stable coronary artery disease undergoing coronary angiography and/or revascularization

Ref.	Year	n	Follow-up (mo)	Mortality	Myocardial infarction	Target vessel revascularization	Renal insufficiency	Vascular complications
Ellis <i>et al</i> ^[14]	1996	3571	12	+	-	-	+	+
Gurm <i>et al</i> ^[15]	2002	3634	60	+	NA	NA	NA	-
Gruberg <i>et al</i> ^[11]	2002	9633	12	+	-	-	+	-
Poston <i>et al</i> ^[19]	2004	1631	12	-	NA	-	NA	NA
Nikolsky <i>et al</i> ^[20]	2005	1301	12	-	-	-	NA	NA
Romero-Corral <i>et al</i> ^[18]	2006	250152	45	+	NA	NA	NA	NA
Oreopoulos <i>et al</i> ^[17]	2009	31021	46	+	NA	NA	NA	NA
Hastie <i>et al</i> ^[16]	2010	4880	60	+	NA	NA	NA	NA
Akin <i>et al</i> ^[21]	2012	5806	12	-	-	-	-	-

NA: Not available.

groups. One-year follow-up revealed no differences in rates of death (3.3% vs 2.4% vs 2.4%; $P = 0.17$), myocardial infarction (2.8% vs 2.3% vs 2.3%; $P = 0.45$), target vessel revascularization (10.9% vs 11.7% vs 11.6%; $P = 0.56$) and major bleeding (2.5% vs 2.1% vs 2.8%; $P = 0.53$) between normal weight, overweight and obese patients, respectively (Table 1).

ACUTE CORONARY SYNDROME

In contrast to stable coronary artery disease an acute myocardial infarction is characterized by a proinflammatory state with different forms of hemodynamic, rhythmogenic and hemostatic disturbances. The phenomenon of an “obesity paradoxon” has also been evaluated in this patient population; yet, data on a potential link between BMI and clinical events in patients with acute myocardial infarction are scarce and inhomogenous in the literature. Data from the PREMIER and TRIUMPH registries including 6359 patients with acute coronary syndrome were taken to look for any relationship between BMI with survival rate^[22]. Similar to patients with stable coronary artery disease there was an inverse relationship between BMI and rate of mortality (9.2% vs 6.1% vs 4.7%; $P < 0.001$) without any interactions of demographic data like age and sex. Similar findings were revealed in the KAMIR registry involving 3824 patients with ST-elevated myocardial infarction^[23]. Baseline characteristics were characterized by the fact that normal weight patients were older, had more impairment of left ventricular ejection fraction, and a higher comorbidity index. Nevertheless, normal weight in this scenario was associated with higher mortality. In contrast of these trials several other trials showed no inverse relationship between BMI and clinical outcome^[24,25]. Our group analyzed 890 patients suffering from ST-elevated myocardial infarction including patients with cardiogenic shock and followed them up to 12 mo; clinical events were not significantly different between all three weight groups, again challenging an obesity paradox. These findings were also seen in patients suffering from cardiogenic shock^[26] (Table 2).

RATIONALE FOR THE “OBESITY PARADOX”

Self-reported obesity increased by 37% from 13.6% to 18.6% among men aged 35-49 since 1970. Simultaneously, epidemiology of other cardiovascular risk factors like arterial hypertension and diabetes increased^[27,28]. However, mortality attributed to coronary events declined during the last 40 years mainly due to decreased cholesterol levels and damaging smoking habits with greatest reduction seen in overweight and obese patients, and to some degree as a result of more frequent revascularization^[29-31]. Nevertheless, overweight and obesity, as part of the metabolic syndrome, are still linked to other cardiovascular risk factors, endothelial dysfunction, and inflammation and are often associated with an increased risk of suffering from atherosclerosis.

The key question to answer is how to explain better survival rates from coronary events despite increasing rates of obesity in light of above mentioned correlation. There is an ongoing debate whether the phenomenon of “obesity paradoxon” is real in the space of coronary artery disease^[10-26].

Close examination of the current literature revealed that some published and most often retrospective data just claimed a U-shaped nonsignificant trend towards lower survival among underweight patients, compared with normal or mildly overweight patients; this however might predominantly result from a technical bias, that cannot be completely corrected by statistical means.

Detailed analyses reveal that up to 2% of patients who were underweight were likely to suffer from comorbid conditions, including malignancies, heart failure, malnutrition, multiorgan dysfunction, and happened to be significantly older than the normal and obese patients^[10,11,15]. There is clear evidence that elderly and frail patients have worse clinical outcomes after any coronary event regardless of reperfusion or reperfusion strategy^[32,33]. It is important to recognise that increasing age with its concomitant comorbidity index results in weight change^[34,35]. Chronic diseases might lead to gradual weight-loss, which is not taken

Table 2 Overview of literature addressing the “obesity paradox” in patients suffering from acute coronary syndrome including cardiogenic shock undergoing coronary revascularization

Ref.	Year	n	Follow-up (mo)	Mortality	Myocardial infarction	Target vessel revascularization	Renal insufficiency	Vascular complications
Kosuge <i>et al</i> ^[25]	2008	3076	Hospital	-	NA	NA	NA	NA
Kang <i>et al</i> ^[23]	2010	3824	12	+	-	-	NA	NA
Camprubi <i>et al</i> ^[24]	2012	824	Hospital	-	NA	NA	NA	NA
Bucholz <i>et al</i> ^[22]	2012	6359	12	+	NA	NA	NA	NA
Li <i>et al</i> ^[31]	2013	1429	12	-	-	-	NA	NA
Shehab <i>et al</i> ^[52]	2014	4379	1	-	-	-	NA	NA
Akin <i>et al</i> ^[26]	2015	890	12	-	-	-	-	-

NA: Not available.

into consideration in presented trials. Along these lines, another important confounding observation was the fact that obese patients tended to be diagnosed and treated at an earlier stage than lean patients^[36]. Furthermore, the “obesity paradoxon” is clearly challenged by a recent survey on > 130000 patients, revealing that adherence to guidelines was better with higher BMI with regards to standard medication such as aspirin, b-blockers, acetylcholinesterase inhibitor and angiotensin II receptor blockers, as well as lipid lowering drugs and also an increased likelihood of receiving invasive diagnostic and treatment^[15,18,21]. Furthermore overweight and obese patients present in a much more stable status with lack of hemodynamic compromise, lower Killip class, and less impairment of ventricular function. Novel theories to explain the “obesity paradoxon” after PCI have included the suggestion that obese patients have “larger vessels” and outcome after PCI is worse in patients with smaller vessel^[37,38]. Antithrombotic medications usually given as standard dosage and not weight adjusted, may be too high in normal weight and underweight patients according to their BMI, resulting in more bleeding events which are associated with higher mortality rate^[39]. Similarly, sheath-to-artery size ratio is different in BMI groups resulting in different rates of vascular complications^[15]. All these differences in periprocedural events can contribute to improved survival among overweight patients^[11,40]. The fact that BMI alone was the only measure of obesity is certainly a limiting factor. There is no information in several trials regarding the distribution of obesity that might be very important, as there is a worse outcome in patients with central obesity^[41]. Moreover additional information regarding waist circumference, waist-to-hip ratio and weight changes are missing in several trials describing the “obesity paradoxon”^[42-45]. Additionally, all trials supporting the “obesity paradoxon” suffer from the inherent limitation of an observational retrospective registry. Potentially confounding variables such as physical inactivity, unintended weight loss and even socioeconomic factors were not analyzed, and may be the source to further bias, not to mention the short follow-up of these registries. Eventually any potential

relation between obesity and in-hospital and short-term survival may be lost the longer patients are followed. Therefore with extended follow-up a cumulative detrimental effect of obesity may manifest over time as increased late mortality^[46,47].

It is sensible to ask what is left to support the so-called “obesity paradoxon”, or is it just a paradoxical concept? Protagonists claim that adipose tissue is being recognized as an endocrine organ^[48] that produces soluble tissue necrosis factor receptor with its protective effect^[49]. Morbidly obese patients (BMI > 40 kg/m²) certainly have higher adjusted rates of post-PCI mortality that might be due to higher levels of prothrombotic factors as well as elevated levels of plasminogen activator inhibitor- I^[50]. On aggregate, while early studies may have suggested an inverse relationship between being underweight with outcomes in heart failure, which led to the assumption of a “obesity paradoxon”, the analysis of the published evidence denies any such “obesity paradoxon” in the context of coronary artery disease and modern coronary interventions. In fact there is no plausible concept to turn away from the classic relationship between risk factors, confounding variables and prognostic outcomes. The limitations of such association studies are not only the lack of a pathophysiologic underpinnings, but moreover the mere association with descriptive notions and the unknown impact of confounding variables. With respect to the neutralizing results from the German DES.DE Registry^[21], the perception of obesity as a protective condition of outcomes after PCI is shattered and the provocative construct of an “obesity paradoxon” evaporates, as such hypothesis was never really substantiated in the clinical setting of coronary artery disease and PCI. Finally, as it turns out, associative studies with little to no statistical evidence lended support to invent a “obesity paradox” which was never supported by biological evidence and seems now shattered by new interpretation of recent clinical data. Any concept will eventually survive only if supported by plausible physiology. In the context of coronary artery disease and PCI there is hardly any plausible explanation and certainly no clinical data to justify an “obesity paradoxon”.

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Lipoprotein-associated phospholipase A2 prognostic role in atherosclerotic complications

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Abstract

Atherosclerosis manifests itself clinically at advanced stages when plaques undergo hemorrhage and/or rupture with superimposed thrombosis, thus abruptly stopping blood supply. Identification of markers of plaque

destabilization at a pre-clinical stage is, therefore, a major goal of cardiovascular research. Promising results along this line were provided by studies investigating the lipoprotein-associated phospholipase A2 (Lp-PLA2), a member of phospholipase A2 proteins family that plays a key role in the metabolism of pro-inflammatory phospholipids, as oxidized low-density lipoproteins, and in the generation of pro-atherogenic metabolites, including lysophosphatidylcholine and oxidized free fatty acids. We herein review the experimental and clinical studies supporting use of Lp-PLA2 activity for predicting cardiovascular events. To this end we considered not only Lp-PLA2 activity and mass, but also *Lp-PLA2* gene variations and their association with incident coronary artery disease, stroke, and cardiovascular mortality. Based on these evidences the major scientific societies have included in their guidelines the measurement of Lp-PLA2 activity among the biomarkers that are useful in risk stratification of adult asymptomatic patients at intermediate cardiovascular risk. The results of two recently published major clinical trials with the Lp-PLA2 inhibitor darapladib, which seem to challenge the pathogenic role of Lp-PLA2, will also be discussed.

Key words: Lipoprotein-associated phospholipase A2; Atherosclerosis; Coronary artery disease; Myocardial infarction; Prognosis

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Core tip: Lipoprotein-associated phospholipase A2 (Lp-PLA2) is a promising new marker of atherosclerotic plaque destabilization, which plays a key role in the metabolism of pro-inflammatory phospholipids and in the generation of pro-atherogenic metabolites. This review focuses on the experimental and clinical studies supporting use of Lp-PLA2 for predicting cardiovascular events considering not only Lp-PLA2 activity and mass, but also *Lp-PLA2* gene variations. Based on current evidences the major scientific

societies have included Lp-PLA2 activity measurement in their guidelines among the biomarkers that are useful in risk stratification of adult asymptomatic patients at intermediate cardiovascular risk.

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INTRODUCTION

Exposure of endothelial cells to damaging stimuli, as smoking, arterial hypertension, diabetes mellitus, dyslipidemia can induce qualitative changes that are collectively defined as “endothelial activation”, and are currently postulated regarded as one of the earliest events in atherogenesis^[1]. An “activated” endothelium expresses adhesion molecules and chemotactic substances, increases its permeability to macromolecules with ensuing variation of the sub-endothelial extracellular matrix composition. As a result, low-density lipoproteins (LDLs), particularly those that are smaller and denser and therefore more pro-atherogenic, penetrate the vessel wall and remain trapped in the sub-intimal space, where they undergo oxidative changes. Oxidized LDLs induce recruitment of monocytes by vascular cells and promote their differentiation into macrophages^[2]. The latter internalize oxidized LDLs and become foam cells^[3], the distinctive feature of the atherosclerotic lesions.

Atherosclerosis manifests itself clinically either when the arterial vessel stenosis prevents the increase of blood flow and oxygen supply during augmented demand (e.g., exercise or digestion) causing the onset of pain (angina pectoris, abdominis or claudication intermittens, depending on the segments involved), or when an unstable plaque undergoes hemorrhage and/or rupture with superimposed thrombosis.

Several studies showed that athero-thrombosis, which is responsible for acute ischemic events, does not correlate strictly with the degree of atherosclerotic plaque narrowing^[4,5], but rather with the plaque features, and, more specifically, with the extent of inflammation, thinning of the fibrous cap, and expression of inflammatory cytokines and metalloproteinases that degrade the fibrous cap^[6,7]. This explains why the atherosclerotic disease might manifest clinically with acute catastrophic events even in patients with apparently mild lesions.

Identification of circulating markers that can be useful to improve the prediction of cardiovascular events is akin the current frontiers of Cardiology. C-reactive protein and cholesterol levels, despite being among the most studied biomarkers^[8,9], bear a rather small predictive value: for example, in the Framingham

Heart Study most of the patients who developed ischemic heart disease during twenty-six years follow-up had “normal” total cholesterol levels comparable to those not developing any cardiovascular disease^[10].

In the Get with the Guidelines study database^[11], which included 231896 patients admitted to 541 United States hospitals with a diagnosis of acute coronary syndrome, 136905 (59%) subjects had the lipid levels determined at admission and 21.1% of them were treated with cholesterol-lowering drugs. The average lipid profile was: LDL cholesterol 104.9 mg/dL (2.17 mmol/L), high-density lipoprotein (HDL) cholesterol 39.7 mg/dL (1.03 mmol/L), and triglycerides 161 mg/dL (1.82 mmol/L). According to this study about half of the patients admitted to the hospital with an acute coronary syndrome had LDL cholesterol levels in the normal range (Figure 1)^[11]. These data provide compelling evidence for the urgent need to perform clinical and laboratory research to identify new biomarkers of imminent plaque destabilization. Along this line, encouraging results were provided by studies investigating the lipoprotein-associated phospholipase A2 (Lp-PLA2), a member of phospholipase A2 proteins family that plays a crucial role in the metabolism of pro-inflammatory phospholipids, such as oxidized LDLs, and in the generation of pro-atherogenic metabolites, such as lysophosphatidylcholine and oxidized free fatty acids (Figure 2).

ROLE OF LP-PLA2 IN ATHEROSCLEROSIS

Lp-PLA2 is a calcium-independent lipase mainly produced by monocytes and macrophages^[12], which catalyze the hydrolysis of the sn-2 acyl chain of the phospholipid substrate^[13] on the surface of LDLs^[14], releasing lysophosphatidylcholine and oxidized fatty acids. The latter are well-established triggers of the inflammatory cascade^[14-16], via stimulation of endothelial cells expression of adhesion molecules and cytokines, induction of chemotaxis of monocytes and leucocytes, and promotion of their entry in the sub-intimal space of the arterial walls.

The accumulation of lysophosphatidylcholine and oxidized fatty acids in the sub-intimal space contributes to the development of the plaque lipid “core”. Moreover, these substrates once taken up by macrophages promote their conversion into foam cells^[17]. In addition, lysophosphatidylcholine induces the production of reactive oxygen species, such as superoxide, by activating the endothelial nicotinamide adenine dinucleotide phosphate oxidase and by inducing the endothelial nitric oxide synthase (eNOS) “uncoupling”^[18,19]. Through the latter mechanism the enzyme becomes a superoxide and peroxynitrite producer, thus contributing to atherogenesis and plaque destabilization, as corroborated by the increased cardiovascular mortality found in coronary artery disease patients carrying an eNOS gene polymorphism that implies enhanced eNOS

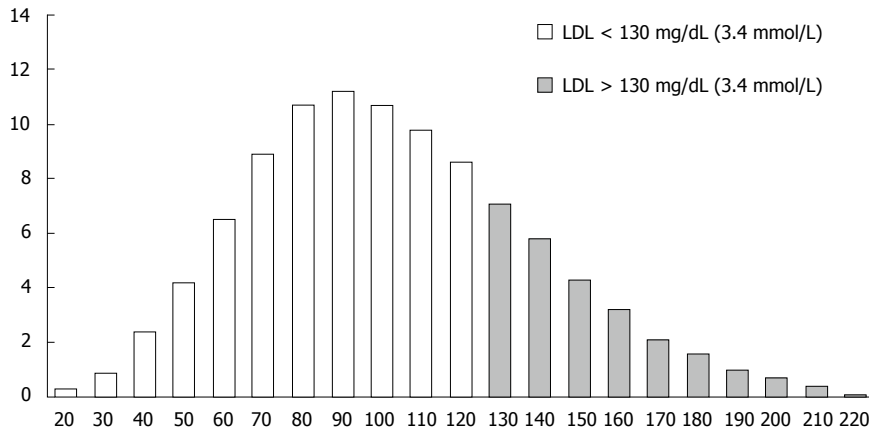
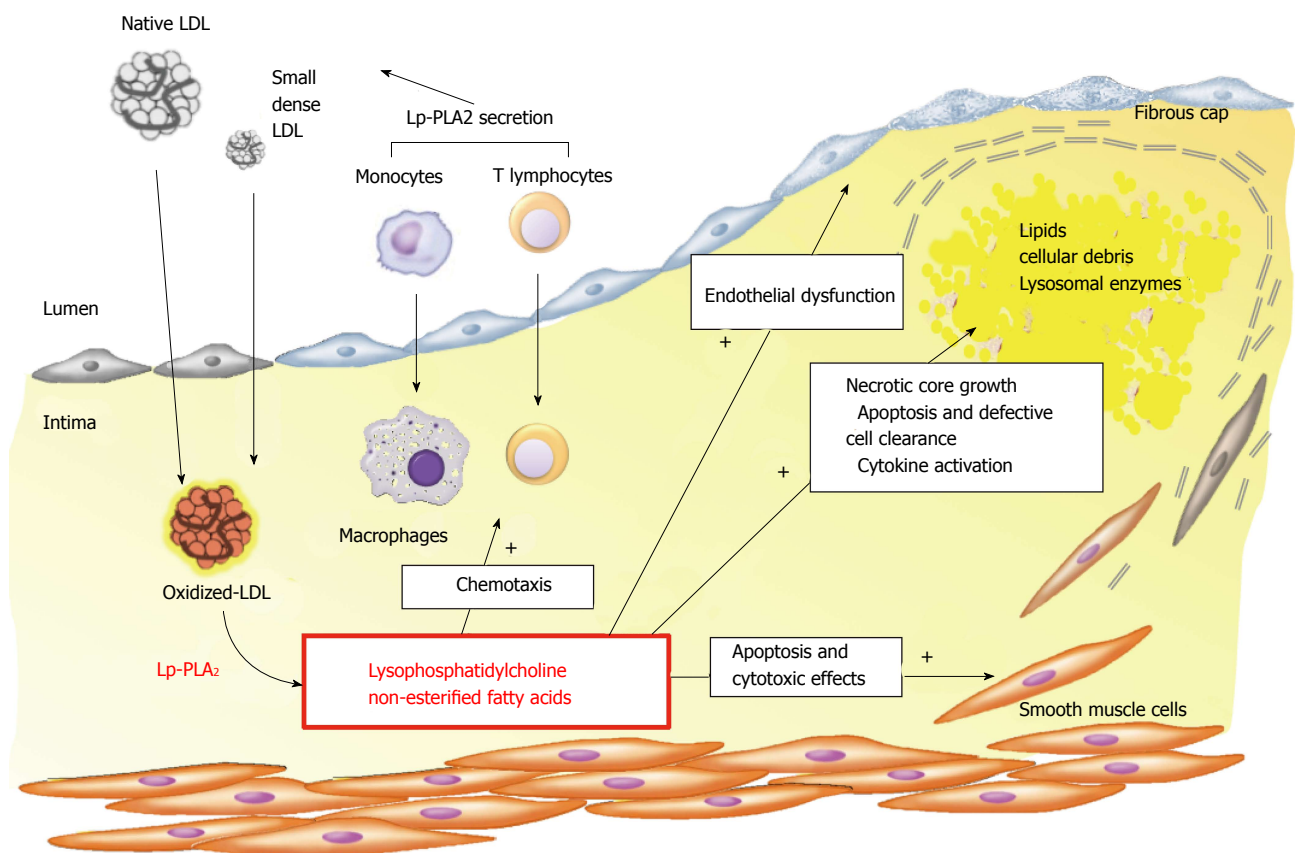


Figure 1 Low-density lipoprotein cholesterol levels on admission in patients with acute coronary syndrome^[74]. LDL: Low-density lipoprotein.



Modified from Steen DL and O'Donoghue ML, *Cardiol Ther* 2013.

Figure 2 Pathogenic role of lipoprotein-associated phospholipase A2 in atherosclerosis development. LDL: Low-density lipoprotein; Lp-PLA2: Lipoprotein-associated phospholipase A2.

expression and reactive oxygen species generation^[20].

In summary, experimental evidences indicate that due to its pro-inflammatory and pro-oxidative effects Lp-PLA2 plays a key role in the pathogenesis of atherosclerosis.

LP-PLA2 SECRETION AND CIRCULATION IN THE BLOOD-STREAM

Hematopoietic (monocytes, macrophages, lymphocytes, mastocytes, and platelets) and hepatic cells (Kupffer's cells)

produce Lp-PLA2; however, its synthesis and release in the circulation occur with monocytes maturation into macrophages^[21] alongside activation by inflammatory mediators^[22].

In the bloodstream Lp-PLA2 circulates by two-thirds bound to the LDLs and by one third to HDLs^[23,24]. It is, however, worth highlighting that with normal LDL-levels the total plasma Lp-PLA2 activity associated with HDL accounts for only 4.9% of the total enzyme activity^[25].

Plasma ultracentrifugation leads to partial separation

of Lp-PLA2 from the lipoproteins, thus indicating that there is a dissociable and a non-dissociable form of the enzyme^[26]. The transition between them might be one of the mechanisms regulating the activity of Lp-PLA2 *in vivo*. The association with HDL and LDL is controlled by post-translational chemical modifications: Glycosylation of specific residues decreases the association of Lp-PLA2 and lipoproteins, even though these changes do not seem to influence the enzyme secretion by the cells^[27].

As regards the relationship of Lp-PLA2 with apolipoproteins, B100 plays a key role in the association of Lp-PLA2 with LDLs, especially its carboxyl terminus, which interacts with the Lp-PLA2 residues Tyr-205, Trp-115, and Leu-116 and, to lesser extent, with the Met-117^[28]. In spite of the fact that Lp-PLA2 preferentially associates with the most dense and electronegative LDLs fractions, even among the latter only 1% of the particles contain Lp-PLA2^[29-31]. As mentioned, only one third of Lp-PLA₂ circulates in plasma with HDLs. Multiple amino-acid residues, as well as the carbohydrate content of the enzyme, appear to play a crucial role for its association with HDL apolipoprotein A-I^[27,32].

Finally, when plasma lipoprotein(a) concentrations are ≥ 30 mg/dL detectable amounts of Lp-PLA2 are associated with this lipoprotein. A major role for its attachment is played by apolipoprotein B-100^[33].

PLASMA LP-PLA2 DETERMINATION

Originally specific tests were developed to determine the Lp-PLA2 plasma concentration (mass) and enzymatic activity. The plasma mass assays were thereafter abandoned due to lack of significant advantages and lower accuracy in patients' risk stratification than enzymatic activity assays. The assessment of Lp-PLA2 activity exploits enzymatic substrates, such as 2 Tio-PAF, whose degradation releases free thiol groups, which are detectable by spectrophotometric reading.

GENETIC DETERMINANTS OF LP-PLA2 ACTIVITY

The prognostic relevance of Lp-PLA2 measurement raised the question whether the enzyme levels and activity are genetically determined ("nature") or influenced by environmental factors ("nurture"). According to one study and a recent meta-analysis Caucasians carry higher Lp-PLA2 activity levels than Hispanics and African-Americans, suggesting that Lp-PLA2 is genetically influenced^[34,35]. Moreover, Lp-PLA2 activity was reported to be 10% lower in females compared to males, possibly due to higher estrogen levels in the former, which down-regulate Lp-PLA2 activity and decrease LDL-cholesterol^[34,35]. A conclusive demonstration of heritability was provided by twins' studies. In fact, genetically identical monozygotic twins showed differences in their plasma levels of Lp-PLA2 much smaller than dizygotic twins, who share only

half of their genes, thus showing that about 62% of the variance of Lp-PLA2 activity levels is under genetic control^[36].

The *Lp-PLA2* gene (*PLA2G7*) is located in chromosome 6p21.2 to 12 and entails 12 exons. Its cDNA was cloned in 1995^[37] and comprises an open reading frame codifying a precursor of 441 amino acids that is cleaved into a 45.4 kDa mature protein^[38]. The *PLA2G7* gene is characterized by non-synonymous polymorphisms that could cause reduction or loss of the enzymatic activity.

The first evidence of the functional relevance of these mutations dates back to the identification of five Japanese families with absent circulating Lp-PLA2, an autosomal recessive trait^[39] linked to a Val279Phe polymorphism on the exon 9^[40]. This variant causes the absence of Lp-PLA2 enzymatic activity because of an amino acid change in proximity of Ser-273 and Asp-296 that is responsible of folding and, thus, functioning of the mature protein.

The Val279Phe polymorphism was associated to atherosclerosis^[41,42], stroke^[43], and dilated cardiomyopathy^[44]. However, these early evidences, which have been produced when Lp-PLA2 was believed to be anti-atherogenic, were not confirmed by subsequent studies that, in fact, showed just opposite results^[45]. Thus, it remains unclear whether the lack of Lp-PLA2 activity is pro- or anti-atherogenic and if carriers of this genetic variant, who are exclusively Asian, could have inherited compensatory mechanisms that change unpredictably the final clinical phenotype.

Other polymorphisms were thereafter identified in Caucasians^[46,47]: Arg92His (exon 4), Ile198Thr (exon 7), Ala379Val (exon 11). In particular, the Ile198Thr variation is located near the Tyr205 residue, a binding site for LDLs, in a position that might decrease the affinity for the substrate, thus explaining the observed reduction of enzymatic activity^[46]. Another polymorphism, the Ala379Val, is located near the residue belonging to the catalytic triad of lipase (His-351), suggesting that it could influence the enzymatic activity^[48].

Ala379Val and Arg92His variants have been associated with coronary artery disease (CAD)^[49], but only the former correlated with the severity of atherosclerosis in a Taiwanese population^[50] and to acute myocardial infarction in two case-control studies^[49,50]. In other studies this association was neither confirmed^[51] nor denied^[52,53]. Two recent meta-analyses^[54,55], which included more than 10000 patients of European ancestry, failed to demonstrate any association between the *PLA2G7* gene polymorphisms and CAD risk. These studies, as well as the meta-analyses that included them, were biased and affected by confounding factors, in that: (1) only a minority of studies used a prospective cohort study design, which is more reliable compared to case-control studies; and (2) the adjustment for potential confounders by multivariate analysis was not consistently performed. Therefore, likely their results

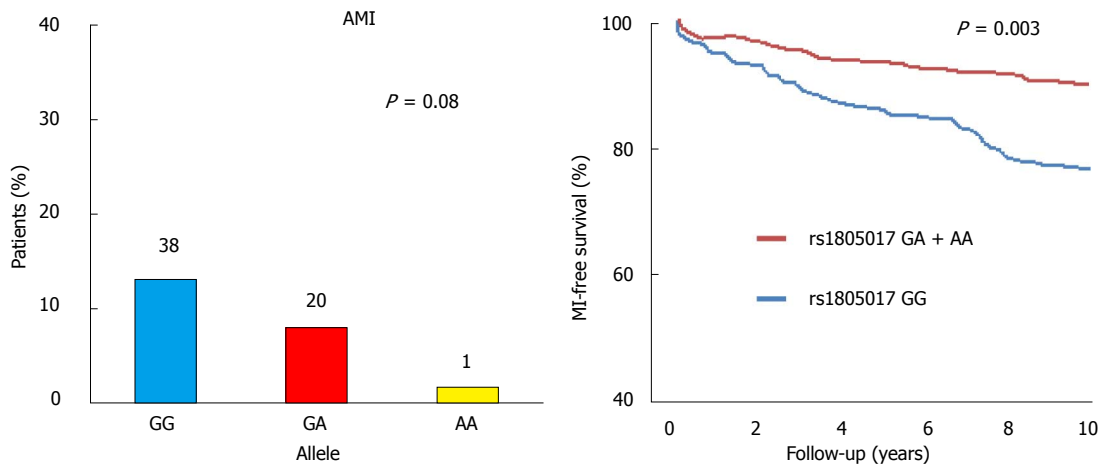


Figure 3 Increased number of acute myocardial infarction depending on the variant gene Arg92His. A: The patients with variant gene GG (Arg92) have a greater number of infarcts compared to the other two variants; B: The Kaplan-Meier curve shows a lower survival free from acute myocardial infarction in patients with variant GG (Arg92). AMI: Acute myocardial infarction.

could not be considered conclusive even despite the large number of patients analyzed.

A recently published prospective cohort study performed with an appropriate prospective cohort design and an utmost care to the role of potential confounders, showed that Arg92His is associated to both high levels of Lp-PLA2 activity, and a 1.75-fold increase of relative risk of acute myocardial infarction (Figure 3)^[56]. Hence, it would appear that genetic predisposition to high Lp-PLA2 activity translates into increased susceptibility to acute coronary events.

LP-PLA2 ACTIVITY AND CARDIOVASCULAR DISEASE

The first study showing an association between elevated Lp-PLA2 plasma levels and cardiovascular events was the West of Scotland Coronary Prevention Study (WOSCOPS)^[57]. Other studies thereafter confirmed Lp-PLA2 to be a predictor of cardiovascular events in different cohorts^[58-66], but the large Women Health Study^[67], which enrolled a healthy female population, found just an opposite association. In apparently healthy populations, three trials demonstrated the Lp-PLA2 prognostic role. In the ARIC study, which enrolled a large cohort of healthy subjects of both genders, those with low LDL cholesterol (< 130 mg/dL) and high Lp-PLA2 levels had an increased relative risk of ischemic heart disease [HR 2.08, 95% confidence interval (CI): 1.20-3.62] compared to those with low levels of Lp-PLA2^[68]. The JUPITER trial also showed that patients with high Lp-PLA2 activity (fourth quartile) had a more than two-fold increased relative risk (HR 2.15, 95%CI: 1.13-4.08) of developing cardiovascular events than those with low activity (first quartile)^[69]. Finally, the Bruneck study also reported that the population in the third tertile of Lp-PLA2 activity had a higher relative risk of incident cardiovascular events (HR 2.2, 95%CI:

1.1-4.8) compared to those in the first tertile^[70].

Lp-PLA2 activity might predict the occurrence of events also in patients at high cardiovascular risk: in the MDCS study, which enrolled healthy subjects, those with metabolic syndrome and high Lp-PLA2 activity had a 1.97 (95%CI: 1.34-2.90) relative risk of cardiovascular events^[71]. The combined analysis of two studies, HPFS and NHS, including patients with diabetes mellitus, showed that those with a high Lp-PLA2 activity had a 1.75 (95%CI: 1.05-2.92) relative risk of cardiovascular mortality and AMI^[72].

The ability of Lp-PLA2 to predict cardiovascular events was also confirmed in subjects with cardiovascular disease. The VA-HIT study, which included patients with ischemic heart disease, the increase of Lp-PLA2 activity levels was associated with a higher relative risk of cardiovascular events (HR 1.17, 95%CI: 1.04-1.32) and death (HR 1.23, 95%CI: 1.01-1.50)^[73]. Similar results were obtained in the LIPID trial that entailed subjects with history of acute coronary syndrome in whom Lp-PLA2 activity was associated to a higher risk of cardiovascular mortality (HR 1.32, 95%CI: 1.00-1.75)^[74]. Another study that included 1051 patients affected by CAD showed that Lp-PLA2 activity predicted the risk of cardiovascular events (HR 2.40, 95%CI: 1.35-4.29)^[63]. Finally, in a large cohort of subjects with CAD of the GENICA Study, we demonstrated that a high Lp-PLA2 activity level predicted both cardiovascular mortality (HR 1.01, 95%CI: 1.00-1.02) and acute myocardial infarction (HR 1.01, 95%CI: 1.00-1.02) (Figure 4)^[75].

Circulating Lp-PLA2 activity levels could be an index of systemic inflammation as suggested by the finding of a direct link between Lp-PLA2 enzymatic activity and activation of lympho-monocytic cells^[76]. These data were confirmed by studies on CAD patients (Rotterdam Study and Ludwigshafen Risk and Cardiovascular Health Study) that demonstrated an association

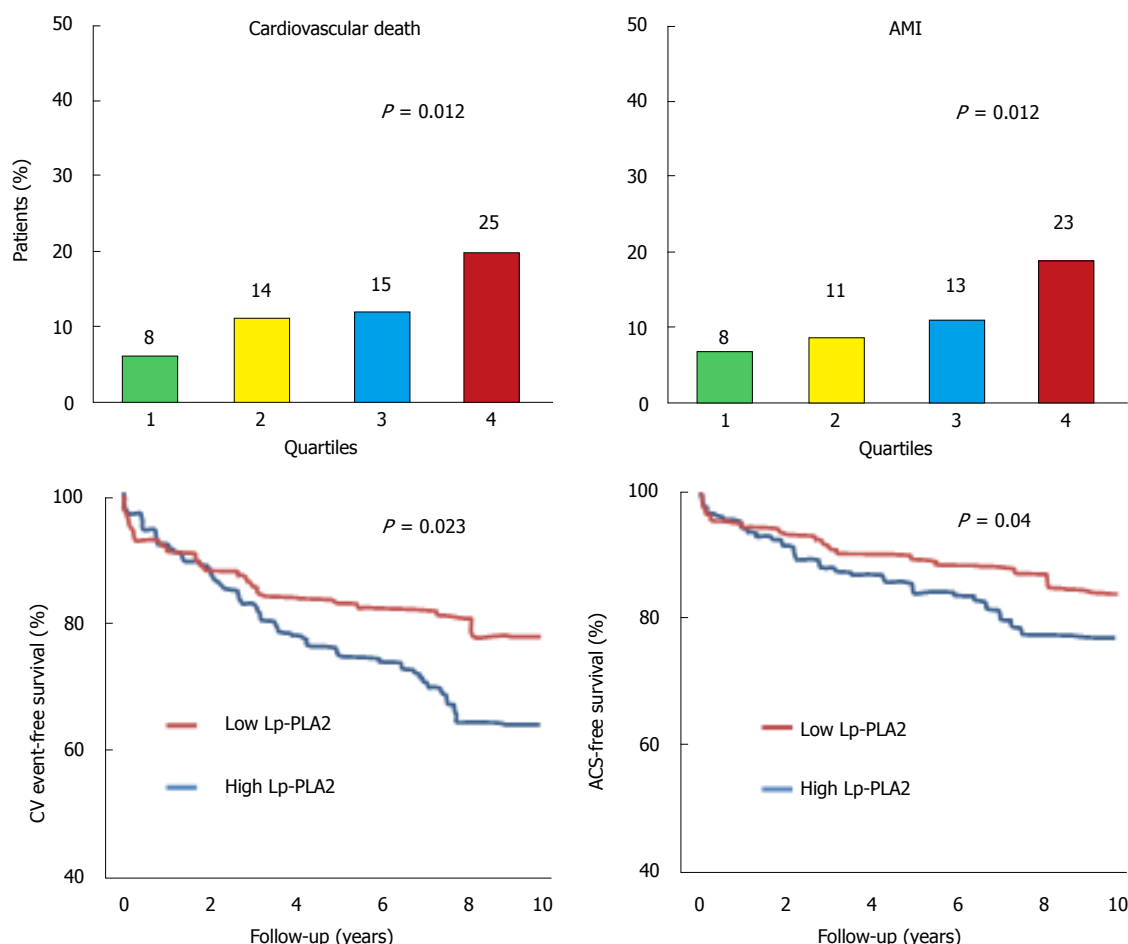


Figure 4 The Kaplan-Meier curves underline a greater survival free from cardiovascular events (death, acute myocardial infarction) in patients with lower lipoprotein-associated phospholipase A2 activity. AMI: Acute myocardial infarction; ACS: Acute coronary syndrome; CV: Cardiovascular.

between Lp-PLA2 enzymatic activity and CAD risk^[77,78].

A meta-analysis that included all prospective studies conducted on Lp-PLA2 including a total of 79036 patients showed a relationship between Lp-PLA2 activity and mass and incidence of CAD, stroke, and cardiovascular mortality^[79].

LP-PLA2 AND GUIDELINES

Based on these evidences, the guidelines of four major international societies, including the European Society of Cardiology, the American College of Cardiology, the American Heart Association, and the American Society of Endocrinology, included the Lp-PLA2 activity measurement among the biomarkers that are useful for risk stratification of asymptomatic adult patients. The use of this marker is particularly advantageous in patients at moderate cardiovascular risk (> 2 risk factors) and in those at high-risk in whom an increase of Lp-PLA2 activity levels should guide the lipid-lowering treatment to reach a target LDL-cholesterol lower than, respectively, 130 mg/dL (< 3.3 mmol/L) or 100 mg/dL (< 2.5 mmol/L) in primary prevention^[80] (Figure 5).

THERAPEUTIC STRATEGY TO REDUCE LP-PLA2 LEVELS

As the majority of plasma Lp-PLA2 is linked to LDLs, a therapeutic strategy aimed at decreasing LDL cholesterol levels could be expected to reduce Lp-PLA2 activity. Accordingly, several cholesterol-lowering treatments, such as statins^[66,74,81,82], fibrates^[81,83], ezetimibe^[81], and omega-3 fatty acids^[84], were found to reduce plasma Lp-PLA2 activity. However, it remained unclear whether the reduction of Lp-PLA2 activity with a lipid-lowering treatment translates into a lower mortality and cardiovascular event rate, and if these benefits could be explained by the reduction of plasma lipids, of Lp-PLA2 activity levels, or of both. This hypothesis has been tested in the LIPID (Long-term Intervention with Pravastatin in Ischemic Disease) study, a double blind multicenter trial that randomized to placebo or pravastatin 9014 patients with CAD^[74]. The levels of many biomarkers, such as cholesterol fractions and Lp-PLA2 activity were determined at "baseline" and after one year of treatment. The study showed that after one-year follow-up, the statins group

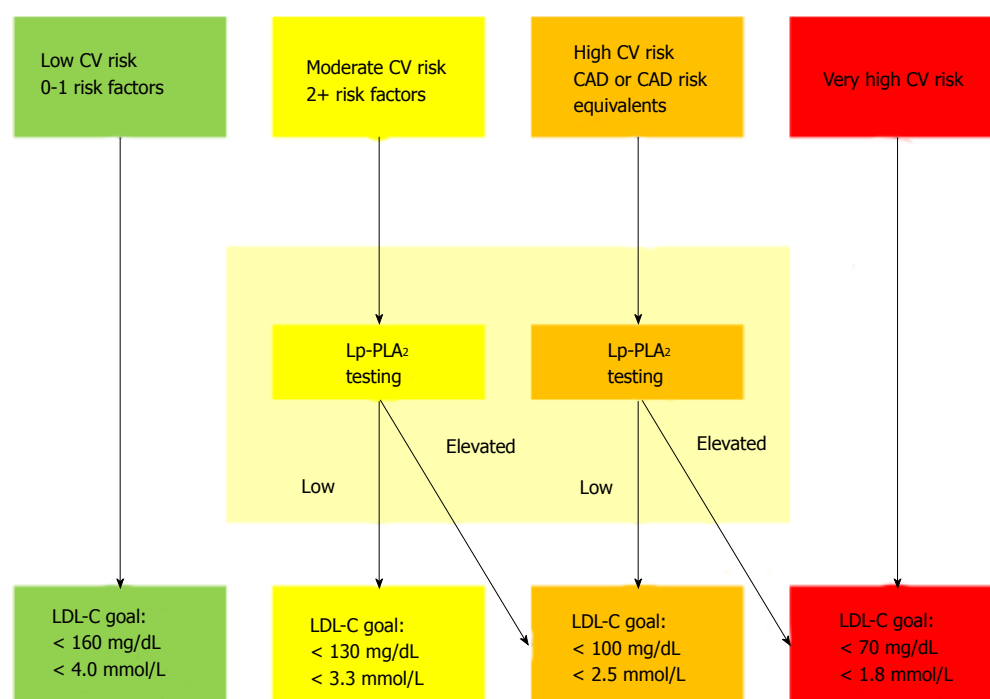


Figure 5 Relevance of measuring of lipoprotein-associated phospholipase A2 activity for risk stratification in adult patients with moderate cardiovascular risk (≥ 2 risk factors) or higher^[80]. Lp-PLA2: Lipoprotein-associated phospholipase A2; LDL: Low-density lipoprotein; CAD: Coronary artery disease; CV: Cardiovascular.

had a reduction Lp-PLA2 activity (50 nmol/min per milliliter, $P < 0.001$) compared to both baseline values and to the placebo group. Similarly to previous studies, the “baseline” values of Lp-PLA2 activity predicted the risk of cardiovascular events, including CAD mortality and acute myocardial infarction, and total mortality; after adjustment at multivariate analysis, the baseline values of Lp-PLA2 activity predicted only CAD mortality. The key finding was that after one year of treatment low Lp-PLA2 levels predicted less major CAD events (HR 0.65, 95%CI: 0.50-0.86, $P = 0.002$), less major cardiovascular events (cardiovascular death, less non fatal acute myocardial infarction or stroke, HR 0.70, 95%CI: 0.55-0.89, $P = 0.003$), and less cumulative cardiovascular events (major cardiovascular events, unstable angina, revascularization, HR 0.70, 95%CI: 0.59-0.83; $P < 0.001$), comparing the first with the fourth quartile of Lp-PLA2 levels. These prognostic value persisted unaltered after adjustment for twenty-three risk factors at enrolling, which led the authors to conclude that the reduction of Lp-PLA2 during treatment with statin was as predictive, or even more predictive, than the decrease of LDL cholesterol^[74]: about 59% of the beneficial effects of pravastatin were explained by a decrease of Lp-PLA2 values. This study could not, however, verify whether the reduction of circulating Lp-PLA2 was associated with a decrease of the enzyme activity into the atherosclerotic plaque, a data that, if confirmed, could explain the observed decrease of events.

RANDOMIZED CONTROLLED CLINICAL TRIALS TESTING THE EFFICACY OF Lp-PLA2 INHIBITORS

This piece of information was, however, obtained, in diabetic and dyslipidemic pigs: darapladib, a Lp-PLA2 inhibitor, reduced the lysophosphatidylcholine levels in coronary artery plaques and decreased macrophage infiltration and necrotic “core” in the plaques^[85]. Moreover, in the IBIS-2 study in humans, darapladib decreased the Lp-PLA2 activity and the necrotic “core” in coronary plaques^[85]. Two randomized trials were conducted to test whether pharmacological inhibition of Lp-PLA2 with darapladib reduces cardiovascular events in stable and unstable CAD and were recently published, the STABILITY^[86] and the SOLID-TIMI 52^[87].

The STABILITY trial randomized 15828 patients with stable CAD to receive darapladib or placebo for a median period of 3.7 years with a composite primary end-point of cardiovascular death, myocardial infarction, or stroke^[86]. The trial fell short of proving its primary end-point and could not demonstrate any beneficial effect of darapladib on each individual component of the primary end-point, or on the overall mortality. However, it demonstrated a beneficial effect of Lp-PLA2 inhibition in that darapladib reduced the rate of major coronary events and total coronary events.

The SOLID-TIMI 52 trial enrolled 13026 patients with an acute coronary syndrome in the last 30 d

before randomization to darapladib or placebo for a median period of 2.5 years with a composite primary end-point of cardiovascular death, myocardial infarction or urgent coronary artery revascularization^[87]. The results did not demonstrate any beneficial effect of darapladib on any of the either primary or secondary endpoints.

These disappointing results might seem to challenge the pathogenic role of Lp-PLA2 in atherosclerosis and plaque destabilization. However, considering the wealth of data demonstrating that Lp-PLA2 predicts cardiovascular events, one could argue that the plasma activity of Lp-PLA2 is only a prognostic marker and does not play a causative role. Another possibility is that the trials, due to the high rate of drug discontinuation (20% in the STABILITY trial and 17% in the SOLID TIMI 52), ultimately lacked the statistical power to challenge the hypothesis that darapladib is efficacious in reducing cardiovascular events. If this was the case the possibility that another, possibly better tolerated, antagonist could improve outcomes needs to be tested. Furthermore, the selection criteria did not include a threshold Lp-PLA2 level, whereas it is well known that the risk of cardiovascular events is dependent on the Lp-PLA2 levels. Moreover, the drug adherence assessment used, *e.g.*, pill count, is well known to underestimate the true therapeutic compliance^[88]. The absence of any report of the Lp-PLA2 levels reached after darapladib administration, testing the therapy efficacy and the patients' compliance, is a missing crucial piece of information in these trials.

At present, from the available data it is possible to speculate that darapladib is not an efficacious therapy. Considering the extensive proof of the Lp-PLA2 prognostic value and the crucial information missing in the completed trials, it is possible to hypothesize that Lp-PLA2 is a marker of disease and does not have a pathogenic role, or that another more efficacious way to inhibit Lp-PLA2 activity by means of new drugs should be investigated.

CONCLUSION

In summary, compelling evidence indicate that high Lp-PLA2 activity levels predict an increased risk of cardiovascular events in the general population, as well as in patients with metabolic syndrome, diabetes, and coronary heart disease^[63,68-75]. Many cholesterol-lowering medications besides decreasing LDL-cholesterol lower circulating Lp-PLA2 levels. Moreover, the Lp-PLA2 levels achieved with pravastatin treatment is a marker of cardiovascular risk and coronary events, even better than the LDL cholesterol level^[74]. The available evidences support the usefulness of the measurement of plasma Lp-PLA2 activity in the clinical practice to stratify the cardiovascular risk, especially in patients at intermediate or high risk. In these subjects Lp-PLA2 activity levels should prompt the physician to pursue

two aims: (1) a more aggressive LDL-cholesterol treatment; and (2) the normalization of Lp-PLA2 levels (Figure 5). For this reason, the scientific societies guidelines introduced the measurement of Lp-PLA2 as a marker of risk in these categories of patients.

The role of Lp-PLA2 as a therapeutic target has been disproven by two large randomized clinical trials thus far. However, due to their intrinsic limitations, it remains unclear if these results depended on the Lp-PLA2 being only a marker of cardiovascular events devoid of a pathogenic role, or on the lack of efficacy of the drug tested in these trials. Further studies are needed to resolve this dilemma.

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Cardioprotection by remote ischemic conditioning: Mechanisms and clinical evidences

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Abstract

In remote ischemic conditioning (RIC), several cycles of ischemia and reperfusion render distant organ and

tissues more resistant to the ischemia-reperfusion injury. The intermittent ischemia can be applied before the ischemic insult in the target site (remote ischemic preconditioning), during the ischemic insult (remote ischemic perconditioning) or at the onset of reperfusion (remote ischemic postconditioning). The mechanisms of RIC have not been completely defined yet; however, these mechanisms must be represented by the release of humoral mediators and/or the activation of a neural reflex. RIC has been discovered in the heart, and has been arising great enthusiasm in the cardiovascular field. Its efficacy has been evaluated in many clinical trials, which provided controversial results. Our incomplete comprehension of the mechanisms underlying the RIC could be impairing the design of clinical trials and the interpretation of their results. In the present review we summarize current knowledge about RIC pathophysiology and the data about its cardioprotective efficacy.

Key words: Remote ischemic conditioning; Ischemic heart disease; Percutaneous coronary intervention; Cardiac surgery; Atherosclerosis

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Core tip: Remote ischemic conditioning (RIC) is a safe, non-invasive, and inexpensive technique that has the potential to protect the heart against the ischemia-reperfusion injury. Its cardioprotective efficacy is currently being evaluated, and diverging results are emerging. It is thus worth resuming current understanding of RIC pathophysiology and clinical efficacy.

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INTRODUCTION

The myocardium can tolerate brief periods (up to 15 min) of severe and even total ischemia. Such ischemic episodes occur in the settings of angina, coronary vasospasm, and balloon angioplasty, and are not associated with concomitant myocyte cell death. With increasing duration and severity of ischemia, greater myocardial damage, and the predisposition to further damage during reperfusion develop. The combined deleterious effects of coronary occlusion and revascularization configure the "ischemia-reperfusion (IR) injury"^[1].

The counterintuitive idea to apply several brief episodes of IR cycles to protect the myocardium against IR injury was firstly advanced in 1986, when Murry *et al*^[2] reported that the infarcted area following a 40-min coronary occlusion was reduced if preceded by four 5-min IR cycles. This phenomenon was called "ischemic preconditioning". Its clinical application is hindered by the unpredictable timing of acute myocardial infarction (AMI), and by the necessity to intervene on coronary vessels^[3]. However, several IR cycles were found to confer cardioprotection even when applied at the onset of coronary revascularization (ischemic postconditioning)^[4,5], both in animals and in human patients undergoing primary percutaneous coronary intervention (PCI).

In 1993, it was demonstrated in anesthetized dogs that 4 episodes of 5-min ischemia and reperfusion in the left circumflex coronary territory, followed by a 1-h occlusion of the left anterior descending coronary artery, significantly reduced the infarct size^[6]. The "remote ischemic conditioning (RIC)" paradigm has been progressively extended^[7]. At present, RIC is defined as the phenomenon by which brief episodes of ischemia and reperfusion in one vascular bed, tissue, or organ render distant sites resistant to the ischemia-reperfusion injury^[7,8]. The IR cycles are effective when applied before myocardial ischemia (remote ischemic preconditioning), during coronary occlusion (remote ischemic perconditioning), and during cardiac revascularization (remote ischemic postconditioning)^[7-12].

The mechanisms conferring protection at distance have not been completely defined^[7], yet their characterization would be relevant to achieve a full comprehension of the phenomenon, and to exploit its full potential in clinical practice. In fact, understanding whether humoral mediators, neural mechanisms, or their combination mediate remote ischemic conditioning would be crucial to determine the optimal number of IR cycles, the better site and timing of their application, to select the patients according to age, comorbidities, and medical treatment, and to optimize the overall therapeutic management of the patient.

In the first part of the present review, we analyze current knowledge of the mechanisms underlying RIC, comparing humoral and reflex-mediated mechanisms.

In the second half of this work, we attempt a critical analysis of the available literature concerning the cardioprotective potential of RIC in different settings.

THE "HUMORAL HYPOTHESIS" OF RIC, AND POTENTIAL CIRCULATING MEDIATORS

The "humoral hypothesis" has been formulated in the setting of remote ischemic preconditioning (RIPC). It postulates that the IR cycles in a distant site cause the local accumulation of mediators which are then released into the bloodstream and finally reach the heart^[7] (Figure 1).

Several data from animal models support this hypothesis. In particular, it has been demonstrated that the effluent from preconditioned hearts could transfer the protection to naïve recipients^[13-15]; this protection seems to be mediated by small hydrophobic proteins whose molecular weight ranges between 3.5 and 15-30 kDa^[16].

Since these humoral mediators must be effective in remote sites after dilution into the bloodstream, their release from the peripheral tissue has to be massive^[16]. The identification of humoral mediators should therefore be relatively easy to perform in animals or humans undergoing a RIPC protocol^[16]. Indeed, proteomic approaches have been attempted in both animals and humans, still they have yielded controversial results^[17-19].

Among the proteins potentially involved, there are kallistatin, apolipoprotein A-I, and stromal-derived factor 1 α (SDF-1 α)^[16].

Kallistatin is a serine protease which reduces inflammation, apoptosis, and oxidative stress in endothelial cells^[20]. It has been recently characterized as a protective factor against renal ischemia-reperfusion injury in mice^[21], and has been found to be increased in the plasma of healthy humans undergoing a RIPC protocol^[16]. However, its role as a humoral mediator of RIPC has not been properly evaluated yet.

Apolipoprotein A-I has anti-inflammatory properties, which could prove useful in the protection against ischemia-reperfusion injury^[18]. In humans, its circulating levels have been found to be either increased^[18] or decreased^[17,22] after a RIPC protocol; therefore, its exact role is still debated.

Finally, SDF-1 α has been proposed to be an important, and possibly the main, mediator of RIPC^[23]. In a study on rats, a 50% plasma increment was detected in rats subjected to a RIPC protocol compared to control animals (890 ± 70 pg/mL vs 590 ± 50 pg/mL; $n = 8$, $P < 0.01$)^[23]. Nevertheless, the administration of a selective inhibitor of SDF-1 α did not completely abrogate the reduction in infarct size following RIPC^[24], suggesting the existence of other mechanisms of cardioprotection^[24].

Several potential other mediators have been identified: among them, there are microRNAs (miRNAs),

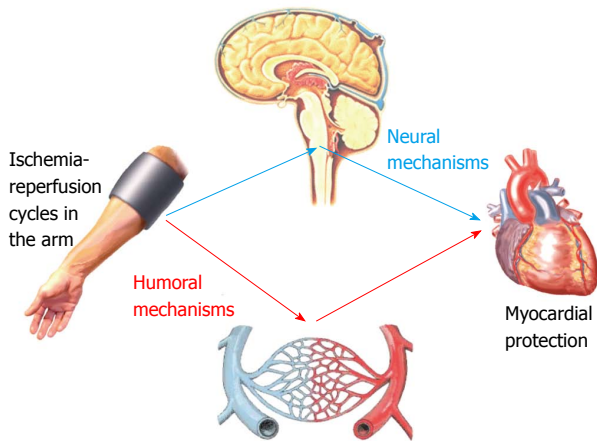


Figure 1 Mechanisms of remote ischemic conditioning. The cardioprotective effects of several ischemia/reperfusion cycles applied in a distant site (most commonly the upper limb) have been ascribed to the activation of humoral and/or neural pathways. The pathogenesis of remote ischemic conditioning is incompletely known, however it is possible that both humoral and neural mechanisms underlie this response.

bradykinin, adenosine, and nitric oxide (NO).

miRNAs have been involved in both muscle ischemia^[25] and protection against myocardial IR injury^[26]. The circulating levels of miR-144 have been found to increase by 1.6 folds in healthy human subjects undergoing a RIPC protocol, even though the exact mechanism of its action is still unknown^[27].

Bradykinin is released by damaged tissues and can activate afferent fibers (see below), possibly contributing to a cardioprotective effect known as "remote preconditioning of trauma"^[28]. A release of bradykinin from ischemic tissues into the bloodstream has been reported^[29]; the involvement of bradykinin in the RIPC response has been postulated, but the data from an animal study were inconclusive^[30].

Finally, both adenosine and NO have been extensively studied in the setting of ischemic preconditioning^[31], and have been considered potential mediators of RIPC as well^[7,32], although their extremely short half-life makes unlikely that they could exert a significant cardioprotective effect.

To summarize, it is quite established the existence of humoral mechanisms underlying RIPC, but the nature of the mediator(s) is currently unclear. Potential humoral mediators should be assessed with respect to their potential mechanism of action, the increase in their circulating levels following a conditioning protocol, and their half-life; in fact, all these parameters should be compatible with a cardioprotective role. Further studies are required to define the existence and the role of humoral mechanisms underlying RIPC, as well as the other forms of remote ischemic conditioning.

NEURALLY-MEDIATED CARDIOPROTECTION

Neural control of the heart

The autonomic nervous system consists of an afferent

pathway, integrating centers located into the central nervous system, and two efferent limbs, the sympathetic and the parasympathetic nervous systems^[33]. In the heart, sensory innervation is provided by afferent neurons located into the nodose and dorsal root ganglia, and projecting to brainstem areas controlling the activity of both sympathetic and parasympathetic nuclei^[33]. Sympathetic efferent fibers innervate the sinoatrial and atrioventricular nodes, the atria, and the ventricles^[33]. Parasympathetic efferent fibers are traditionally believed to control exclusively the nodal tissue and the atria^[33]. Nevertheless, the presence of cholinergic innervations have been detected in both ventricles, and it has been demonstrated that vagal activation decreases the force of ventricular contraction irrespective of its effect on heart rate, in both animals and humans^[33].

The "neural hypothesis" of RIPC, and potential role of the sympathetic system

The "neural hypothesis" of remote ischemic preconditioning (RIPC) postulates that ischemia-reperfusion (IR) cycles in peripheral sites might activate a neural reflex resulting in myocardial protection against a subsequent myocardial insult^[34] (Figure 1).

In animals, cycles of occlusion and reopening of the renal artery, mesenteric artery, or femoral artery resulted in significant cardioprotection; in all these cases, the resection of the afferent fibers projecting to the ischemized territories abolished the cardioprotective effect^[35-37]. In rats, IR cycles in the mesenteric artery conferred a cardioprotection similar in entity to that provided by ischemic preconditioning (*i.e.*, IR cycles of the coronary vessel before sustained occlusion); the systemic administration of hexametonium, a blocker of both sympathetic and parasympathetic ganglia, abolished this effect^[38].

Conflicting results have been provided by Kingma *et al.*^[39], who reported that, in dogs anesthetized with isoflurane, a RIPC protocol conferred robust myocardial protection against a subsequent ischemic injury even during autonomic blockade or surgical denervation of the heart. It should be noted that in this study the animals were anesthetized with isoflurane^[39], which is *per se* a powerful preconditioning agent (see below).

The activation of neural afferents during RIPC has been ascribed to local accumulation of mediators such as calcitonin-gene related peptide (CGRP), adenosine, and bradykinin^[40]. Interestingly, the accumulation of adenosine^[41,42], bradykinin^[43], and other mediators^[44,45] in the exercising muscle has been implied as a determinant of the metaboreflex^[46], which is a neural mechanism coupling sympathetic tone to exercise requirements^[47,48]. It could then be speculated that the IR cycles of a conditioning protocol cause metaboreflex activation. The subsequent increase in sympathetic outflow could confer myocardial protection through the activation of β_1 and/or β_2 adrenergic receptors; this phenomenon has been discovered in animal hearts perfused with β -agonists, and has been named

" β -adrenergic preconditioning"^[49-51].

Evidences of vagal activation following RIPC

As discussed above, a theoretical framework could be provided for sympathetic outflow as the final mediator of RIPC. Nevertheless, a growing body of evidences points to RIPC as a vagal reflex.

The possibility to precondition the heart by the infusion of acetylcholine (ACh) was demonstrated in 1993 by Yao *et al.*^[52] The preconditioning potential of ACh has been confirmed by several other studies^[53-55]. It has been demonstrated that cardioprotection by RIPC is suppressed by spinal cord section, bilateral vagotomy or the systemic administration of the muscarinic receptor antagonist atropine, while vagal stimulation closely recapitulates the effects of RIPC^[56,57].

Using a viral transfer gene approach in rats, Mastitskaya *et al.*^[58] confirmed that an intact parasympathetic outflow is crucial for myocardial protection by RIPC. The neurons in the dorsal motor nucleus of the vagus nerve were selectively silenced, thus abolishing the cardioprotective effect of a RIPC protocol^[58]. The selective activation of the same neurons closely recapitulated the cardioprotective effect of RIPC; this response was suppressed by atropine^[58]. Again in rats, Basalay *et al.*^[59] reported that IR cycles in the limb conferred cardioprotection when applied 25 min prior to myocardial ischemia. The authors then found that the cardioprotective effect was abolished by the denervation of the peripheral ischemic organ or bilateral vagotomy^[59].

Autonomic function in RIPC: What happens in humans?

To our knowledge, only two studies have evaluated the consequences of a RIPC protocol on the autonomic function in humans. In 2005, Loukogeorgakis *et al.*^[60] evaluated the possibility to protect against endothelial ischemia-reperfusion injury by RIPC. A cuff inflation to 200 mmHg for 20 min in the non-dominant arm was used as the ischemic insult; the subsequent endothelial damage was denoted by reduced flow-mediated dilation (FMD)^[60]. When arm ischemia was preceded by a RIPC protocol in the dominant arm, the ischemic insult in the other arm caused no significant reduction in FMD compared to baseline, suggesting endothelial protection by RIPC^[60]. Such response was abolished by the autonomic ganglion blocker trimetaphan^[60]. These results suggested an autonomic activation underlying the endothelial protection by RIPC; however, being trimetaphan an aspecific autonomic blocker, it was not possible to ascertain if either the sympathetic or the parasympathetic system accounted for the protection by RIPC^[60].

Parasympathetic activation was detected as the underlying mechanism by Enko *et al.*^[61] in 2011. After 3 cycles of 5 min ischemia and 5 min reperfusion in the left arm, a significant dilation of the right brachial artery was observed; in the power spectral analysis of heart rate, the high frequency domain

displayed a simultaneous increase, denoting increased parasympathetic outflow^[61].

Remote ischemic preconditioning and postconditioning: does vagal activation play a role?

The first demonstration of remote ischemic preconditioning was provided in 2007: in pigs, four 5-min cycles of lower limb ischemia during a 40-min left anterior descending coronary artery occlusion caused a significant reduction in infarct size, improved indexes of systolic and diastolic function, and less arrhythmic events during the reperfusion phase^[11]. With respect to remote ischemic postconditioning, a cardioprotective effect of IR cycles at the beginning of reperfusion was demonstrated for the first time in 2005^[12], and subsequently corroborated by other animal studies^[62,63].

To our knowledge, only Basalay *et al.*^[59] assessed the pathophysiology of remote ischemic preconditioning and postconditioning. These authors reported that deafferenting the site of IR cycles or cutting both vagus nerves abolished the preconditioning and postconditioning responses in rats, but did not alter the postconditioning effect^[59]. These results suggest that remote ischemic preconditioning relies on neural mechanisms, while remote ischemic postconditioning is mediated by humoral mediators^[59].

Further studies are required to assess this hypothesis. However, it should be noted that neural mechanisms are more qualified than humoral mechanisms to protect the ischemic myocardium in the setting of remote ischemic preconditioning, at least when the coronary flow is completely blocked. In the same setting, an activation of the parasympathetic system would probably be more effective than a sympathetic response.

Excessive concentrations of catecholamines have been detected in the ischemic area during an acute myocardial infarction^[64]. Increased cardiac sympathetic outflow is due to pain, anxiety, and a fall of cardiac output or arterial blood pressure; a further release of catecholamines is promoted by the ischemic damage of nerve endings^[64]. As a result, extracellular norepinephrine reaches up to 100-1000 times its normal plasma concentrations within 30 min of coronary occlusion^[64]. Far from being protective, local concentrations of this magnitude are capable of producing myocardial necrosis even in nonischemic myocardium, and might promote malignant arrhythmias^[64]. This mechanism accounts for the positive effects of early administration of β -blockers during acute myocardial infarction^[65,66], and probably excludes increased sympathetic outflow as the final mediator of cardioprotection by remote ischemic preconditioning.

PROTECTING THE HEART IN THE SETTING OF PCI

Stable coronary artery disease (SCAD) is associated with impaired quality of life, reduced physical endurance,

recurrent hospitalizations and outpatient visits^[67]. Revascularization by either elective PCI or CABG can relieve symptoms, reduce the use of anti-ischemic drugs, improve exercise capacity and quality of life, compared to medical therapy alone^[67]. The efficacy of elective PCI in addition to medical therapy in patients with SCAD has been demonstrated in a large number of randomized controlled trials, meta-analyses, and large-scale registries^[67].

Albeit elective PCI is becoming increasingly safe, balloon inflation during PCI often causes transient ischemia^[68]. Myocardial injury with necrosis may derive from recognizable peri-procedural events such as coronary dissection, occlusion of a major coronary artery or a side-branch, disruption of collateral flow, slow flow or no-reflow, distal embolization, and microvascular plugging; alternatively, the ischemic insult can have no detectable cause^[68]. Myocardial ischemia is attested by a rise and fall of cardiac biomarkers after the procedure, with values rising five or more folds over the 99th percentile being indicative of peri-procedural myocardial infarction (PMI)^[68].

Four recent meta-analyses have demonstrated that RIPC is effective in reducing PMIs in patients undergoing elective PCI^[69-72]. For example, in the meta-analysis by Zografos *et al.*^[71], PMI occurred in 40.3% of patients in the RIPC group and in 51.3% of patients in the control group (odds ratio 0.57).

Several trials have assessed the long-term outcomes after elective PCI. An improvement in prognosis was not found by Prasad *et al.*^[73] over 1 year follow-up. By contrast, in the Cardiac Remote Ischemic Preconditioning in Coronary Stenting study, a significant reduction of major adverse cardiac and cerebral events (MACCE; a composite of all-cause mortality, myocardial infarction, readmission for heart failure, and ischemic stroke or transient ischemic attack) was found at 6 mo^[74]. A recent follow-up study evaluating the same cohort demonstrated that the MACCE rate at 6 years remained lower in the RIPC group^[75].

The significant heterogeneity of the study protocols could be hindering a careful assessment of RIPC efficacy in the setting of elective PCI. For example, the studies assessed in the meta-analysis by Zografos *et al.*^[71] differed with regard to the RIPC procedure (number of IR cycles, duration of the IR periods, site of application of the IR cycles), the percentage of patients with multivessel disease, and the positivity or the negativity of cardiac troponin I (cTnI) before PCI^[71]. By contrast, in all the studies evaluated in this meta-analysis the IR cycles were performed immediately before elective PCI^[74-80], so that a different time span between the IR cycles and the angioplasty procedure cannot be regarded as a potential confounding factor.

On the whole, a protective role for RIPC in the setting of elective PCI is emerging, even though its efficacy seems to be lower than in primary PCI. Nevertheless, only one long-term follow-up study has

been published so far^[75]; the prognostic role of RIPC should therefore receive extensive evaluation, as well as the optimal RIPC protocol to achieve effective cardioprotection.

Remote ischemic preconditioning refers to the application of ischemia-reperfusion cycles in a distant site shortly before revascularization. The first evidences of a protective role of remote ischemic preconditioning in human patients was provided in 2010 by Bøtker *et al.*^[81], who assessed 333 patients with suspected first ST-elevation myocardial infarction. The primary endpoint was myocardial salvage index (MSI), quantified as the proportion of the area at risk preserved by the treatment, 30 d after primary PCI. MSI was significant higher in the conditioning group than in controls; the protective effect of remote ischemic preconditioning seemed to be strongest in patients with more severe infarctions, *i.e.*, presenting with occluded vessels or infarcts in the left anterior descending artery^[81].

The long-term outcome of remote ischemic preconditioning was assessed in the same study population^[82]. A significant reduction of MACCE and all cause mortality was observed in the conditioning group over a median follow-up of 3.8 years. There was also a trend toward reduced myocardial reinfarction, readmission for heart failure, and ischemic stroke or transient ischemic attack^[82].

In 2013, remote ischemic postconditioning was assessed on 232 patients undergoing elective PCI^[83]. In the conditioning group, the patients underwent three 5-min cycles of cuff inflation in the nondominant arm just after the end of the angioplasty. No significant difference was found between the conditioning group and the control group in terms of peak troponin I levels, PMI rate, recurrence of myocardial ischemia^[83]. In another study, the incidence of PMIs was similar between all groups, and no difference was remarked with respect to the creatine kinase (CK) levels or the incidence of acute kidney failure^[84]. In other studies, significant reductions in the incidence of acute kidney failure were observed; the prevention of acute kidney failure is currently regarded as the most promising perspective for the application of remote ischemic postconditioning during PCI^[85,86].

For the details of the studies cited in the present paragraph, see Table 1.

REMOTE ISCHEMIC PRECONDITIONING BEFORE ELECTIVE CARDIAC SURGERY

Elective coronary artery bypass surgery (CABG) stands as an alternative to elective PCI for the management of SCAD^[87]. The safety and efficacy of both techniques are similar, as well as the incidence of PMIs^[87].

In 2007, a randomized controlled study enrolled 57 adult patients undergoing elective CABG surgery^[88]. In the RIPC group, three IR cycles were performed

Table 1 Clinical studies on remote ischemic conditioning in percutaneous coronary intervention

Ref.	Patients <i>n</i> (CTRLS/RIPC)	ST or LT outcome	Conditioning protocol			Primary endpoint	Results		
			I/R cycles	Cuff pressure	Limb		RIPC	CTRLS	<i>P</i>
Remote ischemic preconditioning Prasad <i>et al</i> ^[73] , 2013	48/47	ST	3 × 3'	200 mmHg	Upper	Post-PCI myonecrosis (cTnT ≥ 0.03 ng/dL)	40%	47%	0.42
Ahmed <i>et al</i> ^[76] , 2013	72/77	ST	3 × 5'	200 mmHg	Upper	cTnT 16 h post-PCI	0.02 ng/mL	0.047 ng/mL	0.047
Ghaemian <i>et al</i> ^[77] , 2010	40/40	ST	2 × 5'	Above systolic	Lower	TnT 24 h post- PCI	12.50%	40%	0.01
Hoole <i>et al</i> ^[74] , 2009	117/125	ST	3 × 5'	200 mmHg	Upper	cTnI 24 h post- PCI	0.06 ng/mL	0.16 ng/mL	0.04
Luo <i>et al</i> ^[78] , 2013	104/101	ST	3 × 5'	200 mmHg	Upper	hs-cTnI 16 h post-PCI	0.11 ng/mL	0.21 ng/mL	< 0.01
Xu <i>et al</i> ^[79] , 2014	98/102	ST	3 × 5'	200 mmHg	Upper	hs-cTnI 16 h post-PCI	0.29 ng/mL	0.38 ng/mL	0.256
Davies <i>et al</i> ^[75] , 2013	117/125	LT	3 × 5'	200 mmHg	Upper	MACCE (6 yr follow up)	23	36	0.039
Bøtker <i>et al</i> ^[81] , 2010	167/166	ST	4 × 5'	200 mmHg	Upper	MSI after 30 d	0.75	0.55	0.033
Sloth <i>et al</i> ^[82] , 2014	167/166	LT	4 × 5'	200 mmHg	Upper	MACCE rates (5 yr follow- up)	25.60%	13.50%	0.018
Carrasco-Chinchilla <i>et al</i> ^[83] , 2013	114/118	ST	3 × 5'	200 mmHg	Upper	TnI 24 h post- PCI	0.476 ng/mL	0.478 ng/mL	0.378

cTnI: Cardiac troponin I; cTnT: Cardiac Troponin T; CTRLS: Controls; hs-cTnT: High sensitivity cardiac troponin T; I/R: Ischemia/reperfusion; MACCE: Major adverse cardiac and cerebral events; RIPC: Remote ischemic preconditioning; ST: Short term; LT: Long term.

after the induction of anesthesia, resulting in a 43% reduction in the 72 h area under the curve (AUC) of cTnT compared with the control group^[88]. Other randomized trials confirmed a cardioprotective role of RIPC, in terms of reduced cTnT^[89], cTnI^[90], and CK isoenzyme MB^[91] levels. By contrast, several studies failed to detect significant differences among the RIPC group and the control group^[92-95]; the use of volatile anesthetics with preconditioning potential (isoflurane, enflurane, sevoflurane) possibly accounts for discrepant results^[96-98].

In a meta-analysis, D'Ascenzo *et al*^[99] reported a significant reduction in cTnI and cTnT levels in the RIPC group after elective CABG surgery. Such difference persisted after excluding the trials with potentially confounding factors (among them, the use of isoflurane)^[99]. It has been suggested that the cardioprotective effect of RIPC could be masked by the administration of volatile anesthetics and blunted by the perioperative administration of β -blockers^[99,100]. Indeed, previous studies on animals or isolated human atrial trabeculae had demonstrated that β -blockers could attenuate ischemic preconditioning-induced cardioprotection^[100,101], perhaps since even the activation of β -adrenergic receptor is protective against ischemia-reperfusion injury (β -adrenergic preconditioning; see paragraph The "neural hypothesis" of RIPC, and potential role of the sympathetic system).

Two studies evaluating the long-term efficacy of RIPC provided diverging results. Lucchinetti *et al*^[94] did not find any difference at 6 mo in terms of deaths or revascularizations, whereas Thielmann *et al*^[102] found significantly lower mortality rates in the RIPC group than in controls over a mean 1.54 year follow-up.

With respect to the settings of elective valve replacement surgery and congenital cardiac surgery, a recent meta-analysis detected a significant cardioprotective role for RIPC^[103]. Nevertheless, the small number of studies, and the high heterogeneity among them^[103] might undermine the reliability of these conclusions. Another recent meta-analysis considered cumulatively CABG, valve replacement surgery, and congenital cardiac surgery, and detected a significant reduction in the post-operative cTnI levels among the patients undergoing RIPC^[104]. No subgroup analysis was performed, and the heterogeneity among studies assessing non-CABG surgery was marked^[104]. A third meta-analysis took into consideration the studies evaluating RIPC efficacy in adult patients undergoing "major elective or emergency cardiac or vascular surgery"^[105]. In such a broad and mixed setting, no significant efficacy of RIPC was detected with regard to several outcomes: perioperative death, myocardial infarction, new-onset cardiac arrhythmias requiring treatment, cerebrovascular accidents, renal failure requiring renal replacement therapy, mesenteric

Table 2 Clinical studies on remote ischemic preconditioning before elective coronary artery bypass graft surgery

Ref.	Patients <i>n</i> (CTRLS/ RIPC)	RIPC protocol			Primary endpoint	Results			Notes	
		I/R cycles	Cuff pressure	Limb		RIPC	CTRLS	<i>P</i>	β-blockers	Isoflurane, desflurane
Short terms results										
Hausenloy <i>et al</i> ^[88] , 2007	30/27	3 × 5 min	200 mmHg	Upper	cTnT at				+	+
					6 h	0.31 mcg/L	0.59 mcg/L	0.039		
					12 h	0.37mcg/L	0.69 mcg/L	0.002		
					24 h	0.30 mcg/L	0.52 mcg/L	0.003		
					48 h	0.30 mcg/L	0.52 mcg/L	0.036		
					72 h	0.25 mcg/L	0.48 mcg/L	0.111		
Wagner <i>et al</i> ^[90] , 2010	35/33	3 × 5 min	40 mmHg above systolic pressure	Upper	cTnI at 8 h	2.54 mcg/L	2.90 mcg/L	0.043	-	-
Ali <i>et al</i> ^[91] , 2010	50/50	3 × 5 min	200 mmHg	Upper	CK-MB at					
					8 h	27.22 IU/L	30.24 IU/L	0.026	+	NS
					16 h	33.3 IU/L	37.2 IU/L	0.021		
					24 h	22.74 IU/L	25.22 IU/L	0.052		
					48 h	17.20 IU/L	19.72 IU/L	0.003		
Lomivorotov <i>et al</i> ^[93] , 2012	40/40	3 × 5 min	200 mmHg	Upper	48 h cTnI AUC	54.4 ng/mL	53.3 ng/mL	> 0.05	+	+
Thielmann <i>et al</i> ^[102] , 2013	167/162	3 × 5 min	200 mmHg	Upper	72 h cTnI AUC	266 ng/mL	321 ng/mL	0.022	+	+
Rahman <i>et al</i> ^[95] , 2010	55	3 × 5 min	200 mmHg	Upper	48 h cTnT AUC	30 ng/mL	28 ng/mL	0.721	+	+
Lucchinetti <i>et al</i> ^[94] , 2012	28/27	4 × 5 min	400 mmHg	Lower	72 h hs-cTnT AUC	11708 pg/mL	9574 pg/mL	0.33	+	+
Long term results										
Thielmann <i>et al</i> ^[102] , 2013	167/162	3 × 5 min	200 mmHg	Upper	ACE at 1.54 yr	3	11	0.046	+	+
					death MACCE	8	23	0.005		
Lucchinetti <i>et al</i> ^[94] , 2012	28/27	4 × 5 min	400 mmHg	Lower	ACE at 6 mo death	0	1	1.00	+	+
					rehospitalization	3	3	1.00		

ACE: Adverse cardiovascular events; AUC: Area under the curve; cTnI: Cardiac troponin I; cTnT: Cardiac Troponin T; CTRLS: Controls; hs-cTnT: High sensitivity cardiac troponin T; I/R: Ischemia/reperfusion; MACCE: Major adverse cardiac and cerebral events; NS: Not specified; RIPC: Remote ischemic preconditioning.

ischemia, length of hospital stay and intensive care unit stay^[105].

The few studies evaluating the effects of RIPC in the sole setting of valve replacement yielded conflicting results. For example, Wu *et al*^[106] did not find a significant effect of a standard RIPC protocol on cTnI release after mitral valve replacement surgery, whereas Xie *et al*^[107] reported a significant reduction of the 72 h cTnI-AUC in patients undergoing mitral valve, aortic valve or tricuspid valve surgery.

On the whole, RIPC seems to exert a protective role against PMIs caused by elective CABG surgery, while its long term effects are still uncertain. Furthermore, no definite statement can be made about the RIPC efficacy in other forms of elective cardiac surgery, namely valve replacement surgery and congenital cardiac surgery (Table 2). Finally, volatile anesthetics and β-blockers are emerging as potential confounding factors, although the mechanisms are still unclear.

CONCLUSION

Remote ischemic conditioning (RIC) has been proposed

as a “non-invasive, simple, safe, and cheap”^[108] strategy to protect the heart against ischemic insults. A great research effort has been performed in order to verify the existence of a myocardial protection by RIC, and to evaluate the extent of such protection. Nevertheless, clinical studies have provided conflicting results. A deeper comprehension of the mechanisms underlying RIC is advisable in order to correctly assess the cardioprotective potential of RIC, and to guide future clinical research.

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Cardiovascular disease in human immunodeficiency virus infected patients: A true or perceived risk?

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Abstract

After the successful introduction of highly active antiretroviral agents the survival of patients infected with the human immunodeficiency virus (HIV) in developed countries has increased substantially. This has allowed the surfacing of several chronic diseases among which cardiovascular disease (CVD) is prominent. The pathogenesis of CVD in HIV is complex and involves a combination of traditional and HIV related factors. An accurate assessment of risk of CVD in these patients is still elusive and as a consequence the most appropriate preventive and therapeutic interventions remain controversial.

Key words: Human immunodeficiency virus infection; Atherosclerosis; Cardiovascular risk; Antiretroviral therapy; Dyslipidemia; Hypertension; Smoking; Cardiovascular death

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Core tip: Infection with the human immunodeficiency virus (HIV) was initially universally lethal but with the introduction of highly active antiretroviral therapies (HAART) the life span of HIV infected patients has drastically increased. Along with the lengthening of life span chronic diseases such as non-acquired immunodeficiency syndrome related cancers and cardiovascular diseases surfaced. Currently cardiovascular disease is the primary cause of death among HIV infected patients in industrialized countries and its pathogenesis is very complex. A combination of direct virion injury, chronic low-grade inflammation, adverse cardiometabolic effects of HAART and high burden of traditional risk factors

contribute to this new epidemic.

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INTRODUCTION

In the era of highly active antiretroviral therapy (HAART), the prognosis for human immunodeficiency positive (HIV+) patients in developed countries has dramatically improved^[1,2]. As a consequence HIV infected patients live longer^[3] and the medical care for this population is becoming more focused on the management of non-acquired immunodeficiency syndrome (AIDS) related morbidities, including cardiovascular diseases (CVDs)^[4].

Although CVD is a leading cause of mortality and morbidity in HIV+ patients^[5,6], there remains some controversy as to whether the disease is accelerated (promoted by traditional and non-traditional risk factors for atherosclerosis) or accentuated (greater prevalence of traditional cardiovascular risk factors) in these patients^[7,8]. The prevention and treatment may vary considerably according to which mechanism is the prevailing one. In this review, we address the epidemiology of CVD in HIV+ patients, we discuss the impact of traditional and HIV-related risk factors; we review risk assessment for CVD in HIV and provide a brief overview of future therapeutic approaches to prevention of CVD in patients infected with HIV.

BURDEN OF CVD IN HIV

Epidemiology of CVD in HIV (Table 1)

A large body of evidence supports the notion that the burden of both clinical and sub-clinical CVD is increased in HIV infected patients. Investigators in the Veterans Aging Cohort Study Virtual Cohort (VACS-VC) analyzed data collected in 82459 veterans followed for an average of 5.9 years. The 27350 veterans infected with HIV had a significantly higher risk of myocardial infarction (AMI) compared with uninfected veterans (HR, 1.48; 95%CI: 1.27-1.72)^[6]. The highest AMI risk was recorded among patients with HIV-RNA levels of at least 500 copies/mL and CD4 cell (CD4+) count less than 200 cell/mL; the risk remained elevated among patients who achieved HIV-RNA levels less than 500 copies/mL over time, suggesting that HAART may have contributed to some of the AMI risk^[6]. The increased risk of CVD was noted both in HIV infected men and women^[9]. When the VACS-VC participants were categorized according to the presence or absence of standard risk factors (diabetes mellitus, current smoking, total cholesterol, blood pressure, statins

and antihypertensive medications use), HIV-infected veterans without major CVD risk factors had a 2-fold greater risk of AMI compared with uninfected veterans and the risk increased rapidly with each additional risk factor added^[10]. In a cohort of 3851 HIV-infected patients examined at two Boston health care facilities the rate of AMI was significantly higher than in 1044589 controls after adjustment for age, sex, race, hypertension, and dyslipidemia (RR, 1.75; 95%CI: 1.51-2.02; $P < 0.0001$). Importantly, race appeared to have a different influence on the rate of AMI, with risk being higher in African-Americans and in Hispanics compared to Caucasians^[11]. Although the prevalence of hypertension, diabetes mellitus and dyslipidemia was higher in HIV patients, this study further suggested that traditional risk factors cannot fully account for the increased risk of CVD in HIV^[11]. Other studies performed outside of the US confirmed an increased risk of CVD in infected individuals^[12,13].

The evidence of an increased risk of CVD in HIV extends to studies of subclinical atherosclerosis. Hsue *et al*^[14] measured carotid artery intima-media thickness (IMT), an independent predictor of AMI and stroke^[15,16], in 148 HIV+ patients and 63 age and sex matched controls. They reported that the mean carotid IMT of HIV+ patients was significantly greater and progressed faster in HIV+ patients than in controls. Of note, HIV infection was a predictor of carotid IMT independent of all other risk factors such as age, sex, smoking, HTN, lipid abnormalities and diabetes mellitus; a nadir CD4 count < 200 cells/mL was associated with IMT progression. However, Currier *et al*^[17] failed to show any association between HIV infection and rate of carotid IMT progression.

Coronary computed tomography with and without intravascular iodinated contrast provides information about coronary artery calcium and non-calcified plaques both measures of subclinical atherosclerosis. Post *et al*^[18] showed that HIV-infected men had a greater prevalence of coronary artery plaques compared to uninfected men and more extensive non-calcified plaques. On the contrary, the extent of coronary calcification was similar in the two groups.

In contrast with the above reported increased prevalence of CVD, Klein *et al*^[19] recently reported a decline in incidence of AMI and CVD in HIV-patients. They reviewed data collected among the members of Kaiser Permanente Southern California and Northern California health plans between 1996 and 2011 (24768 HIV-infected patients and 257600 controls). The unadjusted relative risk of AMI for HIV+ patients decreased from 2.0 in 1996-1999 to 1.2 in 2010-2011, and the adjusted RR declined from 1.8 in 1996-1999 to 1.0 in 2010-2011 (Table 2). The decreased incidence of AMI in HIV+ patients may be due to the use of more lipid-friendly HAART medications, earlier initiation of HAART, resulting in lower incidence of severe immunodeficiency, and better control of CVD risk factors. The latter notion was supported by the finding that the

Table 1 Epidemiological studies evaluating the impact of human immunodeficiency virus on cardiovascular disease

Ref.	Size	Follow-up	Findings
Freiberg <i>et al</i> ^[6]	82459 27350 HIV+ 55109 HIV-	5.9 yr	Increased risk of MI among HIV+ patients VACS-VS study ^[6] (HR: 1.48; 95%CI: 1.27-1.72)
Womack <i>et al</i> ^[10]	2187 women 32% HIV+	6 yr	Increased risk of CVD in HIV+ women compared to uninfected women (HR: 2.8; 95%CI: 1.7-4.6)
Paisible <i>et al</i> ^[10]	81322 33% HIV+	5.9 yr	HIV+ veterans without major CVD risk factors had a 2-fold increased risk of MI compared with HIV- veterans without CVD risk factors (HR: 2.0; 95%CI: 1.0- 3.9)
Triant <i>et al</i> ^[11]	3851 HIV+ 1044589 HIV-	8 yr	Increased risk of MI among HIV+ patients (RR: 1.75; <i>P</i> < 0.0001)
Silverberg <i>et al</i> ^[12]	22081 HIV+ 23069 HIV-	13 yr	Higher risk of MI among HIV+ patients with a low recent or nadir CD4 cells (< 200) compared with HIV- subjects (RR, 1.76; 95%CI: 1.31-2.37 for low recent CD4 RR, 1.74; 95%CI: 1.47-2.06 for low nadir CD4)
Lang <i>et al</i> ^[13]	74958 HIV+ FHDH-ANRS CO4	6 yr	The risk of MI was higher in both HIV+ men and women compared with the general population Standardized mortality ratio: 1.4 (95%CI: 1.3-1.6) for HIV+ men and 2.7 (95%CI: 1.8 -3.9) for HIV+ women compared with the general population
Hsue <i>et al</i> ^[14]	148 HIV+ 63 HIV-	1 yr	Higher baseline carotid IMT of HIV+ patients (<i>P</i> = 0.0001) and faster progression (<i>P</i> = 0.002)
Currier <i>et al</i> ^[17]	133 subjects in 45 triads ¹	144 wk	HIV infection and PI use did not contribute to the rate of carotid IMT progression. The median paired difference in IMT change between the PI and non-PI subjects was not statistically significant (<i>P</i> = 0.19). When the HIV+ groups were combined and compared with the HIV- negative group, the difference in progression was also not significant (<i>P</i> = 0.71)
Post <i>et al</i> ^[18]	618HIV+ 383HIV- men cross-sectional study		HIV-infected men had a greater prevalence of CAC [PR: 1.21 (95%CI: 1.08); <i>P</i> = 0.001] and any plaque [PR: 1.14 (CI: 1.05- 1.24); <i>P</i> = 0.001], including non-calcified [PR: 1.28 (CI: 1.13-1.45); <i>P</i> < 0.001] and mixed [PR: 1.35 (CI: 1.10-1.65); <i>P</i> = 0.004] plaque, than uninfected men
Klein <i>et al</i> ^[19]	95687 VACS-VS study 31% HIV+	15 yr	Decline in adjusted MI rate ratio for HIV status over time, reaching 1 (95%CI: 7-1.4) in 2010-2011

¹Each triad consisted of one subject from each of the following categories: (1) HIV+ with continuous PI therapy; (2) HIV+ without prior PI use; (3) HIV- subject. HIV: Human immunodeficiency virus; IMT: Intima-media thickness; MI: Myocardial infarction; PR: Prevalence ratio; CVD: Cardiovascular disease.

Table 2 Crude and adjusted rate ratios (95%CI) of myocardial infarction comparing human immunodeficiency virus infected and uninfected patients during a 13-year time span in the California Kaiser Permanente health system

Year	1996-2011	1996-1999	2000-2003	2004-2007	2008-2009	2010-2011
Crude	1.6 (1.5, 1.8)	2.0 (1.5, 2.8)	2.0(1.6, 2.5)	1.5 (1.2, 1.9)	1.5 (1.1-2.0)	1.2 (0.9-1.6)
Adjusted	1.4 (1.2, 1.6)	1.8 (1.3, 2.6)	1.7(1.4, 2.1)	1.3 (1.0, 1.6)	1.3 (0.9, 1.7)	1.0 (0.7, 1.4)

Modified from Klein *et al*^[19].

Framingham risk scores were lower in HIV+ patients in more recent years compared to the late 90s^[19].

In summary, a considerable body of literature shows a greater incidence of clinical CVD and a greater prevalence of subclinical atherosclerosis in HIV infected patients compared to the general population, supporting the notion that HIV is an independent risk factor for CVD. Whether the recently reported trend reversal is due to a greater awareness and more effective implementation of preventive measures in HIV+ patients remains to be demonstrated in different geographical areas and health delivery settings.

Etiopathogenesis of atherosclerosis in HIV

Infection related factors: The increased CVD risk in HIV may be dependent on a direct role of the HIV virus and on the immunological dysregulations caused by chronic HIV infection (Figures 1 and 2); data from

observational studies provide evidence that both of these elements are involved^[6,10,11].

The importance of continuous suppression of viral replication was investigated in the strategies for management of antiretroviral therapy (SMART) study^[20]. The investigators compared the risk of all-cause death and cardiovascular, renal or hepatic complications in 2720 HIV+ patients receiving intermittent antiretroviral therapy and 2752 HIV+ patients receiving continuous antiretroviral therapy^[20]. After a mean follow-up of 16 mo, the risk of death from any cause and for major cardiovascular, renal and hepatic diseases was significantly higher in patients who received intermittent compared to those who received continuous anti-retroviral therapy (HR for all-cause death: 2.6; 95%CI: 1.9-3.7; *P* < 0.001, HR for major CVD, renal and hepatic disease: 1.7; 95%CI: 1.1-2.5; *P* = 0.009). The authors suggested that the increased CV risk may be

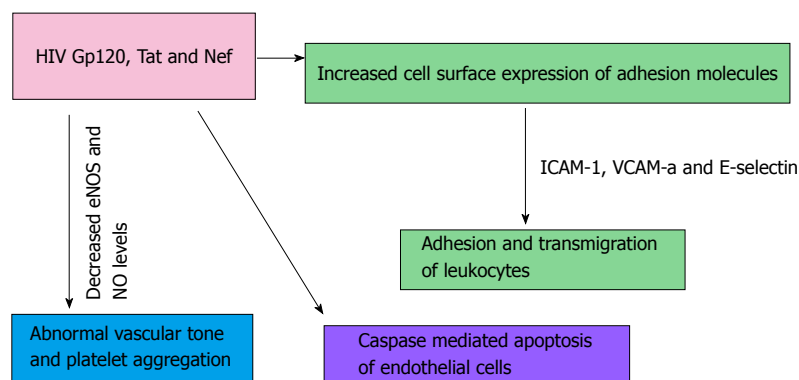


Figure 1 Putative mechanisms by which the human immunodeficiency virus (HIV) increases the risk of atherosclerosis. The virus induces expression of adhesion molecules for leukocytes, reduce the secretion of nitric oxide with reduced vasodilation and induce endothelial cells apoptosis. ICAM: Intercellular adhesion molecule; VCAM: Vascular cell adhesion molecule; eNOS: Endothelial nitric oxide synthase; NO: Nitric oxide; HIV: Human immunodeficiency virus.

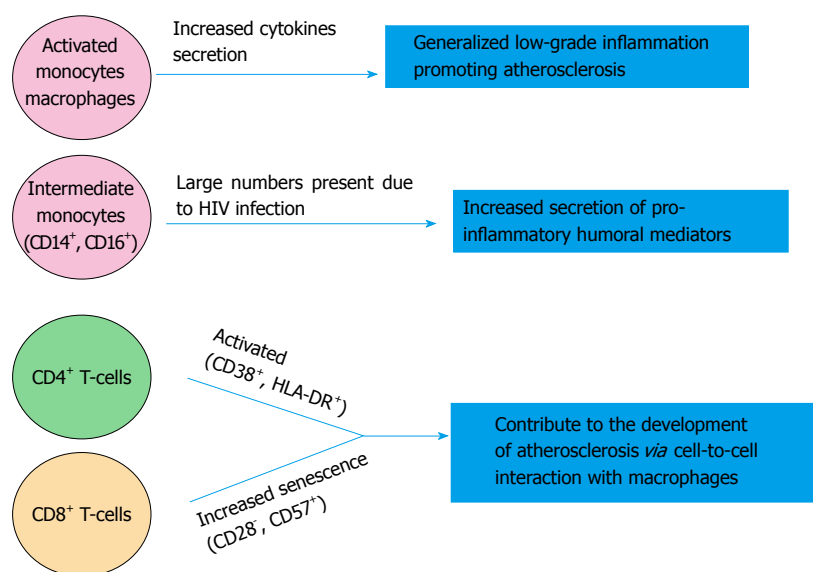


Figure 2 The human immunodeficiency virus promotes a state of low-grade chronic inflammation that increases the risk of atherosclerosis through the activation of lymphocytes, monocytes and macrophages. HIV: Human immunodeficiency virus; HLA: Human leukocyte antigen.

the consequence of alternating low and high CD4 cell numbers and viral loads experienced by the patients while receiving intermittent antiretroviral therapy, hence the importance of early and vigorous control of HIV replication and immune dysfunction^[20].

In a cohort study of 22081 HIV+ and 230069 HIV-adult patients Silverberg *et al.*^[12] showed that the risk of AMI was 44% higher in HIV+ subjects compared with HIV- controls after adjustment for traditional risk factors. HIV+ patients with a nadir CD4 cell count < 200/mcl had a greater risk of AMI compared to controls^[12]. However, the AMI rate of HIV+ subjects with a recent or nadir CD4 cell count \geq 500 /mcl and that of HIV- subjects was the same. In a case-control study of 289 HIV+ patients and 884 HIV-infected controls^[21], a viral RNA level > 50 copies/mL, a low CD4 nadir and a high current CD8 count (> 1150/mm³) were significantly associated with an increased risk of AMI. The ratio of nadir CD4/current CD8 count was the best predictor of an event^[21].

In summary it would appear that a tight control of the HIV reproduction and maintenance of a good CD4 count may be protective against the risk of CV events. However, there exists conflicting evidence as to the actual association between CD4 cell count and the risk of AMI^[22,23], as well as the HIV RNA levels and CV risk.

Some investigators reported a direct association^[21,22], while others were not able to identify one^[24-26]; therefore this aspect of the CV pathogenicity of HIV needs to be clarified further.

The HIV can penetrate into endothelial cells utilizing CD4 receptors, galactosyl-ceramide receptors or chemokine receptors pathway^[27-29], and different components of the HIV may have a role in the pathogenesis of CVD. Gp120 is a glycoprotein exposed on the surface of the HIV envelope and can also be found in the circulation from viral turn over^[30,31]. It mediates HIV-1 entry into human cells by interacting with the HIV receptor for CD4 and co-receptors CXCR4 or CCR5^[32]. Jiang *et al.*^[33] showed that Gp120 and tumor necrosis-alpha (TNF- α) synergistically decrease endothelial nitric oxide synthase (eNOS) and nitric oxide (NO) levels in both porcine and human coronary artery endothelial cells. NO is an important factor in the regulation of vascular tone and inhibition of platelet adhesion and aggregation^[34]. HIV infection is a chronic inflammatory state, characterized by elevated serum levels of factors such as TNF- α and TNF- β , interferon gamma (IFN- γ) and monocyte chemo-attractant protein-1 (MCP-1)^[32,35,36] and Gp120 can magnify the pro-atherosclerotic effects of these mediators. Gp120 can also induce apoptosis by interacting with CXCR4,

a chemokine receptor^[37,38], which is also expressed on vascular endothelium^[39,40].

The trans-activator of transcription (Tat) protein is a regulatory protein that enhances the efficiency of viral transcription^[41]. In a study on the role of Tat on the expression of adhesion molecules in human endothelial cells, Tat was shown to induce the expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin^[42]. In the early phases of atherosclerosis, leukocytes adhere on the surface of endothelial cells and subsequently transmigrate between vascular endothelial cells into the intima layer of the vessel wall. While E-selectin is involved in the initial rolling of leukocytes on the endothelial cells^[40], ICAM-1 and VCAM-1 induce firm adhesion and transmigration of leukocytes across the vascular endothelium^[43]. High levels of soluble ICAM-1, VCAM-1 and E-selectin are associated with and increased risk of AMI in healthy men and women^[44-46]. The levels of ICAM-1, VCAM-1 and E-selectin are elevated in HIV+ patients and there is a correlation between ICAM-1 concentration and the progression of HIV disease as well as the reduction of CD4 count^[47,48]. Similarly to Gp120, Tat can also decrease endothelium dependent vasorelaxation and eNOS secretion in porcine coronary arteries^[49].

Negative factor (Nef) protein is an HIV regulatory protein with an important role in cell apoptosis and enhancement of viral infectivity^[50]. Nef blocks the ATP-binding cassette transporter A1 (ABCA1) pathway, leading to impaired cholesterol efflux from HIV infected macrophages to HDL particles^[51]. As a result, HIV-infected macrophages accumulate lipids turning into foam cells, a step that may contribute to atherosclerosis formation^[51]. Like Gp120 and Tat protein, Nef decreases endothelium-dependent vasorelaxation in porcine pulmonary arteries and reduces eNOS expression in both porcine pulmonary artery and human pulmonary artery endothelial cells^[52]. In the same model Nef increased the levels of reactive oxygen species (ROS)^[52], with an attendant decrease in NO bioavailability^[53]. In endothelial cells, Nef can induce monocyte chemoattractant protein-1 (MCP-1) expression and apoptosis through NF- κ B signaling and ROS-dependent mechanisms, respectively^[54].

In addition to the humoral effects described above, cellular immune activation may play a role in the increased incidence of CVD in infected patients^[35]. Monocytes are readily infected by HIV^[55]; they adhere to the endothelial surface and eventually penetrate in the subendothelial space and intima. Monocytes, especially intermediate monocytes expressing CD14⁺⁺ and CD16⁺, are prone to a greater pro-inflammatory activity once infected with HIV^[56]. Furthermore, Hears et al^[57] showed that infected monocytes and macrophages of HIV+ patients have a reduced phagocytic activity and demonstrate telomere shortening a marker of premature ageing. High levels of monocyte activation

markers, such as soluble CD163, CD14 and MCP-1 have been associated with subclinical coronary artery atherosclerosis, after adjustment for traditional CVD risk factors, in a large cohort of HIV-infected men^[58]. T-lymphocytes are also activated in HIV infection^[59]. In the Women Interagency HIV study, Kaplan et al^[59] showed that HIV infection was associated with significantly elevated levels of activated [CD38⁺ human leukocyte antigen (HLA)-DR⁺] peripheral CD4⁺ and CD8⁺ cells and CD8⁺ senescent cells (CD28⁻CD57⁺). The trend was reduced but not totally reversed after effective viral suppression with HAART. After adjustment for multiple confounders, CD4⁺ and CD8⁺ cell activation and CD8⁺ senescent cells were associated with subclinical carotid artery lesions detected by 2D ultrasound^[59].

Traditional risk factors in HIV: Traditional risk factors are more prevalent in HIV infected patients and likely represent a major driver for CVD in HIV.

(1) Cigarette smoking: The prevalence of cigarette smoking in HIV infected patients has been reported to be higher than in the general population. Analyzing data from 4217 infected (who participated in the Medical Monitoring Project) and 27731 non infected adults (who participated in the National Health Interview Survey in 2009), Mdodo et al^[60] reported that 42.4% of HIV patients (95%CI: 39.7%-45.1%) were current cigarette smokers, while 20.3% (CI: 18.6%-22.1%) were former smokers, and 37.3% (CI: 34.9%-39.6%) had never smoked. Compared with the US adult population, in which an estimated 20.6% of adults smoked cigarettes in 2009, adults with HIV were nearly twice as likely to smoke [adjusted prevalence difference, 17.0% (CI: 14.0%-20.1%)], but were less likely to quit smoking (quit ratio, 32.4% vs 51.7%). A higher prevalence of smoking was also described in the SMART trial^[61] and in the D:A:D study^[62], where the rates of smoking were 40.5% and 51.5% respectively in HIV infected patients. Social and psychological factors such as ethnicity, lower educational level, poverty, illicit drug use, depression are likely contributing to the tobacco epidemic in HIV^[60]. The noxious effects of smoking may be enhanced in HIV patients. Recently Helleberg et al^[63] reported a greater number of life-years lost due to smoking in HIV infected patients compared to smoking controls [12.3 years (95%CI: 11.5-13.0) vs 3.6 years (95%CI: 3.1-4.0), respectively].

(2) Diabetes mellitus: Using data from the Multicenter AIDS Cohort Study (MACS), Brown et al^[64] reported that the incidence of diabetes mellitus in HIV-infected men with HAART exposure was four fold higher than that of HIV-seronegative men. Subsequently De Wit et al^[65] reported an incidence rate of 5.72 per 1000 patient follow-up year (95%CI: 5.31-6.13). The incidence of diabetes increased with cumulative exposure to HAART, and the association remained significant after adjustment for confounding factors. Besides the possible influence of HAART, some investigators reported a higher incidence

of insulin resistance and diabetes mellitus in patients co-infected with hepatitis C virus (HCV). The impact of the directly active agents used in the therapy of HCV to reduce the incidence of diabetes mellitus is currently unknown^[66,67].

(3) Dyslipidemia: The dyslipidemia that develops during HAART is characterized by an increase in total and LDL cholesterol, and triglycerides levels. The effect varies according to the different HAART classes and within each class with different drugs. While this drug-induced toxicity was very common with the older antiretroviral drugs, it has become much less problematic with second generation nucleoside reverse transcriptase inhibitors (NRTI), (namely Rilpivirine) and with integrase inhibitors (Dolutegravir, Elvitegravir and Raltegravir). The HIV is a metabolically active virus capable to alter reverse cholesterol transport *via* modification of the HDL particles functionality and/or impairment of cellular cholesterol efflux. As discussed above, *in vitro* experiments have demonstrated that the HIV-related Nef protein can impair cellular cholesterol efflux through down-regulation of ABCA1^[68,69]. ABCA1 plays a crucial role in stimulating cholesterol export from macrophages. Recently Lo *et al.*^[68] showed that, in the acute phase of HIV infection, HAART can restore the HDL-mediated cholesterol efflux capacity primarily through the suppression of viremia, providing additional evidence that prompt HAART initiation can potentially reduce atherosclerotic risk.

(4) Systemic hypertension: Among HIV infected patients the prevalence and incidence of systemic hypertension ranges from 20%-40% in high-income countries^[70-72] to 11%-28% in low and middle income countries^[73,74]. These trends may reflect the distribution of risk in the general populations of the same regions of the world. As a result, it is currently unclear whether hypertension is more prevalent in HIV infected patients than the general population.

Several studies failed to show a correlation between blood pressure levels and CD4 cell count and between viral load and hypertension. There are sparse and conflicting data on the role of antiretroviral therapy in the pathogenesis of hypertension^[75,76], and the association remains inconclusive.

Impact of antiretroviral therapy on CVD: The first cases of myocardial infarction in HIV-infected patients receiving protease inhibitor were described in the late 1990s; since then several epidemiological studies have examined the association between HIV infection, HAART and the risk of CVD. In 2003 the investigators of the D:A:D study (Data Collection on Adverse Events of Anti-HIV Drugs) reported for the first time an increased incidence of myocardial infarction with longer exposure to combination antiretroviral therapy [adjusted risk rate per year of exposure, 1.26 (95%CI: 1.12-1.41); $P < 0.001$]. Patients with no exposure to therapy had a lower incidence of myocardial infarction than any of the treated groups^[24]. Several years

later the same investigators showed that cumulative exposure to some of the protease inhibitors (Indinavir, Lopinavir-Ritonavir) was associated with an increased risk of myocardial infarction (relative rate per year, 1.12 and 1.13, respectively) after adjustment for the impact of these drugs on lipid metabolism^[77]. This was the first report that suggested that HAART might be responsible for an increased incidence of cardiovascular events independent of their lipid effects. The case of Abacavir remains paradigmatic in this setting. Several observational cohorts and a few randomized clinical trials reported an association between current, but not cumulative exposure of Abacavir and myocardial infarction. Abacavir, as a guanosine analogue, inhibits soluble guanylyl cyclase leading to enhanced platelet adhesion and, ultimately, to increased risk of myocardial infarction^[78].

Numerous other studies implicated Abacavir and protease inhibitors in the development of cardiovascular events, however none of the reports provided conclusive evidence. The adverse effects of these drugs may be particularly harmful in patients with pre-existing high cardiovascular risk, and they should therefore be avoided in those patients.

In spite of early observations reporting an increased risk of CVD in patients receiving HAART, particularly protease inhibitors, more recent evidence suggests a safer cardio-metabolic profile of some categories of HAART^[20,79]. Current strategies to decrease the risk of CVD in HIV infected patients include early initiation of HAART regimens known to be associated with the fewest metabolic adverse effects and careful management of traditional CV risk factors during HAART treatment.

SCREENING ALGORITHMS FOR CARDIOVASCULAR RISK STRATIFICATION IN HIV

Cardiovascular risk assessment in HIV infected patients has traditionally been based on recommendations drafted for the general population. In 2010 Friis-Møller *et al.*^[80] reported on the performance of a new model (derived from the D.A.D. cohort) that included exposure to HAART along with traditional risk factors. Although the new model estimated outcomes more accurately than the Framingham Risk Score (FRS) in the HIV population, it has not been widely adopted.

A new risk prediction algorithm for the general population [atherosclerotic cardiovascular disease (ASCVD)] was introduced in 2013 by the American College of Cardiology/American Heart Association^[81]. In preliminary analyses it appeared that the ASCVD might overestimate CVD risk in the general population^[82]. However the situation is probably the opposite in HIV disease. In a cohort of 2270 HIV infected patients, Regan *et al.*^[83] recently reported that the ASCVD algorithm classified a larger proportion of HIV patients as high-

risk compared to the FRS (25% vs 10%). However, comparing the 5-year observed-vs-predicted event rates, both models underestimated the actual CVD risk in HIV. Similarly, Thompson-Paul *et al.*^[84] compared ASCVD^[81], FRS^[85], the European algorithm known as SCORE^[86] and the DAD score^[80] to predict events in 2392 ambulatory HIV+ patients. After a median follow-up of 6.5 years they recorded 204 events; all models underestimated the actual risk of events. The FRS, ASCVD, and DAD equations showed moderate discrimination (C-statistic range, 0.68 to 0.72), while SCORE showed poor discrimination (C-statistic = 0.59).

Imaging of subclinical atherosclerosis has been suggested as a method to improve risk prediction and implementation of preventive therapies in the general population with variable success. Zanni *et al.*^[87] described a disproportion in HIV infected individuals between presence of subclinical atherosclerosis and statins recommendation. One third of 108 HIV infected patients without known CVD who underwent computed tomography angiography hosted coronary artery plaques with features of high vulnerability. Although a larger number of patients would require statins according to the new ASCVD algorithm compared to the FRS recommendations (26% vs 10%), the majority (74%) of HIV infected patients with subclinical coronary atherosclerosis did not have an indication to receive treatment even with the new algorithm.

Coronary artery calcium (CAC) has been proven to be incremental to risk factors for the prediction of events in the general population^[88,89], but to date it has not been shown to be as useful in HIV infected patients. In a metanalysis Hulten *et al.*^[90] described a similar prevalence of CAC between HIV-positive and uninfected patients (OR: 0.95, $P = 0.851$). In the MACS^[91], among 615 HIV infected men and 332 controls, the adjusted odds ratio for CAC was 1.35 (95%CI: 0.7-2.61). No outcome study has yet been published demonstrating the utility of CAC as an incremental prognostic factor in HIV.

Inflammation plays a central role in the pathophysiology of atherosclerosis and 18-fluoredeoxyglucose positron emission tomography can identify activated macrophages infiltrating the arterial wall. Subramanian *et al.*^[92] compared 27 HIV positive patients receiving HAART without prior CVD, with two non-HIV control groups: one group was matched for age, sex and FRS (non-HIV, FRS-matched controls), while the second one was sex-matched but had known atherosclerotic CVD (non-HIV, atherosclerotic controls). Inflammation in the aortic wall (measured as target to background signal ratio, TBR) was similar between HIV infected patients and non-HIV atherosclerotic controls (2.23; 95%CI: 2.07-2.40 vs 2.13; 95%CI: 2.03-2.23; $P = 0.29$), but was higher than in the non-HIV FRS-matched controls (2.23; 95%CI: 2.07-2.40 vs 1.89; 95%CI: 1.80-1.97; $P = 0.001$).

In HIV infected patients the aortic TBR was associated with the serum concentration of soluble CD163, a

novel marker of activated macrophages ($P = 0.04$)^[93], but not with C-reactive protein ($P = 0.65$) or D-dimer ($P = 0.08$) levels.

More recently, Tawakol *et al.*^[94] performed 18-FDG-PET imaging in 41 HIV+ patients on stable HAART regimen without prior CVD, but with coronary plaques on coronary CT angiography. A higher tracer uptake was correlated with the presence of low-attenuation plaques ($P = 0.02$) and positive remodeling ($P = 0.04$), both features of plaque instability.

Despite these encouraging results, no algorithm to date has incorporated imaging information in risk models for HIV patients. Therefore risk assessment remains reliant on the use of traditional risk factors despite their demonstrated limitations, although there is hope that imaging may add useful information in the future (Figure 3).

MANAGEMENT OF HIV INFECTED PATIENTS TO PREVENT CVD

In view of the information discussed so far it appears true that the CV risk of HIV infected patients is greater than that of the general population; a combination of higher prevalence of traditional risk factors and HIV specific factors likely predispose patients to such increased risk. Four questions emerge regarding risk reduction management in HIV patients: (1) should HIV infected patients be treated more aggressively than the general population for traditional risk factors? (2) As a corollary of the former, should a lower risk threshold be used to initiate treatment? (3) What HAART should be chosen to minimize CV risk in HIV? and (4) Finally, should therapy for HIV infected patients be guided by imaging and/or non-imaging biomarkers?

To date no study has addressed the impact of more aggressive therapy of traditional risk factors in HIV patients to reduce the attendant CV risk. While it would appear that there is a trend toward a relative reduction of CV morbidity and mortality in the past 10-15 years^[95], it is unclear whether this is due to greater physicians' awareness and increased preventive efforts in HIV patients or other as yet unknown factors. The current recommendations for risk assessment and risk reduction in HIV patients mimic those of the general population. However, as discussed above, in spite of a slightly better performance of newer versus older risk assessment algorithms, the majority of patients at risk are not identified as high-risk and are therefore not receiving potentially life saving therapies. The newest algorithm (ASCVD) lowered the threshold for identification of high-risk patients to 7.5% from 20% as recommended in the ATP-III guidelines^[96], but it continues to under perform in HIV patients. In fact, despite the greater proportion of patients at risk identified by the ASCVD, as many as 40% of the patients suffering an acute cardiovascular event would not have met the requirement for prescription

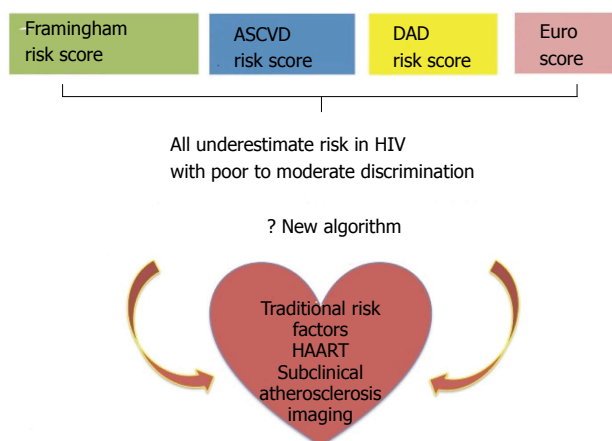


Figure 3 All algorithms currently used to estimate risk of cardiovascular disease in the general population underestimate the actual risk of human immunodeficiency virus positive patients. Although unproven, it is likely that a combination of traditional risk factors, risk linked with some anti-retroviral agents and data on subclinical atherosclerosis collected via imaging, may improve risk prediction in the future. ASCVD: Atherosclerotic cardiovascular disease; HAART: Highly active antiretroviral therapies; HIV: Human immunodeficiency virus.

of statins prior to the event according to two recent studies^[83,97]. Hence, a mere lowering of the threshold for initiation of preventive therapies may be insufficient to effectively lower risk of events in HIV patients. In view of the difficulties highlighted above, a new study was launched^[98] that aims to show that early statin treatment in asymptomatic HIV infected patients will delay the development of inflamed atherosclerotic plaques and reduce cardiovascular events (death, myocardial infarction, angina, stroke and revascularizations).

The choice of HAART and timing of therapy initiation likely carry a significant weight in risk reduction, as some of these drugs appear to have both direct and indirect cardiovascular toxicity while others may lower CV risk. Since the initial observation from the SMART study^[20] reporting a reduced incidence of CV events in patients reaching a stable viral suppression, newer antiretroviral agents have been introduced with improved cardiometabolic toxicity. However, there are currently no long-term studies to demonstrate the safety and effectiveness of new anti-retroviral agents such as integrase inhibitors, although the safety profile of drugs such as atazanavir (a protease inhibitor) and tenofovir (member of the NRTI family) has been established. To address the paucity of prospective follow-up data, the D.A.D. registry has been tasked with the collection of safety data on drugs with a minimum of 30000 person-year follow-up^[99].

Surrogate markers of atherosclerosis and serological biomarkers may provide some insight into the effectiveness of a novel therapeutic agent. In a preliminary communication, Stein *et al.*^[79] reported the effect of various antiretroviral combinations on the progression of the intima-medial thickness (IMT) of the carotid artery bulb in 234 HIV+ patients followed for a maximum of 144 mo from randomization. Atazanavir

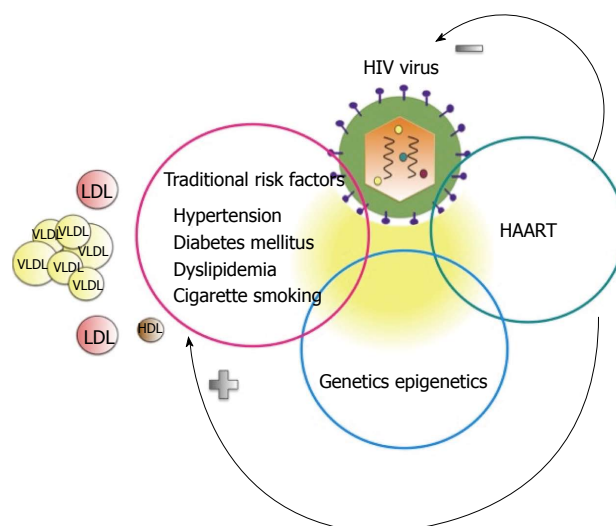


Figure 4 The pathophysiology of human immunodeficiency virus associated atherosclerosis is very complex; a high prevalence of traditional risk factors, direct effects of the human immunodeficiency virus virion and side effects of some the antiretroviral agents, along with yet unknown genetic and epigenetic factors predispose these patients to a high incidence of cardiovascular disease.

The impact of anti-retroviral therapy is particularly difficult to estimate since suppression of viral replication may have an anti-atherosclerotic activity (curved arrow with negative sign) while side effect of some antiretroviral drugs may promote atherosclerosis (curved arrow with positive sign). HAART: Highly active antiretroviral therapies; LDL: Low-density lipoprotein; VLDL: Very low-density lipoprotein; HIV: Human immunodeficiency virus.

(protease inhibitor) was associated with less IMT progression than darunavir (integrase inhibitor), but the progression was similar for atazanavir and raltegravir (first generation integrase inhibitor) despite the better lipid profile of patients receiving raltegravir. Hence, it would appear that only part of the pro-atherogenic effect of HAART may be dependent upon lipid metabolism alterations. Additionally, in a recent publication the authors reported that various HAART regimen did not differ as far as expression of markers of inflammation and immune activation, confirming the notion that the anti- or pro-atherogenic effects of HAART cannot be gauged by their effect on serological biomarkers at the current state of research^[100]. The lack of effect on biomarkers and a not markedly different effect on measures of atherosclerosis suggests that despite a reduction of viral load with HAART, there are residual immunological perturbations and reservoirs of viral replication that may induce atherosclerosis progression. Timing of HAART initiation has recently received renewed interest with the publication of the INSIGHT-START trial results^[101]. In this trial 4685 HIV infected patients were randomized to receive HAART (mainly based on tenofovir, emtricitabine and efavirenz) with a CD4⁺ cell count > 500/cc (median CD4⁺ at initiation 651/cc) or only once the CD4⁺ cell count dropped below 350/cc. The primary end point was a composite of death from all causes, and any serious AIDS related and non-AIDS related events (mostly cardiovascular). After a mean follow-up of 3 years the patient group

that received early HAART demonstrated a significantly reduced event rate (HR: 0.43; CI: 0.30-0.62, $P < 0.001$) compared to the delayed treatment group. These results fuelled an intense debate as to the pros- and cons of early HAART initiation, an approach that needs to be carefully considered in view of the potential side effects of some HAART discussed above.

CONCLUSION

The etio-pathogenesis of CVD in HIV is very complex, with contributions from the retrovirus and the attendant immunologic perturbations, HAART, highly prevalent traditional risk factors and genetics (Figure 4). Therefore a multifaceted approach will be necessary to effectively prevent its development and progression. Since atherosclerosis in HIV patients is characterized by immune activation in association with highly inflamed, non-calcified, potentially vulnerable plaques, these may become the targets of choice in future clinical trials to test the effectiveness of therapy.

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Concepts of hypoxic NO signaling in remote ischemic preconditioning

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Abstract

Acute coronary syndromes remain a leading single cause of death worldwide. Therapeutic strategies to treat cardiomyocyte threatening ischemia/reperfusion injury are urgently needed. Remote ischemic preconditioning

(rIPC) applied by brief ischemic episodes to heart-distant organs has been tested in several clinical studies, and the major body of evidence points to beneficial effects of rIPC for patients. The underlying signaling, however, remains incompletely understood. This relates particularly to the mechanism by which the protective signal is transferred from the remote site to the target organ. Many pathways have been forwarded but none can explain the protective effects completely. In light of recent experimental studies, we here outline the current knowledge relating to the generation of the protective signal in the remote organ, the signal transfer to the target organ and the transduction of the transferred signal into cardioprotection. The majority of studies favors a humoral factor that activates cardiomyocyte downstream signaling - receptor-dependent and independently. Cellular targets include deleterious calcium (Ca^{2+}) signaling, reactive oxygen species, mitochondrial function and structure, and cellular apoptosis and necrosis. Following an outline of the existing evidence, we will furthermore characterize the existing knowledge and discuss future perspectives with particular emphasis on the interaction between the recently discovered hypoxic nitrite-nitric oxide signaling in rIPC. This refers to the protective role of nitrite, which can be activated endogenously using rIPC and which then contributes to cardioprotection by rIPC.

Key words: Remote ischemic preconditioning; Ischemia/reperfusion injury; Nitrite; S-nitrosation; Mitochondria

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Core tip: Therapeutic strategies to treat ischemia/reperfusion injury are urgently needed. Remote ischemic preconditioning (rIPC) appears to exert beneficial effects for patients. The underlying signaling remains incompletely understood. Following an outline of the existing evidence, we will characterize the existing knowledge and discuss future perspectives with particular emphasis on the interaction between the recently discovered hypoxic nitrite-

nitric oxide signaling in rIPC.

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INTRODUCTION

Current guidelines of both European and United States cardiac societies emphasize one primary goal for patients with acute myocardial infarctions - the timely and successful reperfusion^[1]. This concept among many additional pharmaceutical treatments has significantly reduced mortality and morbidity in patients suffering from acute coronary syndromes. Experimental evidence, however, has largely implicated that rapid restoration of coronary perfusion, in turn, may paradoxically harm cardiomyocytes^[2,3]. This phenomenon has been named ischemia/reperfusion (I/R) injury. The underlying signaling is complex and yet incompletely understood. Current concepts for the modulation of myocardial I/R injury have particularly emphasized three major contributors to the final I/R injury: reactive oxygen species (ROS), Ca²⁺, and mitochondria. Particularly the latter are now not only regarded as targets during I/R but also as mediators of cellular injury and death. For a detailed description of the signaling in myocardial I/R injury the reader is kindly referred to the recently published excellent review sources^[2-7]. Experimental and initial clinical studies implicate that all three signaling components of I/R injury may serve as potential targets in attempt to reduce I/R injury. Although early reperfusion therapy has led to a marked decrease in mortality following acute myocardial infarction, application of such cardioprotective strategy might further reduce the burden of cardiovascular disease.

Ischemic conditioning regimens are among the most effective cardioprotective approaches. These techniques are capable of reducing the final infarct size by up to approximately 30%-60%^[8-10]. Brief episodes of sublethal I/R applied to the index organ [ischemic preconditioning (IPC)] - or a remote organ (rIPC) - before, during or after a main ischemic event initiate a powerful cellular signaling cascade rendering the cardiomyocyte capable of protecting itself. The majority of relevant clinical phase II trials have produced favorable results for ischemic conditioning techniques. However, a definitive implementation into the clinical routine is still warranted. The underlying signaling in the scope of rIPC remains incompletely understood. Here we first outline the events leading to myocardial I/R injury, followed by an introduction to ischemic conditioning. Finally, we will put a major focus on the recently discovered nitrite-nitrogen oxides (NO)

signaling, which targets mitochondria during I/R and protects the heart from lethal injury.

MYOCARDIAL I/R INJURY

Reperfusion therapy either by invasive coronary intervention, bypass surgery or pharmacological lysis causes a rapid increase in cellular oxygen levels^[2]. One key pathological consequence of this is an insufficient mitochondrial respiration in consequence of an incomplete electron transport over mitochondrial membranes. This can cause an excess formation of ROS^[11]. While ROS at physiological concentrations contribute to the general cellular homeostasis, higher levels may initiate a deleterious signaling. This contributes to an increase in cell death by necrosis or apoptosis^[12].

The reperfusion phase is not only characterized by increased ROS levels, but also by a reduction in the bioavailability of NO. NO is a gaseous signaling molecule that regulates a wide variety of cardiomyocyte functions including scavenging of radicals, cardiac immune response, improvement of blood flow and left ventricular function^[13-17]. By consequence, these functions are impaired during reperfusion. In addition, interactions between the ROS system and NO signaling may further contribute to the final myocardial I/R injury. This relates to the increase formation of peroxynitrite (an oxidant) in the reperfusion period^[18], which in turn may contribute to nitrosative stress-related cell injury to membranes^[19,20]. The exact role of peroxynitrite and whether peroxynitrite signaling can be effectively modulated in patients remains to be determined.

The second major contributor to cardiomyocyte dysfunction in I/R is a deteriorated Ca²⁺ signaling^[21-24]. Elevated intracellular Ca²⁺ levels can not only trigger arrhythmias, and cardiomyocyte hypercontracture^[25], they may also activate Ca²⁺-dependent signaling pathways. This pertains especially to Ca²⁺-dependent proteases named calpains, which then cleave cellular elements involved, *e.g.*, in apoptosis, mitochondrial respiration or mitochondrial turnover^[26-28]. Hypercontracture and necrosis are not limited to one cell but can also be distributed from cell to cell either by direct contracture or by cell-to-cell progression through intercellular gap junctions^[29]. Off note, high calcium levels may also directly target and disrupt cell membranes^[20]. While both ROS and intracellular Ca²⁺ elevation mark the initial events of myocardial I/R injury, the central target of reperfusion-associated impaired signaling is the mitochondrion.

MITOCHONDRIA IN I/R INJURY

Mitochondrial injury in I/R comprises at least three relevant entities: (1) Destruction of mitochondrial membrane integrity by permeabilization; (2) deficits in mitochondrial structural dynamism, the recently termed mitochondrial fusion and fission and, finally; and (3) the deterioration of mitochondrial respiration^[30].

The exact events leading to mitochondrion-driven cell death by mechanisms of necrosis, apoptosis, or autophagy are incompletely understood. Dysregulated Ca^{2+} signals and ROS concentrations, as found in early reperfusion, provide excellent circumstances to deteriorate mitochondrial integrity^[31].

Apoptosis causes cell shrinkage, cellular fragmentation, and finally phagocytosis. The general characteristics of necrosis include cellular swelling and rupture, and a marked depletion of energy resources. Newer studies argue that apoptosis as well as necrosis are regulated by a complex signaling machinery with overlapping processes^[32]. The critical step for apoptosis is the permeabilization of the mitochondrial outer membrane pore (MOMP). This occurs by activation of pro-apoptotic BH3 proteins, *e.g.*, Bax^[31]. Subsequently, a release of apoptogenic factors is initiated, *e.g.*, cytochrome c or apoptosis-inducing factor (AIF)^[33,34]. By contrast, the key characteristic of necrotic cell death is the permeabilization of the inner mitochondrial membrane causing the formation of a yet incompletely identified mitochondrial permeability transition pore (mPTP).

Mitochondrial morphology and structural dynamism is regulated by fission and fusion^[35]. Interestingly, Bax - previously introduced as major contributor to cell death - is also involved in mitochondrial fusion and fission. As a consequence, Bax supplementation deteriorates mitochondrial structural dynamism in conjunction with a much-increased I/R injury. Taken together both regulation of mitochondria-driven cell death as well as mitochondrial structure is complex but many overlapping yet incompletely defined processes exist that contribute to cardiomyocyte damage in I/R injury.

In case the initial phase of reperfusion is survived the preservation and restoration of mitochondrial function becomes a major goal. Mitochondria comprise one third of the cell mass of cardiomyocytes. Recent evidence suggests that at least three subpopulations exist: Subsarcolemmal (SSM), interfibrillary (IFM) and perinuclear (PNM) mitochondria. Their relative contribution to the function of cardiac cells remains to be completely elucidated^[30,32]. Mitochondria generate a vast amount of adenosine triphosphate per day^[36] and the electron transfer through the complexes of the mitochondrial respiratory chain is under control of a delicate regulatory machinery. I/R may cause a major functional disturbance with a subsequent incomplete respiration and a burst in the generation of ROS^[37]. Many cardioprotective strategies have attempted to reduce these excessive ROS levels. Newer approaches, *e.g.*, rIPC, have focused on regulatory posttranslational modifications of respiratory chain elements and particularly complex I, which we will outline in the following.

THE CONCEPT OF RIPC

rIPC is among the most effective techniques in rendering the myocardium capable of protecting itself against I/R injury^[38]. rIPC-associated protection is

initiated *via* short non-deleterious phases of I/R prior to an index ischemia. The maneuver is applied to an organ or tissue at distance to the one undergoing the main ischemic event^[38,39]. Clinical evidence from recent phase II trials favors a potential translation into clinical routine^[40-42]. The underlying rIPC-related signal mechanism remains under intense debate. Generally, the signal transduction machinery initiated by the rIPC stimulus involves a trigger, the transfer of the trigger to the target organ and a distinct cardiomyocyte signaling leaving the cardiomyocyte protected from I/R^[13]. As triggering pathways both humoral/blood borne factors^[43-45] and neuronal transmission^[46] have been proposed. However, the cardioprotection can be transferred when transfusing blood from rIPC animals to unconditioned littermates^[43,45,47]. This argues in favor of important contribution of a humoral factor. This does not preclude a role for the nervous system to be involved in the modulation of the rIPC response^[48]. However, an intact nervous system is particularly important for the remote and not the target organ^[48]. Off note, the signaling is at least to certain degrees species-specific with some of the pathways that are active in murine studies being irrelevant in large animal models and humans^[48,49].

TRANSFER OF PROTECTION *VIA* HUMORAL FACTORS

It is now generally accepted that rIPC initiates a complex interplay between several mediators not only at the remote site, but also in the circulation as well as in the target cell. Over the past two decades many components have been forwarded, and it is presently unlikely that one single mediator is responsible for the complete protection associated with rIPC. Among the factors which have been evaluated in experimental studies are adenosine, bradykinin, opioids, interleukins, stromal cell-derived factor, hypoxia-inducible factor 1 α and members of the so-called RISK pathway. These studies have in part revealed conflicting results especially when comparing experimental results with those from trials in patients^[6].

We and other groups have recently investigated a potential involvement of hypoxic NO signaling in the course of rIPC. Using a mouse model of warm liver I/R, it was demonstrated that ablation of endothelial NO synthase (eNOS) abrogates the protective effects seen with rIPC on microscopic liver damage^[50]. eNOS generates NO which can then modulate cardiovascular functions either at the place of synthesis or at a distance when transported as nitrite or nitroso species (nitrosated proteins)^[51]. Changes in shear stress, *e.g.*, due to an increase in blood flow as seen in hyperemia after short phases of ischemia, are the strongest physiological stimulus of eNOS activity, which is mirrored in higher circulating NO metabolites^[13]. This led to the hypothesis that nitrite-NO signaling is involved in the pathways

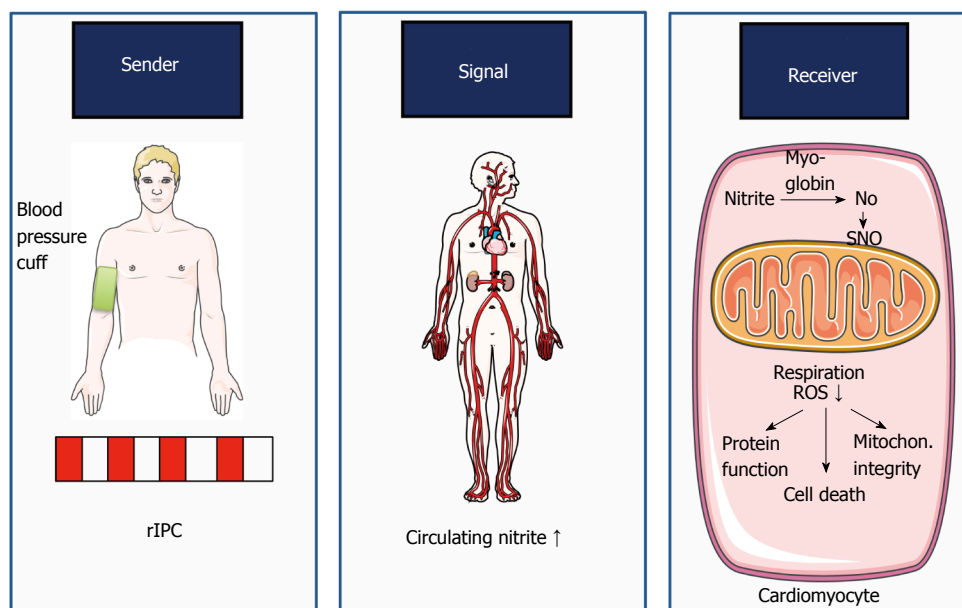


Figure 1 Nitrite/nitrogen oxides related signaling in remote ischemic preconditioning - proposed mechanism. The nitrite/NO signaling involves three components. The sender of the signal is shown in the left panel. In this case the upper extremity receiving rIPC via repetitive in-/deflations of a blood pressure cuff. During this maneuver, the classical L-arginine-NOS-nitrogen oxides (NO) pathway is activated. This includes the activation of eNOS, which generates NO from L-arginine while using several cofactors. Off note, the eNOS system is under tight control of several systems, e.g., the calcium-calmodulin pathway. The middle panel represents the signal transmitted from the sender (remote organ) to the receiver (cardiomyocytes), as nitrite is transmitted from the site of generation via the circulation to the heart. In the receiving target organ, this signal is translated into cardioprotection via myoglobin-dependent nitrite reduction and S-nitrosation of mitochondrial complex I with a protective regulation of mitochondrial functions and reduced reactive oxygen species (ROS). The generation of NO in the last panel activates the alternative pathway for NO generation, which reduces nitrite to NO via hemoglobins, e.g., myoglobin in the heart or hemoglobin in the circulation. The reduction of ROS in turn may help reduce cellular stress as indicated. This relates to an improvement of protein function, cell death and mitochondrial integrity^[8]. rIPC: Remote ischemic preconditioning; NOS: NO synthase.

leading to cardioprotection from the rIPC stimulus.

THE ROLE OF NITRITE/NO SIGNALING IN REMOTE ISCHEMIC PRECONDITIONING

NO is a signaling molecule with a wide variety of functions in the cardiovascular system. Generally, NO protects the heart from I/R injury except for excessive levels during inflammation. However, in the course of I/R the canonical NO generation pathway via the classical L-arginine-NOS-NO signaling pathway becomes impaired due to a lack of molecular oxygen^[52]. Nitrite, formerly regarded a mere NO oxidation product, may serve as an alternative source under these conditions^[14,53]. The majority of the bodily nitrite provision derives from the oxidation of NO, which is enzymatically formed by one of at least three NO synthase (NOS) isoforms^[54]. The remainder of nitrite in the circulation and tissues has been related to nutritional sources^[55]. The half-life of nitrite is quite long in contrast to NO - approximately 35-60 min depending on the models and compartments used to evaluate nitrite distribution^[56]. We have previously shown that the endogenous levels of nitrite can be modified by intravenous infusion of nitrite and certain diets^[14,57,58]. An adequate nitrite production along with sufficient endogenous levels has implications for numerous cardiovascular functions. We have shown that trained athletes with higher nitrite levels performed

superior in exercise tests as compared to those with lower concentrations^[59].

In hypoxia or ischemia, nitrite may be reduced to bioactive NO, thus providing an alternative pathway to the enzymatic formation of NO, which is inactive under these circumstances. Nitrite activation may occur through reaction with hemoglobins such as myoglobin^[60]. With decreasing oxygen gradients, cardiac myoglobin changes its function from an oxygen storage and NO scavenger to an NO producer by reducing nitrite to bioactive NO^[13,15,37,60,61]. This can significantly reduce myocardial I/R injury^[37].

The downstream mechanism following a formation of NO from nitrite largely involves mitochondria. In an experimental approach using exogenous nitrite during myocardial I/R injury we identified an S-nitrosation modification of mitochondrial complex I. This, in turn, regulates myocardial energetics via the modulation of mitochondrial respiration and formation of mitochondria derived ROS. S-nitrosation of complex I is furthermore associated with an adaption of myocardial functions to a reduced O₂ supply. This mechanism has been recognized as hibernation. Finally, nitrite reduction to NO and the downstream signaling cascade contributes to a decrease in myocardial necrosis and apoptosis^[16,37,60,62].

In our recent study using rIPC as protective regimen we revealed that this maneuver also involves a protective nitrite/NO signaling with similarities to the

one described above for exogenous nitrite^[13]. Figure 1 summarizes the general concept. This involves a sender, the transfer of the signal to the target organ, and a receiver - the cardiomyocyte. We propose the sender to be the endothelium of the extremity treated with rIPC. Repetitive phases of in- and deflations of the blood-pressure cuff cause high blood flow and a subsequently increased shear stress. This results in shear-stress dependent eNOS activation and NO formation, which converts to nitrite - the stable oxidation product.

This circulating nitrite is transmitted as signal of cardioprotection to the heart. The stability of nitrite and its half time argues in favor of nitrite^[56]. In transfer experiments, the effects were preserved when infusing conditioned human plasma to isolated mouse hearts. Nitrite scavenging in conditioned human plasma abolished the transmission of cardioprotection to the conditioning-naïve Langendorff hearts.

The receiver of protection is the cardiomyocyte. In the myocardium, cardiomyocyte myoglobin reduces nitrite to NO during I/R with subsequent S-nitrosation modifications of mitochondrial complex I finally leading to cardioprotection. Presently, it is not known whether other signaling molecules regulating for instance apoptosis, necrosis and mitochondrial structure, *e.g.*, the BH3 proteins, are also affected by S-nitrosation modifications by rIPC.

Our recent findings were further evidenced by studies by a group who demonstrated that ischemic conditioning applied locally leads to a wide variety of proteins being S-nitrosated^[63,64]. However, this appears to be a selective process in which SSM mitochondria are favored. This furthermore required connexin-43^[64] to be active. It is tempting to speculate that particularly the subgroup of SSM are largely responsible for cardioprotection during I/R injury. Taken together, current evidence from experimental studies currently implicates a substantial contribution of nitrite/NO to cardioprotection by ischemic conditioning. This involves a specific modulation of mitochondria a targeted S-nitrosation and a reduction in the formation of ROS as well a distinct signaling *via* connexins.

CONCLUSION

In summary, rIPC has been demonstrated to protect the myocardium from I/R injury. Several clinical trials have demonstrated beneficial results for patients. Arguably, the underlying signaling is delicate involving many signaling processes. This pertains to humoral factors, blood cells, neurohumoral mediators and presumably a complex interplay. The data from ours and several other groups implicate a role for hypoxic NO signaling in the course of rIPC^[13,50,64,65]. This requires particularly an S-nitrosation modification of mitochondrial elements. Naturally, many previously forwarded mechanisms will work along-side the proposed one based on NO^[6]. Further studies will be required to elucidate the complex interplay involved in signal generation, signal transfer

and final cardioprotection in the target organ. It will be of particular interest to evaluate the decisive signal transduction pathway in patients treated with rIPC. This is naturally a limitation of the current studies in the field of nitrite-NO signaling in rIPC^[13,50,64,66-68], which involve healthy mice without significant co-morbidities and relevant medications.

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Potential of dietary nitrate in angiogenesis

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Abstract

Endothelial dysfunction with impaired bioavailability of nitric oxide (NO) is the hallmark in the development of cardiovascular disease. Endothelial dysfunction leads to

atherosclerosis, characterized by chronic inflammation of the arterial wall and stepwise narrowing of the vessel lumen. Atherosclerosis causes deprivation of adequate tissue blood flow with compromised oxygen supply. To overcome this undersupply, remodeling of the vascular network is necessary to reconstitute and sustain tissue viability. This physiological response is often not sufficient and therapeutic angiogenesis remains an unmet medical need in critical limb ischemia or coronary artery disease. Feasible approaches to promote blood vessel formation are sparse. Administration of pro-angiogenic factors, gene therapy, or targeting of microRNAs has not yet entered the daily practice. Nitric oxide is an important mediator of angiogenesis that becomes limited under ischemic conditions and the maintenance of NO availability might constitute an attractive therapeutic target. Until recently it was unknown how the organism provides NO under ischemia. In recent years it could be demonstrated that NO can be formed independently of its enzymatic synthesis in the endothelium by reduction of inorganic nitrite under hypoxic conditions. Circulating nitrite derives from oxidation of NO or reduction of inorganic nitrate by commensal bacteria in the oral cavity. Intriguingly, nitrate is a common constituent of our everyday diet and particularly high concentrations are found in leafy green vegetables such as spinach, lettuce, or beetroot. Evidence suggests that dietary nitrate supplementation increases the regenerative capacity of ischemic tissue and that this effect may offer an attractive nutrition-based strategy to improve ischemia-induced revascularization. We here summarize and discuss the regenerative capacity of dietary nitrate on the vascular system.

Key words: Dietary; Nitrate; Vasculature; Regeneration; Hind limb

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or beetroot. Evidence suggests that dietary nitrate supplementation increases the regenerative capacity of ischemic tissue and that this effect may offer an attractive nutrition-based strategy to improve ischemia-induced revascularization. We here summarize and discuss the regenerative capacity of dietary nitrate on the vascular system.

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BREAKDOWN OF NITRIC OXIDE AVAILABILITY IS THE HALLMARK OF CARDIOVASCULAR DISEASE

Nitric oxide (NO) is biosynthesized endogenously from the amino acid L-Arginine (L-Arg) and oxygen by various NO synthases (NOS) which are termed either according to their distribution within the body or allowing for the order where they first purified and cloned. NOS produce NO by catalyzing a five-electron oxidation of guanidino nitrogen of L-Arg that requires binding of five cofactors. These cofactors are: flavin adenine dinucleotide, flavin mononucleotide, heme iron, tetrahydrobiopterin, and calcium-calmodulin^[1]. If any of these co-factors becomes limited, NO production from NOS is restricted and NOS produce superoxide (O₂⁻) instead. This mechanism has been termed "NOS uncoupling"^[2]. Consequently, a physiological oxygen concentration as well as sufficient substrate supply is necessary for a proper NOS function. NO is involved in a wide variety of regulatory mechanisms of the cardiovascular system, including vascular tone (as a major mediator of endothelium dependent vasodilatation), vascular structure (inhibition of smooth muscle cell proliferation), and cell-cell interactions in blood vessels (inhibition of platelet adhesion and aggregation and inhibition of monocyte adhesion). Risk factors like hypercholesterolemia, hypertension, diabetes mellitus or cigarette smoking lead to the inability of the endothelium to produce NO^[3-5]. A decrease of endothelial NO formation due to insufficient oxygen and cofactor supply or inactivation by reactive oxygen species is the hallmark of endothelial dysfunction. Importantly, this is the key element and a facilitative factor in the development of atherosclerosis. Lack of NO in turn promotes aggregation and invasion of inflammatory cells in the vessel wall and aggravates sclerosis of arteries. Thus, a vicious cycle takes place that results in progressive deprivation of blood supply with hypoxia of tissues and organs. Growth of new vessels result as an adaptive mechanism in response to tissue hypoxia or ischemic injury, called angiogenesis.

The physiological repair response, however, is often not sufficient and therapeutic angiogenesis remains an unmet medical need.

ROLE OF NO IN ANGIOGENESIS

Angiogenesis is strongly stimulated in response to tissue hypoxia or ischemic injury and requires several key processes, including dissolution of matrix, endothelial cell proliferation and migration, and organization into tubes followed by lumen formation. One of the most potent angiogenic growth factors is represented by the vascular endothelial growth factor (VEGF) that induces proliferation, migration, survival and permeability of endothelial cells^[6,7]. VEGF upregulates the expression of endothelial NO synthase (eNOS) and stimulates the release of endothelium-derived NO what is believed to play a critical role in the angiogenic action of this factor^[8]. In line with these findings, it could be demonstrated that eNOS gene delivery promotes angiogenesis in animal models of ischemia^[9]. On the contrary, the angiogenic response following hind limb ischemia in mice is impaired in eNOS- deficient mice and this cannot be reversed by VEGF substitution^[10]. From these findings, NO appears to be a downstream mediator of VEGF-induced endothelial cell proliferation and migration and is suggested to even regulate VEGF expression^[11]. However, it should be noted that a major limitation of these investigations is the use of NO donors. The drawback of this approach is that the influence of the released NO might be masked by the NO-independent actions of donating compounds or their derivatives. Likewise, animal models using eNOS overexpression to determine whether the effect of NO on VEGF synthesis could be achieved in ischemic limb neglect that eNOS is dysfunctional in ischemic tissues^[12].

NO GENERATION WITHOUT NOS: THE NITRATE-NITRITE-NO PATHWAY

Since the classical NO-pathway is not functional during ischemia, NOS independent mechanisms must exist to maintain NO homeostasis under hypoxic conditions. The reduction of nitrite, the oxidation product of NO, by several "nitrite-reductases" under hypoxia was identified to be such an alternative pathway (Figure 1 and Table 1)^[13-17]. These nitrite reductases operate along the physiological and pathological oxygen gradient and allow a graded nitrite reduction to NO according to the circulating and metabolic need. The reduction of nitrite to NO reflects a major mechanism by which the NO homeostasis is maintained independent of NOS. New insights evidence that nitrate and nitrite metabolism occurs in blood and tissues to recycle NO and other bioactive nitrogen oxides^[18,19]. Commensal bacteria in the crypts of the tongue own a nitrate reductase enzyme that is utilized for energy metabolism in the absence of oxygen^[20,21]. It was known that nitrate is

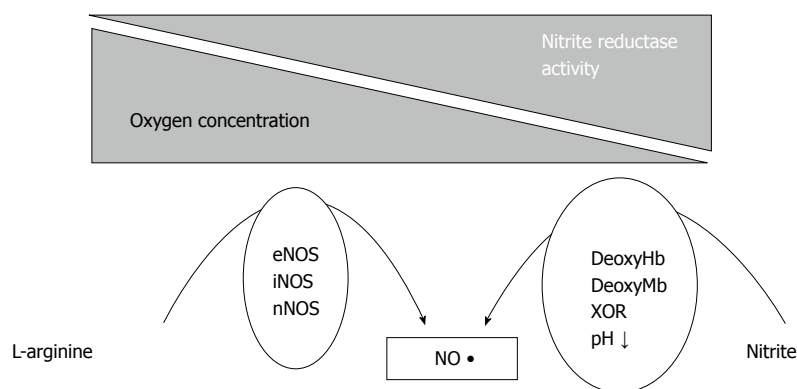


Figure 1 Non-enzymatic nitric oxide formation. One-electron reduction of nitrite (NO_2^-) to NO by ferrous heme proteins like hemoglobin in the blood or myoglobin in the heart can occur under conditions of low oxygen (O_2); the nitrite-reductase activity of these proteins contributes to NOS independent NO formation. eNOS: Endothelial NO synthase; iNOS: Inducible NO synthase; nNOS: Neuronal NO synthase.

Table 1 Nitric oxide generation pathways

Nitrate-nitrite-NO pathway	
NO synthases	Endothelial NO synthase Neuronal NO synthase Inducible NO synthase
Nitrate reductases	Xanthin oxidoreductase Mitochondrial respiratory chain enzymes Cytochrome P-450 Acidic reduction Myoglobin Neuroglobin Hemoglobin

NO: Nitric oxide.

taken up by the salivary glands and concentrated in the saliva. However, the reason for this active process could not be explained until the finding that nitrate serves as substrate for the nitrate reductase enzyme of bacteria in the mouth. These bacteria reduce both plasma extracted nitrate as well as dietary nitrate to form nitrite resulting in salivary nitrite levels that are 1000-fold higher than those found in human plasma^[22]. When nitrite-rich saliva meets the acidic gastric juice after swallowing, nitrite is protonated to form nitrous acid (HNO_2), which then decomposes to NO. This acidic disproportionation takes part in the human defense against pathogens entering *via* the alimentary tract. Furthermore it could provide protection against ulcers from drugs or stress^[23-25]. Beside the intragastric formation of NO it has been demonstrated that ingested nitrite reaches the systemic circulation, thus making it systemically available^[22]. Nitrite in turn can be reduced *in vivo via* numerous pathways to form bioactive NO. These include the reduction *via* deoxygenated myoglobin within the heart muscle, deoxygenated hemoglobin, intracellular xanthin oxidoreductase, enzymes of the mitochondrial respiratory chain, cytochrome P-450 and even *via* the NOS^[13,15,17,26-29]. Thus, several mechanisms exist by which NO is generated in the body, including the NOS enzymes or the non-enzymatically acidic reduction of nitrite. Nitrite mediates hypoxic vasodilation, enhances blood flow and matches oxygen supply to increased

metabolic demands under hypoxic conditions^[30]. Moreover, application of exogenous nitrite bears the potential to reduce myocardial damage after myocardial ischemia and reperfusion injury^[26,31]. In addition, dietary approaches using nitrate to elevate circulating nitrite levels are emerging as a potential treatment regimen for high blood pressure^[32]. Considering the upcoming evidence that nitrite and nitrate mediate cytoprotective effects in human physiology and especially under pathophysiological conditions, it is not unlikely that dietary nitrate and nitrite may positively affect human health and disease. Recognizing that NO is the most important molecule in regulating blood pressure and maintaining vascular homeostasis, food sources rich in NO compounds may provide beneficial effects primarily to the heart and vessels. Although there are clear reports on certain foods and diets that have shown a benefit in terms of preventing cancer and cardiovascular disease, the specific nature of the active constituents responsible for the cardioprotective effects of certain foods is still unknown. Viable candidates are fibers, minerals or antioxidants. High intake of fruits and vegetables is indeed associated with reduced risk for coronary artery disease and apoplectic stroke and the strongest protection against coronary heart disease was seen with high intake of green leafy vegetables^[33]. Dietary intakes of nitrate-rich vegetables lowers blood pressure in subjects with borderline hypertension to the same extend as mono-therapy with a standard antihypertensive drug^[34,35]. Likewise, blood pressure lowering effects could be demonstrated for dietary nitrate and ingestion of beetroot juice respectively^[32,36]. We could recently demonstrate that dietary supplementation with inorganic nitrate improves prognostic relevant outcome measures that have been shown to predict cardiovascular events, namely endothelial dysfunction, vascular stiffness and systolic blood pressure in the elderly with moderately increased cardiovascular risk^[37]. Improvements in blood pressure following the nitrate rich diet were associated with reductions of pro-inflammatory cytokines, which points to the potential anti-inflammatory actions of the nitrate-nitrite-NO pathway^[38,39]. The hypothesis that dietary nitrate might provide cardiovascular benefit is further encouraged by animal models of myocardial infarction,

where dietary supplementation of these anions provided beneficial effects on I/R injury^[40]. Interestingly, a diet rich in vegetables, such as the Mediterranean and the traditional Japanese diets, contains more nitrate than the recommended acceptable daily intake by the World Health Organization^[41]. Even a portion of spinach consumed in one serving of salad can exceed the acceptable daily intake for nitrate^[22]. Taken together, the current evidence supports the conclusion of the European Food Safety Authority that benefits of vegetable and fruit consumption outweigh any perceived risk of developing cancer from the consumption of nitrate and nitrite in these foods. The outlined above data from observational epidemiologic and human clinical studies support the hypothesis that nitrates and nitrites of plant origin play essential physiologic roles in supporting cardiovascular health.

REGENERATIVE CAPACITY OF DIETARY NITRATE ON VASCULATURE

Increased oxygen radicals (ROS) and subsequent reduced NO bioavailability impair vascular growth and remodeling, which highlights important targets for therapeutic angiogenesis in ischaemic myocardial infarction, peripheral vascular disease, as well as stroke. Significant advances have been made with respect to quantitative analytical methods to detect specific ROS and NO species. This must be employed in future studies to enhance the understanding of the relationship between ROS levels and stimulation of vascular remodeling in conjunction with NO bioavailability. Moreover, studies indicate impaired eNOS function in diabetes and atherosclerosis as a result of increased vascular generation of ROS and reduced NO bioavailability^[42,43]. Despite endeavors to promote blood vessel formation by administration of pro-angiogenic factors, gene therapy or targeting of microRNAs, clinically applicable strategies have not been developed yet or are still in a preclinical phase^[44-47]. In this context, dietary nitrate becomes an attractive candidate in the field. A nitrate rich diet can be achieved *via* consumption of leafy green vegetables, as spinach or lettuce, or by consumption of beet-root. However, for comparative reasons, most studies have used nitrate in the pure chemical form of sodium nitrate or potassium nitrate. Mechanistically, nitrate and nitrite can be viewed as stable storage pools for NO-like bioactivity. To further determine the mechanisms of a healthy diet through dietary nitrate on the vasculature we investigated the age-related changes that occur at a molecular level and determined the age-related vascular transcriptome altered by dietary nitrate^[48,49]. Intriguingly, a chronic nitrate supplementation was shown to act as a modifier of gene expression, highlighting the plethora of putative mechanisms, which dietary nitrate influences^[49]. Our results highlight the potential of a dietary approach

counteracting the compromised cardiovascular system. Further investigations in ischemic tissues applying the hind limb model in mice highlighted the potential of dietary nitrate in angiogenesis^[50]. A cytoprotective role of nitrite in the setting of myocardial, liver, kidney, and brain ischemia-reperfusion (I/R) injury has previously been demonstrated and continuous pharmacological intervention with nitrite injections increases vascular density in hind limb models^[51-55]. We could show that dietary nitrate supplementation strongly augments perfusion recovery in chronic hind limb ischemia *in vivo* *via* a significant increase in capillary density. This improvement was associated with an increase in circulating nitrite concentrations, an elevated mobilization of CD34⁺/Flk-1⁺ cells and migration of bone marrow-derived CD31⁺/CD45⁻ cells into ischemic tissue^[50]. The mobilization of circulating angiogenic cells following dietary nitrate supplementation was recently supported by a phase I clinical study^[56]. A further effect of the dietary nitrate supplementation to drinking water was an attenuated apoptosis in myoblasts in chronic hind limb ischemia. Intriguingly, disruption of the nitrate-NO pathway by chronic eradication of the oral bacteria completely abolished beneficial effects of dietary nitrate supplementation and likewise effectively suppressed circulating nitrite levels, which were observed after intake of nitrate-rich food or dietary nitrate supplementation in drinking water. In line with these findings, the results of this study further point to a distinct contribution of dietary nitrate supplementation on tissue viability. Dietary nitrate ameliorated the remarkable capacity of adult skeletal muscles to regenerate myofibers after damage. This rapid repair process is mainly carried out by satellite cells (SCs) with contribution of NO^[57,58]. Quiescent SCs become active and proliferate upon injury and display the regenerative capacity of the muscle. Committed daughter cells, the myoblasts, continue to proliferate followed by definite differentiation as initialized by a coordinated cellular signaling^[59]. The precise pathways that are influenced by the nitrate-nitrite-NO pathway are under intensive investigations and not fully understood yet.

In summary dietary nitrate supplementation increases the regenerative capacity of ischemic tissue and may offer an attractive nutrition-based strategy to improve ischemia-induced revascularization.

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Is there a rationale for short cardioplegia re-dosing intervals?

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Abstract

While cardioplegia has been used on millions of patients during the last decades, the debate over the best technique is still going on. Cardioplegia is not only meant to provide a non-contracting heart and a field without blood, thus avoiding the risk of gas emboli, but also used for myocardial protection. Its electromechanical effect is

easily confirmed through direct vision of the heart and continuous electrocardiogram monitoring, but there is no consensus on the best way to assess the quality of myocardial protection. The optimal approach is thus far from clear and the considerable amount of literature on the subject fails to provide a definite answer. Cardioplegia composition (crystalloid *vs* blood, with or without various substrate enhancement), temperature and site(s) of injection have been extensively researched. While less frequently studied, re-dosing interval is also an important factor. A common and intuitive idea is that shorter re-dosing intervals lead to improved myocardial protection. A vast majority of surgeons use re-dosing intervals of 20-30 min, or even less, during coronary artery bypass graft and multidose cardioplegia has been the "gold standard" for decades. However, one-shot cardioplegia is becoming more commonly used and is likely to be a valuable alternative. Some surgeons prefer the comfort of single-shot cardioplegia while others feel more confident with shorter re-dosing intervals. There is no guarantee that a single strategy can be safely applied to all patients, irrespective of their age, comorbidities or cardiopathy. The goal of this review is to discuss the rationale for short re-dosing intervals.

Key words: Myocardial protection; Del Nido cardioplegia; Continuous cardioplegia; Intermittent cardioplegia; Single-shot cardioplegia; Multidose cardioplegia; Crystalloid cardioplegia; Blood cardioplegia; Custodiol®; Histidine-ketoglutarate-tryptophan

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Core tip: During myocardial ischemia, cardioplegia is the preferred method of myocardial protection. However, decades after its implementation, there is still no consensus on the optimal re-dosing interval. Shorter re-dosing (15-30 min) has been preferred to longer intervals (45-60 min), but the choice of one approach over another relies more on the surgeon's preference than on clear advantages. As the interest for one-shot

cardioplegia has been increasing recently, we intend to discuss the rationale, if any, for short cardioplegia re-dosing interval.

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INTRODUCTION

The successful curative treatment of certain congenital or acquired heart diseases requires a method allowing direct vision of the open heart. For decades, cardioplegia has been the preferred and best method to provide a non-contracting heart and a field without blood. However, despite its use on millions of patients, the debate over the ideal cardioplegic technique is still going on. Strictly speaking, cardioplegia means paralysis of the heart: the visual appearance of standstill heart and continuous monitoring of the electrocardiogram allow for an easy control. But cardioplegia is also the major component of myocardial protection. There is no consensus on the best tools for myocardial protection assessment. First, there is no way to routinely perform real-time myocardial protection evaluation. Second, myocardial protection is clinically assessed post-operatively *via* a number of indirect factors such as troponin I/T or creatine kinase MB levels, ischemic electric signs on EKG or myocardial infarction, stroke, atrial fibrillation, myocardial function on echo, low cardiac output state, inotropic support, intra-aortic balloon pump, extracorporeal membrane oxygenation, as well as time to extubation and length of stay in intensive care unit^[1-6]. These indirect factors are affected by several causes including surgery, anesthesia, intensive care, and, in the case of complex cardiopathies, resultant physiology following surgical palliation or cure. It is impossible to determine the role played by each cause. Myocardial protection is a concept without clear and specific clinical signs.

Experimental works on cardioplegia aiming to assess myocardial protection use time-consuming, invasive and often expensive approaches such as continuous monitoring of intra-myocardial pH, myocardial lactate production, or myocardial biopsy for ATP dosage, which are unrealistic in routine clinical practice^[7-9]. However, there is a huge amount of publications on cardioplegia trying to find the best technique for cardiac arrest and myocardial protection. The composition of the cardioplegic solution (blood vs crystalloid, with various substrate enhancement), the injection site(s) (antegrade, retrograde or both), the temperature (cold, tepid or warm) and the re-dosing intervals were the main factors explored. The large number of combinations of these factors makes studies difficult to compare.

Furthermore, the type of cardiac repair interferes with re-dosing intervals. During coronary artery surgery, re-dosing is usually done after each distal anastomosis, *i.e.*, every 10 ± 5 min, but 60-min re-dosing intervals have also been described^[10]. During valvular or complex procedures, re-dosing intervals are variable from one surgeon to another^[2,11-22].

Shorter re-dosing intervals could be a more critical factor than total aortic cross-clamp time in terms of myocardial protection, which would imply that shorter intervals lead to improved protection^[23]. This intuitive observation was supported by experimental and clinical works^[24-26]. Unexpectedly though, other works suggested that single-shot cardioplegia was better or at least equivalent to multidose cardioplegia^[27-30].

We have learned from heart transplantation that myocardial ischemia may be tolerated for several hours^[31]. The upper limit for donor heart ischemic time, which was 4 h in the early days of heart transplant surgery, was progressively increased to 6-8 h^[32]. Successful heart transplantation with a donor heart ischemic time of 13 h was published, but in this clinical case, the follow-up was limited to three months^[33]. Cold blood cardioplegia^[34], histidine-tryptophan-ketoglutarate (HTK) solution or Custodiol[®], a solution developed for organ preservation in transplantation^[35,36], and Del Nido cardioplegia were all proposed for single-shot cardioplegia^[37,38].

The debate over the best re-dosing interval is not new but while multidose cardioplegia has been the "gold standard" for decades, one-shot cardioplegia is becoming more commonly used and is likely to be a valuable alternative. This is particularly true for minimally invasive vascular surgery^[39,40]. However, the use of single-shot cardioplegia relies more on the surgeons' preference than on true advantages in terms of immediate outcomes^[41].

The goal of this work is to discuss the rationale for short re-dosing intervals.

FROM CONTINUOUS BLOOD CARDIOPLEGIA TO SINGLE-SHOT CARDIOPLEGIA *VIA* INTERMITTENT CARDIOPLEGIA

From a theoretical point of view, a continuous injection of normothermic oxygenated blood containing the arresting agent is the best way to perform cardiac arrest and optimal myocardial protection. Gott *et al*^[42] proposed this technique of aerobic arrest in 1957, but it was long to establish itself. In 1989, a clinical case was published on retrograde continuous blood cardioplegic warm infusion *via* the coronary sinus during mitral surgery. After a cross-clamp time of 393 min, the patient was easily weaned from bypass without intra-aortic balloon pump or inotropic support. It is noteworthy that the cardiac output was higher

immediately after bypass than before surgery^[43]. The same team used the same technique of aerobic arrest in 308 consecutive procedures with warm surgery. Twenty-two of these patients needing an aortic cross-clamp time greater or equal to 3 h had excellent results^[44]. The technic never became really popular for several reasons. First, it is technically more demanding and blind insertion of the cannula into the coronary sinus may be uneasy. Second, drawbacks were described, such as hyperkalemia, coronary sinus damage, catheter misplacement, migration or dislodgment in the right atrium^[45-47]. Third, a major concern was the distribution of cardioplegia to the right hypertrophic ventricle or when the right coronary vein is next to the coronary sinus^[48,49]. Last, for coronary artery bypass graft, a micro blower delivering compressed air is often needed for completion of distal anastomosis^[50].

To overcome constraints related to retrograde continuous cardioplegia, intermittent, antegrade warm blood cardioplegia was proposed as an alternative. From November 5, 1990 to December 31, 1992, a study was conducted at three adult cardiac surgical centers of the University of Toronto on 720 patients operated on for aortocoronary. Warm blood cardioplegia was interrupted during 5-15 min for enhancing visualization during distal anastomosis. The longest ischemic time, equivalent to the longest time off cardioplegia (LTOC, in minutes per patient) and the total duration of ischemic time as a proportion of the aortic cross-clamp time were collected. The quality of myocardial protection was assessed on post-operative mortality at 30 d (or in-hospital deaths for patients with a length of stay > 30 d), myocardial infarction by enzyme criteria and low-output syndrome. The authors postulated that short periods of normothermic ischemia should be well tolerated if followed by adequate cardioplegic reinfusion. Their study aimed to evaluate the relation between intermittency of cardioplegia and cardiac events.

The results suggested that the longest ischemic time was more important than the cumulative ischemic time and that prolonged LTOC > 13 min was a risk factor for adverse outcomes^[23]. For a vast majority of surgeons, thirteen minutes is a reasonable time for distal anastomosis construction. In summary, intermittent, antegrade warm blood cardioplegia is a valuable alternative to antegrade cold blood cardioplegia when the time off cardioplegia remains under 13 min. In a clinical scenario, this time off cardioplegia is more realistic than the maximal cardioplegia halted time of 5 min proposed by Menasché *et al*^[51] in 1992. In 1995, a simpler technique was described, allowing a re-dosing interval of 15 min. Two groups of 250 patients undergoing elective coronary artery bypass grafting were compared using either intermittent warm blood cardioplegia or intermittent cold blood cardioplegia. For the surgeon, the two types of cardioplegia were similarly demanding. After an initial injection in the aortic root, re-dosing was done after each distal anastomosis or after 15 min. This maximal ischemic

time was chosen to be long enough for a difficult distal anastomosis. The outcome was superior in the warm group, with significantly less cardiac-related deaths and a dramatic decrease in morbidity. There was lactate washout in both groups 1 min after aortic cross-clamping, but lactate production was still present 20 min after reperfusion in the cold group, while there was evidence of normal lactate extraction in the warm group, suggesting a rapid restoration of a normal metabolism. In summary, in this study, intermittent warm cardioplegia was superior to intermittent cold blood cardioplegia, with lower morbidity, mortality and decreased length of stay in ICU and hospital^[52]. The re-dosing interval was later increased to 20-30 min without complication^[53,54]. Other authors confirmed the good tolerance of 30-min warm ischemia time in 1996 and 2000^[55,56]. In 2009, a comparison between intermittent warm blood cardioplegia and single-shot warm blood cardioplegia was published. The study was done from January 2001 to December 2006 and included 4014 patients: 1708 had single-shot cardioplegia and 2306 had intermittent warm blood cardioplegia with a 20-min re-dosing interval. There was statistical insignificance for mortality, intra or postoperative intra-aortic balloon pump, postoperative inotropics or postoperative arrhythmia. Single-shot had a favorable effect on post-operative myocardial infarction and an unfavorable effect on intraoperative inotropics and postoperative dialysis. Authors found that the first shot of warm cardioplegia may safely exceed 20 min and, in case of short cross-clamping (35 to 40 min), cover the whole cross-clamping time without increased risk^[57]. However, there is probably an individual threshold for tolerance due to pre-, intra- and post-operative factors.

Interestingly, the same evolution in re-dosing interval was described in pediatric cardiac surgery. We introduced this technique in 2002 with a cardioplegia protocol identical to the one described in adults in 1995: warm oxygenated blood was diverted from the arterial line via a roller pump and St Thomas' solution was added downstream the pump with an electrical syringe. The blood to arresting agent ratio was 60:1, therefore the hydric balance of cardioplegia was negligible and it was named microplegia^[58]. The re-dosing interval was 15 min and a nomogram was developed for volume and duration of the first injection and re-injection^[59].

Another group implemented the same approach of intermittent warm blood microplegia, except for the re-dosing interval (10 min). They also suggested, on a group of arterial switch, that intermittent warm blood microplegia was a valid alternative to intermittent cold blood cardioplegia^[60].

Following our initial experience on 1400 pediatric patients, we demonstrated that the technique was safe for long aortic cross-clamp time on 38 patients with a cross-clamp time > 90 min^[61]. Using the same protocol for cardioplegia with a 15-min re-dosing interval, a group from Brussels used cardiac biopsies to demonstrate a significant increase in myocardial ATP

stores during the first cardioplegic ischemic time and a return to initial values after coronary reperfusion. This reproducible method was considered safe, with low morbidity and mortality and a similar quality of cardiac repair^[62].

The technique was gradually implemented in several European units and more than 10000 cases with re-dosing intervals varying from 10 to 25 min were published in 2010^[63]. As in adult surgery, intervals gradually increased between 2001 and 2013, from 10 to 35-40 min, without any adverse effect^[64,65]. However, the major issue remains: how long is too long? It is likely that re-dosing intervals cannot be indefinitely increased without inducing adverse cardiac events.

The merits of single-dose vs multidose cardioplegia in the infant heart were described in animal experiments several decades ago^[27-30], but there was a certain lack of enthusiasm for its clinical use. However, in 1988, a clinical study was published, comparing two groups of arterial switch operated on with single-dose or multidose cold blood cardioplegia with a 15-min re-dosing interval. The incidence of mortality and ST-T changes was significantly higher in the multidose group. The conclusion was that single-dose is as good as, or better than, multidose cardioplegia^[34]. In 1989, another group confirmed the efficiency of single-shot cold crystalloid cardioplegia in arterial switch procedure^[66].

There is increasing interest in HTK or Custodiol® single-dose cardioplegia. In 1998, Sakata suggested that single high-volume HTK provided a more adequate myocardial protection for mitral surgery than multidose cold blood cardioplegia^[67]. In 2001, the benefit of single-dose HTK cardioplegia over multidose cold crystalloid cardioplegia was also suggested in a clinical work comparing two groups of 15 patients. The incidence of arrhythmia and inotropic support decreased significantly in the HTK group and so did the ICU length of stay^[68]. The feasibility and safety of single-shot HTK cardioplegia was suggested in adult and pediatric surgery^[35,36,69,70]. A new modified HTK solution named Custodiol-N, likely to enhance the organ protective potential of the previous solution, was tested on animals during a 60-min hypothermic cardiac arrest^[71,72].

Del Nido cardioplegia has been used for two decades at the Boston Children's Hospital, generally in a single-dose fashion^[73]. Its use was expanded to adult surgery, triggering a growing interest in this solution^[74]. Recent works in adults and pediatrics suggest that it is a safe and valuable alternative to conventional multidose cardioplegia^[37].

WHERE ARE WE? WHERE DO WE GO FROM HERE?

A review of the literature published prior to 1975

concluded that a 20-min ischemic period at 32 °C could be tolerated by the heart without the need for inotropic support, while the anoxic safe period was extended to 30 min when temperature was lowered to 16 °C-20 °C^[75]. Forty years later, despite ample evidence that the ischemia time can be safely increased, even during warm surgery, a vast majority of surgeons use re-dosing intervals of 20-30 min, or even less, during coronary artery bypass graft. Some surgeons prefer the comfort of single-shot cardioplegia while others feel more confident with shorter intervals. How can we explain the myocardial tolerance to anoxia? Is it due to the composition of the arresting solution, the temperature of cardioplegia, or both?

We have seen that for cold and warm blood cardioplegia, short-term outcomes are equivalent with identical re-dosing intervals, just as they are identical for cold blood and cold crystalloid cardioplegia. The temperature is likely to have little effect, if any, but studies focused on aortic cross-clamp times < 90 min. We have also seen that different cardioplegic solutions can be safely used for hypothermic single-shot cardioplegia^[34-38]. The composition did not seem to be critical, or, at least, different solutions can be used for single-shot cardioplegia and comparisons between these solutions are missing.

It is probably a fool's errand to look for a universal gold standard. The best cardioplegia with optimal re-dosing interval is likely to vary with different patients having different pathologies, and different aortic cross-clamp time. Dr J Vaage's stated: "If you had a clamp time of < 60 min, you could actually use whatever cardioplegia or myocardial protection you wanted, you could always get to the shore, so to say"^[57]. It is probably true, but we are still looking for the optimal cardioplegia, for simple and complex cardiopathies. The goal is not just to get to the shore, but also to use the best, simplest, fastest and cheapest way to deliver optimal results to our patients. Furthermore, we intend to prevent cardiac events not just during the initial outcome - the one that allows getting to the shore - but also during mid- and long-term outcomes^[76]. Myocardial fibrosis could be a late side effect of cardioplegia^[77]. This is more challenging and less extensively studied.

CONCLUSION

This review does not solve the issue on the rationale for short-term re-dosing interval. However, facts are facts, and many works suggest or demonstrate that short-term re-dosing intervals are not critical for every patient. There is probably no rationale to use the same re-dosing interval for all patients needing aortic cross-clamping for surgical cardiac repair. Despite the lack of consensus on cardioplegia composition, temperature, way of administration and re-dosing interval, the outcomes of adult and pediatric cardiac surgery are continuously improving.

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Role of platelet-rich plasma in ischemic heart disease: An update on the latest evidence

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Abstract

Myocardial infarction is the most common cause of congestive heart failure. Novel strategies such as directly reprogramming cardiac fibroblasts into cardiomyocytes are an exciting area of investigation for repair of injured myocardial tissue. The ultimate goal is to rebuild functional myocardium by transplanting exogenous stem cells or by activating native stem cells to induce endogenous repair. Cell-based myocardial restoration, however, has not penetrated broad clinical practice yet. Platelet-rich plasma, an autologous fractionation of whole blood containing high concentrations of growth factors, has been shown to safely and effectively enhance healing and angiogenesis primarily by reparative cell signaling. In this review, we collected all recent advances in novel therapies as well as experimental evidence demonstrating the role of platelet-rich plasma in ischemic heart disease, focusing on aspects that might be important for future successful clinical application.

Key words: Platelet-rich plasma; Ischemic heart disease; Myocardial infarction; Myocardial regeneration; Cardiac repair

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Core tip: Tissue regeneration requires precise coordination among endothelial, epithelial and mesenchymal morphogenesis. Growth factor-induced angiogenesis plays a key role in recovery from ischemic disease and organ regeneration. Recent studies show that stem-cells and PRP together have opened new horizons in the myocardial infarction treatment.

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INTRODUCTION

Coronary infarction is the most frequent cause of death globally^[1]. The loss of cells during ischemia and resultant fibrosis are the main reasons for cardiac failure^[2].

As cardiac muscle has very little potential to create new cells, methods of heart regeneration have been studied further as repair modalities for failing myocardium after acute coronary infarction or chronic ischemia^[3].

In this article, we investigate current advances and demonstrate approaches such as the upcoming challenges of platelet-rich plasma (PRP) application as well as opportunities to develop its role.

We have gathered all experimental and clinical studies in which PRP was used as a therapy post-MI, and have focused on aspects that might be important for future successful clinical application. The PubMed database was searched for articles using the terms "platelet-rich plasma" and keywords "ischemic heart disease", or "myocardial infarction", or "coronary disease".

NOVEL REGENERATIVE THERAPIES

The majority of patients survive a myocardial infarction (MI). Their outcome, however, is negatively influenced by several events, such as loss of viable cardiomyocytes due to a post-MI inflammatory response, eventually resulting in heart failure and/or death. Regenerating the human heart is a challenge that has engaged researchers around the globe almost a century. Although the human cardiac muscle has not been regenerated yet, decades of experimental progress have guided us onto a promising path^[4].

Stem cell approach has become a promising tool for cardiac regeneration^[5]. The main target is to repair functional myocardial tissue by implanting exogenous or by activating native stem cells.

Cardiac stem progenitor cells (CS/PCs) are one kind of adult stem cell with the ability to differentiate into heart lineages. Induced pluripotent stem cells (iPSCs) may differentiate into the needed cells in order to repair injured myocardium. These two types of stem cells play a key role in cardiac regeneration. Two main delivery modes of stem cells (percutaneous intramyocardial or intracoronary) are used today for patients with recent acute MI or ischemic cardiomyopathy^[6]. Other delivery routes, such as intravenous *via* coronary sinus or

peripheral veins and surgical have also been used with less success^[6].

While further studies intent to increase the efficacy of current approaches, experimental protocols using new methods such as exploiting paracrine effect and tissue engineering could enhance repair of injured human heart.

Various chemical methods, including both microRNA and anti-microRNA approaches, proteins and modified peptides demonstrate serious potential^[7].

Takahashi *et al*^[8] investigated pluripotent stem cells (iPSCs) revealing in a new horizon of cellular reprogramming in organ regeneration. It has been shown that iPSCs can be differentiated efficiently into multiple cell types that may be used in the future for regenerative strategies^[9].

Finally, transmyocardial laser revascularization (TMR) is a controversial therapeutic technique that relieves angina but can't create a significant effect on heart function^[10]. It improves the clinical status without confronting the underlying atherosclerotic disease. Therefore, TMR offers palliation and not cure^[10].

ROLE OF PRP

What is PRP

Autologous PRP is an increased amount of platelets in a small portion of plasma^[11]. This is why the term PRP is preferred to plasma-rich growth factors (PRGFs), platelet concentrate or platelet-rich gel. PRP is a source of autologous growth factors, including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), epithelial growth factor and transforming growth factor beta 1 (TGF- β 1), that is secreted by platelets in order to trigger the healing cascade^[11,12]. Structurally similar to the natural fibrin clot, it can be used as scaffold for cells infiltration and assembly of vascular networks.

One of the most crucial questions regarding methodology, refers to the ideal mechanisms of intramyocardial delivery of PRP. Surgical (epicardial) application is performed into ischemic areas with a thin needle, allowing for multiple injections within and especially around the infarct area. The other less invasive interventional delivery route is the transendocardial catheter injection.

PRP and neovascularization

Neovascularization plays a significant role post-ischemia regeneration and organ repair.

It has been reported that the mixture of angiogenic factors in an certain percentage is crucial for the creation of functional blood vessels^[13,14]. Angiogenesis-induced vessels, not only deliver nutrients and oxygen but also provide instructive regulatory signals to surrounding tissue affecting organ regeneration^[15,16].

Neovascularization involves multiple complex events such as the maturation and enlargement of size of the preexisting small vessels through vascular remodeling

(arteriogenesis), sprouting of pre-existing resident endothelial cells (angiogenesis) and the recruitment of bone marrow derived endothelial progenitor cells (vasculogenesis)^[17].

Angiogenesis involves both microvascular and macrovascular mechanisms. At the microvascular level, neovascularization is the genesis of capillaries, which, however, regress after pause of basic fibroblast growth factor (bFGF) triggering if pericytes are not gathered efficiently. Therefore, the stabilization of newly formed capillary networks by pericytes, known to be recruited by PDGF-BB, is crucial for therapeutic angiogenesis^[18]. The enhancement of blood vessel maturation is one of the main modalities implemented to treat such patients.

According to the above-mentioned issues, a limited portion of plasma enriched in platelets, is an attracting attention as a safe and cost-effective source of various growth factors^[19]. PRP, by containing these various cytokines, plays an important role in repairing damaged tissue^[20]. As we have already discussed, little is known about the mechanism of PRP-related regeneration of damaged tissue. Successful reperfusion of ischemic tissue depends not only on stimulation of angiogenesis but also on arteriogenic activity. Different growth factors in PRP have different roles in angiogenesis and restoration of blood flow following ischemia^[21]. It has also been shown that PRP effectively restores blood flow by significant increase of the number of capillaries (angiogenesis) as well as mature vessels (arteriogenesis) in the murine hind limb ischemia, which was confirmed by double staining with endothelial marker and pericytes marker respectively^[22].

The VEGF, TGF- β and PDGF-BB, have a significant effect as pro-angiogenic stimulators. Evidence shows that PDGF-BB has a potential as arteriogenic factor, promoting differentiation of endothelial cells^[17,23]. VEGF is known to trigger post-ischemia neovascularization^[24], and TGF- β enhances cell mitosis^[25]. Other reports, however, demonstrated that many growth factors, such as TGF- β and PDGF-BB, inhibit the angiogenic ability of bFGF^[26]. These studies evaluated the angiogenic impact using combined solutions of growth factors. Within growth factors, PDGF-BB is the one that allows blood vessels to grow functionally^[27,28]. According to this fact, multiple releases of prostaglandin F₂ alpha metabolite and bFGF will improve the maturation of blood vessels. It is also demonstrated that the mixed release of VEGF and PDGF promoted the maturation of newly created blood vessels against VEGF release alone^[29].

VEGF is the principle stimulatory factor of angiogenesis after ischemia^[30]. However, VEGF enhances the creation of unstable capillaries^[31]. It promotes mural cell accumulation, presumably through the release of PDGF-BB. It also causes endothelial cell proliferation and migration, resulting in capillary sprouting or angiogenesis. Lastly, it recruits hematopoietic stem cells to ischemic site from bone marrow *via* circulation.

Basic FGF and PDGF are chemoattractants to smooth

muscle cells. Those are also causes of growth of smooth muscle cell as well as enlargement of vessel (formation of mature vessels or arteriogenesis). These stem cells produce a capillary plexus and eventually form mature vessels. All of these together cause formation of new vessels for vascular supply in ischemic limbs. So, combined administration of different growth factors may lead to potentially therapeutic angiogenesis^[31,32]. PDGFR-beta are needed for vascular stabilization by gathering of mesenchymal progenitors. Absence of PDGF leads to fragile neovasculature^[33], indicating that PDGF-BB has potent arteriogenic effect after ischemia.

Insulin-like growth factor-1 (IGF-1) triggers angiogenesis and myogenesis, the pro-angiogenic impact, however, seems to be less efficient than that of other angiogenic factors^[34]. Finally stromal cell-derived factor 1 (SDF-1) has direct or indirect (*via* certain secondary cytokines) effects on endogenous angiogenesis^[35]. There is also cross talk between VEGF and bFGF; bFGF and PDGF-BB to induce post-ischemia angiogenesis^[36]. Finally, inhibition of Ang1-Tie2 signaling suppresses angiogenic ability of the PRP *in vivo* and PRP-induced angiogenesis *in vitro*.

Experimental evidence

Inspite of a large amount of evidence on PRP's usefulness, limited work has been conducted using PRP in myocardium.

Gallo *et al.*^[37] evaluated histological and morphological impact of the injection of PRP in ischemic sheep myocardium. Noteworthy was the formation of new blood vessels in hematoxylin-eosin-stained sections and factor VIII in PRGF-treated myocardia. According to this report, implantation of platelet growth factors in previously infarcted sheep hearts promoted neovascularization.

Hargrave *et al.*^[38] utilized the technique of nanosecond pulsed electric fields (nsPEF) in order to determine the efficiency of a protocol involving the *in vivo* treatment of the ischemic and reperfused myocardial cells in culture with PRP in rabbits. The left ventricle had faster contraction/relaxation rate and the size of the infarct was diminished in PRP-treated hearts compared to saline-treated. Mitochondrial depolarization and reactive oxygen species (ROS) production were reduced in PRP-treated cells. These facts show that PRP contributes in cardiac protection by stabilizing the mitochondria and reducing ROS generation of the ischemic-reperfused heart.

Mishra *et al.*^[39] used permanent ligation, in order to find out whether PRP, enhances cardiac function in an ischemia-reperfusion model as measured by left ventricular ejection fraction (LVEF).

Compared with phosphate-buffered saline (PBS) controls, PRP-treated animals had a higher LVEF after ischemia, while PRP-treated animals who underwent ischemia-reperfusion had higher LVEF after ischemia. Histology revealed increased granulation in the control group vs the PRP group. In the same time, magnetic resonance imaging (MRI) revealed a positive impact

Table 1 Summary of the effects of platelet-rich plasma in ischemic heart disease

Ref.	Type of study	Animal model	Delivery method	Effect
Gallo <i>et al</i> ^[37]	Experimental	Sheep	Implantation	Increased formation of new vessels
Hargrave <i>et al</i> ^[38]	Experimental	Rabbit	Intramyocardial injection	Reduced reactive oxygen species generation Stabilized the mitochondria of the ischemic/reperfused heart
Mishra <i>et al</i> ^[39]	Experimental	Murine (Mouse)	Intramyocardial injection	Higher left ventricular ejection fraction after ischemia
Vu <i>et al</i> ^[40]	Experimental	Porcine	Intramyocardial injection	Attenuated adverse cardiac remodeling
Yu <i>et al</i> ^[41]	Experimental	Murine (Rat)	Intramyocardial injection	Decreased infarct size Increased ventricular wall thickness Improved cardiac function and reperfusion
Li <i>et al</i> ^[42]	Experimental	Murine (Rat)	Intramyocardial injection	Limitation of ventricular expansion, Attenuated myocardial hypertrophy in the noninfarct region Facilitated angiogenesis and arteriogenesis in the infarct.
Sun <i>et al</i> ^[43]	Experimental	Murine (Rat)	Intramyocardial injection	Improved LV performance
Wehberg <i>et al</i> ^[10]	Clinical	-	Intramyocardial injection	More efficacious at relieving angina Improved myocardial function

of PRP on left ventricular function in both ligation and ischemia/reperfusion murine model.

Vu *et al*^[40] attempted a translational, large-scale restorative but minimally invasive approach in a porcine model, aiming at both structurally stabilizing the LV wall and improving function following ischemic injury.

In this study, a combination of PRP, anti-oxidant and anti-inflammatory factors with intramyocardial injection of hydrogel had the potential to structurally and functionally enhance the injured heart muscle while attenuating adverse cardiac remodeling after acute myocardial infarction.

Yu *et al*^[41] conducted a study in order to investigate the impact of direct myocardial injection of PRP on cardiac function, ventricular remodeling and myocardial perfusion in rats. EF was significantly higher and myocardial perfusion significantly improved in the PRP group. Histological examination also confirmed that PRP treatment can decrease infarct size, increase ventricular wall thickness and improve cardiac function.

Li *et al*^[42] demonstrated that a platelet-mediated paracrine effect may accelerate the healing process after myocardial infarction in rats. According to this experimental protocol, implantation of thrombin-activated PRP into the ischemic myocardium lead in enhancement of ventricular remodeling and accelerated repair, as shown through the limitation of ventricular expansion, facilitation of neovascularization, arteriogenesis in the infarct and attenuation of myocardial hypertrophy in the noninfarct part.

Sun *et al*^[43] reported that adipose-derived mesenchymal stem cells (ADMSC) in a platelet-rich fibrin (PRF) scaffold were superior to direct ADMSC injection in enhancing LV function and diminishing LV remodeling in a post-MI animal model.

PRP and TMR

TMR induces a reconfiguration of the microcirculation, with blood shunted from epicardial to endocardial areas. Current literatures propose a synergistic effect among TMR and exogenously delivered growth factors.

Wehberg *et al*^[10] assessed the impact of PRP intramyocardial injection combined with TMR. Angina relief was similar in both groups (TMR-alone and TMR + PRP); the TMR + PRP group, however, had a decreased average angina score and more were angina free compared to the TMR-alone group. EF improved significantly in the TMR + PRP group compared to the TMR-alone group. This study suggested that intramyocardial injection of PRP and TMR may be more effective at treating angina and enhancing heart function than TMR alone.

All above-mentioned studies are summarized in Table 1.

CONCLUSION

While stem-cell therapies and cellular reprogramming hold promise, the use of PRP emerges as an additional modality for repairing cardiac muscle.

Development of tissues is based on accurate coordination among epithelial, mesenchymal and endothelial morphogenesis. Furthermore, growth factor-induced angiogenesis is significant in organ regeneration after ischemia. Recent tissue engineering researches suggest that cells and PRP-derived growth factors together into biomaterials have opened new horizons in the myocardial infarction treatment.

PRP should be investigated for its potential regenerative properties and its use as a therapeutic modality for ischemic myocardium.

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Basic Study

Enhanced caveolin-1 expression in smooth muscle cells: Possible prelude to neointima formation

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Abstract

AIM: To study the genesis of neointima formation in pulmonary hypertension (PH), we investigated the role of caveolin-1 and related proteins.

METHODS: Male Sprague Dawley rats were given monocrotaline (M, 40 mg/kg) or subjected to hypobaric hypoxia (H) to induce PH. Another group was given M and subjected to H to accelerate the disease process (M + H). Right ventricular systolic pressure, right ventricular hypertrophy, lung histology for medial hypertrophy and the presence of neointimal lesions were examined at 2 and 4 wk. The expression of caveolin-1 and its regulatory protein peroxisome proliferator-activated receptor (PPAR) γ , caveolin-2, proliferative and anti-apoptotic factors (PY-STAT3, p-Erk, Bcl-xL), endothelial nitric oxide synthase (eNOS) and heat shock protein (HSP) 90 in the lungs were analyzed, and the results from M + H group were compared with the controls, M and H groups. Double immunofluorescence technique was used to identify the localization of caveolin-1 in

pulmonary arteries in rat lungs and in human PH lung tissue.

RESULTS: In the M + H group, PH was more severe compared with M or H group. In the 4 wk M+H group, several arteries with reduced caveolin-1 expression in endothelial layer coupled with an increased expression in smooth muscle cells (SMC), exhibited neointimal lesions. Neointima was present only in the arteries exhibiting enhanced caveolin-1 expression in SMC. Lung tissue obtained from patients with PH also revealed neointimal lesions only in the arteries exhibiting endothelial caveolin-1 loss accompanied by an increased caveolin-1 expression in SMC. Reduction in eNOS and HSP90 expression was present in the M groups (2 and 4 wk), but not in the M + H groups. In both M groups and in the M + H group at 2 wk, endothelial caveolin-1 loss was accompanied by an increase in PPAR γ expression. In the M + H group at 4 wk, increase in caveolin-1 expression was accompanied by a reduction in the PPAR γ expression. In the H group, there was neither a loss of endothelial caveolin-1, eNOS or HSP90, nor an increase in SMC caveolin-1 expression; or any alteration in PPAR γ expression. Proliferative pathways were activated in all experimental groups.

CONCLUSION: Enhanced caveolin-1 expression in SMC follows extensive endothelial caveolin-1 loss with subsequent neointima formation. Increased caveolin-1 expression in SMC, thus, may be a prelude to neointima formation.

Key words: Endothelial cells; Neointima; Pulmonary hypertension; Smooth muscle cells

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Core tip: Neointima in pulmonary hypertension (PH) is associated with poor prognosis. Caveolin-1, a cell membrane protein has a critical role in PH. We investigated the association of caveolin-1 and neointima formation in monocrotaline (MCT) + hypoxia-treated rats, and in human PH lung sections. The progressive caveolin-1 reduction in endothelial cells is followed by an increased caveolin-1 expression in smooth muscle cells (SMC). In human PH as well as in the MCT + hypoxia model, neointima was observed only in the arteries exhibiting an increased caveolin-1 expression in SMC. Thus, the increased caveolin-1 expression in SMC may in part, facilitate neointima formation.

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INTRODUCTION

Pulmonary hypertension (PH) is a rare, but a progressive disease with a high morbidity and mortality rate. Although considerable progress has been made in the field; the pathogenesis of PH, however, is not yet fully understood, which makes the design of preventive and curative treatment a daunting challenge. The advances in therapeutic modalities have improved the life expectancy as well as the quality of life; the pulmonary vascular remodeling, however, remains progressive^[1]. A number of diverse diseases can develop PH, and several PH-associated gene mutations are known to significantly increase the risk of familial PH^[2,3]. Irrespective of the underlying disease, severe PH is typically characterized by endothelial dysfunction, impaired vasodilatation, increased vasoconstriction, cell proliferation, medial wall thickening, PH and right ventricular hypertrophy (RVH)^[4]. The development of neointima and plexiform lesions in pulmonary arteries associate with poor outcomes although whether or not they are causative of disease or result from an abnormal hemodynamic milieu remains unclear in the human PH^[5].

In the monocrotaline (MCT) model, endothelial caveolin-1 loss and the activation of proliferative and anti-apoptotic pathways are observed before PH becomes evident. Concurrent loss of several endothelial cell (EC) membrane proteins including PECAM-1, soluble guanylate cyclase and Tie2 is suggestive of an extensive EC membrane damage. At 2 wk post-MCT, PH and RVH are observed, accompanied by a further disruption of EC as indicated by the loss of cytosolic proteins such as heat shock protein (HSP) 90, Akt and I κ B- α ^[6-8]. Importantly, preventive measures restore endothelial caveolin-1 resulting in the inhibition of proliferative pathways and attenuation of PH^[9,10]. Caveolin-1 is a major scaffolding protein of caveolae (50-100 nm), a subset of lipid rafts in the plasma membrane of a number of different cell types including EC and smooth muscle cells (SMC). It plays a pivotal role in maintaining vascular homeostasis. It directly interacts with transducing molecules within caveolae and stabilizes them in an inactive form. It regulates cell proliferation, apoptosis, cell differentiation, cell cycle, and also eNOS function^[11-13].

The presence of pulmonary arterial hypertension (PAH) in patients with CAV-1 mutation associated with reduced endothelial caveolin-1 expression, further supports a critical role of caveolin-1 in the lung vasculature^[14,15]. Importantly, the loss of endothelial caveolin-1 and vWF accompanied by an increased caveolin-1 expression in SMC has recently been reported in children and adults with PAH associated with drug toxicity, congenital heart disease and idiopathic PAH (IPAH)^[16-18]. Furthermore, pulmonary arterial SMC from the patients with IPAH revealed increased capacitative Ca²⁺ entry and DNA synthesis; both could be attenuated by silencing caveolin-1^[18]. Thus, caveolin-1 switches from being an anti-proliferative to a pro-proliferative factor. Interestingly, the dual role of caveolin-1 is a known phenomenon in cancer^[19].

Studies with rat models of PH using “VEGF receptor blocker (Sugen) + hypoxia”^[20], MCT + pneumonectomy^[21] and MCT + hypoxia^[22] have shown severe PH with neointima and plexiform lesions, closely mimicking human PH. In these models, underlying EC damage is an important initial phase. We hypothesized that the extensive EC damage and/or loss might be a prerequisite for the increased caveolin-1 expression in SMC and subsequent development of neointima. To test this hypothesis, we treated rats with MCT and exposed them to hypobaric hypoxia (MCT + hypoxia) to accelerate the disease process. Hemodynamic data, lung histopathology, the expression of caveolin-1, and proliferative and anti-apoptotic factors, endothelial nitric oxide synthase (eNOS) and HSP90 proteins were examined. We evaluated the expression of caveolin-2 because it co-localizes with caveolin-1^[23], and the expression of peroxisome proliferator-activated receptor (PPAR) γ , because it regulates caveolin-1 expression^[24,25], and its loss is implicated in the pathogenesis of PH^[26,27]. In addition, we examined caveolin-1 expression in the lung tissue from patients with IPAH and heritable PAH (HPAH).

MATERIALS AND METHODS

Male Sprague-Dawley rats (150-175 g, Charles River Wilmington, MA) were maintained at 22°C on a 12 h light and dark cycle in the Animal Facility. They were allowed to acclimatize for 5 d, with free access to laboratory chow and water. The Protocols were approved by the Institutional Animal Care and Use Committee at New York Medical College (IACUC # 4-1-0113), and conform to the guiding principles for the use and care of laboratory animals of the American Physiological Society, and the National Institutes of Health. Rats were divided into 4 groups: Gr1, Control rats maintained in room air; Gr2, rats received MCT (40 mg/kg, sc), and kept in room air; Gr3, rats subjected to hypobaric hypoxia (atmospheric pressure 380 mmHg); and Gr4, rats received MCT 40 mg/kg and were subjected to hypobaric hypoxia starting on day 1. The hypoxia chamber was opened twice per week for 15 min to weigh the rats, replenish food and water, and to provide clean bedding similar to the other rats in room air. At the end of 2 and 4 wk, these rats were studied.

Human lung tissue was obtained from PAH patients at the time of post-mortem autopsy or lung transplantation; control tissue was obtained from healthy subjects who died due to traumatic injuries. Vanderbilt Pulmonary Hypertension Research Cohort study participants were recruited *via* the Vanderbilt Pulmonary Hypertension Center. The Vanderbilt University Medical Center Institutional Review Board approved all study protocols (IRB #9401). All participants, or their surrogate custodians as appropriate, gave informed written consent to participate in genetic and clinical studies. PAH was defined either by autopsy results showing plexogenic pulmonary arteriopathy in the absence of other causes

such as congenital heart disease, or by clinical and cardiac catheterization criteria. These criteria included a mean pulmonary artery pressure ≥ 25 mmHg with a pulmonary capillary wedge or left atrial pressure ≤ 15 mmHg, and exclusion of other causes of PH in accordance with accepted international standards of diagnostic criteria^[2]. HPAH was considered the type of PAH if a subject met one or both of the following criteria: (1) family history of two or more subjects with confirmed PAH according to international standards of diagnostic criteria; or (2) detection of a mutation in a PAH-specific gene, such as *BMPR2*. The majority of lung tissue specimens available for this study from PAH patients were from subjects deceased prior to the discovery of the *BMPR2* gene and other genes that could be considered PAH-specific genes which are mutated in association with HPAH. Included in this study were 7 patients: 3 with IPAH and 4 with HPAH. The age ranged from 29 to 55 years except for one patient who was 6 years old diagnosed with HPAH.

Chemicals and antibodies

All chemicals including MCT were purchased from Sigma Aldrich, St Louis, MO. Antibodies: caveolin-1 α (sc894), PPAR γ (sc7273), HSP90 (sc13119) purchased from Santa Cruz laboratories, Santa Cruz, CA. PY-STAT3 (Tyr705, 9145), Bcl-xL (2764), p-Erk (Thr202/Tyr204, 4370), and Erk (4695) from Cell Signaling, Beverly, MA, β actin (A5441) and α -actin (C6198) from Sigma, caveolin-2 (610684), eNOS (610297) and STAT3 (610190) from BD Transduction, Palo Alto, CA.

Measurement of right ventricular systolic pressure

Rats anesthetized with pentobarbital (60 mg/kg, *ip*), were ventilated through a tracheostomy (roughly equivalent to 70-80 breaths/min)^[6]. A thoracotomy was performed; and right ventricular systolic pressure (RVSP) measured with a small needle attached to a tubing (PE50). After perfusing the lungs with normal saline, heart and lungs were removed. Right lung was frozen and stored at -80°C. The heart and the left lung were kept in 10% buffered formaldehyde.

Estimation of right ventricular hypertrophy

The ratio of the right ventricle (RV) and the left ventricle including septum (LV) was used to assess right ventricular hypertrophy (RVH)^[6,7]. In addition, the ratio of RV (mg)/final body weight (FBW, g) and the ratio of LV (mg)/FBW (g) were calculated.

Estimation of protein expression

Proteins (50-100 μ g) from lung supernatants were used to examine the expression of proteins of interest^[6,7]. The antibodies used were caveolin-1 (1:5000), Caveolin-2 (1:500), PPAR γ (1:100), PY-STAT3 (1:200), Bcl-xL (1:200), p-Erk (1:2000), eNOS (1:400), or HSP90 (1: 3000). Loading protein was evaluated using β actin (1:10000), STAT3 (1: 2000) or Erk

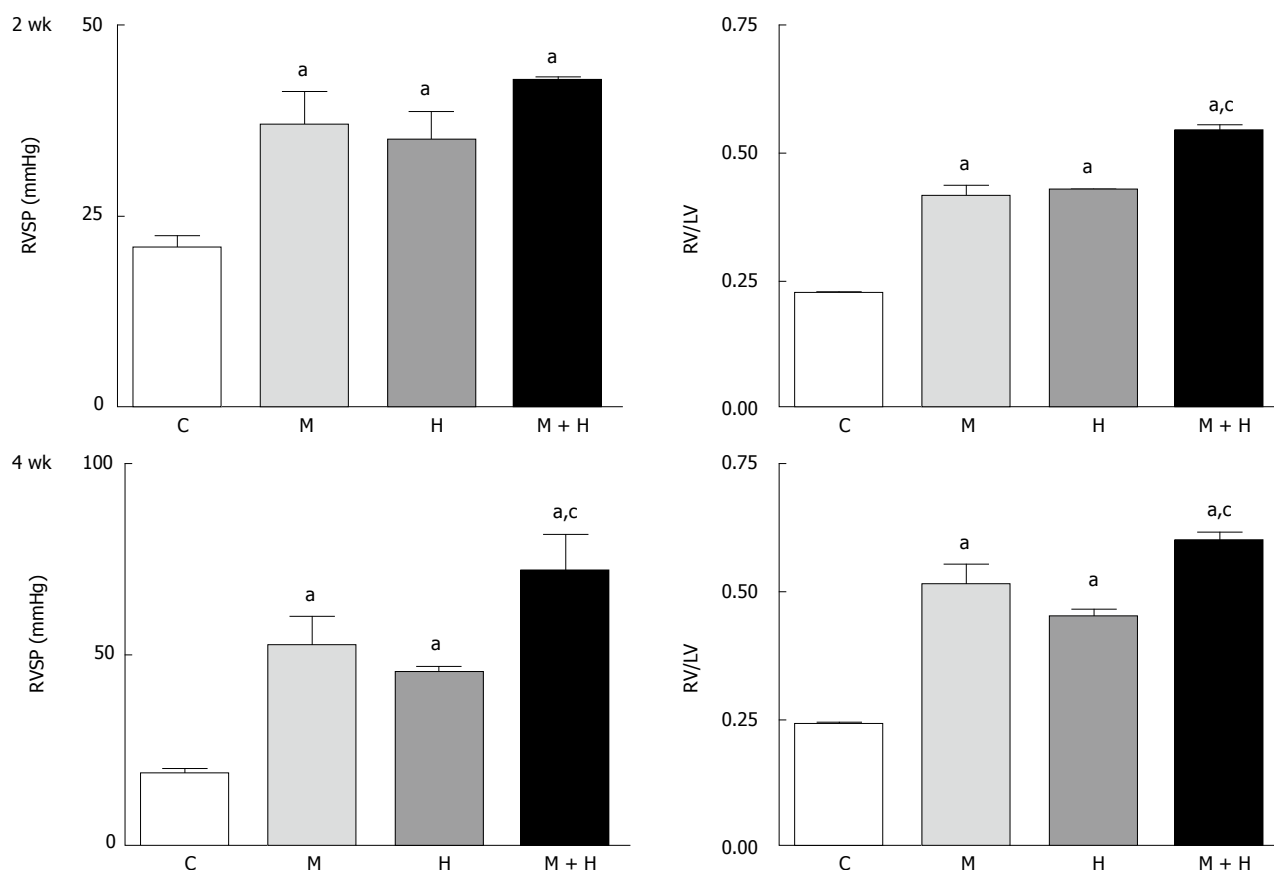


Figure 1 This figure depicts right ventricular systolic pressure and right ventricular hypertrophy in controls, monocrotaline, hypoxia and monocrotaline + hypoxia at 2 ($n = 5-8$) and 4 wk ($n = 6-10$). ^a $P < 0.05$ vs C, ^c $P < 0.05$ vs M and H. RVSP: Right ventricular systolic pressure; C: Controls; M: Monocrotaline; H: Hypoxia; M + H: Monocrotaline + hypoxia.

(1:2000) as appropriate. Protein bands visualized by chemiluminescence are expressed as % normal.

Lung histopathology and double immunofluorescence

Five to 6 μ m lung sections were cut from the paraffin blocks, which were processed from the lung tissue preserved in 10% formaldehyde. Hematoxylin/eosin and elastic van Gieson stains were used for histopathological evaluation. Double immunofluorescence study (on all sections) was carried out at New York Medical College Facility, using caveolin-1 and α -actin antibodies as described previously^[6,7]. Immunofluorescence was evaluated using a laser scanning confocal microscope.

Statistical analysis

The data are expressed as means \pm SEM. Differences among multiple means were determined by one way Anova analysis using SPSS program. Specific differences were determined using Scheffe's test with < 0.05 as significant.

RESULTS

Weight gain

At 2 wk ($n = 5-8$), the weight gain in the MCT and hypoxia groups was lower compared with the controls

(controls, 63 ± 3 g; MCT, 38 ± 3 g^a; hypoxia, 39 ± 2 g^a). In the MCT + hypoxia group, there was a further reduction in the weight gain (6 ± 7 g^{a,c}). There was no mortality in any of the groups. ^a $P < 0.05$ vs controls, ^c $P < 0.05$ vs MCT and hypoxia groups.

At 4 wk ($n = 7-11$), the mortality in the MCT and the MCT + hypoxia groups were 22% and 30% respectively, but none in the hypoxia alone group. Weight gain in the hypoxia group was comparable to the controls (97 ± 7 g vs hypoxia 94 ± 4 g, $P = \text{NS}$). The weight gain in the MCT group was significantly reduced (68 ± 7 g^a) and a further reduction was noted in the MCT + hypoxia group (45 ± 5 g^{a,c}). ^a $P < 0.05$ vs controls, ^c $P < 0.05$ vs MCT.

Hemodynamic data

At 2 wk, RVSP and RV/LV ratio were significantly higher in the MCT, hypoxia and MCT + hypoxia groups compared with the controls (Figure 1, top panel); with a further increase at 4 wk (Figure 1, bottom panel). The ratios of RV (mg)/FBW (g) confirmed increased RVH in the MCT + hypoxia groups at 2 and 4 wk compared with the MCT and hypoxia alone groups. RV (mg)/FBW (g) ratio: 2 wk; C, 0.5 ± 0.01 , MCT, 1.02 ± 0.57^a , Hypoxia, 1.19 ± 0.057^a , MCT + Hypoxia, $1.54 \pm 0.04^{a,c}$, 4 wk; C, 0.55 ± 0.019 , MCT, 1.15 ± 0.56^a ,

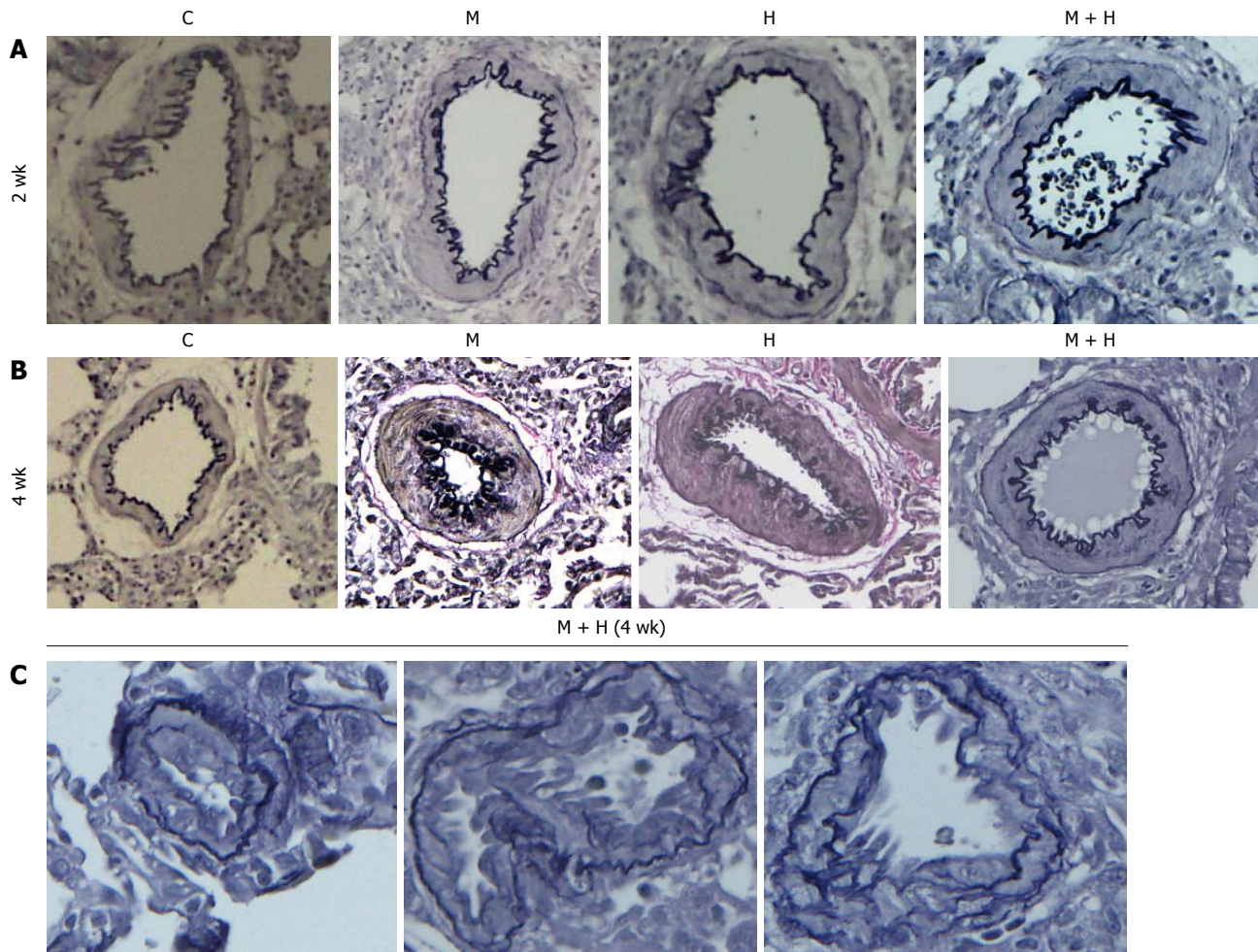


Figure 2 Pulmonary arteries (experimental groups). A and B: Pulmonary arteries (size 200-317 μm) from the controls and different experimental groups (elastic van Gieson stain): At 2 and 4 wk, arteries from MCT (M), hypoxia (H) and MCT + hypoxia (M + H) exhibit increased medial wall thickening compared with the control (C). Magnification = $\times 100$; C: Arteries (size 100-155 μm) from 4 wk M + H group showing the presence of neointima. Fragmentation of internal elastic lamina can be seen in these arteries. Magnification = $\times 400$.

hypoxia, 1.05 ± 0.08^a , MCT + hypoxia, $1.59 \pm 0.01^{a,c}$. $^aP < 0.05$ vs C, $^cP < 0.05$ vs MCT or hypoxia group. The LV (mg)/FBW (g) ratio, however, was not different in any of the experimental groups compared with the controls (data not shown).

Histopathology

Experimental groups: Increased pulmonary arterial medial wall thickening is present in all the experimental groups at 2 and 4 wk (Figure 2, panels A and B). Panel C shows neointima in small arteries at 4 wk in the MCT + hypoxia group.

Humans: Pulmonary arteries from IPAH and HPAH patients show varying degrees of medial wall thickening, neointima and luminal narrowing (Figure 3).

Caveolin-1 and caveolin-2 expression

The expression of both caveolin-1 and caveolin-2 was significantly reduced in the MCT and MCT + hypoxia groups at 2 wk. In the hypoxia alone group, caveolin-1 expression was not reduced; however, the caveolin-2 expression was slightly but significantly reduced

compared with the controls (Figure 4).

At 4 wk, caveolin-1 and caveolin-2 were significantly reduced in the MCT group. In the hypoxia group, the expression of caveolin-1 was comparable to the controls; however, the expression of caveolin-2 was reduced, but not as low as seen in the MCT group. Importantly, in the MCT + hypoxia group, caveolin-1 expression was significantly increased compared with the MCT group ($81\% \pm 3.9\%$ vs $17\% \pm 3.6\%$, $P < 0.05$), although still low compared to the controls ($81\% \pm 3.9\%$ vs $100\% \pm 0\%$, $P < 0.05$). However, despite an increased caveolin-1 expression in this group, caveolin-2 showed a further reduction (Figure 4).

Localization of caveolin-1

Experimental groups: At 2 wk post-MCT, only $23\% \pm 0.87\%$ of arteries exhibited the presence of endothelial caveolin-1. Consistent with previous observations^[7]; in the current study, the endothelial caveolin-1 loss at 2 wk was not associated with an increased caveolin-1 expression in SMC. The MCT + hypoxia group showed a further reduction in the endothelial caveolin-1 expression ($11\% \pm 1\%$). A few

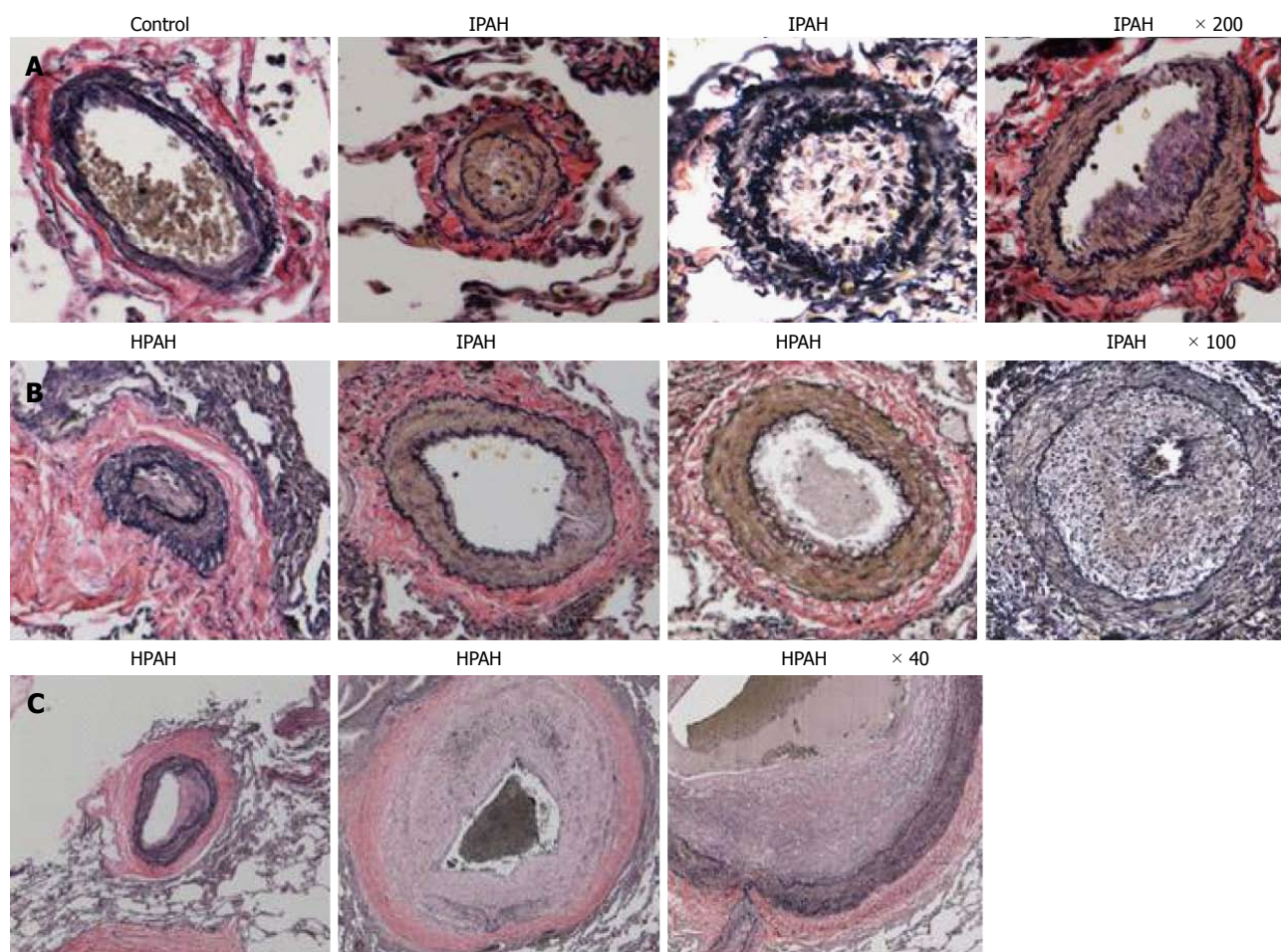


Figure 3 Pulmonary arteries (Human). A and B: Pulmonary arteries (size 134-323 μm) from a control, IPAH and HPAH patients. Control artery is thin walled. The arteries from patients exhibit varying degrees of muscular thickening, neointima and significant narrowing of the lumen; C: Larger arteries exhibiting vascular remodeling, extensive neointima formation and narrowing of the lumen.

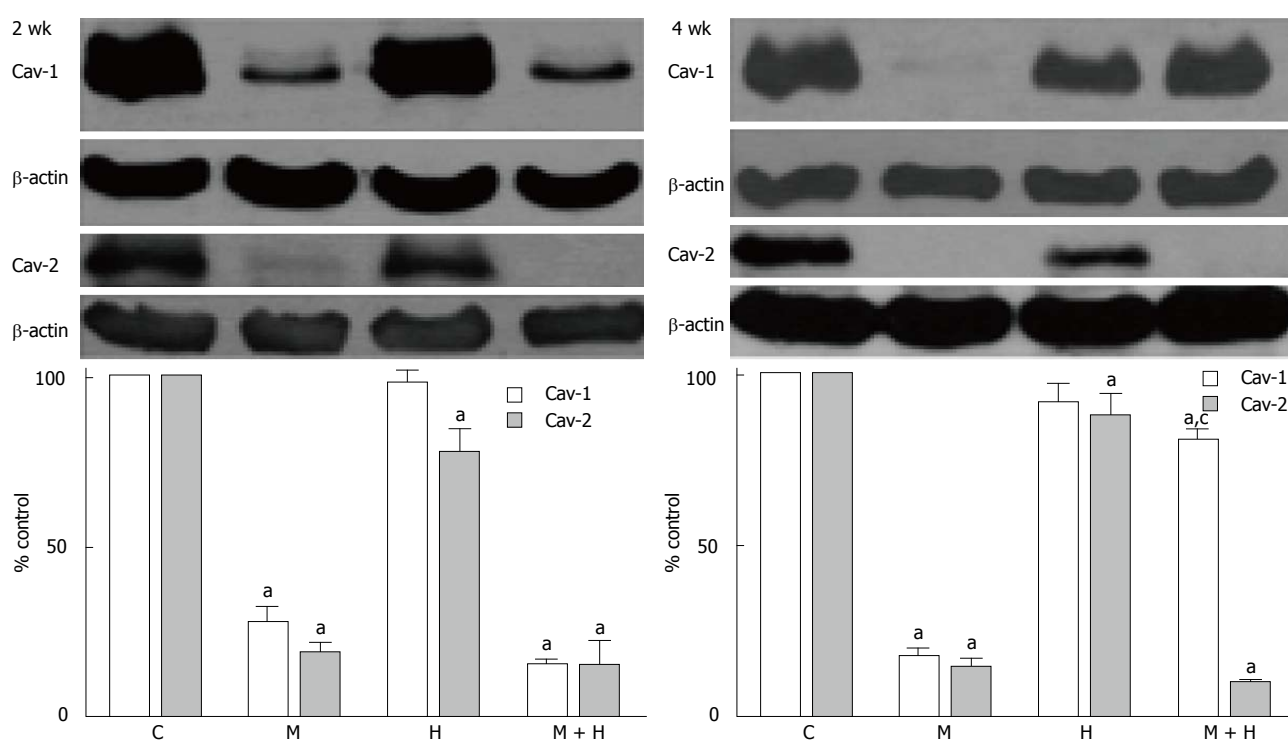


Figure 4 Western blots and bar graphs showing the expression of caveolin-1, caveolin-2 and β actin in controls, monocrotaline, hypoxia and monocrotaline + hypoxia at 2 ($n = 3-6$) and 4 wk ($n = 5-8$). ^a $P < 0.05$ vs C, ^c $P < 0.05$ vs M. C: Controls; M: Monocrotaline; H: Hypoxia; M + H: Monocrotaline + hypoxia.

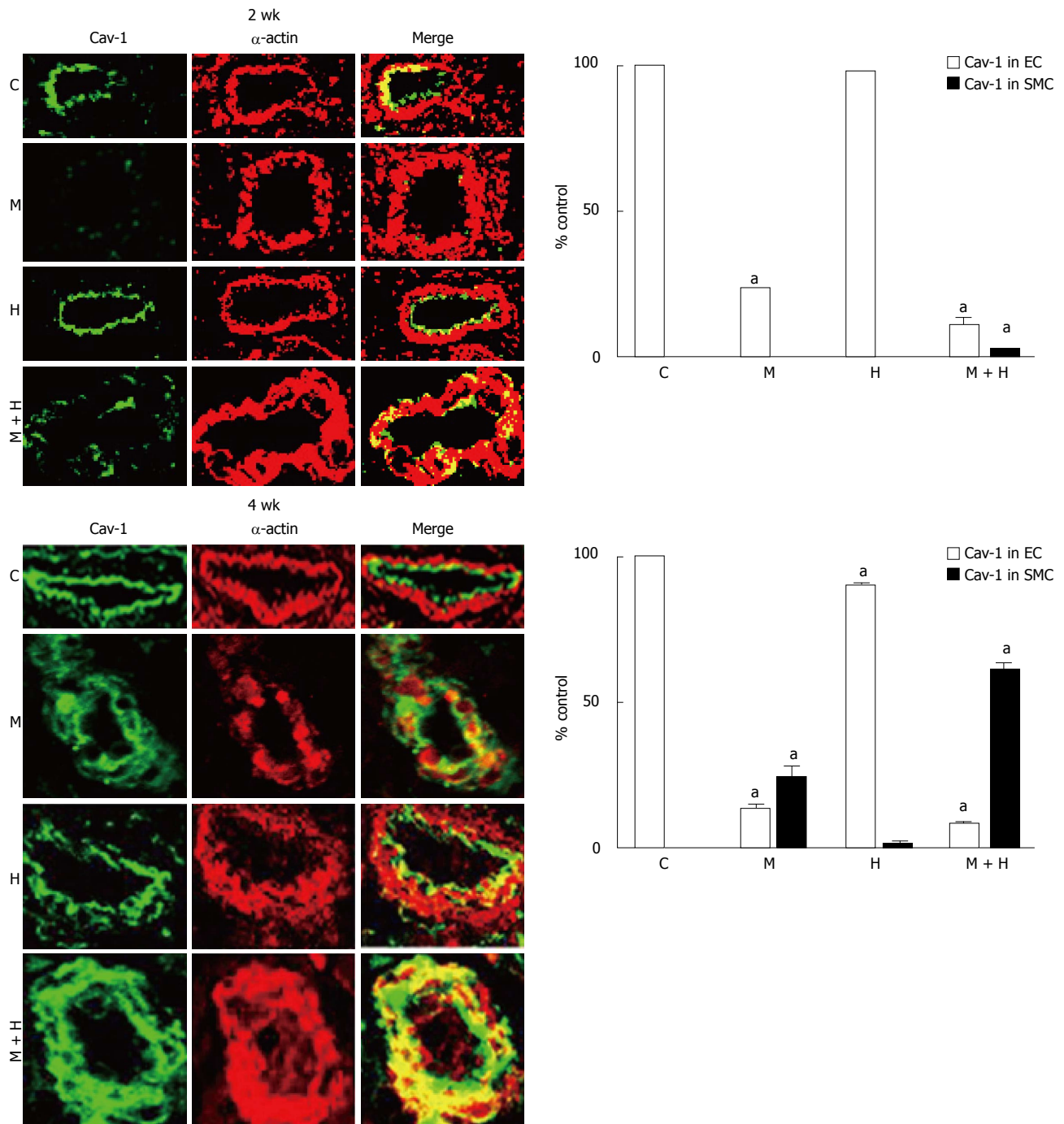


Figure 5 Immunofluorescence study depicting the expression of caveolin-1 (green) and smooth muscle α actin (red) in pulmonary arteries from controls, monocrotaline, hypoxia and monocrotaline + Hypoxia groups at 2 and 4 wk. The accompanying bar graphs ($n = 4-5$) shows the % arteries exhibiting the presence of caveolin-1 in endothelium (EC) and in smooth muscle layer (SMC). ^a $P < 0.05$ vs C. C: Controls; M: Monocrotaline; H: Hypoxia; M + H: Monocrotaline + hypoxia.

arteries displaying endothelial caveolin-1 loss exhibited increased expression of caveolin-1 in SMC ($2.9\% \pm 0.25\%$). Expression of endothelial caveolin-1 in the hypoxia group, however, was not different compared with the controls (Figure 5, top panel).

At 4 wk, in the MCT and MCT+ hypoxia groups, endothelial caveolin-1 was expressed in $13\% \pm 1.4\%$ and $8\% \pm 0.79\%$ of arteries respectively. In the MCT group, increased caveolin-1 expression in SMC was

observed in $24\% \pm 3.5\%$ of arteries. Importantly, in the MCT + hypoxia group, $61\% \pm 2\%$ of arteries displayed increased caveolin-1 in SMC, consistent with the observed increase in total caveolin-1 expression in the lungs. However, the neointimal layer revealed scant expression of caveolin-1. Interestingly, in the hypoxia group, there were a few arteries with endothelial caveolin-1 loss ($90\% \pm 0.89\%$ vs C, $100\% \pm 0\%$, $P < 0.05$); and a smaller number of arteries (1.2%

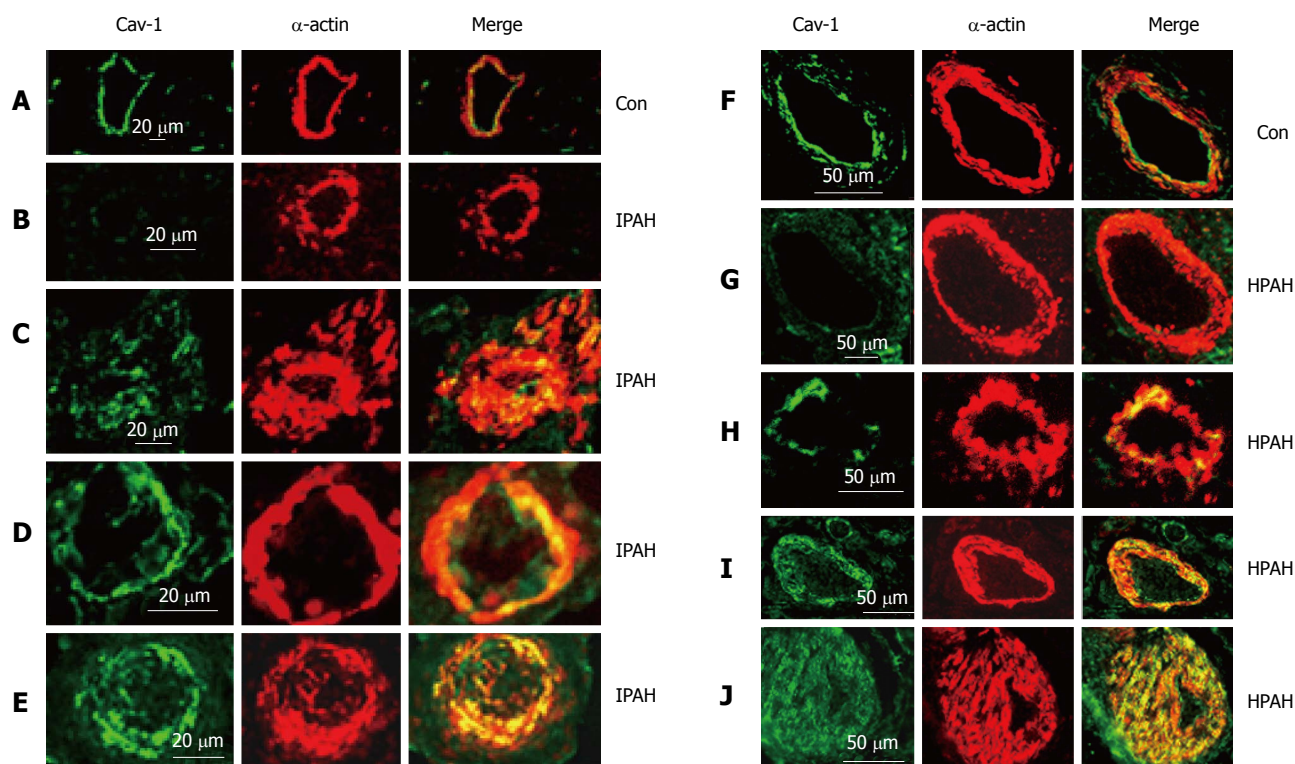


Figure 6 Immunofluorescence study showing the expression of caveolin-1 (green) and smooth muscle α -actin (red) in pulmonary arteries from the controls (A and F), and from the patients with idiopathic pulmonary arterial hypertension (B-E) and with heritable pulmonary arterial hypertension (G-J). In controls, endothelial caveolin-1 is well preserved and there is no enhanced expression of caveolin-1 in smooth muscle layer. Two arteries each from patients, IPAH (B and C), HPAH (G and F) show loss of endothelial caveolin-1 in B and G, and the appearance of increased expression of caveolin-1 in SMC in C and H. The next panels D, E, I and J from 4 different patients show loss of endothelial caveolin-1 and enhanced expression of caveolin-1 in SMC. PAH: Pulmonary arterial hypertension; IPAH: Idiopathic PAH; HPAH: Heritable PAH; SMC: Smooth muscle cells.

$\pm 0.58\%$) with endothelial caveolin-1 loss, exhibited an increased caveolin-1 expression in SMC (Figure 5, bottom panel).

Human lungs: The control pulmonary arteries showed well preserved caveolin-1 in the endothelial layer. Arteries from IPAH and HPAH patients showed varying degrees of alterations in caveolin-1 expression not unlike what was noted in the 4 wk MCT + hypoxia group, such as endothelial caveolin-1 loss, increased caveolin-1 expression in SMC and the presence of neointima (Figure 6).

Caveolin-1 and PPAR γ expression

At 2 wk, caveolin-1 loss in the MCT and MCT + hypoxia groups was accompanied by an increase in the expression of PPAR γ ($P < 0.05$ vs controls, Figure 7). Since our previous studies had shown caveolin-1 loss at 48 h after MCT injection, we investigated the expression of PPAR γ and caveolin-1 at 48 h ($n = 4$) and 1 wk ($n = 4$). At 48 h post-MCT, caveolin-1 expression was reduced to $56\% \pm 1.4\%$ ($P < 0.05$ vs controls) associated with a PPAR γ expression of $118\% \pm 9\%$ ($P = \text{ns}$ vs control). At 1 wk post-MCT, a further reduction in caveolin-1 ($38\% \pm 1\%$) was associated with an increase in the expression of PPAR γ ($203\% \pm 22\%$, $P < 0.05$ vs control). No alterations were observed in the

expression of PPAR γ in the 2 wk hypoxia group (Figure 7).

At 4 wk, in the MCT group, a reciprocal increase in PPAR γ expression accompanied the caveolin-1 loss. Importantly, in the MCT + hypoxia group, the increased total caveolin-1 expression in the lungs correlated with a reduction in the expression of PPAR γ . In the hypoxia group, the expression of caveolin-1 was slightly decreased ($90\% \pm 0.89\%$), and the PPAR γ expression, however, was not altered (Figure 7).

Proliferative and anti-apoptotic pathways

As shown in Figure 8, both at 2 and 4 wk, the activation of p-Erk and PY-STAT3, and increased Bcl-xL expression were present in all experimental groups.

eNOS and HSP90 expression

Although eNOS expression in the 2 wk-post MCT group was not significantly reduced compared with the controls, the expression of HSP90, however, was reduced ($P < 0.05$ vs controls). Expression of eNOS was increased in the hypoxia group, but the HSP90 expression was unaltered. In the MCT + hypoxia group, an increased eNOS expression, and a normal HSP90 expression were observed (Figure 9).

At 4 wk, in the MCT group, eNOS and HSP90 levels were reduced. In the hypoxia and MCT + hypoxia

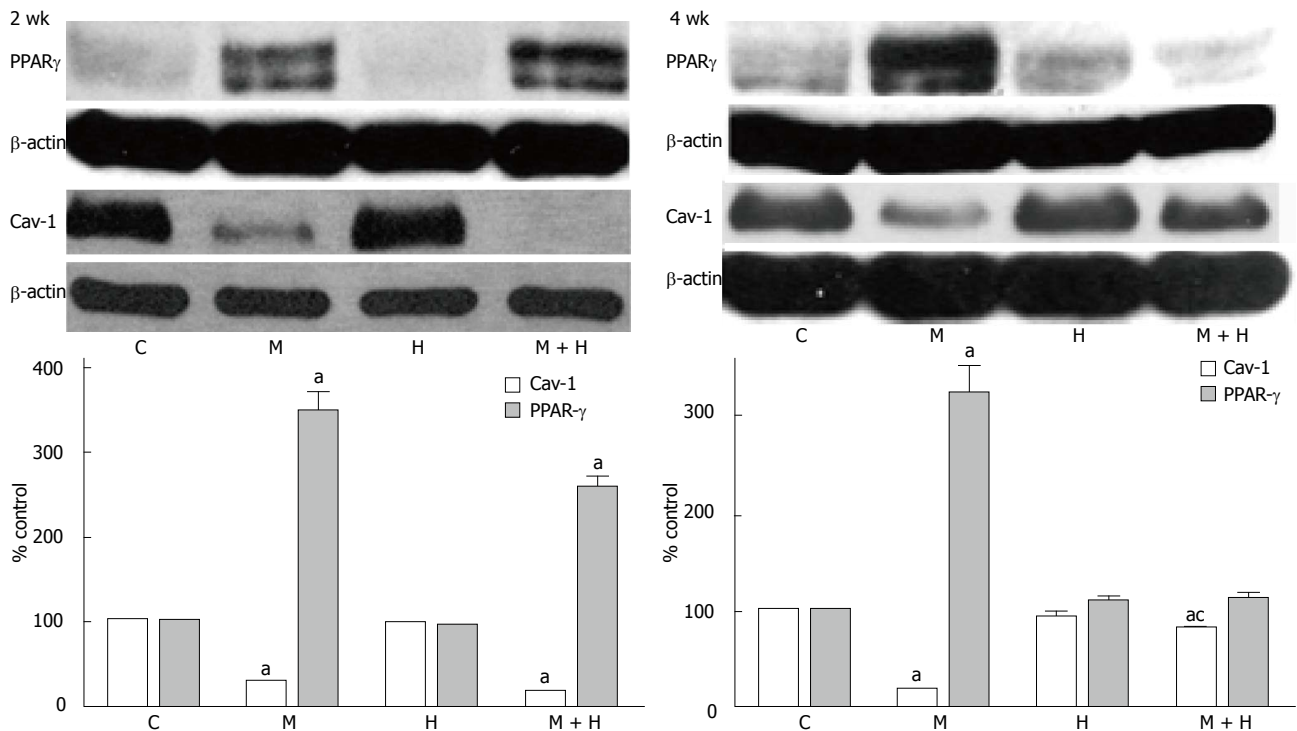


Figure 7 Representative western blots and bar graphs depicting the expression of caveolin-1 and peroxisome proliferator-activated receptor γ in controls, monocrotaline, hypoxia and monocrotaline + hypoxia groups at 2 ($n = 5-8$) and 4 wk ($n = 5-8$). ^a $P < 0.05$ vs C, ^c $P < 0.05$ vs M. C: Controls; M: Monocrotaline; H: Hypoxia; M + H: Monocrotaline + hypoxia; PPAR: Peroxisome proliferator-activated receptor.

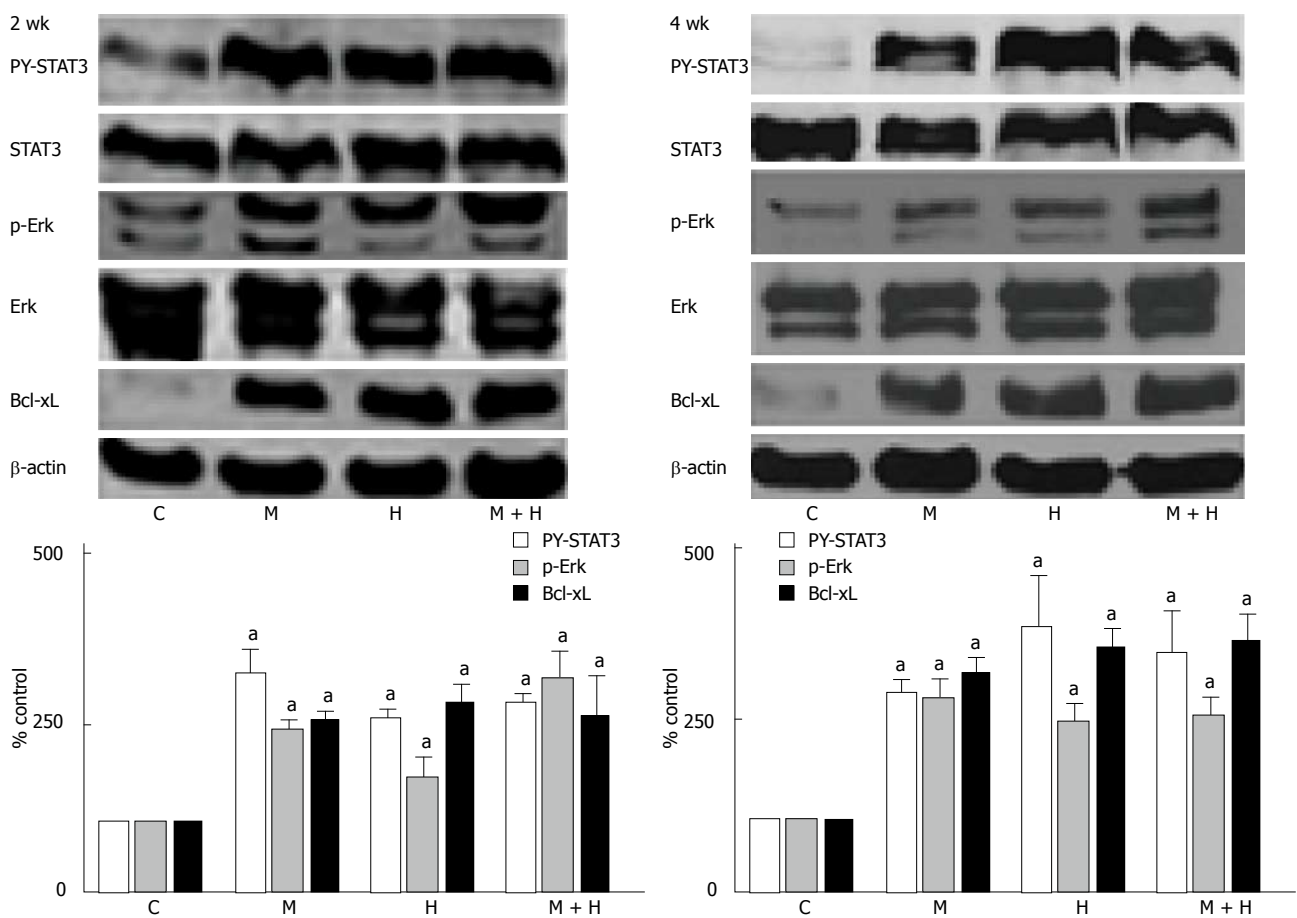


Figure 8 Representative western blots and bar graphs depicting the expression of PY-STAT3, p-Erk and Bcl-xL in controls, monocrotaline, hypoxia and monocrotaline + hypoxia at 2 ($n = 4-7$) and 4 wk ($n = 5-8$). STAT3, Erk and β -actin were used to assess the protein loading. ^a $P < 0.05$ vs C. C: Controls; M: Monocrotaline; H: Hypoxia; M + H: Monocrotaline + hypoxia.

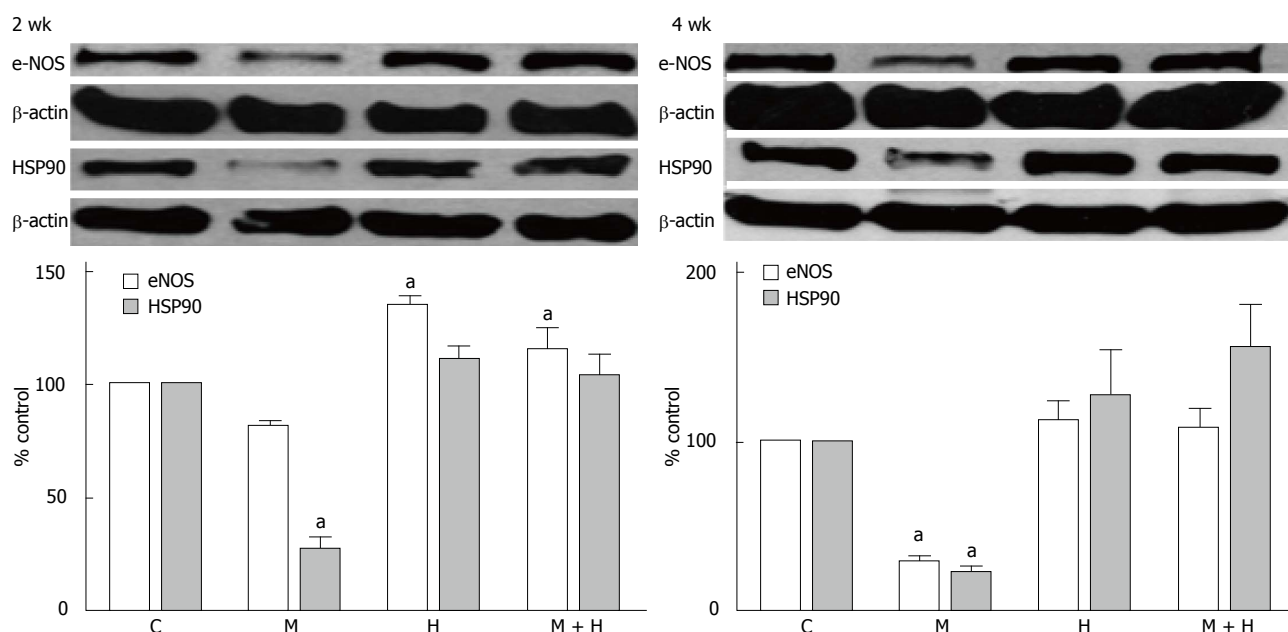


Figure 9 Representative western blots and bar graphs depicting the expression of endothelial nitric oxide synthase, HSP90 and β -actin in controls, monocrotaline, hypoxia and monocrotaline + hypoxia at 2 ($n = 3-6$) and 4 wk ($n = 4-7$). ^a $P < 0.05$ vs C. C: Controls; M: Monocrotaline; H: Hypoxia; M + H: Monocrotaline + hypoxia.

groups, eNOS and HSP90 levels were not altered (Figure 9).

DISCUSSION

The significant aspect of our study is the progressive disruption and loss of endothelial caveolin-1, activated proliferative pathways leading to PH in the MCT model. By 4 wk, a further reduction in endothelial caveolin-1 is accompanied by an increased caveolin-1 expression in SMC, observed in 24% of the arteries. The total caveolin-1 expression, however, remained significantly low. Exposure of MCT-treated rats to hypoxia accelerated the disease process. An increased number of arteries exhibited augmented caveolin-1 expression in SMC associated with an increase in total caveolin-1 expression. Importantly, some of the arteries exhibiting an increased caveolin-1 expression in SMC displayed neointima with scant caveolin-1. Furthermore, lung sections from patients with IPAH as well as HPAH showed similar changes, *i.e.*, endothelial caveolin-1 loss, increased caveolin-1 in SMC. Neointimal lesions were seen only in arteries with increased caveolin-1 expression in SMC.

Neointima and plexiform lesions have been described in rodent PH models such as Sugen + hypoxia and pneumonectomy + MCT^[20,21,28]. In the Sugen + hypoxia model, the initial EC apoptosis is followed by cellular proliferation and angiogenesis deregulation resulting in plexiform lesions with significantly reduced caveolin-1 expression^[29,30]. The reduced expression of caveolin-1 in plexiform lesion is supported by the electron microscopic examination showing a lack of caveolae^[31]; the total caveolin-1 protein levels in the lungs, however, are

not decreased^[32]. *In-vitro* studies have shown that in response to cyclic stretch, caveolin-1 in SMC shifts to non-caveolar sites, mediates Erk activation and participates in cell proliferation. Interestingly, SMC not expressing caveolin-1 fail to proliferate when subjected to cyclic stretch^[33,34]. It is likely, that the extensive damage and/or loss of EC, leads to the exposure of SMC to direct shear stress and pressure, resulting in the caveolin-1 shift from caveolae to non-caveolar sites, thus altering caveolin-1 function.

In the hypoxia group, at 2 wk, there was no endothelial caveolin-1 loss, indicating that there was no physical disruption of EC. During hypoxia, caveolin-1 forms a tight complex with eNOS^[19,35], leading to the dysfunction of both factors. Removal of hypoxia^[36,37] or eNOS/caveolin-1 complex disruption attenuates PH^[38]. At 4 wk, the total caveolin-1 expression in the lungs was not altered, but immunofluorescence studies revealed a small loss in endothelial caveolin-1 accompanied by 1.2% of arteries exhibiting increased caveolin-1 expression in SMC. It is noteworthy that in infants with respiratory distress syndrome or bronchopulmonary dysplasia, PH in the absence of EC disruption, does not lead to endothelial caveolin-1 loss or increased caveolin-1 expression in SMC. However, accompanying inflammation results in endothelial cell membrane disruption and endothelial caveolin-1 loss with subsequent increased caveolin-1 expression in SMC^[16]. These studies suggest that the endothelial disruption and the endothelial caveolin-1 loss may be necessary for the increased caveolin-1 expression in SMC.

Caveolin-2 loss concomitant with caveolin-1 loss has been shown in the experimental models of PH,

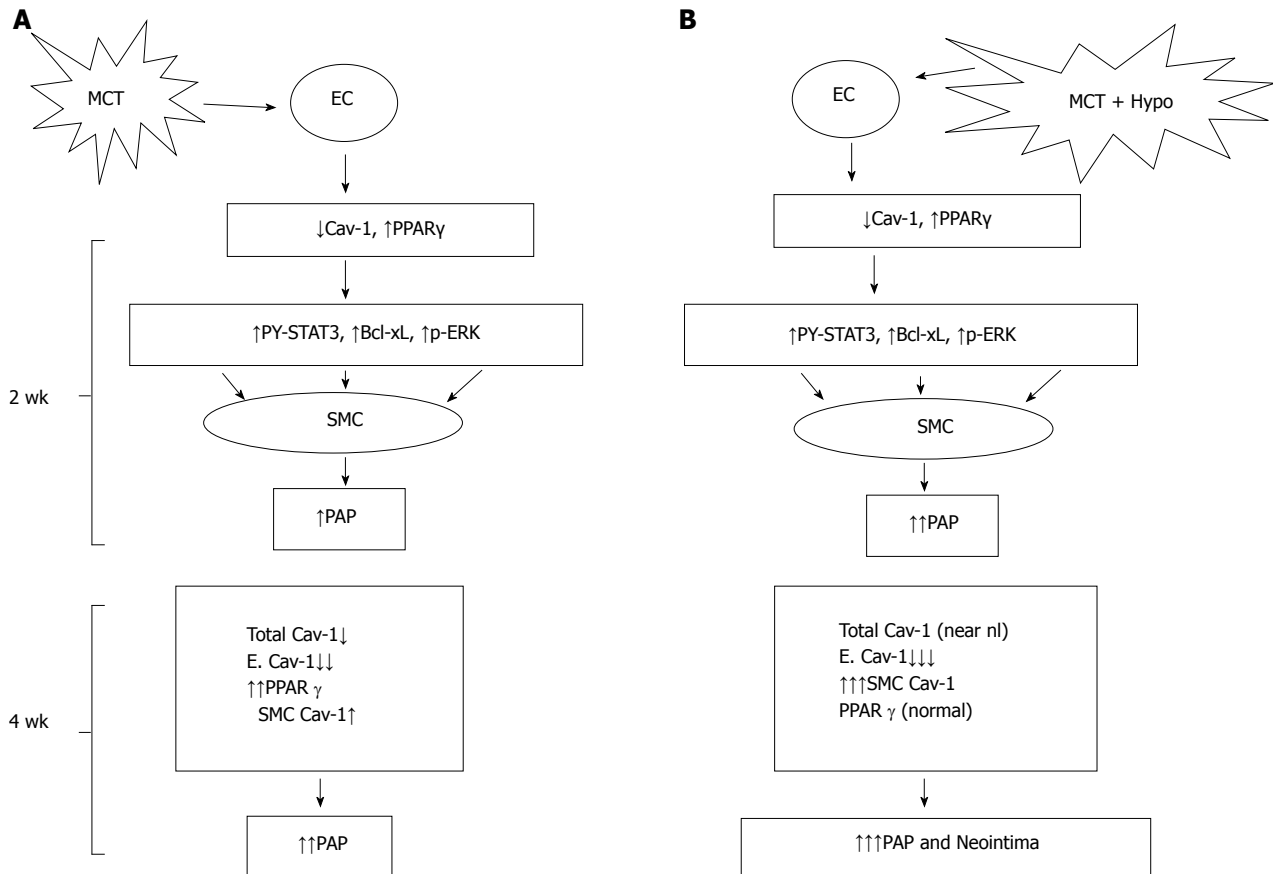


Figure 10 Monocrotaline injury to endothelial cells resulting in the loss of caveolin-1 and the activation of the proliferative pathways (PY-STAT3, Bcl-xL, p-ERK) leading to PH at 2 wk; and a reciprocal relationship between caveolin-1 and peroxisome proliferator-activated receptor expression (A) and MCT + hypoxia (MCT + Hypo) accelerates the disease process (B). At 4 wk, there is a further loss of endothelial caveolin-1 (E. Cav-1) and enhanced expression of cav-1 in smooth muscle cells (SMC), however, the total cav-1 levels remain low (17% vs C, 100%). These alterations are accompanied by a further increase in pulmonary artery pressure. Panel B shows MCT + hypoxia (MCT + Hypo) accelerates the disease process. At 2 wk, extensive endothelial caveolin-1 is accompanied by the activation of proliferative pathways and PH (higher pulmonary artery pressure compared with MCT alone group). At 4 wk, a further loss of E. Cav-1 is accompanied by significantly increased expression of caveolin-1 in SMC compared with MCT alone group. At this stage total caveolin-1 level is closer to normal (81% vs C, 100%), and neointimal lesions can be seen. MCT: Monocrotaline; EC: Endothelial cells; PPAR: Peroxisome proliferator-activated receptor.

and the rescue of caveolin-1 restores caveolin-2 expression^[10,39]. Caveolin-2 is expressed in a number of cell types including EC and SMC, and it colocalizes with caveolin-1 and necessitates caveolin-1 for its transport to caveolae^[23]. However, caveolin-2 is not necessary for caveolar localization of caveolin-1; but the co-expression of caveolin-1 and 2 results in a more efficient formation of caveolae^[40,41]. In the present study, MCT-treated rats exhibited a significant loss of caveolin-2 concomitant with the loss of caveolin-1. In the MCT + hypoxia group at 4 wk, despite an increase in the total caveolin-1 expression, a significant loss of caveolin-2 was present, which supports the view that the major part of caveolin-1 in SMC may not be localized in caveolae. In the hypoxia group, despite the presence of caveolin-1, some loss of caveolin-2 was observed, suggesting that a part of caveolin-1 may not be available for caveolin-2 localization.

All experimental groups (MCT, hypoxia and MCT + hypoxia) at 2 and 4 wk revealed the activation of PY-STAT3, pERK1/2 and Bcl-xL. Caveolin-1 is a well known inhibitor of pro-proliferative and anti-apoptotic

factors^[11,42]; and the rescue of caveolin-1 as a preventive measure in the MCT model, inhibits the activation of proliferative pathways and attenuates PH^[9,10]. Interestingly, in the presence of caveolin-1 in hypoxia groups and MCT + hypoxia group at 4 wk, proliferative pathways were activated; which strongly suggest that caveolin-1 is dysfunctional in these groups.

In the 4 wk MCT group, the expression of eNOS and HSP90 was significantly reduced, but was normal in the MCT + hypoxia groups. In addition, caveolin-1 expression in native EC and in neointimal cells was sparse in the latter group. Strong eNOS expression and low caveolin-1 expression have been reported in the plexiform lesions^[39,43], besides, oxidant stress is a critical feature in patients with IPAH^[44]. The major cause of PH in caveolin-1 knockout mice is thought to be eNOS uncoupling and subsequent oxidative and nitrosative stress; and PH is attenuated by caveolin-1 re-expression, eNOS inhibition or treatment with superoxide dismutase mimetic^[45,46]. Furthermore, EC from patients with IPAH show caveolin-1 degradation induced by sustained eNOS and Src signaling^[47]. It

is important to note, that caveolin-1 regulates eNOS-derived NO and superoxide, and NOX activity. Caveolin-1 sequesters uncoupled eNOS, inhibits superoxide formation and prevents eNOS oxidase activity^[48,49]. These observations support a pivotal role for caveolin-1 in preventing oxidative and nitrosative stress.

Protein and mRNA expression of PPAR γ is described to be low in IPAH, Sugen + hypoxia^[26] and the shunt^[27] models of PH, but not in chronic obstructive pulmonary disease patients^[26]. PPAR γ , a ligand-activated transcription factor belongs to the nuclear hormone superfamily. In several cell systems, PPAR γ has been shown to upregulate caveolin-1 expression^[24,25,50]. In the present study, PPAR γ levels revealed an inverse relationship with caveolin-1 in the MCT groups; initial low endothelial and total caveolin-1 levels were associated with increased PPAR γ levels. At 4 wk in the MCT + hypoxia group, an increase in total caveolin-1 was associated with a decrease in PPAR γ levels. The increased expression of PPAR γ may be a compensatory mechanism to upregulate the caveolin-1 expression during the initial phase of PH associated with significantly reduced caveolin-1 levels. In the hypoxia group, however, the PPAR γ levels were not altered. A thiazolidinedione (TZD) compound (PPAR γ activator) has been reported to attenuate hypoxia-induced PH^[51]. Some of the TZD compounds are reported to have cholesterol disruptive function independent of PPAR γ ^[52]. Interestingly, cholesterol lowering statins in the hypoxia model of PH has been shown to disrupt the tight complex of eNOS and caveolin-1 resulting in the restoration of eNOS function and the attenuation of PH^[38]. Recent studies have shown that increased PPAR γ expression portends poor prognosis in some forms of cancer^[53,54]. In view of these observations, increasing PPAR γ levels as a therapeutic measure in PH is of some concern. It is possible that PPAR γ activation may be beneficial in some forms of PH or at some stage during the disease; or a selective increase in PPAR γ expression in EC may be useful. In any case, further studies are necessary to ascertain the roles of PPAR γ and caveolin-1, and their interrelationship in PH.

In conclusion, addition of hypoxia to MCT-treated rats results in an acceleration of the disease process. Extensive endothelial damage, progressive endothelial caveolin-1 loss, and increased caveolin-1 expression in SMC accompanied by an augmented total caveolin-1 protein expression in lungs is followed by neointima formation. In addition, caveolin-1 and PPAR γ revealed an inverse relationship (Figure 10). Importantly, lung sections from IPAH and HPAH patients showed similar alterations in caveolin-1 expression, *i.e.*, endothelial caveolin-1 loss and increased caveolin-1 expression in SMC. Both in humans and the MCT + hypoxia group, neointimal lesions were observed only in the arteries exhibiting increased caveolin-1 expression in SMC. Since increased caveolin-1 expression in SMC has been shown to be actively pro-proliferative, this alteration in caveolin-1 expression may be a prelude to neointima

formation. In the hypoxia group, in the absence of endothelial disruption or the endothelial caveolin-1 loss, there was neither an increased expression of caveolin-1 in SMC nor neointima. These results suggest that the endothelial cell integrity may be an important factor that determines the course of the disease.

ACKNOWLEDGEMENTS

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COMMENTS

Background

Neointima formation in pulmonary hypertension (PH) portends poor prognosis. Despite major advances in the field, the mechanism/s of pathogenesis is not yet clear, which makes the therapeutic measures a challenge.

Research frontiers

Caveolin-1, a membrane protein plays a significant role in pulmonary vascular homeostasis and in the pathogenesis of PH.

Innovation and breakthroughs

An important aspect of the study is that caveolin-1 plays a dual role in the pathogenesis of PH, *i.e.*, as an anti-proliferative and a pro-proliferative factor. This change in function of caveolin-1 is similar to what has been reported in cancer. Loss of endothelial caveolin-1 leads to the activation of proliferative pathways, vascular remodeling and PH. As the disease progresses, the resulting extensive endothelial caveolin-1 loss associated with endothelial cell damage is followed by an enhanced expression of caveolin-1 in smooth muscle cells (SMC). The authors have shown that by subjecting monocrotaline-treated rats to hypoxia accelerates the disease process; by 4 wk, a large number of arteries exhibit enhanced expression of caveolin-1 in SMC. This caveolin-1 becomes pro-proliferative and facilitates cell proliferation, cell migration and neointima formation. In the experimental models and humans, neointima is observed only in the arteries exhibiting extensive endothelial damage and enhanced expression of caveolin-1 in SMC.

Application

The results would lead to further research in the role of caveolin-1 in SMC in PH, and in assessing the effects of modulation of caveolin-1 expression.

Peer-review

The present investigation is well written and interesting.

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Retrospective Cohort Study

Renal function assessment in atrial fibrillation: Usefulness of chronic kidney disease epidemiology collaboration *vs* re-expressed 4 variable modification of diet in renal disease

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Abstract

AIM: To compare the performance of the re-expressed Modification of Diet in Renal Disease equation *vs* the new Chronic Kidney Disease Epidemiology Collaboration equation in patients with non-valvular atrial fibrillation.

METHODS: We studied 911 consecutive patients with non-valvular atrial fibrillation on vitamin-K antagonist. The performance of the re-expressed Modification of Diet in Renal Disease equation *vs* the new Chronic Kidney Disease Epidemiology Collaboration equation in patients with non-valvular atrial fibrillation with respect to either a composite endpoint of major bleeding, thromboembolic events and all-cause mortality or each individual component of the composite endpoint was assessed using continuous and categorical ≥ 60 , 59-30, and < 30 mL/min per 1.73 m^2 estimated glomerular filtration rate.

RESULTS: During 10 ± 3 mo, the composite endpoint occurred in 98 (10.8%) patients: 30 patients developed major bleeding, 18 had thromboembolic events, and 60 died. The new equation provided lower prevalence of renal dysfunction < 60 mL/min per 1.73 m^2 (32.9%),

compared with the re-expressed equation (34.1%). Estimated glomerular filtration rate from both equations was independent predictor of composite endpoint (HR = 0.98 and 0.97 for the re-expressed and the new equation, respectively; $P < 0.0001$) and all-cause mortality (HR = 0.98 for both equations, $P < 0.01$). Strong association with thromboembolic events was observed only when estimated glomerular filtration rate was < 30 mL/min per 1.73 m^2 : HR is 5.1 for the re-expressed equation, and HR = 5.0 for the new equation. No significant association with major bleeding was observed for both equations.

CONCLUSION: The new equation reduced the prevalence of renal dysfunction. Both equations performed similarly in predicting major adverse outcomes.

Key words: Atrial fibrillation; Anticoagulants; Follow-up studies; Kidney; Prognosis

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Core tip: In atrial fibrillation, renal dysfunction entails more adverse events. Limited data exist on the performance and prognostic value of the re-expressed Modification of Diet in Renal Disease equation vs the new Chronic Kidney Disease Epidemiology Collaboration equation in atrial fibrillation. We compared the performance of both equations at predicting major outcomes in patients with non-valvular atrial fibrillation. The study encouraged the use of the new equation as it decreased the prevalence of patients with renal dysfunction, in a real world cohort of patients with non-valvular atrial fibrillation and at the same time showed similar prognostic impact like the re-expressed equation.

Abumuaileq RRY, Abu-Assi E, López-López A, Raposeiras-Roubin S, Rodríguez-Mañero M, Martínez-Sande L, García-Seara FJ, Fernández-López XA, González-Juanatey JR. Renal function assessment in atrial fibrillation: Usefulness of chronic kidney disease epidemiology collaboration vs re-expressed 4 variable modification of diet in renal disease. *World J Cardiol* 2015; 7(10): 685-694 Available from: URL: <http://www.wjgnet.com/1949-8462/full/v7/i10/685.htm> DOI: <http://dx.doi.org/10.4330/wjc.v7.i10.685>

INTRODUCTION

Renal dysfunction is a common comorbidity observed in patients with atrial fibrillation (AF). Patients with AF and renal dysfunction are more likely to develop thromboembolic (TE) events compared to those with AF and normal renal function^[1,2]. The presence and severity of renal dysfunction is also a recognized predictor in the bleeding risk scores used commonly to estimate the hemorrhagic risk in anticoagulated patients with AF^[3,4].

Therefore, accurate assessment of renal function is of paramount importance as it will help inform the decision making process aiming for optimizing the management of patients with AF. Current recommendations advocate the estimation of renal function by means of estimated glomerular filtration rate (eGFR) using the validating prediction equations instead of serum creatinine^[5].

Until recently, the two most commonly used creatinine based equations estimating GFR were the 4 variable Modification of Diet in Renal Disease (MDRD-4) Study^[6] and the Cockcroft-Gault (C-G) equation^[7]. The MDRD-4 equation was re-expressed to be used in the current era of standardized serum creatinine assay, whereas the C-G equation was not updated, and its use is not recommended currently^[8]. More recently, a new equation, the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation^[9], has been proposed as an alternative equation to replace the widely used re-expressed MDRD-4 formula in routine clinical use, on the basis that it estimates measures of GFR more accurate than the re-expressed MDRD-4 equation.

Several studies have demonstrated the higher accuracy of the new CKD-EPI at estimating the true renal function, thus enabling it to provide better clinical risk prediction in different disease contexts^[10-12]. However, it is currently unknown if the better estimates from the new CKD-EPI would be translated into better risk prediction in the particular context of patients with AF, since very few patients in the derivation cohort of the new CKD-EPI formula had AF^[9].

In this study, we aimed to comparatively evaluate the re-expressed MDRD-4 and the new CKD-EPI formulas at predicting the occurrence of major adverse outcomes in a real world cohort of patients with non-valvular AF (NVAFA) who are recently on vitamin K antagonists (VKA).

MATERIALS AND METHODS

Patient's sample

Retrospectively, we identified all consecutive patients of ≥ 18 years of age with a confirmed diagnosis of AF on VKAs attending outpatient cardiology consultations of a tertiary hospital between January 2011 and February 2013. Only patients who fulfilled the following criteria were included in this study: Patients with permanent or paroxysmal AF recently started on VKAs (*i.e.*, not more than 8 mo passed since the beginning of their VKAs therapy), and who have regular visits for INR measurements. Patients with prosthetic valve ($n = 452$), rheumatic heart disease ($n = 43$), active cancer ($n = 41$), dementia ($n = 26$), and/or interrupted vitamin K antagonist > 3 d ($n = 73$) were excluded. Thus, the final analyzed cohort consisted of 911 patients. A detailed medical history was recorded for each patient, and the basal clinical characteristics at study entry together with information on follow up were carefully

gathered by cardiologists.

The vast majority of patients were on acenocoumarol (93%); and the remaining patients were on warfarin).

The study was approved by the Clinical Research Ethics Committee of our hospital.

Calculation of eGFR

For each patient, Serum creatinine was measured by the modified kinetic Jaffe method in a single clinical laboratory in our institution. All creatinine measurements were performed with an isotope dilution mass spectroscopy (IDMS)-traceable enzymatic assay that has previously been shown to provide very reliable eGFR results compared with the measured GFR^[13]; these measurements were analyzed automatically using the ADVIA 2400 Chemistry System (Siemens Diagnostics, Tarrytown, NY, United States).

We calculated the eGFR using the IDMS-traceable version of the MDRD-4 equation^[8]: $175 \times [\text{standardized serum creatinine (mg/dL)}]^{-1.154} \times \text{age}^{-0.203} \times (0.742 \text{ if female}) \times (1.212 \text{ if black})$.

The new CKD-EPI equation was also used^[9]: $141 \times (\text{minimum of standardized serum creatinine (mg/dL)/}\kappa \text{ or } 1)^{\alpha} \times [\text{maximum of standardized serum creatinine (mg/dL)/}\kappa \text{ or } 1]^{-1.209} \times 0.993^{\text{age}} \times (1.018 \text{ if female}) \times (1.159 \text{ if black})$. Where κ is 0.7 for females and 0.9 for males and α is -0.329 for females and -0.411 for males.

We categorized the eGFR obtained from each formula into three categories: ≥ 60 mL/min per 1.73 m^2 (normal or mild renal dysfunction), 30–59 mL/min per 1.73 m^2 (moderate renal dysfunction) and < 30 mL/min per 1.73 m^2 (severe renal dysfunction). No patients were on renal replacement therapy.

Endpoints and definitions

Patients were followed up to 1-year after the enrolment. The primary endpoint of the present study was a composite endpoint of major bleeding, TE complications, or death; whichever comes first. The secondary endpoint was each individual component of the composite endpoint.

Data on major bleeding, and TE complications were gathered from the cardiology clinic visits and records, and through hospital files as well as through primary care centers reports.

We used the 2005 International Society on Thrombosis and Haemostasis (ISTH) criteria to define major bleeding^[14]. Thus, a major bleeding event was adjudicated if one of the following criteria was met: fatal bleeding and/or symptomatic bleeding in a critical area or organ (e.g., such as intracranial, intraspinal, intraocular, retroperitoneal, atraumatic intraarticular, pericardial, or intramuscular with compartment syndrome); and/or bleeding causing drop of hemoglobin of ≥ 2 g/dL, or leading to transfusion of ≥ 2 units of whole blood or packed red blood cells.

A TE complication was defined as the occurrence of

ischemic stroke, transient ischemic attack, or peripheral embolism (including fatal TE events). Diagnosis of stroke or transient ischemic attack required an acute neurological deficit lasting for more or less than 24 h, respectively, which could not be explained by other causes and with at least 1 image test (computed tomography or magnetic resonance) compatible with the diagnosis, as well as confirmation from a neurologist. A diagnosis of peripheral embolism was defined as non-central nervous system embolism leading to an abrupt vascular insufficiency associated with clinical or radiographic evidence of arterial occlusion in absence of another mechanism such as atherosclerosis, instrumentation, or trauma.

Statistical analysis

Qualitative data were expressed as frequencies and percentages while quantitative data were summarized as mean and standard deviation. Comparison between qualitative data was performed using the χ^2 test or the Fisher exact test, as appropriate. The *t*-Student test was used to compare quantitative data.

The relationship between the primary endpoint and eGFR according to both formulas was evaluated using separate Cox proportional hazard regression models. The candidate variables to construct the multivariate Cox models were those variables presented $P < 0.10$ in the univariate Cox analysis, or those co-variables of recognized prognostic value in the medical literature. Once the initial Cox models had been established, they were simplified by stepdown elimination. Thus, the final Cox models to determine the adjusted effect of eGFR on the composite endpoint, included: age, sex, previous stroke, basal hemoglobin, chronic obstructive pulmonary disease, diabetes mellitus, congestive heart failure or left ventricular ejection fraction $\leq 40\%$, history of malignant disease and coronary artery disease.

The association between eGFR formulas and the individual endpoints of either major bleeding or TE events was determined using competing-risks regression based on Fine and Gray's proportional subhazards models. The Fine and Gray models were adjusted for HAS-BLED score^[4] in the case of testing the relationship between eGFR formulas and major bleeding, and for CHA₂DS₂-VASc score^[15] in the case of testing the relationship between eGFR formulas and TE events. For all-cause mortality, we used a Cox regression model. Once the initial Cox model for predicting all-cause mortality had been established, it was simplified by stepdown elimination; and finally included the following covariables: age, sex, diabetes mellitus, and history of malignant disease, previous stroke, basal hemoglobin, and congestive heart failure or ejection fraction $\leq 40\%$.

The discriminatory capacity of each formula at predicting either the primary or secondary endpoint was determined by calculating the c- statistic. We used

Table 1 Baseline characteristics *n* (%)

Age (yr)	73 ± 11
Men	605 (66.4)
Systolic blood pressure at study entry	139 ± 28
Hypertension	678 (74.4)
Current smoking	77 (8.5)
Diabetes mellitus	220 (24.1)
Heart failure	343 (37.7)
Peripheral arterial disease	92 (10.1)
History of stroke or TIA	103 (11.3)
Coronary artery disease	127 (13.9)
COPD	183 (20.1)
CHA2DS2-VASc:	
= 0	62 (6.8)
≥ 1	849 (93.2)
≥ 2	772 (84.7)
History of malignancy	135 (14.8)
HAS-BLED	
0	47 (5.2)
1	160 (17.6)
2	365 (40.1)
3	261 (28.6)
4	69 (7.6)
5	6 (0.7)
6	3 (0.3)
Alcohol consumption ≥ 40 g/daily	81 (8.9)
Prior bleeding	115 (12.6)
Anemia	178 (19.5)
Abnormal liver function ¹	9 (1)
PINRR	58% ± 18%

¹Defined as cirrhosis or elevated liver transaminases enzymes > 3 times higher than the upper limit of normal and elevated total bilirubin > 2 times higher than the upper limit of normal. CHA2DS2-VASc: Congestive heart failure, hypertension, age ≥ 75, diabetes mellitus, stroke, vascular disease, female sex category; COPD: Chronic obstructive pulmonary disease; HAS-BLED: Uncontrolled Hypertension: systolic > 160 mmHg, Abnormal renal/liver function, Stroke, Bleeding history or predisposition, Labile international normalized ratio, elderly > 65 years, drugs/alcohol concomitantly; TIA: Transient ischemic attack; PINRR: Percentage of INRs in therapeutic range.

the Delong test to compare the c-statistic values from each formula.

The calibration of the model was assessed with the Grønnesby and Borgan goodness-of-fit test. This test determines how closely the predicted event rate approximates the observed event rate over a range of scores. A significant value of *P* indicates a lack of fit.

The estimated coefficients were expressed as the hazard ratio (HR) with the respective 95%CI. A 2-sided *P* < 0.05 was considered statistically significant for all analyses.

Finally, we also assessed the incremental prognostic value of using one equation over another; using the concept of net reclassification improvement (NRI) as described by Pencina *et al.*^[16], to determine whether the reclassification of patients by one of the formulas regarding to each other, would result in a more accurate risk estimation.

All the analyses were performed with STATA 13, and by using the MedCalc statistical software version 12.2.1.

The study was reviewed by our expert Biostatistic

RESULTS

Mean age was of 73 ± 11 years, male patients constitute 66.4% of the studied population. Baseline characteristics are summarized in Table 1.

Assessment of renal function according to the formula used

The mean eGFR was higher when computed by the new CKD-EPI than with the re-expressed MDRD-4 (69.8 ± 23, 67.2 ± 19 mL/min per 1.73 m²), respectively (*P* < 0.0001 for comparison).

There was lower prevalence of eGFR < 60 mL/min per 1.73 m² with the new CKD-EPI than with the re-expressed MDRD-4 (32.9% vs 34.1%).

Events throughout the follow-up

During a follow up of 10 ± 3 mo, the composite endpoint occurred in 98 (10.8%) patients: 30 (3.3%) patients developed major bleeding, 18 (2%) had TE events, and 60 (6.6%) patients died.

Relation with the composite endpoint

The rate of the composite endpoint increased monotonically from the higher to the lower eGFR categories for both formulas (Figure 1).

Significant association was observed between the eGFR using both formulas as continuous variables and the composite endpoint. The adjusted hazard ratios of eGFR by each formula on the composite endpoint were: 0.98 (95%CI: 0.967-0.988) and 0.97 (95%CI: 0.963-0.987) for the re-expressed MDRD-4 and the new CKD-EPI, respectively (Table 2).

Similarly, the eGFR as a categorical variable was a strong independent predictor of the occurrence of the composite endpoint regardless of the formula used (Table 3).

The discriminative capacity of both formulas at predicting the composite endpoint, were quite similar, regardless of the eGFR was used as continuous (0.683 vs 0.695 for the re-expressed MDRD-4 and the new CKD-EPI, respectively; *P* = 0.748) or categorical variable (0.632 vs 0.639 for the re-expressed MDRD-4 and the new CKD-EPI, respectively; *P* = 0.45) (Table 4).

Relation with major bleeding

There was a step increase in the major bleeding rate, as the eGFR declines, independently of the formula used to calculate the eGFR (Figure 1).

After adjusting for HAS-BLED bleeding risk score, the re-expressed MDRD-4 eGFR as well as the new CKD-EPI eGFR, as continuous variables, showed a tendency to predict major bleeding: HR for both formulas = 0.98 (95%CI: 0.965-1.000; *P* = 0.07) (Table 2).

No significant association was observed between

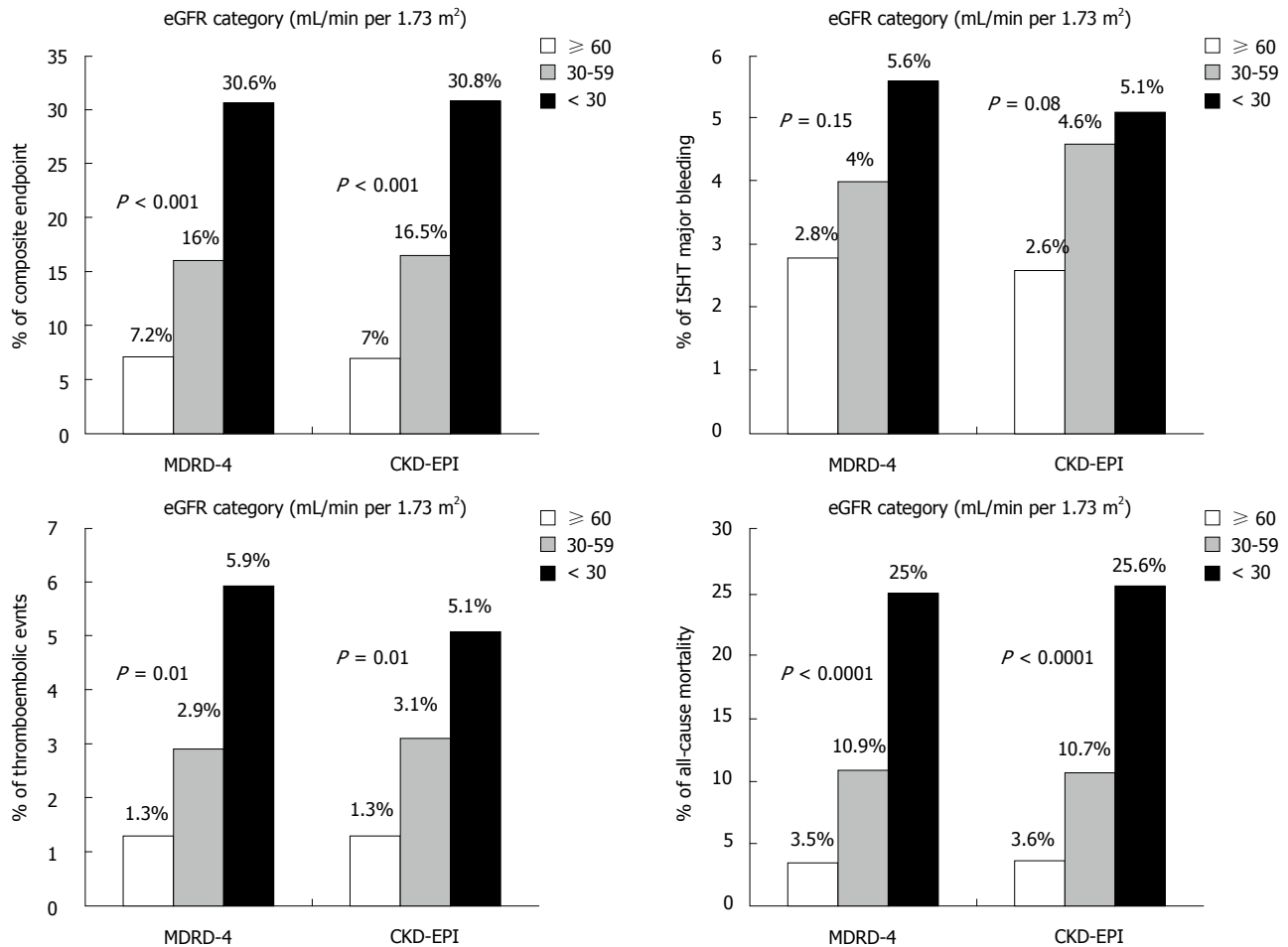


Figure 1 Distribution of major cardiovascular events according to the categories of estimated glomerular filtration rate using the re-expressed Modification of Diet in Renal Disease-4 and the new Chronic Kidney Disease Epidemiology Collaboration equations. eGFR: Estimated glomerular filtration rate; MDRD-4 indicates: Four variables Modification of Diet in Renal Disease; CKD-EPI: Chronic Kidney Disease Epidemiology Collaboration.

Table 2 Unadjusted and adjusted effect (HR) on outcomes of continuous estimated glomerular filtration determined by the re-expressed Four variables Modification of Diet in Renal Disease and the new Chronic Kidney Disease Epidemiology Collaboration equations

n (%)	MDRD-4		CKD-EPI	
	Unadjusted HR (95%CI)	Adjusted HR (95%CI)	Unadjusted HR (95%CI)	Adjusted HR (95%CI)
Composite endpoint, 98 (10.8)	0.97 (0.958-0.977)	0.98 ¹ (0.967-0.988)	0.96 (0.955-0.975)	0.97 ¹ (0.963-0.987)
P value	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Major bleeding, 30 (3.3)	0.97 (0.951-0.985)	0.98 ² (0.965-1.000)	0.97 (0.949-0.984)	0.98 ² (0.965-1.000)
P value	< 0.0001	0.07	< 0.0001	0.07
Thromboembolism, 18 (2)	0.98 (0.959-1.003)	0.98 ³ (0.965-1.000)	0.97 (0.948-0.996)	0.98 ³ (0.965-1.001)
P value	0.09	0.15	< 0.0001	0.22
All-cause mortality, 60 (6.6)	0.96 (0.948-0.973)	0.98 ⁴ (0.965-0.995)	0.96 (0.947-0.971)	0.98 ⁴ (0.965-0.995)
P value	< 0.0001	< 0.0001	0.02	0.001

¹Adjusted for age, sex, previous stroke, basal hemoglobin, chronic obstructive pulmonary disease, diabetes mellitus, congestive heart failure or left ventricular ejection fraction ≤ 40%, history of malignant disease and coronary artery disease; ²Adjusted for HAS-BLED risk score [Hypertension (uncontrolled: systolic >160 mmHg)], abnormal renal function, abnormal liver function, stroke, bleeding history or predisposition (anemia), labile international normalized ration (INR), elderly > 65 years, and Drugs/alcohol concomitantly; ³Adjusted for CHA2DS2-VASc score [Cardiac failure or dysfunction, Hypertension, Age ≥ 75 years (doubled), Diabetes, Stroke (doubled), Vascular disease, Age 65 to 74 years, and Sex category (female)]; ⁴Adjusted for age, sex, diabetes mellitus, history of malignant disease, previous stroke, basal hemoglobin and congestive heart failure or ejection fraction ≤ 40%. CKD-EPI: Chronic Kidney Disease Epidemiology Collaboration; MDRD-4: 4-variable Modification of Diet in Renal Disease Study equation.

categorical eGFR from both formulas and major bleeding, either in the unadjusted or by using the adjusted competing-risk models (Table 3).

At predicting major bleeding, the discriminative ability of the continuous re-expressed MDRD-4 eGFR was modest: 0.666; quite similar to that obtained from

Table 3 Unadjusted and adjusted effect (HR) on outcomes of categorical estimated glomerular filtration rate determined by the re-expressed four variables Modification of Diet in Renal Disease and the new Chronic Kidney Disease Epidemiology Collaboration equations

<i>n</i> (%)		MDRD-4		CKD-EPI	
		Unadjusted HR (95%CI)	Adjusted HR (95%CI)	Unadjusted HR (95%CI)	Adjusted HR (95%CI)
Composite endpoint, 98 (10.8)	≥ 60		1.00 (Reference)		
	30-59	2.43 (1.592-3.703) <i>P</i> < 0.0001	1.7 ¹ (1.11-2.78) <i>P</i> = 0.02	2.51 (1.642-3.827) <i>P</i> < 0.0001	1.8 ¹ (1.1-2.8) <i>P</i> = 0.02
	< 30	6.99 (3.585-13.649) <i>P</i> < 0.0001	3.3 (1.6-6.9) <i>P</i> = 0.001	7.4 (3.871-14.125) <i>P</i> < 0.0001	3.6 (1.8-7.4) <i>P</i> < 0.0001
Major bleeding, 30 (3.3)	≥ 60		1.00 (Reference)		
	30-59	1.53 (0.715-3.260) <i>P</i> = 0.30	1.01 ² (0.46-2.25) <i>P</i> = 0.95	1.87 (0.883-3.948) <i>P</i> = 0.1	1.2 ² (0.58-2.75) <i>P</i> = 0.58
	< 30	3.56 (0.811-15.580) <i>P</i> = 0.09	1.03 (0.22-4.95) <i>P</i> = 0.93	3.65 (0.827-16.074) <i>P</i> = 0.08	1.1 (0.25-5.35) <i>P</i> = 0.9
Thromboembolism, 18 (2)	≥ 60		1.00 (Reference)		
	30-59	2.04 (0.734-5.649) <i>P</i> = 0.17	1.4 ³ (0.49-4.15) <i>P</i> = 0.15	2.13 (0.767-5.917) <i>P</i> = 0.15	1.4 ³ (0.50-4.25) <i>P</i> = 0.50
	< 30	8.01 (1.664-38.555) <i>P</i> = 0.009	5.1 (1.04-25.4) <i>P</i> = 0.045	7.84 (1.625-37.825) <i>P</i> = 0.01	5 (1.0-24.9) <i>P</i> = 0.04
All-cause mortality, 60 (6.6)	≥ 60		1.00 (Reference)		
	30-59	3.34 (1.909-5.827) <i>P</i> < 0.0001	2.6 ⁴ (1.4-2.7) <i>P</i> = 0.002	3.14 (1.793-5.481) <i>P</i> < 0.0001	2.4 ⁴ (1.3-4.5) <i>P</i> = 0.005
	< 30	10.64 (4.843-23.359) <i>P</i> < 0.0001	4.9 (2.0-11.9) <i>P</i> < 0.0001	10.89 (5.122-23.166) <i>P</i> < 0.0001	5.2 (2.2-12.3) <i>P</i> < 0.0001

¹Adjusted for age, sex, previous stroke, basal hemoglobin, chronic obstructive pulmonary disease, diabetes mellitus, congestive heart failure or ejection fraction ≤ 40%, history of malignant disease and coronary artery disease; ²Adjusted for HAS-BLED risk score [Hypertension (uncontrolled: systolic > 160 mmHg)], Abnormal renal function, abnormal liver function, stroke, bleeding history or predisposition (anemia), labile international normalized ration (INR), elderly > 65 years, and drugs/alcohol concomitantly); ³Adjusted for CHA2DS2-VASc score [Cardiac failure or dysfunction, Hypertension, Age ≥ 75 years (doubled), Diabetes, Stroke (doubled), Vascular disease, Age 65 to 74 years, and Sex category (female)]; ⁴Adjusted for age, sex, diabetes mellitus, history of malignant disease, previous stroke, basal hemoglobin and congestive heart failure or ejection fraction ≤ 40%. CKD-EPI: Chronic Kidney Disease Epidemiology Collaboration; MDRD-4: 4-variable Modification of Diet in Renal Disease Study equation.

Table 4 Calibration and discrimination abilities of the re-expressed four variables Modification of Diet in Renal Disease and the new Chronic Kidney Disease Epidemiology Collaboration equations

		MDRD-4	CKD-EPI	<i>P</i> value
Composite endpoint	Calibration, χ^2 (<i>P</i> value)		1.7 (0.79)	3.5 (0.48)
	c-statistic (95%CI)	eGFR continuous	0.683 (0.629-0.737)	0.695 (0.643-0.747)
		eGFR categorical	0.632 (0.600-0.664)	0.639 (0.607-0.670)
Major bleeding	Calibration, χ^2 (<i>P</i> value)		5.9 (0.20)	5.4 (0.25)
	c-statistic (95%CI)	eGFR continuous	0.666 (0.581-0.751)	0.677 (0.596-0.759)
		eGFR categorical	0.550 (0.443-0.658)	0.571 (0.465-0.679)
Thromboembolism	Calibration, χ^2 (<i>P</i> value)		0.13 (0.99)	1.9 (0.76)
	c-statistic (95%CI)	eGFR continuous	0.616 (0.584-0.648)	0.644 (0.612-0.675)
		eGFR categorical	0.617 (0.585-0.649)	0.622 (0.590-0.654)
All-cause mortality	Calibration, χ^2 (<i>P</i> value)		0.83 (0.94)	1.5 (0.82)
	c-statistic (95%CI)	eGFR continuous	0.715 (0.684-0.744)	0.722 (0.691-0.750)
		eGFR categorical	0.679 (0.647-0.709)	0.678 (0.646-0.708)

eGFR: Estimated glomerular filtration rate; CKD-EPI: Chronic Kidney Disease Epidemiology Collaboration; MDRD-4: 4-variable Modification of Diet in Renal Disease Study equation.

using the continuous new CKD-EPI eGFR: c-statistic = 0.677 (*P* = 0.85).

When eGFR was considered as a categorical variable, the discriminative capacity of each formula at predicting major bleeding was of 0.550 and of 0.571 for the re-expressed MDRD-4 and the new CKD-EPI, respectively (*P* = 0.79) (Table 4).

Relation with thromboembolic event

As shown in Figure 1, the distribution of the TE event rate in the different eGFR categories, demonstrated a

consistent gradient of risk, regardless of the formula used.

After adjusting for the CHA₂DS₂-VASc risk score, no significant association was observed between eGFR as a continuous variable and TE events: HR = 0.98 (95%CI: 0.965-1.000) and 0.98 (95%CI: 0.965-1.001), for the re-expressed MDRD-4 and the new CKD-EPI, respectively (Table 2).

When eGFR was considered as a categorical variable, only significant association existed between eGFR < 30 mL/min per 1.73 m² and the TE complications, after

controlling for CHA₂DS₂-VASc score: HR = 5.1 (95%CI: 1.04-25.4) for the re-expressed MDRD-4, and HR = 5.0 (95%CI: 1.0-24.9) for the new CKD-EPI (Table 3).

The discriminative power of GFR estimates determined by both formulas was also modest. For continuous eGFR, the c-statistic values were of 0.616 and 0.644 for the re-expressed MDRD-4 and the new CKD-EPI, respectively, ($P = 0.27$), and for categorical eGFR, the c-statistic values were 0.617 and 0.622 when using the re-expressed MDRD-4 and the new CKD-EPI, respectively, ($P = 0.76$) (Table 4).

Relation with all-cause mortality

The rate of all-cause mortality increased progressively from the higher to the lower eGFR values for both formulas (Figure 1).

Continuous eGFR calculated by either the reexpressed MDRD-4 or the new CKD-EPI was an independent predictor of all-cause mortality; adjusted HR = 0.98; ($P < 0.01$) (Table 2).

A strong association was also found between categorical eGFR and all-cause mortality after adjusting for several confounders (Table 3).

Good discrimination was obtained from continuous eGFR: c-statistic = 0.715 for the re-expressed MDRD-4 and 0.722 for the new CKD-EPI ($P = 0.52$).

The discriminative power of eGFR as a categorical variable in terms of c-statistic was: 0.679 and 0.678 when using the re-expressed MDRD-4 and the new CKD-EPI, respectively, ($P = 0.91$) (Table 4).

Estimated GFR from both formulas demonstrated good calibration for the major cardiovascular events with P value > 0.1 (Table 4).

The NRI analysis did not significantly favor the new CKD-EPI over the re-expressed MDRD-4 whether for predicting the composite endpoint, major bleeding and all-cause mortality (NRI = 2.13%, 4.35%, and 0.9%, with $P = 0.27$, 0.19, and 0.7, respectively).

However, at predicting the TE event, the NRI favored the new CKD-EPI formula with NRI of 1% (95%CI: -0.08 to +2.0, $P = 0.07$) indicating a strong tendency to reclassify better the patients according to their risk of developing TE event, compared with the re-expressed MDRD-4.

DISCUSSION

In this real world cohort of patients with NVAf on VKAs, the new CKD-EPI formula classified lower percentage of patients as having eGFR < 60 mL/min per 1.73 m² than the re-expressed MDRD-4 equation did. This means that the use of the new CKD-EPI formula results in lower prevalence of renal dysfunction. We also found that renal dysfunction assessed either by the re-expressed MDRD-4 or the new CKD-EPI was strongly associated with the composite endpoint of major bleeding, TE event and all-cause mortality, and with all-cause mortality, as well.

Patients with NVAf are often elderly with multiple comorbidities which require pharmacotherapy of growing complexity, and this makes the reliable estimation of renal function to be undeniably a critical issue. Moreover, the availability of the new oral anticoagulants have renewed the great interest toward the accurate evaluation of renal function in patients with NVAf^[17,18].

Up to our knowledge, this is the first study comparing the prognostic performance of the re-expressed MDRD-4 and the new CKD-EPI formulas used for estimating GFR in a real world population of patients with NVAf on VKAs who have a full range of eGFR.

In this cohort, the new CKD-EPI formula classified lower percentage of patients as having eGFR < 60 mL/min per 1.73 m² (32.9% with new CKD-EPI vs 34.1% with re-expressed MDRD-4). This reasonable ability of the new CKD-EPI formula to reduce the rate of patients with renal dysfunction could be highly appreciated by the clinicians in daily clinical practice which usually needs close attention to the status of renal function to reach the optimal management, and more safe use of renally excreted medications and nephrotoxic contrast agents, in patients with NVAf. Our finding is consistent with that found in the derivation cohort of the new CKD-EPI^[9] and to the findings obtained from multiple studies in different clinical settings^[12,19-21].

In our analysis, renal dysfunction determined by GFR estimates using both formulas was a significant predictor of the composite endpoint and all-cause mortality. Similar findings have been shown in previous study used the MDRD-4^[22], but until now, no study has compared the prognostic usefulness of these formulas in a real world patients with NVAf. In this study, we did not find any significant difference in the prognostic impact between the new CKD-EPI and the re-expressed MDRD-4 at predicting major adverse cardiovascular outcomes.

In our analysis, we found that both formulas with the eGFR as a continuous variable and after controlling for HAS-BLED risk score^[4], showed a tendency to predict major bleeding. Previous association between renal dysfunction and major bleeding were found in AF studies^[22,23]. However, the prior tendency was lost when the eGFR using both formulas was tested as categorical variables; this may be explained by the small number of events (30 events, 3.3%) that could limit the detection of significant relationship from the data.

TE prevention remains the primary cornerstone in the management of patients with NVAf. In dealing with this great aim, there are conflicting data about the ability of renal dysfunction to predict this major catastrophe. Several studies demonstrated significant association between reduced eGFR and TE event^[22-24], conversely, in other studies, decreased eGFR did not show significant relationship with TE event^[25,26]. These differences could be explained by the differences in the formula used to estimate GFR, sample size, patients

characteristics (*i.e.*, from a real world or clinical trial population), and/or the disparities in duration of follow up between the studies. Therefore, there is a strong need for further evaluation of that uncertainty in a real world population. Regarding this important issue, in our real world cohort of patients with NVAf, and after adjusting for the CHA₂DS₂-VASc risk score^[15] there was a significant association between eGFR as categorical variable and TE event only when the eGFR was < 30 mL/min per 1.73 m² (*i.e.*, severe renal dysfunction category) with similar prognostic impact of both the re-expressed MDRD-4 and the new CKD-EPI. Furthermore, the NRI analysis showed a tendency of the new CKD-EPI to reclassify better the patients according to their risk of developing TE event, compared with the re-expressed MDRD-4.

It should be kept in mind that the eGFR formulas were designed to most accurately estimate renal function and not to predict major adverse outcomes. Indeed, the relative performance of the two different GFR estimating equations in our study can be explained by their respective compositions (*i.e.*, the difference of mathematical modeling and how specific variables are coded and weighted by each equation). Also, the relative variance in performance between both formulas can be explained by the differences in their respective derivation populations. The MDRD-4 formula was originally developed in patients with established renal dysfunction^[6]; for this, the re-expressed MDRD-4 formula may be less applicable to patients from the real world with full range of GFR. In contrast, the new CKD-EPI equation could be more precise in our community-based cohort of patients with NVAf, as the new CKD-EPI was developed in population with and without renal dysfunction^[9].

Although, many laboratories are preparing their installation to use the new CKD-EPI equation instead of the re-expressed MDRD-4 formula according to the current guideline^[27] and a consensus document^[28], however, old habits die hard. Our assessment of the prognostic performance of both formulas in the particular clinical context of AF might be of great importance as it could help convince the clinicians and mitigate the doubts and obstacles regarding the adoption of the new CKD-EPI.

Really, patients with NVAf and renal dysfunction continue to represent a complex management problem in relation to decision making for thromboprophylaxis. With respect to the overall concept, the data obtained from our analysis, state that the new CKD-EPI formula reduced the prevalence of patients with renal dysfunction (*i.e.*, eGFR < 60 mL/min per 1.73 m²), and at the same time continued to have prognostic impact similar to that of the re-expressed MDRD-4 equation at predicting the major adverse events. Taken together, our notable results from a real world cohort encourage the use of the new CKD-EPI equation to assess renal function in patients with NVAf and reinforce the current

recommendation^[9,27,28] for the use of the new CKD-EPI formula in all clinical situations.

It is clear that our study presents an analysis of a modest sized cohort of patients with NVAf on VKAs from the real world, and the prevalence of patients with eGFR < 60 mL/min/1.73 m² was just reduced by 1.2% when using the new CKD-EPI formula. However, our cohort might give a good reflection of the general population with millions of patients having NVAf, in whom the percentage of 1.2% would be highly significant.

Limitations

The main limitation of our study is its retrospective design, but it has interesting strong points as it reflects real world practice by enrolment of consecutive patients with NVAf who have full range of eGFR and were attending our outpatient cardiology clinics with the advantage of careful follow up and data collection by cardiologists.

The sample size might be another limitation of our study that could limit the likelihood of detecting small effects or significant relationships from the data. Important to mention here that we did not have the direct measured GFR, so we cannot determine the extent to which the two formulas reflect the GFR as determined by the gold standard method. However, eGFR is the practical way to estimate renal function which has been used in several patient populations. The fact that we have only one serum creatinine measure for every patient could limit the verification of the acute vs chronic nature of the renal dysfunction in some patients, but this limitation was present in several related studies^[23-25]. The lack of cystatin C data might be considered a limitation of our study. However, it should be taken into account that all the creatinine measurements in our study cohort were performed with the IDMS-traceable enzymatic assay method, which has been shown to provide very reliable eGFR results^[13] and is considered the standard method to assess renal function^[29].

Finally, all of the enrolled patients in our cohort have Caucasian race, so the applicability of our findings in other populations with different races should be addressed in other studies.

The new CKD-EPI reduced the prevalence of patients with renal dysfunction, in a real world cohort of patients with NVAf on VKAs. Renal dysfunction reflected by GFR estimates from the re-expressed MDRD-4 or the new CKD-EPI was an independent predictor of the composite endpoint and all-cause mortality. Both formulas had similar prognostic impacts regarding the prediction of composite endpoint, major bleeding, TE events and all-cause mortality. Our analysis indicates that the more widespread adoption of the new CKD-EPI instead of the re-expressed MDRD-4 may improve the management of patients with NVAf.

COMMENTS

Background

Renal dysfunction is a frequent comorbidity seen in patients with atrial fibrillation. Moreover, renal dysfunction is a strong predictor of thromboembolic event and also of bleeding event (when the patients are anticoagulated). This reflects the need for more accurate estimate of renal function to guarantee the optimal management of patients with atrial fibrillation. The standard way to assess renal function is the glomerular filtration rate. Among the available equations to estimate the glomerular filtration rate are: the re-expressed Modification of Diet in Renal Disease equation which is still the commonly used equation by many laboratories all over the world and the new Chronic Kidney Disease Epidemiology Collaboration equation which has been recently proposed to be used instead of previous equation in daily practice as the new equation has an assumed ability to reduce the prevalence of patients with renal dysfunction and better reclassification of patients. There is limited information about the performance of both equations in patients with atrial fibrillation.

Research frontiers

The authors think that the new Chronic Kidney Disease Epidemiology Collaboration equation to estimate glomerular filtration rate must have a wide diffusion as an alternative to the re-expressed Modification of Diet in Renal Disease equation. In this paper the authors provide support to the hypothesis, reporting the superiority of the new Chronic Kidney Disease Epidemiology Collaboration equation over the re-expressed Modification of Diet in Renal Disease equation in the clinical context of patients with atrial fibrillation on anticoagulation.

Innovations and breakthroughs

The results derived from our analysis, state that the new Chronic Kidney Disease Epidemiology Collaboration equation reduced the prevalence of patients with renal dysfunction (*i.e.*, estimated glomerular filtration rate < 60 ml/min per 1.73 m²), and at the same time continued to have the prognostic impact similar to the re-expressed Modification of Diet in Renal Disease equation at predicting the major adverse events. Although there are still some concerns about the performance of the new equation in subgroups of elderly and obese patients, the study from a real world cohort encourages the cardiologists to use of the new Chronic Kidney Disease Epidemiology Collaboration equation to assess renal function in patients with atrial fibrillation and increase the confidence to use it in all clinical situations.

Applications

The millions of patients with atrial fibrillation will get benefit and better management if there is wide spread adoption of the new Chronic Kidney Disease Epidemiology Collaboration equation instead of the re-expressed Modification of Diet in Renal Disease equation, giving the ability of the new equation to correctly reclassify patients in comparison with the re-expressed equation.

Terminology

The Chronic Kidney Disease Epidemiology Collaboration equation (CKD-EPI) was published in May 2009 as a reliable tool to estimate glomerular filtration rate. It was developed in an effort to create an equation more accurate than the re-expressed Modification of Diet in Renal Disease equation. Researchers pooled data from multiple studies to develop and validate this new equation. They used 10 studies that included 8254 participants, randomly using 2/3 of the data sets for development and the other 1/3 for internal validation. Sixteen additional studies, which included 3896 participants, were used for external validation. The CKD-EPI equation performed better than the Modification of Diet in Renal Disease equation, as the prevalence of chronic kidney disease was 11.5% vs 13.1% according to the National Health and Nutrition Examination Survey data in the United States of America.

Peer-review

First of all I would like to congratulate the authors with their achievement. In this retrospective study including relatively limited sample size of Caucasian subjects, the findings encourage the use and application of the new CKD-EPI equation for assessment not only of renal function in patients with non-valvular atrial fibrillation

but also in all clinical situations. For the first time, Abumuaileq RRY *et al* evaluated the re-expressed MDRD-4 and the new CKD-EPI formulas at predicting the occurrence of major adverse outcomes in a real world cohort of patients with non-valvular atrial fibrillation on anticoagulation. The study was well conducted and clinically relevant.

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Percutaneous pulmonary valve implantation in a single artery branch: A preliminary experience

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Abstract

To describe preliminary experience of percutaneous

pulmonary valve implantation, in a single pulmonary branch position. Two procedures in 2 patients from a single center are described, where implantation of percutaneous valves within a single pulmonary artery branch was technically successful. The procedural indication was pulmonary valve regurgitation and/or residual stenosis. The 2 patients were symptomatic. An Edwards Sapien™ valve (Patient 1), and a Medtronic Melody™ valve (Patient 2) were implanted. Both pts were discharged with an excellent valve function. In this report it is underlined that this modality is technically feasible and may be considered an option in patients with congenital heart defect under special circumstances.

Key words: Congenital heart disease; Tetralogy of fallot; Pulmonary atresia; Percutaneous pulmonary valve; Grown-ups with congenital heart diseases

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Core tip: Today MelodyVR valve (Medtronic, Minneapolis, MN) and the SAPIENT™ transcatheter heart valve valve (Edwards Lifesciences LLC, Irvine, CA) are available to use in patients with a conduit connecting the right ventricle to the main pulmonary arteries (PA). However, given the anatomic variability of the right ventricular outflow tract and the concomitant occurrence of branch PA disease frequently encountered in this patient population, alternative approaches to valve replacement needs to be explored. In order to solve this problem we use a different approach implementing percutaneous pulmonary valve implantation, in a single pulmonary branch position.

Chessa M, Butera G, Giugno L, Micheletti A, Negura DG, Carminati M. Percutaneous pulmonary valve implantation in a single artery branch: A preliminary experience. *World J Cardiol* 2015; 7(10): 695-699 Available from: URL: <http://www.wjgnet.com/1949-8462/full/v7/i10/695.htm> DOI: <http://dx.doi.org/10.4330/wjc.v7.i10.695>

INTRODUCTION

The first successful percutaneous pulmonary valve implantation (PPVI) was described by Bonhoeffer *et al*^[1]. Currently two balloon expandable transcatheter valves are available for PPVI: the MelodyVR valve (Medtronic, Minneapolis, MN) and the SAPIENTM transcatheter heart valve (Edwards Lifesciences LLC, Irvine, CA).

Both valves are indicated in patients with a conduit connecting the right ventricle to the main pulmonary arteries (PA). However, given the anatomic variability of the right ventricular outflow tract (RVOT) and the concomitant occurrence of branch PA disease frequently encountered in this patient population, alternative approaches to valve replacement have been explored^[2-4]. We report our preliminary experience of PPVI in a single pulmonary branch.

CASE REPORT

Two patients (Table 1) underwent PPVI in a single branch between 2013 and 2014.

Patient 1 had a complete repair of tetralogy of Fallot in 2001, followed by a re-operation in August 2003 for residual ventricular septal defect (VSD) + right ventricular outflow tract obstruction (RVOTO). During the second intervention a trans-annular patch + VSD closure + Tricuspid Valve plasty and patch enlargement of a hypoplastic left pulmonary artery (LPA) were performed. In 2006, the patient underwent a cardiac catheterization that showed absence of the LPA, confirmed in 2013 with a cardiac magnetic resonance (CMR) patient 2 had a left modified Blalock-Taussig Shunt, and then at age 1 year, underwent a complete correction of her PA-VSD + MAPCAs unifocalization and FreeStyle 14 mm RV-PA conduit implantation. The conduit was changed when she was 5-year-old using a 23 mm FreeStyle conduit associated with PA bifurcation plasty.

A CMR showed a dilated RV (EDVI = 157 mL/m²) with severe pulmonary valve regurgitation (PVR) (RF = 43%), moderate RVOTO (peak gradient 40 mmHg), preserved RV systolic function, and absence of the right pulmonary artery (RPA) branch.

Informed consent was obtained for both two patients. Femoral venous and arterial accesses were obtained under general anesthesia. A complete left and right heart catheterization was done in both pts.

The RV angiography in patient 1 showed a dilated RVOT with a diameter of 32 mm (Figure 1A-C), too large for classical PPVI. At the origin of the RPA, there was the evidence of a waist (Figure 1D) with a diameter of 24 mm; a pre-stenting of the PA branch was made with a 43 mm ANDRA XXL stent mounted on 25 mm × 50 mm Crystal Balt balloon (Figure 1E).

The RV angiography in patient 2 showed a dilated, calcified, and moderate stenotic conduit (Figure 2A and B). A pre-stenting of the conduit was made using 2

premounted covered CP stents (45 mm length, 8 zigs and dilated with a BiB balloon 22 mm × 55 mm) (Figure 2C-E). The final stented conduit had a proximal angle which was considered not perfectly suitable (Figure 2F) for a traditional implant of the percutaneous valve. The valve was implanted at the origin of the LPA where the landing zone was better.

After a standard valve preparation including a thorough washing protocol and crimping protocol, a 26 mm Edwards Sapien valve in patient 1, and a 22 mm MelodyVR valve in patient 2 were deployed.

There were no immediate procedure-related complications. The trans-thoracic echo (TTE) performed the day after showed excellent valve function with no regurgitation and no stenosis. Therefore the patient 2 complained, 48 h later, about an abrupt onset of chest pain, shortness of breath, and hypoxia. She immediately underwent a computed tomography angiography (CTA) and the final diagnosis was micro pulmonary embolism with evidence of filling defects in the distal pulmonary branches in the latero-basal segment of the lower lobe + in the inferior segment of the lingula.

She was immediately transferred to the ICU and she was started on Eparine.

The patient recovered immediately, the saturation became normal and she was discharged at home 10 d after.

At the last follow-up (range 6-12 mo) both pts were asymptomatic, with neither pulmonary valve regurgitation nor residual stenosis.

Patient 1 repeated the CMR 1 year after and the EDVI was 123 mL/m².

DISCUSSION

Robb *et al*^[5], reported an animal study, with a Melody valve implantation in the pulmonary artery branches. The Authors demonstrated that RVOT dilation and distortion consistent with transannular patch repair of ToF could be reliably mimicked with an animal model. Secondly, it was shown that PPVI into the right and left branch PAs resulted in a significant reduction in pulmonary regurgitation, with preserved biventricular function demonstrated by MRI and catheterization.

Qureshi *et al*^[6], reported a case of a transcatheter placement of the Melody valve in the proximal left pulmonary artery of a patient with acquired right pulmonary artery occlusion.

In our patients the valve was inserted in the only pulmonary artery branch available either because the RVOT was judged to be too large to allow a PPVI in a regular position, or the previous implanted conduit had a better angle (after pre-stenting) in the distal part, in the area in which there was a conjunction with the left pulmonary artery.

The complication experienced by Patient 2 could have occurred to the fact that some microemboli originate in the lower part of the conduit just under the pulmonary valve. Since this zone in which there is a

Table 1 Patients' characteristics

Patient	DoB	Weight (kg)	Diagnosis	Surgery	L/R PAB	PVR (yes/no)	RVOTO (yes/no)	Previous Caths (year)	CMR/CT scan	Valve	Prestenting	Complications	Follow-up
1 st (male)	June-2000	43	ToF	TAP	RPAB	Yes	No	2006	CMR	Sapient 26	Yes	No	12-mo
2 nd (female)	July-1994	56	PA-VSD-MAPCAs	RV-PA conduit FreeStyle 14 mm and 23 mm (1999)	LPAB	Yes	Yes (moderate)	2012	CMR	Melody 22	Yes	Yes	6-mo

PA-VSD-MAPCAs: Pulmonary arteries-ventricular septal defect-major aortopulmonary collateral arteries; TAP: Trans-annular patch; RVOTO: Right ventricular outflow tract obstruction; CMR: Cardiac magnetic resonance; PVR: Pulmonary valve regurgitation.

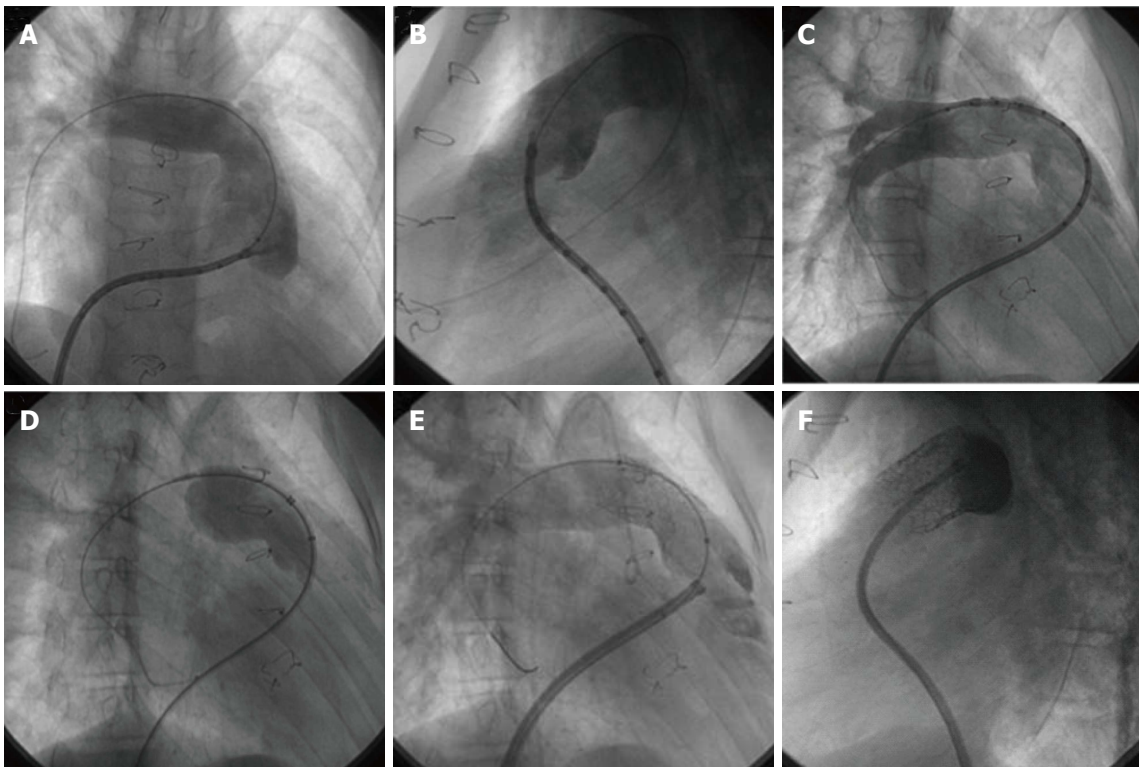


Figure 1 Angiograms in anterior-posterior, LL, right anterior oblique projections of the percutaneous pulmonary valve implantation using a Melody valve. A-C: Injections in the RV and origin of the RPA showing a dilated RVOT and stenosis at the origin of the RPA; D: Evidence at the balloon sizing of a waist at the origin of the RPA; E: Pre-stenting of the PA branch with a 43 mm ANDRA XXL; F: Good final result after Valve implantation with no evidence of PVR. PPVI: Percutaneous pulmonary valve implantation; RVOT: Right ventricular outflow tract; PA: Pulmonary arteries; RPA: Right pulmonary artery; AP: Anterior-posterior.

flow is not contractile, it could have favored a slowing down of the flow, creating a favorable condition for the formation of microemboli. It is difficult to support the idea that these emboli were related to the large sheaths used, because the episode was 48 h later. The pt was anticoagulated with a Heparine bolus of 100 IU/kg and the ACT was > 200 during all the procedure-time: She was just on antiplatelet therapy (ASA: 100 mg as in our protocol), after the procedure. Anticoagulation prophylaxis vs standard platelet antiaggregation should be take into consideration for these specific pts.

This brief report shows that PPVI can be performed in Pts with a congenital heart defect, and with a single

pulmonary artery branch.

The use of a percutaneous valve in a branch pulmonary artery is not to be proposed for all the pts with a large or with a complex RVOT anatomy; what can be underlined with this report is that this modality is technically feasible and it may be considered as an option in high-risk patients under special circumstances.

COMMENTS

Case characteristics

Patient 1: Dyspnea and low stress tolerance; and Patient 2: Chest pain, shortness of breath, and hypoxia.

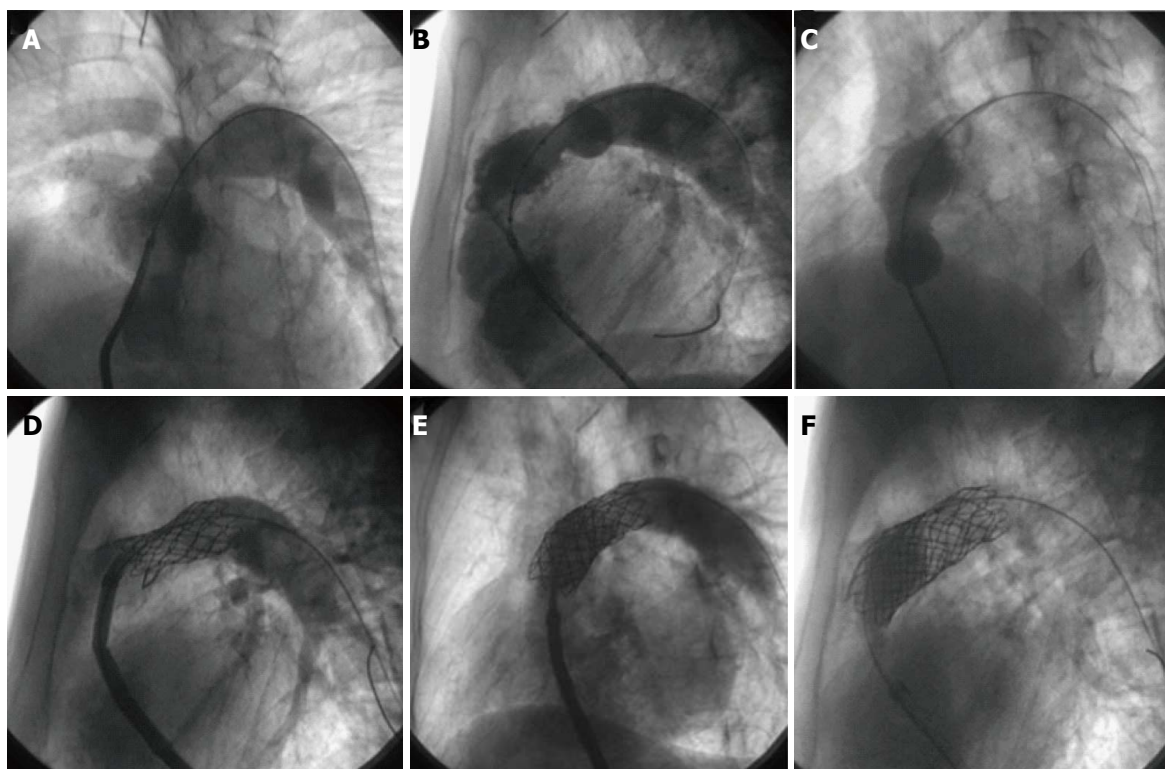


Figure 2 Angiograms in anterior-posterior, LL, projections of the percutaneous pulmonary valve implantation using a Sapien valve. A, B: Injections in the RV showing a dilated and calcified conduit with some degree of stenosis at the PA edge; C: A compliant AGA 34 mm ASD sizing balloon was inserted into the Freestyle conduit showing the stenotic area; D: Prestenting of the conduit using a premounted covered CP stent 45 mm; E: Second covered CP stent was implantation; F: After a re-dilation by utilizing an ATLAS 22 × 40 balloon dilatated at 14 Atm a final stented conduit was obtained with a proximal angle not suitable for a PPVI in a regular position. PPVI: Percutaneous pulmonary valve implantation; RV: Right ventricular; PA: Pulmonary arteries.

Clinical diagnosis

Patient 1: Hypoplasia of left pulmonary branch; and Patient 2: Occlusion of right pulmonary branch.

Laboratory diagnosis

Patient 1: WBC: 7650/mm³ (N: 66%; L: 18%; M: 10%). Hb: 13.3 g/dL, Hct: 38.4%; PLT 216000/mm³; PCR: 2.8 mg/dL; PCT: 0.05 ng/mL; Patient 2: Hb: 10.2 g/dL, Hct: 31.1%; RBC: 3.78 × 10⁶ U/L; INR: 2.12.

Imaging diagnosis

Patient 1: Cardiac magnetic resonance (MR), chest X-ray, echocardiography; Patient 2: Cardiac TC multi-slide, cardiac MR, chest X-ray, echocardiography.

Pathological diagnosis

Patient 1: The right ventricular angiography showed a dilated right ventricular outflow tract with a diameter of 32 mm and at the origin of right pulmonary artery there was an evidence of a waist with a diameter of 24 mm; Patient 2: A cardiac RM show a dilated right ventricle (EDVI = 157 mL/m²) with severe pulmonary valve regurgitation (PVR) (RF = 43%), moderate right ventricular outflow tract obstruction (RVOTO) (peak gradient: 40 mmHg), preserved RVC systolic function.

Treatment

Patient 1: 26 mm Edward Sapien Valve implantation in left pulmonary branch; Patient 2: 22 mm MelodyVR valve implantation.

Related reports

Patient 1: ToF s/p complete surgical repair and following surgical treatment of residual Ventricular septal defect + RVOTO with trans annular patch (TAP) + VSD closure + Tricuspid valve plasty and patch enlargement of hypoplastic left pulmonary artery (LPA). In 2006 a cardiac catheterization showed the absence of LPA, confirmed in 2013 with a cardiac MR that show hypoplasia of left

pulmonary branch; Patient 2: pulmonary atresia + VSD and MAPCAs s/p a left modified Blalock-Taussing shunt (MBTS) and following complete correction with unifocalization and 14 mm RV-PA conduit implantation that five years later was change with a 23 mm FreeStyle conduit + PA bifurcation plasty.

Term explanation

PPVI is the Percutaneous Pulmonary Valve Implantation that can be done in case of congenital heart disease in a GUCH population (Grown Ups with Congenital Heart Diseases) with RVOTO and/or PVR.

Experiences and lessons

Valve implantation in pulmonary branches could be a useful approach in high-risk patients under special circumstances.

Peer-review

This article provided the innovative approach to a particular kind of diseases in critical patients and gave the good results at the last follow up.

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Intra-His bundle block in 2:1 atrioventricular block

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Author contributions: Hong SP, Park YW and Lee YS designed and wrote the report; Lee YS performed the electrophysiological study and pacemaker implantation.

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phenomenon, but it is important for the development of advanced or complete AV block. We observed a 77-year-old female patient with the 2:1 AV block due to an intra-hisian block. In this case we tried to detect the block site, but an alternating pattern of the AH conduction was noted on the His-electrogram in the electrophysiological study (EPS). The cause of the confusing finding might have been the instability of the catheter to record a His potential. We could detect a splitting of the His-electrogram with an intra-hisian block after minimal manipulation of the catheter. The authors' observations suggest that catheter stability is important for a precise recording in the EPS and radiofrequency catheter ablation procedure.

Key words: Cardiac electrophysiologic techniques; Bundle of His; Atrioventricular block

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Core tip: Intra-hisian atrioventricular (AV) block associated with 2:1 AV block is an uncommon phenomenon, but it is important for the development of complete AV block. We observed a 77-year-old female with 2:1 AV block due to an intra-hisian block. An alternating pattern of the AH conduction was noted on the His-electrogram. The cause of that confusing finding might have been the instability of the catheter for recording the His potential. We could detect a splitting of the His-electrogram with intra-hisian block after minimal manipulation of the catheter. The authors' observations suggest that catheter stability is important for a precise recording.

Hong SP, Park YW, Lee YS. Intra-His bundle block in 2:1 atrioventricular block. *World J Cardiol* 2015; 7(10): 700-702
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Abstract

Intra-hisian atrioventricular (AV) block is not a common

INTRODUCTION

Catheter stability is very important for achieving a precise

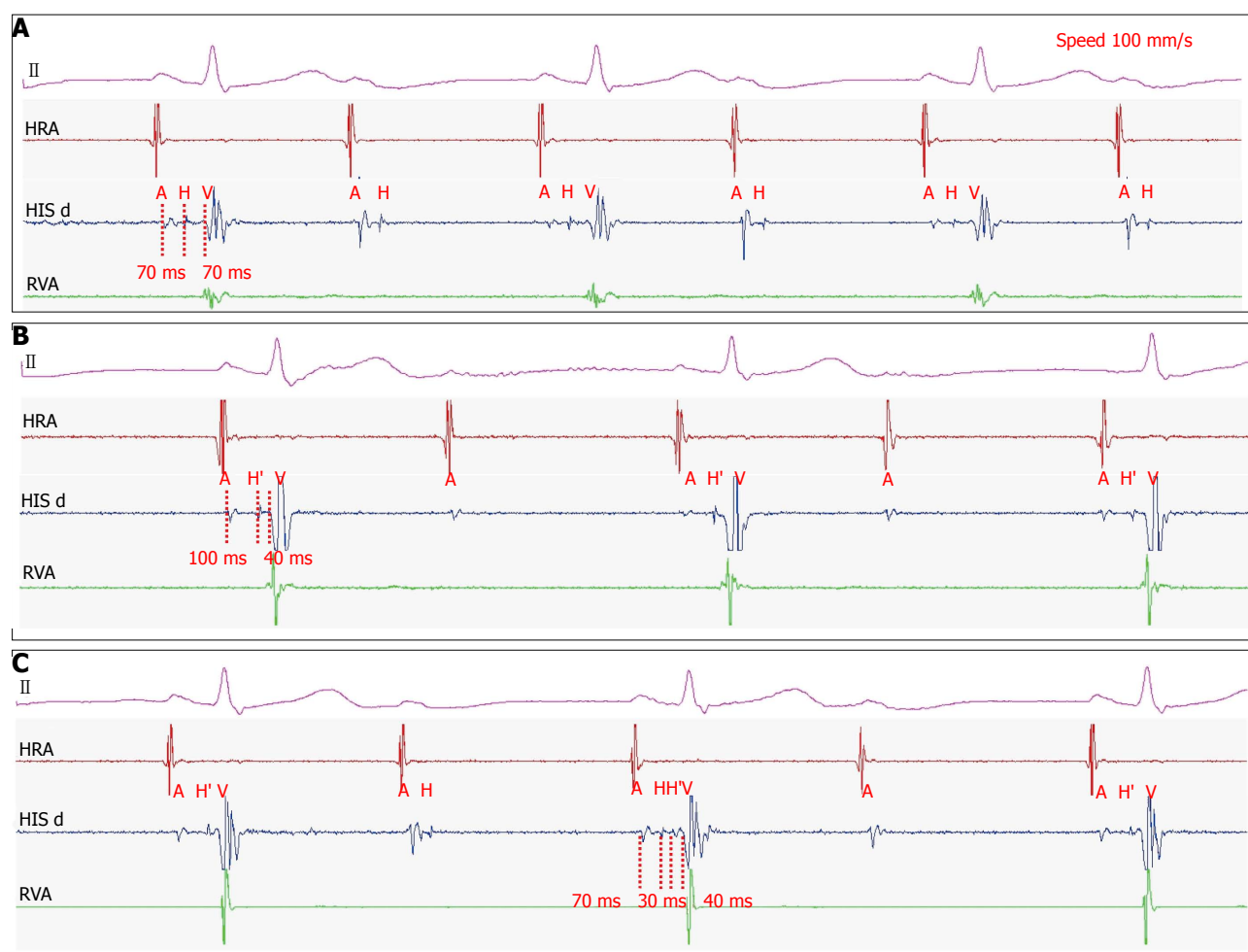


Figure 1 Surface electrocardiograms with intra-cardiac electrocardiograms in sweep speed of 100 mm/s. Surface ECG shows 2:1 atrioventricular block with a right bundle branch block. A: Intra-cardiac ECG was located the right ventricular apex (RVA), His bundle (HIS), and right ventricular apex (RVA). Surface ECG revealed 2:1 atrio-ventricular block. Intra-cardiac ECG revealed infra-hisian block and short A-H interval (70 ms); B: Intra-cardiac ECG revealed supra-hisian block and long A-H interval (100 ms); C: The intracardiac ECG shows the splitting of the His potential (H-H') during AV conduction and only the proximal activation of the His potential during the AV block, which means an intra-hisian block. A: Potential of right atrium; H: Potential of proximal His bundle; H': Potential of distal His bundle; V: Potential of right ventricle; ECG: Electrocardiogram.

electrophysiological study (EPS); 2:1 atrioventricular (AV) block is usually a disease of various levels such as supra-, intra- and infra-hisian^[1,2]. We report a case with some confusion caused by the instability of the catheter used for the His-electrogram recording during 2:1 AV block due to an intra-hisian block.

CASE REPORT

A 77-year-old female complained of dizziness. Her past history had hypertension, and had been taking dihydropyridine calcium channel blockers for 10 years. The chest radiograph showed cardiomegaly. The echocardiography revealed a normal function and wall motion. A resting electrocardiogram (ECG) revealed 2:1 AV block and right bundle branch block. After an intravenous injection of atropine and an exercise treadmill test, the ECG exhibited persistent 2:1 AV block.

The patient underwent an EPS to determine the position of the conduction block. EPS catheters located

in right ventricle, His bundle and right atrium. Firstly, we found an infra-hisian block with a normal A-H interval (70 ms) and long H-V interval (70 ms) on the intracardiac ECG (Figure 1A), and then a supra-hisian block with longer A-H interval (100 ms) and shorter H-V interval (40 ms) (Figure 1B). After a while, another intracardiac ECG revealed a splitting of the His potential (H-H') in the A-V conduction and only the proximal activation of the His potential during A-V block, which meant an intra-His bundle block (Figure 1C).

The patient was performed the implantation of permanent pacemaker as a result of the intra-His bundle block. The patient has no other symptoms until now.

DISCUSSION

In our case, we tried to detect the block site but an alternating pattern of the AH conduction was noted in the His-electrogram during the EPS. The cause of

this confusing finding might have been due to the instability of the catheter to record a His potential. However, the His potential was distinct in the first recoding of the His bundle (Figure 1A). In addition, the His potential spontaneously moved and the AH interval prolonged during the AV conduction, which concluded that the catheter was not stable.

A consistent intracardiac electrogram reflects a stable catheter position. Among the several catheters used during the EPS, the catheter used for the His-electrogram recoding is the most unstable in a beating heart. Catheter stability is important for obtaining a precise recording during the EPS and radiofrequency catheter ablation procedure. Instability of catheters can contribute to a misdiagnosis and procedural complications especially with AV nodal reentrant tachycardia. Recently, a remote magnetic navigation system is able to provide better catheter stability during the radiofrequency catheter ablation procedure^[3]. However, that system cannot be used during diagnostic procedures such as in our study because of the high cost. Fortunately, we could detect the splitting of the His-electrogram with an intra-hisian block after a minimal manipulation of the catheter.

Intra-hisian AV block during 2:1 AV block is not a common phenomenon, but it tends to develop into advanced or complete AV block^[4]. We should try to obtain a precise His electrogram and find the exact block site when conducting an EPS for 2nd degree high grade AV block.

COMMENTS

Case characteristics

A 77-year-old female complained of dizziness.

Clinical diagnosis

Intra-hisian atrioventricular (AV) block detected a splitting of the His-electrogram after a correction of the catheter instability during the electrophysiological study (EPS).

Differential diagnosis

Second-degree Mobitz 1 block with 2:1 AV is easily misdiagnosed as general

two to one conductive rate regardless of block site.

Laboratory diagnosis

The laboratory test results were unremarkable.

Imaging diagnosis

The intracardiac electrocardiogram revealed splitting of the His potential of the AV conduction during the EPS.

Treatment

A permanent pacemaker was implanted as a result of intra-hisian AV block.

Related reports

To best of our knowledge, intra-hisian AV block is an uncommon phenomenon.

Term explanation

Intra-hisian AV block in His-electrogram exhibited a splitting of the His potential during the AV conduction and only the proximal activation potential of the His bundle during the AV block, which meant an intra-His bundle block.

Experiences and lessons

The authors should try to achieve a precise recording of the His electrogram and find the exact block site when conducting an EPS for 2nd degree high grade AV block.

Peer-review

It is an interesting case report.

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Late endocarditis of Amplatzer atrial septal occluder device in a child

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Abstract

Bacterial endocarditis following atrial septal defect closure using Amplatzer device in a child is extremely rare. We report a 10-year-old girl who developed late bacterial endocarditis, 6 years after placement of an Amplatzer atrial septal occluder device. Successful explantation of the device and repair of the resultant septal defect was carried out using a homograft patch. The rare occurrence of this entity prompted us to highlight the importance of long-term follow up, review the management and explore preventive strategies for similar patients who have multiple co-morbidities and a cardiac device. A high index of suspicion is warranted particularly in pediatric patients.

Key words: Endocarditis; Atrial; Septal; Device

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Core tip: Bacterial endocarditis following atrial septal defect closure using Amplatzer device in a child is extremely rare.

We report a 10-year-old girl who developed late bacterial endocarditis, 6 years after placement of an Amplatzer atrial septal occluder device. This case report demonstrates the need for long-term follow up of patients with intracardiac device especially those with multiple co-morbidities or who have vulnerability to infection due to poor general condition or extremes of age such as our's. A high index of suspicion for device complication is required if sepsis or embolic phenomenon found. Incomplete endothelialization of the prosthetic devices may be linked to endocarditis and needs to be explored.

Jha NK, Kiraly L, Murala JSK, Tamas C, Talo H, El Badaoui H, Tofeig M, Mendonca M, Sajwani S, Thomas MA, Al Doory SA, Khan MD. Late endocarditis of Amplatzer atrial septal occluder device in a child. *World J Cardiol* 2015; 7(10): 703-706 Available from: URL: <http://www.wjgnet.com/1949-8462/full/v7/i10/703.htm> DOI: <http://dx.doi.org/10.4330/wjc.v7.i10.703>

INTRODUCTION

Bacterial endocarditis of intracardiac devices including Amplatzer atrial septal occluder in pediatric population is extremely rare. In view of the limited experience, this is an opportunity to highlight such cases in order to review management and explore preventive strategies for a successful outcome in the future. We, therefore, presenting herewith a child with multiple co-morbidities who developed late bacterial endocarditis of an Amplatzer device following closure of secundum atrial septal defect and underwent successful management.

CASE REPORT

A 10-year-old girl was referred to us with a high-grade intermittent fever of 2 wk duration being treated for septic shock and altered sensorium. She also had a pyopericardium which was drained. She required ventilatory and minimal inotropic support. She had generalised anasarca, bilateral mild pleural effusions and generalised muscle spasticity. Additionally, there was cellulitis on the chest wall requiring surgical debridement. Blood and wound cultures were positive for *Streptococcus Pyogenes*, sputum for *Pseudomonas* and urine for *Escherichia coli*. She was managed with appropriate antibiotics. Other medications included diuretics, anti-convulsive therapy and ACE-inhibitors. She was known to have global developmental delay associated with cerebral atrophy and epilepsy with clonic seizures. In the past, at the age of 4 years, she underwent device closure of atrial septal defect in a hospital abroad. However, the details of the procedure were not available.

Routine blood tests revealed elevated markers of infection in addition to evidence of hepato-renal dysfunction. Computerized tomography of the chest revealed minor effusions in the pleural cavities and an

Amplatzer device in the atrium.

A two dimensional echocardiogram confirmed moderately depressed biventricular function, thickened pericardium and a prosthetic device in the inter-atrial septum with attached mobile vegetation (Figure 1). Other cardiac structures were normal.

It was suspected that the atrial septal occluder device was infected and possibly was a source of multiple systemic embolization and persistent bacteraemia. Therefore, we proceeded for surgical removal of the device.

Surgery was performed through median sternotomy under systemic heparinization, standard cardiopulmonary bypass using aortic and bicaval cannulation, aortic cross-clamping and cardioplegic arrest at moderate systemic hypothermia via right atrial approach. The pericardium was thickened and adherent all around featuring constrictive pericarditis. The Amplatzer device's surface was partially covered with the soft tissue (endothelialized) with patchy bare areas. However, there was no active vegetation found (Figure 2). Other cardiac structures including valves were grossly normal. After explantation of the device, a resultant atrial septal defect was repaired using a patch obtained from a pulmonary homograft.

The patient had slow but steady recovery. The post-operative echocardiography showed improved cardiac function without residual defects. There was a constant decline in the levels of inflammatory markers. She recovered fully except that generalized spasticity persisted. The histopathological examination of the tissue attached to the device showed evidence of severe acute and chronic inflammation in the connective tissue (Figure 3). However, a stain for fungal organism and culture of the tissue within the device was negative. Further studies to investigate the tissue infection within the soft tissue such as biofilm study or electron microscopy was not available.

DISCUSSION

Transcatheter occlusion technique using Amplatzer device has become a preferred approach for atrial septal defects in selected patients. The common complications associated with occluding devices are mal-positioning or migration of device, thromboembolism, arrhythmias or endocarditis^[1-5]. Bacterial endocarditis of an Amplatzer septal occluder device in the pediatric population is very rare. However, few reports have described early and late endocarditis associated with such device in adult population^[2-4].

Early device infection could be due to inoculation of organisms during implantation. However, hematogenous infection is the primary source of late endocarditis. In our patient, the source of infection could have been cellulitis or respiratory infection. In addition, there was purulent pericarditis. This combination suggests a hematogenous spread of infection leading to prosthesis endocarditis. In the only published report in a 4-year-old

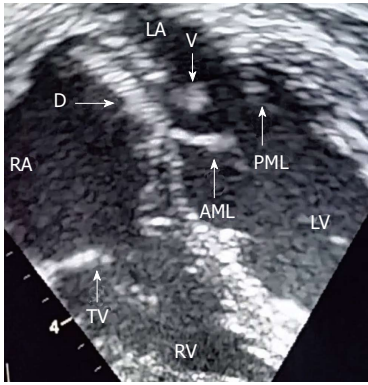


Figure 1 Two dimensional echocardiography image showing a large mobile vegetation (V) attached to the lower surface of an Amplatzer device (D) near the mitral valve. AML: Anterior mitral leaflet; PML: Posterior mitral leaflet; LV: Left ventricle; RV: Right ventricle; LA: Left atrium; TV: Tricuspid valve; RA: Right atrium.



Figure 2 Explanted Amplatzer device showing embedded soft tissue and bare metal surface.

child, authors have proposed incomplete endothelialization of the device as a mechanism of late endocarditis^[3]. Upon closer look of the explanted device, we also have noticed gross evidence of incomplete endothelialization in the form of exposed metallic surface of the device in places without soft tissue coverage.

There are no established guidelines for the management of late endocarditis involving intra cardiac devices. We suggest that intensive management involving prolonged antibiotic therapy, monitoring of inflammatory markers and frequent blood cultures may be the first step. However, surgery is warranted if there is evidence of septal perforation, dehiscence, fistula formation, vegetation or embolization^[6,7]. The relative indication may include persistent positive blood cultures in spite of maximal medical therapy^[6,7]. The homograft patch may be the preferred choice for repair of resultant septal defect after explantation of the device in this situation presumably due to the resistant nature of the homograft tissue against infection and better antibiotic penetration as compared to synthetic materials. Bovine pericardium may be an alternative.

In the clinical and experimental studies, it has been

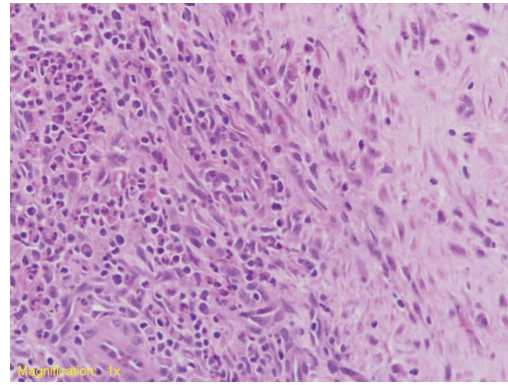


Figure 3 Microphotograph of the tissue within the explanted device showing connective tissue with dense, mixed, acute and chronic inflammatory infiltrates.

demonstrated that it takes 3-6 mo for complete neo-endothelialization of the device^[3]. Therefore, appropriate length of bacterial endocarditis prophylaxis for patients with atrial septal device closure was arbitrarily determined and usually extends from 6 mo to 1 year after implantation^[2-4]. We hope that in future, additional investigations, imaging techniques or biochemical markers will allow identification of patients with incomplete endothelialization who warrant long-term endocarditis prophylaxis.

This case report demonstrates the need for long-term follow up of patients with intracardiac devices especially those with multiple co-morbidities or who have vulnerability to infection due to poor general condition or extreme of age. A high index of suspicion for device complication is required, if sepsis or embolic phenomenon is found. An Intensive medical or surgical management and prolonged follow up is warranted for successful outcome.

COMMENTS

Case characteristics

A 10-year-old girl presented with late bacterial endocarditis of a cardiac Amplatzer device in addition to multiple co-morbidities and pancarditis.

Clinical diagnosis

Late bacterial endocarditis of cardiac prosthesis with cellulitis and pancarditis.

Differential diagnosis

Primary endocarditis of cardiac device, septicemia, pericarditis.

Laboratory diagnosis

Leucocytosis and mildly elevated hepato-renal function markers.

Imaging diagnosis

CT scan of the chest and an echocardiography showed evidence of a cardiac prosthesis (Amplatzer atrial septal occluder device) with a large vegetation.

Pathological diagnosis

The histopathology of the soft tissue attached to the explanted cardiac device showed the presence of acute and chronic inflammatory infiltrates. In addition, gross examination of the explanted Amplatzer device confirmed the presence of

bare metal exposed to the surface and the blood within the device.

Treatment

The patient was treated with intravenous antibiotics according to the culture and sensitivity reports of the blood, urine and pericardial fluids. Additionally, explantation of the atrial septal occluder device was done on cardiopulmonary bypass on urgent basis in order to remove the source of persistent bacteraemia and to avoid thromboembolism.

Related reports

The biofilm and electron microscopic studies were not available which may have a precise-diagnostic value to prove the presence of specific infection within the device.

Term explanation

Cardiopulmonary bypass is a term used to commonly indicate open heart surgery using a cardiopulmonary bypass machine with an oxygenator.

Experience and lessons

This case report not only represents a very rare occurrence of the late endocarditis of the cardiac device in association with pancarditis and multiple co-morbidities in a child but also provides authors an opportunity to focus their attention on the mechanism and prevention of this pathology for a better outcome in future. They have substantiated the hypothesis of the role of exposed bare metal surface and deficient soft tissue coverage (incomplete endothelialization) as a cause of prosthetic endocarditis. This fact not only guides us to explore the preventive measures while designing cardiac devices in future but also to be aware of a possibility of endocarditis in similar patients with multiple comorbidities and low resistance especially in pediatric population in order to have a cautious long-term follow-up and antibiotic prophylaxis.

Peer-review

The case report is very interesting and well described.

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