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Life After GWAS: Functional Genomics in Vascular Biology
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The Use of High-Throughput Technologies to Investigate Vascular Inflammation and Atherosclerosis

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***Abstract*—**The greatest challenge of scientific research is to understand the causes and consequences of disease. In recent years, great efforts have been devoted to unraveling the basic mechanisms of atherosclerosis (the underlying pathology of cardiovascular disease), which remains a major cause of morbidity and mortality worldwide. Because of the complex and multifactorial pathophysiology of cardiovascular disease, different research techniques have increasingly been combined to unravel genetic aspects, molecular pathways, and cellular functions involved in atherogenesis, vascular inflammation, and dyslipidemia to gain a multifaceted picture addressing this complexity. Thanks to the rapid evolution of high-throughput technologies, we are now able to generate large-scale data on the DNA, RNA, and protein levels. With the help of sophisticated computational tools, these data sets are integrated to enhance information extraction and are being increasingly used in a systems biology approach to model biological processes as interconnected and regulated networks. This review exemplifies the use of high-throughput technologies—such as genomics, transcriptomics, proteomics, and epigenomics—and systems biology to explore pathomechanisms of vascular inflammation and atherosclerosis. **(*Arterioscler Thromb Vasc Biol*. 2012;32:182-195.)**

Key Words: atherosclerosis ■ coronary artery disease ■ vascular biology ■ high-throughput ■ systems biology

As the underlying pathology of cardiovascular disease, atherosclerosis entails a chronic inflammation of the vascular wall, which is initiated by vascular structural alterations and the subendothelial accumulation of low-density lipoprotein (LDL). On oxidation, oxidized LDL elicits the activation of endothelial cells (ECs), mediating leukocyte recruitment. Intimal neutrophils, T cells, and monocyte-derived macrophages contribute to sustained inflammation, causing early fatty streak lesions to progress into advanced fibroatheromatous plaques, which, on gradual thinning of the protective smooth muscle cell (SMC) cap, transform into rupture-prone lesions.[1] Although atherosclerosis starts in childhood, it is mostly diagnosed only at a late stage. Optimized treatment requires the understanding of the disease-associated mechanisms. However, atherosclerosis is a multifactorial disease and results from an intricate interplay of multiple genes, signaling pathways, cells, and tissues. Also, many environmental factors, such as hyperlipidemia and hypertension, have been identified as risk factors for atherosclerosis, which further increases the complexness of the disease. This review discusses the use of high-throughput approaches to study atherosclerosis, and also briefly addresses cerebrovascular diseases (focusing on stroke) and peripheral artery disease (PAD). Although the latter is a direct consequence of atherosclerosis, cerebrovascular diseases comprise a variety of disorders (eg, vascular dementia or subarachnoid hemorrhage), with stroke being the most numerous. About 80% of strokes are ischemic, and atherosclerosis, but cardioembolism mostly triggered by atrial fibrillation—and small-vessel disease are also very common causes.[2,3] The multifaceted pathomechanisms underlying all these diseases necessitate a holistic study approach. Therefore, in vitro experiments, which lack the complex organization of an organism, need to be combined with clinical and animal studies. In atherosclerosis research and many other disorders, mice are the gold standard, and although they do not meet all criteria of human diseases, they offer great advantages because of the relatively low cost, easy access, high fertility, and availability of numerous biological tools, including genetic modifications.[4–6] However, not only mice but also other animals, such as rabbits, hamsters, pigs, and dogs, have been used to study atherosclerosis and vascular inflammation.[7,8]

Importantly, animal models provide the opportunity to apply high-throughput technologies, which enable the generation of large-scale data on DNA, RNA, and protein levels, to a broad variety and high number of cell and tissue samples, including those that are not available from humans (eg, genetically modified background). Furthermore, computational tools have been developed to integrate all these

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"omics"-based data for improved information extraction. In addition, biological processes are increasingly being approached from a systems biology point of view, aiming to model them as interconnected networks, and this has revealed information on disease-associated mechanisms that would never have been discovered with a classical approach.[9]

This review outlines the use of high-throughput technologies in the research field of vascular inflammation and cardiovascular disease. The widespread application of genomics, transcriptomics, and proteomics in biomedical research makes it impossible to address these techniques in a detailed way. Instead, we discuss selected studies to exemplify the context of their usage, their advantage versus classical small-scale approaches, and their current limitations. In addition, we address the rising field of epigenomics in cardiovascular disease.

We sincerely apologize to all scientists whose important contributions to the field could not be cited because of space constraints.

Genomics

Genomics involves the genome-wide sequencing and annotation of an organism's DNA, including the identification of open reading frames and regulatory elements, such as transcription factor binding sites. The sequencing of the complete human genome and the rise of high-throughput technologies as DNA microarray multiplex genotyping, enabling genome- and population-wide screens of single-nucleotide polymorphisms (SNPs) or copy number variations, has triggered an explosion of genome-wide association studies (GWASs), aiming to identify disease-associated genetic loci using disease quantitative trait locus (QTL) mapping.[10] Furthermore, metaanalyses of multiple GWAS data sets have considerably increased the power to detect genetic disease associations.[10] In this way, many susceptibility loci have been identified for coronary artery disease (CAD) and myocardial infarction,[1,11–13] as discussed in more detail in another review of this series. Also, chromosomal loci associated with stroke, PAD, and serum lipid levels have been revealed.[13–17] However, many of the identified loci still require independent replication to avoid false-positive results.[18–20] Studies examining PAD-associated chromosomal loci to unravel disease mechanisms are scarce.[21–23] Notably, several approaches have been made to open up the way for successful GWAS in addressing PAD with the help of electronic medical records, including the storage of DNA and plasma samples, as well as demographic data and laboratory values.[24,25]

Despite the recent explosion of GWAS-generated disease-associated susceptibility loci, single genetic loci or even their combination can only explain a small fraction of the risk for multifactorial diseases, such as atherosclerosis.[9] In addition, GWASs have the considerable drawback that the identified susceptibility loci mostly do not reveal direct information on disease genes or affected pathways, which is crucial for therapeutic drug discovery. Therefore, human GWAS-generated susceptibility loci have been combined with corresponding mouse disease QTLs in a cross-species comparative genomics approach to narrow down susceptibility intervals.[26] In addition, complementation of clinical trait QTL data with transcriptomics[17,27,28] and expression QTL (eQTL) mapping[29–32,] which aims to map chromosomal loci that are strongly associated with RNA expression (expression SNPs)-, can considerably facilitate candidate disease gene identification. For example, combination of GWAS and mouse clinical QTLs identified LSq1, a locus on mouse chromosome 7, to be responsible for the infarct volume of ischemic stroke in mice, and the risk allele of SNP rs4783244 is significantly associated with decreased plasma adiponectin levels, pointing to an increased risk of ischemic stroke.[33,34] Furthermore, a genome-wide SNP scan and atherosclerosis QTL mapping in intercrosses of mouse strains with different atherosclerosis-susceptibility were combined with macrophage gene expression profiling and transcription factor binding site analysis to reveal genes and promoter elements associated with atherosclerosis susceptibility. The genes *Wbscr* and *Aps*, which were differentially expressed in macrophages from mice with high versus low atherosclerosis susceptibility, mapped close to a specific atherosclerosis QTL on chromosome 5, indicating these genes as potential candidates influencing atherosclerosis risk.[35] In a comparable approach, QTL mapping for atherosclerotic plaque size in a segregating mouse population with variable atherosclerosis susceptibility was combined with transcriptome analysis of liver and adipose tissue and subsequent eQTL mapping, allowing a prioritization of the atherosclerosis QTL-associated candidate genes based on colocalization with *cis*-acting eQTLs.[29] Similarly, eQTLs affecting gene expression in human liver were screened for correlation with CAD-associated QTLs identified by GWASs, revealing the genes *SORT1*, *PSRC1*, *CELSR2*, and *SYPL2* on chromosome 1p13.3 as potential candidates for CAD development. Transcriptional analysis of these genes in the liver of a segregating mouse population with varying atherosclerosis risk furthermore indicated expression levels of *Sort1*, *Psrc1* and *Celsr2*, but not *Sypl2*, to be significantly associated with plasma LDL cholesterol levels, a risk factor for CAD. Combination of genotypic and transcriptomic data enabled the modeling of a genome-wide gene network for both human and mouse and revealed the 3 genes to colocalize in a species-conserved subnetwork causally associated with cholesterol levels and atherosclerosis.[31]

The combination of clinical trait QTL mapping with expression profiling or eQTL mapping in a network-based framework is called systems genetics or integrative genetics, and it has also been successfully applied to identify genes and pathways underlying other complex diseases, such as obesity.[36,37]

Network modeling is further discussed below, but a more comprehensive application of comparative genomics is outlined in a recent review.[26] Whether genetic screening will also be useful beyond cardiovascular disease gene identification (eg, for disease classification, outcome prediction, or therapy guidance) remains unclear.[17,38,39]

Transcriptomics

Transcriptomics entails the genome-wide study of RNA expression, mostly through microarray analysis. It is a widely applied technique for studying mechanisms underlying diseases, and it has also extensively been used to study expression profiles of atherosclerotic-prone or atherosclerotic ves-

sels,[40] predominantly in human[41–43] and mouse,[44–46] but also in rat,[47] monkey,[48] and pig.[49] Macrodissection[50–53] and laser-capture microdissection[54–59] have furthermore enabled scientists to isolate and analyze specific subregions within atherosclerotic vessels or lesions, such as, eg, plaque area versus media and adventitia,[55] fibrous cap,[50,51] lesion shoulder,[56] or adventitial aortic tertiary lymphoid organs.[57] In addition, these methods have allowed the enriched isolation of particular cell types, such as ECs[49,53,58] or macrophages,[59] for subsequent genome-wide expression profiling, enhancing our understanding of cell type-specific functions in atherogenesis. Given that metabolic disorders, such as dyslipidemia, obesity, and diabetes, are risk factors for CAD, it is not surprising that also transcriptome analysis of organs relevant to energy metabolism has increased our insight into molecular processes associated with the presence or risk of CAD, as shown for liver[60,61] and fat.[30,32,62] For example, using expression profiles from human atherosclerotic arterial wall, carotid stenosis tissue and visceral fat, an atherosclerosis-associated set of genes was identified, which was linked with the transendothelial leukocyte migration pathway and with the transcriptional cofactor LDB2 as a potential hierarchical regulator. Associated expression SNPs of this gene set showed an increased association with CAD.[62] In addition, integration of genotype, liver and adipose tissue gene expression profiles and aortic lesion size in a segregating mouse population identified 292 genes as potentially causal for aortic lesions. This causality was confirmed for *C3ar1* in a mouse knockout study, and the candidate causal genes, especially those from adipose tissue, were shown to strongly overlap with a plaque progression signature derived from transcriptional profiling of atherosclerotic aortic arches from 2 mouse models.[32] Interestingly, also expression profiles of peripheral blood cells, crucial inflammatory mediators and key players in atherosclerotic lesions, have been shown to correlate with the occurrence and extent of CAD,[63–65] and a gene expression-based blood test was recently developed to measure obstructive CAD in nondiabetic patients.[66] And although in vitro transcriptome studies fail to provide information on complex interactions between cells, organs and environment, they also have considerably contributed to our understanding of atherosclerosis-associated signaling pathways and biological processes in diverse cell types, as ECs,[53] SMCs[67] and monocytes.[68] For example, microarray analysis revealed an upregulation of *Wnt2* and *Wnt4* in platelet-derived growth factor–BB–induced, proliferating vascular SMCs (VSMCs) compared with unstimulated, resting VSMCs, and further in vitro studies showed Wnt4 to trigger VSMC proliferation through Frizzled 1. The role of Wnt4 in VSMC proliferation was confirmed in a *Wnt4*-deficient mouse model, demonstrating a significantly reduced intimal thickening and VSMC proliferation after carotid artery ligation.[67] In a similar approach, transcriptional profiling of human aortic ECs with knockdown of microRNA10a, which was found to be downregulated in the endothelium of atherosclerosis-susceptible regions of the aortic arch in swine, revealed nuclear factor-κB–mediated inflammation as a key upregulated process. This could be confirmed in swine aortic arch ECs,[53] validating in vitro experiments as a useful approach to gain insight into disease-related mechanisms. In addition, in vitro studies can provide first line indications of cellular responses to drugs. As such, transcriptional analysis of human ECs, SMCs, and hepatocarcinoma cells treated with orvastatin or pitavastatin showed statins to affect genes involved in coagulation, cell growth, and vascular constriction, providing insight into statins' cellular effects.[69]

Finally, profiling of microRNAs, which are major regulators of mRNA expression, can also offer insights in atherosclerosis-related mechanisms, and has been performed on injured vascular walls,[70] plasma,[71] blood,[65] and vascular cell types.[53] For instance, Fichtlscherer et al detected a significant reduction in the plasma concentration of vascular and inflammatory cell-derived microRNAs in patients with CAD, whereas cardiac muscle-microRNAs showed an increased trend.[71] Whether specific microRNAs could also be used as biomarkers to predict the course of disease or classification remains to be further examined.

In the context of cerebrovascular disease and stroke, transcriptomic analysis was mainly set up to gain insight in the blood transcriptome after injury. Stroke-associated gene expression changes, for example, may be reflected within this transcriptome and therefore mediate cerebral inflammatory changes. In addition, animal models comparing changes in gene expression in cerebral tissue revealed an important role for blood microRNAs, which have also gained interest as biomarkers for brain injury.[72,73] As an example, microRNA-124 has recently been described as biomarker for cerebral infarction.[74,75] Another study explored the transcriptomic profiling of cellular pathways being regulated after an in vivo transient cerebral ischemic stroke in mice mediated in the presence and absence of glutathione peroxidase 1. Glutathione peroxidase 1 is an enzyme that is important in reduction of oxidative stress, and its lack has been implicated in neurodegenerative disorders and stroke. Here, the authors were able to show that glutathione peroxidase 1 plays a crucial role in the protection against oxidative stress and inflammation during ischemia-reperfusion injury.[76]

Regarding PAD, Zhao et al performed a microarray analysis of 30 femoral artery samples identifying, for example, MAP4K4 as being differentially upregulated in intermediate and advanced lesions. Surprisingly, many of the differentially regulated genes in this study have not been reported to be related to atherosclerosis before.[77] A similar approach was used by Evans et al, who classified 335 genes as differentially regulated between normal and diseased arteries. Moreover, they were able to acknowledge a gene expression signature for PAD and an associated diabetic phenotype in PAD.[78]

Proteomics

Dynamic proteins are key regulators of biological functions. The total protein complement of an organism and its genome is called proteome. Methods trying to unravel protein structure, expression, modification, localization and protein-protein interactions are referred to as proteomics.[79–81] Most important proteomic methods comprise 2-dimensional gel electrophoresis (2-DE) or 2D differential in-gel electrophoresis, which is an improved 2-DE, including the fluorescent labeling of the different protein suspensions investigated. Moreover, gel–liquid chromatography–tandem mass spec-

trometry and shotgun (gel-free) proteomics are used to explore proteomic functions. Using gel-based proteomics, proteins are first separated by 1- or 2-DE before enzymatic digestion and mass spectrometry (MS) analysis. Shotgun proteomics uses the digested protein mixture directly without the need for gel separation.[79,82]

In the past decade, several attempts were made to exploit the VSMC[83–85] and EC[86–88] proteomes in different species; however, identification of signaling networks is complex.[80] For example, proteomic studies, including Western array analysis, screened human carotid artery specimens for key proteins and identified numerous genes which are differentially expressed under atherosclerotic conditions, eg, apoptosis linked gene 2 (*ALG2*) or Annexin 1.[89,90] Furthermore, metabolic and proteomic investigations of SMCs from *Apoe*$^{-/-}$ mice under normoxic versus hypoxic conditions showed a differential regulation of key enzymes in glucose metabolism, resulting in faster glucose consumption under hypoxia and a compensatory reduction in baseline interleukin (IL)-6 secretion, which was associated with a marked upregulation of insulin-like growth factor binding protein 3.[91] However, direct conclusions identifying signaling pathways remain elusive in the above-mentioned studies. Nevertheless, other studies, addressing more specific questions, did investigate signaling pathways by proteomic approaches: 2-DE identified the correlation of the strongly anti-apoptotic heat shock protein (HSP)-27 phosphorylation level with cardiovascular diseases, by identifying LDL as an important regulator of HSP27 phosphorylation involving p38–mitogen-activated protein kinase signaling in VSMCs.[92] Moreover, 2-DE and MS revealed that paracrine secretion of platelet-derived growth factor–BB by ECs is implicated in the pathophysiological vascular remodeling induced by low shear stress, involving the phosphorylation of extracellular signal-regulated kinases (ERK)1 and 2, which belong to the mitogen-activated protein kinase family.[93] In addition, the importance of ERK1 and ERK2 phosphorylation was shown in vitro in bovine aortic ECs after stimulation with oxidized phospholipids using liquid chromatography-mass spectrometry analysis.[94] Supplementally, proteomics has also been proven to be of emerging interest in atrial fibrillation–associated stroke,[3] cerebral infarction,[95] and focal cerebral ischemic injury,[96] identifying potential marker proteins such as HSP2 or HSP60. Furthermore, proteomic screening of prediagnostic plasma samples from postmenopausal women receiving a hormone therapy described B2M and insulin-like growth factor binding protein 4 as risk markers for CAD and stroke, respectively.[97] Considerable effort was also made to discover key proteins in PAD, which lead to the recognition of B2M and cysteine C as being associated with PAD, independently of the traditional risk factors of age, diabetes mellitus, hyperlipidemia, hypertension, and tobacco use.[98,99]

In summary, proteomic techniques are helpful to identify marker proteins of vascular inflammation and to unravel inflammation-associated signaling cascades, especially protein phosphorylation signaling networks, which can severely affect cellular processes.[100]

Metabolomics describes the (quantitative or qualitative) measurement of the endogenous metabolome, defined as the entire set of metabolites (small molecules, representing intermediates of the metabolism) within 1 organism.[81,101] Furthermore, metabolomics involves the investigation of the complex interactions of metabolites with each other (metabolism) or with other molecules (eg, genes or proteins). Metabolites, as the smallest entity of metabolomics, reflect dynamic and unique aspects of cellular homeostasis, which may provide insight into intricate molecular processes. Analytical methods to measure metabolites are MS and nuclear magnetic resonance spectroscopy.[102] However, to date, there are only a limited number of studies using these methods to unravel complex disease mechanisms and subsequent therapeutic options in cardiovascular disease.[103,104] It is noteworthy that Shah et al presented the first evaluation of heritable metabolite profiles in humans, investigating 117 individuals and 66 metabolites by quantitative MS-based metabolomics. Surprisingly, the heritabilities for many metabolites were very high, even higher than for conventional risk factors, implying a strong genetic impact on clustering of metabolic signatures in families burdened with CAD.[105] Another metabolomic study, combining nuclear magnetic resonance spectroscopy and gas chromatography–MS, investigated the metabolism in liver and other organs to examine the consequences of peroxisome proliferator–activated receptor (PPAR)-α loss with the help of Ppar-α-deficient mice. Ppar-α partially controls hunger and the feeling of satiety, and its lack resulted in hepatic steatosis and changes in lipid and glucose metabolism also affecting the cardiovascular system.[106] In addition, recent liquid chromatography-mass spectrometry metabolomics identified the plasma molecules choline, betaine, and trimethylamine *N*-oxide, all metabolites of the dietary lipid phosphatidylcholine, as risk factors for cardiovascular disease, which triggered the identification of a link between dietary lipid intake, intestinal microflora, and atherosclerosis.[107] Other recent metabolomic approaches expanded this finding, showing that gut microbiota affects host energy, systemic lipid metabolism, and hepatic metabolic profiles, altogether highlighting the role of gut microflora and its role in the development of metabolic diseases.[108–110] Furthermore, metabolomics were also used to study and introduce metabolic biomarkers of stroke and cerebrovascular diseases. Jung et al were able to demonstrate, that plasma of stroke patients was characterized by the increased excretion of lactate, pyruvate, glycolate, and formate, whereas the excretion of glutamine and methanol was decreased.[111] Other recent approaches using nuclear magnetic resonance–based metabolomic analysis of cerebrospinal fluid and serum showed reduced levels of serum betaine in patients with cerebrovascular disease, perhaps indicating a population at high risk for stroke, because betaine, a methyl donor, reduces levels of homocysteine, a known risk factor for cerebrovascular disorders. Interestingly, cerebrospinal fluid metabolite profiles did more accurately predict the diagnosis of multiple sclerosis than anticipated cerebrovascular disease.[112]

Hence, metabolomics will provide further insight into the mechanisms of cardiovascular physiology and may lead to improved therapeutic approaches.

A main risk factor for atherosclerosis is hyperlipidemia, caused by an imbalanced lipid metabolism of multifactorial

origin. The lipidome of an organism comprises all lipid molecules, which are additionally divided into lipid classes, and their associated interacting factors. Therefore, lipidomics is of outstanding interest for atherosclerotic research, also with regard to lipid distribution within cells and biochemical mechanisms mediated by lipids. The most important methods to enable high-throughput and high-quality lipid analysis are MS and lipid biochemistry, which make simultaneous identification and quantification of hundreds of molecular lipids within 1 species possible.[113,114] For example, comparing fractionized serum samples of diabetic patients versus healthy controls by liquid chromatography-mass spectrometry indicated that the serum concentrations of specific triacylglycerol acids are more accurate markers for insulin resistance and abdominal obesity than total serum triacylglycerol concentrations. Thus, fatty acid characteristics may become important markers identifying individual risk profiles for various diseases.[115] Furthermore, 1 example of how lipids may affect pathway signaling has been nicely discussed by Wheelock et al, who revealed 77 biochemical pathways to be differentially affected between low and high fat cholesterol diet in mice, by combining liver transcriptomics and lipidomics data and mapping them onto a metabolic pathway database.[116] System biology approaches like those introduced by Wheelock et al[116] are very helpful to process high-throughput data, which is further discussed below. In addition, changes in lipid profiles, including phospholipids, ceramides, sphingomyelin, cerebrosides, cholesterol, and their oxidized derivatives, may also affect or be indicative of ischemic stroke, as indicated by Fonteh and Fisher[117] and by Fonteh et al.[118]

Protein microarrays are an emerging tool, competing with traditional gel-based methods (eg, 2-DE), offering a higher reproducibility and the possibility of high-throughput approaches. These microarrays mostly consist of thousands of immobilized proteins spotted to a glass slide and enable simultaneous analysis of various protein-protein interactions at once.[119,120] Another example are cytokine antibody arrays, which use membrane-spotted capture antibodies to investigate the cytokine profile of biological fluids, a technique that became famous in cancer research to detect tumor markers.[121] As an example of its utility in vascular inflammation, the use of a cytokine array detecting 36 proteins released from vascular endothelial growth factor (VEGF)–stimulated ECs revealed a VEGF receptor 2–PKD1 axis-dependent production of IL-6, IL-8, and growth regulated oncogene-α in ECs but not in leukocytes, pointing at differential pathways induced by VEGF-dependent signaling in different cell types.[122] Furthermore, human cytomegalovirus, known to be proatherogenic by, eg, stimulation of angiogenesis, induced IL-6, IL-8, and granulocyte-colony stimulating factor secretion by infected ECs, as evidenced by human cytokine array screening for 174 different proteins in EC supernatants.[123] Again, modeling protein networks or pathways from here is still challenging and a mathematical exercise. However, there are already studies that apply quantitative mathematical models to dynamic immune interactions, although not explicitly for vascular biology.[124,125] Overall, proteomic techniques are promising tools to unravel complex network formation.

RNA Interference

The term RNA interference (RNAi) describes an RNA-dependent gene silencing process, which is initiated by short double-stranded RNA, resulting in a reduction of targeted mRNA levels. RNAi molecules comprise sets of double-stranded RNA, short hairpin (sh)RNA, silencing RNAs (siRNAs) or endoribonuclease-prepared siRNAs. These small molecules are able to induce a reduction-of-gene-function, which may lead to a phenotypically detectable variation. In turn, this variation may be monitored by specific assays. RNAi offers the possibility of performing both small-scale studies targeting 1 gene or high-throughput approaches investigating numerous genes and their function at once, though of course careful data interpretation is needed.[126] Although there are recent reviews[126–129] and original studies[130,131] summarizing and investigating high-throughput RNAi screening to unravel cellular signaling in general, studies addressing signaling cascades in vascular inflammation rather concentrated on knockdown of specific gene functions instead of investigating on large-scale basis. For example, silencing of bone morphogenetic proteins/Sma and Mad related proteins signaling in human aortic ECs,[132] downregulation of tumor necrosis factor–dependent nuclear factor-κB activation in ECs,[133] or silencing of PI3-AKT signaling in human umbilical vein ECs[134] was shown to have atheroprotective effects on the vascular endothelium. Furthermore, it was shown that simultaneous downregulation of the VEGF receptor 2/3-, CXCR4-, and CCR7-dependent signaling axes via AKT, ERK1/2, and p38 signaling pathways had a greater inhibitory effect on angiogenesis and cell growth than the blockade of each individual axis.[135] These results may be of special interest regarding atherosclerosis because the CXCR4/CXCL12 axis in ECs seems to play a protective role in atherosclerosis.[136,137] One other recent study of outstanding interest identified candidate genes involved in cellular cholesterol metabolism by transcriptional profiling of sterol-depleted HeLa cells, literature mining and a "multiple-gene" RNAi screen, which combined reverse siRNA transfection on siRNA microarrays with high-content screening microscopy and quantitative image analysis software to analyze effects on total cholesterol levels and LDL uptake efficiency.[138] Thus, these findings may offer subsequent therapeutic approaches in terms of atherosclerosis or even Niemann-Pick disease type C, which is an inherited lipid storage disorder, characterized by a defect in intracellular trafficking of exogenous cholesterol and glycosphingolipids, and in which regulators of cellular cholesterol accumulation have also been studied through an RNAi screen.[139] In addition, RNAi was helpful to unravel mechanisms important in cerebral ischemia-induced vascular and brain injury. Here, Yin et al. were able to show that Pparδ expression in mouse aortic VSMCs can prevent ischemic brain injury by inhibition of matrix metalloproteinase-9 activation and attenuation of postischemic inflammation in mice. RNAi-mediated knockdown of matrix metalloproteinase-9 in Pparδ knockout mice phenocopied these effects, rendering pharmacological activation of Pparδ a promising therapeutic tool for stroke-induced vascular and neuronal damage.[140] Similarly to matrix metalloproteinase-9, silencing of caspase-3 in mice by siRNA

treatment also exhibited beneficial effects in the cure of stroke.[141] Another approach, investigating the plasma concentrations of circulating angiogenesis-related factors like VEGF, placenta growth factor, and thrombospondin-1 in 184 PAD patients and in 330 paired healthy controls demonstrated an increase of TSP-1 plasma levels in the disease group. Additional in vitro studies, knocking down TSP-1 with siRNA in human endothelial colony-forming cells, unraveled a potential mechanism by which increased TSP-1 levels might be responsible for inadequate neovascularization. Hence, targeting TSP-1 ex vivo or in vivo might have the potential to modulate angiogenesis in PAD patients.[142] Overall, RNAi is a powerful tool to investigate inflammatory networks and cellular signaling, although it is still mostly used on a small scale in the context of vascular inflammation and cardiovascular disease.

Epigenomics

Epigenomics involves the study of phenotypic or gene expression changes caused by inheritable mechanisms independent of DNA sequence, eg, DNA methylation and histone posttranslational modifications. Also, noncoding RNA alterations can be classified as epigenetic mechanisms, as they have been shown to mediate epigenetic DNA and histone modifications.[143] Epigenetic marks are increasingly being recognized as important elements underlying phenotypic variation, biological processes, inflammation, and diseases such as cancer, and large-scale epigenome projects have been set up in an attempt to create reference human epigenome maps.[144] High-throughput techniques enabling epigenetic analysis on a genome-wide scale include, eg, shotgun bisulfite sequencing and pyrosequencing to analyze DNA methylation,[145] and genome-scale chromatin immunoprecipitation with antibodies recognizing specific histone modifications or DNA methylation, followed by microarray analysis or high-throughput sequencing.[146,147] Alternative methods to examine DNA methylation are differential methylation hybridization, which encompasses methylation-sensitive DNA restriction followed by microarray analysis of the restriction-protected, hypermethylated DNA,[148] bead array methylation analysis of bisulfite-treated DNA,[149] and base-specific cleavage combined with matrix-assisted laser desorption ionization/time of flight.[150] However, epigenetics is cell dependent and subjected to environmental factors, urging disease-specific studies to uncover epigenetic changes associated with disease. Compared with, for example, cancer research, the cardiovascular field is still in its infancy of epigenetic research. Initial studies have addressed vascular inflammation–related epigenetic changes on a global level (examining global changes in epigenetics without linkage to specific gene promoters) or have focused on specific genes. For example, genomic DNA in human atherosclerotic lesions has been shown to be hypomethylated, whereas inconsistent results have been obtained in peripheral blood lymphocytes from patients with cardiovascular disease.[151–153] Hypermethylated DNA has been detected in ischemic brain tissue after middle cerebral artery occlusion in mice and may promote cell death.[154,155] Immunohistochemistry for specific histone methylation modifications revealed prenatal exposure to apolipoprotein E deficiency and postnatal hypercholesterolemia to be associated with cell-specific histone methylation changes in carotid artery of mice.[156] Furthermore, oxidized LDL treatment of cultured human ECs induced histone phosphorylation and acetylation, both globally (as detected by Western blot analysis) and on the promoters of *MCP-1* and *IL-8* (as shown by chromatin immunoprecipitation).[157] Interestingly, histone modifications could be reversed by statin pretreatment.[157] Also methylation changes have been detected in the promoters of genes involved in atherogenesis, such as estrogen receptor α, endothelial nitric oxide synthase, and 15 lipoxygenase, as comprehensively reviewed in recent reports.[158,159] In contrast, only few studies are available examining the relationship between epigenetics and vascular inflammation on a high-throughput, genome-wide level. For example, Breton et al investigated the effect of prenatal exposure to tobacco smoke, a risk factor for cardiovascular disease, on global and genome-wide gene-specific DNA methylation in children, using bisulfite sequencing of DNA repetitive elements and gene methylation bead array analysis of child buccal cell DNA.[160] Eight genes, including *AXL* and *PTPRO*, were shown to be differentially methylated on maternal smoking.[160] In a comparable approach, array-based genome-wide methylation profiling of peripheral blood DNA revealed that heavy smokers displayed a significantly reduced methylation of *F2RL3*, a gene with known functions in cardiovascular disease.[161] Furthermore, differential methylation hybridization discovered 204 gene promoter regions to be differentially methylated in the fetal liver genome of mouse embryos with prenatal low-protein diet. Among these was the promoter of liver X receptor (Lxr) α, a nuclear receptor involved in cholesterol metabolism. *Lxr*α hypermethylation was linked to reduced expression of Lxrα and its target genes, speculating long-term effects on lipid metabolism and potentially cardiovascular disease.[148] Movassagh et al analyzed DNA methylation in human end-stage cardiomyopathy samples using methylated DNA immunoprecipitation and microarray analysis, and identified 3 angiogenesis-related genetic loci that were differentially methylated.[162] Expression of the genes was furthermore shown to be significantly altered between diseased and control hearts.[162]

Much more research is required in the field of cardiovascular epigenetics to increase our insights into disease-associated epigenetic patterns, and to clarify whether epigenetic changes truly contribute to pathogenesis or whether they are merely a result of pathological processes. Global hepatic DNA methylation in postmortem samples of young adults, measured by bisulfite sequencing, did not indicate an association of global DNA methylation with subclinical atherosclerosis.[163] However, this does not exclude the possibility that specific epigenetic marks are causally associated with pathogenesis, which could offer therapeutic options given the reversible nature of epigenetic modifications.

Analysis and Integration of High-Throughput Data: Network Modeling and Systems-Based Biology

All these "-omics" techniques generate an enormous wealth of data, urging computational methods to extract all con-

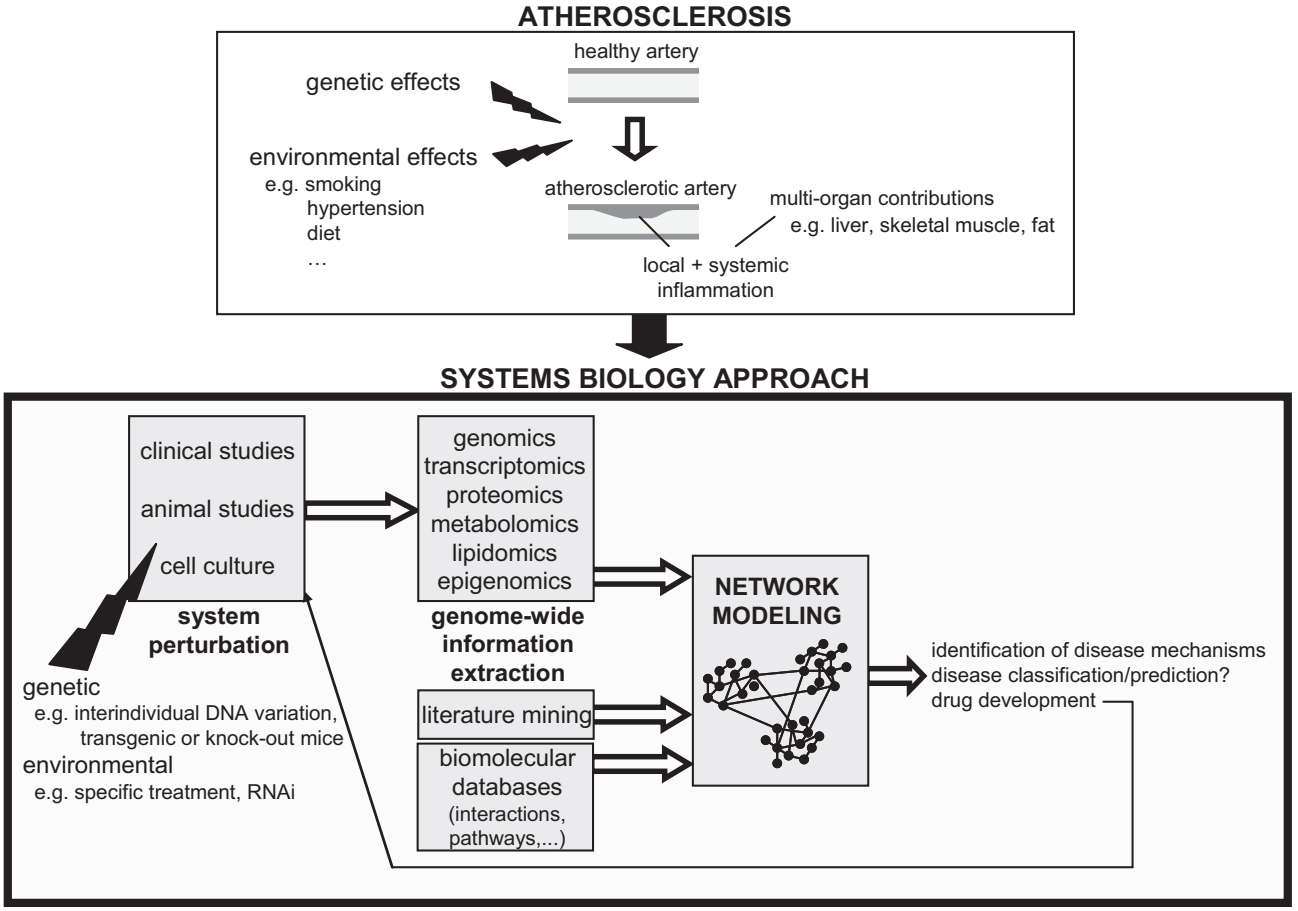

Figure. A systems biology approach to atherosclerosis. To uncover mechanisms underlying multifactorial diseases, such as atherosclerosis, high-throughput "omics" techniques are applied in clinical studies and experimental models, including animal studies and in vitro experiments. Integration of the resulting large-scale data sets in combination with intensive literature and database mining allows the modeling of complex biological networks representing biological processes, which on perturbation can shift from a healthy to a diseased state. Such a systems biology approach has an unprecedented power to uncover disease-associated mechanisms, which in turn provide a strong base for therapeutic drug design. It can also trigger the discovery of new disease biomarkers in the hope of enabling improved disease classification and prediction, bringing personalized medicine a step closer. RNAi indicates RNA interference.

cealed information. Systems biology aims to model biological processes as networks, generating graphical maps consisting of nodes and edges representing the individual system components and their relations, respectively. For a protein network, for example, edges can represent physical protein interactions, functional similarities, or the regulation of 1 protein by another. A high internal connection between a subset of nodes results in their clustering into modules, which represent key control points within the network. By combining multiple high-throughput data sets generated by in vitro and in vivo studies, with intensive literature mining and biomolecular databases of, eg, gene classification, protein interactions and pathways, network analysis has the strength to reveal disease-associated components and connections that would not have been discovered with classical approaches.[164,165] Furthermore, mathematical models can be developed to simulate network behavior on perturbations. Iterative optimization of the model by additional, hypothesis-driven system perturbations and data integration is essential to increase its precision and its ability to accurately predict the dynamics of the system studied (for example, a patient's response to drugs, or disease progression) (Figure).[164,165] Although the development of such mathematical, disease-predicting models is still in its infancy, network analysis of high throughput data sets has already proven a powerful tool to increase our insights into complex diseases.

In the context of vascular inflammation and atherosclerosis, network analysis combining transcriptomics and literature mining revealed candidate gene sets involved in human in-stent restenosis[41] and coronary and carotid atherosclerosis.[43,166] Transcriptional profiling of lesions in atherosclerosis-prone mice with a human-like hypercholesterolemia and a genetic switch to lower plasma LDL cholesterol revealed a set of cholesterol-responsive atherosclerosis genes, of which a subset could be modeled into a gene regulatory network of foam cell formation based on additional expression profiling of acetylated LDL-exposed macrophages targeted with specific siRNAs.[45] In the same context, network modeling and pathway analysis of transcriptomics data from oxidized LDL-treated macrophages identified biological processes relevant to foam cell formation, such as regulation of macrophage differentiation and lipoprotein catabolic processes.[164] A pathway analysis approach has also been successfully applied on CAD-associated GWAS data, identifying perturbations in

VSMC contraction, among others, as a key process in atherosclerosis.[167] Furthermore, gene expression profiling, promoter sequence analysis, and temporal expression comparison between transcription factors and gene targets were integrated to model the dynamic transcriptional response of Toll-like receptor signaling in macrophages.[168,169] In atherosclerosis-prone mice, network modeling based on liver transcriptome and lipid metabolome data sets revealed a close link between liver inflammation and lipid metabolism on dietary cholesterol intake, and identified transcriptional regulators coregulating both processes.[60] In addition, a systems biology approach combining transcriptional profiling, proteomics, cytokine arrays, and pathway analysis revealed genes, proteins, and biological processes associated with late-phase responses of lipopolysaccharide-stimulated rat ECs and showed lipopolysaccharide to induce, for instance, nuclear factor-κB-mediated inflammatory signaling, in addition to self-protective responses by upregulating mediators of anti-inflammation, antiapoptosis, and antioxidation.[170]

Using transcriptional profiling of swine EC samples isolated from arterial regions with variable atherosclerosis susceptibility, gene coexpression networks were modeled on the basis of coregulated expression and suggested the presence of endoplasmic reticulum and oxidative stress in susceptible sites.[49] Also interindividual genetic variability can be exploited to subdivide genes into coregulated modules. For example, intercrosses of inbred mouse strains with dramatic differences in high-density lipoprotein metabolism were used to model gene coexpression networks and identify modules that were significantly associated with high-density lipoprotein cholesterol levels.[171] Interestingly, some of the genes within these modules had previously been identified as candidate genes associated with plasma lipids in human GWASs.[17] Comparably, coexpression networks based on expression profiles of EC cultures from multiple heart transplant donors resulted in the identification of pathways, novel gene functions and regulatory relationships in the human EC response to the oxidized phospholipid oxidized palmitoyl arachidonyl phosphatidylcholine.[28,172] For instance, the unfolded protein response and the transcription factor ATF4 were revealed as important mediators of inflammatory IL-8 production in oxidized palmitoyl arachidonyl phosphatidylcholine-stimulated ECs,[172] and GPR39 was found to control the expression of heme oxygenase 1, a known regulator of inflammation, by combination of the modeled network with CAD-associated GWAS data sets.[28] The latter furthermore exemplifies that the integration of expression profiling or eQTL mapping with GWAS data increases the power to detect disease-associated genes, as also discussed above, and the simultaneous analysis of genotype, transcriptome, and clinical trait can even enable the construction of networks that can predict causal relationships between genes and disease.[36]

A systems biology approach has also been used to meet the complexity of the proangiogenic function of the VEGF family, not only in terms of CAD, but also for stroke and PAD.[173] In addition, a predictive network model, evaluating the transcriptome of whole blood from a mouse model of neuroprotection in ischemic stroke, was described, and it proved to be able to precisely predict mammalian system behavior under novel conditions.[174]

Far from being complete, these examples demonstrate how network modeling has contributed to the analysis of high-throughput data sets and the identification of new cardiovascular disease-associated genes and pathways that would not have been discovered with the traditional, single-analysis approaches. In this way, networks could trigger the development of new diagnostic markers (eg, of atherosclerosis) by revealing targets that accurately predict lesion vulnerability.[44] Accordingly, systems biology and predictive network modeling may be used to develop concrete clinical applications helping to improve, for example, patient selection or monitoring of stroke preventive intervention.[174] In addition, the subdivision of networks of multifactorial diseases in subnetworks or modules indicates that multiple genes and pathways probably need to be tackled together to treat the disease as efficient as possible, and could offer help in the design of a combination therapy. For more detailed information on network modeling, and an overview of currently available tools and databases, we refer to previous in-depth reviews.[116,164,165,175]

Evaluation and Conclusions

High-throughput technologies comprising all omics techniques, as well as RNAi, offer great advantages to profile the complex pathophysiology of human cardiovascular disease. The combination of clinical trait QTLs and eQTLs enables the matching of a specific chromosomal locus to a phenotypic trait through gene expression. In addition, proteomic techniques such as 2-DE or LC/MS offer the possibility of identifying altered or differentially expressed proteins, which influence cellular signaling cascades or protein phosphorylation. In the same context, protein arrays explore protein contents of biological fluids. Moreover, metabolomics permits insight into dynamic and unique aspects of cellular homeostasis and general metabolism, similar to lipidomics, which helps to understand imbalances in lipid metabolism, the latter being of outstanding interest in atherosclerosis. In addition, approaches using RNAi arrays enable the specific knockdown of numerous target genes, leading to the detection of signaling pathways and subsequent phenotypic changes. Finally, epigenomics is rapidly gaining interest. It is not only expected to fill the gaps in our knowledge of cardiovascular disease risk but also represents an interesting area for drug discovery, given the reversible nature of epigenetic patterns.

It is expected that the refinement, expansion, and cost reduction of high-throughput methods in all these fields will increase data generation even more. However, the main challenge will be to extract all hidden information to enhance our insights in the pathophysiological processes of cardiovascular disease, and to trigger clinical application. For example, genomic information is not yet widely applied in clinical cardiology, in contrast to clinical practice in oncology. One reason could be the missing heritability of GWAS-identified risk SNPs, which may be due to low-frequency variants and epigenetics. Another reason is the lack of functional annotations of these SNPs and their links with cardiovascular disease. To fulfill the requirements of a clinical use, for example as a biomarker, these polymorphisms would have to meet the criteria of a preclinical marker, which is able to indicate pathogenic mechanisms for concrete diagnostic or

interventional approaches. Likewise, epigenetic changes require translation into associated alterations in gene regulation and cellular signaling. Therefore, integration of all omics techniques is of extreme importance to extract functional links between omics-generated data sets and pathological processes, and to open the way for novel drug discovery. In this context, combination of genomics with transcriptomics has indeed proven to be very successful in identifying disease-associated genes and pathways. Enhanced integration of multidimensional data sets will unequivocally require systems biology approaches. Ultimately, it is hoped that accurate network modeling of diseases in such systems biology approach can be used to predict therapeutic responses and potential side effects and to trigger personalized medicine by integrating complex genetic and environmental aspects of multifactorial diseases, such as cardiovascular disease. Given these extreme challenges, the future success of systems biology requires a continuous expansion and refinement of software for high-throughput data acquisition, storage and integrative analysis through network-based approaches. Obviously, clear ethical guidelines how to deal with results, also in terms of, for example, life insurance, will be indispensable.

Overall, high-throughput techniques are promising tools to unravel the complex biological networks underlying vascular inflammation and atherosclerosis.

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