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REVIEW

Translational medicine

Novel methodologies for biomarker discovery in atherosclerosis

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Identification of subjects at increased risk for cardiovascular events plays a central role in the worldwide efforts to improve prevention, prediction, diagnosis, and prognosis of cardiovascular disease and to decrease the related costs. Despite their high predictive value on population level, traditional risk factors fail to fully predict individual risk. This position paper provides a summary of current vascular biomarkers other than the traditional risk factors with a special focus on the emerging −omics technologies. The definition of biomarkers and the identification and use of classical biomarkers are introduced, and we discuss the limitations of current biomarkers such as high sensitivity C-reactive protein (hsCRP) or N-terminal pro-brain natriuretic peptide (NT-proBNP). This is complemented by circulating plasma biomarkers, including high-density lipoprotein (HDL), and the conceptual shift from HDL cholesterol levels to HDL composition/function for cardiovascular risk assessment. Novel sources for plasma-derived markers include microparticles, microvesicles, and exosomes and their use for current omics-based analytics. Measurement of circulating micro-RNAs, short RNA sequences regulating gene expression, has attracted major interest in the search for novel biomarkers. Also, mass spectrometry and nuclear magnetic resonance spectroscopy have become key complementary technologies in the search for new biomarkers, such as proteomic searches or identification and quantification of small metabolites including lipids (metabolomics and lipidomics). In particular, pro-inflammatory lipid metabolites have gained much interest in the cardiovascular field. Our consensus statement concludes on leads and needs in biomarker research for the near future to improve individual cardiovascular risk prediction.

Keywords Atherosclerosis • Clinical biomarker • Risk prediction • Systems biology • Mass spectrometry • HDL • Micro-RNA

Biomarkers: state of the art

Preventive cardiovascular risk assessment relies on established risk factors, including smoking, hypertension, dyslipidaemia, and diabetes; however, approximately half of the people developing coronary heart disease (CHD) have been classified as having low or intermediate risk based on current risk algorithms.[1–4] Although biomarkers seem to be a rather novel research field the term 'biomarker' was already introduced in 1980.[5] In fact, 'biomarker' in the broad sense, being 'a characteristic that is objectively measured and evaluated as an indication of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention',[6]

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covers also traditional risk factors that are used for the above-mentioned risk algorithms. Hence, 'biomarkers' will be used in the following as markers measured in biological specimens, such as cells or serum.

The clinical value of serological biomarkers for the diagnosis and prediction of clinical manifestations of atherosclerotic disease has been assessed in numerous clinical studies. Meta-analyses and reviews are widely available in international literature summarizing diagnostic and predictive properties of both, cardiac-specific markers (e.g. produced/released by the cardiac muscle and hence likely reflecting coronary atherosclerosis) and non-cardiac-specific markers (systemic markers such as lipids, creatinine, glycaemia and glycated haemoglobin, essentially reflecting metabolic risk factors). In spite of the large number of biomarkers that have been tested, the European Society of Cardiology (ESC) guidelines only recommend the use of troponin for the diagnosis and prognosis in the management of acute coronary syndromes (ACS),[7,8] along with the assessment of lipid profile, creatinine, and glycaemia. The development of high-sensitivity (hs) assays for troponin I and T has improved the diagnostic sensitivity for acute myocardial infarction (MI), decreased the time to diagnosis and led to quicker rule-out of myocardial ischaemia. In addition, elevated hs-troponin has been associated with adverse outcomes in patients with stable CHD and in the general population. However, troponin does not have sufficient independent prognostic value to advise systematic measurements in patients with stable CHD.[9–12] In fact, in this condition, the current guidelines do not recommend testing any biomarkers beyond lipids, creatinine, glycaemia and glycated haemoglobin, adding the organ specific BNP or NT-proBNP only if heart failure is suspected.[9] Specifically, the use of hsCRP or any other novel biomarker is not recommended.[7–9] However, the number of routinely measured biomarkers that can be used to predict MI or presence of clinically silent atherosclerotic disease is rather limited and systemic markers may have some limitations, because although atherosclerosis can be considered a systemic disease, in some cases it may be progressing at different rates in different arterial beds or individuals depending on variables such as age, ethnicity,[13,14] etc. Thus, tentative new biomarkers need to be robust enough to be able to indicate progression of disease even if it happens in a relatively small part of the arterial tree.

Guidelines for biomarker use to assess presence of atherosclerotic disease in the absence of an acute event are scarce. The reasons for this may be found in our limited scientific understanding of biomarkers so far or the described limited added value of biomarkers on top of the predictive value of traditional risk factors for prediction of adverse events. This is in sharp contrast with biomarker guidelines for the diagnosis of heart failure where NTpro-BNP is an accepted standard.[15]

For the prediction of incident cardiovascular events, markers with strong potential are mainly associated with lipids and lipoproteins. For recurrent cardiovascular events, markers are mostly associated with ischaemia.[15] There is an ongoing debate which biomarkers should be applied in patients with low, intermediate, and high 5- to 10-year risk for MI. For example, the National Academy of Clinical Biochemistry guidelines from 2009 discussed the use of the most often used commercially available biomarkers such as hsCRP and fibrinogen.[16] Their conclusion was that there is no need for further biomarker screening in low-risk patients and in case of intermediate risk much is left to the discretion of the medical practitioner.

Biomarkers that reflect the inflammatory state are not recommended for routine use in non-high-risk subjects. Recommendations for hsCRP screening slightly differ between US and EU standards. While both American Heart Association (AHA) and ESC recommend hsCRP measurements in patients with moderate or unusual CHD risk profile, asymptomatic high-risk patients, and patients with hypertension categorized as intermediate risk by Framingham criteria to assess 10-year CHD risk,[4,17,18] the AHA also recommends screening of asymptomatic low-risk patients.[17]

The clinical value and appreciation of biomarkers may be hampered by many determinants such as intra-individual variability, lack of tissue specificity, inter-lab variability, analytical sensitivity and accuracy,[19] age, weight, renal function, gender differences, or differences among ethnicities. Also, progression of atherosclerosis may not be homogeneous in different areas, and different degrees of peripheral artery disease have been described for similar degrees of CHD.[14] This fact could limit theoretically the information given by a cardiac biomarker on the progression of atherosclerosis in other areas and vice versa. Finally, unmonitored and unaccounted differences in pre-analytical sample handling (e.g. time from collection to storage, isolation protocol, and room temperature) and marker stability may be additional limitations. This strongly depends on storage conditions; number of freeze–thaw cycles, etc., and is molecule specific and thus not generalizable.[20]

Moreover, biomarkers with causal involvement (*Table 1*) are usually regarded more valuable for risk stratification as they may also be used in testing drug efficacy or applied as companion diagnostic. However, Mendelian randomization studies have shown that some of the most widely applied biomarkers for cardiovascular disease are not causally related with disease progression.[21,22]

Thus, overall there is no consensus regarding the value of many current, mostly 'serological' biomarkers for risk prediction of MI or stroke. In the last years, new approaches are being used to search for novel biomarkers. These approaches have two distinctive features. First, they do not focus only in proteins, but they also assess other molecules (*Table 2*). Second, they are able to analyse large numbers of these molecules instead of a few of them, as happened with the traditional approaches. In fact, large EU-granted programs are using these new technologies to search novel biomarkers. Among these are the EPIC-CVD study[23] and the Biomarker for Cardiovascular Risk Assessment in Europe (BiomarCaRE). The latter is a European collaborative research project that integrates clinical and epidemiological biomarker research throughout Europe and validates the biomarker effectiveness in large, well-defined primary and secondary prevention cohorts from 13 European countries.[24] These studies will provide an overview of the clinical value of biomarkers including geographical differences of outcomes.

In the following, we will provide an overview of current and future biomarkers, changing paradigms and novel technologies, including their differences and pros and cons (*Table 2*). A particular focus will be on the discovery and measurements of biomarkers applying new –omics technologies. Although the relevance of genetic variants should not be underestimated, they cannot be held responsible

Table 1 Potential causal involvement in disease progression of discussed novel circulating biomarkers for atherosclerosis

Biomarker	Causality	Source or anticipated mechanism of action
(High-sensitivity) troponin	None	Consequence of myocardial necrosis
(NT-pro) BNP	None	Consequence of myocardial damage
HDLc	Causal factor?	Insufficient evidence that HDLc-raising therapeutic interventions are beneficial[187]
HDL-function	Likely causal factor	Capacity to exhibit cardioprotective effects (e.g. cholesterol efflux)[40,187]
Endothelial or leukocyte microparticles	Both	Consequence of cellular damage and/or regulated mechanism of inter-cellular communication[44,47–51]
Cardiac or endothelial miRNAs	Both	Consequence of cellular damage and/or regulated mechanism of inter-cellular communication[119,120]
HSP-27	Causal factor	Atheroprotective protein, low plasma levels are associated with cardiovascular disease[188–192]
sTWEAK	No evidence shown	Member of the tumor necrosis factor superfamily, low plasma levels are associated with cardiovascular disease[193–197]
Eicosanoids	Causal factor	Enhanced synthesis of inflammatory lipid mediators[198,199]
Polyunsaturated cholesteryl esters with long-chain fatty acids	Likely causal factor	Thought to contribute to foam cell formation and progression of atherosclerosis[200]

Table 2 Comparison of novel circulating biomarkers for atherosclerosis and current technologies used for their identification

Type of biomarker	Screening technology	Sample throughput[a]	Limitations[a]	Future challenges
HDL	HPLC, NMR	Low to high	Single biomarker approach, limited predictive value	To identify predictive components of HDL (e.g. lipoproteins), shift towards alternative indexes such as particle size, subclass distribution, HDL functionality
Microparticles	Flow cytometry	Low	Lack of standardized measurement	To define measurement standards and develop novel analytical tools to reduce lower particle size limit of detection
miRNA	PCR-based array	Intermediate	Lack of validated internal controls for PCR-based approaches, sensitive to confounding factors, e.g. medications	To improve sensitivity of more specific technologies without amplification steps (e.g. probe-based arrays) for low-abundant miRNAs
Proteins	MS-based proteomics	Low	Requirement of large sample amount and complicated sample preparation	To improve sensitivity and simplify pre-analytical sample preparation steps
Metabolites	MS- or NMR spectroscopy-based metabolomics	Intermediate to high	High per-sample cost of detailed MS-based analysis detecting many metabolites vs. cost-effective NMR-based high-throughput analysis of abundant biomarkers	To improve cost-effectiveness and sample throughput of MS-based approaches, combination of NMR and MS in large population-based cohorts
Lipids	MS-based lipidomics	Intermediate	Mostly, targeted analysis of selective lipid classes and metabolites involved in known pathways	To perform holistic epidemiological studies including all lipid classes

MS, mass spectrometry; NMR, nuclear magnetic resonance spectroscopy.
[a]Referring to currently used technologies.

for the vast majority of heredity (~90%) in cardiovascular medicine[25,26] and are therefore beyond the scope of this review.

Before going into details of specific technologies, it should be noted that general −omics study design follows similar principles as standard epidemiology. Current −omics technologies can be directly applied in existing large epidemiological sample collections. Quantitative −omics that can provide marker concentrations in physiological units can be analysed using the same statistical

methods, without requiring special knowledge on the underlying technology, as any other biomarker assay data.

High-density lipoprotein: shifting paradigms

The classically named high-density lipoproteins (HDLs) are clinically measured in plasma by analysing the amount of cholesterol contained in the particles, i.e. HDL cholesterol (HDLc). HDLc has been epidemiologically and clinically linked to cardiovascular disease presentation with low HDLc independently associated to high cardiovascular risk,[27,28] whereas high levels were related to cardiovascular disease protection in the primary prevention setting.

High-density lipoprotein particles are complex lipoproteins and the pioneering studies evaluating the HDL vasculo-protective effects did not use HDL carrying high levels of cholesterol but used protein rich-HDL (nascent ApoA1-rich lipoprotein)[29,30] or ApoA1 overexpression.[31] These studies showed that HDL exerted atheroprotection. A key role of HDL is to promote cholesterol efflux and reverse cholesterol transport from the periphery to the liver. High-density lipoprotein cholesterol efflux capacity was recently shown to inversely correlate with the incidence of cardiovascular events in a population-based cohort, suggesting its potential use as a novel biomarker for atherosclerosis.[32] A plethora of additional potential beneficial vascular properties have also been consistently attributed to HDL including antioxidant, anti-apoptotic, anti-inflammatory, anti-thrombotic/fibrinolytic, and vasodilatory effects.[33] The variable composition of HDL has emerged as the possible cause of these diverse functional effects. Indeed, the HDL particles not only contain ApoA1 and transport cholesterol but they are carriers of >85 proteins,[34,35] numerous lipid species and small molecules.

Even with this complex nature, HDLc is still a good biomarker in the primary prevention setting. A recent study in over 35 000 patients has shown that for every 5 mg/dL increase of HDLc the risk of hospitalization for cardiovascular disease decreased 6%. In addition, if the levels of HDLc increased 6.5 mg/dL cardiovascular disease decreased 8%; on the contrary, if HDLc decreased 6.5 mg/dL cardiovascular risk increased 11%.[36] In fact, in acute percutaneous coronary intervention studies for every 5 mg/dL increase in HDLc concentrations, the risk of periprocedural acute MI decreased by 20%.[37] However, in some patient populations with established chronic coronary artery disease or chronic kidney disease the inverse association between HDL cholesterol levels and cardiovascular events is attenuated.[38,39]

Recent studies have revealed that the occurrence of coronary disease is associated with a reduction in HDL antioxidant and anti-inflammatory potential indicating the variable and complex nature of these particles[40] that are easily remodelled during their metabolic life span. Interestingly, recent data with drugs developed to specifically raise HDLc levels have questioned the assumed protective role of raising the HDL particles carrying high levels of cholesterol.[41] Human genetic analysis has also shown that genetic alterations of HDL cholesterol levels are not uniformly associated with the risk of coronary disease.[22]

In summary, HDL particles express a multitude of molecular complexity and the measurement of cholesterol levels in particles alone is not the only biomarker of HDL function. Although HDLc is still used clinically in primary prevention, new tests to better measure HDL functionality[42] including cholesterol efflux capacity[32] are being actively sought.

Microparticles

Microparticles (MPs; often also called microvesicles) belong to the family of extracellular vesicles released from activated or apoptotic cells. Microparticles (~100–1000 nm in diameter) stem from the cellular plasma membrane, whereas exosomes, which are <100 nm, originate from intracellular multivesicular bodies (for reviews, Refs 43–46). However, MPs are not only surrogate markers of cellular injury as they can affect the function of target cells and therefore influence the course of cardiovascular diseases (for reviews, Refs 44,47–51).

Microparticles of different cellular origin, as well as exosomes, circulate in human plasma and other body fluids.[52–54] The major fractions in plasma stem from platelets, red blood cells, and leukocytes, whereas in most studies circulating endothelial MPs are less abundant.[52] So far, the cellular origin of circulating exosomes remains elusive, although their role in inter-cellular signalling and carriers of RNA, especially micro-RNAs (miRNAs), receives increasing attention.[55,56]

Detection of MP subpopulations in human plasma has gained increasing interest in the past two decades for their potential as biomarker. Considered as remnants of parental cell injury, they are easily accessible for measurement in the circulation. Their identification relies on the presence of externalized phosphatidylserine and specific markers from the parental cell membrane.[57] Plasma MP levels increase in subjects with cardiovascular risk factors and in patients with atherosclerosis or other cardiovascular disorders (for reviews, Refs 58–61). Interestingly, local levels of circulating MPs increase in culprit coronary arteries of patients with ST-segment elevation MI,[62] and even further in those from patients with sudden cardiac death,[63] suggesting that changes in circulating MP levels reflect an increased release of microvesicles in atherosclerotic vascular disease. Finally, patients on lipid-lowering treatment with statins have lower numbers of circulating MPs despite similar plasma cholesterol levels.[64]

Microparticle quantification remains challenging; the pros and cons of each method have been reviewed previously.[65,66] Standardized methods are being developed and will certainly foster the application of MP assays in clinical settings. Both the standardization of the pre-analytical steps and the sensitivity of MP flow cytometry analysis have greatly improved in the past decade[67–75] resulting in numerous investigations of the potential benefit of using plasma MPs as biomarkers.

So far, two specific subpopulations of circulating MPs have received most attention for use as CHD biomarkers: endothelial and leukocyte MPs.

Plasma levels of endothelial MPs expressing either CD144 or CD31 inversely associate with the degree of endothelium-dependent vasodilation in humans.[76–81] Therefore, they reflect acute or chronic endothelial dysfunction and vascular injury in general. Elevation of endothelial MP subsets predicts atherosclerotic plaque instability in patients undergoing endarterectomy.[82]

Furthermore, endothelial MPs expressing either CD144, CD31 or CD62E are independent predictors of cardiovascular outcome in patients with heart failure,[83] coronary artery disease,[83,84] end-stage renal failure,[85] stroke history,[86] or other cardiovascular diseases.[83,87] Combining endothelial MP detection with classical biomarkers strategy improves risk stratification for cardiovascular events in patients at risk of CHD.[88] No data are available yet in the general population regarding the prognostic value of circulating endothelial MPs.

Levels of circulating CD11a expressing MPs associate with atherosclerotic plaque burden in asymptomatic patients.[89] In patients with ACS, $CD11b^+$ MPs inversely associate with the early recurrence of cardiovascular events, possibly because these MPs are consumed during thrombus formation.[90] Recent studies demonstrate that plasma MPs might be useful to assess the composition and the vulnerability of atherosclerotic plaques. In patients with severe carotid stenosis (>70%), plasma levels of leukocyte $CD11b^+$ $CD66b^+$ MPs are associated with plaque instability.[91] In patients with familial hypercholesterolemia, levels of $CD45^+CD3^+$ lymphocyte MPs help to discriminate lipid-rich plaques from fibrous lesions.[92]

Taken together, these findings indicate that MPs from endothelial cells and leukocytes could provide useful tools to identify patients at high risk for future cardiovascular events. Furthermore, the complex MP composition (proteins, lipids, and nucleic acids) might be an interesting source for −omics.[93–96]

Micro-RNA

Micro-RNAs are small non-coding RNAs that control gene expression by binding to target mRNAs, thereby inducing mRNA degradation or repression of protein translation. Besides their important intracellular functions and potential value as therapeutic targets,[97–99] extracellular miRNAs have also been detected in various body fluids including the blood. The levels of circulating miRNAs are modulated in disease states and, therefore, yield potential value as cardiovascular disease biomarkers.

Initially, researchers found elevated levels of miRNAs that are highly expressed in the myocardium ('cardiac miRNAs') in acute MI patients, e.g. miRNA-1, miRNA-133, miRNA-208a/b, and miRNA-499.[100–104] Meanwhile, multiple additional circulating miRNAs were shown to be enhanced following MI or angina pectoris.[101,103,105–114] Several cardiac-enriched miRNAs accumulate in plasma early after MI or transcoronary ablation of septal hypertrophy[115] with similar kinetics as conventional cardiac injury biomarkers.[103,105,115] Their increase in transcoronary gradients of ACS patients[104] suggests that they are indeed released from damaged cardiac myocytes. Circulating cardiac miRNAs were proposed to improve the diagnostic value, when combined with traditional markers like high sensitive troponin T,[116] and circulating cardiac miRNAs were associated with poor prognosis in first studies.[117] More recently, combinations of miRNAs, such as miRNA-132, miRNA-150, and miRNA-186, were shown to facilitate the diagnosis of unstable angina[118] and changes in miRNA-126, miRNA-223, and miRNA-197 expression predicted subsequent MI.[96] Recent studies revealed that miRNAs also circulate within microvesicles and may contribute to cardiac pathophysiology by targeting vascular and cardiac cells[119,120] and can predict subsequent heart failure.[121]

Less is known about the use of circulating miRNAs as biomarkers to detect early stages of atherosclerosis or atherosclerotic plaque characteristics. Patients with stable coronary artery disease exhibit reduced levels of the endothelial cell-enriched and vasculoprotective miRNA-126 and members of the miRNA-17-92a cluster.[122] Lower levels of circulating miRNA-126 were also observed in patients with diabetes.[123] Moreover, the smooth muscle-enriched miRNA-145-5p was significantly lower in patients with CHD.[122] Circulating miRNAs may thus have the potential to reflect endothelial function or provide non-invasive insights into plaque vulnerability. Recent evidence suggests that the passenger strands of miRNAs that normally undergo degradation also deserve attention as potential biomarkers, since they have been reported to regulate endothelial regeneration[98,119] or circulate in fibroblast-derived exosomes to target cardiac cells.[124]

However, miRNA measurements currently depend on PCR, making them sensitive for confounding factors and normalization is challenging due to the lack of valid house-keeping miRNAs. Furthermore, the origin of miRNAs cannot be unequivocally determined as many circulating miRNAs derive from platelets[125] and the discrimination between vessel wall- and platelet-derived miRNAs is difficult if not impossible. Moreover, several pharmacological interventions, such as platelet inhibitors and heparin were shown to affect miRNA measurements.[125–127]

While circulating miRNAs may be promising CHD biomarkers, the field is still in its infancy and is challenged by confounding factors that interfere with miRNA measurement. Solutions to current methodological problems may include: (1) the use of multi-miRNA panels, (2) technical improvements in miRNA measurements (i.e. replacing PCR by hybridization technologies). Large-scale studies to document the ability of circulating miRNAs to detect early atherosclerosis or plaque vulnerability remain to be performed.

Proteomic technologies

Proteomic technologies allow comparing the expression of hundreds or thousands of proteins from two biological specimens, including fluids, tissue, or cells. For instance, arteries with and without atherosclerosis can be compared or the effect of different therapies can be assessed.[128] Over the past decade, proteomics analyses have evolved from protein separation by two-dimensional electrophoresis to mass spectrometry (MS)-based approaches.[128] At present, a variety of proteomic platforms are available. Their selection depends on the specimens and the type of proteins to explore (for reviews, Refs 128–130).

In general, there are two MS approaches: First, the untargeted discovery approach, in which samples are analysed without *a priori* assumptions and peptides are prioritized for fragmentation based on their relative abundance. This approach is limited by its bias towards abundant proteins since there is currently no technological platform to resolve the entire human plasma proteome.[130,131] The result of an untargeted discovery proteomic experiment is a list of proteins, among which potential biomarker candidates are selected according to their highest statistical significance and relevance. Second, targeted MS offers an alternative approach, in which

a pre-selected panel of proteins is measured with high precision. This method is termed using multiple reaction monitoring and was selected by Nature Methods as technology of the year in 2012.[132]

Although multiple reaction monitoring offers better sensitivity than untargeted proteomics, higher abundant plasma proteins are still more readily quantified and for now antibody-based methods remain the method of choice for the quantitation of less abundant proteins (i.e. cytokines, chemokines, growth factors, etc., which are present at picograms to nanograms/mL plasma). A combination of an untargeted with a targeted approach provides the most comprehensive strategy to discover new biomarkers by proteomics.

Blood proteomics

Blood is an excellent source for the discovery of new biomarkers in atherosclerosis, as it exchanges molecules with the arterial wall and several blood cell populations are causally involved in atherosclerosis. However, proteomic studies of plasma or serum are complicated by the presence of high-abundant proteins, such as albumin or immunoglobulins, that may mask important biomarker candidates present at lower concentrations.[128,131] By using adequate methods to remove these high-abundant proteins, different candidate biomarkers have been identified.[133,134] Nevertheless, this approach is not without risk as removing albumin from the sample may also remove albumin bound proteins with potential value.

The proteome of circulating cells may yield relevant information comparing either patients with different clinical pictures of atherosclerosis or testing the effect of anti-atherosclerotic drugs in subjects with this disorder.[135,136] The potential value of post-translational protein modifications as biomarkers of CVD can also be evaluated by proteomic analysis, as reported in blood of ACS patients.[137,138] A different study used nuclear magnetic resonance (NMR)-based technology to identify a protein glycan that is associated with incident CHD.[139] Finally, urine may be a potential source of biomarkers as it contains proteins filtrated from plasma with low-molecular weight that can be analysed avoiding excessive manipulation.[140]

Proteomics of atherosclerotic lesions

Another complementary approach is to study whole tissue specimens. For instance, patients with higher atheroma content of osteopontin are at increased risk of developing cardiovascular events.[141] However, studying the whole tissue could result in identification of structural proteins not involved in atherosclerosis. In this case, the study of the secretome may be instrumental.[142] In this procedure, a tissue specimen is cultured, allowing it to release molecules into the medium, mimicking the secretion of biomarkers from atheroma into the blood. Thereafter, the supernatant is analysed avoiding the presence of structural proteins. Comparing the secretome of carotid endarterectomy specimens and healthy arteries, low plasma levels of HSP-27 (Heat Shock Protein-27) and sTWEAK (soluble tumour-necrosis factor-like weak inducer of apoptosis) have been identified as diagnostic markers of atherosclerosis,[143,144] although their prognostic value remains controversial.[145–147] Another possibility is to assess the effect of drugs when added to the cultured tissue.[148] Moreover, retrieved coronary thrombi of MI patients have been recently analysed by proteomics, revealing potential new candidates for biomarkers of atherothrombosis.[149,150]

Proteomic approaches allow screening to detect differences in protein expression between different biological specimens. Although this approach is not free of limitations, it has considerably increased our ability to discover novel biomarkers of atherosclerosis as untargeted or targeted protein analysis can be performed by MS without *a priori* assumptions and without the need for the availability of good antibodies to a specific protein of interest.

Metabolomics

Detailed profiling of metabolic status, termed metabolite profiling or metabolomics, can provide insights into the molecular mechanisms underlying atherosclerosis.[151–156] The quantification of large numbers of circulating metabolites across multiple pathways may also identify metabolic changes prior to the onset of overt disease, and hereby potentially lead to earlier and more accurate identification of individuals at high cardiovascular risk.[156–158] Two technological platforms are used: nuclear magnetic resonance spectroscopy[156] and MS.[158,159] The former has an advantage in throughput but is limited in sensitivity. Vice versa, MS can identify many more metabolites but often with limited throughput.

Coronary heart disease biomarkers

Technological improvements in sample throughput now allow for metabolite profiling of extensive epidemiological cohorts, rather than case–control settings, to enhance biomarker discovery and replication.[160,161] Metabolite profiling has been successful in identifying biomarkers for the development of type 2 diabetes.[162–167] Circulating biomarkers for diabetes are more directly associated with the disease than those for atherosclerosis.[168,169] However, few metabolite biomarkers have been consistently associated with future cardiovascular events across multiple studies.[154,156–158,170,171]

Many common systemic metabolites, such as amino acids show consistent associations in properly powered epidemiological studies. For example, it has been demonstrated that there are prospective associations of some amino acids with carotid intima-media thickness, a subclinical measure of atherosclerosis.[156,172] Nuclear magnetic resonance-based metabolite profiling in large prospective cohorts recently identified phenylalanine, monounsaturated and polyunsaturated fatty acids as biomarkers for CHD risk.[171] The association strengths of some of these new biomarkers, as of phenylalanine, are comparable with those of established risk factors, e.g. LDL cholesterol, and they remain predictive even when adjusted for standard lipids and glycaemic traits.[156,171]

Key issues in metabolic profiling in epidemiology

Quantitative metabolic profiling can aid biomarker discovery in an unbiased and unsupervised manner by providing molecular information across multiple pathways: all metabolic measures can then be separately tested for the potential disease association or incidence. This should be followed by appropriate independent replication of the candidate biomarkers identified in the discovery cohort. Unfortunately, the promise of metabolomics in biomarker discovery has

not been fully realized; even though various papers have been published, there is very little consistency and rigor in the metabolomics works in this area as recently pointed out.[173] We call for a stringent attention to statistics and replication in the field of metabolomics to strengthen the scientific value of the work, irrespective of the analytical platform used. Particularly when aiming for clinical applications, recent frameworks are recommended to strengthen the methodological rigour and quality for the prediction models.[174,175]

Lipidomics

Lipidomics is a specific subset of metabolomics, which refers to a systems-based study of all lipids.[176] This approach could potentially go beyond the analysis of cholesterol and triglycerides for assessment of cardiovascular risk and response to therapy. In addition, lipidomics can be used to detect several lipid mediators involved in cellular homeostasis as well as in inflammation initiation and resolution[177] and whose circulating levels may give insight into pathophysiological processes of atherosclerosis. However, the simultaneous detection of lipids is impeded by the heterogeneity of the lipid molecules reflecting different metabolic pathways originating from structurally different lipid species. The theoretical number of different molecular lipid species has been estimated to 180 000.[178] To facilitate the lipid classification, the Lipid Maps consortium[179] categorized the human plasma lipidome into six major classes, namely fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, and prenol lipids.

In addition to structural diversity, it should also be considered that the concentrations may differ between different lipid classes, which also should be taken into account when using lipidomics for the identification of circulating biomarkers. For example, several of the eicosanoids are active at nanomolar concentrations at specific G-protein-coupled receptors,[180] and the local response to these lipid mediators at the site of an atherosclerotic lesion may not necessarily be reflected in their circulating levels. Selective lipidomic analysis of eicosanoids released by carotid atherosclerotic lesions has characterized the metabolites of 5-, 12-, and 15-lipoxygenase as the predominant eicosanoids produced by the atherosclerotic lesion, compared with cyclooxygenase products. For example, 12-hydroxyeicosatetraenoic was the most abundant eicosanoid, at levels ≈40-fold greater compared with a prostacyclin metabolite.[181]

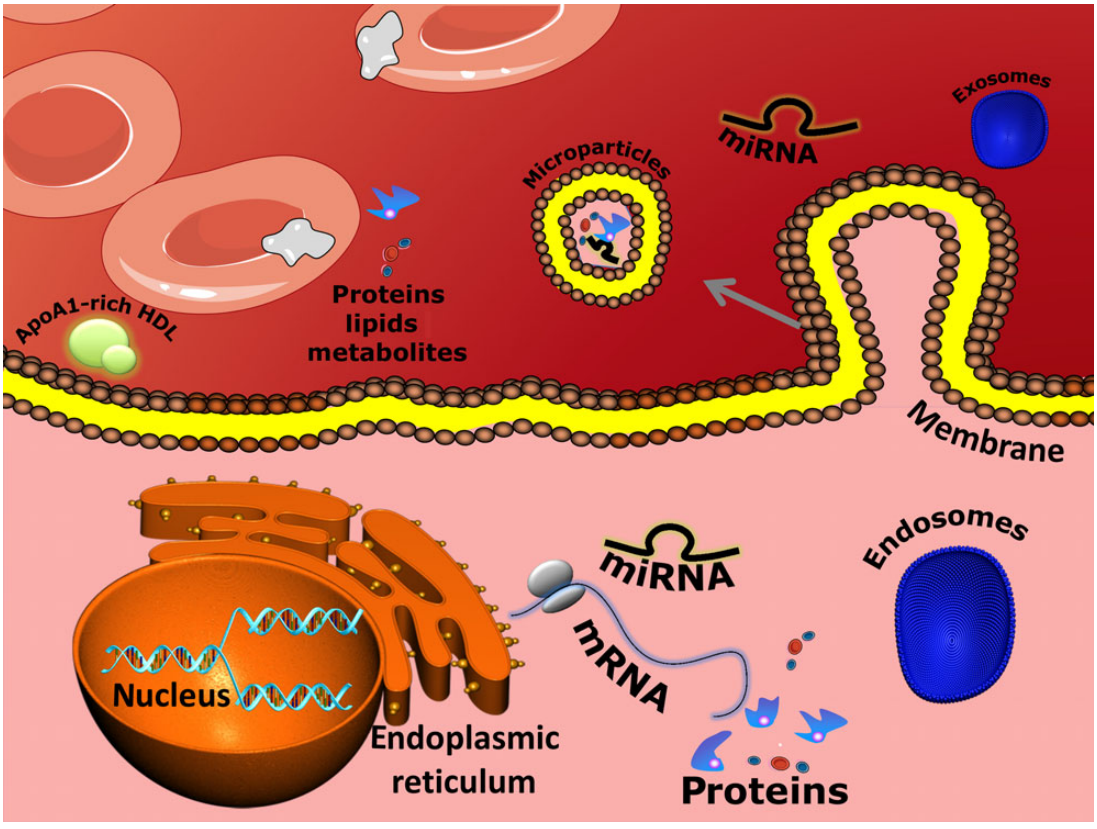

Figure 1 Circulating biomarkers for cardiovascular disease. The transcriptome, proteome, metabolome, and lipidome analysis of cells, blood, and serum is increasingly identifying circulating biomarkers for cardiovascular disease originating from the cellular and extracellular compartments. These include high-density lipoprotein particles and high-density lipoprotein-related proteins (e.g. apoA1), small circulating particles enveloped in cell membranes, such as microparticles (which stem from the cellular plasma membrane) and exosomes (which derive from intracellular multivesicular bodies such as endosomes), intracellular molecules such as short regulatory sequences of gene transcription (miRNA), as well as proteins, peptides and lipid metabolites. These might circulate freely or be carried by microparticles and exosomes.

Broader lipidomic analyses have identified members of several different lipid classes in human atherosclerotic lesions.[182,183] Comparing the local lipidome of atherosclerotic lesion with that observed in plasma has revealed that polyunsaturated cholesteryl esters with long-chain fatty acids and certain sphingomyelin species exhibited the greatest relative enrichment in plaques compared with plasma and formed part of a lipid signature for vulnerable and stable plaque areas within the same lesion.[158,184] Despite these differences in terms of lipid profiles within atherosclerotic plaques and the plasma lipidome, qualitative and quantitative characterization of circulating lipid species by means of lipidomics may offer additional tools for patient risk stratification. For example, the assessment of 305 different plasma lipid species added incremental value in classifying coronary patients and matched healthy controls.[185] In addition, multiple lipid species were shown to distinguish unstable from stable coronary disease.[185] However, some of the reported associations were rather unexpected, i.e. the inverse association of cholesteryl esters with unstable lesions, and could potentially have been confounded by the higher proportion of statin and heparin use in patients with unstable compared with stable coronary disease. Those initial findings in patient cohorts were recently extended by the assessment of 135 lipid species in a prospective population-based study.[158] The latter study highlighted that a shift in the fatty acid chain length of cholesteryl esters, sphingomyelins, and triacylglycerides exhibited the strongest and most consistent association with cardiovascular disease. A similar fatty acid chain length shift has previously been linked to the risk of type 2 diabetes.[163]

The top scoring lipids significantly improved the risk discrimination for cardiovascular disease during a 10-year follow-up, hence providing a first piece of evidence for a predictive value of circulating biomarker identified by means of plasma lipidomics.[158] Finally, the possibility of using lipidomics to predict response to lipid-lowering therapy has received some support by studies of, for example, statins and fibrates.[186]

Consensus statement

For decades, the endeavours to find new biomarkers for prediction, prevention, diagnosis, and prognosis of cardiovascular events have focused on a rather small number of molecules. Technological improvements in automated analytical methodologies, for example in terms of sensitivity and sample throughput, have revolutionized biomarker research in the past 10 years. Integration of multiple complementary platforms, such as micro-RNA, transcriptome, proteome, metabolome, and lipidome analysis, allows unprecedented biological details across multiple pathways and is leading to a conceptual shift from individual markers to multi-marker panels for cardiovascular risk prediction (*Figure 1*). However, the vast amount of data generated also calls for bioinformatics expertise to handle and combine the data. In the case of new technologies, it is also essential to critically assess their advantages and disadvantages, and keep their potential limitations in mind (*Table 2*). For example, the sensitivity of a method inherently affects the biomarker panel obtainable. Reliable interpretation of improved molecular details may also call for more stringent analytical and clinical sample quality standards—collection and storage conditions safe for traditional biomarkers may not be such for some new biomarkers and, say, the molecular effects of diet and medication can be more noticeable than with traditional biomarkers. Explanatory research related to technical, analytical, and practical aspects of new technologies is therefore essential and expected to increasingly commence in the near future. Ultimately, the application and value of new candidate biomarkers will depend on their predictive power over traditional risk assessment, on their reproducibility in multiple cohorts and on the practicalities and the cost-effectiveness of their integration into clinical routines and laboratories.

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