Recommendation on Design, Execution, and Reporting of Animal Atherosclerosis Studies

A Scientific Statement From the American Heart Association

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Abstract—Animal studies are a foundation for defining mechanisms of atherosclerosis and potential targets of drugs to prevent lesion development or reverse the disease. In the current literature, it is common to see contradictions of outcomes in animal studies from different research groups, leading to the paucity of extrapolations of experimental findings into understanding the human disease. The purpose of this statement is to provide guidelines for development and execution of experimental design and interpretation in animal studies. Recommendations include the following: (1) animal model selection, with commentary on the fidelity of mimicking facets of the human disease; (2) experimental design and its impact on the interpretation of data; and (3) standard methods to enhance accuracy of measurements and characterization of atherosclerotic lesions. **(*Arterioscler Thromb Vasc Biol*. 2017;37:e131–e157. DOI: 10.1161/ATV.0000000000000062.)**

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Atherosclerosis is a major cause of morbidity and mortality in many populations that contributes to coronary artery disease, stroke, and peripheral vascular diseases. Given the magnitude of the devastating impact on public health, there have been substantial efforts to garner insights into both the mechanisms of the process and development of pharmaceutical approaches for attenuating evolution of lesions. However, providing insight into the progression of atherosclerosis in humans has many significant barriers. One difficulty is chronicity of the disease. Although atherosclerotic lesions are detected as early as the second decade of life, overt clinical manifestations do not usually occur until many decades later. Another impediment is the complexity of the disease as it profoundly changes the arterial wall over decades of evolution. Finally, because arterial tissue can only be obtained either at autopsy or a single selected phase of life during surgery, there is limited direct access to evolving stages of atherosclerosis. Therefore, imaging modalities are the only means of obtaining sequential measurements of atherosclerosis. Although selected imaging modalities have evolved to enable measurement of lesion dimensions with reasonable precision, there are limitations in providing in-depth analysis on lesion characteristics.

The natural history of the pathological progression of human atherosclerotic lesions has been derived from studies that acquired arterial tissue at a single time point per person. Information on the composition of atherosclerotic lesions in the initial decades of life has generally relied on tissue acquisition from individuals dying in traumatic circumstances, such as warfare or automobile accidents.[1–3] Although these studies provide valuable insight into lesion initiation and early progression, they also illustrate the difficulties of defining pathology with precision, in part because of the heterogeneity of

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this disease. Other studies have acquired knowledge on lesion composition by arterial tissue acquisition at more advanced stages. These studies and others have been used as the foundation of consensus statements, such as those from the American Heart Association (AHA), on the initiation and progression of human atherosclerosis.[4–6] However, limitations of the data provide alternative avenues for interpretation of pathology for initiation and progression of human atherosclerosis lesions.[7] Animal models of atherosclerosis have the potential to overcome many of the inherent restrictions of human research. Indeed, there have been many decades of research in which atherosclerosis has been induced in animals ranging from mice to monkeys. These animal studies enable acquisition of atherosclerotic tissues at many stages of lesion progression.

The profound health impact of atherosclerosis has led numerous researchers to perform studies to determine the effects of pharmaceutical or dietary interventions in humans. A multitude of studies have examined the genetic basis of human atherosclerosis.[8,9] These studies commonly assess clinical end points to atherosclerosis-related disease, such as occurrence of heart disease. Such studies are restricted by logistic complexities and expenses related to the recruitment of large numbers of people entered into multiyear longitudinal trials. Although these human studies are a requirement for ultimate validation of an intervention or genetic effect on atherosclerosis, progress in the field requires the completion of meaningful animal studies to provide proof of concept.

Overall, there is a dire need for further studies to expand our knowledge on mechanisms by which atherosclerotic lesions are initiated, progress, and cause overt clinical manifestations. Inherent shortcomings of human atherosclerosis research require that appropriate animal models be used in rigorously designed studies that are developed with standardization. Indeed, animal studies have already provided an extensive insight into atherosclerosis. For this expansive literature to provide meaningful extrapolation to the human disease, there are many aspects of these studies that should be deliberated.

Insights Provided by Animal Models

There has been a long history of using animal models to provide insight into mechanisms of human atherosclerosis. A large number of animal studies have contributed to the notion that lowering plasma cholesterol concentrations is an effective mode of reducing atherosclerosis,[10] which has led to therapeutic approaches for reducing plasma cholesterol concentration to effectively reduce atherosclerosis-related diseases in humans.[11]

Although a wide range of animal models have been used in atherosclerosis research, the majority of contemporary studies are performed in mice. The similarities of mouse genes to those identified as promoting risk in human genome-wide association studies enhance the potential to extrapolate findings in mouse models to the human disease.[12,13] Also, mouse-based models have been particularly useful for elucidating effects of individual genes on atherosclerosis, with subsequent confirmation of human relevance in many instances. Salient examples include the elucidation of genes regulating triglyceride-rich lipoprotein metabolism in mice and their impact on atherosclerosis, such as *apoCIII*[14] and *apoAV*.[15] Recent human genetic studies confirmed a similar impact on triglycerides and coronary heart disease in humans,[16] which led to new therapeutic targets and the confirmation of the independent role of triglyceride-rich lipoproteins in atherogenesis.[17] Another example is the effects of a human apoAI (apolipoprotein AI) transgene expression in hypercholesterolemic mice in reducing atherosclerosis.[18,19] These observations contributed to the development of high-density lipoprotein (HDL) infusion therapies in humans.[20] Similarly, mouse studies provided the first evidence that PCSK9 (proprotein convertase subtilisin/kexin type 9) increases plasma low-density lipoprotein (LDL) cholesterol concentrations by reducing expression of LDL receptors and showed that PCSK9 was secreted by the liver, which implied that circulating PCSK9 could potentially be targeted by a neutralizing monoclonal antibody approach.[21,22] This finding provided key information that contributed to the development of PCSK9 antibodies as a therapy for high LDL cholesterol levels and atherosclerosis in humans. The efficacy of PCSK9 antibody administration in lowering LDL cholesterol concentrations has now been proven.[23]

Although there are many favorable aspects of mouse studies, there also needs to be an awareness of differences between mouse and human atherosclerosis.[24,25] These include common assessment of atherosclerosis in mice in vascular locations other than the most common beds that cause disease in humans, such as coronary arteries. Animal models have greater vascular inflammation than found in human disease. Numerous animal atherosclerosis studies have shown the complexities of the immune system and inflammation in atherogenesis.[26–30] Inflammation and related signaling pathways have therapeutic benefits in mice, but these have not been translated to humans. Some animal models lack or have only minimal calcification, whereas calcification in human atherosclerosis not only predicts future events but is observed in >80% of cases at sites of plaque rupture.[31] A pivotal difference is that lesion rupture is not routinely reproduced in animal models.

In general, despite differences of atherosclerotic characteristics from the human disease, over the past several decades, animal models, especially genetically modified mouse models, have provided significant insights into our understanding of the development and mechanisms of atherosclerosis.[32] To enhance the data reproducibility of atherosclerosis in mouse models and improve experimental and data quality, the purpose of this statement is to provide guidelines for development and execution of experimental design and interpretation in animal studies. Recommendations include the following: (1) animal model selection, with commentary on the fidelity of mimicking facets of the human disease; (2) experimental design and its impact on the interpretation of data; and (3) standard methods to enhance accuracy of measurements and characterization of atherosclerotic lesions.

Animal Model Selection

In this section, recommendations are provided for selection of animal models in atherosclerosis studies. Large (rabbits, pigs, and monkeys) and small (mice) animals are individually

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discussed according to their merits in research, and suggestions are provided for appropriate models to be used in specific situations.

Considerations in Selecting a Specific Model

The vast majority of contemporary atherosclerosis studies are performed in genetically engineered mice. Mice offer several advantages and are usually regarded as a proof-of-principle model to examine overexpression or deletion of specific gene targets or pharmaceutical agents on atherosclerosis. Although mice offer rapid answers and a reasonable cost structure, there are several disparities with regard to lesion location, formation, atherosclerotic susceptibility, and progression in mice versus humans. Therefore, large-animal models (rabbits, pigs, and nonhuman primates) are potentially more relevant with regard to preclinical studies of drug toxicology and potency as they relate to combating atherosclerosis in humans.

Atherosclerotic model selection is influenced by several factors, including breeding, genetic pliability, animal size, duration of study, types and locations of lesions, human genomic/structural compatibility, and economics. Listed below are the pros and cons of individual species in experimental models of atherosclerosis.

Mice

Mouse models are the most common species used in atherosclerosis studies. The primary advantages of mouse models are the relatively low cost and ease of maintenance and breeding, genetic and transgenic pliability, and the rapid formation of atherosclerosis. Mice are unique because they can be manipulated using whole or partial gene deletions or additions, as well as conditional deletions or insertions through specific promoters. These gene deletions allow for development of coronary atherosclerosis and myocardial infarction when mice are doubly deficient in apoE (apolipoprotein E) and Ldlr (LDL receptor)[33] or apoE and SR-BI (scavenger receptor BI).[34]

One of the most commonly used mouse models of atherosclerosis is apoE$^{-/-}$ mice.[35] apoE$^{-/-}$ mice spontaneously develop hypercholesterolemia because of increased chylomicron remnant, very low-density lipoprotein (VLDL) and LDL cholesterol, with total cholesterol concentrations ranging from 300 to 500 mg/dL.[36,37] Although the rate of atherosclerotic lesion development varies greatly between facilities, macrophage foam cells can be present in the aortic root within a month. At later stages, there are complex lesions containing spindle-shaped smooth muscle cells, increased extracellular matrix, and overlying fibrous caps.[38] The addition of a saturated fat-enriched diet (21% wt/wt saturated fat, 0.2% wt/wt cholesterol; commonly referred to as Western diet) accelerates atherosclerosis and results in elevated total plasma cholesterol concentrations of >1000 mg/dL. Western diet feeding results in a more foam cell–rich atherosclerosis, with lesions containing cholesterol crystals, necrotic cores, and calcifications. However, a fat-enriched diet can alter the outcomes of atherosclerosis because of the induction of pronounced hypercholesterolemia. For example, recombination-activating gene 1–deficient (Rag1$^{-/-}$) mice on an apoE$^{-/-}$ background have attenuated atherosclerosis when fed a normal laboratory diet, but lesion size did not change when fed a Western diet compared with Rag1$^{+/+}$ mice.[39] Although apoE$^{-/-}$ mice are commonly used for atherosclerosis studies, they have some attendant disadvantages. For example, apoE has many roles in addition to its effects on lipoprotein metabolism. This includes roles in macrophage biology, immune functions, and adipose tissue biology, and this must be considered in data interpretation.[40] Furthermore, apoE is expressed in bone marrow–derived cells, as demonstrated by increased aortic root atherosclerosis without concomitant changes in plasma lipids, by bone marrow transfer from apoE$^{-/-}$ mice into apoE$^{+/+}$ recipients. This result and others suggest that macrophage-derived apoE has an independent role in lesion development, potentially because of cholesterol efflux from the macrophage foam cell. Similarly, this complicates transfer of apoE$^{+/+}$ bone marrow into apoE$^{-/-}$ mice because of the correction of cholesterol efflux and normalization of plasma cholesterol concentrations.[41,42]

The other most commonly used atherosclerotic model is the LDL receptor–deficient mouse.[43] Deletion of the Ldlr gene is uncomplicated compared with apoE, because its primary function is to clear LDL in the liver. LDL receptor deficiency influences lipoprotein uptake and clearance, which results in it being modestly increased in animals fed a normal diet; however, normal diet–fed Ldlr$^{-/-}$ mice only develop small lesions, even at an advanced age (beyond 6 months).[43,44] Therefore, this mouse strain is fed a Western diet to accelerate atherosclerosis. Ldlr$^{-/-}$ mice fed a Western diet accumulate larger VLDL/remnant lipoproteins, with elevated total plasma cholesterol concentrations >1000 mg/dL. Atherosclerotic lesions are mostly foamy compared with mice fed a normal laboratory diet. However, no systematic pathological analysis of lesion development in normal diet–fed mice has been reported in this model, in contrast to apoE$^{-/-}$ mice.[38,45] Another benefit is derived from being able to use Ldlr-positive donors in bone marrow transplantation experiments, because the receptor rapidly becomes downregulated, even in expressing cells, as plasma cholesterol concentrations increase.[46–48] Although atherosclerosis induced in the Ldlr-deficient model is generally less severe than in apoE$^{-/-}$ mice, LDL receptor deficiency coupled with either an apoB100 editing enzyme apoBEC-1 deficiency or the human apoB100 transgene causes mice to develop spontaneous atherosclerosis similar to apoE$^{-/-}$ mice fed a normal laboratory diet.[49,50]

Although there are many advantages in using mouse models for atherosclerosis studies, it is important to note the many differences between mice and humans. For example, mice do not exhibit HDL subsets or apoE isoforms, do not express CETP (cholesterylester transfer protein), are relatively small (allowing for minimal tissue collection), and are not good drug corollaries to humans with regard to metabolism or pharmacokinetics/dynamics. However, this deficiency can be overcome by using mice that transgenically express CETP or human apoE isoforms.[51,52] With regard to responses mediated or modulated by the immune system, species differences should be recognized.[53] In addition, mouse models of atherosclerosis differ with regard to their lesion distribution and lipoprotein profile compared with humans. For example, mice accumulate lesions mostly in the aortic root, the aortic arch, and side branches such as innominate and carotid arteries,

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with many fewer lesions in the coronary arteries. Other differences include thin aortic medial layers, accelerated heart rates (> 600 beats/minute), and low total plasma cholesterol concentrations (50–100 mg/dL). Furthermore, mice transport cholesterol primarily in HDL (versus LDL in humans) and have many other differences in important aspects of lipoprotein metabolism. For example, although LXR (liver X receptor) agonists being developed as an atherosclerosis treatment did not raise LDL cholesterol levels in mice, they were found to substantially increase LDL cholesterol concentrations by inducing IDOL (which promotes degradation of the LDLR) in monkeys and humans, which precluded further clinical development.[54] Therefore, although native mice have marked differences in lipoprotein metabolism compared with humans, genetically manipulated mice have been invaluable to our understanding of molecular mechanisms and the underlying pathways involved in atherosclerosis.

Rabbits

Rabbits are the initial species in which experimental atherosclerosis was induced when Anitschkow demonstrated that feeding eggs led to atherosclerotic lesion formation.[10] There are 2 common rabbit models of atherosclerosis.[55] The most common is feeding New Zealand White rabbits a cholesterol-enriched diet. New Zealand White rabbits have a similar lipoprotein profile to humans and express CETP; however, rabbits are hepatic lipase deficient, which results in β-migrating VLDL being the primary circulating lipid constituent during cholesterol feeding. Furthermore, rabbits have low plasma concentrations of apoAII, which has been linked to increased atherosclerosis susceptibility, circulating free fatty acids, body fat, and insulin resistance (reviewed in Castellani et al[56]). Further limitations of this model include the predominance of foam cell lesions, similar to mice. An additional detriment is the tendency to develop fatty livers and jaundice with longer-term cholesterol feeding.

The other common model, Watanabe hereditary hypercholesterolemic (WHHL) rabbits, exhibit familial hypercholesterolemia and have been used frequently in atherosclerosis studies.[57,58] A defect that leads to absence of LDL receptors, caused by premature degradation, results in increased plasma LDL cholesterol concentrations, evolution of progressive intimal lesions, fatty streaks, raised foam cell lesions, medial lipid deposition, and atheroma in the aorta and coronary arteries, with the additional presence of subcutaneous xanthomas.[59] The lesions can progress to advanced humanlike atherosclerosis. In WHHL heterozygous rabbits fed diets enriched with 0.5% wt/wt or 1% wt/wt for 12 to 24 weeks, lesions develop similarly to those in patients with familial hypercholesterolemia with atherosclerotic necrosis, cholesterol clefts, and calcification.[60] Unlike cholesterol-fed New Zealand White rabbits, WHHL rabbits do not develop fatty livers. Limitations to using WHHL rabbits include few suppliers, breeding difficulties, and limited availability.[61]

In addition to these 2 models, a substrain of WHHL was created for examination of robust coronary atherosclerosis.[62] There are also several rabbit transgenic models that modify atherosclerosis, including apoE deficiency.[63]

Overall, rabbits can be cost equivalent to mice at purchase, although they are more expensive to house and feed. The bigger size of rabbits can provide some advantages over mouse models, including by facilitating noninvasive arterial analysis, providing sufficient arterial tissues and atherosclerotic lesions for harvest, and enabling implantation of stents for biomechanical or pharmaceutical design and testing. Under certain circumstances, rabbit atherosclerotic models provide advantages over mouse models. For example, rabbits express CETP that more closely mimics human lipoprotein metabolism.[64]

Pigs

Pigs represent a model of atherosclerosis that has more similarity to humans in terms of anatomy, physiology, lipoprotein profile, and site of lesion formation. Although lipoprotein profiles in pigs have similarities to those in humans, like mice, CETP activity is absent in their plasma.[65] Pigs fed a normal laboratory diet have low plasma concentrations of LDL cholesterol and high HDL and do not develop mature atherosclerotic lesions. Atherosclerotic lesion formation can be accelerated by feeding a diet supplemented with high fat and cholesterol.[66] Furthermore, pigs are large enough to elucidate hemodynamic changes in arteries and endothelial dysfunction.[67] Similar to humans, pigs develop lesions at arterial bends characterized by low shear stress and flow separation.[68]

The most common model used today is the domestic crossbred farm pig (*sus scrofa domestica*) fed a high-cholesterol (1.5% wt/wt) and high-fat (19.5% wt/wt lard) diet.[69] This model results in hypercholesterolemia that primarily involves increased plasma concentrations of LDL cholesterol. After 15 weeks of this enriched diet feeding, pigs develop predominantly foam cell atherosclerotic lesions in coronary arteries and the abdominal aorta, extending into iliac arteries. After 30 weeks, plaques become more complex, with a frequent occurrence of a fibrous cap, underlying necrotic lipid core, lipid-laden smooth muscle cells, and foam cells.[70] Although advanced lesions that resemble human coronary atherosclerotic lesions occur (thick fibrous cap, necrotic lipid core, calcification, and hemorrhaging), it can take up to 2 years to form in this model at great cost and size, because these pigs can reach up to 200 kg in weight.

Some researchers use the inherited hyper-LDL cholesterolemia pig model, which has a mutation in several apolipoprotein genes that results in elevated LDL and spontaneous hypercholesterolemia even when fed a cholesterol-free, low-fat diet.[71] The size and composition of this LDL differs from that of the high-fat and high-cholesterol–fed pig (LDL is cholesterol ester rich and buoyant compared with LDL in normocholesterolemic swine). This model develops similar lesions to the normal pig fed a high fat and cholesterol diet; however, advanced lesions occur as quickly as 12 to 18 months. The atheroma is more advanced and humanoid in these pigs, characterized by complicated stenotic lesions containing fibrous caps, necrotic cores, cholesterol clefts, granular calcium deposits, and neovascularization deep within the lesion of the major coronary arteries by 24 months. Similar to the high-fat, high-cholesterol pig model, these hypercholesterolemic pigs are a long-term model with dramatic expenses and a size that presents challenges.

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There are also other pig models available, including miniature pigs (Yucatan, Hanford, Gottingen, and Sinclair Hormel breeds), pigs with streptozotocin-induced diabetes mellitus to accelerate atherosclerosis,[72,73] and familial hypercholesterolemic transgenic pigs.[74,75]

These pig models develop extensive atherosclerosis in several arterial regions, including the coronary. The nature of the lesions extends from primarily lipid-laden macrophages to more advanced stages of evaluation that include necrotic core formation, extensive fibrosis, calcification, angiogenesis, and intralesion hemorrhage.

Nonhuman Primates

Nonhuman primates are the closest humanoid model of atherosclerosis on the basis of anatomy, physiology, lipoprotein profile, and site of lesion formation. Primates have a predominance of non-HDL lipoproteins and express humanlike HDL subclasses. In the most common monkey models, atherosclerosis is first seen in the aorta, carotid, and iliac arteries. Atherosclerotic lesions form in the proximal and then distal coronary arteries after a prolonged period. Fatty streaks are the most common, but other lesions range from uncomplicated fibrous plaques to complex pathology containing necrotic cores, mineralization, hemorrhage, and medial destruction. Furthermore, rhesus monkeys consuming an atherogenic diet have a relatively high frequency of myocardial infarction.[76] Coronary artery remodeling is similar between monkeys and humans with regard to the Glagov hypothesis of remodeling.[77]

Several original studies in nonhuman primates were performed in new world monkeys, such as Squirrel, Woolly, and Cebus monkeys[78]; however, these monkeys have a high incidence of chronic renal disease, which can modulate both their lipoprotein metabolism and atherosclerotic incidence. Current models focus on the old world monkeys, such as African Green, Rhesus, and cynomolgus monkeys, fed a high-fat/cholesterol diet for prolonged periods of time. Similar to humans, males develop more atherosclerosis than reproductively active females after being fed a high-fat/cholesterol diet.[79] Also comparable to humans, monkeys have an age-dependent susceptibility to atherosclerosis. In a study of cynomolgus monkeys, although prepubescent and postpubescent monkeys had equivalent plasma HDL cholesterol and total cholesterol concentrations, postpubescent monkeys had accelerated susceptibility to coronary artery atherosclerosis compared with almost complete protection in their prepubescent peers.[80] Social support also has a dramatic outcome on atherosclerosis in monkeys. Cynomolgus monkeys housed in a group that provides social support have higher HDL cholesterol concentrations and lower atherosclerosis than singly housed monkeys.[81] In support of this, dominant males in an unstable environment had twice as much atherosclerosis caused by large increases in heart rate and blood pressure during decision-making situations compared with males in a stable environment or subordinate position.[82] Therefore, the social and behavior aspects of monkeys make them unique compared with other atherosclerosis models.

In addition to the benefits stated above, the size of the monkey facilitates tissue availability for atherosclerosis analysis and provides large enough vessels and appropriate lesions for stent validations, noninvasive measurements of atherosclerotic lesions, and invasive angiogram or measurements. The drawbacks of this model are the great expense with regard to feeding, housing, and amount of drug for pharmaceutical studies, the difficulty of genetic modification, and the limited genetic information available compared with other animal models. This model is also expensive, because monkeys usually need to remain on modified diets for extended intervals. Irrespective of these disadvantages, nonhuman primates are the most comparable model to mimic atherosclerosis in humans.

Other Species

Atherosclerosis has also been induced in many other species. These include other mammals such as rats,[83] hamsters,[84] and dogs.[85,86] Atherosclerosis has been developed in avian (pigeons[87]) and fish (zebrafish[88]) species. These species have not been studied extensively. Therefore, there is a lack of compelling evidence that these species furnish advantages or insight that is not provided by the more commonly used species.

Comorbidities

Several chronic disease processes influence the initiation and natural progression of atherosclerosis in humans via exacerbation of inflammatory, metabolic, or biomechanical processes. These chronic disease states most often include diabetes mellitus, renal failure, high blood pressure, and obesity.

Diabetes Mellitus

Patients with either type 1 or type 2 diabetes mellitus are prone to increased cardiovascular disease outcomes, particularly atherosclerotic cardiovascular disease. Although there are several hypotheses concerning why this occurs, the basis for the correlation between diabetes mellitus and increased atherosclerosis remains incompletely understood, especially whether hyperglycemia per se accelerates the development of atherosclerosis.[89,90] Several models of diabetes mellitus have been developed in mice, rabbits, pigs, and nonhuman primates, as reviewed in detail elsewhere.[91] The most common is the streptozotocin injection model, which mimics a type 1–like form of diabetes mellitus in animals. Type 2 diabetes mellitus is most often created by high-fat diet feeding of selective strains of mice, rabbits, pigs, and nonhuman primates or by genetic manipulation of leptin or leptin receptor in mice. Unfortunately, these type 1 and type 2 models of diabetes mellitus only increase atherosclerosis in a few selected animal models, which is complicated by species-specific hyperlipidemia and the inability to parse effects of insulin resistance versus hyperglycemia in increasing atherosclerosis. However, there are a few recent models of type 1 diabetes mellitus that circumvent these hyperlipidemic complications in mice by directly targeting the insulin receptor. One model used insulin receptor/insulin receptor substrate-1 double heterozygous apoE$^{-/-}$ mice fed a Western diet. These mice had no increases in plasma cholesterol concentrations but had an increased atherosclerotic burden with impaired insulin signaling and hyperinsulinemia.[92] Another model uses Ldlr$^{-/-}$ mice with an insulin promoter–driven lymphocytic choriomeningitis virus

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that results in destruction of β-islet cells, rapid development of type 1 diabetes mellitus, progression of arterial inflammation, and lesion initiation without diabetic dyslipidemia.[93] Overall, although the mechanisms have not been defined, the current literature supports that diabetes mellitus augments development and progression of atherosclerosis.

Renal Failure

End-stage or chronic renal failure is associated with significantly higher levels (10- to 20-fold higher) of atherosclerosis and vascular calcification when populations are stratified for age, sex, and presence of diabetes mellitus.[94] Chronic renal failure is associated with several traditional (diabetes mellitus, hyperlipidemia, and hypertension) and uremia-related (hyperhomocysteinemia and anemia) risk factors for atherogenesis.[95] Therefore, it is unclear whether renal dysfunction imparts atherosclerosis through its direct contribution or its associated conditions.

Chronic renal failure in animals was developed by partial nephrectomy, radiation nephropathy, or unilateral ureteral obstruction.[96] To address effects of chronic renal failure on atherosclerosis, Bro and colleagues subjected apoE$^{-/-}$ mice to a fifth or sixth nephrectomy, resulting in uremia. These mice had augmented atherosclerosis compared with apoE$^{-/-}$ mice that had unilateral nephrectomy.[97] The proposed mechanisms include that chronic renal dysfunction induces dyslipidemia, macrophage lipid homeostasis, and renin-angiotensin activation[98,99]; however, no definitive mechanisms have been defined.

High Blood Pressure

High blood pressure is the leading risk factor–related cause of death throughout the world and has been strongly associated with both coronary heart disease and stoke deaths.[100,101] As such, the AHA regards blood pressure as a powerful, consistent, and independent risk factor for atherosclerosis-based cardiovascular diseases in humans.[102] There are several methods to induce transient or stable increases of blood pressure in mice by genetic, pharmacological, or surgical approaches (reviewed in Lu et al[103]). There are 2 accepted methods to measure blood pressure in conscious mice: the noninvasive tail-cuff system and by implantation of a radiotelemetry device, as described in the AHA's "Recommendations for Blood Pressure Measurement in Humans and Experimental Animals."[104] The most commonly used method is the tail-cuff system, which is amenable to screening a large number of mice and detecting substantial increases in systolic blood pressure. Concerns associated with the tail-cuff method include the lack of dynamic measurements (eg, it is not 24 hours a day), stress induction, accuracy of the data, and user variability[104]; however, reproducible data can be achieved by using rigorous protocols.[105] Radiotelemetry is considered more accurate, with uninterrupted blood pressure measurements derived directly from the carotid artery in unhindered mice; however, the expense can be extreme, and the duration of data acquisition cannot usually extend beyond 2 weeks. Because most atherosclerosis experiments range from 8 to 16 weeks in length, use of a tail cuff–based system is recommended for long-term and high-output studies in mice.

Some publications have demonstrated a correlation of blood pressure with atherosclerosis in mouse models (reviewed in Lu et al[103]), whereas others have suggested atherosclerosis can be affected without changes in blood pressure, and conversely, changes in blood pressure do not affect atherosclerosis. For example, endothelial nitric oxide synthase and apoE compound–deficient mice have increased systolic blood pressure (30 mm Hg) and aortic atherosclerosis in both males and females.[106] However, although blood pressure in these mice was reduced to baseline levels with hydralazine, atherosclerosis was not attenuated. In another example, when candesartan (an angiotensin receptor antagonist) or amlodipine (a calcium channel blocker) was administered to apoE$^{-/-}$ mice in doses to achieve equivalent reductions in blood pressure, amlodipine did not reduce aortic atherosclerosis, whereas candesartan significantly attenuated atherosclerosis.[107] Combined with several other studies, it does not appear that elevated systolic blood pressure per se is associated with increased lesion development in mice, but rather the stimulus for hypertension appears to be the major determinant of atherosclerosis.[103]

Obesity

Obesity is considered the driving force behind metabolic syndrome, a multifaceted risk factor that doubles risk for atherosclerosis.[108] It is thought that obesity is a risk factor for atherosclerosis independent of the standard risk factors; however, the link between obesity and atherosclerosis is controversial in the current literature. The Seven Countries Study demonstrated little correlation between body weight and incidence of atherosclerosis,[109] whereas the Framingham Heart Study has consistently shown that increasing levels of obesity are accompanied by augmented rates of atherosclerosis.[110] However, multivariate analysis of the Framingham Heart Study suggests the relationship between body weight and atherosclerotic risk is mediated through major risk factors (eg, blood pressure, hypercholesterolemia, and diabetes mellitus).[111] In contrast, there are also studies that have concluded that moderate overweight is beneficial, but severe obesity augments atherosclerotic heart disease.[112] Therefore, reports from human studies suggest that obesity might not be linearly associated with atherosclerosis. Several models exist to examine whether obesity is related to atherosclerosis in animals, and these are summarized elsewhere[113]; the most common are high-fat diet–fed apoE$^{-/-}$ or Ldlr$^{-/-}$ mice or ob/ob or db/db mice crossed onto an apoE$^{-/-}$ or Ldlr$^{-/-}$ background.[114,115] However, as with diabetic models, obesity in these models does not impact atherosclerosis unless there are concomitant changes in lipids.[78] Hence, the relationship between obesity and atherosclerosis remains controversial. This lack of clarity can be attributed to differences in study participant characteristics, study design, and statistical analysis.

Source of Animals

There are numerous reputable sources that breed common research models of mice, rabbits, and rats, including The Jackson Laboratories (Bar Harbor, ME), Charles River Laboratories (Wilmington, MA), Taconic Farms (Hudson, NY), Harlan Laboratories (Indianapolis, IN), and independent collaborating investigators. Pigs and nonhuman primates are available from a more restricted number of vendors.

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The most popular strains of atherosclerotic mice, apoE$^{-/-}$ (B6.129P2-Apoe$^{tm1UNC/J}$, strain #002052) and Ldlr$^{-/-}$ (B6.129S7-Ldlr$^{tm1Her/J}$, strain #002207), often originate from colonies at The Jackson Laboratory. Once obtained from these facilities, mice are often crossbred with a gene mutant of interest and may stay in investigator-run facilities for several years. However, caution should be taken when obtaining apoE$^{-/-}$ or Ldlr$^{-/-}$ mice from independent investigators because of the phenomena of genetic drift and substrain divergence when breeding constantly for years at a time. These conditions could confound research conclusions and lead to potentially inaccurate or misleading results. The Jackson Laboratory constantly combats genetic drift by using a formulated genetic stability program that refreshes breeding stocks with cryopreserved embryos every 5 generations.

Experimental Design

There is growing concern regarding the numerous contradictions in experimental findings, low reproducibility of results, poor predictability of animal models for human diseases, and the relative paucity of extrapolating preclinical experimental studies successfully to therapeutic targets in humans.[116,117] In recent years, several antiatherosclerotic agents have failed in clinical trials despite promising results in animal models. Poor design, execution, and reporting of preclinical research have been blamed. To optimize the predictive value of preclinical research, the AHA journals are endorsing a recent report from the National Institutes of Health on "Principles and Guidelines for Reporting Preclinical Research."[118] To facilitate interpretation and reproducibility of experimental atherosclerosis studies, it is highly recommended that the key standards for appropriate design, execution, and reporting of preclinical research be followed, as summarized in Table 1. Animal experiments require ethical considerations that differ in different institutes and different countries. Some of these ethical concerns can affect features of experimental design that could influence data interpretation and reproducibility. Other factors that should also be considered include the preanesthetic fasting and water deprivation interval, which can affect plasma profiles such as glucose and triglycerides, and constant pain and distress with known (some manipulations or procedures) or unknown causes, which can have potential effects on the development of atherosclerosis.

In this section, recommendations are provided for experimental design to execute atherosclerosis studies that will yield robust data. This will include suggestions on reporting to facilitate interpretation and reproducibility. Given that the vast predominance of literature uses mice as the model of atherosclerosis, the recommendations primarily focus on this species.

Genetic Background

The inbred background of mice has profound effects on atherosclerotic development. In fact, background differences were exploited in studies to identify genes responsible for development of lesions.[119–121] Marked effects of strain background have also been shown in apoE$^{-/-}$ mice. For example, lesion formation is dramatically less in C3H and FVB/N strains of apoE-deficient mice than in the C57BL/6 strain despite a similar magnitude of hypercholesterolemia.[121,122] The need to clearly define the source of the strain is illustrated by C57BL/6 mice, which have substrains both within a single vendor and between vendors.[123] The effect of strain difference has potential importance in studies in which atherosclerosis in mice with a single gene deletion is compared to lesions in mice with compound gene deficiency. Even after extensive backcrossing, there remains the potential for "passenger" genes to be present.[124] Therefore, an important issue in the development of compound deficient mice is ensuring strain equivalency with the control mice, because interpretation of an effect, when comparing compound and singly deficient mice, is contingent on strain equivalency. The impact of strain background engenders reservations in performing studies that

Table 1. Recommendations for a Core Set of Reporting Standards for Rigorous Study Design

Randomization
1. Animals should be assigned randomly to experimental groups, and the method of randomization should be reported.
2. Data should be collected and processed randomly or appropriately blocked.
Blinding
1. Allocation concealment: Investigators should be unaware of the group to which the next animal taken from a cage will be allocated.
2. Blinded conduct of the experiment: Animal caretakers and investigators conducting the experiments should be blinded to the allocation sequence.
3. Blinded assessment of outcome: Investigators assessing, measuring, or quantifying experimental outcomes should be blinded to the intervention.
Sample-size estimation
1. An appropriate sample size should be computed when the study is being designed and the statistical method of computation reported.
2. Statistical methods that take into account multiple evaluations of the data should be used when an interim evaluation is performed.
Data handling
1. Rules for terminating data collection should be defined in advance.
2. Criteria for inclusion and exclusion of data should be established prospectively.
3. Outliers should be defined and handled in a manner that should be decided when the experiment is being designed, and any data removed before analysis should be reported.
4. The primary end point should be prospectively selected. If multiple end points are to be assessed, then appropriate statistical corrections should be applied.
5. Investigators should report on data missing because of attrition or exclusion.
6. Pseudo replicate issues need to be considered during study design and analysis.
7. Investigators should report how often a particular experiment was performed and whether results were substantiated by repetition under a range of conditions.

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compare mice that are only cousin lines or using controls that are purchased.

Two major strategies can be used to ensure changes in lesion formation are attributable to a single gene when using compound–deficient mice. The most commonly used approach is to backcross into an equivalent strain. The most common background strain in atherosclerosis studies is C57BL/6. Using random breeding approaches, it is usual to backcross ≥10 times. The number of backcrosses can be reduced by using the technique of speed congenics, in which offspring from each mating are selected on the basis of the greatest genetic similarity to the target strain. Although this saves time to development of strain-equivalent mice, it is a significant technical barrier for many laboratories. However, such service is now offered on a contract basis at The Jackson Laboratory and Charles River Laboratories. The major disadvantage of the approach of backcrossing is the time and expense of generating the mice. An alternative approach is the breeding of atherosclerosis-susceptible mice from parental strains that are heterozygous for a gene of interest. The littermates from this mating strategy can be compared for atherosclerotic lesion characteristics. Although there is likely to be genetic variance within these groups, this should be random. Therefore, any difference in lesion size of littermates should be the consequence of the single gene. Confidence in the interpretation of such studies is assisted by the use of large group numbers and a large effect on atherosclerotic lesion formation. Although a specific number of mice cannot be provided, confidence in the reproducibility of a study would be enhanced by the statistical analysis not only representing the confidence level but also the power.

When transgenic mice are created with genetically hybrid embryos (eg, C57BL/6.FVB), the inserted transgene carries a block of genes from either FVB or C57BL/6 in linkage disequilibrium with the targeted locus, which could systematically bias the results.[124] There are several approaches to this problem. One is to create the transgenic strain using C57BL/6 ES cells or embryos, and another is to demonstrate reversal of the phenotype of interest using a rescue transgene. Additionally, emerging CRISPR technology offers the possibility of creating transgenic strains using genetically uniform embryos.

Sex

Sex can exert major effects on the outcome of atherosclerosis studies. Early studies demonstrated that atherosclerotic lesions in the aortic root were larger in female mice[125–127]; however, this observation has not been a consistent finding across studies or in analysis of arterial regions other than the aortic root.[128] More importantly, there are several examples of manipulations that have sex-specific effects. Examples of studies that have demonstrated a sex-specific difference include those that have manipulated the function of peroxisome proliferator-activator receptor-γ, lipoic acid synthase, and interferon-γ.[129–132] Considering the potential for sex differences in lesion size and pathological mechanisms, it is unclear whether there are distinct advantages for studying one sex over the other. Therefore, it is advisable to include sufficient mice of both sexes to permit sex-specific analysis of atherosclerosis. It is strongly recommended that reported data be segregated by sex.

Manipulations for Determining Progression Versus Regression

The vast majority of mouse atherosclerosis studies define the effects of a genetic manipulation or pharmacological intervention that is initiated before or concurrent with development of atherosclerotic lesions. This has provided valuable information on mechanisms of atherosclerosis initiation and propagation; however, it might not be relevant to treatment in the clinical scenario, which requires changes to existing lesions either to retard subsequent growth or even to promote regression. One of the practical issues in performing studies on regression mechanisms is the protracted interval needed for the completion of these studies. In addition, these studies require additional experimental groups to determine the change in lesions from time of intervention to end of study. Also, although pharmacological interventions are readily amenable to regression studies, the use of genetic manipulations is more limited. Fortunately, there are increasing options for influencing gene expression in a temporal manner for regression studies. One that has already been used is adenoviral vector–based expression.[133,134] Intravenous injection of these vectors leads to virtually exclusive expression in the liver, and hence, its use is most commonly restricted to making changes in peripheral mechanisms rather than directly at the level of the arterial wall. However, development of systems to control temporal and spatial expression of genes should provide mechanistic insight into lesion regression.[134]

We briefly review the most established mouse models currently used for regression studies, as well as some emerging ones.

Aortic Transplantation

This model was introduced in 2001[135] and revised in 2003.[136] In its simplest approach, lesions develop in atherosclerosis-susceptible mice (for example, $apoE^{-/-}$ or $Ldlr^{-/-}$), then aortic segments containing these plaques are transplanted into recipient mice with a regression-promoting environment (eg, a wild-type mouse of the same strain as the recipient). In this way, the systemic environment of lesions is changed rapidly, with an opportunity to sustain this new environment for as long as desired. In the current iteration, for example,[137] when aortic arches from $apoE^{-/-}$ mice fed a Western-type diet for 16 weeks to develop complex lesions were transplanted into wild-type recipients, rapid (<1 week) changes in content and phenotypic state of cells positive for CD68 (a macrophage marker) were apparent. To assess effects after the change in lesion environment, mice treated identically as donor mice are needed to provide baseline data, and transplants from a donor mouse into the same type of mouse (eg, $apoE^{-/-}$ to $apoE^{-/-}$) are necessary to control for the surgical procedures. Although the aortic transplantation model is relatively cumbersome, it has a number of advantages. For example, as noted above, changes in lesions are typically rapid. Also, the nature of the donor or recipient mice can be varied by genetic alterations, bone marrow transplantation, adenovirus, antisense oligonucleotide, or small-molecule therapies. Finally, by using

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the mismatch in the leukocyte marker CD45 (CD45.1 versus CD45.2), trafficking of cells into and out of lesions can be readily accomplished.[137,138]

Reversa Mouse

This model was introduced originally in 2003 as a model of delayed progression of atherosclerosis[139] and was later adapted for a study of regression in 2011.[140] It is based on an Ldlr$^{-/-}$ platform but has been genetically manipulated to express only apoB100 and to be homozygous for a conditional allele for *Mttp* (the gene for microsomal triglyceride transfer protein) and the inducible *Mx1-Cre* transgene. Thus, before the deletion of the *Mttp* gene, feeding the mice a Western-type diet results in hypercholesterolemia and atherosclerosis progression, but after the injection of polyinosinic-polycytidylic acid, which induces *Mx1-Cre* recombinase expression, VLDL (and hence, LDL) production from liver is reduced. In the ensuing weeks, lesion regression occurs. One major disadvantage of this model is the relative inconvenience of introducing new factors genetically by crossbreeding, because a number of alleles that would have to be reconstituted to homozygosity. In some cases, however, when only changes in myeloid-derived cells are of interest, bone marrow transplantation can be used to circumvent this issue.

Viral-Based Expression Systems

Since the late 1990s, a number of gene therapy approaches based on adenoviral or adenovirus-associated vectors have been used. These genetic therapies include expression of apoE in apoE$^{-/-}$ mice,[133,141] apoAI in Ldlr$^{-/-}$ mice,[117] and LDL or VLDL receptors in Ldlr$^{-/-}$ mice, respectively.[142,143] Two problems with the early viral vectors were the limited duration and amplitude of expression of the candidate gene, but progress in viral vectors has greatly improved performance in these 2 areas.[144] One major benefit of this approach is that a gene of interest can be introduced without the need for crossbreeding; however, a limitation is that the regression factor must be effective on predominately hepatic expression.

PCSK9 regulates LDL receptor homeostasis.[145–147] Gain-of-function mutations of PCSK9 lead to hypercholesterolemia.[148,149] Therefore, an adenovirus-associated vector technique has been applied to introduce a gain-of-function mutation of human PCSK9D374Y or its mouse equivalent, D377Y, in mice,[150–153] which results in profound increases of plasma cholesterol concentration with lipoprotein distribution comparable to that of Ldlr$^{-/-}$ mice fed a Western diet. This approach is rapid and efficient and mimics the Ldlr-deficient condition, augmenting atherosclerosis within a short period in C57BL/6 mice.[150–152,154] However, the C57BL/6 mouse strain, but not other normolipidemic mouse strains such as FVB, 129, and BALB/C, responds to enhanced expression of PCSK9 activity.[151,152] There is also evidence that PCSK9 has effects independent of LDL receptor regulation.[155] Therefore, use of an adenovirus-associated vector approach for enhancing PCSK9 activity should take into account both the mouse strain and potential LDL receptor–independent effects.[156,157]

Pharmacological Approaches

Approaches that have been adopted to decrease hypercholesterolemia in humans can also be adapted to mouse studies. These include use of MTP inhibitors or apoB antisense oligonucleotides.[158,159]

Duration

Atherosclerosis studies require prolonged housing of mice and are labor intensive. Consequently, the vast majority of studies are performed with lesions measured at a single interval defined by mouse age or duration of the atherogenic stimulus. This is commonly in the 10- to 12-week range. If the extent of disease being studied that has been caused by a manipulation is either unaltered or consistently changed, a single time point will provide an accurate evaluation; however, it is possible that the effect of the manipulation is only transient. There are examples of transient effects on atherosclerosis. For example, endothelium-specific overexpression of 15-lipoxygenase[160] and a deficiency of mature lymphocytes[161] led to transient increases and decreases in lesion size, respectively, that were not sustained. There is also the potential for a manipulation to only affect lesions at later stages of the disease process or to have opposite effects in early and late lesions. For example, genetic mutations that increase macrophage apoptosis are associated with decreased lesion area and cellularity in early lesions because of efficient phagocytosis of apoptotic macrophages by healthy macrophages (efferocytosis), whereas in advanced lesions, the failure of efferocytosis can lead to postapoptotic necrosis and increased lesional necrotic core formation.[162] Therefore, an early versus a late-stage evaluation would determine differences missed in either stage. Although no clear guidelines can be provided for the determination of a drug or genetic effect compared with control groups, it would be beneficial to determine atherosclerosis at >1 interval.

Diet

Dietary manipulations are used commonly to induce hypercholesterolemia and atherosclerosis in a spectrum of animals. For all species, diets used to generate lesions have enhanced content of saturated fat, cholesterol, or both.[37,72,163,164]

Before the development of genetically manipulated atherosclerosis-susceptible mice, a diet was developed to induce modest hypercholesterolemia in a range of mouse strains. The diet used in initial studies was enriched in saturated fat (21% wt/wt), cholesterol (1.25%), and cholate (0.5%), and it is colloquially referred to as the Paigen diet.[165] This study showed that the induction of inflammatory gene expression was primarily associated with the very high cholesterol content of the diet, whereas cholate was associated with fibrotic gene expression. Although the use of cholate is controversial, its inclusion is not needed to produce hypercholesterolemia in genetically manipulated mice.[166] Overall, there have not been extensive systematic studies examining the role of different fats and cholesterol contents on development of atherosclerosis in the most commonly used mouse models of atherosclerosis. Therefore, dietary decisions are often based on empirical considerations. The most commonly used diet is enriched in saturated fat to 21% wt/wt, with cholesterol of 0.2 % wt/wt. This diet has become colloquially known as the Western diet, given that it approximately mimics the average dietary composition consumed by humans in the Western hemisphere.

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Genetic manipulation of some mouse strains can eliminate the need for additional stimuli for lesion formation. For example, apoE−/− mice are spontaneously hypercholesterolemic and do not need further manipulation for lesion development. However, different diets have been used to accelerate lesion formation, particularly those enriched in saturated fat and cholesterol. By gross pathology, the inclusion of saturated fat and cholesterol in the diet has been shown to promote the same atherogenic process as occurs during the feeding of a normal laboratory diet.[38]

Because dietary constituents have a profound effect on atherosclerosis, reporting of the sources and catalogue number of the diet is required. Because most diets are formulated from natural ingredients, they are likely to have batch-to-batch variation. Unfortunately, this is not a variable that can be controlled, and this encourages the use of the same batch in the execution of a single experimental study. Finally, the information provided should include whether the diets are irradiated or not, because this could affect the fatty acid or microbial composition of the diet. A potential mode of minimizing the dietary effects of batch variations is to use semipurified diets to induce hypercholesterolemia.[167]

For consideration of the effects of variations in diets on atherosclerosis in rabbits, the systematic studies of Kritchevsky and colleagues, who have provided data on the effects of fats, protein, and carbohydrates,[168,169] should be consulted.

Numbers per Group

Determination of appropriate group sizes for a statistically reliable study requires knowledge of the variance of the control groups and the predicted change of a manipulation. Data from other laboratories can be a poor gauge for estimation of the extent and variance of lesion size, because there is considerable variance among laboratories. If there are no preliminary data or previous experience, data from other laboratories can be helpful to determine the group size for an initial study. Typically, a group size of 10 to 20 is found in publications of studies. Although most atherosclerosis studies are performed on inbred mice in controlled environments with standardized diets, there is usually high variability in the extent of atherosclerosis formed. This is apparent in publications that provide data on atherosclerotic lesion size for individual mice. Furthermore, the distribution of lesion areas from individual mice within a group frequently is not gaussian, and this impacts the statistical test that can be applied. This could require the use of nonparametric statistical tests, which are generally less sensitive in determining significant differences among groups. For group size estimations to enable statistically reliable results, it is recommended that a biostatistician be consulted for complex groups that involve multiple factor considerations or if you are not familiar with these calculations.

Vivarium Conditions

Although not well documented, environmental conditions have major effects on atherosclerosis development. For example, differences in atherosclerotic lesion size have been associated with the health status of vivarium housing.[170] Also, differences in atherosclerosis development in response to gene deletion have been noted in mice housed in barrier versus conventional facilities. However, housing effects are ill-defined, and it is not possible to attribute the effects to any specific variable. Some of the potential variables include barrier versus nonbarrier facility, housing density, bedding material, water source, noise-induced stress, and light/dark cycle. Reporting of these variables is consistent with the ARRIVE guidelines (Animals in Research: Reporting In Vivo Experiments).[116] An emerging consideration is the temperature of the vivarium, which has been demonstrated to alter the development of mouse atherosclerosis.[171,172] Mice are usually housed in rooms that are ~20°C, whereas their thermoneutrality is ~30°C. Although maintaining mice under thermoneutral conditions is not usually practical, these recent publications highlight the need to monitor room temperatures throughout the course of the study.

Gut microbiota composition has been shown to influence atherosclerosis development in mice,[173,174] and this is dependent on environmental factors. If mice are imported into a vivarium from a vendor, the microbiome will likely change over time and might not reach a steady state for some generations. In addition, the impact of a genetic intervention can result in a phenotypic difference that is caused by changes in the microbiome rather than in the host per se. The totality of the host genome and the microbiome is referred to as the metagenome and must be taken into account in the analysis of experimental data.[175] Because mice eat each other's feces, one practical way to deal with this issue is to cohouse mice of all groups included in the experimental design. For example, cohousing littermate mice that do or do not contain an induced mutation would be a simple way to control for both host genetic background differences and differences in the microbiome.

Bone Marrow Transplantation

Before the extensive use of cell-specific genetic approaches, bone marrow transplantation was a common approach for studying effects of genes in leukocytes on the development of atherosclerosis.[41] As noted above, repopulation of irradiated apoE$^{-/-}$ mice with cells from apoE$^{+/+}$ donors normalizes plasma cholesterol concentrations and ablates atherosclerotic lesion formation.[41,42] In contrast, the LDL receptor phenotype of donor cells does not significantly impact the size of atherosclerotic lesions, except under modest hypercholesterolemic conditions.[176–178] Therefore, it is not necessary to take the complex step of developing compound deficient mice for donors. Although the LDL receptor genotype of donor cells has no documented effects in LDL receptor–deficient recipients, the bone marrow cell LDL receptor genotype affects lesion size in C57BL/6 mice.[177,178] An interpretative issue is the effect of the bone marrow transplantation procedure on lesion development. One study that directly compared the effects of irradiation in mice noted a site-specific effect on lesion formation.[179] Mice irradiated and repopulated with donor stem cells had decreased lesion sizes in the thoracic aorta compared with mice that did not undergo the procedure. In the aortic sinus, the converse occurred, with lesions being smaller in mice subjected to the transplantation procedure. In addition to the effects of the procedure influencing lesion size, the procedure

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also changes lesion composition. Currently, there is no insight into mechanisms of this procedural effect and how this can be interpreted in defining atherogenic mechanisms.

A major interpretative issue has arisen in defining cell types within atherosclerotic lesions that are repopulated by transplanted bone marrow cells. Bone marrow–derived cells can be differentiated into myeloid and lymphoid lines. Initial studies usually interpreted their results in terms of macrophage metabolism, largely based on the predominant cell type in mouse atherosclerotic lesions being of myeloid origin. However, in addition to macrophages, circulating platelets, lymphocytes, and other cell types are present in lesions and can contribute to the development of atherosclerosis. There are also studies reporting that smooth muscle cells and endothelial cells in atherosclerotic lesions are derived from hematopoietic precursors,[180,181] but this hypothesis has been refuted.[24,182,183]

Methodology

Plasma Constituents

Many biological molecules have been measured in plasma and serum in experimental atherosclerosis studies. The focus of this statement is to provide recommendations on plasma constituents in the interpretation of atherosclerosis studies. In this regard, an exclusive requirement of such studies is the need to induce some form of dyslipidemia. Therefore, interpretation of data requires inclusion of plasma cholesterol concentrations, especially under circumstances in which plasma cholesterol concentrations are altered. Mechanistic interpretation of lesion formation is enhanced by determination of the major lipoprotein fractions that transport cholesterol.

Plasma Cholesterol Concentrations

Current total plasma cholesterol and triglyceride assays are colorimetric assays that are rapid and relatively cheap. Most of the assays use small volumes of plasma (1 to 10 μL). These measurements, combined with plasma lipoprotein cholesterol concentrations, are a major determinant of atherosclerotic lesion size.[184] Therefore, inclusion of this measurement is critical for interpretation. Given the simplicity of the assay and the small volume of plasma or serum needed for measurement, there is an expectation that plasma cholesterol concentrations will be obtained from every study animal.

Lipoprotein Quantification and Characterization

Lipoprotein measurements are most commonly performed in 3 different modes. Lipoproteins are classically defined by flotation characteristics of plasma and serum that are subjected to ultracentrifugation.[185] Although ultracentrifugal resolution of lipoprotein fractions has been considered the "gold standard" previously, it is time consuming. It is more difficult to use this technique when only small amounts of plasma or serum are available, which is common in mouse studies.

A second technique is the use of precipitation, which under specific conditions provides a measurement of HDL cholesterol concentrations and apoB-containing lipoproteins. This involves measurement of total cholesterol before and after incubation of the plasma with materials such as heparin/manganese or dextran sulfate. The accuracy of this measurement relies on the complete precipitation of apoB-containing lipoproteins, which might not occur in plasma that is highly hyperlipidemic, as is common in experimental animal atherosclerosis studies. Hence, it is important to validate the assay for completeness of apoB precipitation in experimental studies.

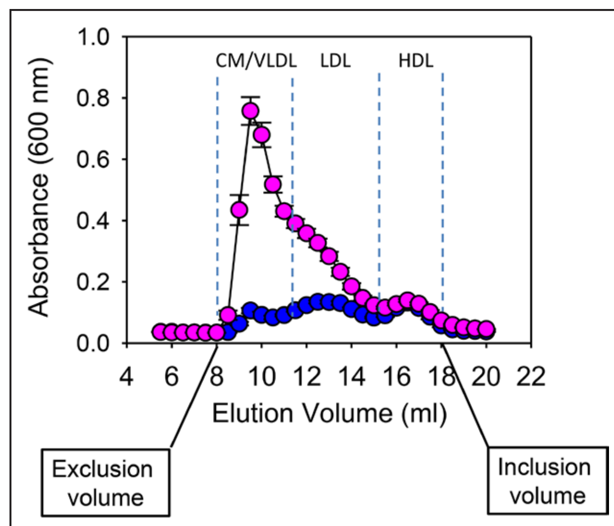

Figure 1. Analysis of lipoprotein-cholesterol distribution by size-exclusion chromatography. In the example shown in this Figure, profiles were derived from resolution of 50 μL of serum by a single Superose 6 column eluted at 0.5 mL/min. These data are derived from analysis of groups of low-density lipoprotein (LDL) receptor–deficient mice (4 per group) fed either a normal laboratory diet (blue circles) or a saturated fat–enriched diet (cyan circles) to illustrate examples of modest and profound hypercholesterolemia. Chylomicrons (CM)/chylomicron remnants and very low-density lipoprotein (VLDL) particles both elute at the exclusion volume and cannot be resolved by Superose columns. High-density lipoprotein (HDL) particles elute at the inclusion volume. Further resolution of HDL particles can be achieved by elution through a Superose 12 column connected in tandem with a Superose 6 column. Baseline resolution for cholesterol of different lipoprotein fractions is not commonly achieved by size-exclusion chromatography. The lack of baseline separation engenders the need for devolution analysis to provide informative cholesterol concentrations in each designated lipoprotein fraction.

The third commonly used technique is size-exclusion chromatography, such as fast protein liquid chromatography (Figure 1). This method is able to partially separate VLDL, LDL, and HDL fractions and can be performed with relatively small amounts of plasma (≤50 μL). Under some conditions in mice, a large apoE-rich HDL particle can appear in the LDL fraction, which requires additional studies for interpretation. For any firm conclusion to be drawn on lipoprotein cholesterol distribution, it is recommended that this analysis be performed in a mode that accounts for biological and technical variability. A single determination for a group using a pooled plasma sample provides little or potentially inaccurate information on either of these variables, and it is recommended that fast protein liquid chromatography be performed on plasma samples from individual mice in each group. Size-exclusion chromatography seldom provides baseline separation of major lipoprotein fractions; therefore, determination of the cholesterol content in major lipoprotein fractions might require the

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application of software that deconvolutes the profile into individual components.[186]

Measurement of Atherosclerotic Lesions

Aortic root is the most common region for atherosclerosis quantification, as reported in the literature. Historically, this was the only area in which atherosclerosis was consistently present in all pertinent models. With the advent of genetically manipulated mice, atherosclerotic lesion development occurs in other vascular areas. The use of en face analysis of lesions on the intimal surface of the aorta is convenient,[127] as routinely performed in larger-animal models of atherosclerosis.[74,187] Technical details of the quantification of lesions in these regions are provided elsewhere.[188–190] Table 2 provides recommendations for measurement of atherosclerotic lesions.

Table 2. Recommendations for Measurement of Atherosclerotic Lesions

1. Quantification of lesion sizes in tissue sections should be based on multiple sections that account for variable thickness along the artery.
2. It is preferable to measure lesion size in multiple regions. However, because the mechanism of lesion development could be region specific, this is not an absolute requirement.
3. Because lesion development can be region specific, the measurement of lesion size and tissue characteristics must be performed in the same region.
4. Measurement of plasma lipids is required to enable interpretation.

Selection of Vascular Beds

A major decision in developing an experimental design is whether atherosclerotic lesions in ≥1 vascular beds should be quantified. Because atherosclerosis can develop nonuniformly in different vascular regions,[191–193] it can be informative to obtain data in several atherosclerosis-prone regions. If an intervention exhibits similar effects on lesion size in all regions quantified, this offers a straightforward interpretation of its effect on atherogenesis. Divergence in the effects of a manipulation on lesion sizes in different vascular beds provides challenges for interpretation. There is a good overall correlation between lesion area measured in the aortic root and the whole aorta in $Ldlr^{-/-}$ and $apoE^{-/-}$ mice with advanced lesions, which suggests common pathogenic mechanisms.[127] There are also several examples in which interventions have no effects on lesion size in the aortic root but do have effects on lesion size by the en face assay, and vice versa.[192–194] Thus, measurements of lesion size by both methods can be informative in mice with more advanced lesions.

The issue of relating the mechanisms of atherosclerosis in mice to those that occur in humans is unclear. Regions in which atherosclerosis is commonly quantified in mice are not those responsible for overt cardiovascular disease in humans. In fact, great vessels of the coronary circulation and branches of carotid arteries responsible for heart disease and stroke, respectively, in humans, are not disease-prone sites in mice.[195] For example, lesions found in the coronary arteries of older $apoE^{-/-}$ mice fed a normal laboratory diet are extensions of lesions originating in the aortic sinus.[195] The basis for differences of lesion location between mice and humans remains to be defined.

Tissue Sectioning of Aortic Root and Innominate Artery

Measurement of atherosclerotic lesion size was initially described in detail by Paigen and colleagues.[188] Briefly, this method entails sequential tissue sectioning of the aortic root, namely, from the origin of the aortic valves to the ascending aorta. There is considerable regional difference in size of lesions in the aortic root[188]; therefore, it is recommended that measurement of lesion size be performed throughout the aortic root. This can be achieved by acquisition of sequential sections throughout the region. For example, cutting sections (usually ~10 µm if using a cryostat) with a sequential arrangement of slides provides sections throughout the region at ~80- to 100-µm intervals on a single slide. This is accomplished by placing the initial section in the lower left corner on each of multiple slides. Sequential sections are then placed serially on these multiple slides. This placement strategy for each slide creates serial sections of the aortic root (Figure 2).

Lesions can be easily visualized with several types of staining. Lesion size can be quantified by image analysis software, preferably by 2 individuals who are blinded to the experimental design and each other's results. Area is defined by the internal elastic lamina to the luminal edge of the lesion. This can enable automated measurements if staining permits distinctive boundaries. However, automated quantification of lesion size is compromised by the inability of a stain to uniformly cover the entire area of the lesion. For example, Oil Red O staining usually provides inconsistent staining within a lesion. This might be expected for a stain that only visualizes the neutral lipid component of lesions and does not account for regions of tissue sections occupied by constituents such as extracellular matrix and unesterified cholesterol. Another common mode of visualizing atherosclerotic lesions is immunostaining; however, immunostaining is also unlikely to consistently define the margins of lesions. Simple histological stains, such as hematoxylin and eosin or Verhoeff van Gieson, are an effective means of distinguishing the lesion boundaries of the internal elastin lamina and the lumen surface.

There is no agreement on the number of sections that need to be measured for authentic quantification of lesion size. Because lesion sizes vary throughout the aortic root, any measurement needs to procure tissue from the equivalent region in the root. The mode of analysis described above measures the lesion area of serial sections throughout the aortic root.[189] One potential mode of orientating sections across mice is the use of the first appearance of the valve leaflets as a reference point; however, the initial appearance of the aortic valves can be an imprecise reference point. Alternatively, sections can be orientated relative to the disappearance of the aortic valve cusps to represent lesion area throughout the root.[196] The definition of lesion size throughout the aortic root is preferable, to prevent data being inadvertently prejudiced by selection of sections that might come from different regions of the root. Overall, quantification of lesions in the aortic root is a commonly used method in mice. One of the major drawbacks of this technique is the technical skill and time required to acquire serial sections throughout the entire aortic root.

Another preferred site for lesion development in mice is the innominate artery (also called the brachiocephalic artery). It is the first arterial branch in the aortic arch that extends to

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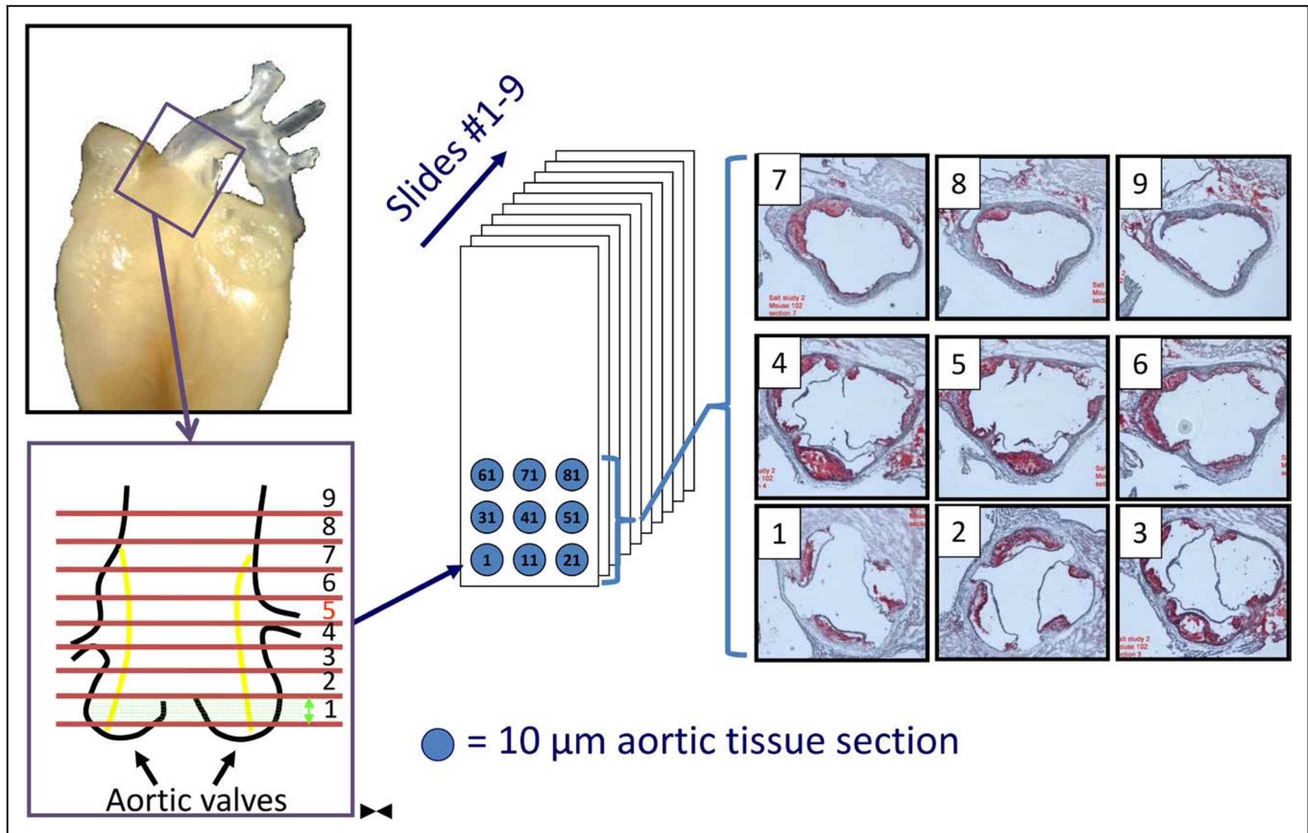

Figure 2. A recommended scheme for acquisition of tissue sections throughout atherosclerosis-susceptible region of the aortic root and ascending aorta. Tissue sections (10 µm thickness) are acquired from the initial appearance of the aortic valves. Serial sections are distributed between consecutive slides. The number of sections will increase with increased atherosclerosis. This mode of analysis creates multiple slides containing adjacent aortic tissue sections that are separated by 100 µm. This enables measurements throughout the entire region that contains lesions, in which area varies by location.

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the right carotid artery. Measurable lesions develop at this site in normal laboratory diet–fed apoE$^{-/-}$ mice at 16 to 20 weeks of age. In older apoE$^{-/-}$ and Ldlr$^{-/-}$ mice, lesions in the innominate artery progress to relatively advanced lesions containing large areas of necrosis, fibrosis, and calcification.[197–200] Very advanced lesions in the innominate artery often contain intraplaque hemorrhage as a result of fissures along the lateral margins of the plaques.[197] A drawback to evaluating lesions in the innominate artery is the very small size of this artery (generally <1 mm) and the difficulty with dissection and proper orientation while embedding. It is recommended that serial cross sections through the entire innominate artery be generated and that several sections be measured.

En Face Technique

Briefly, this technique involves removal of the entire length of aorta from heart to iliac bifurcation, and adventitial tissue is removed. The intimal surface is exposed by a longitudinal cut through the inner curvature and down the anterior aspect of the aorta. A cut is also made through the greater curvature of the aortic arch to the subclavian branch. One issue that requires careful attention in mouse studies is that atherosclerotic lesions can be easily displaced during tissue processing. This is particularly the case for arterial tissues extracted from mice in which bone marrow transplantation has been performed. Lesion dislodgment occurs primarily during handling of tissues, such as during the process of removing adventitia and cutting it open to expose the intimal surface. Therefore, it is preferable to use a dissecting microscope. The tissue is then pinned on a dark surface. If lesions are sufficiently mature, they are clearly visible without staining. Lesions can also be visualized after neutral lipid staining. This offers a limited advantage in large lesions but can aid in the visualization of small lesions (Figure 3).

Measurements of the surface area of atherosclerotic lesions can be acquired by manual tracing using imaging analysis software and are usually represented as percent lesion area of the total intimal surface area. Image analysis software also has the potential to automate measurements of lesion area; however, the parameters used by the software to acquire measurements need to be stated in detail. For example, if the presence of lesions is defined by a threshold of color, there should be a description of how the threshold was determined and standardized. Also, if the tissue stained for neutral lipid, the acquisition needs to be cognizant of the potential confounding staining of any adventitial adipose that was not removed during tissue processing.

Because this requires some arbitrary decisions by the observer concerning lesion boundaries, it is preferable for at least 2 individuals to quantify lesions independently. Even high-quality image capture equipment frequently produces some ambiguity in visualization of lesions. Therefore, it is recommended to observe the pinned aorta under a dissecting

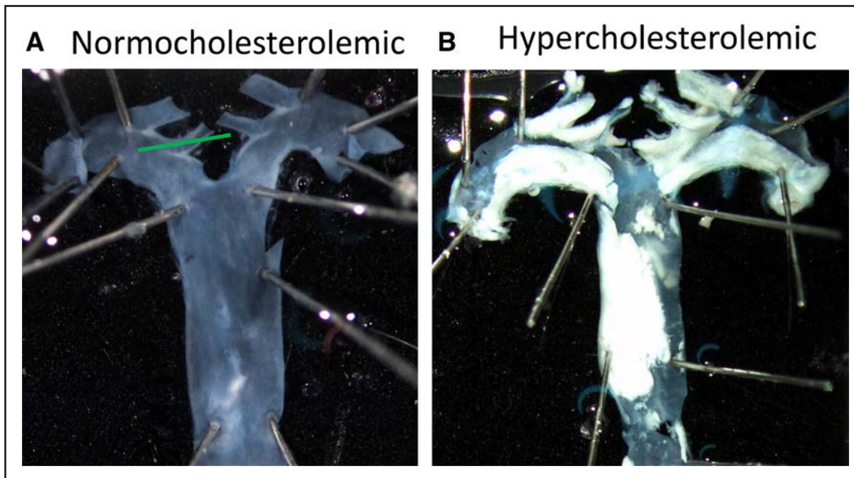

Figure 3. Representation of a mouse aorta arranged for optimal measurement of lesion area by en face analysis. Examples are shown of aortic arches from **(A)** a normolipidemic mouse in which no atherosclerosis is present and **(B)** a hypercholesterolemic mouse with extensive atherosclerotic lesions that are readily discernable without staining. Atherosclerotic lesions are usually largest on the inner curvature and at the branches of the innominate and left carotid arteries.

microscope during the analysis of images to ensure that only lesions are being outlined.

Lesion size is routinely represented as absolute or percent lesion area of the entire intimal surface area. The meaningful interpretation of this requires that a standardized area of intima is quantified. Therefore, care should be taken to ensure consistency in dissecting, opening, and pinning aortas. There also should be consistency in defining the intimal region. Generally, lesions initially develop in the ascending and arch regions and are only present in measurable amounts in other aortic regions in animals with more advanced disease. Therefore, sparse lesion presence in the thoracic and abdominal aorta might preclude the acquisition of any meaningful lesion measurement in these regions.

Measurement of lesions by this approach provides a 2-dimensional surface area without taking into account the thickness of lesions. We have noted previously that lesion area was not a good indicator of lesion burden, in which thickness and volume should be considered.[201] Lesion thickness can be evaluated by histological sectioning, although this would be time consuming.

Noninvasive Imaging

Multiple imaging systems have been used to quantify experimental atherosclerosis size or to characterize components of lesions. Experimental atherosclerosis has been quantified and characterized by several modalities, including ultrasound,[202] magnetic resonance imaging,[203,204] and positron emission tomography, sometimes in combination with computed tomography.[205,206] There have also been applications to experimental atherosclerosis using multimodality imaging such as positron emission tomography/magnetic resonance imaging.[207] Although noninvasive imaging holds promise, the resolution of these modalities provides a barrier to accurately determining lesion size in experimental atherosclerosis, particularly in mice. Therefore, although development of noninvasive measurement should be encouraged,[208] current imaging systems do not provide reliable acquisition of lesion size and characteristics for mouse atherosclerotic lesions in vivo.

Cellular and Biochemical Constituents

Clinical evaluation of human atherosclerosis has provided evidence that the size of an atherosclerotic lesion is not the primary factor defining the overt clinical consequence of the disease.[7,209] Instead, there is a consensus that the majority of cases of acute clinical manifestation of atherosclerosis are attributed to thrombosis induced by rupture. The propensity to rupture is based on structural and chemical characteristics of lesions that are independent of size. Hence, there is now a greater emphasis on providing composition analysis of experimental atherosclerotic lesions.

Tissue Sterol Content

The sterol content of atherosclerosis can provide mechanistic insight into the response to therapeutic approaches. For example, intra-arterial deposition of sterols can form complex structures both extracellularly and intracellularly.[210,211] In addition to providing an alternative index of atherosclerosis severity, the ratio of unesterified to esterified cholesterol can provide insight into the mode of lipoprotein delivery and processing within the arterial wall.[212–215]

The sterol content of atherosclerotic lesions can provide insight that complements pathological analysis. For analysis of tissue sections, 3-dimensional reconstructions have been reported but are not commonly used because this technique is labor intensive. For atherosclerotic lesions that are predominantly lipid laden, measurement of sterols can be more informative under conditions in which area measurements are not changed but lesions are markedly thicker.[201]

One issue in the consideration of tissue sterol measurement is the mode of normalization. In larger animals, it has been common to normalize to wet weight of tissue.[201] In small-animal models, such as mouse, the very small size of tissue can render difficulties in accuracy of determining a wet weight of aortic tissue (the most common area used). It is recommended that sterol measurements be normalized to either intimal area or total protein concentrations.

Composition of Atherosclerotic Lesions

Atherosclerotic lesions are complex and heterogeneous. Although whole tissue analyses can provide information on the presence of specific biochemical entities, in the absence of information on spatial distribution, this measurement provides limited insight into the disease process. Therefore, compositional analysis of tissue sections is a common and desirable feature of experimental atherosclerosis studies. Historically,

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Table 3. Recommendations for Compositional Analysis of Atherosclerotic Lesions

1. Validation of authenticity of antibody reactivity needs to be provided.
2. Because atherosclerotic lesions are heterogeneous, selection criteria for tissue section analysis need to be stated. Recommendations are to determine multiple sections per lesion.
3. Quantitation of immunostaining must include complete details on the mode of data capture.
4. Compositional data should be referred to by objective descriptors and not by inferential descriptors (eg, *vulnerable* or *unstable*).
5. Considering phenotypic plasticity, simultaneous use of multiple cell type–specific markers enhances the accuracy of data acquisition.

these pathological techniques have been reported primarily with images of representative examples of staining. More recently, techniques based on image analysis software have been applied to provide quantitative information from tissue staining techniques. In this section, recommendations will be provided on the rigor that should be applied to the staining of tissues and reporting of this form of analysis to enable reproducibility and interpretation (Table 3).

Histological Staining

The composition of atherosclerotic lesions can be evaluated in a variety of ways and is fundamental in defining the stages and properties of the disease process. One common example is defining lesion composition relative to the potential for rupture. Although this is not appropriate with mouse lesions that do not rupture to the extent that they induce occlusive thrombosis, this has become a widespread practice and is based on the presence or absence of macrophages, large necrotic areas, encapsulation of necrotic areas, and connective tissue as a measure of fibrosis. The presence of connective tissues such as collagen, elastin, and proteoglycans can be evaluated with histological stains such as Verhoeff–Van Gieson, Masson trichrome, or modified Movat's, which provide color contrasts between different connective tissue components. However, quantifying the amount of a particular connective tissue protein on the basis of histological staining is difficult and often subjective. This is also the case when measuring the thickness of fibrous caps and the size of necrotic zones. Therefore, a simple quantitative approach is to set a series of parameters for defining the lesion simply on the basis of the presence or absence of different components. Examples of these parameters include the following:

1. Presence of a large necrotic core, defined as being >50% of the volume of the lesion. A necrotic core is often visualized as an empty area where lipid has been extracted or an area devoid of intact cells with cellular debris, cholesterol crystals, and calcification (may require calcium- or phosphate-specific stains, such as Von Kossa stain or Alizarin red).
2. Presence of a thick or thin fibrous cap. A fibrous cap in mouse lesions is often difficult to define and may simply be a thin layer of connective tissue overlaying a necrotic zone rather than concentric layers of cells within a connective tissue matrix.
3. Presence of intraplaque thrombus or hemorrhage. Hemorrhage is easy to recognize on the basis of the presence of intact red blood cells. However, red blood cells often are not intact, thus requiring additional staining for hemoglobin (Dunn Thompson) or iron (Perls' Prussian Blue iron). The presence of an intraplaque thrombus is based on demonstration of the presence of fibrin/fibrinogen, which stains pink with hematoxylin and eosin or blue with Weigert Gram stain. However, there is no definitive way to demonstrate presence of thrombus except at the ultrastructural level, where platelets and fibrin strands can be resolved. Even immunostaining with antibodies cannot distinguish between fibrin and fibrinogen.
4. Predominance of aggregates of intact foam cells.
5. Highly fibrotic lesion (absence of cells and necrotic zones).
6. Highly calcified lesion (containing multiple or contiguous areas of calcification). With this approach, serial sections can be evaluated for the frequency with which each of these parameters occurs, and mean values for the entire lesion can be calculated and used as the basis of comparison among groups.

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Immunostaining

Controls for Immunostaining. Immunostaining is a common technique to define presence and spatial distribution of antigens within atherosclerotic lesions. This technique assumes that under controlled conditions, any positive color development after immunostaining represents identification of the antigen to which the antibody was developed. Although this technique can be highly informative, it might also provide confounding data because of nonspecific staining (Figure 4). A commonly used tissue immunostaining format is to identify the area of positive staining after incubation with a biotinylated secondary antibody, an avidin-biotin amplification complex, and chromogen. All these steps have the potential for nonspecific binding.[216] Fortunately, the extent of nonspecific binding of these components is easy to define by removal of the primary antibody or both the primary and secondary antibodies in the incubation scheme. For tissue immunostaining using fluorescent-labeled antibodies, a required control is a tissue section not exposed to antibody and acquired under identical conditions as a staining tissue. This step determines the contribution of autofluorescence.

The basis for the nonspecificity of antibodies used to stain tissues has not been defined, but it does not appear to be related to Fc interactions with receptors.[217] The potential for nonspecific interactions of primary antibodies is a recognized concern in immunostaining tissue. Unfortunately, there are no standard protocols in this technique to determine whether a primary antibody is reacting exclusively with the antigen.[216] However, there is increasing awareness that apparent immunostaining does not necessarily represent authentic reactivity of many antibodies, even those that have been used in many publications.[218,219] To comply with National Institutes of Health guidelines, there should be some validation of the specificity of antibody reactivity.

There are several controls for determining the specificity of a primary antibody.[216] These include controls using tissue sections from antigen of interest-deficient mice, preabsorption with the immunogen, and colocalization of 2 different antibodies to the same antigen (preferable for 2 epitopes

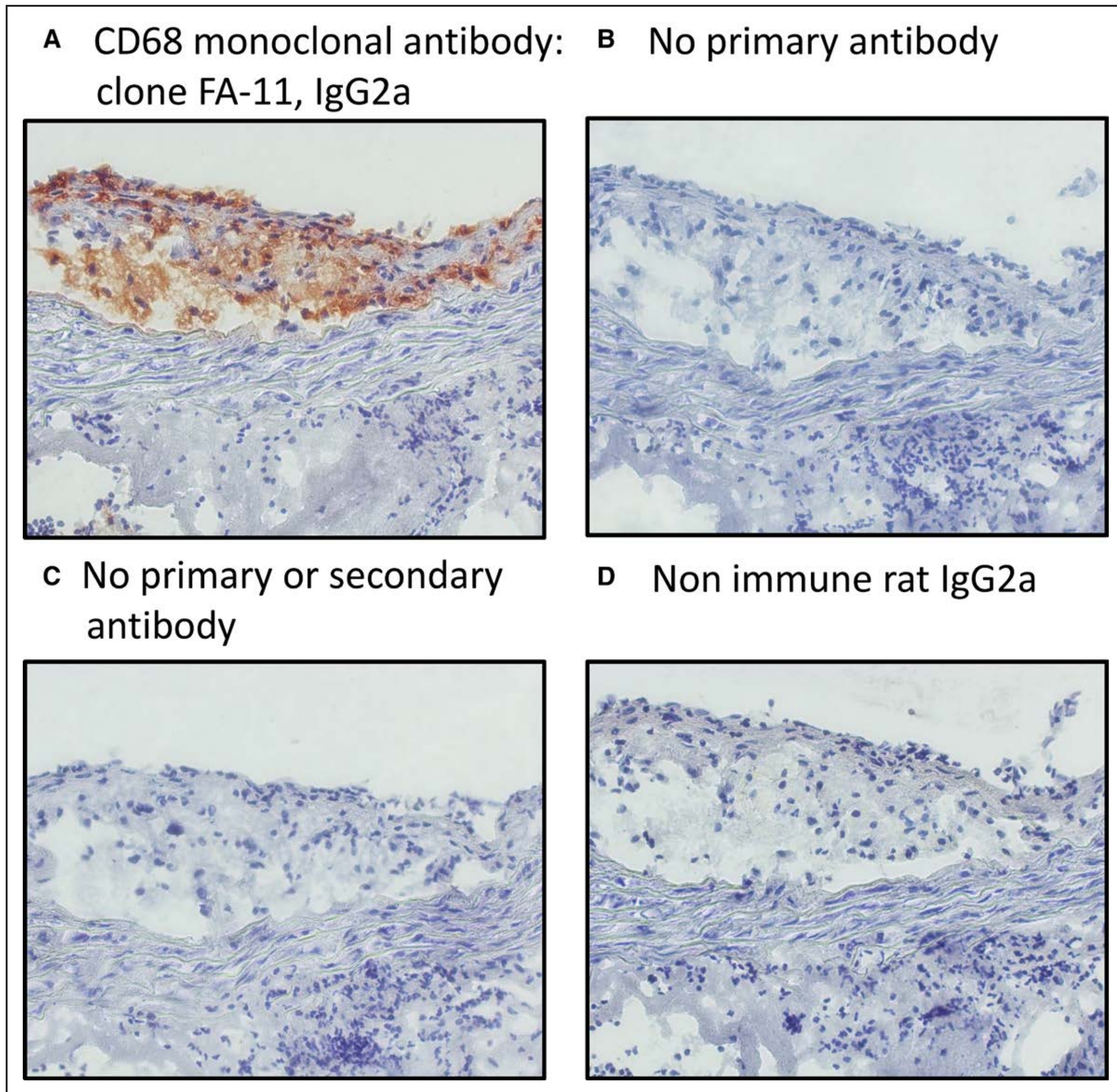

Figure 4. Appropriate controls for determining specificity of immunostaining of atherosclerotic tissue. Given the increased awareness of the potential for immunohistochemical techniques to produce nonspecific staining, there is a need for increased rigor to demonstrate authentic reaction of the antibody with the antigen. This image represents minimal controls for immunostaining of CD68 in mouse atherosclerotic tissue **(A)**, with controls of no primary antibody **(B)**, with no primary and secondary antibodies **(C)**, and incubated with an isotype match nonimmune antibody **(D)** at the same concentrations as used for the CD68 antibody. All controls were performed concurrently on adjacent tissue sections from the same mouse.

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within the antigen). For monoclonal antibodies, an isotype control (usually IgG1, IgG2A, or IgG2B) can be substituted for the antigen-targeted antibody to determine nonspecificity. For antibodies harvested from animals, nonimmune serum or IgG is often used. In the development of an affinity-purified antibody, the flow through the purification column can also serve as a negative control antibody. This permits comparison of immunostaining with 2 antibodies (specific versus nonspecific) from the same source. Another ideal control is to use tissue sections from a mouse with genetic deletion of the protein of interest. However, some genetic manipulations result in truncated proteins; therefore, meaningful interpretation requires knowledge of the antigen location within the protein, with a determination of whether this epitope exists in the truncated protein.

Data reports should include the source of the antibody and include catalogue number, because some suppliers provide multiple antibodies to a single antigen. Also, there should be inclusion of the dilutions used for antisera, as well as concentrations for purified IgG fractions.

Analysis of Cellular Composition Based on Staining. Immunostaining for cell-specific antigens is the primary method to determine cellular compositions in atherosclerotic lesions. However, there is increasing ambiguity about assigning an immunostain to a specific cell type, given the growing awareness that there is a high degree of plasticity for different cellular phenotypes.[220] Traditionally, cellular composition has been based only on immunostaining for predominant cell types such as smooth muscle, macrophages, endothelial cells, and lymphocytes. In most cases, α-actin has been used as

the marker for smooth muscle cells; Mac-2 (galectin-3) or F4/80 as markers for macrophages; von Willebrand factor, PECAM-1 (platelet/endothelial cell adhesion molecule 1), or VE (vascular endothelial)-cadherin for endothelial cells; and CD3 as a pan lymphocyte marker. The number of cells stained positive for each marker can be counted and normalized to the total number of cells (Nuclei Staining). Frequently, the area of the amplified immunostaining for each antigen is measured (Image Analysis of Tissue Sections). Although this approach enables comparisons among groups, it is not an accurate measurement of the cellular composition in lesions. It is further complicated by frequent smearing of reaction products, which leads to a diffuse pattern of staining. Thus, for accuracy and reproducibility, it is essential that counterstaining for nuclei be performed and that the number of immune-positive cells be counted and normalized to the total number of cells. As noted, in recent years the complexity of evaluating the cellular composition of lesions has increased with the recognition of multiple and interchangeable phenotypes for the major cell types. Smooth muscle cells could undergo a process of "modulation" whereby a "contractile" phenotype could become a "synthetic" phenotype.[221] However, this has become exceedingly more complex, because mesenchyme-derived cell types can interconvert from smooth muscle cells to adipocytes, chondrocytes, and osteoblasts, as well as fibroblasts.[222] In both mouse and human lesions, the presence of cell types involved in active ectopic calcification, such as osteoblast-like and chondrocyte-like cells that express markers such as alkaline phosphatase, collagen 4, and RUNX2, has been documented.[223] There is evidence that smooth muscle cells within lesions can express the markers and functionality of macrophages.[220,224] Lineage tracing methods have been established such that cells expressing specific markers at one point in time can be traced as they gain or lose markers of the same or other cell types.[220] Thus, a more complete evaluation of cellular composition in lesions requires simultaneous immunostaining of the same or serial sections with multiple cell type–specific antibodies and careful mapping of the spatial distribution by superimposing images.

Nuclei Staining

Counterstaining of nuclei in sections of atherosclerotic lesions is essential for normalizing data in studies of cellular proliferation (bromodeoxyuridine or labeled thymidine incorporation), apoptosis (TUNEL [terminal deoxynucleotidyl transferase dUTP nick-end labeling] or activated caspase), cellular protein by immunostaining, or mRNA expression by in situ hybridization. There are a variety of dyes for staining of nuclei on paraffin-embedded and frozen sections of lesions. These methods include commonly used stains such as hematoxylin, toluidine blue, methyl green, and Hoechst. Staining of nuclei enables counting of the total number of cells within the lesions and normalization of data for cells positive for proliferation, apoptosis, or protein and mRNA expression as a percentage of the total cell number. Normalization in this way facilitates the comparison of data on lesions from humans or animal models. The counting of nuclei also provides a measure of the cellularity of the lesion and can be normalized to the volume of the entire lesion or area of interest.

Image Analysis of Tissue Sections

The sections above describe issues related to visual detection of pathological analysis of atherosclerotic lesions that have been reported commonly in atherosclerosis studies. Unfortunately, the Methods sections of manuscripts generally provide scant details of how these analyses were performed to permit replication and interpretation. It is recommended that the following details be provided in each manuscript.

Sample Numbers

Because size and components of atherosclerotic lesions are heterogeneous, sample selection influences data acquisition. Although no definitive guidelines can be provided, minimal requirements in reporting should include the number of sections analyzed per animal, the basis for selection of analyzed section(s), and the location (which is identified by the same landmark of tissue sections) of sections within lesions.

Mode of Selecting Region of Interest

Definition of the region of interest (ROI) within a tissue section can greatly influence data acquisition. For example, many images are acquired at a high magnification that only permits a selected small area of a tissue section to be quantified. Therefore, when immunostaining is heterogeneous across tissue sections, the mode of defining the ROI needs to be clearly stated, preferably with some explanation to avoid selection bias. To enable interpretation, it is recommended that the minimum reporting requirements are a clear definition of the ROI and an explanation for the basis of selecting it. In concert with reporting of the number of sections used to derive data, there should also be clear numerical data provided on the number of ROIs measured per section.

Definition of Area for Inclusion in Image Analyses

Data on constituents of atherosclerotic lesions are most commonly expressed as a percent of the staining (immunostaining or histological staining) in an ROI. Parameters used to define the area for measurement are not commonly stated. Most studies have set a threshold based on an RGB color mode to define areas of positive staining; however, for many stainings, there is not a precipitous change in areas that can be deemed to be positive versus negative. Rather, there is frequently a spectrum of color intensity. Therefore, the definition of the threshold has a major influence on deriving the measurement of positive areas. To provide insight into how data were derived, reports should clearly describe how the area of positivity was defined and what approaches were taken to overcome the inherent subjectivity of this assessment.[225]

Spatial Data

The acute clinical manifestations of atherosclerosis are attributable to interactions of a lesion with blood to precipitate an obstructive thrombus. The 2 principal modes of precipitating thrombus formation are rupture and erosion.[226] Thrombus formation is a consequence of both the composition of the lesion and the spatial distribution of its constituents. For example, rupture-prone plaques have been described as containing variably sized lipid-rich necrotic cores encased by a thin fibrous cap. The fibrous cap contains a high density of leukocytes, in contrast to the very low density that is commonly present in the other lesional regions. Rupture-prone lesions are also

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characterized by neovascularization, hemorrhage, adventitial leukocyte accumulation, and patches of calcification.[227–230]

Given the importance of the spatial distribution of lesional elements to acute thrombus formation, meaningful compositional analysis of lesions (chemical, biochemical, cellular, etc) is needed to enhance the relevance of experimental models to the human disease. In experimental atherosclerosis, the occurrence of atherosclerotic lesion-associated thrombus is controversial,[231–233] but it is clearly not a common event reported in the literature. In addition, the preponderance of published images of experimental atherosclerotic lesions do not mimic the composition of the rupture-prone lesions described herein. Therefore, unlike human data, in which thrombus-associated lesions are used to determine lesions that are defined as vulnerable, the paucity of this information in mouse atherosclerosis studies discourages the use of inferential terms such as *vulnerable* and *stability*.

Section Thickness

Most compositional analysis is a 2-dimensional measurement of a 3-dimensional object. Therefore, data derived can be influenced by the third dimension, the thickness of the tissue section, with greater thicknesses increasing the apparent area of a staining. For example, analysis of a different cell type in a thin section can provide spatial discrimination; however, sections of greater thickness have an increased propensity for cell types to apparently occupy the same space, because the 2-dimensional image cannot discriminate the variable locations in the third dimension. Although the accuracy of the so-called positive area would be enhanced by the use of thin sections, there are practical issues to consider. For instance, immunostaining frequently has to be performed on unfixed non–paraffin-embedded tissues, and cryostat sectioning of fibrous tissue such as arteries commonly requires ~10 µm of thickness per section. However, interpretation of the data should take into account that the deduced area of staining can be variable based on tissue section thickness, and reports should take into account the precision of this technique.

Cell Isolation and FACS Analyses

Quantification of cell population numbers by immunostaining of tissue sections has constraints, as described previously. To overcome these issues, several publications have isolated cells from atherosclerotic mouse aortas and performed flow cytometry.[234] Flow cytometry–based approaches have the benefit of providing relatively accurate counting of specifically labeled cells isolated from atherosclerotic lesions.[234] This technique digests extracellular matrix of mouse aortas, and released cells are labeled with fluorescent antibodies. One potential caveat with this approach is the potential for contamination of cells derived from the adventitial aspect of the aorta. There also needs to be a validation for quantitative recovery of all cells from the atherosclerotic lesion, because cell counting by flow cytometry has the potential to select subpopulations. An example would be foam cells, which may be too fragile to survive the isolation process.[235] If adventitial cell contamination is minimal and cell recovery is high, this approach provides meaningful insight into cell population numbers present in lesions.

Laser Capture Microdissection

Atherosclerotic plaques are composed of multiple cell types. The use of laser capture microdissection to analyze specific cell types from particular locations in atherosclerotic plaques was introduced in 2002.[236] A number of method articles have been published that provide extensive technical details.[237–239] Although there are a number of technical variations to laser capture microdissection based on the particular instrument, the common approach is to use light microscopy to initially identify cells of interest, then a laser beam of sufficient energy to remove cells without damaging their macromolecular contents. The selected cells are then lysed, and the material of interest is purified. In plaques, macrophages, the easiest cells to select, are also recognized as key players in the atherosclerotic process. The same approach for selecting macrophages can be applied to other cell types, such as vascular smooth muscle cells.[236] Endothelial cells present some logistical challenges given the thinness of this cell type in tissue sections, although endothelial cells have been analyzed by this technique.[240] Analysis of endothelial cells can be accomplished by other methods that remove this cell type, such as removal of an en face preparation or by aortic digestion.[241]

Typically in atherosclerosis experiments, gene expression analysis is the goal, and it is possible to obtain sufficient high-quality RNA from laser capture microdissection–selected cells for direct quantification by reverse-transcription polymerase chain reaction[236] or amplification for transcriptome analysis by microarray.[242] The likelihood of achieving a good yield of high-quality RNA is highest when the original aortic tissue is not perfused or fixed in formalin but is placed after phosphate-buffered saline perfusion into OCT compound and processed as frozen sections. Using a number of proprietary kits, however, some RNA can be obtained from fixed tissues, and this would be particularly helpful to interrogate archived tissues (eg, human lesions from a tissue bank) at a molecular level.

mRNA Profiling

Transcript levels can be quantified with quantitative polymerase chain reaction, expression arrays, or RNA sequencing. The most commonly used expression arrays are from Affymetrix, Agilent, and Illumina, and results are roughly comparable.[243–245] RNA sequencing is replacing expression arrays for many applications because it provides additional information about long noncoding RNAs, RNA splicing, and allele-specific expression.[246] RNA sequencing is considerably more expensive, usually costing at least several hundred dollars per sample, depending on the depth of sequencing required. Statistical analysis is another issue critical in expression profiling. Normalization is generally used to remove systematic biases.[247] Profiling has been conducted in conjunction with laser capture microdissection to study defined populations of plaque cells.[242]

Interpretation and Presentation of Data

The basis of statistics is to provide an objective assessment of data to assess whether defined measurements are the same or different among groups.[248] In the biological world, there has been broad agreement that a probability value of <0.05 (5%)

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is ascribed as being statistically significant. There are numerous statistical tests to determine whether the null hypothesis is true or false for specific data sets; however, many of these tests have constraints that limit their applicability, and their inappropriate use can provide the wrong conclusions. In fact, a previous analysis of cardiovascular publications has stated that half of the literature has applied statistical tests incorrectly.[249] One of the most common mistakes is the use of the incorrect test for multiple groups, such as the Student *t* test, when other tests, such as ANOVA, are more appropriate. Another common mistake is the use of parametric tests while failing to determine whether the data set is appropriate for the analyses required for their application, such as by virtue of having normal distribution and equivalence of variance.

To overcome these concerns, it is recommended that the test for statistical analysis used on each data set be clearly defined in the manuscript. A recent report from the National Institutes of Health on principles and guidelines for reporting preclinical research has been endorsed by the AHA journals.[118] The primary emphasis of these guidelines is to provide sufficient detail for other investigators to be able to replicate the studies.

Concluding Statements

Atherosclerosis is the most prevalent cause of morbidity and mortality in developed nations. It is imperative that substantial research efforts be undertaken to determine novel approaches for attenuating the development and progression of this disease. Given the inherent difficulties and expense of performing studies in humans, animal models are a major tool to understand the complexity and mechanisms of this disease. To provide insight into the human disease, animal studies must be rigorously designed and executed, with data being interpreted within a realistic framework of extrapolation from the species to humans. We hope that the recommendations within this document will provide a framework for continuous animal research that assists in the development of new and effective therapies.

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Writing Group Disclosures

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*Modest.

†Significant.

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Shenghan Lai	Johns Hopkins School of Medicine	None	None	None	None	None	None	None

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