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Translating molecular discoveries into new therapies for atherosclerosis

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Atherosclerosis is characterized by the thickening of the arterial wall and is the primary cause of coronary artery disease and cerebrovascular disease, two of the most common causes of illness and death worldwide. Clinical trials have confirmed that certain lipoproteins and the renin–angiotensin–aldosterone system are important in the pathogenesis of atherosclerotic cardiovascular disease, and that interventions targeted towards these are beneficial. Furthermore, efforts to understand how risk factors such as high blood pressure, dysregulated blood lipids and diabetes contribute to atherosclerotic disease, as well as to understand the molecular pathogenesis of atherosclerotic plaques, are leading to new targets for therapy.

During atherosclerosis, the arterial wall gradually thickens to form an atherosclerotic plaque, resulting in the narrowing of the lumen of the artery. Consequently, the amount of blood supplied to the organ is reduced, most commonly affecting the heart and the brain. Plaques can abruptly rupture, causing a blood clot and often myocardial infarction (heart attack) or stroke. Intensive study of the cellular and molecular mechanisms that underlie atherogenesis (that is, the formation of atherosclerotic plaques) and plaque rupture has led to a consensus view of these processes[1] (Fig. 1). Initiation and progression of the lesion are highly complex processes, and many aspects of atherogenesis remain incompletely understood. Furthermore, in most cases, mechanistic insights have yet to be translated into therapeutic approaches. In this review, we discuss the most exciting advances in atherosclerosis research since 2000, emphasizing new findings that have translational and therapeutic implications. For a review of earlier findings, see ref. 2. At present, the two main conceptual approaches to therapy for atherosclerosis are manipulation of plasma lipoprotein metabolism or cellular cholesterol metabolism, and manipulation of inflammatory processes. Here we discuss both approaches, focusing on how recent findings might lead to new types of therapy. We set the scene with a discussion of how new therapeutic targets are identified and validated and then finish by looking at how genome-wide association studies are rapidly altering the way in which atherosclerosis is understood and might be treated.

Identification of therapeutic targets in humans and mice

Perhaps the most convincing evidence for a potential therapeutic target is provided when a human genetic condition arising from simple mendelian genetics is found to be associated with altered risk of atherosclerotic disease. An example is homozygous familial hypercholesterolaemia, which is caused by mutations in the gene encoding the low-density lipoprotein (LDL) receptor. The observation that this disease is associated with markedly premature atherosclerosis led to an understanding that increased concentrations of LDL cholesterol in plasma can cause atherosclerosis. This observation also led to the general concept that intervening to increase LDL-receptor expression would reduce LDL concentrations and thus the risk of atherosclerosis. However, classic mendelian disorders are not associated with most genes of interest, and even when they are, the prevalence of these disorders is usually too low to provide strong evidence of an association with atherosclerosis. By examining extended families, linkage studies have identified loci that seem to be important determinants of premature coronary artery disease, but it has often been challenging to identify the specific genes that cause disease. One notable recent success was the identification of a mutation in the gene encoding LDL-receptor-related protein 6 (LRP6) in a large family as responsible for autosomal dominant premature coronary artery disease accompanied by features of the metabolic syndrome (which is a group of risk factors that are commonly associated with coronary artery disease, including hyperlipidaemia, hypertension and insulin resistance)[3]. 'Candidate genes' are frequently tested by genotyping single-nucleotide polymorphisms (SNPs) in large cohorts (or groups) of patients and examining whether particular SNPs are associated with atherosclerotic disease. Unfortunately, many of the published association studies have not been subjected to rigorous replication[4]. Most recently, genome-wide association studies have been used in an attempt to identify genes that are significantly associated with atherosclerotic disease and its risk factors (discussed later).

Studies of genetically modified mice are also commonly used to identify and validate potential therapeutic targets, as well as to investigate atherosclerotic disease mechanisms in detail. The bidirectional flow of information between mouse and human studies has been crucial for furthering knowledge of atherosclerosis, as well as for validating new therapeutic targets. However, the relevance of mouse studies for understanding the pathophysiology of atherosclerosis in humans needs to be carefully considered. There are important differences between mice and humans with respect to two of the main processes involved in atherogenesis: lipoprotein metabolism and inflammatory pathways. In addition, there are many inconsistencies between the various studies of atherosclerosis in mice, and the basis of these discrepancies is often unclear. Strain differences might, in part, be responsible; indeed, there can be substantial genetic variation between control and experimental mice even after extensive backcrossing of both into the same strain. A lack of standardization in measuring lesion size in mice might also contribute to these discrepancies. Furthermore, there is an increasing recognition that lesion composition, rather than size, determines the acute complications of atherosclerotic disease in humans. However, compositional analysis of lesions in mice is not routine or standardized, and the implications of differing lesion composition for disease

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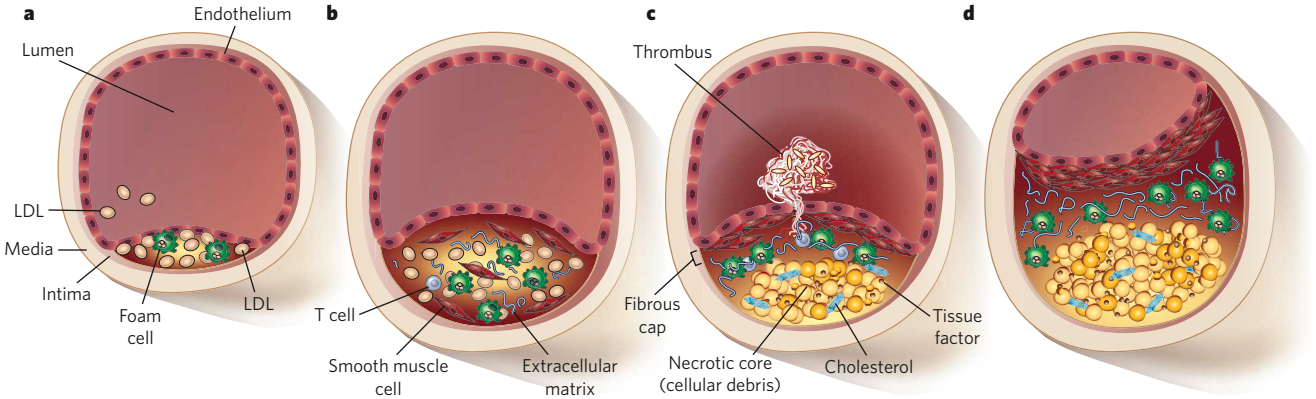

Figure 1 | Initiation and progression of atherosclerosis. Atherosclerosis occurs at sites in the arterial tree where laminar flow is disrupted. A lesion begins as a fatty streak (**a**) and can develop into an intermediate lesion (**b**), and then into a lesion that is vulnerable to rupture (**c**) and, finally, into an advanced obstructive lesion (**d**). A more detailed description of this process follows. **a**, Atherogenic lipoproteins such as low-density lipoproteins (LDLs) enter the intima, where they are modified by oxidation or enzymatic activity and aggregate within the extracellular intimal space, thereby increasing their phagocytosis by macrophages. Unregulated uptake of atherogenic lipoproteins by macrophages leads to the generation of foam cells, which are laden with lipid. The accumulation of foam cells leads to the formation of fatty streaks, which are often present in the aorta of children, the coronary arteries of adolescents, and other peripheral vessels of young adults. Although they cause no clinical pathology, fatty streaks are widely considered to be the initial lesion leading to the development of complex atherosclerotic lesions. **b**, Vascular smooth muscle cells — either recruited from the media into the intima or proliferating within the intima — contribute to this process by secreting large amounts of extracellular-matrix components, such as collagen. The presence of these increases the retention and aggregation of atherogenic lipoproteins. In addition to monocytes, other types of leukocyte, particularly T cells, are recruited to atherosclerotic lesions and help to perpetuate a state of chronic inflammation. As the plaque grows, compensatory remodelling takes place, such that the size of the lumen is preserved while its overall diameter increases. **c**, Foam cells eventually die, resulting in the release of cellular debris and crystalline cholesterol. In addition, smooth muscle cells form a fibrous cap beneath the endothelium, and this walls off the plaque from the blood. This process contributes to the formation of a necrotic core within the plaque and further promotes the recruitment of inflammatory cells. This non-obstructive plaque can rupture or the endothelium can erode, resulting in the exposure of thrombogenic material, including tissue factor, and the formation of a thrombus in the lumen. If the thrombus is large enough, it blocks the artery, which causes an acute coronary syndrome or myocardial infarction (heart attack). **d**, Ultimately, if the plaque does not rupture and the lesion continues to grow, the lesion can encroach on the lumen and result in clinically obstructive disease.

progression and outcome are not well understood. Another weakness of mouse studies is their focus on the mechanisms of lesion initiation and early progression instead of on the mature disease stages, which are the main stages targeted for therapy in humans. In addition, it is uncertain whether the vascular regions in which atherosclerosis is measured in mice have relevance to the human disease. These considerations translate into a lack of confidence that the effects of pharmacological interventions on atherosclerosis in mice will be reproducible in humans[5].

Lipoprotein metabolism

Lipoproteins transport lipids, including cholesterol, in the blood, and their metabolism is closely interrelated with the initiation and progression of atherosclerosis. The two most abundant lipoproteins in the plasma are LDLs and high-density lipoproteins (HDLs). Targeting aspects of their metabolism is one of the main interventions for preventing and treating atherosclerotic cardiovascular disease.

LDL metabolism

Both human studies and animal studies have shown that lipoproteins that contain apolipoprotein B (apoB) — for example, LDLs — are required for the development of atherosclerosis. The progression of atherosclerosis and the incidence of coronary and cerebrovascular events is significantly reduced, regardless of baseline LDL concentrations, after administration of inhibitors of HMG-CoA reductase (3-hydroxy-3-methylglutaryl coenzyme A reductase). These drugs, known as statins, inhibit cholesterol biosynthesis and result in accelerated clearance of plasma LDLs (the main lipid component of which is cholesterol) by the liver. The development of statins is one of the great translational successes in the atherosclerosis field and illustrates how research in biochemistry, cell biology, animal models, and human physiology and genetics can converge to produce a highly effective therapy.

The success of statins has inspired further efforts aimed at understanding the molecular mechanisms that regulate plasma LDL concen-

LDL receptor present at the hepatocyte surface is one of the most important factors influencing plasma LDL concentration. Recent research indicates, however, that LDL-receptor regulation is substantially more complex than previously thought. Genetic mutations that cause the rare disease autosomal recessive hypercholesterolaemia result in disruption of LDL-receptor recycling and in a substantial reduction in the number of LDL receptor molecules at the hepatocyte surface, thus markedly increasing plasma cholesterol concentrations[6]. The protein that is encoded by the mutated gene in individuals with this disease normally functions as a modular adaptor for the LDL receptor, either chaperoning the receptor to coated pits, where it binds to LDL, or anchoring the receptor in these pits during internalization.

More recently, another complexity was uncovered by genetic linkage analysis. Mutations in the gene encoding proprotein convertase subtilisin/kexin type 9 (PCSK9) are associated with a form of autosomal dominant hypercholesterolaemia[7]. An independent study found that mice on a high-cholesterol diet had a reduced expression of *Pcsk9* in the liver[8]. By contrast, hepatic overexpression of *Pcsk9* in mice results in marked hypercholesterolaemia[9], leading to the conclusion that the human mutations that cause hypercholesterolaemia have gain-of-function properties. Subsequently, PCSK9-deficient mice were found to have lower cholesterol concentrations than wild-type mice[10], and humans heterozygous for loss-of-function mutations in *PCSK9* were shown to have substantially reduced LDL concentrations[11], accompanied by a marked reduction in their lifetime risk for coronary artery disease[12]. Mechanistic studies show that after catalysing its own cleavage, PCSK9 is secreted and binds to cell-surface LDL receptors, thereby targeting them for degradation rather than recycling[13]. *PCSK9* is a sterol-responsive gene, the expression of which is upregulated by statin treatment, with the effect of blunting the reduction in LDL-cholesterol concentrations associated with statin therapy. PCSK9 is thus a highly attractive target for reducing LDL-cholesterol concentrations (Table 1).

Plasma concentrations of atherogenic lipoproteins such as LDLs are

lipoproteins (VLDLs), the metabolic precursor of LDLs. Indeed, hepatic overproduction of VLDLs is a common finding in individuals with insulin resistance or type 2 diabetes and is also the basis of familial combined hyperlipidaemia, a common genetic lipoprotein disorder. Upstream transcription factor 1 (USF1) has been genetically associated with familial combined hyperlipidaemia[14], although the molecular mechanisms underlying the effect of USF1 on VLDL production are unclear. Studies of humans with low LDL concentrations have also provided important insights into the regulation of VLDL and LDL production. Mutations in the gene encoding apoB — the key structural protein component of VLDLs and LDLs —can result in low LDL concentrations, at least in part by reducing VLDL production. Patients with abetalipoproteinaemia have loss-of-function mutations in the gene encoding microsomal triglyceride transfer protein (MTP), which is required for loading triglycerides onto apoB. These mutations therefore result in markedly impaired VLDL assembly and secretion, and an absence of plasma LDLs. LDL concentrations in humans have been successfully lowered by inhibiting either the production of apoB-100 (the form of apoB that is produced by the liver and is present in LDLs) with antisense oligonucleotides or the activity of MTP with small molecules[15,16], and these strategies are in clinical development (Table 1). Genome-wide association studies are likely to identify other potential targets for decreasing LDL concentrations (discussed later).

HDL metabolism

As is the case for LDLs, the main lipid component of HDLs is cholesterol. However, in contrast to LDL cholesterol, plasma concentrations of HDL cholesterol are inversely associated with atherosclerotic disease. HDL metabolism is complex and is influenced by numerous factors (Fig. 2). HDLs have long been considered an important endogenous factor that protects against atherosclerosis and are thus an attractive therapeutic target[17]. In animals, overproduction or repeated infusion of the major protein in HDLs, apoA-I, reduces the extent of atherosclerosis[17], and two small clinical trials suggest that apoA-I has a similar effect in humans[18,19]. However, the 'HDL hypothesis' — that raising HDL concentrations has beneficial therapeutic effects — has been difficult to prove in humans because of the lack of interventions that substantially increase HDL-cholesterol concentrations. Some Japanese individuals have extremely high HDL concentrations, and the finding that this results from a genetic deficiency in the cholesteryl ester transfer protein (CETP) spurred the development of CETP inhibitors, which have been shown to raise HDL concentrations in humans[20]. However, the development of the first CETP inhibitor to advance into phase III clinical trials, torcetrapib, was terminated because of increased mortality and cardiovascular events[21]. Torcetrapib increases blood pressure and aldosterone concentrations through effects unrelated to inhibiting CETP, confounding the interpretation of the clinical trial and leaving hope for the development of a 'clean' CETP inhibitor[22]. Other approaches to increasing HDL concentration — such as inhibition of endothelial lipase (which breaks down HDLs) (Fig. 2) — are also of interest (Table 1). New targets could also be identified from ongoing studies of the molecular physiology of HDL metabolism and function, as well as from genome-wide association studies searching directly for genes that affect HDL concentrations (discussed later).

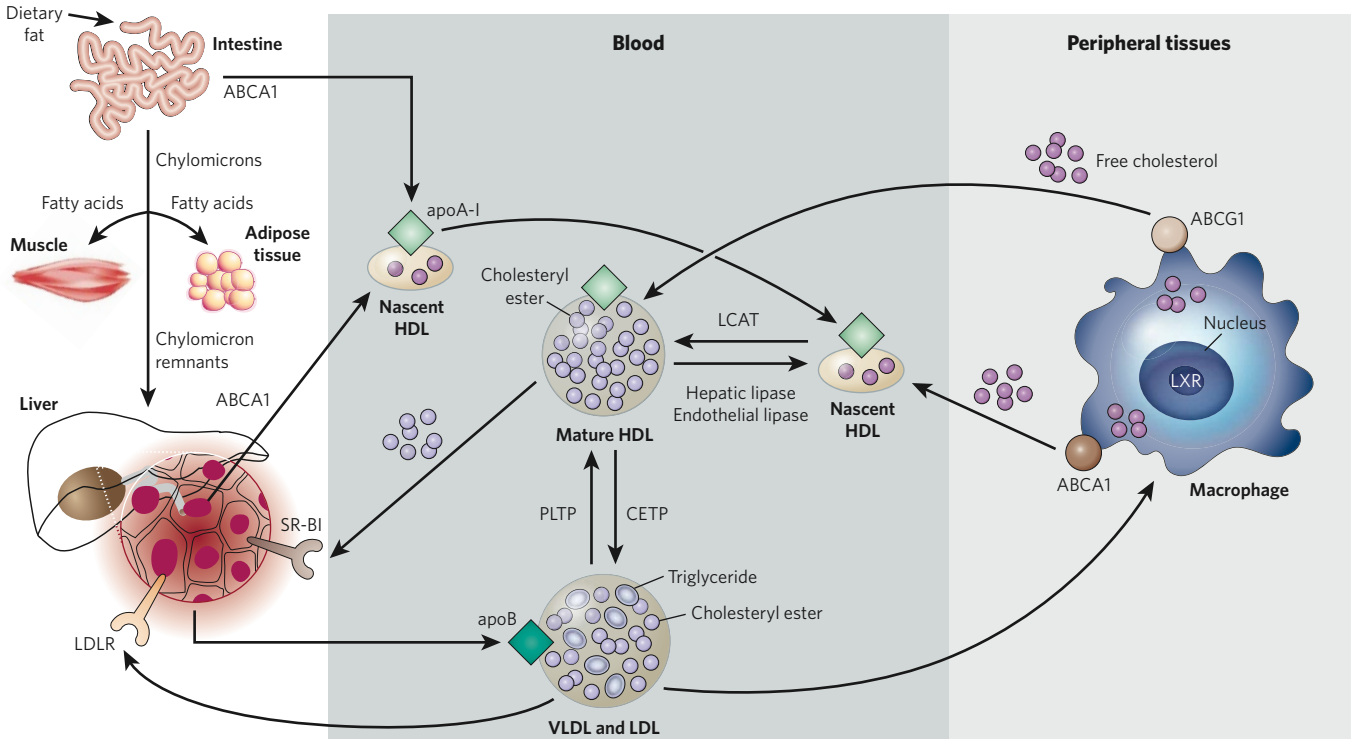

Figure 2 | Lipoprotein metabolism. Lipoprotein metabolism has a key role in atherogenesis. It involves the transport of lipids, particularly cholesterol and triglycerides, in the blood. The intestine absorbs dietary fat and packages it into chylomicrons (large triglyceride-rich lipoproteins), which are transported to peripheral tissues through the blood. In muscle and adipose tissues, the enzyme lipoprotein lipase breaks down chylomicrons, and fatty acids enter these tissues. The chylomicron remnants are subsequently taken up by the liver. The liver loads lipids onto apoB and secretes very-low-density lipoproteins (VLDLs), which undergo lipolysis by lipoprotein lipase to form low-density lipoproteins (LDLs). LDLs are then taken up by the liver through binding to the LDL receptor (LDLR), as well as through other pathways. By contrast, high-density lipoproteins (HDLs) are generated by the intestine and the liver through the secretion through the actions of the transporter ABCA1, forming nascent HDLs, and this protects apoA-I from being rapidly degraded in the kidneys. In the peripheral tissues, nascent HDLs promote the efflux of cholesterol from tissues, including from macrophages, through the actions of ABCA1. Mature HDLs also promote this efflux but through the actions of ABCG1. (In macrophages, the nuclear receptor LXR upregulates the production of both ABCA1 and ABCG1.) The free (unesterified) cholesterol in nascent HDLs is esterified to cholesteryl ester by the enzyme lecithin cholesterol acyltransferase (LCAT), creating mature HDLs. The cholesterol in HDLs is returned to the liver both directly, through uptake by the receptor SR-BI, and indirectly, by transfer to LDLs and VLDLs through the cholesteryl ester transfer protein (CETP). The lipid content of HDLs is altered by the enzymes hepatic lipase and endothelial lipase and by the transfer proteins

The experience with torcetrapib has focused increasing attention on new therapies that improve HDL function, as distinct from those that increase plasma HDL concentrations. The most well-established mechanism by which HDLs protect against atherosclerosis is by promoting cholesterol efflux from macrophages and transporting the cholesterol to the liver for excretion in bile and faeces, a process termed reverse cholesterol transport (Fig. 2). Although this process is difficult to quantify *in vivo*, circumstantial evidence supports the idea that reverse cholesterol transport contributes to HDL-mediated inhibition of atherogenesis. The molecular pathways of cholesterol efflux from macrophages and the molecular nature of the lipoprotein acceptor particles have been extensively investigated, as have the pathways by which cholesterol is returned to the liver for excretion. A seminal discovery was the identification of mutations in the gene encoding the transporter protein ABCA1 in individuals with Tangier disease[23]. ABCA1 promotes the efflux of cholesterol from cells, including macrophages, to lipid-poor apoA-I-containing particles[23], which are HDL precursors present in plasma. Cholesterol efflux from macrophages to mature HDLs was subsequently found to occur through a different transporter, ABCG1 (ref. 24). Both ABCA1 and ABCG1 have been shown to contribute to

Table 1 | Selected new therapeutic targets for atherosclerosis or its risk factors

Target	Human genetics*	Types of therapy	Biomarkers†	Phase‡
Lipoprotein metabolism				
Squalene synthase	None	Inhibitor	LDL cholesterol	III
MTP	Abetalipoproteinaemia	Inhibitor	LDL cholesterol, apoB	II/III
apoB	Hypobetalipoproteinaemia	Antisense oligonucleotide	LDL cholesterol, apoB	II
PCSK9	*PCSK9* gain of function and loss of function	Antisense oligonucleotide, small interfering RNA	LDL cholesterol	Preclinical
Thyroid-hormone receptor-β	None	Agonist	LDL cholesterol, triglycerides and HDL cholesterol	II
Farnesoid X receptor	None	Agonist	Triglycerides	I
		Antagonist	LDL cholesterol	Preclinical
Lipoprotein lipase	Familial chylomicronaemia, association	Gene-replacement therapy	Triglycerides	I/II
CETP	*CETP* deficiency, association	Inhibitor	LDL cholesterol, HDL cholesterol	II/III
Cannabinoid receptor 1	None	Antagonist	Triglycerides, HDL cholesterol and body weight	III
LXR	None	Agonist	*ABCA1* expression	I
Niacin receptor (GPR109A)	None	Agonist	Free fatty acids, triglycerides and HDL cholesterol	I
apoA-I	*APOA1* mutations	Full-length protein	apoA-I	I/II
		Mimetic peptides	Cholesterol efflux, anti-inflammatory function	I/II
		Upregulator	apoA-I	I
Lecithin cholesterol acyltransferase (LCAT)	*LCAT* deficiency	Agonist peptide	HDL cholesterol	II
Endothelial lipase	Association	Inhibitor	HDL cholesterol	Preclinical
Biologically active lipids				
TXA_2 receptor	None	Antagonist	TXA_2	Preclinical
mPGES1	None	Inhibitor	PGE_2	Preclinical
PGE_2 receptors (EP1 and EP3)	None	Antagonist	None	Preclinical
PGD_2 receptor (DP1)	None	Antagonist	Niacin-associated flushing	III
5-LO	Association	Inhibitor	LTB_4	Preclinical
FLAP	Association	Inhibitor	LTB_4	II
LTA_4 hydrolase	Association	Inhibitor	LTB_4	Preclinical
LTB_4 receptors (BLT1 and BLT2)	None	Antagonist	None	Preclinical
15-LO	None	Inhibitor	LTE_4	Preclinical
Myeloperoxidase	None	Inhibitor	Myeloperoxidase activity	Preclinical
Secretory PLA_2 (groups IIa, V and X)	Association	Inhibitor	Secretory PLA_2 activity	II
Lipoprotein-associated PLA_2	Association	Inhibitor	Lipoprotein-associated PLA_2 activity	II
Leukocyte recruitment and retention				
VCAM1	Association	Antagonist	Soluble VCAM1	Preclinical
ICAM1	Association	Antagonist	Soluble ICAM1	Preclinical
P selectin	Association	Inhibitor	Soluble P selectin	Preclinical
CD44	None	Inhibitor	Soluble CD44	Preclinical
CCR2	Association	Antagonist	None	Preclinical
CX_3CR1	Association	Antagonist	None	Preclinical
CCR7	None	Agonist	None	Preclinical
Extracellular-matrix turnover and plaque rupture				
MMPs (for example, MMP9)	Association	Inhibitor	MMP activity	Preclinical
Cathepsins (for example, cathepsin S)	None	Inhibitor	Cathepsin activity	Preclinical

*Human genetics refers to the existing data on mendelian disorders or candidate gene association that help to validate the target. †Biomarkers refers to the biomarker that might be used to determine efficacy and optimal dosing. ‡Phase refers to the current phase of drug development and is based on public domain information that can change rapidly.

the efflux of cholesterol from macrophages and to reverse cholesterol transport *in vivo*[25]. Transcription of the genes encoding ABCA1 and ABCG1 is stimulated by liver X receptor (LXR), a nuclear receptor that is activated by oxysterols (oxidized derivatives of cholesterol). Accordingly, LXR agonists promote cholesterol efflux from cultured macrophages[26], accelerate reverse cholesterol transport *in vivo*[27], and lead to substantial retardation or even regression of atherosclerosis in mice[26,28]. Thus, LXR is a target for the development of therapies focused on promoting reverse cholesterol transport to treat atherosclerotic disease (Table 1).

HDLs have various other properties that could contribute to their antiatherogenic properties. For example, HDLs can promote the activity of nitric-oxide synthase 3 (NOS3; also known as eNOS) and thereby increase the bioavailability of nitric oxide[29]. In addition, HDLs have anti-inflammatory effects both *in vitro* and *in vivo*, and these have been the subject of intensive study[30]. Intriguingly, the many activities of HDLs might have evolved from an original role in innate immunity. HDLs bind to lipopolysaccharide, a component of bacterial cell walls, and protect mice from lipopolysaccharide-induced mortality[31]. More recent work has shown that HDLs function as a platform for the assembly of a complex that contains apoL-I and haptoglobin-related protein and is highly lytic for a species of trypanosome[32]. Indeed, systematic proteomic analysis has revealed that a large number of proteins are bound to human HDLs, including proteins involved in inflammation, complement regulation and innate immunity[33], and variation in the protein composition of HDLs might affect HDL function. Protein components of HDLs have also been targeted as a therapy. For example, as indicated earlier, the therapeutic potential of peptides based on the sequence of apoA-I that can mimic the cholesterol-efflux-promoting and/or anti-inflammatory properties of HDLs has been pursued[34].

Inflammatory processes

Inflammation is crucial for the development of atherosclerotic plaques. Inflammatory pathways such as those involving biologically active lipids, the renin–angiotensin–aldosterone system and cellular processes within atherosclerotic plaques are involved in atherosclerosis, and components of these pathways are the targets of interventions (both in use and in development) for treating atherosclerotic cardiovascular disease.

Biologically active lipids

Biologically active lipids activate receptors — usually G-protein-coupled receptors — and, consequently, induce an intracellular signalling cascade. These lipids have been implicated in the pathogenesis of atherosclerosis, so the enzymes that generate them and the receptors that mediate their actions are attractive targets for therapy.

Prostaglandins are a family of biologically active lipids that are generated from arachidonic acid, which is present in the plasma membrane. The first step in prostaglandin synthesis is carried out by cyclooxygenase (COX), for which there are two distinct isozymes, COX1 and COX2. The cardioprotective effects of aspirin are thought to result from inhibition of COX1 in platelets, which reduces concentrations of the prothrombotic prostaglandin thromboxane A_2 (TXA_2). The efficacy of low-dose aspirin in the secondary prevention of myocardial infarction and stroke attests to the importance of prostaglandins in human cardiovascular disease. Findings that COX2-selective inhibitors have the opposite effect (that is, they increase the risk of atherothrombotic cardiovascular events[35]) indicate that prostaglandins have a highly complex role. These experiments in both mice and humans showed that COX2-selective inhibitors suppress the formation of another prostaglandin, prostacyclin (PGI_2), without affecting the COX1-mediated formation of TXA_2 (ref. 35). PGI_2 is atheroprotective in mice, and deletion of the gene encoding the PGI_2 receptor accelerates the development of atherosclerosis[36,37]. Conversely, TXA_2 is proatherogenic: deficiency or antagonism of the TXA_2 receptor results in reduced progression of atherosclerosis in mice[36]. Multiple mechanisms are likely to be involved in the effects of prostaglandins and their receptors on atherosclerosis, including control of not only platelet activation but also the vessel wall. These findings[35–37] suggest that selective agonism of the PGI_2 receptor or antagonism of the TXA_2 receptor might have beneficial therapeutic effects for individuals with atherothrombotic disease.

Another prostaglandin, prostaglandin E_2 (PGE_2), is generated by several dedicated enzymes, including microsomal PGE synthase 1 (mPGES1; also known as PTGES). Experiments in mice suggest that mPGES1 promotes atherosclerosis: upregulation of expression of the gene encoding mPGES1 occurs in atherosclerosis, whereas deficiency in this gene results in reduced development of atherosclerosis, together with increased PGI_2 (but not TXA_2) biosynthesis (owing to diversion of the precursor PGH_2 from PGE_2 to PGI_2 generation)[38]. Thus, inhibiting mPGES1 could be a new approach to treating atherosclerosis. An alternative could be to block the activity of PGE_2 by preventing it from binding to its receptors. PGE_2 can activate four receptors: EP1, EP2, EP3 and EP4. In particular, antagonizing EP1 or EP3 — both of which are present at the cell surface of macrophages and have pro-inflammatory effects when activated — might have antiatherosclerotic effects.

PGD_2 is produced in atherosclerotic lesions by macrophages, as well as by mast cells (which can also be found in these lesions). The PGD_2 receptor (DP1) is expressed in the vasculature, but the effects of its activation on atherogenesis are unknown. An antagonist of DP1 has efficacy in reducing allergy-induced nasal congestion, as well as the cutaneous flushing associated with nicotinic acid (niacin) administration[39], and it is in clinical development for reducing niacin-associated flushing.

The leukotrienes are another family of biologically active lipids derived from arachidonic acid[40]. Leukotriene A_4 (LTA_4) is generated by the action of the enzyme 5-lipoxygenase (5-LO) with the aid of the 5-LO-activating protein (FLAP). LTA_4, in turn, is metabolized by the LTA_4 hydrolase to LTB_4. LTB_4 can then bind to and activate its receptors BLT1 and BLT2, which are expressed by vascular cells and leukocytes, promoting the recruitment of leukocytes into the vessel wall. Alternatively, LTA_4 can be conjugated to glutathione, yielding cysteinyl leukotrienes; these lipids bind to the receptors CysLT1 and CysLT2, which are also expressed by vascular cells and leukocytes. Both protein and lipid components of the 5-LO–LTB_4 pathway have been identified in atherosclerotic plaques in humans, and the concentrations of these components are higher in unstable plaques than in stable plaques[41]. In the past few years, genetic studies in humans have implicated the 5-LO–LTB_4 pathway in atherosclerotic cardiovascular disease. In a candidate gene association study, specific polymorphisms in the promoter of the gene *ALOX5AP* (which encodes FLAP) were found to be associated with variation in carotid intima-media thickness — a marker of atherosclerotic disease — and systemic markers of inflammation[42]. Furthermore, a study of large Icelandic pedigrees showed linkage of *ALOX5AP* to risk of myocardial infarction. This finding was replicated in other cohorts, in which a particular haplotype of this gene was found to be associated with a twofold increase in the risk of myocardial infarction and stroke[43]. Further investigation of candidate genes in the 5-LO pathway yielded evidence that certain polymorphisms in the gene encoding LTA_4 hydrolase were significantly associated with myocardial-infarction risk, an effect that was particularly notable in individuals of African descent[44]. In mice, an early study suggested that deficiency in 5-LO reduces the development of atherosclerosis in mice[45]; however, this conclusion was not supported by a subsequent study[46]. In addition, antagonism of the LTB_4 receptors BLT1 and BLT2, or deletion of the genes encoding these, was reported to reduce atherosclerosis in mice[40]. Thus, inhibition of the 5-LO–LTB_4 pathway could be a therapeutic approach to atherosclerosis. Indeed, a FLAP inhibitor has entered clinical development for the treatment of atherosclerotic cardiovascular disease[47].

The unsaturated fatty acid at the *sn*-2 position of phospholipids is prone to oxidation in the arterial intima, and a growing body of evidence indicates that the resultant oxidized phospholipids are highly pro-inflammatory and contribute to atherogenesis[48]. There is substantial interest in identifying the specific enzymes — such as lipoxygenases, NADPH oxidases and myeloperoxidase — that create the environment of increased oxidant stress that promotes lipid peroxidation.

enzymatic catalyst of lipid peroxidation, particularly at sites of inflammation such as the atherosclerotic lesion[49]. Myeloperoxidase also catalyses reactions that modify proteins (such as nitration, halogenation and carbamoylation[50]) and can influence protein function and promote atherogenesis. Phospholipases that cleave parent and oxidized phospholipids within the atherosclerotic plaque might also contribute to inflammation and therefore could be targets for inhibition. The family of secretory phospholipase A_2 (PLA_2) enzymes (particularly group IIa, group V and group X) has been implicated in atherogenesis[51]. Lipoprotein-associated PLA_2 might also contribute to inflammation; this enzyme cleaves oxidized phospholipids that have a short-chain oxidized fatty acid at the *sn*-2 position (for example, platelet-activating factor) and might generate pro-inflammatory and proatherogenic products[52]. Inhibition of secretory PLA_2 enzymes and lipoprotein-associated PLA_2 is actively being pursued, with compounds targeting these enzymes in clinical development (Table 1).

Oxidized phospholipids can also elicit a beneficial immune response, as shown by a study in which immunization of atherosclerotic mice with oxidized forms of LDL generated antibodies that reduced the size of lesions[53]. This beneficial response was found to be mediated by the antibody T15 (which is a 'natural' antibody', because it can be produced in the absence of immunization); T15 recognizes the head group of oxidized phospholipids and inhibits the accumulation of modified lipoproteins in macrophages. Immunization with oxidized forms of LDL leads to increased production of interleukin-5 (IL-5), which stimulates B1 cells to secrete T15 (ref. 54). In humans, plasma concentrations of oxidized phospholipids, as determined by binding to T15, are strongly correlated with plasma concentrations of lipoprotein (*a*) — an independent cardiovascular risk factor of unknown function — and are independently predictive of angiographic coronary disease[55]. The implications of these findings for the development of new therapies have yet to be determined.

Renin–angiotensin–aldosterone system

The renin–angiotensin–aldosterone system, long recognized to be a crucial regulator of blood pressure, has consistently been shown to have a prominent role in atherogenesis in both humans and experimental animals. Effective therapies for atherosclerotic cardiovascular disease based on inhibition of this system were developed from research in multiple fields, similar to the development of statins. Several lines of evidence indicate that the proatherogenic effects of activating this system are not solely the result of increases in blood pressure. Chronic infusion of angiotensin II promotes atherosclerosis in hyperlipidaemic mice independently of changes in arterial blood pressure[56,57]. Conversely, in a wide range of experimental animals, pharmacological inhibition or genetic deficiency of components of the renin–angiotensin–aldosterone system effectively reduces the development of atherosclerosis independently of blood-pressure reduction[58]. Many inhibitors of the renin–angiotensin–aldosterone system are clinically approved for reducing blood pressure, including drugs that target renin (which catalyses the first step in the pathway, cleavage of angiotensinogen to generate angiotensin I), angiotensin-I-converting enzyme (ACE, which converts angiotensin I into angiotensin II), the AT1 receptors (receptors for angiotensin II), and the mineralocorticoid receptor (the receptor for aldosterone). In humans, ACE inhibitors have a beneficial effect on atherosclerotic disease, even in individuals who do not have high blood pressure[59]. In addition to angiotensin II, a family of biologically active angiotensin-I-derived peptides has recently been identified; these peptides have a broad range of actions on all of the main cell types in atherosclerotic lesions. Further understanding of the mechanisms and consequences of the actions of these peptides could lead to the identification of new therapeutic targets.

Cellular processes in atherosclerotic lesions

Endothelial cells form a continuous monolayer on the luminal surface of atherosclerotic lesions. Hence, these cells have a crucial role

lesions is a prominent feature of atherosclerosis (Fig. 3). Knowledge of the molecular mechanisms that lead to leukocyte recruitment is continually being refined[60]. Endothelial cells display several adhesion molecules at the cell surface, and a deficiency in these molecules has been shown to decrease lesion formation in mice. Deficiency in vascular cell-adhesion molecule 1 (VCAM1) has the most marked effects, whereas deficiency in intercellular adhesion molecule 1 (ICAM1) or platelet selectin (P selectin) has less of an effect[61]. In addition, expression of the gene encoding the adhesion molecule CD44 is upregulated specifically in atherosclerotic lesions, and deletion of this gene results in reduced monocyte recruitment and less development of atherosclerosis[62]. Thus, blockade of one or more key adhesion molecules might be an effective strategy to reduce lesion formation.

An important property of endothelial cells is their ability to sense changes in vascular flow dynamics[63]. In normal (laminar) flow conditions, endothelial cells produce small amounts of adhesion molecules, but production of these is increased in non-laminar or turbulent flow. There has been substantial interest in understanding the molecular mechanisms by which these flow changes are sensed and trigger changes in intracellular signalling and gene expression. Recent findings suggest that Kruppel-like factor 2 has a central role in endothelial mechanotransduction, by regulating the transcriptional response to changes in flow dynamics[64]. Kruppel-like factor 2 affects the expression of a wide variety of genes involved in atherosclerotic lesion development and is therefore a plausible target for therapy.

The recruitment of monocytes to the intima and their differentiation into macrophages are the primary cellular events during the initiation of a lesion, and this recruitment continues during the expansion and progression of the atherosclerotic lesion (Fig. 3). Numerous mediators that attract monocytes to developing lesions have been identified. One of the early steps in this process is the binding of the chemoattractant cytokine (chemokine) CCL2 to its receptor (CCR2). Recent studies have defined distinct subsets of monocytes that are preferentially recruited to lesions[65,66]. Using an antibody directed against cell-surface molecules called Ly6 antigens, a subset of monocytes was found to accumulate preferentially in atherosclerotic lesions: these cells display large amounts of Ly6C at the cell surface, as well as the chemokine receptors CCR2 and CX_3CR1. Thus, it might be possible to inhibit selectively the recruitment of discrete monocyte subpopulations into the arterial wall, avoiding the potentially adverse consequences of broadly inhibiting monocyte recruitment.

Within the intima of the atherosclerotic lesion, monocyte-derived macrophages have many activities, including intracellular accumulation of lipids and secretion of (potentially) a wide range of chemokines, other cytokines and proteases (Fig. 3). Like the monocytes they are derived from, macrophages are heterogeneous[67]. In the presence of cytokines such as interferon-γ, tumour-necrosis factor (TNF) and granulocyte–macrophage colony-stimulating factor, macrophages are activated through the classical pathway; this class of activated macrophage (sometimes referred to as M1 cells) produces inducible nitric-oxide synthase and secretes IL-1β, IL-6 and TNF. By contrast, in the presence of various other stimuli, including IL-4 and IL-13, macrophages are activated through the alternative pathway; this class of activated macrophage (sometimes referred to as M2 cells) assists in resolving inflammation through increased endocytic activity, which is mediated by the class A scavenger receptor (also known as MSR1) and the macrophage mannose receptors[68]. The relative importance of different macrophage classes in atherogenesis is, however, uncertain at present.

Macrophage function is regulated by Toll-like receptors (TLRs), which are pattern-recognition receptors that are involved in initiating innate immune responses. The genes encoding several TLRs — including TLR1, TLR2, TLR4 and TLR5 — are expressed in atherosclerotic lesions. Hyperlipidaemic mice that are deficient in TLR2 or TLR4 (refs 69, 70) — or in the main TLR adaptor protein, MyD88 (refs 69, 71) — have smaller atherosclerotic lesions. Modified lipids or heat-shock proteins that are present in lesions have been suggested to function as

studies have provided evidence that TLRs are important in human atherosclerosis[73]. But whether targeting TLRs is a viable approach for treating atherosclerosis remains to be determined.

The role of leukocytes other than monocytes and macrophages in atherosclerosis continues to be debated[74]. Although there is a substantial body of research on the involvement of lymphocytes, the complexities of these cells have confounded a definition of their precise roles[75]. Recent studies have begun to investigate how natural killer cells[74], mast cells[76] and platelets[77] might also contribute to atherosclerosis. Of particular interest is the finding that dendritic cells can exit from lesions (Fig. 3); this egress depends on CCR7 signalling and might promote lesion regression, through the removal of lipid from the lesion[78,79]. These findings provide a new model for how lesion size can be modulated and indicate that activation of CCR7 could promote regression of atherosclerosis.

Another area of intense recent interest concerns the possibility that bone-marrow-derived endothelial and smooth muscle progenitor cells are recruited to lesions, raising the exciting possibility that a cell-based approach could be used to treat atherosclerosis. Some evidence suggests that the recruitment of these cells contributes considerably to atherosclerotic lesion formation[80]. However, this concept is controversial, with some studies finding large numbers of bone-marrow-derived endothelial cells and smooth muscle cells in atherosclerotic lesions in experimental models[81], and others finding only a small number of such cells[82].

Atherosclerosis-associated thrombosis

Atherosclerotic lesions trigger acute cardiovascular disease such as myocardial infarction and stroke only when an occlusive thrombus (or clot) forms (see page 914). The formation of a thrombus has been ascribed to at least two different types of event: first, rupture of the surface of the lesion, exposing a thrombogenic subendothelial layer of the blood vessel; and second, erosion of the fibrous cap of the lesion (which consists of smooth muscle cells)[83]. Elucidating how lesions rupture or erode and testing interventions that could prevent these events would be greatly assisted by using an animal model. However, there is no widely accepted animal model. Ruptured atherosclerotic lesions have been found in apoE-deficient mice, but the extent to which this pathology reproduces that responsible for acute cardiovascular events in humans is a matter of intense debate[84,85]. In addition, mice deficient in both apoE and the class B scavenger receptor SR-BI have accelerated atherosclerosis and spontaneous myocardial infarctions[86]; however, it is unclear whether these infarctions are caused by thrombi that form as a consequence of atherosclerosis.

Elucidating the mechanisms underlying plaque rupture has been the main focus of research on atherosclerosis-associated thrombosis. Plaque rupture is associated with the degradation of the extracellular-matrix components collagen and elastin, so it is logical to infer that extracellular proteases are involved in this process. Many members of the matrix metalloproteinase (MMP) class of enzymes have been found in atherosclerotic plaques and have been suggested to be involved in rupture (see ref. 87 for a review). Most of these studies showed increased production of specific MMPs at the shoulder regions of lesions, where plaques usually rupture. MMP9, which is produced by macrophages, is the most commonly detected MMP. This enzyme is functionally important for plaque rupture in mice: expression of a gene encoding an activated form of MMP9 in apoE-deficient mice results in the disruption of atherosclerotic lesions in the brachiocephalic artery[88]. Another class of proteases, cathepsins, has also been suggested to contribute to lesion disruption. Although cathepsins are lysosomal proteases, which function in the acidic conditions of the lysosome, they maintain the ability to degrade elastin and collagen in the extracellular environment, which is pH neutral. In mice, deficiency in particular types of cathepsin results in an increased abundance of extracellular-matrix components, and this is thought to decrease the propensity of plaques to rupture[89].

The death of cells in atherosclerotic lesions is thought to increase the risk of plaque rupture because, at late stages of disease, increased leukocyte recruitment to plaques is associated with increased levels of necrosis and apoptosis[90]. Studies in mice have shown that the apoptosis of macrophages in atherosclerotic lesions is triggered by endoplasmic-reticulum stress and

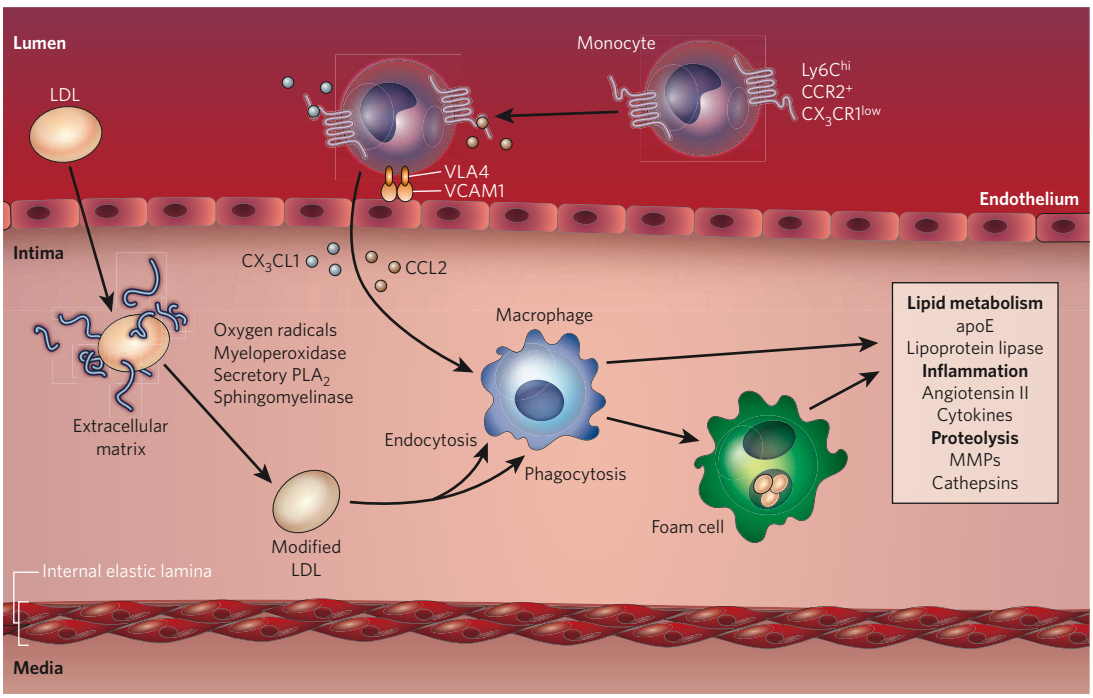

Figure 3 | Recruitment of monocytes and formation of foam cells. LDLs in the blood enter the intima, where they are retained through binding to the extracellular matrix. LDLs are then modified by oxygen radicals, myeloperoxidase, secretory phospholipase A_2 and sphingomyelinase. This results in the generation of pro-inflammatory biologically active lipids that initiate and maintain an active inflammatory process in the intima (not

CX$_3$CL1 and CCL2, which recruit subsets of monocytes to the intima. These monocytes then differentiate into macrophages, which take up modified LDL through endocytosis or phagocytosis and become foam cells (which are loaded with cholesterol). Macrophages secrete various factors involved in propagating the atherosclerotic plaque, including factors involved in lipid metabolism, inflammation and proteolysis. VLA4, very late activation

signalling through multiple receptors, including the class A macrophage scavenger receptor and TLR4, leading to Jun amino-terminal kinase (JNK)-dependent apoptosis[91]. The death of vascular smooth muscle cells might also contribute to rupture. For example, the induction of apoptosis specifically in vascular smooth muscle cells in apoE-deficient mice has no effect on atherosclerotic lesion size but affects the cellular composition of lesions, and such changes in lesion composition are expected to make the lesions more prone to rupture[92]. The role of cell death in atherogenesis remains poorly defined at all stages of lesion development and is an important area for future investigation.

Genetics of atherosclerosis in humans

Although animal models of atherosclerosis provide valuable information, their relevance to human disease remains uncertain. The current rapid progress in human genetic studies could, however, provide unprecedented mechanistic insight into human pathophysiology.

Atherosclerotic cardiovascular disease is clustered in families and has a strong genetic component, but identifying the genes that contribute to risk, beyond those affecting the 'traditional' risk factors, has been difficult. Linkage studies in large pedigrees with premature coronary artery disease have generally failed to identify causal genes definitively, with rare exceptions such as *LRP6*. By contrast, genome-wide association studies have emerged as a potentially powerful method of identifying genes underlying complex traits such as coronary artery disease. A striking and consistent finding is the highly significant association of a locus on chromosome 9p21 with myocardial infarction or coronary artery disease[93–95]. The risk-associated allele is common, with a frequency of almost 50%, and each copy of this allele increases the risk of myocardial infarction by about 25%. The genes nearest to the SNPs that are most highly associated with risk encode cyclin-dependent kinase inhibitors: *CDKN2A* (which encodes INK4A) and *CDKN2B* (which encodes INK4B). The encoded proteins are members of the INK4 family of cell-cycle suppressors, which regulates the G1–S cell-cycle checkpoint and has a role in transforming growth factor-β (TGF-β)-mediated growth inhibition, a process implicated in the pathogenesis of atherosclerosis[96]. The roles of *CDKN2A* and *CDK2NB* in atherogenesis remain to be determined, but these genetic findings[93–95] strongly implicate cell-cycle regulation in the pathogenesis of atherosclerotic cardiovascular disease.

A genome-wide association study has also implicated genes involved in cell proliferation in the risk of myocardial infarction[95]. This study presented convincing evidence that three genes (*PSRC1*, *MIA3* and *SMAD3*) encoding cell-growth regulators are significantly associated with myocardial-infarction risk. Notably, SMAD3 is an intracellular signalling molecule that links activation of the TGF-β receptor to transcriptional regulation. This study also identified a locus near the gene encoding the chemokine CXCL12 as significantly associated with myocardial-infarction risk. This chemokine was originally thought to regulate the homing of haematopoietic stem cells to the bone marrow and is now recognized to have a role in the mobilization, homing and differentiation of vascular progenitor cells in response to vascular injury[97]. Another locus identified in this study is near the gene encoding methylenetetrahydrofolate-dehydrogenase-1-like protein (the mitochondrial C1-tetrahydrofolate synthase), but how this protein might affect myocardial-infarction risk is unclear.

Genome-wide association studies can also be used to probe the genetic basis of risk factors for atherosclerosis, such as type 2 diabetes, high blood pressure and dyslipidaemia[98]. The Diabetes Genetics Initiative, for example, was designed to investigate type 2 diabetes, but 18 other phenotypes, including plasma lipid concentrations, were analysed as secondary traits[99]. Several of the loci that are significantly associated with changes in lipid concentrations are in or near genes in which mutations have been shown to cause mendelian syndromes affecting lipid concentrations (namely genes that encode the proteins apoE, ABCA1, apoA-V, CETP, lipoprotein lipase and hepatic lipase). However, other associations were also uncovered; for example, a SNP in the gene *GCKR*, which encodes glucokinase regulatory protein, was found to have a highly significant association with enzyme in the glycolytic pathway, and it can affect hepatic triglyceride synthesis. The subsequent analysis, accompanied by extensive replication of results, of three genome-wide association studies for type 2 diabetes[100] found that loci near the genes *ANGPTL3* (which encodes angiopoietin-like 3, a protein that affects triglyceride metabolism in mice) and *MLXIPL* (which encodes carbohydrate-response-element-binding protein, a transcription factor that connects hepatic carbohydrate flux with fatty-acid synthesis) are significantly associated with triglyceride concentrations. In addition, loci on chromosome 1p13 (near the genes *CELSR2, PSRC1* and *SORT1*), 19p13 (near *CILP2* and *PBX4*) and 8q24 (near *TRIB1*) were found to be significantly associated with LDL-cholesterol concentrations. The locus on 1p13 is noteworthy because a genome-wide association study of myocardial infarction also identified this locus as being significantly associated with myocardial infarction[95]. *SORT1* encodes the protein sortilin 1, a multi-ligand cell-surface receptor, which could plausibly affect lipoprotein metabolism and thus atherosclerosis. Finally, the gene *GALNT2* — which encodes *N*-acetylgalactosaminyltransferase 2, an enzyme involved in O-linked glycosylation — was found to be significantly associated with HDL-cholesterol concentrations. Thus, genome-wide association approaches are beginning to yield new biological insights and potential therapeutic targets for atherosclerosis and its risk factors.

Perspectives

Atherosclerosis has been the subject of an immense amount of basic and applied research, resulting in substantial advances in understanding the molecular pathogenesis of the disease. These insights have led to the development of successful therapeutic interventions for atherosclerotic cardiovascular disease; for example, reducing plasma concentrations of atherogenic lipoproteins (notably with statins) and blocking renin–angiotensin–aldosterone system activity (notably with ACE inhibitors and angiotensin-receptor blockers). Results from studies in cell biology, whole-animal physiology, human genetics and mechanism-based research in humans will need to be integrated carefully to choose the next generation of therapeutic targets. LDL-cholesterol concentrations and blood pressure are likely to continue to be acceptable surrogate end points in clinical trials for the registration of new drugs; however, the registration of new therapies for which the efficacy cannot be tracked by these end points will require proof of efficacy in morbidity and mortality trials. Decisions regarding which drugs and which doses to advance into large clinical end-point trials will be difficult, and new biomarkers (see page 949) and non-invasive imaging modalities (see page 953) will be needed to improve this process. Ultimately, the huge investment of the biomedical community in multidisciplinary research in atherosclerosis is likely to pay major dividends with the development of a new generation of therapies, ranging from those that can prevent disease to those that can cause its regression. ■

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