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# The Molecular Basis of Predicting Atherosclerotic Cardiovascular Disease Risk

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## Abstract

Atherosclerotic cardiovascular disease (ASCVD) proceeds through a series of stages: initiation, progression (or regression), and complications. By integrating known biology regarding molecular signatures of each stage with recent advances in high-dimensional molecular data acquisition platforms (to assay the genome, epigenome, transcriptome, proteome, metabolome, and gut microbiome), “snapshots” of each phase of ASCVD development can be captured. In this review, we will summarize emerging approaches for assessment of ASCVD disease risk in humans using peripheral blood molecular signatures and molecular imaging approaches. We will then discuss the potential (and challenges) for these snapshots to be integrated into a personalized movie providing dynamic readouts of an individual’s ASCVD risk status throughout the life course.

### Keywords

Cardiovascular disease risk prediction; prevention; genomics; proteomics; metabolomics; Biomarkers; Cardiovascular Disease; Primary Prevention; Atherosclerosis

## INTRODUCTION

Atherosclerotic cardiovascular disease (ASCVD) remains the leading cause of mortality worldwide accounting for approximately 17.6 million deaths annually.[1] At the population level, traditional clinical risk factors explain a large proportion of the attributable risk,[2] but their ability to predict future CVD events in individuals is more limited.[3] Combining recent advances in our understanding of the molecular biology of atherosclerosis development in experimental models[4] with the emerging capability to ascertain corresponding molecular data *in vivo* in humans may herald the next frontier for ASCVD risk assessment.[5]

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Atherosclerotic CVD can be conceptualized in three phases: initiation, progression, and complications.[4] "Snapshots" of each phase can be ascertained by integrating known biology with information gained through high-throughput, high-dimensional molecular data acquisition platforms, genomics, and molecular imaging. The challenge (and opportunity) is to integrate these static snapshots into a dynamic movie that details the evolution of ASCVD over the life course. In this review, we will summarize the primary biological mechanisms underlying ASCVD, discuss *state-of-the-art* approaches for assessment of disease risk and activity in humans, and offer projections for how molecular insights may be integrated into clinical risk prediction approaches in the coming years.

## ATHEROSCLEROSIS INITIATION

### Molecular Biology

Initiation of atherosclerosis fundamentally involves three processes: atherogenic lipid deposition, pro-inflammatory conditions, and endothelial dysfunction (Figure 1). Atherogenic apolipoprotein B-containing lipoproteins (mainly low-density lipoprotein cholesterol, LDL-C) initiate atherosclerosis by depositing in the arterial intima.[4,6] This deposition is directly related to circulating levels of atherogenic lipoproteins, with recent evidence suggesting that atherosclerosis would probably not occur with LDL-C levels not in excess of physiologic needs (10–20 mg/dL).[7] Retained LDL-C particles contribute to atherogenesis via promotion of macrophage transition to atherogenic foam cells, by stimulating immunologic responses, and through the formation of reactive oxygen species and other inflammatory mediators.[4,8,9] LDL-C in the arterial wall also directly binds to intimal proteoglycans and lipid-proteoglycan aggregates enter smooth muscle cells to further support growth of the developing atheroma (plaque).[10,11] Other lipid particles are involved in atherogenesis. Triglycerides are causally linked with atherosclerosis[12] and may act primarily through pro-inflammatory pathways.[13] Despite strong inverse associations for high-density lipoprotein cholesterol (HDL-C) with atherosclerotic events in observational studies,[14] its causal role in protecting against atherosclerosis has been questioned by some studies of genetically-mediated HDL-C levels, and by trials of HDL-C raising drugs that have not yet proven to be clinically beneficial.[4] Lipoprotein(a), which has a similar structure to the LDL-C particle but with the addition of an apolipoprotein(a) molecule, has both pro-inflammatory and pro-atherogenic effects[15] that explain its causal relationship with atherosclerosis.[16]

Inflammation is integral to the initiation of atherogenesis. High levels of circulating LDL-C promote arterial wall inflammation and stimulate the conversion of the pro-inflammatory mediator interleukin-1ß to its bioactive cytokine.[17] Indeed, the promotion of systemic inflammation represents an important mechanistic link between clinical risk factors and ASCVD. High blood pressure, smoking, and greater amounts of visceral adiposity – which correlates with insulin resistance and type 2 diabetes mellitus – all lead to upregulation of systemic inflammatory responses.[4,18] This systemic inflammatory response can be measured by pro-inflammatory markers such as C-reactive protein (CRP), which is robustly – but perhaps not causally[19] – linked to CVD risk.[20] One end-result of a heightened inflammatory milieu is the activation of vascular endothelium to express chemoattractants and adhesion

molecules, thereby leading to leukocyte migration and adhesion.[21] Adaptive immunity also plays an important role in atherogenesis and both B and T lymphocytes can be identified within the microarchitecture of developing plaques.[4,22] The observation that certain helper T-cell subsets promote atherosclerosis, while others counteract it,[17] suggests that adaptive immunity may be a key modulator of the atherosclerotic process.

The vascular endothelium represents the interface between circulating blood and the arterial intima, where atherosclerosis formation occurs. Endothelial damage and dysfunction (due to exposure to risk factors) contribute directly to atherogenesis. Lower bioavailability of endothelial nitric oxide results from endothelial damage and impairs the maintenance of laminar blood flow and vascular hemostasis.[23] Dysfunctional endothelium expresses vascular adhesion molecules that attract leukocytes and further contribute to the development and growth of plaques.[23,24]

## Assessing atherosclerosis initiation in humans

Atherosclerosis initiation, therefore, relies on a systemic pro-atherogenic milieu that may be particularly amenable to interrogation via detailed profiling of circulating molecules and their genetic determinants. Evidence supporting the measurement of various “omics” levels to improve prediction and identification of atherosclerosis CVD development are discussed in the following section.

**Genetics—**Identification of genetic predispositions to ASCVD have in many cases validated decades of observational and experimental data. Principally, investigations of genetic determinants of lipid levels confirm the powerful role of elevated LDL-C in atherosclerosis development.[25] Loss of function mutations in the LDL-C receptor lead to reduced clearance of LDL-C from the blood and resulting high circulating levels of LDL-C. Individuals inheriting LDL-C receptor mutations from one parent (heterozygous) often have LDL-C levels >200 mg/dL and can have premature clinical CVD events in their third and fourth decades of life, and individuals inheriting two copies of the defective LDL-C receptor genes (homozygous familial hypercholesterolemia) have LDL-C levels of 650–1000 mg/dL and can experience CVD events in their teens or early twenties.[26,27] Conversely, loss of function mutations of the proprotein convertase subtilisin/kexin type 9 protein (PCSK9), which removes LDL-C receptors from the surface of hepatocytes, lead to ≈28% reductions in LDL-C cholesterol levels and accordingly are associated with an 88% percent relative risk reduction in coronary heart disease.[28] The causal role for LDL-C in ASCVD pathogenesis is further supported by Mendelian randomization studies demonstrating that each genetically-determined 38.7 mg/dl lower LDL-C is associated with a 55% reduction in coronary heart disease.[29]

Common variant genetic association studies have also provided evidence supporting causal roles for other putative contributors to atherosclerosis initiation such as atherogenic lipid particles (triglycerides), pro-inflammatory pathways (insulin resistance and inflammation), and endothelial dysfunction (cell adhesion and proliferation, nitric oxide signaling, and vascular remodeling).[30–32] For example, loss of function variants in the *ANGPTL4* gene,

which encodes the angiopoietin-like 4 protein, result in 35% lower triglyceride levels and reduced coronary artery disease (CAD) risk.[33]

In addition, loss of function mutations in the nitric oxide receptor component guanylate cyclase 1, soluble, alpha 3 (GUCY1A3) are associated with early CVD[34] attesting to the central role of endothelial dysfunction in promoting atherosclerosis. Similarly, multiple lines of evidence including genome wide association,[35] Mendelian randomization,[36] and mechanistic studies[37,38] implicate loss of function mutations in the CXCR4/CXCL12 pathway (which is integral to maintaining vascular integrity) as causal for ASCVD development.

On the other hand, the majority of CVD-associated variants are located in non-coding chromosomal regions and their mechanistic links to CVD pathogenesis can be challenging to elucidate.[30] One example of this is the Chr9p21 risk locus, which is consistently observed to be the most highly significant ASCVD risk locus in genome-wide association studies.[39] The Chr9p21 locus contains no protein-coding genes, but does include some exons of the antisense noncoding RNA in the INK4 locus (*ANRIL*).[30] Recently, studies have shown that variants affecting *ANRIL* abundance and splicing may contribute to atherogenesis, but the exact mechanisms linking variants in the Chr9p21 region with ASCVD risk remain elusive.[39]

With the use of ever-larger discovery datasets, the current list of genetic variants associated with ASCVD now numbers >150.[30] As the effect size for each of these variants is low, combining many (or even all) of the potential risk variants in polygenic risk scores may provide the ability to more comprehensively assess an individual's CVD risk.[40–43] In addition to including genetic variants reaching genome-wide statistical significance, several groups have recently expanded the concept of polygenic risk to include all risk-predicting variants across the human genome.[44–46] With this approach, a genome-wide polygenic score comprising >6 million single nucleotide polymorphisms can identify 8% of the population to be at >3-fold higher risk of CAD, which rivals the risk for rare monogenic mutations.[44]

Whereas the discovery of genetic determinants of ASCVD have validated experimental observations of contributors to atherosclerosis initiation, and identified important druggable targets,[28] their translation to clinical risk prediction is currently limited. For elevated lipid levels, understanding the genetic risk is scientifically important, but it is unlikely to provide incremental predictive information beyond lipid measurement itself. Polygenic CVD risk scores may be useful in identifying individuals who are most likely to benefit from lipid-lowering therapies,[40] but the ability of polygenic risk scores to provide incremental information to clinical risk factors alone for clinical prediction and management of CVD events has not been fully established.[47,48] Several other unanswered questions regarding the clinical translation of polygenic risk scores remain including optimal modeling strategies, most appropriate ages for assessment, and how to account for gene-gene, gene-environment, or gene-risk factor interactions.[49] Moreover, polygenic risk scores have primarily been derived and validated in populations of European descent; calculation of polygenic risk scores in other racial and ethnic groups is necessary before they can be used widely for ASCVD risk assessment.[30,50,51]

**Epigenetics—**Epigenetic modifications both reflect and contribute to the development of ASCVD and, therefore, represent intriguing biomarkers for risk prediction.[52] Variability in DNA methylation (binding of a methyl group to the 5' carbon of cytosine in cytosine-guanine dinucleotide sequences) is heritable, relates to aging, and can be altered by environmental exposures and CVD risk factors.[53] Differential DNA methylation also exerts important regulatory effects on gene expression, which can affect drivers of atherosclerosis such as lipid levels[54] and pro-inflammatory cytokines.[55] While analysis of DNA methylation requires collection and preparation of cells (frequently circulating white blood cells), non-coding RNAs serve as epigenetic biomarkers that are released into the bloodstream and are readily detectable in peripheral blood samples. MicroRNAs (miRNAs) are small, single-stranded, noncoding RNAs that modulate myriad biological processes including lipid metabolism,[56,57] endothelial cell dysfunction, and inflammation,[56] and may prove as useful biomarkers for CVD risk assessment.[58–60] MiRNA profiling and methylation signatures can be combined to provide an even broader view of epigenomic regulation of gene expression in relation to ASCVD risk.[61]

**Transcriptomics—**Numerous studies have sought to identify gene expression signatures of atherosclerosis and CVD risk by measuring messenger RNA (mRNA) in peripheral blood, but few candidate transcripts have validated across studies.[62] In one intriguing investigation, Yao and colleagues integrated genetic and transcriptomic data to identify 13 CVD-related gene expression modules that were associated with inter-individual phenotypic variation in clinical risk factors.[63] While these observations require further validation, they demonstrate the potential to combine genetics and transcriptional profiling of circulating cells to more precisely characterize CVD risk phenotypes.

Transcriptional profiling of individual cell types has the potential to provide dynamic insights regarding atherosclerosis initiation and development. In experimental models, single-cell RNA sequencing of cell types within plaques, such as macrophages, reveal unique cellular populations with distinct gene expression profiles and different relations to stages of atherosclerosis.[64,65] Methods are improving to harvest specific cell types directly from humans enabling more targeted transcriptional profiling or single-cell RNA sequencing.[66] In one example of the information that can be gained from transcriptional profiling of specific types of cell, activated human platelet phenotypes in circulating blood can be detected in response to circulating inflammatory mediators,[67,68] potentially identifying "higher risk" platelet phenotypes that may be amenable to targeted interventions.

**Proteomics—**Multiplexed, high-throughput proteomic profiling platforms are now capable of assaying 1,000 to 5,000 of the ≈20,000 proteins in the human proteome. Human plasma represents the '*largest and deepest version*' of the human proteome (the secretome reflects 15% of the body's proteins).[69,70] Blood, therefore, is one of the best '*reporter systems*' for detecting disease onset and progression. Blood tests are easy to obtain, inexpensive, and can be repeated even in frail, elderly people.[71] Yet, measuring blood proteins is challenging. The concentration range of plasma proteins spans ten orders of magnitude resulting in the propensity for high-abundance proteins (range of g/ml) to mask low-abundance proteins (e.g., cytokines, with levels in μg/ml to pg/ml). The advent of microfluidics, multiplexing,

and miniaturization have enabled ‘lab-on-chip’ tests compatible with point-of-care testing and population screening. Aptamer array platforms using affinity capture methods[72,73] now facilitate automated multiplexed quantification of thousands of very low abundance proteins with small sample volumes, high throughput, and rapid scalability enabling profiling of large samples. These platforms have excellent analytic and clinical validity, with very high correlations with conventional laboratory-based assays for a variety of low abundance markers.[74–76]

These newer proteomic technologies have been applied to the discovery of novel protein biomarkers of ASCVD risk in the blood,[74] with identification of proteins related to ASCVD risk factor burden,[77] varied measures of cardiovascular health,[78] and clinical CVD events.[79] Indeed, multi-protein scores may predict CVD with better accuracy than risk scores based solely on traditional risk factors.[79,80] Thus far, these broad, unbiased, proteomic profiling efforts have identified novel ASCVD risk proteins, some that have been associated with atheroma biology in experimental models and some for which the function is unknown and require further investigation. Initial efforts in proteomic discovery highlight the possibility that by assaying thousands of circulating proteins, we may obtain a more precise “snapshot” of an individual’s health status.

**Metabolomics—**Small molecule chemical intermediates (metabolites) representing different chemical categories such as amino acids, lipids, or by-products of drug or food metabolism can be detected in biological samples.[81] Several of these metabolites have been demonstrated to relate to cardiometabolic risk in humans. Higher levels of circulating branched chain amino acids, for example, are correlated with insulin resistance,[82,83] decrease with weight loss,[84] are associated with future development of diabetes in community-dwelling individuals,[85] and relate to future ASCVD risk.[86] Metabolite profiling methods can also be used to assay a large number of bioactive lipids, thereby enabling more precise quantification of the atherogenic lipidome.[87] Higher levels of circulating monoglycerides produced via hydrolysis of triglycerides, for instance, are associated with higher CVD risk, while higher levels of circulating lysophosphatidylcholines may inhibit macrophage biosynthesis and cellular cholesterol accumulation and therefore provide cardioprotection.[88,89] Certain types of the bioactive sphingolipid ceramide and sphingomyelin species are also associated with higher CVD risk.[90,91]

The circulating metabolome may also contain exogenous substances reflecting relevant external exposures.[92] For example, Breitner *et al.* demonstrated that metabolite concentrations are affected by ambient particulate matter and ozone concentration in hospitalized patients.[93] Such assessments could enable precise quantification of individual exposures (exposome), and host-exposure interactions.

Hence, circulating metabolite levels reflect multiparametric host responses and external exposures, and can, therefore, integrate a large amount of potential information for ASCVD risk assessment. One important application of metabolomics in clinical practice may be its ability to explain some of the inter-individual variability in the links between clinical risk factors and future clinical ASCVD. In obese individuals, metabolite profiles can help distinguish between metabolically healthy and metabolically unhealthy obesity

subphenotypes.[94] Beyond improving the precision of traditional risk factor assessment, metabolomic profiling can also be used to define novel phenotypes. An example of this is the emerging endophenotype termed “metabolic flexibility,” which represents an organism’s adaptive capability to maintain homeostasis under a variety of metabolic conditions or differences in the availability of energy sources.[95] An important challenge to the application of metabolomic data to clinical use is the relatively high intra-individual variation that can be observed in metabolite levels over time.[96]

**Gut microbiome—**Atherosclerotic plaques contain DNA from the same bacteria taxa as that found in the gut and oral cavity,[97,98] indicating that microbial communities might contribute to and affect plaque formation. The composition of the gut microbiota can also impact host lipid levels, especially triglycerides.[99–102] One product of microbial metabolism – trimethylamine *N*-oxide (TMAO), the hepatic oxidation product of the microbial metabolite trimethylamine (TMA) – has been shown to have a particularly strong association with atherosclerosis risk,[103] however there are conflicting reports of inverse association of TMAO and ASCVD.[104,105] TMA is rapidly oxidized to TMAO by flavin monooxygenase enzymes in the liver and released into the circulation.[106] Circulating TMAO levels are associated with the presence of atherosclerotic plaques[99] and CVD events.[107] The causal role of TMAO in atherosclerosis is supported by a recent observation that a small molecule inhibitor of TMA lyase activity suppressed TMAO formation, macrophage foam cell formation, and atherosclerosis in mice.[108] It remains to be seen whether profiling of gut microbiome features and/or TMAO levels may prove to be an important adjunct to current risk prediction methods.

**Clonal hematopoiesis of indeterminate potential—**Clonal hematopoiesis of indeterminant potential (CHIP) characterizes somatic mutations in bone marrow hematopoietic stem cells that confer a proliferative advantage and thereby promote clonal proliferation of myeloid lineages in the peripheral blood. CHIP clones can be identified via genetic sequencing of peripheral blood. They are present in 10% of individuals above age 70 years and are known to promote thrombosis and accelerate atherosclerosis.[109,110] Although precise mechanisms are incompletely elucidated, CHIP is theorized to contribute to ASCVD risk via stimulation of inflammation and innate immunity. Given its association with ASCVD independent of clinical risk factors,[109] CHIP represents a promising avenue for further study to assess its ability, if any, to improve clinical ASCVD risk prediction.

## ATHEROSCLEROSIS PROGRESSION

### Molecular Biology

Developing atherosclerotic plaques can be influenced by a number of diverse features serving to either promote or inhibit their growth and evolution (Figure 1). Atherosclerotic plaque progression traditionally proceeds through continued accumulation of lipid and formation of lipid-engorged cells.[4] Migration of smooth muscle cells from the vascular media into the intima support the growing plaque and release extracellular matrix macromolecules,[111] which bind lipoproteins causing extracellular lipid accumulation.[4] The growing plaque remodels eccentrically initially driven partially by the release of proteinases

(such as matrix metalloproteinase 3) by smooth muscle cells.[112] Cysteine cathepsins are a family of proteins that perform varied functions in developing plaques including promoting extracellular matrix degradation, mediating LDL-C aggregation and accumulation and foam cell formation, and increasing immune cell recruitment.[113] A growing number of resident leukocytes localize within the plaque through both infiltration and proliferation.[114] Leukocytes are retained within growing plaques by locally secreted proteins such as *semaphorins*.[115] Specific plaque characteristics are determined by diverse features such as programmed cell death of leukocytes, resulting in a growing necrotic core.[116] Both micro- and macro-calcifications deposit in growing plaques as a result of impaired clearance and dysregulated deposition of extracellular matrix components.[117] Within developing plaques, macrophages regulate inflammation, clear apoptotic cells, and determine plaque stability by secreting matrix metalloproteinases and processing and storing lipids.[118] Depending on the local environment, these macrophages can 'polarize' to diverse cellular phenotypes, which serve either to promote or inhibit atherogenesis.[118] Developing plaques therefore have specific characteristics and can be broadly categorized as fibrous plaques (fibroatheroma) or fibrocalcific plaques. Plaques displaying features that have been shown to predispose to rupture are termed 'vulnerable plaques,' which are characterized by large lipid cores with thin fibrous caps.[4] Other high risk plaque features include expansive positive (outward) vessel remodeling and scattered (not dense) calcifications.[119,120]

## Assessing atherosclerosis progression in humans

Within individuals, and even within the same blood vessel, many different plaques exist simultaneously, each with distinctive structural and molecular characteristics. Indeed, atherosclerosis proceeds heterogeneously across vascular beds and among different plaques within a given vascular territory. The atherosclerotic process is influenced both by the systemic factors reviewed previously and by the local microarchitecture of individual plaques. In the following section, we review methods to identify plaque characteristics through circulating markers and focused molecular imaging techniques, with a particular focus on identifying features of high-risk plaques.

**Circulating molecular profiles—**Detection of molecular signatures of unstable or higher risk plaques in the blood have been reported. By measuring >300 different circulating lipid molecules, Meikle et al. identified distinct lipid profiles of stable versus unstable CAD.[121] In individuals with established ASCVD, Ganz *et al.* used broad proteomic profiling to identify 9 novel proteins that predicted CVD events over the following 4 years.[79] Several of the proteins highlighted by this study, such as angiopoietin-2 and matrix metalloproteinase-12, are likely to reflect heightened metabolic activity among remodeling plaques.[122] Moreover, features of the gut microbiome identified by metagenomic sequencing have been shown to differ in individuals with stable versus unstable plaques: persons with unstable plaques demonstrated lower fecal levels of the genus Roseburiam.[123] Higher circulating TMAO levels have also been found to correlate with unstable plaque morphology.[124]

In addition to systemic profiles associated with adverse plaque morphology, methods are evolving to examine cell-specific molecular profiles that may provide insight into individual plaque biology. Single cell molecular profiling enables comprehensive study of distinct cell

types. This approach has been used to interrogate different leukocyte species within atherosclerotic plaques, demonstrating that the higher proportion of memory T-cell lineages within carotid plaques is more predictive of future CVD events than macrophage number.[65] Cell typing by flow cytometry has also been used to analyze and compare different leukocyte cell types in humans, with relevance for plaque biology.[125] The next steps are to use these single cell profiling techniques to leverage the distinct proteomic,[126] epigenetic,[127,128] transcriptomic,[129] and other molecular profiles of cellular constituents of high risk plaques to improve ASCVD risk prediction.

**Molecular imaging—**Cardiovascular imaging modalities presently used in clinical practice are focused primarily on the diagnosis and treatment of the symptoms and complications of ASCVD. One modality that has been shown to add complementary information to traditional risk factor assessment is coronary calcium scoring, which can be used to reclassify, and in some cases refine, risk prediction estimates.[130] Coronary computed tomography angiography (CTA), and assessment of carotid intima-media thickness and ankle-brachial index are also used to assist with the diagnosis of subclinical ASCVD and can aid in ASCVD risk assessment.[131] However, such techniques rely primarily on anatomical quantifications of plaque burden and, therefore, do not assess plaque 'activity' or high-risk plaque features.

New imaging approaches can harness molecular probes to assess plaque biology with real-time *in vivo* individualized assessments. Molecular tracers targeted to pathological processes of interest are labeled with an imaging reporter and injected into the body where they accumulate at sites of increased disease activity.[132] Theoretically, any disease process can be imaged with this technique once suitable tracers and imaging platforms are identified, but regulatory approval and cost remain prohibitive in many cases.[132] Here we review examples of molecular imaging probes that are being used to assess atherosclerosis progression with the goal of highlighting individual plaque characteristics that can be identified in real time. Detailed and comprehensive reviews are available elsewhere.[132,133]

18F-fluorodeoxyglucose (18F-FDG) is a PET tracer and glucose analog that is taken up by metabolically active cells.[132] It is useful in identifying vascular inflammation as macrophages utilize glucose at a higher rate than surrounding cells,[134] and FDG-PET activity has been found to correspond with macrophage content in excised carotid plaques.[135] Endarterectomy specimens demonstrate correlations between areas of increased 18F-FDG uptake and regions of inflammation, microvascular permeability and microvascularization.[136] In a retrospective study of 513 patients undergoing PET imaging for evaluation of cancer, 18F-FDG uptake in the aorta was associated with CVD events over a mean follow up of 4.2 years.[137] Vascular 18F-FDG PET studies are time-efficient and relatively low cost.[132] While aortic imaging is relatively simple, 18F-FDG uptake in the coronary arteries is more challenging to image. Glucose is the predominant energy source of the myocardium, which increases the "background" signal and renders coronary artery 18F-FDG uptake difficult to identify. Other PET tracers with more optimal characteristics for imaging coronary inflammation are in development.[132]

Ultrasmall superparamagnetic particles of iron oxide (USPIO) are removed from circulating blood by the reticuloendothelial system and accumulate in macrophages present in atherosclerotic plaques, thereby serving as a marker of plaque activity.[132] Plaques can then be imaged using T2*-weighted magnetic resonance imaging (MRI) sequences.[138,139] Intriguingly, the USPIO signal intensity has been shown to decrease with 3 months of statin therapy, suggesting that it may be a useful tool to assess plaque regression in response to treatment.[140]

As opposed to vascular macrocalcification, which may represent plaque chronicity and stability, microvascular calcification is associated with active inflammation and higher risk features.[117] 18F-fluoride binds to calcified deposits within atherosclerotic plaques with high selectivity and differentially binds to micro- as opposed to macrocalcification, thereby identifying calcium deposits too small to be recognized by CT imaging.[141,142] In a study of 40 patients with recent MI, culprit plaque uptake of 18F-fluoride was elevated in 37 individuals.[143] One challenge to applying this technique more broadly is that microcalcification of the vascular media (i.e., medial sclerosis) can occur, especially in the setting of chronic kidney disease or diabetes, but is not localized to atherosclerotic plaques and, therefore, may therefore distort ASCVD risk assessments based solely on vascular microcalcification.[144]

Other techniques such as near-infrared fluorescence (NIRF) imaging and MR spectroscopy hold promise for improving imaging of molecular characteristics of atherosclerotic plaques.[133,145] MR spectroscopy, in particular, combines the spatial imaging of MRI with spectral analysis to detect chemical composition of cardiovascular tissue.[145] MR spectroscopy of carotid arteries, for instance, can quantify cholesterol esters within atherosclerotic plaque.[145]

As molecular imaging techniques become more widely available, their use to further delineate not only the presence and burden of atherosclerotic plaques, but also their biological activity, and to combine this with other molecular ‘snapshots’ of an individual’s risk profile has the potential to potently improve the precision of individual-level risk prediction. For this vision to reach clinical care, there will need to be consensus on optimal imaging modalities and isotopes, standardized protocols, and better delineation of the associations of imaging findings with clinical outcomes.

## ATHEROSCLEROSIS COMPLICATIONS

### Molecular Biology

Atherosclerotic plaques lead to overt events when they limit myocardial blood flow via progressive stenosis or thrombus formation (Figure 1). Plaque growth may eventually impair coronary arterial perfusion, resulting in ischemia during rest or exercise (i.e., angina pectoris). The most common cause of myocardial infarction is atherosclerotic plaque rupture.[146] High-risk (or “vulnerable”) plaque features that predispose to rupture include large lipid cores with a thin fibrous cap.[4] Inflammation-mediated dysregulation of extracellular matrix homeostasis disrupts the normal balance between collagen synthesis and removal, with resultant degradation of the protective fibrous cap by interstitial collagenases.[146,147] Intraplaque hemorrhage often precedes full *plaque rupture* and may derive from

plaque neovascularization or from small ruptures of the plaque's fibrous cap causing blood to infiltrate the plaque from the vessel lumen.[148,149] When *plaque rupture* occurs, the thrombogenic material in the plaque core is exposed to the blood compartment leading to platelet aggregation and the polymerization of fibrin. An alternative thrombotic mechanism is *plaque erosion*, which typically occurs in plaques with few inflammatory leukocytes, lower lipid concentrations, and a rich extracellular matrix without a thin fibrous cap. Innate immune activation and the participation of polymorphonuclear leukocytes may be particularly important in fostering plaque erosion.[4,150] Alternatively, plaques may undergo favorable remodeling leading reduced risk of rupture or erosion. Plaque stabilization or regression has mostly been demonstrated in response to lipid lowering therapy.[151]

## Assessing atherosclerosis complications in humans

The primary complication of ASCVD is myocardial infarction (MI), which results from inadequate blood flow to the myocardium usually due to an occlusion or stenosis of a coronary artery. Early identification of impending MI is essential to mitigate the adverse effects and consequences. Here, we review evolving methods to improve the upstream diagnosis of MI using molecular profiling.

**Genetics/Epigenetics—**Genetic markers and epigenetic modifications related to platelet reactivity[152] and thrombus formation may identify individuals who are more likely to have atherosclerotic CVD complications. These may include transcriptional profiling to recognize gene expression signatures of higher risk (or hyperreactive) platelets.[153,154] Moreover, methylation signatures of pro-thrombotic hemostatic profiles can be identified in the blood representing markers of heightened thrombotic risk.[155] Circulating MiRNAs may also prove useful as early indicators of myocardial damage.[156] Studies have also investigated miRNA biomarkers of plaque instability (vulnerable plaques)[157–159] and have shown that miRNAs may be important biomarkers for future CVD events in patients with established CVD.[59,128,160]

**Proteomics—**Profiling of lower concentration circulating proteins may enable upstream detection of MI prior to manifest heart damage. High-sensitivity troponin assays are now deployed clinically to facilitate early and accurate identification of myocardial infarction, often enabling treatment before clinically relevant myocardial damage has occurred.[161] Efforts are ongoing to identify novel proteins that provide complementary information regarding early indicators of MI. Broad proteomic profiling approaches have been applied to detection of myocardial damage identifying a number of novel proteins that may improve the diagnostic accuracy, and perhaps the prevention, of myocardial infarction,[80,162] and its complications.[163] In one example, Jacob et al. measured nearly 5000 circulating proteins after MI and found 29 mostly intracellular proteins that were increased post-MI, which require further study regarding their clinical utility in MI diagnosis. In another study, plasma levels of biliverdin reductase B were shown to correlate with *intraplaque hemorrhages* that antedate clinical MI symptoms, and which may therefore enable even earlier detection of plaque rupture events prior to coronary occlusion.[164]

**Metabolomics—**Metabolite profiling is similarly being investigated to identify circulating signatures of impending/evolving MI prior to their detection by traditional methods. For example, peripheral blood metabolite profiles can predict the presence of CAD and associate with future events in individuals undergoing coronary angiography.[165,166] Metabolomic profiling of circulating blood can also be used to identify evidence of myocardial injury,[167,168] the extent of ischemia-reperfusion injury,[169] and potentially to distinguish between stable and unstable CAD.[121]

## CONSIDERATIONS FOR INCORPORATING MULTI-DIMENSIONAL DATA INTO RISK PREDICTION

This review has primarily focused on highlighting the tremendous progress in elucidating molecular signatures of ASCVD risk. However, there are a number of challenges and important considerations that must be addressed in the process of transitioning these discoveries to clinical use (Table 1 and Supplemental Table), several of which are discussed below.

### Single points in time

Traditional observational methods for biomarker discovery rely on demonstrating an association between a biomarker of interest and the presence or development of the outcome. Accordingly, biomarker discovery often begins with measurements made at one timepoint. Longitudinal assessments to measure the cumulative burden, growth trajectory, or variability of biomarkers can add incremental value for risk assessment. In addition, the timing of the measurement within the patient's overall risk profile and health trajectory is of paramount importance. A straightforward example that illustrates this point is the vastly different implications of an elevated high-sensitivity troponin level for a 30-year-old who has just run 5 miles versus a 65-year-old with chest pain or a 50-year old patient with end-stage renal disease and mild heart failure. This point holds true even for perhaps the most commonly assessed cardiovascular risk biomarker: LDL-C. Current guidelines support distinct recommendations for LDL-C lowering therapy depending on the individual's global ASCVD risk assessment.[170] The same LDL-C level will carry distinctive prognostic significance and, as a consequence, different treatment recommendations in individuals with very different ASCVD risk profiles.[171] For LDL-C, this approach has largely evolved from an effort to balance the risk of adverse medication effects with the absolute risk reduction expected to be derived from lipid-lowering therapy. Similar calculations will have to be considered for incorporation of novel risk markers in clinical care.

### Considerations of differences based on sex, race, and geography

Inherent in risk prediction efforts is the assessment of how molecular risk markers vary according to patient characteristics. Widespread efforts must be made to replicate and compare findings across diverse populations to define specific normative limits based on age, sex, racial/ethnic background, and geographic location. Not only are the distribution of risk factors across populations important, however, but the association with the outcome must be assessed iteratively. For example, sex differences in high-sensitive troponin levels have been described with important considerations for clinical care.[172]

### Rigor and reproducibility of measurements

The accuracy and precision with which novel molecular markers are identified is of substantial importance in their translation to the clinic. Novel assays provide the opportunity to measure molecules at a scale never before seen but the assay accuracy and methods of quantification must be validated. Moreover, protocols need to be standardized for pre-analytic biosample processing and quantification methods across laboratories.

### Modeling techniques

In most populations, traditional risk factors can explain ~80% of the variation in CVD development.[2,173] This is acceptable from a population level but the extrapolation of this variance-explained to treatment implications for an individual is a major challenge.[3] A clear illustration of this challenge is exemplified by the observation that despite the strong association of high blood pressure with CVD events, the majority of individuals experiencing CVD events in the US have a normal blood pressure.[174] Personalized, precise, risk assessments that account for variability in the individual's response to risk factor burden are necessary therefore to improve individual-level risk prediction.

## INTEGRATION OF MULTI-DIMENSIONAL MOLECULAR DATA FOR LIFE-COURSE RISK PREDICTION

Major progress is being made, as summarized in this review, in identifying molecular signatures that can be used to improve CVD risk assessment. In coming years, we anticipate that these multi-dimensional assessments will be integrated into a life-course risk assessment framework that harnesses the growing knowledge of the molecular biology of atherosclerotic plaques with dynamic assessments of a patient's molecular profile (Figure 2).

Genetic risk is the obvious starting point for such assessments as it is the only "omic" domain that is predetermined and fixed. Genetic risk can be broadly divided into two categories. Modifiable genetic risk markers may affect known biological pathways, such as lipid levels, that can be measured and modulated with lifestyle and drug interventions. These genetic risk markers should not necessarily alter ASCVD risk prediction as they are not independent of known traditional risk factors. Other, non-modifiable genetic risk markers should be incorporated in global CVD risk prediction as they represent risk above what is routinely assessed.

Dynamic assessments of the epigenome, trancriptome, metabolome, proteome, and gut microbiome in circulating blood may be performed throughout the life course to gauge immediate health risks and identify targets for treatment (i.e., intermediate phenotypes). Molecular imaging and tissue-level molecular signatures could then serve as adjunct modalities in individuals identified as having sufficient risk for near-term events to warrant further evaluation of more precise markers of disease activity and potentially, to gauge therapeutic responses. Lastly, high-sensitivity, multi-dimensional markers of disease activity can facilitate identification of impending ASCVD events prior to symptom development or clinical relevance, allowing targeted preventative therapies. We foresee a new paradigm of multi-parametric, longitudinal, risk assessments that would adapt iteratively throughout an

individual's health course, with different sets of markers being more relevant at specific points in atherosclerosis progression.

## FUTURE DIRECTIONS AND NEXT STEPS

Despite the tremendous progress to date in identifying and measuring molecular signals of atherosclerosis, much work is still required if we are to reach the aspirational vision for molecular targeting of CVD prevention depicted in the previous section.

The discovery phase of identifying novel risk markers and modalities relies primarily on studies of one or two "omics" domains at a time. Optimal risk assessment is likely to draw on trans-omics assessments integrating proteomic and metabolite responses with genetic and epigenetic information, for example, in a broad multi-omics multi-marker approach. This approach will be facilitated by advances in machine learning and big data analytics. An essential question that will arise is the optimal approach to ASCVD risk prediction for clinical care. Are "black box" machine learning approaches that arrive at the best prediction formula using obscure methodologies optimal, or will methods that are driven by empiric observations and known biology provide more actionable clinical insights?

Current risk prediction methods draw primarily on assessments made at a single time point in the resting state. Future models will incorporate lifetime burden, variability, and trajectories of risk features (which is already known to be predictive for a number of CVD risk factors including BMI, smoking and LDL-C[175,176]). In addition to the growing precision with which molecular markers of essential biological processes can be identified, more precise quantification of the exposome will be available in the future, facilitated by smartphone technologies, chemo-, mechano- and biosensors, and identification of the imprint of extrinsic factors in circulating blood.

Finally, it is our opinion that focusing our prevention efforts on specific organ-based disease systems may be a narrow perspective. Atherosclerosis, for example, leads not only to coronary artery disease, but also culminates in cerebrovascular disease and stroke, carotid disease, aortic disease, and peripheral vascular disease. Unifying the approaches to detection and monitoring of atherosclerosis development across multiple vascular beds and related organ systems is an important next step in comprehensive promotion of vascular health. In addition, many risk factors and circulating risk markers are not unique to CVD but are shared among other diseases of aging such as dementia and cancer. As such, a broader framework of chronic *disease prevention* as opposed to *CVD prevention* alone is likely to provide the most widespread health benefits for individuals and populations.

## Summary

The molecular diagnosis of ASCVD is advancing at a rapid pace benefiting from tremendous progress in elucidating the pathobiology of atherosclerosis development and in high-throughput profiling of high-dimensional molecular data. Integration of multi-parametric molecular assessments has the potential to provide a longitudinal "snapshot" of an individual's current status and risk profile and may enable more precise risk prediction and treatment approaches.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## ABBREVIATIONS

| | |
|---|---|
| **ASCVD** | atherosclerotic cardiovascular disease |
| **CVD** | cardiovascular disease |
| **LDL-C** | low-density lipoprotein cholesterol |
| **HDL-C** | high-density lipoprotein cholesterol |
| **CRP** | C-reactive protein |
| **CAD** | coronary artery disease |
| **miRNA** | micro RNA |
| **mRNA** | messenger RNA |
| **CHIP** | clonal hematopoiesis of indeterminant potential |
| **CTA** | computed tomography angiography |
| **18F-FDG** | 18F-fluorodeoxyglucose |
| **USPIO** | ultrasmall superparamagnetic particles of iron oxide |
| **MRI** | magnetic resonance imaging |
| **NIRF** | near-infrared fluorescence |
| **MI** | myocardial infarction |

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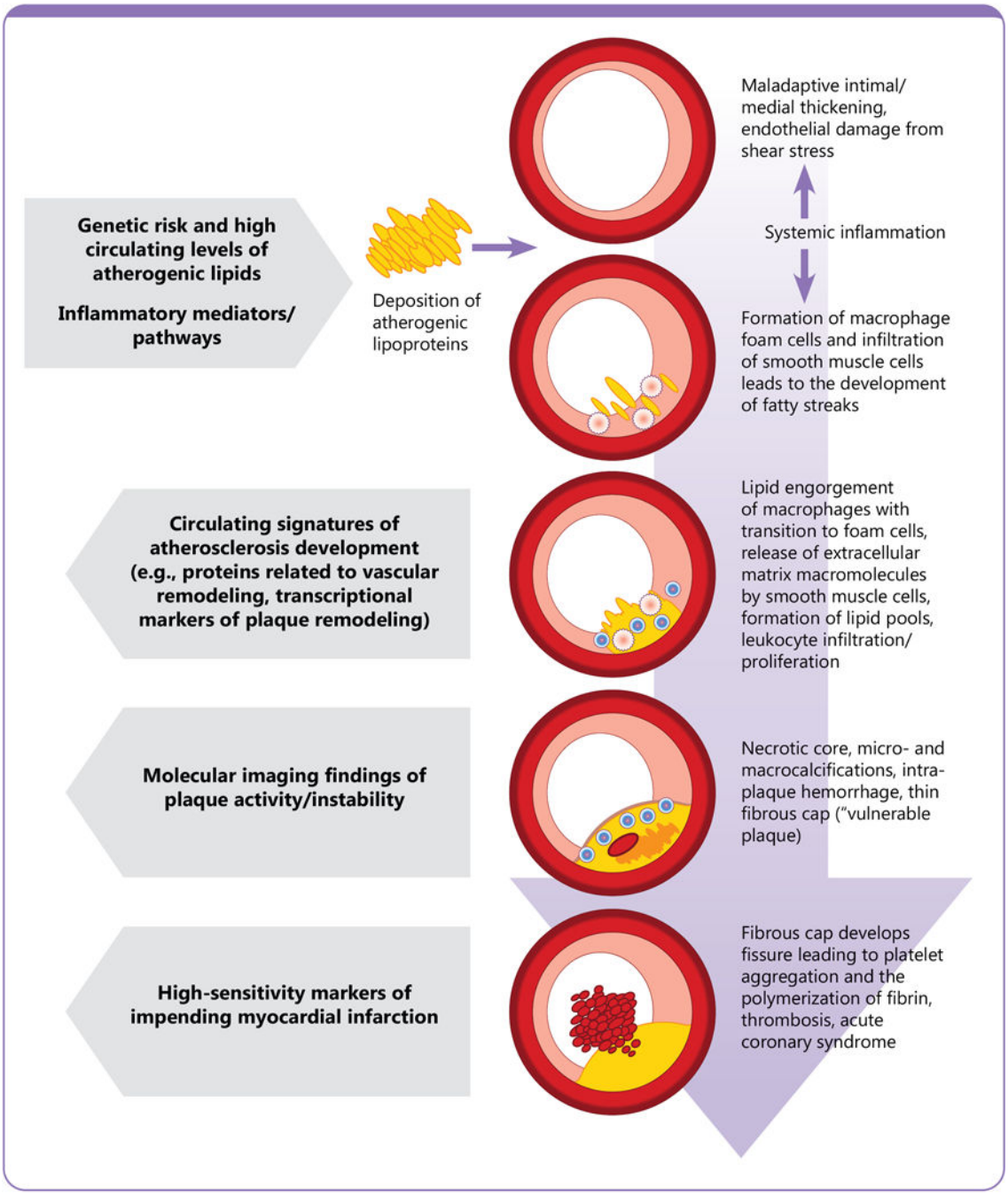


**Figure 1. The molecular biology of ASCVD and its peripheral blood signature.**

*Initiation* of atherosclerosis commences with a pro-atherogenic, lipid rich, inflammatory milieu leading to focal inflammation, maladaptive intimal and medial thickening, and endothelial damage. Markers of elevated blood lipid values and systemic inflammation (and their genetic and epigenetic determinants) can be detected in circulating blood. As atherosclerosis *progresses*, fatty streaks develop into atherosclerotic plaques through migration of smooth muscle cells and lipid accumulation. This process is heavily influenced by the functions of different leukocyte species including macrophage polarization and

transition to foam cells and lymphocyte infiltration and proliferation within developing plaques. Programmed leukocyte cell death and micro-calcifications can promote a necrotic core, which, coupled with a thin fibrous cap, are characteristic of ‘vulnerable plaques’ more prone to rupture. Epigenetic, proteomic, and metabolomic fingerprints of developing plaque characteristics can be detected in circulating blood. Molecular imaging and cell-type specific profiling via cell sorting and single cell sequencing may also identify adverse plaque features. *Complications* of ASCVD primarily occur as a result of critical blockages and/or plaque rupture events (frequently preceded by subclinical intra-plaque hemorrhages) leading to thrombosis and acute organ ischemia. Identification of early signs of vessel occlusion prior to symptom development may be facilitated by assaying high-sensitivity transcriptomic, proteomic, and metabolomic markers in circulating blood.

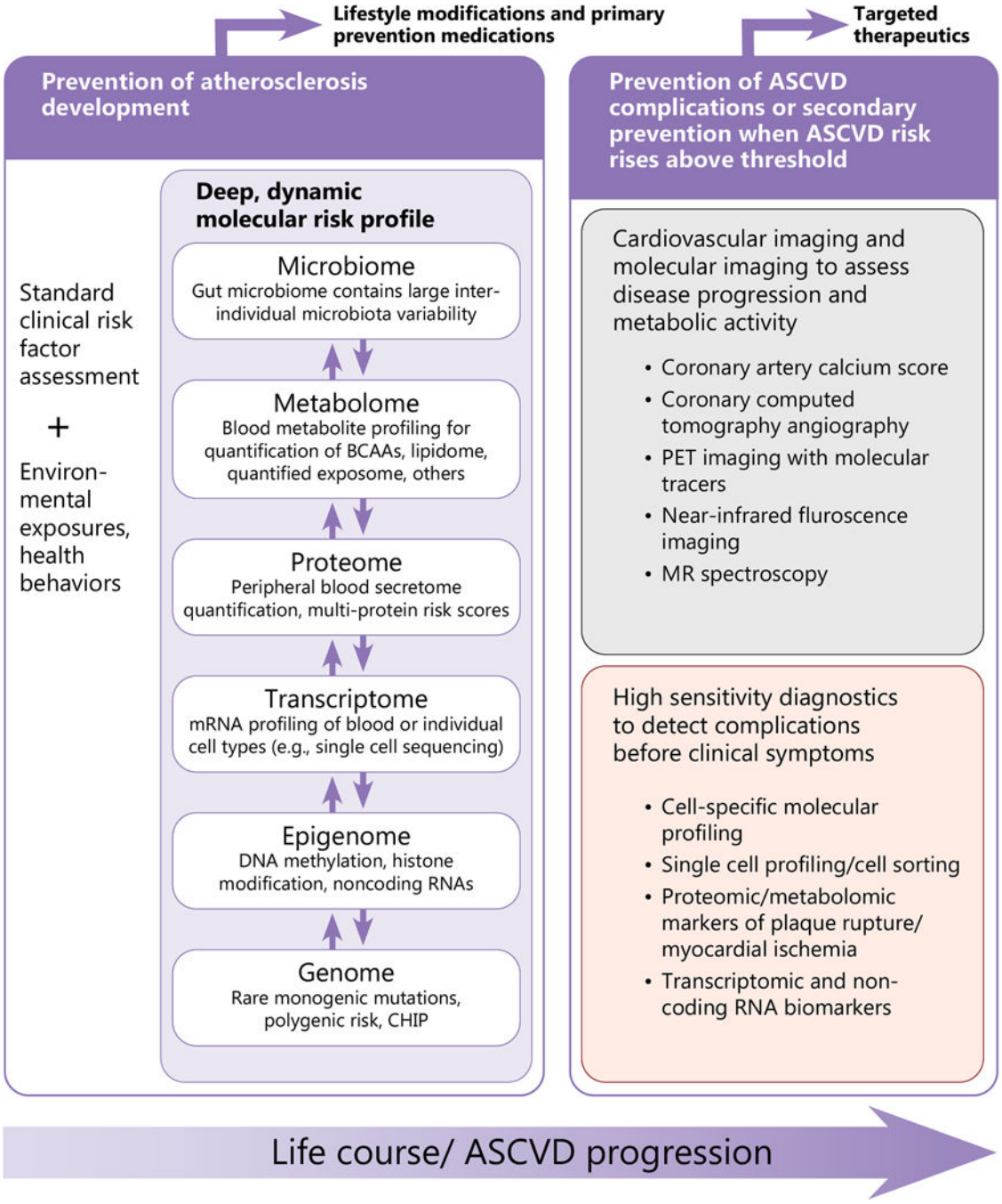


**Figure 2. Life course integration of multi-dimensional molecular data for ASCVD risk prediction.**

A conceptual view of how molecular diagnosis of ASCVD may proceed through the life course. Prevention of atherosclerosis development begins early in the life course with monitoring of standard clinical risk factors, environmental exposures, and deep, dynamic molecular risk profiles integrating information from the various “omics” layers. The connections between different “omic” layers displayed here is simplified for visualization purposes; the ‘post-genetic’ layers (epigenome, transcriptome, proteome, metabolome, microbiome) also influence each other bi-directionally. During early adulthood to mid-life,

when atherosclerosis typically develops, personalized molecular risk profiles can inform risk status, leading to personalized treatment recommendations. In individuals with sufficient risk (typically in middle age and older adults), molecular imaging and cell-specific molecular profiling can be deployed to more precisely risk profile individual plaque features, with the goal of informing targeted therapeutic approaches. Lastly, high-sensitivity molecular diagnostics may reveal signs of impending ASCVD complications (e.g., plaque rupture) before clinical symptoms develop, enabling upstream treatment and prevention approaches.

**Table 1.**

Challenges to clinical translation of molecular biomarkers

| Challenges | Potential considerations/solutions |
|---|---|
| Threshold for abnormal values vary according to clinical context, background risk profile, medications, comorbidities | • Incorporation of biomarkers into multivariable equations using standard ASCVD risk markers and other relevant clinical information<br>• Clinical utility studies, serial measures<br>• Biomarker testing in variety of clinical contexts and patient samples |
| Differences in specificity of biomarkers in blood vs. tissue | • Blood assays require egress from tissue or overt injury for markers to appear in circulation<br>• Novel methods (e.g., molecular imaging, cell sorting) to measure biomarkers within tissues |
| Differences in prognostic significance of biomarkers based on age, sex, race, geography | • Replication of the results in independent multi-ethnic samples<br>• Careful study design with inclusion of appropriate controls, adequate sample size |
| Rigor and reproducibility | • Standardization of pre-analytical biosample processing<br>• Analytical standardization, especially in studies with multiple batch runs and longitudinal samples<br>• Analytical validation using orthogonal methods |
| Optimal design relative to role of biomarker investigated | • Clearly defined purpose of the study to address a specific clinical question<br>• Distinct study designs are needed to assess screening role, cross-sectional disease correlates, prognostic significance, predictive capability, clinical utility, and potential to guide treatment<br>• Time horizons over which utility of biomarkers are assessed |