

## Review Article

# Biobanks and the search for predictive biomarkers of local and systemic outcome in atherosclerotic disease

Rob Hurks<sup>1,2</sup>; Wouter Peeters<sup>1-3</sup>; Wouter J. M. Derksen<sup>1</sup>; Willem E. Hellings<sup>1,2</sup>; Imo E. Hoefer<sup>1</sup>; Frans L. Moll<sup>2</sup>; Dominique P.V. de Kleijn<sup>1,3</sup>; Gerard Pasterkamp<sup>1</sup>

<sup>1</sup>Experimental Cardiology Laboratory, University Medical Center Utrecht, Utrecht, The Netherlands; <sup>2</sup>Vascular Surgery, University Medical Center Utrecht, Utrecht, The Netherlands; <sup>3</sup>Interuniversity Cardiology Institute of the Netherlands, Utrecht, The Netherlands

### Summary

Multiple risk factors have been associated with progression of atherosclerosis. To identify the individual patient who is at risk for disruption of a vulnerable plaque, leading to a cardiovascular event, remains a major challenge. Current screening methods, based on traditional risk factors, do not allow risk stratification on an individual level. The discovery of new biomarkers would aid in identifying specific patient groups at risk for adverse cardiovascular events due to atherosclerotic disease progression. The current definition of the vulnerable plaque, e.g. atheromatous inflammatory plaque with a thin fibrous cap, has been based on cross-sectional post-mortem studies. The predictive value of these histological characteristics of the vulnerable plaque is likely to be low, because they are also frequently observed at multiple locations in symptomatic and asymptomatic patients.

### Keywords

Atherosclerosis, biobank, vulnerable plaque, biomarker, plaque signature

The Athero-express study follows a new concept to search for the atherosclerotic patient who may suffer from adverse events. In this study, we investigate the predictive value of local plaque composition for adverse events in other vascular territories, regarding the plaque as a concentrated expression of this systemic disease. First results from this longitudinal biobank study show that the local plaque hides strong predictive value for cardiovascular events elsewhere in the vascular tree. Longitudinal biobank studies will facilitate the identification of novel local plaque markers. The search for the plaque protein signature that is predictive for adverse events might enable patient stratification that will allow individualized tailor made medicine and subsequently guide the choice for therapeutic interventions.

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### Introduction

Atherosclerosis is the principal cause of mortality in high-income countries with ischaemic heart disease and cerebrovascular disease accounting for 27.3% of total deaths, and is becoming the number one killer in low- and middle-income countries with an incidence of 21.3% (1, 2). To identify patients eligible for (primary) prevention of clinical manifestations of atherosclerosis, it is crucial to define risk factors and biomarkers with strong prognostic and diagnostic value. Traditional risk factors, combined with risk scores such as the Framingham score, can predict outcome for groups of patients but lack discriminative power to identify individual subjects who are at risk for a cardiovascular event in the near future (3).

The knowledge on the pathophysiologic mechanisms of atherosclerotic plaque formation and destabilization is rapidly

increasing due to newly developed animal models and technology development that allows high throughput analysis and storage of plaques, often referred to as biobanking. Biobanks are well-organized resources of biological samples with associated clinical characteristics used for scientific investigation (4). Biobanks of atherosclerotic plaques with subsequent detailed immunohistochemical studies have resulted in major steps in the understanding of the pathogenesis of atherosclerosis. However, the natural history of atherosclerotic plaque progression and complications are still unclear owing to the inherent drawback of the descriptive nature of pathology-based research and the lack of large animal models with spontaneous atherosclerotic disease that mimic advanced atherosclerosis in humans. In addition, atherosclerotic disease progresses over decades, which makes *in vivo* follow-up studies impractical. Still, the pressure is growing to identify plaques at risk for disruption and patients at risk to

Correspondence to:  
Gerard Pasterkamp, MD, PhD  
Experimental Cardiology Laboratory, Suite G02.523  
Heidelberglaan 100, 3584CX Utrecht, The Netherlands  
Tel.: +31 88 755 7155, Fax: +31 30 252 2693  
E-mail: g.pasterkamp@umcutrecht.nl

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suffer from a cardiovascular event, such as myocardial infarction or stroke. The development and validation of plaque-stabilizing therapies is one of the major challenges of the pharmaceutical industry, but it is hampered by the lack of surrogate endpoints to prove efficacy of treatment. It is of importance to identify markers of progression of atherosclerotic disease that will facilitate drug development and drug efficacy in humans.

## The vulnerable plaque

It is important to identify the individual patient in whom disruption of a vulnerable plaque is likely to occur, subsequently leading to a cardiovascular event. The vulnerable plaque (also called: unstable or high-risk plaque) can cause local thrombosis and thereby unstable clinical syndromes like myocardial infarction or stroke (5). Multiple definitions of the vulnerable plaque have been documented but three different microscopical descriptions of atherosclerotic plaque appearances have been proposed that could lead to thrombotic arterial occlusion (6). The typical, and most common, type of vulnerable plaque is described as a plaque with a large lipid core, covered by a thin fibrous cap. The fibrous cap is prone to rupture, resulting in thrombosis when exposed to the blood. A second type of vulnerable plaque is described as a plaque with superficial erosion of the endothelial cells, which results in direct contact of the blood with the thrombogenic sub-endothelial connective tissue. A third type of vulnerable plaque encompasses a calcified nodule protruding into the lumen, which is considered to induce thrombosis. Typical histological features of the vulnerable plaque are besides the large lipid core and thin fibrous cap, infiltration of inflammatory cells, particularly macrophages, neovascularization with intra-plaque haemorrhage and outward remodeling (5, 7–12).

Vulnerable plaque characteristics like large atheromatous plaques can be detected by high resolution imaging which could facilitate the identification of vulnerable lesions that are likely to rupture, subsequently giving adverse clinical symptoms. With intravascular ultrasound (IVUS) it is possible to identify lipid core and calcifications (13, 14). With optical coherence tomography (OCT), lipid core size can be measured accurately, and it is becoming feasible to perform more advanced plaque characterization such as macrophage infiltration in the fibrous cap (15, 16). If the histological features of the vulnerable plaque would have predictive power for future events then these imaging technologies would be clinically applicable for treatment decisions using intra-procedural assessment of predicting plaque composition. However, it merits careful consideration that the natural history of atherosclerotic lesion progression is unknown.

## Vulnerability of the vulnerable plaque concept

It is unknown if the above mentioned vulnerable plaque characteristics hide positive predictive value to identify plaques that are prone to rupture. Current evidence is largely based on cross-sectional and retrospective studies and prospective evidence is lacking. Autopsy studies provided insights in the prevalence of histological characteristics, such as increased macrophage infiltration, large lipid core and the presence of a thin fibrous cap which have been associated with increased plaque vulnerability.

In addition, these observational studies showed that the predictive value of the vulnerable plaque characteristics is probably minimal because lipid-rich inflammatory plaques are also frequently observed in asymptomatic patients, and plaques lacking typical vulnerable histopathological characteristics are able to cause clinical events and plaque rupture itself is asymptomatic (17–22). These cross-sectional biobank studies are mainly descriptive and therefore do not allow inferences regarding causality.

Pathological studies have also been executed to examine the systemic distribution of vulnerable plaque characteristics. Post-mortem studies revealed that the presence of inflammatory cells in the cap of the plaque does not seem associated with the presence of cap inflammation at another location in the arterial system. This observation indicates that plaque inflammation is not homogeneously distributed throughout the circulation, independent of its prevalence. On the other hand, post-mortem studies demonstrate that the presence of a large lipid core in one artery is associated with its presence in a contralateral artery. In summary, the phenotype of the vulnerable plaque remains to be elucidated since prospective studies have not been performed (23).

## The vulnerable patient

To identify individual subjects at risk for developing future adverse cardiovascular events, many other factors, besides local plaque characteristics, can be considered. For instance, diabetes mellitus, hypertension, smoking and hypercholesterolemia, have been established risk factors for a long time. Naghavi et al. (24) introduced the term “the vulnerable patient” who could be determined by different factors like the vulnerable plaque, thrombogenic blood (vulnerable blood) and electrical instability of myocardium (vulnerable myocardium). According to Naghavi et al., plaques with similar characteristics may reveal different clinical presentations because of the properties of the vulnerable blood and/or the vulnerable myocardium. Atherosclerosis is a multi-system chronic disease; therefore, it is essential for accurate risk assessment to appreciate the total patient vulnerability and not just the characteristics of a single vulnerable plaque. The traditional risk assessment strategies have already shown to predict for long-term outcome of atherosclerotic disease. An integrated approach of defining vulnerable plaque characteristics, traditional risk factors, systemic biomarkers and genetic profiling may facilitate personalized medicine and predict risk for the individual patient. Ideally, screening for the vulnerable patient should be inexpensive, non invasive, reproducible, applicable to an asymptomatic patient population and it should add predictive value to the measurements of the established risk factors (24, 25).

## Biomarkers

Current screening for patients who are at risk for adverse events due to progression of atherosclerotic disease is based on traditional risk factors. However, this approach does not allow specific risk stratification on an individual level to determine who will or will not suffer from a clinical event such as myocardial infarction or stroke.

One of the most considered approaches for risk assessment is executing tests on peripheral blood samples for the presence of a

specific atherosclerosis marker. Because atherosclerosis is a systemic inflammatory disease, known inflammatory markers and acute phase reactants have been studied for this purpose. These reactants are mainly produced by hepatocytes, and the increased expression is driven by cytokines, which are produced by activated macrophages and other cells (26). According to the United States National Institutes of Health a biomarker is defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, or pharmacological responses to therapeutic intervention (27). One of the most extensively studied serum biomarkers for cardiovascular disease is C-reactive protein (CRP), which is highly elevated in patients suffering from most forms of inflammation, infection or trauma. In the absence of these events it has a value as a biomarker for atherosclerosis, since the concentration was directly correlated to the presence and severity of coronary, cerebral and peripheral artery atherosclerosis (28). CRP is a robust marker because of its analytical stability, reproducible results in clinical studies, and high sensitivity assays with good precision are commercially available (29). It was shown that an increased production of CRP was common in patients with angina and turned out to be associated with an increased risk of myocardial infarction and sudden death (30). CRP has been associated with traditional risk factors, such as smoking, obesity and serum triglycerides, but it demonstrated an independent predictive value for the occurrence of cardiovascular events. Treatment with statins, which are known to reduce plaque inflammation, leads to decreased levels of CRP (31). Despite this, the predictive value of CRP as a biomarker for atherosclerosis is only moderate and less compared to traditional risk factors and clinical application is therefore not widely accepted (32). In addition, many other inflammatory markers have been identified in relation to atherosclerosis. For instance circulating interleukin(IL)-6 levels predicts future coronary events in patients with acute coronary syndromes (33, 34).

A good biomarker needs to be specific for disease development or progression, to have a high predictive value and should reflect successful treatment. Atherosclerosis is a multi-factorial disease and therefore one single biomarker will not be sufficient to reach these objectives. However, with the currently available circulating biomarkers, even the use of multiple biomarkers only adds moderate predictive value to the traditional cardiovascular risk factors. In the Framingham heart study, a cohort of 3,209 patients was analyzed to evaluate if this multimarker approach could enhance risk stratification with currently available and previously reported individual biomarkers (35). A combination of 10 biomarkers was assessed, including: CRP, B-type natriuretic peptide, N-terminal proatrial natriuretic peptide, aldosterone, rennin, fibrinogen, D-dimers, plasminogen-activator inhibitor type 1, homocysteine, and the urinary albumin-to-creatinin ratio. After a follow-up of 7.4 years the hazard ratio for cardiovascular events was only 1.84 for the people in the highest quintile scores, compared to the two lowest quintiles. The C statistic, used to measure the ability to classify risk, increased only slightly not reaching significance (0.76 to 0.77). This paper has been criticized for the limited number of major events and for its definition of these major events, when including heart failure and coronary insufficiency (36).

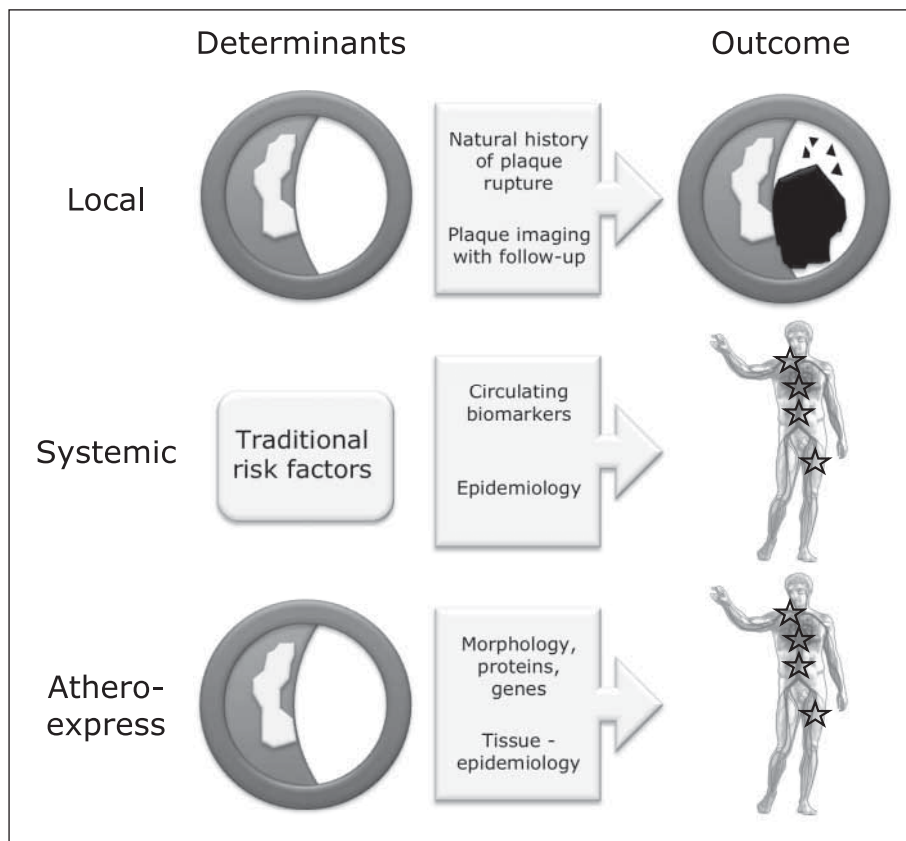
Another approach was proposed by investigators of a community-based cohort of 1,135 elderly Swedish men (37), it was

stated that current risk factors do not directly reflect myocardial cell damage, left ventricular dysfunction, renal failure and inflammation, all being clinical conditions associated with an increased risk on cardiovascular disease and death. They tested the added value to patients risk stratification of a combination of biomarkers involved in named pathophysiological processes, including troponin I, N-terminal pro-brain natriuretic peptide, cystatin C and high-sensitivity CRP. The C statistic increased significantly (0.664 to 0.766) when these four biomarkers were added in a model containing established risk factors, thereby suggesting an improved risk assessment in the cohort of elderly Swedish men. The absence of validation of these findings in other (independent) patient groups makes it hard to generalize found results to other age groups, women and ethnic groups. Some concerns were raised concerning possible confounding illnesses in the enrolled elderly men, such as chronic liver disease, potentially raising the levels of the tested biomarkers (38).

Given these results there is a pressing need for more specific and prognostic biomarkers to be added to the established risk factors to optimize risk prediction. Due to the lack of longitudinal studies, the local atherosclerotic plaque has not been considered as a source for biomarkers to predict future adverse cardiovascular events. Longitudinal studies (including imaging) will be necessary for the discovery of novel local plaque markers and circulating (cell) markers: the assembly of multiple plaque biomarkers may lead to the discovery of protein plaque signatures that will facilitate the identification of different patient groups. Until recently, atherosclerotic pathological biobanks have not been considered as a source for biomarkers in a longitudinal study design. The Athero-express study is the first atherosclerotic pathological biobank that will elucidate the positive and negative predictive power of local histological plaque characteristics and potential biomarkers, and determine whether plaque markers hide prognostic value for future events. An overview of study designs for identifying markers to predict future cardiovascular events is shown in Figure 1.

## Athero-express

In two Dutch hospitals the Athero-express study started in 2002. This study is an ongoing prospective cohort study collecting both blood and endarterectomy specimens of the carotid and femoral artery. All cohort members will be followed for carotid restenosis for two years and the occurrence of adverse cardiovascular events for a minimum of three years. In January 2008 over 1,000 plaques were included from patients who underwent a carotid endarterectomy. The main objective is to determine the predictive value of local morphological plaque characteristics and biomarkers as determinants for local restenosis or future cardiovascular events elsewhere in the body, such as myocardial infarction, stroke or peripheral intervention (39). The concept is based on the fact that atherosclerosis is a systemic disease and it is hypothesized that not just one single plaque but that all plaques in the vascular system share information about the stability of the atherosclerotic lesions irrespective of the vascular territory. In the Athero-express study dissected carotid plaques are stored and after a three-year follow-up, cases and control patients are defined. Subsequently plaques from cases and controls are com-



**Figure 1: Overview of study designs for identifying markers to predict future cardiovascular events.** Traditionally, systemic risk factors are used to define the risk of cardiovascular events (systemic determinants to predict systemic outcome; epidemiology). An upcoming approach is the identification of vulnerable plaques with plaque imaging coupled to follow-up (local determinants for local outcome; natural history of plaque rupture). The Athero-express study follows a third and novel approach aiming at identification of local plaque characteristics (morphology, protein expression, genes) that are predictive of systemic outcome (local determinants for systemic outcome; tissue epidemiology).

pared on a histological and protein level. Characteristics that demonstrate differential expression between cases and controls are validated in another cohort of patients that encompasses about 300 patients who underwent femoral endarterectomy with a three-year follow-up.

The first observations based upon the Athero-express biobank were cross-sectional studies and revealed differences in plaque phenotype from symptomatic patients compared to asymptomatic patients (40). Symptomatic lesions demonstrated predominantly an atheromatous plaque phenotype in comparison with asymptomatic lesions which showed a more fibrous phenotype. Subsequent studies demonstrated that atherosclerotic lesions from women are associated with a more stable plaque phenotype, which might suggest that women may benefit less from a carotid endarterectomy (41). In the Athero-express biobank also restenotic lesions have been harvested that had developed after previous endarterectomy procedures. Restenotic lesions that became haemodynamically significant after five years had an inflammatory plaque phenotype, characterized by macrophage infiltration and large lipid core size, comparable to the plaques of primary symptomatic lesions (42).

### Predicting local atherosclerotic disease progression

One of the objectives of the Athero-express was to examine whether local dissected plaques hide characteristics that could predict restenosis. If so, then these plaque features could serve as

prognostic markers and serve as surrogate markers for future restenosis. Hellings et al. (43) analyzed the progression of restenosis after carotid endarterectomy as a function of immuno-histochemical plaque composition. Carotid plaques of patients undergoing primary carotid endarterectomy were collected and subjected to histological examination. Patients underwent duplex follow-up to assess patency of the target vessel at one year and clinical follow-up at one to three years after surgery. Results of 500 patients showed that vessels with previous stable fibrous plaques (low macrophage and lipid content) are more prone to develop restenosis after endarterectomy. In contrast, lipid and macrophage content were associated with lower rates of restenosis. This finding was somewhat unexpected, since it is generally assumed that progression of restenotic lesions is driven by inflammation (44, 45). Early restenosis is mainly caused by smooth muscle cells and collagen deposits, which is different from late restenosis which resembles primary atherosclerosis (42). Inflammatory arteries may be protected from early restenosis due to increased expansive geometrical remodeling of the vessel (43). This was the first time that it was demonstrated that locally dissected plaques still hide prognostic value for ongoing vascular occlusive disease.

### Predicting systemic atherosclerotic disease progression

A concept that has gained much attention is the identification of the vulnerable patient as described above (24, 25, 46). Each year,

many patients suffer from their first cardiovascular event, such as a stroke or myocardial infarction. If it were possible to identify these patients before they become symptomatic, this might dramatically decrease the burden of cardiovascular disease. For patients with manifest atherosclerotic disease, it is important to predict the chance of a second vascular event. Since secondary cardiovascular events can occur anywhere in the vascular tree, the research focus shifts from natural history and determinants of the local vulnerable atherosclerotic plaque to markers of systemic cardiovascular vulnerability.

In the search for new biomarkers that predict major adverse events, one of the approaches is to develop a serological test for molecules that are known to be present in unstable plaques or suggested to be involved in the mechanisms of plaque destabilization. High serum levels of these “plaque-markers” could provide a fingerprint of the vulnerable patient. This concept was supported by studies which discovered that IL-6 was increased in blood samples distally from the coronary plaque after percutaneous intervention of the coronary artery, suggesting secretion of IL-6 from the plaque (47). However, the choice for these biomarkers should be considered with great care since they mostly originate from observational pathological studies. If a protein is co-expressed in ruptured lipid-rich plaques, then it is unknown whether the protein is related with the cause or consequence (repair!) of plaque destabilization or an innocent aspecific bystander.

Still, major biomarker studies have been initiated and are ongoing based on expression profiles that are associated with the current concepts of the vulnerable plaque. The search for the set of biomarkers with a strong risk prediction, is still ongoing.

Instead of searching for histological characteristics that are associated with plaque rupture or systemic biomarkers that are predictive for adverse systemic events, there is increasing evidence that local plaque characteristics could also be predictive for cardiovascular events elsewhere in the vascular tree. Several studies showed that the instability of the vascular wall is a systemic process rather than only local inflammation and that the molecular structure of the atherosclerotic vascular wall at one side could hold information about the stability of the whole system (48–50). Thus, local atherosclerotic lesions, harvested with surgical endarterectomy, may hide predictive biomarkers for adverse systemic cardiovascular events in any vascular territory.

## Identification of the vulnerable patient

Since sero-epidemiological research (e.g. systemic markers predictive for systemic outcome) has not yielded biomarkers that are strong enough for individual risk stratification, other approaches have been attempted. As described, there is large interest in prospective imaging trials to predict local plaque instability (local determinants for local outcome). In the Athero-express study, we follow a third and novel approach. We investigate the predictive value of the local plaque composition for systemic cardiovascular outcome, regarding the plaque composition as a concentrated expression of a systemic disease. We hypothesize that local plaques contain molecular information that is predictive for atherothrombotic events in other vascular territories (6). In this case the local atherosclerotic plaque may act as a source to

identify prognostic biomarkers for all adverse cardiovascular events. A nested case-control study design has been applied in the first discovery phase. Proteomics studies have been executed to compare plaque proteins between 100 patients that had developed (multiple) adverse cardiovascular events during their follow-up and 100 controls without events during follow-up. Preliminary observations clearly show that local plaque proteins are a source for biomarkers with strong predictive value for future cardiovascular events in all vascular territories (51). Many potential markers have been discovered using this proteomics approach and these are now being validated in the whole cohort. In this protein biomarker discovery, the traditional concept of the vulnerable plaque is not taken into account. These proteins with biomarker properties are likely expressed in both stable and unstable plaques and can be considered as sensors for stability of the atherosclerotic process throughout the vascular system. This is not surprising as individual plaques may undergo stabilization and destabilization over time. The ideal biomarker is expressed in plaques that are active (inflammatory, unstable) and can become activated (stable plaques that can become destabilized). Further research should be focused at identifying the best independent protein markers in the plaque. Markers that are validated positively will undergo external validation in femoral or other plaques. The next step will then be to combine multiple markers into a plaque protein signature, in order to improve risk prediction and identify the vulnerable patient. The first results have stimulated the project group to set an ambitious threshold for risk prediction. We hypothesize that within one year we will be able to identify which 25% of the patients who undergo an endarterectomy have a 70% risk of suffering from a secondary adverse event within three years. On the other hand we will then also be able to identify which patients will suffer from a less than 5% risk for a secondary event. This will be a great leap forward to identify the vulnerable patient. In addition, genomic analyses are being executed, and bioinformatics will assist us to identify subgroups of patients who are at risk. The above mentioned genomic and proteomic approaches in atherosclerotic plaques could introduce the discovery of a whole new set of prognostic biomarkers that will be specific for certain types of adverse cardiovascular events.

The predictive value of local plaque characteristics is currently limited to patients who undergo vascular surgery. To make the predictive value of the plaque characteristics available to more patients and make the step towards primary prevention, the targets identified in our study should be translated to markers which can be measured in patients who do not undergo surgery. The first possibility is to measure the circulating levels of the protein signature identified in the plaque. Proteins from atherosclerotic carotid plaques can be secreted into the systemic circulation and measured in a peripheral blood sample (47). Circulating levels might also be predictive for future cardiovascular events, but secretion of the specific protein by other tissues adds noise to the signal of plaque-derived proteins. Nevertheless, when a strongly predictive signature can be obtained in the plaque, the circulating levels of these proteins may still possess good predictive value for future vascular events. Another option that will be considered is the identification of the plaque protein markers in circulating cells.

## Future perspective

There are also other possibilities to determine the Athero-express biomarkers in patients for (primary) prevention. In theory, plaque proteins with predictive value could be imaged with contrast agents coupled to specific antibodies. Although not yet clinically applicable, developments in the nanochemistry of magnetic resonance imaging (MRI) contrast agents have brought molecular imaging closer (52). In the context of the non-invasive detection of biomarkers, present possibilities include imaging agents that may be directed to atherosclerotic sites using specific targeting of cell surface receptors that are either expressed at the vasculature or inside plaques with neovascularisation (53, 54). This is realized by conjugating an imaging probe with one or several targeting ligands. These ligands can potentially be chosen to target specific biomarkers. Current limitations of these techniques restrict its use to biomarkers that are being expressed in the cell membrane. Besides this, toxicity and clearance of the used products is an important issue for this field in research. For this, SPECT analysis might be a quicker route to the clinic, as toxicity and clearance is less of a problem and quantification is easier. It is evident that a plaque biomarker that is detected in one or a few plaques and that has strong predictive value for events in all vascular territories has an enormous potential, since this would bring non-invasive imaging (of the carotid artery) much closer to predicting risk of a coronary event.

Other clinical applications for discovered predictive biomarkers are to serve as surrogate endpoints in clinical trials. The intima-media thickness is used as a surrogate endpoint in clinical trials investigating the effects of lipid-lowering drugs. If plaque

molecular imaging or plaque biopsies would be optional, then the local plaque biomarkers could serve as a strong surrogate marker for drug efficacy. Plaque biopsies could also be executed in non stenotic segments, e.g. femoral artery when a coronary catheterization is executed. In United States about four million vascular invasive interventions are executed on an annual basis in which plaque biopsy could be considered.

Due to the massive amount of data gathering from pathological atherosclerotic biobanks (genomics, proteomics and forthcoming biomarkers) there is an increasing need for extending biobank analyses to multiple disciplines (55). Systems biology coupling clinical, genetic and protein data is likely to be the next phase in the search for the vulnerable plaque and patient. The use of bio-informatics will lead us to a systems biology approach and will provide new possibilities to obtain an optimal predictive protein profile. This will require integration of skills in pathology, bioinformatics, molecular biology and genetics.

## Conclusions

It is clinically important to identify the individual patient who is at risk for both local and systemic cardiovascular events. For this purpose identifying biomarkers and plaque characteristics that are predictive for events is crucial. Combining biomarkers from plaques in a longitudinal study might lead to the discovery of plaque signatures for different patient groups, and will be useful for clinical decision making and therapeutic interventions. Prospective longitudinal biobank studies may fulfill a pivotal role in this first step towards personalized medicine.

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