Pathophysiology of the proatherothrombotic state in the metabolic syndrome

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TABLE OF CONTENTS

1. ABSTRACT

The metabolic syndrome (MetS) is defined by the presence of at least three of the following abnormalities: glucose intolerance, hypertension, abdominal obesity, low HDL-cholesterol levels and hypertriglyceridemia. Obesity and insulin resistance are very frequently associated to the MetS and play a pivotal role in the development of type 2 diabetes mellitus (T2DM), which in turn increases the risk of cardiovascular disease. Although it varies among ethnic groups, the worldwide prevalence of MetS is 23% in young adults and increases with age. Remarkably, the prevalence of MetS is expected to increase during the next decades due in part to the acquisition of unhealthy life-style habits (e.g., sedentarism, smoking, unhealthy diet, etc). A major pathological alteration present in the MetS is a prothrombotic state as a result of endothelial dysfunction and hypercoagulability produced by a dysbalance of coagulation factors and proteins involved in the regulation of fibrinolysis. Although intensive research in recent years has permitted the identification of a number of prothrombotic alterations in MetS patients, a better understanding of the molecular mechanisms underlying the relationship between MetS and atherotrombosis is required to improve preventive and therapeutic strategies. In this review we discuss the main alterations in the endothelial function, coagulation cascade, fibrinolysis and platelet function that promote atherothrombosis in MetS patients. We also review available mouse models exhibiting alterations in atherothrombosis.

2. PATHOPHYSIOLOGY OF THE METABOLIC SYNDROME

MetS is a heterogeneous complex disease that is clinically associated to increased risk of T2DM, atherosclerosis and associated cardiovascular disease (CVD) (e.g., myocardial infarction and stroke). The diagnosis of MetS is based on the presence of at least three factors among glucose intolerance, insulin resistance (IR), visceral abdominal obesity, hypertension, increased triglyceridemia, and decreased plasma levels of high-density lipoprotein (HDL) cholesterol (1). Epidemilogical studies based on the National Cholesterol Education Programme criteria have shown that between 20% and 30% of the adult population in industrialized countries are affected by the MetS (2). The condition is progressive, beginning with borderline risk factors that eventually progress to categorical risk factors (3-5). Obesity and IR are very frequently associated to the MetS and play a pivotal role in the development of T2DM, which further increases the incidence of CVD in humans (6). The MetS is becoming one of the most relevant cardiovascular risk factors since its prevalence is expected to increase by 165% in the next 40 years, representing the health plague of the 21st century (7). Population aging and acquisition of sedentary lifestyle patterns (e.g., obesity and physical inactivity) are major driving forces behind this syndrome.

Dyslipidemia, including plasmatic accumulation of small dense low-density lipoprotein (LDL) particles, triglycerides, and apolipoprotein B (apoB), and low level of HDL, and dysglycemia (glucose intolerance and elevated fasting glucose) are among the metabolic abnormalities found in MetS subjects. Individuals with the MetS are also characterized by a prothrombotic state (increased coagulability and reduced fribrinolytic activity), as well as a proinflammatory state that increases plaque instability and favors formation and persistence of thrombus (Figure 1). Endothelial dysfunction and platelet

reactivity are considered key determinants of the proatherothrombotic state in the MetS (8-10). On the other hand, abdominal visceral obesity and IR are two of the predominant risk factors behind the MetS (3). The adipose tissue has emerged as an active endocrine and paracrine organ which secretes adipokines, such as adiponectin, leptin, resistin and proinflammatory mediators like tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1) and IL-6, and plasminogen activator inhibitor-1 (PAI-1). These molecules have profound effects on several physiological functions including carbohydrate and lipid metabolism, endothelial function, vascular homeostasis and inflammatory processes (11, 12). Other coagulation factors, like tissue factor (TF), are upregulated in the MetS due to the stimulation of proinflammatory cytokines and other endothelial mediators enhancing CVD risk and favoring thrombus formation following plaque rupture (13). Cellular and epidemiological studies have also linked IR with endothelial dysfunction, possibly as a consequence of glucotoxicity, lipotoxicity and inflammatory processes which characterize the MetS (14).

This review discusses the pathophysiology of the prothrombotic state associated to MetS, covering aspects of endothelial dysfunction, alterations of coagulation/fibrinolysis cascade, platelet activation and existing murine models of atherotrombosis and MetS.

3. ENDOTHELIAL DYSFUNCTION IN THE METABOLIC SYNDROME

3.1. Evidence of endothelial dysfunction in the metabolic syndrome and underlying mechanisms

The endothelium, which functions as a physical barrier between blood and the vascular wall, is a strategically located organ with several endocrine and paracrine functions. Under physiological conditions, the endothelium releases vasoactive substances which inhibit vascular constriction, leukocyte adhesion, vascular smooth muscle cell (VSMC) growth and platelet aggregation (15-17). Besides its function as a regulator of vascular tone, the endothelium plays an important role in the maintenance of vascular homeostasis due to its antiinflammatory and antiatherogenic properties (16, 17). Indeed, a common feature of IR states, MetS and atherothrombosis is the establishment of endothelial dysfunction characterized by a deficiency of nitric oxide (NO) production in response to physiological stimuli, increased expression of adhesion molecules on the endothelial cell (EC) surface, and inflammatory changes that underlie early processes of atherosclerosis and CVD (18). Decreased NO bioavailability in IR states leads to vasoconstriction and leukocyte activation, promotes thrombosis and VSMC proliferation, and is linked to hypertension, hypercholesterolemia, atherosclerosis and aging, thus contributing to the development of CVD (19).

A variety of humoral substances that alter endothelial function, including free fatty acids, adipokines and prooxidant molecules such as oxidized LDL (oxLDL), have been identified in the MetS. These mediators activate signaling kinases and are also closely linked to the endothelial production of reactive oxygen species, a hallmark in the inflammatory milieu that contributes to endothelial dysfunction and atherogenesis in MetS and T2DM patients (20-23). Multiple mechanisms have been implicated in the development of endothelial dysfunction in the setting of IR, such as inflammation and vascular production of the reactive oxygen species superoxide and peroxynitrite, which scavenge free NO reducing its bioavailability and promote EC apoptosis (24-31). The divergent effects of IR on the insulin signaling pathway may also be a crucial determinant of endothelial dysfunction. The sequential phosphorylation of the insulin receptor (INSR), insulin receptor substrates (IRS), PI-3 kinase and Akt constitute a common pathway by which insulin stimulates glucose uptake in metabolically active cells and promotes NO production by ECs (14). However, this pathway has been shown to be selectively down-regulated by IR in animals and humans leading to a reduction in NO production (32, 33).

Hyperglycemia is another common trait of the MetS that has a significant impact on endothelial function, particularly on vessel relaxation, through a mechanism involving advanced glycation end-products (AGEs). Targets of AGEs are the endothelium-derived relaxing factors NO and prostacyclin (PGI2). On the other hand, glycated serum albumin decreases *in vitro* the production of PGI2 by microvascular ECs (34) and induces the production of the potent vasoconstrictor endothelin-1 through nuclear factor-κB activation (35). The accumulation of AGEs in the extracellular matrix might result in NO depletion (36). In addition, a marked reduction in protein and mRNA levels of endothelial nitric synthase (NOS) occurs as a result of decreased serine phosphorylation of this enzyme and increased rate of mRNA degradation (37-39).

The increased risk of CVD in patients with MetS has been partially attributed to the inflammatory state provoked by visceral obesity (Figure 1). For example, endothelial dysfunction in MetS patients has been related to the secretion by visceral adipose tissue of proinflammatory and proatherosclerotic adipokines, including TNF-α, leptin, IL-6, and angiotensinogen (40). TNF-α contributes to IR by inhibiting insulin signaling. Leptin activates the immune system and increases blood pressure. IL-6 stimulates in obese subjects the hepatic production of C-reactive protein (CRP), an acute inflammatory protein. Angiotensin II (produced from angiotensinogen) exerts its adverse endocrine effects via the angiotensin II type 1 receptor (AT1), leading to oxidative stress, vasoconstriction, aldosterone secretion, renal sodium resorption, vasopressin release, PAI-1 expression and thrombosis (41). In contrast, plasmatic levels of adiponectin, a protein secreted by the adipose tissue into the circulation at relatively high levels in healthy human subjects, are markedly reduced in MetS. Circulating levels of adiponectin correlate negatively with the parameters characteristics of IR (e.g., percent body fat, central fat distribution, fasting plasma insulin, and oral glucose tolerance), and positively with parameters related to glucose intolerance (e.g., glucose disposal during euglycemic insulin clamp) (41). Plasma adiponectin levels are also significantly lower in patients with coronary artery disease compared

with control subjects (42, 43). Studies in animal models and human subjects have demonstrated an association between circulating adiponectin levels and endothelial function. Forearm blood flow during reactive hyperemia in humans is inversely correlated with adiponectin, indicating that this cytokine is closely associated with endothelium-dependent vasodilation (44-46). Moreover, independently of a correlation with insulin sensitivity, circulating adiponectin levels in humans are positively associated with arterial vasodilation in response to nitroglycerine, a measure of endothelium-independent vasodilation (47).

The induction of endothelial dysfunction has also been associated with the presence of dyslipidemia (41). Specifically, circulating free fatty acids, which are characteristic of the dyslipidemia in MetS states, have been shown to have a deleterious effect on endothelial functions (48).

The biochemical abnormalities associated with IR mentioned above, including reduced NO bioavailability, increased production of reactive oxygen species and downregulation of PI-3 kinase-mediated signaling, may impair vascular repair by endothelial progenitor cells (EPCs) (49, 50), which are blood borne bone marrow-derived cells that contribute to neoangiogenesis induced by tissue ischemia (51-53). It has been suggested that proinflammatory factors associated to the MetS lead to cell damage and impaired regeneration within the vessel wall (54). In addition, the accumulation of cardiovascular risk factors is associated with reduced number and dysfunction of EPCs (55, 56). Therefore, a poor regenerative ability of the vessel wall may contribute to increased risk of atherotrombosis in MetS states.

3.2. Mouse models of endothelial dysfunction based on nitric oxide synthase-deficiency

As discussed in the previous section, reduced bioavailability of NO may contribute to endothelial dysfunction in the MetS. This gas can be produced by neuronal (nNOS, type I), inducible (iNOS, TypeII), and endothelial (eNOS, Type III) NOS (57, 58). These isoforms exhibit complex and overlapping patterns of expression. nNOS is found in neurons in the brain and in the nonadrenergic, noncholinergic, autonomic nervous system. iNOS can be induced in macrophages in response to infection or tumor immunity. eNOS is found in ECs and is the isoform involved in endothelium-dependent relaxation. nNOS and eNOS are constitutively produced and their activity is regulated by intracellular calcium concentrations. In contrast, iNOS is induced by a variety of stimuli such as TNF and interferon.

Studies with genetically-modified mice have helped clarify the role of the three NOS isoforms (59). *nNOS*-knockout mice are resistant to global and focal cerebral ischemia, confirming a role for neuronal NO in cellular toxicity after stroke. In contrast, *eNOS*-knockout mice are more prone to stroke consistent with a protective effect of NO on the brain vasculature. Furthermore, *eNOS*-knockout mice are hypertensive, lack endothelium-dependent relaxing factor activity and respond to vascular injury with increased neointimal proliferation, consistent with a physiological role for NO as a suppressor of VSMC proliferation (59). Finally, *iNOS*-deficient mice exhibit altered immune responses, increased susceptibility to bacterial and viral pathogens and resistance to sepsis-induced hypotension (58).

Interestingly, nephrotic diabetes is a characteristic of mice lacking all three NOS isoforms (*n/i/eNOS*-deficient mice) (60), which also develop severe coronary arteriosclerosis, thrombosis and myocardial infarction (61). Characterization of *n/i/eNOS*-deficient mice demonstrated that they develop a phenotype similar to the MetS in humans, featuring visceral obesity, hypertension, hypertriglyceridemia and impaired glucose tolerance. Remarkably, oral treatment of *n/i/eNOS*-deficient mice with an angiotensin II type 1 receptor blocker significantly reduces both coronary atherosclerosis and the occurrence of myocardial infarction and ameliorates metabolic abnormalities, thus demonstrating that disruption of the NOS system impairs cardiovascular and metabolic homeostasis at least in part through activation of angiotensin II type 1 receptor. The occurrence in *n/i/eNOS*-deficient mice of acute events along with metabolic abnormalities makes this animal model a suitable tool to study the proatherothrombotic state associated to the MetS.

Additional mouse models have highlighted a link between dysfunction of the endothelial NOS system with the MetS and atherothrombosis. Studies in *Insr*-null mice, which exhibit mild IR and endothelial dysfunction, support a role for endothelial cell superoxide production as a mechanism underlying the early reduction in NO bioavailability (62). It has been also suggested that the toll-like receptor-4, a main receptor of innate immunity, plays a relevant role as a mediator of the deleterious effects of free fatty acids on endothelial NO signaling and on insulin-dependent stimulation of eNOS, thus providing a mechanism by which diet-induced obesity promotes vascular inflammation and IR (63).

4. ALTERATIONS IN PLAQUE STABILITY, COAGULATION AND FIBRINOLYSIS SYSTEMS IN THE METABOLIC SYNDROME

4.1. Hypercoagulation and hypofibrinolysis states

The increased risk of CVD in MetS patients is linked to a hypercoagulable and hypofibrinolytic state (64). In this sense, augmented plasma levels of several factors involved in clotting and fibrinolysis have been associated to the MetS (41, 65-67) (68, 69). For example, MetS and IR patients have increased cardiovascular risk associated to elevated plasma levels of factor VII, fibrinogen, factor XIII B-subunit, factor VIII, von Willebrand factor (vWF) and PAI-1 (70-72). Moreover, IR strongly correlates with factor XIII B-subunit (72).

The superoxide radicals, which accumulate in the MetS, are related to endothelial inflammation and the unsuing activation of monocytes (73). The microparticles (MP) liberated from monocytes during cellular activation or apoptosis can expose anionic phospholipids, P-selectin (CD62P), CD42a and also TF (74, 75), a primary cellular initiator of blood coagulation whose expression in the vasculature promotes thrombosis. TF is expressed at high levels by VSMC and macrophages within atherosclerotic plaques, is upregulated by cytokines favoring thrombus formation after plaque rupture and therefore its activity is associated with an increase in CVD risk in the MetS (13, 76). TF activity is inhibited by various agents, including TF pathway inhibitor (TFPI), whose activity reduces thrombosis in animal models (77, 78). Notably, TFPI activity has been negatively correlated with parameters characteristic of the IR and the MetS (79) as well as body mass index (80). In contrast, factor VII activity positively correlates with both body mass index and triglyceride levels (41) and has been shown to increase during postprandial hyperlipidemia (81). The simultaneous increase in soluble TF and factor VII activates the coagulation protease cascade, thus enhancing the risk of acute events in the MetS by leading to fibrin deposition and activation of platelets (13).

MetS patients exhibit hypofibrinolysis (6, 9, 82). This alteration in fibrinolysis associated to the MetS is mainly due to an increment in the serum levels of PAI-1, which reflect dysfunction of the fibrinolytic system that can lead to arterial thrombosis and CVD (83-86). In addition, increased levels of PAI-1, which have been found in T2DM patients (87, 88) and strongly correlate with IR, are linked to increased risk of atherothrombosis in humans (87, 89). A number of proinflamatory cytokines characteristic of the MetS, like TNF-α, leptin, IL-6 and angiotensinogen, have been demonstrated to increase the serum levels of PAI-1 due to its overexpression in adipose tissue (90-92). Recent findings also suggest that PAI-1 may be involved in adipose tissue development and may contribute to obesity through indirect effects on insulin signaling, adipocyte differentiation and recruitment of inflammatory cells in adipose tissue (93). In agreement with these studies, healthy diet, weight loss and lifestyle modifications decrease inflammation and the levels of PAI-1 (92, 94-96). Finally, decreased plasma tissue plasminogen activator activity is related to IR in patients with MetS (97) and a positive correlation between insulin levels and the thrombin-activable fibrinolysis inhibitor has been observed (98), thus favoring thrombus persistance. These changes contribute to attenuation of plasminogen conversion, resulting in a hypofibrinolytic state (41).

The reduced level of circulating HDL-cholesterol may also contribute to the prothrombotic phenotype of MetS patients. Indeed, HDL-cholesterol enhances the anticoagulant protein C pathway by stimulating the generation of activated protein (99) and reduced levels of circulating activated protein C are associated with the extent and severity of coronary atherosclerosis and a higher risk of myocardial infarction, which might be related to the anticoagulant and antiinflammatory properties of anticoagulant protein C (100).

It has been suggested that PAI-1 polymorphisms probably interact with known environmental risk factors (chronic hyperglycaemia, obesity, etc.) to induce a more severe insulin-resistant metabolic profile in overweight subjects, and to further increase risk for coronary heart disease in diabetic patients (101).

4.2. Platelet activation

In thrombotic arterial disease, the increased reactivity of platelets plays a major role in thrombus formation induced by atherosclerotic plaque rupture (102, 103). Subjects with increased platelet reactivity have an increased risk of suffering coronary events and death (104-106). It has been shown that patients with MetS and diabetes exhibit higher degree of platelet activation (64, 107, 108), and markers of platelet activation such as P-selectin (CD62P), CD63, PAC-1 and annexin V are increased in diabetic subjects (109). P-selectin allows the recruitment of leukocytes to areas of inflammation thus favoring the formation of lesions (110). Furthermore, platelets are known to adhere to activated or damaged ECs both *in vitro* (111) and *in vivo* (112, 113). On the other hand, platelets also produce TF (114), a factor which is upregulated in the MetS and promotes thrombosis (13). However, the contribution of platelets to MetS pathophysiology seems to be more complex as these cells may also contribute to atherosclerotic plaque development (Figure 1).

In addition to the direct role of platelets in arterial thrombus formation, they also contribute to the process through the release of platelet microparticles (PMP), which contain phospholipids with procoagulant function (115, 116). In agreement with this, elevated levels of PMP have been detected in patients with myocardial infarction (117) and diabetes (109, 115). It has been shown that PMP promotes thrombus formation by binding to the subendothelial matrix, which may act as a substrate for further platelet binding in a GP IIb/IIIa–dependent manner (116). PMP may also activate ECs and leukocytes by releasing arachidonic acid and other molecules (118), and also cause or worsen endothelial dysfunction (119).

Other abnormalities of the MetS, including dyslipidemia, enhanced oxidative stress and the generation of biologically active oxidized lipids are associated with a prothrombotic phenotype. Podrez *et al* (120) demonstrated that the interaction of platelet CD36, a major platelet glycoprotein, with specific endogenous oxidized lipids promotes platelet activation and thrombosis. By using multiple murine models of thrombosis, the authors demonstrated that deletion of CD36 protects mice from hyperlipidemia-associated enhanced platelet reactivity and thrombosis.

Other studies have also suggested a role of the circulating activated platelets in atherosclerosis development, which enhances the risk of thrombosis. In this sense, circulating activated platelets have been shown to increase P-selectin (CD62P)-

mediated monocyte arrest on the surface of incipient atherosclerotic lesions and exacerbate atherosclerosis, possibly due to the interaction between GPIIb-IIIa expressed by activated platelets and endothelial ICAM-1 (121) as well as to the binding of P-selectin from activated platelets to endothelial P-selectin glycoprotein ligand 1 (PSGL-1) (122).

Activated platelets may also play a role in thrombosis by enhancing a proinflammatory state. Thus, upon activation, platelets release adhesive and proinflammatory molecules from α granules, which cause endothelial dysfunction (123). Platelets store and express CD40L on their surface. Once they are activated, platelets release CD40L which induces endothelial expression of cell adhesion molecules such as E-selectin, ICAM-1 and VCAM (124). Furthermore, the expression of CD40L on the platelet surface favors their adherence to the endothelium and stimulates the release of IL-8 and monocyte chemoattractant protein 1 (MCP-1) from platelets, promoting leukocyte recruitment to incipient lesions (124, 125). Activated platelets also secrete the EC activator IL-1 β, thus promoting the release of IL-6 and IL-8 and expression of cell adhesion molecules (126, 127).

Chemokines produced by platelets also enhance monocyte recruitment. Platelet factor 4 (PF-4) is released in high amounts by activated platelets, promoting chemotaxis of monocytes and LDL retention by ECs through inhibition of LDL receptor (LDLr) degradation in the endothelium (128). The chemokine RANTES (regulated on activation normal T cell expressed and secreted) secreted by activated platelets is involved in vascular recruitment of monocytes (129). Furthermore, the presence of PF4 enhances the arrest of RANTES-stimulated monocytes and monocytic cells on activated ECs, and binding of PF4 to the surface of monocytes is increased by RANTES (129). Circulating PMPs (see above) can also provoke the release of RANTES, thus favouring atherosclerotic plaque development (130).

Multiple mechanisms contribute to atheroma development, including abnormal VSMC proliferation and migration, two processes that are coordinately regulated by p27 (131-133), a growth suppressor which inhibits murine atherosclerosis (134, 135). Platelet-derived growth factor (PDGF) released during platelet activation also contributes to atherosclerosis by stimulating VSMC proliferation and migration and monocyte recruitment (136).

5. MOUSE MODELS TO STUDY PLAQUE VULNERABILITY AND THROMBUS FORMATION

The initial event in coronary thrombosis is typically provoked by coronary atherosclerotic plaque rupture or plaque ulceration as a consequence of disruption of the fibrous cap that protects the lesion (137). The definition of plaque stability, vulnerability and rupture in animal models is an unresolved issue. Moreover, the mechanisms underlying atherothrombosis are ill defined, in part due to the lack of 'true' models that mimic the disease in humans (137-141). Major progress into the understanding of the molecular mechanisms underlying atherosclerosis has been achieved through the generation and phenotypic characterization of genetically-engineered mice, mainly the apolipoprotein E-deficient (*apoE-/-*) and the LDLr-deficient (*LDLr-/-*) mouse models of atherosclerosis (142). Nonetheless, these murine models do not seem to reliably reproduce the later stages of atherosclerosis in humans, including spontaneous plaque rupture and hemorrhage (143). Recent studies, however, have permitted significant advances in modeling these processes in the mouse. Jackson *et al.* defined plaque rupture in mice as *'a visible defect in the cap accompanied by intrusion of erythrocytes into the plaque below'* (144). The existing mouse models of plaque rupture and thrombosis, which are mainly based on the induction of the atheromatous plaque rupture and thrombosis by different means, typically in *apoE-/-* and *LDLr-/-* mice, have been the subject of excellent reviews (e.g.) (138, 140, 143, 145, 146). This section summarizes key findings derived from some of these models.

Mice doubly deficient for *apoE* and the high-density lipoprotein receptor *SR-BI* exhibit features characteristic of human coronary heart disease, including spontaneous multiple myocardial infarction, cardiac dysfunction, and coronary atherosclerotic plaques displaying cholesterol clefts and extensive fibrin deposition, which indicate the presence of hemorrhage and clotting (147).

The combination of ligation and subsequent cuffing of the carotid artery in *apoE-/-* mice results in disruption of the macrophage-rich neointima, intralesional hemorrhage and thrombosis (148). Likewise, 8 weeks of fat feeding leads to acute plaque rupture in the brachiocephalic artery of male *apoE-/-* mice (149). In this animal model, pravastatin treatment inhibits early plaque rupture and is also effective when begun after unstable plaques have developed (149).

Manipulation of apoptosis (150, 151) and matrix metalloproteinase (MMP) activity in preexisting atherosclerotic lesions (152) have been also shown to provoke plaque rupture and hemorrhage in *apoE-/-* mice. Indeed, induction of atherosclerotic plaque vulnerability and rupture has been reported in this animal model upon adenovirus (Ad)-mediated transfer of the proapoptotic factors p53 (150) and Ad-FasL (151). Treatment with a vasopressor compound increases spontaneous rupture in Ad-p53-transferred mice (150), and thrombotic rupture, intraplaque haemorrhage, buried caps and iron deposits are seen in carotid artery lesions of Ad-FasL-transferred mice (151). By analyzing *apoE/MMP3, apoE/MMP7, apoE/MMP9* and *apoE/MMP12* doubly-deficient mice, Johnson *et al.* (152) provided evidence that different MMPs play divergent roles on atherosclerotic plaque development and stability in brachiocephalic arteries, since MMP-7 has no effect on plaque growth or stability, although it is associated with reduced VSMC neointimal content, MMP-12 supports lesion expansion and destabilization, and MMP-3 and MMP-9 limit plaque growth and promote a stable plaque phenotype (152).

However, using a perivascular collar placement model of atherosclerosis, de Nooijer *et al.* (153) reproted that lesional overexpression of MMP-9 promotes intraplaque hemorrhage in advanced lesions but not at earlier stages of atherogenesis.

Additional murine models of thrombosis include genetically-manipulated mice with defects in the platelet/coagulation system (138, 146). For example, homozygosity for the Factor V Leiden mutation in *apoE-/-* mice leads to enhanced arterial thrombosis and atherosclerosis (154). Regarding manipulation of the plasminogen system in *apoE-/-* mice, loss of plasminogen greatly accelerates atherosclerotic lesion formation (155), and overexpression of PAI-1, a key regulator of tissue-type plasminogen activator (tPA) which may promote plaque progression by inhibiting fibrinolysis and predispose to ischemic heart disease, leads to enhanced coronary arterial thrombosis (156). However, PAI-1 deficiency results in both detrimental and protective effects on atherosclerotic lesion development. On one hand, Eitzman *et al.* (157) reported prolonged time to occlusive thrombosis following photochemical injury to carotid atherosclerotic plaque in *apoE/PAI-1* doubly deficient mice compared to *apoE-/-* counterparts. On the other hand, Luttun *et al.* (158) find that loss of PAI-1 in *apoE-/-* mice promotes the growth of advanced atherosclerotic plaques coincident with enhanced extracellular matrix deposition, collagen fiber disorganization and degradation.

A major role of TF in atherothrombosis has been demonstrated in murine models (13, 159-161). First, thrombosis is markedly reduced in a carotid artery injury model in $TF^{floxed/floxed}SM22\alpha$-*Cre* mice harboring VSMC-specific deletion of TF (13). Second, *apoE-/-* mice with only one allele of TFPI inactivated exhibit enhanced TF activity within the atherosclerotic plaque, increased atherosclerosis burden and enhanced thrombosis measured as decreased time to occlusive thrombosis after photochemical carotid plaque injury compared to *apoE-/-* controls (159). CRP has been also implicated in the pathogenesis of arterial thrombosis, since CRP-transgenic mice subjected to two different models of arterial injury show an expedited and higher rate of thrombotic occlusion (160). Moreover, CRP overexpression increases TF and decreases TFPI expression by murine VSMCs *in vitro* and *in vivo*, and increases arterial thrombosis and intimal hyperplasia after femoral artery injury (161).

Recently, Moura *et al.* (162) reported accelerated atherosclerotic plaque maturation in *apoE-/-* mice lacking thrombospondin-1, a protein implicated in various inflammatory processes (163). The authors suggested that defective thrombospondin-1-mediated phagocytosis enhances plaque necrotic core formation and accelerates inflammation and macrophage-induced elastin degradation by MMPs, thus accelerating plaque maturation and vessel wall degeneration.

6. MOUSE MODELS OF ATHEROTHROMBOSIS AND METABOLIC SYNDROME

Several animal models featuring MetS/IR and accelerated atherosclerosis have been generated in recent years by means of three main strategies (164, 165): 1) induction of obesity, IR and atherosclerosis with 'diabetogenic' or 'atherogenic' diets in susceptible mouse strains, 2) analysis of mice carrying naturally occurring mutations that produce obesity and diabetes ($Lep^{ob/ob}$ and $Lep^{db/db}$) in combination with additional genetic alterations, and 3) generation of genetically-modified mice affecting genes related to glucose homeostasis and β-cell function, in combination with diets and/or additional genetic alterations.

Relevant functional links between the MetS/IR and the acceleration of atherosclerosis have been obtained from the manipulation of *Inrs* and *Irs*, two key genes in the insulin-signaling pathway. Mice with total ablation of *Irs2* superimposed on the absence of *apoE* (*apoE-/-Irs2-/-* mice) exhibit a MetS/IR phenotype featuring glucose intolerance, hyperinsulinemia and enhanced atherosclerosis compared to similarly hypercholesterolemic *apoE-/-* counterparts with intact *Irs2* (166, 167). Doubly-deficient *apoE-/-Irs2-/-* mice reveal a positive correlation between atherosclerotic burden and circulating insulin levels (167), a finding also observed in $Lep^{ob/ob}$*:LDLr-/-* and $Lep^{ob/ob}$*:apoE-/-* mice, two additional models of obesity-induced hyperlipidemia/IR/MetS and accelerated atherosclerosis (168). Notably, accelerated atherosclerosis associated to MetS-like manifestations (e.g., glucose intolerance and mild hyperinsulinemia) has also been found in fat-fed *apoE-/-Irs2+/-* mice with only one allele of *Irs2* inactivated (169). By analyzing aortic tissue and primary VSMCs and macrophages from these mice, as well as circulating leukocytes from MetS patients with and without IR, we have recently reported that enhanced levels of MCP1 resulting from reduced IRS2 expression and accompanying defects in Akt2 and Ras/Erk1/2 signaling pathways may contribute to accelerated atherosclerosis in MetS states (169). Of note, atherosclerosis burden is similar in *apoE-/- and apoE-/-Irs2+/-* mice fed control chow (169), indicating that reduced *Irs2* expression accelerates atherosclerosis only when combined with severe hypercholesterolemia, a frequent trait of the MetS. IR combined with severe hypercholesterolemia is also associated with severe atherosclerosis in fat-fed mice with liver-specific deletion of *Inrs* (166). Recently, Lloyd *et al.* have characterized two novel models exhibiting the phenotypes of the MetS (e.g., obesity, hyperinsulinemia, hyperlipidemia, hypertension, and atherosclerosis) by crossing *apoB48-/-*$Lep^{ob/ob}$ mice with either *apoE-/-* or *Ldl-r-/-* mice (170).

Studies of macrophage-specific inactivation of the insulin-signaling pathway have revealed a potential mechanism causing enhanced atherosclerosis in the MetS, as transplantation of *Inrs-/-* bone-marrow into irradiated *LDLr-/-* mice aggravates atherosclerosis coincident with increased lipid uptake through the upregulation of CD36 and SRA (171). Consistent with this, studies in mice (172) and humans (173, 174) have linked defective insulin signaling to increased foam-cell formation through CD36 receptor enhanced expression, and murine *apoE-/-Irs2+/-* macrophages exhibit higher expression of SRA and CD36 and increased uptake of acetylated LDL compared to *apoE-/-* controls with intact *Irs2* (169).

Although the aforementioned mouse models have provided new insight into the mechanisms of MetS-accelerated atherosclerosis, the occurrence of atherosclerotic acute events, like myocardial infarction, plaque rupture and thrombosis, have not been reported in these animal models. The triple *n/i/eNOS*-deficient mouse (see section 3.2.) maybe an appropriate model to study atherothrombosis in the MetS as these animals display the syndrome and severe coronary arteriosclerosis and myocardial infarction (61).

7. CONCLUSIONS AND PERSPECTIVES

Clinical and epidemiological studies have conclusively established a correlation between MetS and increased risk of arterial thrombosis and associated myocardial infarction and stroke. It has been well established that an increase in endothelial dysfunction, hypercoagulability and hypofibrinolysis produced by a dysbalance of coagulation factors, enhanced platelet reactivity and increased plaque vulnerability play a major role in the prothrombotic state induced by MetS, although understanding the precise mechanisms underlying this association requires further research.

In recent years, mouse models of atherothrombosis are being generated mainly through genetic manipulation of factors involved in the coagulation and fibrinolysis pathways. Studies with these animal models and primary cells derived from them are shedding light into the molecular mechanisms governing arterial thrombosis. Additionally, by crossing these animals with available mouse models of atherosclerosis and MetS, we are improving our knowledge into the mechanisms by which the MetS augments the risk of thrombosis and acute ischemic events. Special mention deserves the triple *n/i/eNOS*-deficient mice which develop a phenotype similar to the MetS in humans, including visceral obesity, hypertension, hypertriglyceridemia and impaired glucose tolerance, leading to severe coronary arteriosclerosis, thrombosis and myocardial infarction. While animal models pose obvious limitations, we expect that the study of genetically-modified mice will help understand the molecular mechanisms underlying MetS-induced thrombosis in humans, thus facilitating the development of novel diagnostic tools for the identification of the vulnerable plaque and therapeutic strategies for the treatment of patients at high risk of thrombosis and acute ischemic episodes.

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Key Words: metabolic syndrome, endothelial dysfunction, haemostasis, thrombosis, plaque vulnerability, platelet activation, genetically-modified mice.

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Figure 1. Pro-atherothrombotic state in metabolic syndrome. This figure illustrates mechanisms by which the MetS induces endothelial dysfunction and the underlying inflammatory response. Platelet adhesion to ECs occurs through interactions between ligands and their receptor (e.g., PSGL-1/P-selectin, GPIb/FVW, integrins), which in turn mediates the release of bioactive molecules, either from platelets or ECs, thus further enhancing platelet-monocyte and endothelium-monocyte interactions. Ultimately, increments in both fibrinogen and PAI-1 and plaque rupture can trigger a thrombotic event. PMP: platelet microparticles; MMP: monocyte microparticles.