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SPOTLIGHT REVIEW

Experimental animal models of coronary microvascular dysfunction

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Abstract

Coronary microvascular dysfunction (CMD) is commonly present in patients with metabolic derangements and is increasingly recognized as an important contributor to myocardial ischaemia, both in the presence and absence of epicardial coronary atherosclerosis. The latter condition is termed 'ischaemia and no obstructive coronary artery disease' (INOCA). Notwithstanding the high prevalence of INOCA, effective treatment remains elusive. Although to date there is no animal model for INOCA, animal models of CMD, one of the hallmarks of INOCA, offer excellent test models for enhancing our understanding of the pathophysiology of CMD and for investigating novel therapies. This article presents an overview of currently available experimental models of CMD—with an emphasis on metabolic derangements as risk factors—in dogs, swine, rabbits, rats, and mice. In all available animal models, metabolic derangements are most often induced by a high-fat diet (HFD) and/or diabetes mellitus via injection of alloxan or streptozotocin, but there is also a wide variety of spontaneous as well as transgenic animal models which develop metabolic derangements. Depending on the number, severity, and duration of exposure to risk factors—all these animal models show perturbations in coronary microvascular (endothelial) function and structure, similar to what has been observed in patients with INOCA and comorbid conditions. The use of these animal models will be instrumental in identifying novel therapeutic targets and for the subsequent development and testing of novel therapeutic interventions to combat ischaemic heart disease, the number one cause of death worldwide.

Keywords Coronary microvascular dysfunction • Animal model • Metabolic derangements • Endothelial dysfunction • INOCA

This article is part of the Spotlight Issue on Coronary Microvascular Dysfunction.

1. Introduction

Common risk factors for cardiovascular disease, including diabetes mellitus (DM), dyslipidaemia, hypercholesterolaemia, and chronic kidney disease (CKD), are independently, but especially in combination, well-known risk factors for the development of coronary artery disease (CAD) of both large epicardial arteries and smaller coronary arteries.[1–4]

While it is well-established that obstructive CAD is a major cause of myocardial ischaemia,[5] there is increasing evidence that coronary microvascular dysfunction (CMD) also contributes to myocardial ischaemia, not only in the presence of obstructive CAD[6–8] but also in patients without obstructive CAD, a situation referred to as 'ischaemia and no obstructive coronary artery disease' (INOCA).[2,9,10] Clinical studies have shown that INOCA is present in approximately one-third of men and

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two-thirds of women undergoing angiography for suspected ischaemic heart disease.[11,12] Importantly, cardiovascular death or myocardial infarction occurred in 6.7% of the patients without any signs of CAD and in 12.8% of patients with non-obstructive CAD.[11,12]

Since INOCA has only recently been recognized as a separate clinical entity, its exact definition and the underlying pathophysiology are not well-established yet.[9] The potential multitude of factors underlying ischaemia in these patients underscores the complexity of the disease and simultaneously presents a diagnostic and therapeutic challenge.[13] The current diagnostic workup for chest pain is not optimized for determining the different INOCA aetiologies, and INOCA is currently mainly used as a diagnosis *per exclusionem* in patients with non-obstructive CAD. Recently, the 'CORonary MICrovascular Angina (CorMicA)' study and the working group of INOCA of the American College of Cardiology have proposed a similar diagnostic flowchart.[9,13] According to these experts, a diagnostic flowchart for INOCA encompasses a three-step approach, including invasive coronary angiography for the evaluation of coronary obstructions with invasive diagnostic fractional flow reserve (FFR) if needed, coronary flow reserve (CFR) measurements for the evaluation of microvascular dysfunction, and a vasoreactivity test to acetylcholine and a nitrate for the assessment of endothelial dysfunction with/without vasospasm. Such an approach could discriminate patients with epicardial vasospastic angina vs. microvascular angina and enables evaluation of a tailored treatment between these groups. Although studies with non-invasive techniques, including positron emission tomography (PET), transthoracic echo-Doppler, and cardiac magnetic resonance imaging, have shown some promising results, invasive testing is currently still considered the gold-standard.[9]

An important limitation of patient studies is that the disease is only diagnosed when patients present with overt complaints, and hence the differentiation into the various angina subtypes, based on coronary function, typically occurs at a later stage, at a time when the (potentially synergistic) contributions of individual risk factors, including diabetes, hypercholesterolaemia, CKD, hypertension, and even sedentary lifestyle are difficult to assess. Longitudinal, mechanistic invasive studies considering individual comorbidities, age and sex, should be performed to identify the different patient subgroups. However, such studies in patients are very difficult given the complexity of the disease and co-occurrence of risk factors. In addition, structural microvascular alterations, including arteriolar remodelling and capillary rarefaction that can contribute to impaired CFR and myocardial oxygen delivery, are also difficult to assess in clinical studies. For this purpose, animal models are instrumental, as influences of metabolic factors, genetic predisposition, sex and age on the development of perturbations in coronary microvascular function and structure, as well as the progression of CMD, can be thoroughly studied.

In this review, we focus on the different animal models for CMD, which is a critical hallmark of INOCA. Since each animal model has its advantages and disadvantages, the specific research question should be the prime determinant of the animal model of choice. It is therefore important to take the (pitfalls for) translation to the clinical setting into account when selecting an animal model. Here, we present an overview of different models for studying CMD in the setting of metabolic derangements in commonly used animal species. We discuss the different ways to induce metabolic derangement, the resulting microvascular dysfunction, and the underlying mechanisms for each individual model. Subsequently, the models are evaluated and compared with respect to their translational capacity for the study of INOCA.

2. Animal models: anatomical and metabolic considerations

A variety of animal species and models has been employed to study the effects of different risk factors on the development and progression of CMD. The cardiovascular system of each species has evolved differently in order to meet the demands of that species and has specific similarities and differences with the human cardiovascular system. In this section, we will provide an overview of the most important similarities and differences in terms of coronary anatomy and body and myocardial metabolism.

2.1 Anatomical considerations

Large animal species (canine and porcine) have been widely used to study ischaemic heart disease. Dogs, pigs, and humans have been shown to vary with respect to the anatomic distribution of their coronary arteries. In all these species, the coronary arteries and their main branches run on the epicardial surface. The coronary vasculature in humans is mostly right dominant, implying that in most humans the right coronary artery supplies the right ventricle as well as the posterior wall of the left ventricle with blood, whereas the anterior and lateral wall of the left ventricle are perfused via the left anterior descending coronary artery and the left circumflex coronary artery. Humans have no separate septal artery, and the ventricular septum is supplied through perforating vessels that originate from the interventricular branches.[14] Similarly, the porcine coronary circulation is right dominant, while dogs are left dominant.[15] Humans and swine are also similar with respect to the vascularization of the interventricular septum, which is supplied by anterior and posterior septal branches arising from the left and right coronary arteries, respectively. In addition, the atrioventricular node and the bundle of His in both humans and swine are irrigated predominantly by the posterior septal branch.[16] This anatomical resemblance implies that ischaemia-associated injury to the conduction system of the swine heart is analogous to that in humans, contrary to the canine model, in which the blood supply originates from the anterior septal artery.[17] Importantly, while an innate coronary collateral circulation is negligible in humans and swine,[18,19] there is an extensive pre-existing collateral circulation in the dog heart, which—depending on the dog breed—can supply as much as 40% of the blood flow distal to an occluded coronary artery.[20] In rabbits, the left coronary artery is always the dominant artery, from which the septal artery originates. In rodents, the coronary anatomy differs markedly from that in large animals. Thus, coronary arteries run deeper in the myocardium and contain fewer layers of smooth muscle cells. In the mouse, the heart is supplied by two coronary arteries, left and right, each perfusing the corresponding ventricle. Studies have demonstrated a single major septal coronary artery arising either from a separate ostium from the right sinus of Valsalva or as a proximal branch of the right coronary artery.[21] The mouse septal coronary artery courses along the right side of the interventricular septum and provides perfusion to this region of the myocardium.[21] In the rat, the coronary anatomy is similar to that in mice, although coronary angiography in Lewis rats indicates that the septal artery branches off either from the proximal part of the left coronary artery (60% of the animals) or the right coronary artery (40%).[22] Furthermore, coronary collateral blood flow capacity is low, but not negligible, in rabbits,[23] rats,[19] and mice.[24]

2.2 Metabolic considerations

Blood pressure is similar among mammalian species, independent of body weight.[25] The heart is the most energy-requiring organ of the

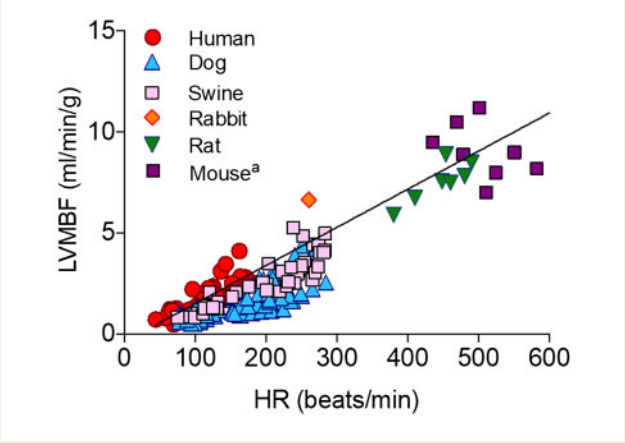

Figure 1 Relation between HR and LVMBF at rest and during exercise in dogs, swine, rats, mice, and humans. For awake rabbits only a single resting measurement is available.[34] [a]There are no measurements available in awake mice. Note that the regression line is based on the human data points. Adapted with permission from Duncker *et al.*[35] HR, heart rate; LVMBF, left ventricular myocardial blood flow.

body, with heart rate being the most important determinant of oxygen consumption. There are significant intrinsic differences between rodents and the large mammals in terms of heart rate, but also oxygen consumption, and metabolic activity, partly mediated by species-specific activity of thyroid hormones.[26] Humans, dogs, and pigs have similar heart rates, 60–80 b.p.m. in rest up to 200 b.p.m. (humans) or 300 (dog, pig) during maximal exercise,[27] while resting heart rates are 200–250 b.p.m. in rabbits,[28,29] 350–400 b.p.m. in rats,[25] and 500–600 b.p.m. in mice.[30,31] Dynamic exercise increases left ventricular coronary blood flow (CBF) in proportion to the increase in heart rate (*Figure 1*).[30–36] CBF measurement during exercise is very difficult to perform in mice, and to our knowledge, no exercise blood flow data are available. However, the few studies performed in anaesthetized mice[30,31] as well as in awake rats[35] indicate high flow values already under resting conditions, i.e. five to six times higher than in humans or large mammals, which appears to be entirely due to the higher resting heart rates (*Figure 1*).[35]

In adult fasting mammals, 60–80% of the cardiac energy metabolism relies on the oxidation of free fatty acids, the rest being accounted for by glucose, lactate, and ketone bodies.[37] However, there are species differences, as mice rely more on glucose, lactate, and ketone bodies and much less (30–40%) on fatty acids.[38] In addition, also in terms of lipid transporters in the blood—with low-density lipoprotein (LDL) and high-density lipoprotein (HDL) being the predominant carriers of cholesterol—there are major species differences in the proportion of LDL and HDL particles in plasma. Thus, pigs and rabbits transport most of the cholesterol in LDL particles—as do humans (~60% LDL, ~40% HDL)—while rodent species carry the majority of cholesterol in HDL, making rodents virtually resistant to atherosclerosis and less appropriate as models for dyslipidaemia and atherosclerosis.[39]

In line with the high heart rate in mice, basal metabolic rate per gram body weight is also seven times greater in mice than in humans. Such differences in metabolic rate have a major effect on reactive oxygen species (ROS) production in these species.[40,41] One of the consequences of high metabolism in mice is that, in response to tissue hypoxia, mice are capable of reducing their oxygen utilization by down-regulating the activity of mitochondrial uncoupling proteins (responsible for a significant part of overall O_2 consumption), without affecting ATP production. Hence, interventions targeting or affecting cellular metabolism in mouse models may not predict efficacy in humans.[42] Rats have a larger blood volume when compared with mice, however, they are particularly resistant to oxidative stress due to their high activity of tissue antioxidant enzymes. Furthermore, nitric oxide (NO) metabolites in blood are 10–20 times higher than those in humans, potentially limiting translational impact of studies involving NO and ROS in this species.[43] Given the importance of NO and oxidative stress in the regulation of coronary microvascular function, such interspecies differences are highly relevant in the choice of animal model for the study of CMD.

3. Large animal models

3.1 Canine models of CMD

The dog represents a large animal model that has traditionally been employed to study CBF regulation in health and disease[32,44,45] including in studies pertaining to the influence of cardiovascular risk factors on CBF and regulation of coronary microvascular tone, (see Supplementary material online, Table S1). To induce metabolic derangement in dogs, alloxan-induced DM and/or HFD-induced obesity that is associated with dyslipidaemia, hypertension, and insulin resistance, have been used. These canine models are highly relevant for the study of metabolic derangements and their influence on microvascular function.

3.1.1 Canine models with alloxan-induced DM

Insights in some of the alterations produced by longer exposure to metabolic derangement is offered by studies in mongrel dogs with alloxan-induced DM. A strength of this model is that measurements can be performed before and after DM induction, allowing for assessment of DM effects within the same animal. Alloxan is typically administered in a dose of 40–60 mg/kg i.v., which results in robust hyperglycaemia and hypoinsulinaemia without the need of insulin treatment.[46] Following the induction of DM, which stabilizes within 1 week of alloxan infusion, resting CBF gradually decreased to ~60% of its pre-DM value, at 5 weeks of follow-up.[46] Vasodilation in response to adenosine and acetylcholine was impaired in the absence of narrowing of the large coronary arteries, suggesting the presence of coronary microvascular endothelial dysfunction. Tune *et al.*[47] studied the haemodynamic alterations after only 1 week of alloxan-induced DM at rest and during graded treadmill exercise. Although resting CBF was unaltered 1 week post-alloxan, the exercise-induced increase in CBF was progressively impaired. The limitation of myocardial oxygen delivery during exercise elicited an increase in myocardial oxygen extraction, thereby resulting in lower coronary venous oxygen tensions at each level of exercise.[47] The authors demonstrated in a subsequent study that α-adrenoceptor blockade augmented the exercise-induced increase in CBF and attenuated the decrease in coronary venous oxygen tension to a greater extent in DM than in non-DM dogs, indicating that α-adrenoceptor-mediated coronary vasoconstriction was increased in diabetic dogs, particularly during increased metabolic demand.[48] Adenosine triphosphate-sensitive potassium (K_{ATP}) channel blockade with glibenclamide[49] blunted the coronary hyperaemia during exercise in DM, but not in healthy dogs, suggesting that K_{ATP} channels exert an increased coronary vasodilator influence in DM dogs, acting to maintain CBF during increased myocardial metabolism and increased sympathetic activation during exercise.

3.1.2 Canine models with diet-induced metabolic derangement

Several studies in dogs of either sex have investigated the effects of chronic metabolic derangement produced by 5–6 weeks of HFD on the coronary microvasculature. Such exposure to HFD resulted in features of the metabolic syndrome (MetS) with obesity, moderate hypertension, dyslipidaemia, and insulin resistance. These factors impaired myocardial perfusion, especially during exercise, by decreasing coronary vascular conductance, although myocardial ischaemia was absent.[50] The tonic constriction of the coronary vasculature in this setting was mediated by metabolic derangement-associated neurohumoral alterations, including increased vasoconstriction mediated by angiotensin II (ANGII),[51] activation of the sympathetic nervous system,[52] and the endothelium-derived vasoconstrictor, endothelin 1 (ET-1),[53] as tested in a series of consecutive studies in male and female dogs.

Zhang *et al.*,[51] investigated the involvement of prediabetic metabolic derangement-associated activation of the renin–angiotensin–aldosterone system (RAAS) on CBF regulation. Metabolic derangement was associated with increased plasma renin activity and elevated ANGII levels, resulting in a significantly increased ANGII-induced vasoconstriction, mediated by angiotensin II receptor type 1 (AT_1) receptors in isolated arterioles. Further interrogation of this mechanism *in vivo* supported the *in vitro* data, showing an impaired exercise-induced increase in CBF that was alleviated by AT_1 receptor blockade. Taken together, these data suggested that in the canine model of prediabetic metabolic derangement, chronic activation of the RAAS contributes to coronary vascular dysfunction, via increase in circulating ANGII and/or increases in coronary arteriolar AT_1 receptor density. The increase in sympathetic activity associated with the metabolic derangement and its effects on coronary circulation were investigated in the same model by Dincer *et al.*[52] Although baseline CBF was not altered *in vivo*, increased plasma epinephrine concentrations were associated with augmented α_1-adrenoceptor-mediated coronary vasoconstrictor responses in anaesthetized dogs. Sensitization of α-adrenoceptor signalling represents a potentially important contributor to impaired control of CBF. A third important vasoconstrictor involved in the control of CBF and shown to be increased in metabolic derangement is ET-1. Knudson *et al.*[53] tested the hypothesis that prediabetic metabolic derangement augments endothelin receptor A (ET_A)-mediated vasoconstriction, thereby limiting coronary perfusion. Interestingly, despite a reduction in ET_A expression in the coronary microvasculature, coronary vasoconstrictor responses to ET-1 were not different between HFD-fed dogs and normal dogs, while circulating ET-1 levels were unaltered, suggesting either a sensitization of the ET_A receptors or an increased contribution of endothelin receptor B (ET_B)-mediated vasoconstriction. The reduction in ET_A receptor expression may represent an early compensatory mechanism acting to maintain CBF in the face of increased ANGII- and α_1-adrenoceptor-mediated coronary vasoconstrictor influences.

Adipokine production is altered in the MetS, and to investigate the potential contribution of adipokines to CMD, Tune and colleagues performed a series of experiments in which they investigated the coronary microvascular responses to a variety of adipokines. Payne *et al.*[54] investigated the effect of various endogenous adipose-derived factors on coronary endothelial function, by infusing adipose-tissue-conditioned buffer in healthy, lean dogs. Although baseline CBF remained unaltered, coronary endothelial dysfunction was observed both *in vivo* and *in vitro*. Endothelial dysfunction was the result of reduced NO bioavailability, possibly via selective inhibition of endothelial nitric oxide synthase (eNOS), independent of oxidative stress. Although the specific adipokine(s) responsible for the alterations in the latter study were not identified, these data show that adipokine administration does result in acute endothelial dysfunction, by altering different endothelial vasodilator mechanisms, suggesting that chronic exposure to circulating adipose-tissue-derived factors can potentially result in vascular dysfunction. To further delineate the precise adipose-tissue-derived factor(s), Dick *et al.*[55] set out to test whether acute infusion of resistin, an adipokine implicated in endothelial dysfunction in obesity affected CBF *in vivo* in healthy dogs, by increasing oxidative stress. At concentrations observed in obese, type 2 DM (T2DM) patients, resistin did not affect CBF. However, it did produce endothelial dysfunction, both *in vivo* and in isolated coronary arterioles, which was likely endothelium-derived hyperpolarizing factor (EDHF)-mediated, as the bradykinin-induced vasodilation was impaired, while acetylcholine (ACh)-induced response was maintained. Furthermore, this response was independent of oxidative stress. Knudson *et al.*[56] investigated the direct effects of leptin, an adipokine involved in several biological processes including glucose metabolism and inflammation, on the coronary circulation and specifically on coronary endothelial function in healthy dogs as well as dogs with metabolic derangement. In normal healthy dogs, leptin produced coronary microvascular endothelial dysfunction characterized by a blunted vasodilator response to acetylcholine. In contrast, and despite increased leptin plasma concentration, prediabetic high-fat-fed dogs had normal coronary microvascular vasodilator responses to acetylcholine. Interestingly, the coronary vasodilator response to ACh was not affected by leptin. These findings suggest that resistance to leptin-induced endothelial dysfunction may represent a protective mechanism at this early stage of the disease.

3.1.3 Summary: canine models of CMD

Taken together, alloxan-induced DM or exposure to HFD in dogs of either sex, for up to 5–6 weeks results in altered coronary microvascular tone control, involving increased coronary vasoconstrictor mechanisms such as α-adrenoceptor-mediated and ANGII-mediated coronary vasoconstriction. These increased vasoconstrictor influences result in perturbations in CBF regulation and myocardial oxygen delivery but are partly mitigated by compensatory reductions in ET_A receptor density and by increased leptin resistance and K_{ATP} channel activation (*Table 1*). Future studies are required to determine whether long-term exposure to these cardiovascular risk factors produces progressive perturbations in coronary microvascular function during disease progression.

3.2 Porcine models of CMD

Swine are widely used in translational cardiovascular research, as they share many similarities with humans with respect to coronary and cardiac anatomy and physiology. Furthermore, swine are omnivores and have a human-like myocardial metabolism.[57] Compared with dogs, domestic swine have the advantage of lower cost and societal pressure. Unfortunately, the fast growth rate of domestic swine limits study duration and favours utilization of juvenile swine, for a disease that occurs in an ageing human population. These pitfalls can be circumvented by the availability of different strains of mini-swine. Different porcine models of CMD have been generated over the past 20 years, typically by exposure to risk factors that are common in patients with ischaemic heart disease including dyslipidaemia, DM, CKD, and hypertension. In addition, several swine strains with increased genetic susceptibility to developing metabolic derangement—particularly when exposed to these risk factors—

Table 1 Main mechanisms involved in CMD per animal model

	Functional							Structural	
	Endothelium-dependent				Neurohumoral		VSMC	Arteriolar	Capillary
	↓NO	↑ROS	↑ET-1	↓PGI_2	↑RAAS	↑SNS	↓function	↑ Media thickness	↓Density
Canine models									
Alloxan	+	NA	NA	NA	NA	+	+	NA	NA
High-fat diet	NA	NA	-	NA	+	+	-	NA	NA
Adipokine infusion	~	-	NA	-	NA	NA	-	NA	NA
Porcine models									
Induced domestic	+	+	~	NA	NA	NA	~	NA	+
Induced Yucatan	~	NA	NA	-	NA	NA	+	NA	NA
Rapacz FH	+	NA	NA	NA	NA	NA	-	NA	NA
Ossabaw	~	NA	NA	NA	NA	NA	~	NA	+
Rabbit models									
Alloxan	+	NA	NA	+	NA	NA	-	NA	NA
High-fat diet	~	+	+	NA	NA	+	~	+	NA
WHHL	NA	NA	NA	NA	NA	NA	-	NA	NA
Rat models									
Streptozotocin	~	-	NA	-	+	NA	~	~	~
High-fat diet	+	NA	NA	NA	+	NA	~	NA	-
Zucker	~	+	-	~	NA	~	-	-	NA
OLETF	~	+	+	NA	+	NA	-	+	NA
GK	~	NA	NA	-	NA	+	+	+	-
Murine models									
db/db	+	+	NA	NA	+	NA	~	~	~
ob/ob	+	NA	NA	NA	NA	NA	-	NA	-
Induced T1 + 2DM	+	+	+	NA	NA	+	+	+	NA
apoE	+	+	NA	-	NA	NA	-	NA	NA

Overview of models of coronary microvascular dysfunction per species and what features are present (+), absent (-), ambiguous results (~), or not investigated (NA; not assessed).
apoE, apolipoprotein E knockout mouse; db/db, leptin receptor deficient mouse; ET-1, endothelin 1; FH, familial hypercholesterolaemia; GK, Goto-Kakizaki non-obese diabetic rat; NO, nitric oxide; ob/ob, leptin deficient mouse; OLETF, Otsuka Long-Evans Tokushima Fatty rat; PGI_2, prostacyclin; RAAS, renin–angiotensin aldosterone system; ROS, reactive oxygen species; SNS, sympathetic nervous system; T1 + 2DM, type 1 and 2 diabetes mellitus; VSMC, vascular smooth muscle cell; WHHL, Watanabe heritable hyperlipidaemic rabbit; Zucker, Zucker obese and diabetic fatty rats.

have been identified, characterized, and inbred and are now being used in a variety of experimental studies into coronary microvascular function in health and disease. Finally, a transgenic porcine model of diabetes has recently been established.[58]

3.2.1 Domestic swine

For over 40 years, domestic swine have been employed in studies focusing on the regulation of CBF in health and ischaemic heart disease.[32,45] Studies pertaining to the effects of different risk factors on coronary microvascular function have for the most part been published in the past 10 years (Supplementary material online, *Table S2*). In these studies, different methods to induce risk factors have been used, including high-fat/high-sugar diets either as single intervention or in combination with streptozotocin (STZ)-induced hyperglycaemia and/or CKD-associated hypertension.

Gerrity *et al.*[59] and Ditzhuijzen *et al.*[60] demonstrated that in young (12 weeks of age) male domestic swine, <20 weeks of HFD (1–1.5% cholesterol and 15-25% lard), or a combination of HFD and STZ-induced DM did not result in flow-limiting coronary lesions. We also observed that although 10 weeks of HFD and DM by low-dose STZ in domestic swine did not induce coronary arterial lesions, CMD was already present at this early timepoint.[61] Thus, epicardial conduit artery function was unaltered, but isolated coronary small arteries (~300 μm in diameter) showed impaired endothelial function in DM swine that were fed a HFD. CMD was characterized by impaired NO bioavailability, while EDHF-dependent mechanisms were not affected, and the overall vascular smooth muscle cell (VSMC) sensitivity to NO was preserved.[61] The endothelial dysfunction-associated reduction in vasorelaxation was accompanied by a markedly reduced ET_A-receptor-mediated vasoconstrictor response to ET-1, possibly serving as a compensatory mechanism for the increase in circulating ET-1 levels and the loss of NO bioavailability, at this early stage of the disease. Furthermore, 10 weeks of DM+HFD resulted in increased microvascular passive stiffness, potentially further contributing to perturbations in coronary microvascular function *in vivo*.[61] Indeed, Mannheim *et al.*[62] observed impaired CBF responses to intravenous adenosine in swine after approximately 3 months of HFD, suggesting that the early alterations in coronary microvascular control mechanisms as observed *in vitro* can indeed translate into impaired CBF responses *in vivo*.

Interestingly, in our initial study, no changes in coronary microvascular responses to bradykinin or ET-1 were observed in non-DM animals fed the same HFD.[61] The latter observation appears to be in contrast to an

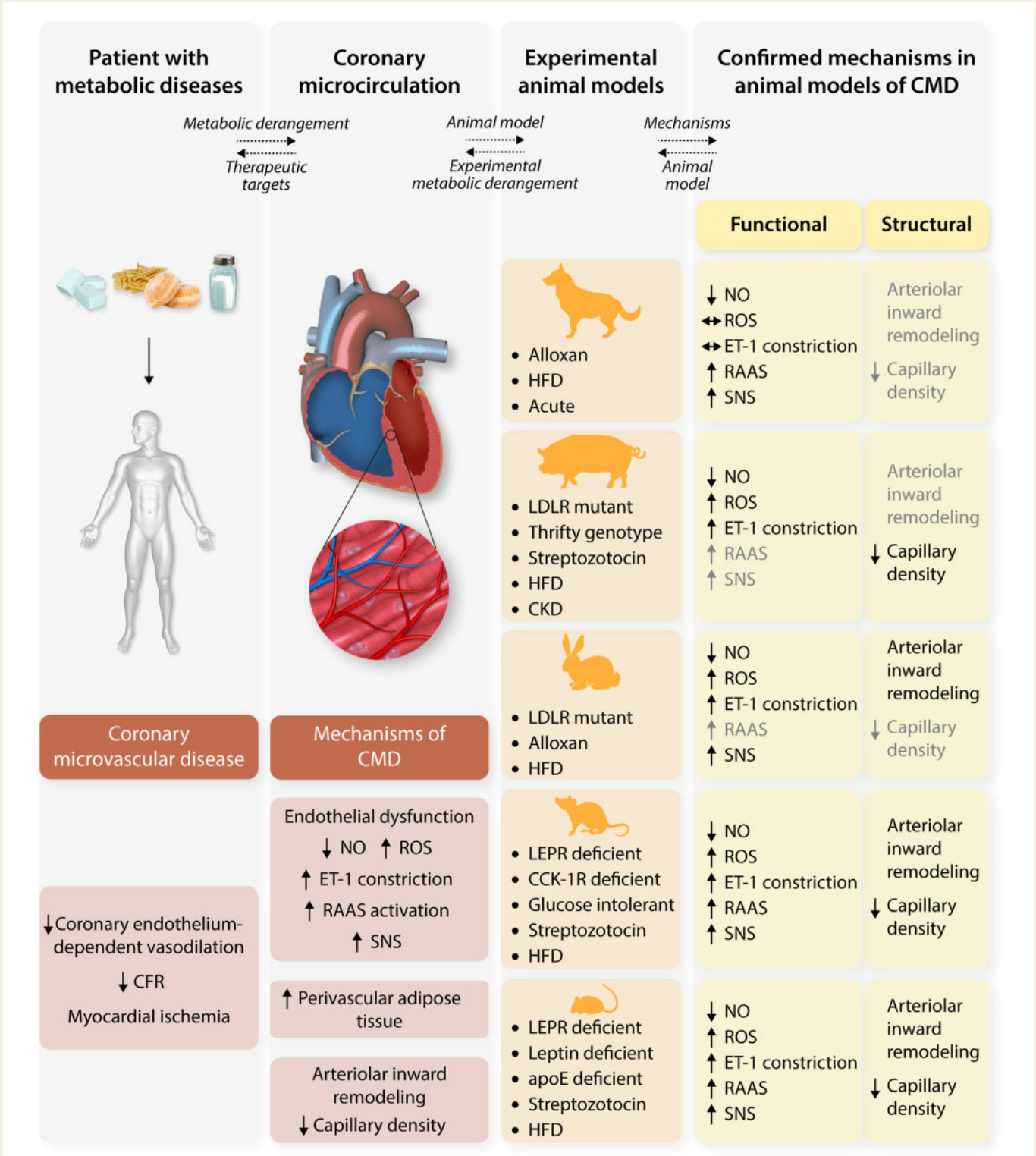

Figure 2 Animal models for CMD in the presence of metabolic derangement: large and small animal models for CMD, either spontaneous, inducible, or inbred/engineered for the development of metabolic risk factors have been employed to study the mechanisms leading to CMD. Alterations in microcirculatory endothelial function leading to a disturbed vasodilator/vasoconstrictor balance as well as structural modifications both at the arteriolar and capillary level have been described, mimicking the human pathology including reduced basal and maximal coronary perfusion and myocardial ischaemia. apoE, apolipoprotein E; CBF, coronary blood flow; CCK-1R, cholecystokinin-1 receptor; CFR, coronary flow reserve; CKD, chronic kidney disease; ET-1, endothelin-1; HFD, high-fat diet; LDLR, low-density lipoprotein receptor; LEPR, leptin receptor; NO, nitric oxide; RAAS, renin–angiotensin–aldosterone system; ROS, reactive oxygen species. Right-sided panel presents the mechanisms of CMD which have been recapitulated in at least one animal model/species (black), which have been investigated but were not found to be present (black ↔) or which have not been investigated (grey) in the various species.

early study by Hasdai *et al.*,[63] who reported increased vasoconstriction of small arteries (~500 μm diameter) to ET-1 *in vitro*, in swine after 10–13 weeks of HFD. These different results are not readily explained but may well stem from differences in vessel size (300 μm[61] vs. 500 μm[63]), sex (male[61] vs. female[63]), or the diet composition (1% cholesterol, 25% saturated fats, 20% fructose/20% sucrose[61] vs. 2% cholesterol, 20% lard, and 1% hog bile extract[63]).

In a subsequent study, we subjected male swine with or without STZ-induced DM to HFD for 15 months and observed significant functional and structural alterations in the coronary vascular bed in both large and small arteries.[64] Thus, at this stage of the disease, plaques were found in epicardial conduit arteries (albeit not flow-limiting; i.e. <30% plaque burden) and in coronary small arteries, while the latter also showed increased passive stiffness. Moreover, microvascular tone control studies showed a normal endothelium-dependent bradykinin-induced vasorelaxation that was accompanied by an enhanced ET_B-receptor-mediated vasoconstrictor response to ET-1.[64] Interestingly, these alterations were principally the result of the HFD and independent of the presence of DM. Taken together these two studies reveal a surprising shift from an early blunting of endothelium-dependent vasorelaxation at 10 weeks[61] towards a late 'normalization' of endothelium-dependent vasorelaxation at 15 months,[64] that was accompanied by a shift from an early blunting[61] to a late augmentation[64] of the vasoconstrictor responses to ET-1. These results underscore the importance of performing longitudinal studies, as the mechanisms of microvascular dysfunction were highly dependent on the duration of exposure to the cardiovascular risk factors.

When CKD was combined with hypercholesterolaemia and metabolic derangement for 4–5 months, sustained inflammation and oxidative stress were associated with impaired coronary vasodilation to adenosine and bradykinin, suggestive of endothelial dysfunction.[65,66] These early microvascular functional alterations were accompanied by a reduced myocardial capillary density, and together may lead to impaired CBF and oxygen delivery, thereby contributing to INOCA.

Further studies directed at a complete characterization of CMD at different stages of the disease are needed, to potentially provide new therapeutic strategies aiming at alleviating microvascular disease and improving myocardial perfusion. However, such studies are difficult to conduct in domestic pigs, as their body growth limits follow-up time. Mini-swine may offer an alternative due to their limited growth rate and size at maturity.

3.2.2 Yucatan mini-swine

Yucatan mini-swine are commonly used for the study of CAD, due to their ability, similar to domestic pigs, to reproduce the neointimal formation and thrombosis as observed in humans.[67] As with domestic swine, metabolic derangement can be induced using chemical destruction of the pancreatic beta cells and/or a HFD.[68] Twenty weeks of HFD resulted in dyslipidaemia in male Yucatan mini-swine without changes in plasma glucose.[69] At this time point, isolated coronary arterioles (~100 μm in diameter) showed only very limited endothelial dysfunction as assessed by the responses to bradykinin, adenosine diphosphate (ADP) and flow-mediated dilation, despite increased microvascular spontaneous tone in HFD animals and lower eNOS protein content. These modest microvascular perturbations were alleviated by exercise training.[69]

The addition of alloxan-induced DM as an additional cardiovascular risk factor resulted in significant decreases in both basal and hyperaemic CBF in response to adenosine and resulted in endothelial dysfunction as assessed with bradykinin *in vivo*.[70] While exercise training alleviated the impairment in basal perfusion and CFR, endothelial dysfunction was not affected by exercise training.[70] These studies show that Yucatan mini-swine subjected to DM in combination with a HFD are an excellent model for long-term studies of the pathophysiology of and therapeutic interventions for CMD.

3.2.3 Ossabaw swine

Ossabaw swine represent a unique model for the study of MetS and CAD. These swine have a 'thrifty genotype' (propensity to obesity) that enabled them to survive long periods of scarce food conditions on Ossabaw Island off the coast of Savannah, Georgia. Consumption of excess kcal (i.e. HFD) causes these animals to manifest components of the MetS, including central (intra-abdominal) obesity, insulin resistance, impaired glucose tolerance, dyslipidaemia, and hypertension, and progress towards T2DM and eventually coronary atherosclerosis.[71] When studied in parallel, male Ossabaw swine had higher glucose-intolerance and insulin resistance after 43 weeks of HFD vs. male Yucatan swine.[68] Furthermore, CFR in response to intracoronary adenosine was impaired and endothelial dysfunction, as evidenced by the response to intracoronary bradykinin infusion, was greater in Ossabaw compared with Yucatan swine either on normal chow or on HFD. In addition, Ca^{2+} efflux was impaired in coronary smooth muscle cells from HFD fed Ossabaw vs. Yucatan swine. These coronary vascular alterations were accompanied by diffuse CAD in Ossabaw but not in Yucatan swine on HFD.[68]

Also in 7–10 weeks old male Ossabaw swine, a short (9 weeks) exposure to a high-fat, high-fructose diet resulted in early-stage MetS, with obesity, hyperglycaemia and dyslipidemia.[72] However, at this early stage, CBF both at baseline and during intracoronary adenosine infusion remained unchanged, as was the response of isolated coronary arterioles (~100 μm in diameter) to adenosine or the NO donor sodium nitroprusside, suggesting preserved VSMC function despite early alterations in adenosine A_2 receptors and K_{ATP} channels expression.[72] With prolongation of diet duration, myocardial perfusion became significantly impaired.[73] The contribution of the voltage-gated potassium (K_V) channels to metabolic control of CBF at rest and during exercise was studied in lean Ossabaw swine vs. obese Ossabaw swine with MetS produced by 4 months of HFD.[73] MetS swine showed a 30–35% reduction in CBF both at rest and during treadmill exercise and a reduction in coronary vascular conductance. This CMD was the result of a blunted contribution of K_V channels to CBF control during increased metabolic demand in MetS.[73] Subsequent studies revealed that coronary large conductance Ca^{2+}-activated potassium (BK_{Ca}) channel dysfunction (both *in vivo* and *in vitro* in isolated arterioles ~100μm in diameter) was associated with increased L-type Ca^{2+} channel-mediated constriction, which also contributed to CMD after 3–6 months of MetS.[74] Furthermore, after 6 months of HFD in Ossabaw swine, isolated coronary microvessels showed increased myogenic tone, which was associated with inward hypertrophic remodelling, indicating that longer-term MetS can also result in structural changes in the coronary microvasculature.[75] In addition, capillary rarefaction was present which may have further contributed to the impaired CFR.[75] The addition of renovascular hypertension (by unilateral renal artery stenosis) to this model, resulted in further impairment of maximal myocardial perfusion, as the MetS and hypertension synergistically suppressed the adenosine-induced hyperaemic response almost completely.[76] This response was associated with impaired eNOS expression and hypertrophic remodelling in the coronary microvasculature and resulted in left ventricular diastolic dysfunction, making this animal

model with multiple cardiovascular risk factors also suitable for the study of microvascular involvement in heart failure with preserved ejection fraction.[76]

3.2.4 Rapacz familial hypercholesterolaemic swine

Downsized Rapacz familial hypercholesterolaemic (FH) swine have been inbred at the University of Wisconsin Swine Research and Teaching Center by Drs Rapacz and Hasler-Rapacz to yield a swine model of high plasma cholesterol levels and accelerated atherosclerosis. This was achieved by a spontaneous mutation in the LDL-receptor gene on chromosome 2; the protein product of this gene normally removes LDL from the circulation.[77] This model is especially suitable for the study of coronary vascular dysfunction associated with high levels of circulating LDL, as seen in FH patients prior to or in the presence of obstructive coronary artery lesions. In 20-month-old Rapacz FH swine, 5 months of HFD resulted in marked hypercholesterolaemia and diffuse coronary atherosclerosis, associated with CMD.[78] This study demonstrated once again the presence of microvascular endothelial dysfunction both *in vivo* and in isolated coronary arterioles (~100 μm in diameter) prior to obstructive plaque development. The endothelial dysfunction appeared to be mediated by impaired EDHF-dependent vasodilation as well as by impaired NO bioavailability that was compensated for by an increased sensitivity to NO. These perturbations in the regulation of microvascular tone resulted in impaired CBF and myocardial oxygen delivery—especially during exercise—that was associated with a shift towards anaerobic myocardial metabolism particularly during increased myocardial metabolic demand.[78]

3.2.5 Summary: porcine models of CMD

In conclusion, several swine models of CMD in the presence of comorbidities are currently available, showing clear evidence of CMD depending on the duration of exposure to cardiovascular risk factors. The mechanisms involved in the development and progression of CMD are summarized in *Table 1*. While domestic pigs are readily available and cheap, young animals are typically used in cardiovascular studies which makes chronic treatments, including exercise training, difficult to assess due to rapid body growth of the animals, thereby limiting follow-up time. The use of inbred mini-pigs, including Yucatan, Ossabaw, and Rapacz enables the study of adult animals, prolongation of diet duration and mechanistic studies at different time points, opening opportunities for the development of therapeutic targets aimed at alleviating CMD.

3.3 Rabbit models of CMD

For nearly a century, rabbits have been utilized to investigate the pathophysiology and therapy of atherosclerosis including endovascular stents.[79] Both in terms of cardiac physiology and body size, rabbits represent an intermediate between large animals (pigs and dogs) and small rodents (rats and mice), that are large enough to place human endovascular stents while still relatively easy to house and handle. Although the rabbit has a higher metabolic rate, lipid profiles and chemical composition of plasma in rabbits show greater resemblance to human lipid metabolism, making them especially suitable for atherosclerosis research.[79] Most commonly used are the domestic rabbit breeds, which all originate from the European rabbit (*Oryctolagus cuniculus*) including Japanese White rabbits and New Zealand White rabbits.[80] In several of these models, investigators have studied the effects of cardiovascular risk factors on coronary microvascular function and structure (Supplementary material online, *Table S3*).

3.3.1 Spontaneous hyperlipidaemic rabbit models

The Japanese veterinarian, Yoshio Watanabe, discovered that a male Japanese White rabbit developed spontaneous hyperlipidaemia on normal chow, due to an inherited recessive trait. This mutation results in a defective LDL-receptor, resembling human-like familial hypercholesterolaemia. Selective inbreeding was used to generate the Watanabe heritable hyperlipidaemic (WHHL) rabbit strain, which has 8- to 14-fold higher serum cholesterol levels than normal Japanese White rabbits and develops hypertriglyceridaemia (300–600 mg/dL).[81] A different inbred strain was developed at the St. Thomas Hospital, which also shows hypercholesterolaemia but with normal LDL-receptor function and mildly elevated levels of triglycerides. This model resembles the human familial hyperlipidaemia more closely and appears therefore more suitable for evaluating the relation between hypertriglyceridaemia and insulin resistance.[82] Both models are used in combination with normal chow as well as a high (~1%) cholesterol diet. Whereas, to date, these models have principally been used to study atherosclerosis, they also represent interesting models for the assessment of CMD in response to hyperlipidaemia, although an early study in the WHHL rabbits did not reveal any changes in either basal CBF or CFR.[83]

3.3.2 Transgenic rabbit models

To study alterations in lipid compositions other than the specific phenotype of spontaneous hyperlipidaemic rabbit models, transgenic rabbit models with modifications in the genes involved in lipid metabolism have been developed. The transgenes include human apolipoproteins (hapo), specifically the A-I/C-III/A-IV gene clusters (hapoA-I/C-III/A-IV gene cluster), hepatic lipase (hHL), lecithin: cholesterol acyltransferase (hLCAT), lipoprotein lipase (hLPL), and scavenger receptor class B type I (hSRB-I).[79,84] Similar to the inbred strains, levels of cholesterol and/or triglycerides are increased.[84] However, to our knowledge, these transgenic rabbit models have not yet been used in studies of CMD.

3.3.3 Rabbit models with induced metabolic derangement

Given the limited availability of spontaneous or transgenic rabbit models for dyslipidaemia, induction of risk factors has been used to model metabolic derangement in rabbits. Similar to other animals, induction of risk factors such as DM or dyslipidaemia can be achieved in multiple ways. Supplementary material online, *Table S3* presents an overview of the studies that induced hyperlipidaemia to study alterations in coronary microvascular function. In addition, DM has been created through injection of alloxan. Alloxan was used because older studies showed that rabbits, similar to guinea pigs, are resistant to the diabetogenic effects of STZ, and thus is not feasible to use STZ in rabbits as opposed to other animal models.[85,86] In rabbits with alloxan-induced DM, the isolated perfused heart setup was used to study coronary microvascular function *ex vivo*.[87] Nine to 12 weeks of exposure to type 1 DM (T1DM) had no effect on baseline CBF or CFR and showed similar vasodilator responses to papaverine compared to euglycaemic conditions. However the responses to serotonin and adenosine were attenuated in hyperglycaemic and hyperosmotic conditions. In addition, the coronary vasodilator response to hypoxia was reduced, which was not due to alterations in adenosine-mediated vasodilation but was mediated through an altered contribution of cyclooxygenase products.[87]

Multiple studies have used a HFD to induce metabolic derangement to study coronary (micro)vascular function in rabbits. These studies vary in the cholesterol content of the diet (ranging from 0.8% to 2%) as well as duration of exposure (ranging from 4 to 16 weeks). Endothelial

dysfunction was evidenced by impaired relaxation to acetylcholine, substance P, and ADP in the hypercholesterolaemic group in most[88–90] but not all[91,92] studies. Conversely, smooth muscle responsiveness to NO was preserved after 8–12 weeks of hyperlipidaemia.[89–92] In addition, vasoconstriction to norepinephrine as well as serotonin was enhanced in coronary arteries from hypercholesterolaemic animals.[88] Furthermore, the vasodilator response to acidosis was impaired which was due to an impairment upstream of the K_{ATP} channels, as the response to the K_{ATP} channel opener levcromakalim was unaltered.[92] In line with these findings, Pongo *et al.*[93] showed that the effect of protein kinase C—acting through K_{ATP} channels—on vasomotor control in hypercholesterolaemic coronary arterioles was lost but was restored after farnesol supplementation. Finally, the response to ischaemia-induced paracrine vasodilator factors was attenuated in hypercholesterolaemic animals, as a result of increased oxidative stress.[91] Histological examination of small coronary arteries and arterioles of rabbits with hypertension (induced by removal of the left kidney and partial ligation of right renal artery) and hypercholesterolaemia (by 0.8% cholesterol diet for 16 weeks), showed structural changes such as hyalinization and/or intimal hyperplasia.[94]

3.3.4 Summary: rabbit models of CMD

Taken together, the usage of rabbits in studying CMD in metabolic derangement is viable and has distinct advantages when compared with rodent models. Genetically modified models are available, although they have not been used yet to specifically investigate CMD. Induction of metabolic derangement is possible and although previous research utilized a large variety of diet compositions and durations, all studies report the presence of CMD (*Table 1*).

4. Rodent models of CMD

4.1 Rat models for CMD

A variety of rat models, exhibiting a range of comorbid conditions, have been utilized to interrogate mechanisms of CMD (Supplementary material online, *Table S4*). The most prevalent models include the STZ-induced model of T1DM, the HFD or western diet (WD)-fed and obese Zucker rat (OZR) models of obesity and insulin resistance, the Zucker diabetic fatty (ZDF) rat, the Otsuka Long-Evans Tokushima Fatty (OLETF) rat model of obesity, progressive insulin resistance, and T2DM and the Goto-Kakizaki (GK) non-obese model of T2DM. Each of these models typically exhibit hyperglycaemia and some recapitulate additional aspects of human metabolic disease. Specifically, the HFD/WD-fed rat and OZR models exhibit obesity, hyperinsulinaemia, and hypercholesterolaemia. The ZDF and OLETF rat models are also obese with hypercholesterolaemia, however, hyperglycaemia in these models is associated with insulin resistance in younger rats followed by progressive impairment of insulin secretion. Lastly, the GK rat exhibits hypercholesterolaemia and, such as the ZDF and OLETF rats, hyperglycaemia owing to insulin resistance and eventual impairment of insulin secretion independent of obesity. With several exceptions,[95–100] available studies evaluating coronary microvascular function in these models report similar blood pressures between rats with metabolic derangement and matched controls.[101–114]

Despite the various metabolic phenotypes of these models, available evidence suggests similarities in the nature of the associated CMD. A common finding across these models is altered coronary endothelial function, however, important distinctions in the manifestation of this dysfunction are worth noting. First, significant impairment of endothelium-dependent vasodilation (typically to acetylcholine) occurs early in some models or later in the disease process of others due to compensatory changes in the underlying mechanism of vasodilation. For instance, most studies in STZ rats, HFD/WD-fed rats, and young OZR report maintained coronary vasodilation to acetylcholine.[95,101,102,105,106,115,116] Further examination of mechanisms of coronary endothelium-dependent vasodilation in these models, however, has revealed compensatory up-regulation of BK_{Ca} channel expression/activity,[105,115] up-regulation of endothelial small (SK_{Ca}) and intermediate (IK_{Ca}) conductance K_{Ca} channel expression/activity,[106] increased basal phosphorylation of eNOS and Akt,[108] and increased VSMC soluble guanylate cyclase activity[116] and reduced phosphodiesterase 5 activity[109] in models of metabolic derangement. Thus, early endothelial dysfunction in these models is masked by compensatory mechanisms not seen in the OLETF or GK rat models of metabolic derangement. Indeed, impaired endothelium-induced vasodilation has been reported at the earliest time points examined in OLETF[111] and GK[117] rats. An interesting point in this regard relates to the OZR model in which insulin-induced vasodilation, also endothelium-dependent, is impaired earlier than dilation to acetylcholine.[98,107,118] Therefore, insulin-induced vasodilation may serve as a functional biomarker of early CMD in metabolic disease and vascular insulin resistance may be an early mechanism of dysfunction in these disease states.[119] Furthermore, increased basal and stimulated coronary or cardiac oxidative stress is reported in these models[97,107,112,118,120,121] and may serve as an initial trigger of dysfunction/compensation. Scavenging of reactive O_2 species restores impaired endothelium-dependent vasodilation in OZR and ZDF rats.[98,118] Taken together, when using rat models to evaluate endothelial function, it should be kept in mind that different rat models represent different disease stages. Therefore, rat models need to be carefully selected based on whether the hypothesis being tested is focused on 'early' or 'late' coronary endothelial dysfunction with the genetic models (i.e. OLETF, GK, and older OZR) appearing to represent later stages of disease with more pronounced endothelial dysfunction.

Studies evaluating the impact of metabolic derangement on coronary microvascular VSMC function in rats are quite disparate and perhaps context- or agonist-dependent. For instance, coronary vasoconstrictor responses in OZR are similar in most,[105,107,108,120,122] but not all,[109,123,124] studies compared with lean Zucker rat as controls. Several studies report reduced vasoconstrictor responses to several agonists (e.g. ET-1, KCl) in this model.[123,124] In addition, in endothelium-denuded coronary arterioles from OZR, vasoconstrictor responses to insulin,[118] and hydrogen peroxide (H_2O_2)[120,121] are similar, however, H_2O_2-stimulated production of cyclooxygenase-2-derived prostanoids and H_2O_2-induced VSMC Ca^{2+} entry and mobilization are increased.[120,121] Conversely, in the OLETF rat model, evaluation of ET-1-induced coronary vasoconstriction reveals progressively increased vasoconstriction with age.[111] In addition, older GK rats exhibit impaired coronary myogenic vasoconstriction due to defective Rho-kinase activity.[114] Together, these data demonstrate a lack of correlation between the endothelial and VSMC phenotypes underlying CMD in rat models of metabolic derangement. Interestingly, in the STZ rat model of T1DM, inhibition of NOS/COX *in vivo* has been reported to reveal focal stenosis and segmental vasoconstriction by *in vivo* synchrotron imaging that was alleviated by Rho-kinase inhibition.[95,101] Thus, these data may suggest altered coronary VSMC function possibly preceding oxidative stress and impaired endothelial function in this particular model.

Beyond changes in vascular cell function in metabolic diseases, rat models have been utilized to examine changes in cardiac perfusion and

flow control associated with CMD. In general, when viewed across studies, available evidence suggests that changes in cardiac perfusion or coronary flow control occur after the development of oxidative stress and endothelial dysfunction in metabolic diseases. In the OLETF rat model, for instance, a time course study demonstrated reduced CFR *in vivo* at 15 weeks of age,[100] while a separate study reported endothelial dysfunction as early as 5 weeks of age in this model.[111] Impaired metabolic hyperaemia was reported in OZR at 8–12 months of age[97] but the relationship to endothelial dysfunction is unclear as no earlier time points were assessed. The impact of HFD and WD feeding on cardiac perfusion is ambiguous as both a trend for increased baseline perfusion[104] and a reduction in estimated perfusion[103] have been reported, respectively. Results in these models likely differ depending on the composition of the experimental diet and the length of feeding utilized. Lastly, both reduced baseline coronary flow[125] and impaired CFR[126] have been reported in GK rats and this dysfunction may be greater in females.[127] To our knowledge, very few studies have examined sex differences in the coronary microvascular phenotype of rat models of metabolic derangement. This is a critical gap in knowledge in light of evidence that intermediate-to-high-risk women, but not men, with reduced CFR experience greater cardiovascular events.[128]

The pathogenic mechanisms initiating CMD, which may serve as appropriate therapeutic targets, in these rat models remain incompletely understood. However, one approach that has been examined in several models with promising results is inhibition of the RAAS. Indeed, angiotensin-converting enzyme (ACE) inhibition prevented reduced capillary length density in STZ rats[96] and acutely restored bradykinin-induced vasodilation in coronary arterioles from HFD-fed rats.[129] Furthermore, inhibition of aldosterone-binding mineralocorticoid receptors (MRs) reversed coronary vasodilator dysfunction in OZR[107] and OLETF[111] rats, however, enhanced vasoconstriction in OLETF rats was not altered by MR blockade. Increased acetylcholine-induced vasoconstriction and coronary perivascular fibrosis (i.e. transforming growth factor β1, plasminogen activator inhibitor 1, collagen I and III, and fibrin expression) in the OLETF rat were improved by angiotensin receptor inhibition.[99,130] These data are supported by clinical evidence that ACE inhibition and MR blockade improve CFR in patients with DM.[131–133] An additional intervention that successfully prevented coronary vasomotor dysfunction in the STZ rat is chronic *in vivo* inhibition of the sodium-hydrogen exchanger.[134] Much work remains, however, to better understand the precipitating mechanisms of CMD in these rat models as well as their translatability to mechanisms implicated in human disease.

4.1.1 Summary: rat models of CMD

There is wide variety of rat models that show CMD including non-genetic (DM and HFD) and genetic (Zucker, OLETF, and GK) models. All these models display CMD, the underlying mechanisms being summarized in *Table 1*.

4.2 Murine models of CMD

The use of murine models for the study of the coronary microcirculation in health and disease has gained interest over the past 20 years. Since some of the initial reports of endothelial dysfunction in leptin receptor deficient (db/db) and leptin deficient (ob/ob) mice,[135,136] there have been several publications showing the impact of risk factors on endothelial and vascular function in mice. Supplementary material online, *Table S5* summarizes several of these murine models that have been used in the study of the coronary microcirculation.[30,135–148]

Db/db mice, such as the OZR model, have a deficient leptin receptor and as a consequence display polyphagia and obesity, resulting in T2DM. Several studies in this mouse model of obesity have demonstrated reduced acetylcholine[135,137,138,149] and flow-mediated[135,138,149] vasodilator responses, and either maintained[135,149] or reduced[138] sensitivity to NO. In addition, there is evidence of inward hypertrophic remodelling of arterioles,[150] with variable effects on microvascular densities.[150,151] Ob/ob mice are deficient in leptin production and as a result display the same phenotype as the db/db mice. Studies in this mouse model have shown maintained basal coronary flow velocities but reduced hyperaemic coronary flow velocities and coronary flow velocity reserve.[152,153] Bender *et al.*[140] used a WD to produce obesity and T2DM in wild-type mice, and observed reduced baseline coronary vascular resistance in isolated perfused mouse hearts that was accompanied by reduced NO bioavailability and blunted VSMC sensitivity to NO. Trask *et al.*[154] compared T1DM—induced with STZ—and T2DM in db/db mice, and observed inward coronary arteriolar remodelling in T2DM but not in T1DM. Finally, ApoE knockout mice are mostly used for studies of atherosclerosis but also display coronary endothelial dysfunction as demonstrated in isolated arterioles[155] or isolated buffer perfused hearts,[156] with maintained VSMC sensitivity to NO (Supplementary material online, *Table S5*).

Mouse models have both advantages and disadvantages—like any preclinical model—but one major advantage is the ability to use genetically modified animals, which creates a unique platform enabling investigators to address very precise questions. For example, Saitoh *et al.*[157] reported that the drug 4-aminopyridine (4-AP) attenuated coronary metabolic dilation in a large animal model. 4-AP is an antagonist of voltage-gated potassium channels, leading the authors to conclude that these channels were involved in metabolic coronary hyperaemia. However, the K_v-channel family is large with 40 genes encoding for 12 K_v channel families. Although 4-AP may have some preferential antagonism for certain K_v channels, it is impossible to unequivocally establish the particular channel responsible for metabolic vasodilation using a standard pharmacological approach. This limitation underscores the rationale for using murine models, to engender a more precise conclusion and, in this situation, to enable determination of the specific K_v channel (or channels) that are linked to metabolic coronary vasodilation. Within this context, Ohanyan *et al.*[30] demonstrated in a genetically modified murine model that $K_{v1.5}$ channels are critical to coronary metabolic dilation. Thus, deletion of these channels compromised the connection between cardiac work and myocardial blood flow. Because the knockout was global, the investigators also created a reconstituted rescue model that had the $K_{v1.5}$ channel (on the null background) expressed only in vascular smooth muscle upon induction with a tetracycline. This model, with the reconstituted channel, restored normal metabolic dilation, i.e. re-established the connection between myocardial blood flow and cardiac work.[30]

4.2.1 Summary: murine models of CMD

Genetic and non-genetic mouse models of metabolic derangement support the concept of CMD as an early abnormality in the disease process, with clear coronary microvascular endothelial dysfunction (*Table 1*). There is no question that the murine model has its limitations—including a high heart rate, high rate of metabolism, and sympathetic dominance—but it provides a route enabling the interrogation of specific questions in coronary microvascular physiology and pathophysiology. Genetically modified mice have enhanced our understanding of the control of the coronary microcirculation and will enable further 'proof of concept experiments' whereby genes that are linked to a human pathology can

be expressed in a mouse to determine if the particular gene is causal in the process.

5. Translational value of the different animal models of CMD and concluding remarks

5.1 Translational value to clinical setting

Since INOCA is more often diagnosed in post-menopausal women than in men, studies of sex differences in pathophysiology are important and animal studies of CMD and INOCA should preferably take sex differences into account. Although rodents are more often studied in a comparative manner (young vs. old and male vs. female) than large animals, most studies in the field of metabolic dysregulation and its effects on coronary perfusion have been performed in young animals (e.g. 8–12 weeks old mice, 3–6 months old pigs, Supplementary material online, *Tables S1–S5*) and have principally investigated a single sex. Yet, obesity and T2DM are strongly associated with maturation and ageing, and several differences have been reported between young and old animals, as well as between males and females. Thus, beta-cell replicative capacity strongly decreases with age in mice, and young rats do not develop insulin resistance in response to nutrient infusion, whereas older animals do.[158] Also in male Gottingen minipigs studied from 6 to 24 months of age, increases in plasma glucose, fructosamine, and triglycerides were observed with age, while plasma cholesterol levels decreased with age.[159] Importantly, oestrogen is known to have positive effects on metabolism and to be protective against the development of obesity, insulin resistance and hyperglycaemia,[158] as well as against endothelial dysfunction,[160] so that the use of animals of only one sex and one menopausal state in case of female animals is likely to introduce a bias. These limitations should be kept in mind when choosing an animal model, and limitations such as young age and single sex should, when possible, be circumvented in the study of INOCA, especially when testing novel therapies.

Another important aspect pertaining to the translational value of animal models is their ability to utilize clinically relevant methodological approaches to diagnose and treat the disease. PET studies and invasive microvascular function measurements have recently been proposed to be the best approach in stratifying patients with INOCA for a tailored therapy.[9] Such measurements, especially when involving invasive techniques, are not easily performed in smaller animal models (Supplementary material online, *Tables S3–S5*). However, large animal models may also pose some challenges. For example, while FFR measurements and acetylcholine and adenosine infusions can easily be performed in dogs and swine, these tests need to be adapted from the clinical protocol. For instance, in the porcine coronary circulation acetylcholine induces muscarinic vasoconstriction requiring use of another endothelium-dependent vasodilator. Finally, while the use of isolated small arteries/arterioles is very instrumental for the study of perturbations in specific mechanisms regulating microvascular tone, it should be noted that—irrespective of the chosen animal model—isolated small arteries/arterioles represent only one segment of the coronary microvasculature and interactions with the surrounding myocardium are lacking. Hence, while specific mechanisms involved in CMD can easily be studied in isolated coronary small arteries or arterioles, the evaluation of the coronary microcirculation as a whole, including microvessels of all sizes, is impossible using an *in vitro* technique. Hence, *in vivo* studies are ultimately required to assess coronary function in an integrated manner.

5.2 Concluding remarks

Animal models of coronary microvascular disease yield important insights into the genetic and environmental basis of human coronary pathophysiology in ischaemic heart disease and provide translational models for preventive and therapeutic interventions (see *Figure 2*). In the past 50 years, animal models have been instrumental in advancing our knowledge pertaining to CBF regulation in health and ischaemic heart disease. Notwithstanding the undisputable merits of experimental animal models, researchers need to carefully consider the choice of a specific animal model.[161,162] It is imperative to acknowledge that no single animal model perfectly emulates the human disease, nor has a perfect translational capacity to the clinical setting (*Table 1* and *Figure 2*). In addition, there are a number of financial and logistical considerations that need to be considered, including costs, infrastructure, and the requirement for specialized personnel. Small animal models have the advantage of being relatively cheap, easy to breed and handle, have short reproductive cycles and large litter sizes, a well-defined genome and a relative ease of genetic modification to explore pathophysiological mechanisms with great molecular precision. Disadvantages include different metabolic and lipoprotein profiles compared to humans and resistance to atherosclerosis development, requiring genetic modification, and technical challenges to study the coronary microcirculation particularly *in vivo*. Conversely, large animal models have the advantage of human-sized hearts and coronary blood vessels, allowing the application of clinical diagnostic and therapeutic tools. Moreover, from a coronary anatomical and physiological perspective, large animals, especially swine models, approximate the human heart and its coronary circulation more closely, have similar lipoprotein profiles and develop similar metabolic derangement. In addition, the possibility of awake measurements in large animals is of great importance, as neurohumoral and cardiac dysfunction especially in early disease—possibly not detectable at rest or under deep anaesthesia—might be unmasked by exercise. As summarized here, several animal models have been developed for the study of CMD and characterized in detail. These models—depending on number and severity of, and duration of exposure to risk factors—show perturbations in coronary microvascular (endothelial) function and structure, similar to what has been observed in patients with non-obstructive CAD and comorbid conditions. The use of these animal models, with careful selection based on the specific research question, will be instrumental in identifying novel therapeutic targets and for the subsequent development and testing of novel therapeutic interventions to treat CMD.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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