The multi-variable and continuous nature of atherosclerotic disease development as a novel paradigm in cardiovascular disease risk assessment

Abstract

Cardiovascular disease is the number one cause of death world wide. Although the death rates of cardiovascular disease (CVD) have declined, the burden of the disease remains high. Furthermore, a growing population has an increased risk for CVD. Therefore, there is a strong possibility that the decline of the CVD death rate will come to a halt and the medical burden soon will start to increase again. Identification of patients at risk for a cardiovascular event is, therefore, still the highest concern among healthcare workers. Currently, CVD risk is estimated through models that predict the 10-year risk of cardiovascular disease related events or death. This is only a long-term risk estimation and monitoring disease progression is not possible. The individuals at the highest levels of risk gain the most from risk factor management recommended by these models; however the most deaths in a community come from those patients at lower levels of risk. This emphasizes the important need for individual based short-term risk assessment and CVD disease monitoring.

The common risk factors are mostly static variables or have low dynamics; therefore they are better suited to predict long-term risk than near-term risk. Novel risk factors that reflect acute processes influencing atherosclerotic plaque progression and rupture are needed. New CVD risk assessment models should include continuous multi-marker profiles that take biomarker kinetics into consideration. Multi-marker dynamics could predict trends toward a clinical manifestation and thereby enable disease monitoring and short-term risk assessment.

This overview will summarize potential targets for cardiovascular disease biomarkers. Furthermore, a potential platform to analyze these targets will be discussed. Finally, the shortcomings of current CVD risk prediction models and the potential to develop new multi-biomarker dynamical models which can be used for individual based short-term cardiovascular risk assessment will be discussed.

Abbreviations: Cardiovascular Disease (CVD), Body Mass Index (BMI), Framingham Risk Score (FRS), Systemic COronary Risk Evaluation (SCORE), C-reactive protein (CRP), N-terminal pro-brain natriuretic peptide (NT-proBNP), Low density lipoproteins (LDL), Apolipoprotein B100 (Apo B100), Apolipoprotein A-I (Apo A-I), Coronary Artery Disease (CAD), Interleukin (IL), Macrophage colony-stimulating factor (MCSF), Familial Hypercholesterolemia (FH), Soluble lectin-like oxidized low-density lipoprotein receptor-1 (sLOX-1), acute coronary syndrome (ACS), Troponin T (TnT), Heart-type fatty acid binding protein (H-FABP), Intima-media thickness (IMT), Pattern-recognition receptors (PRR), Pathogen-associated molecular patters (PAMPs), Toll-like Receptors (TLR), Percutaneous Coronary Intervention (PCI), free flow rate (FFR), Tumor Necrosis Factor- α (TNF-α), Angiotensin Converting Enzyme (ACE), Matrix Metalloproteinases (MMPs), Tissue Inhibitor of Metalloproteinases (TIMPs), Unstable Angina Pectoris (UAP), Granulocyte-macrophage colony stimulating factor (GM-CSF), γ-interferon (IFN-γ), Osteopontin (OPN), Systolic blood pressure (SBP).

Introduction

According to the latest annual heart disease and stroke statistics report (2011 update) from the American Heart Association; the death rates of cardiovascular disease (CVD) have declined, yet the burden of the disease remains high.(2) In 2007 the overall death rate from CVD was 251.2 per 100.000 which is a decline of 27.8% since 1997, however in the year 2007 CVD still accounted for 33.6% of all deaths in the United States. (2) The decline in CVD death rate should not be interpreted as a victory or a gain in control over the most abundant cause of death world wide. With 67.3% of the US adults (≥20 years) being overweight, 33.7% of the US adults being obese (body mass index (BMI) ≥30 kg/m^2), an increasing prevalence of early childhood obesity and in parallel an over time dramatically increasing prevalence of diabetes mellitus, which are all risk factors for CVD, a growing population has an increased risk for CVD. (2) Therefore, there is a strong possibility that the decline of the CVD death rate will come to a halt and the medical burden soon will start to increase again. (1) Prevention of cardiovascular disease, identification of patients at risk for a cardiovascular event and early intervention are thus still the highest concerns among healthcare workers.

The current most common approach by physicians to target the highest-risk patients for intensive treatment is based on a multivariable assessment such as the Framingham Risk Score (FRS) or Systemic COronary Risk Evaluation (SCORE). (1, 3) These CVD risk evaluation models estimate the 10-year risk of cardiovascular disease related events or death. In such models the multivariable nature of CVD is used in an attempt to simplify the estimation of total CVD risk. Although these models provide an accurate estimation for the risk of cardiovascular disease related events or death, with these models we are only capable of long-term risk estimation and monitoring disease progression is not possible. Beside this, the individuals at the highest levels of risk gain the most from risk factor management recommended by these models; however the most deaths in a community come from those patients at lower levels of risk. This is explained by the fact that individuals at lower levels of risk are more numerous compared with high risk individuals who, paradoxically, develop fewer events in absolute terms. (1) This is called the prevention or Rose paradox. Individual based short-term risk assessment and disease monitoring in cardiovascular medicine could possibly reduce the number of deaths within this large population by better and personally adapted prevention strategies or early intervention.

Individual short-term risk assessment and disease monitoring is thus of high importance to reduce the medical burden of CVD, however this is not possible with the classical CVD risk factors, like: hypertension, smoking, diabetes mellitus, and gender. The most common risk factors are all little dynamical conditions because they are either fixed or chronic. Therefore, they are better suited to predict long-term risk than near-term risk. Novel risk factors that reflect the acute processes influencing atherosclerotic plaque progression and rupture need to be found. Biomarkers that are easy to measure and make it possible to screen large numbers of individuals for near-term risk would be of most value. (3)

A large effort has already been put in the search for new cardiovascular disease related biomarkers and many biomarkers, that could independently be associated with cardiovascular events, have been identified (e.g. C-reactive protein (CRP), troponin I, N-terminal pro-brain natriuretic peptide (NT-proBNP)). (4, 5) As single

biomarkers they do not improve the risk estimation for cardiovascular events. However, the simultaneous addition of CRP, troponin I and NT-proBNP to models of established risk factors substantially improves the risk stratification for fatal and non-fatal cardiovascular events. (4, 5) This improvement on 10-year cardiovascular disease risk assessment emphasizes the potential of new biomarkers to make individual based short-term risk assessment possible. However, care must be taken to distinguish those biomarkers that are truly predictors of future events from those that are secondary markers emanated from “target organ” damage. (3) Plasma troponin levels are an example of such a secondary marker. Troponin is a marker of myocardial damage and is used as a diagnostic marker to determine whether a symptomatic patient has suffered from a cardiovascular event. However, in the primary prevention setting, troponin has proven little prognostic value. (3) New biomarkers should therefore be developed from a more transitory perspective.

Because multi-biomarker panels are one of the goals of individual based short-term cardiovascular risk assessment, it is highly important to develop biomarker panels that are possible to adopt in daily practice. The panels should be easy to obtain, have specificity to the disease process, have a high predictive power, and as easy clinical measurement is a considerable requirement, should be measurable in a simple assay with one single analyzing tool. (3)

As any biological component (e.g. (soluble, intracellular, or membrane bound) proteins, DNA or (m)(i)RNA) which is related to a disease process can function as a biomarker, selecting one single platform that can process them all seems to be a difficult task. However, since the development of flow cytometry and the further improvement of this technique, flow cytometry has the potential to become a major diagnostic tool in cardiovascular disease risk assessment.

This overview will highlight potential targets for short-term cardiovascular disease risk assessment. Furthermore, flow cytometry as a potential analytical tool to analyze these markers will be discussed. Finally, the shortcomings of current CVD risk prediction and the potential to develop new dynamic biomarker models which can be used for individual based short-term cardiovascular risk assessment will be discussed.

Atherosclerosis

The underlying pathology of cardiovascular events is atherosclerosis which mostly becomes clinically manifest when it causes either an occlusive or subocclusive thrombus. The thrombus is caused by a culprit plaque that may be ruptured or non-ruptured. Accounting for 70% of all fatal acute myocardial infarctions and/or sudden coronary deaths, plaque rupture is the major type of plaque complication. (6)

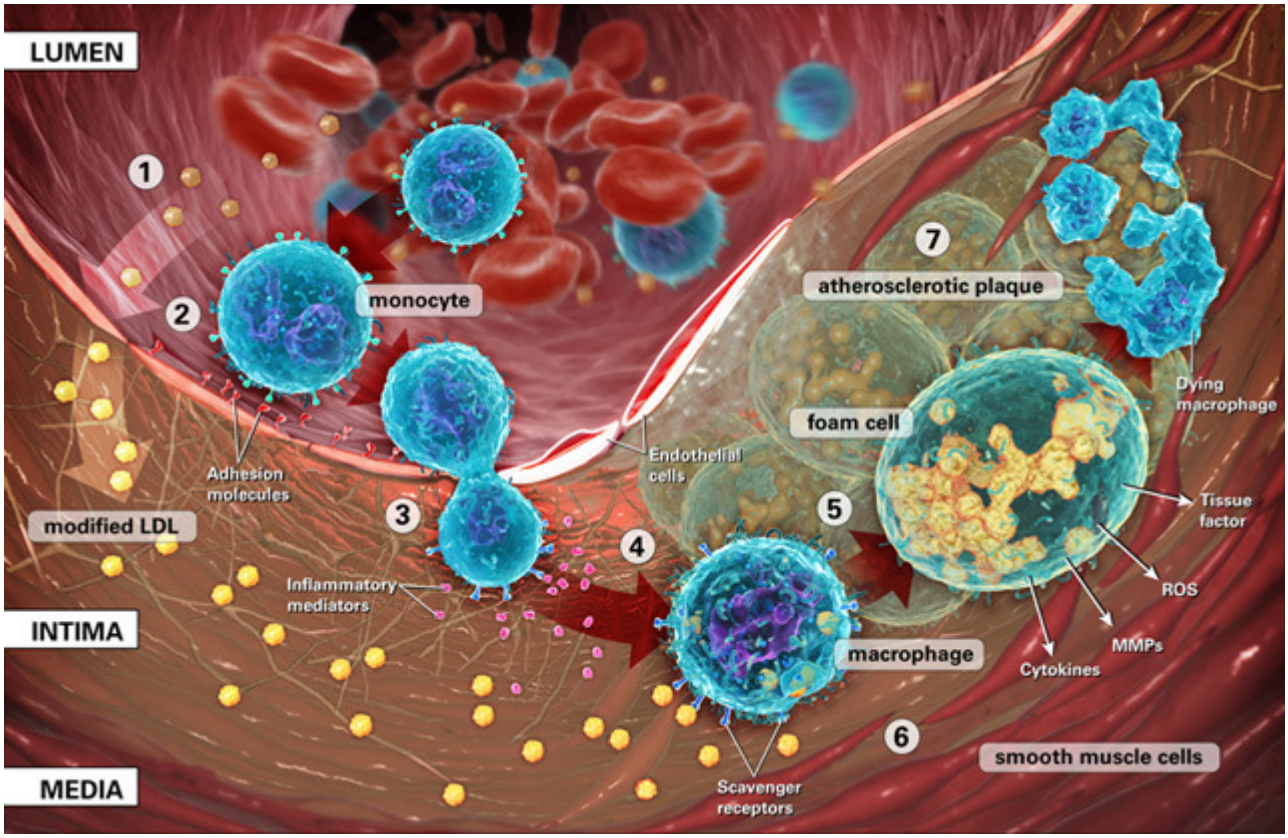

Figure 1 The process of atherosclerotic plaque formation. (1) Oxidized LDL particles cause endothelial cell activation. (2) Activated endothelial cells express adhesion molecules and chemokines that promote monocyte migration. (3) Monocytes adhere to endothelial cells and migrate into the intima. (4) Monocytes differentiate in macrophages and enhance their scavenger receptor expression. (5) Cholesterol accumulation turns the macrophages in foam cells. (6) Smooth muscle cells contribute to the formation of a fibrous cap composed of collagen. (7) The foam cells die and form the necrotic core of the lesion. (Adapted from InViVo Communications)

Atherosclerotic plaque formation is a multi-factorial process which starts at early childhood and propagates as people age. Oxidative stress followed by endothelial cell activation is the initial event which causes atherosclerotic plaque formation (Figure 1, 1). The activated endothelial cells enhance expression of adhesion molecules and chemokines that promote monocyte migration into the intima (Figure 1, 2-3). In the intima the monocytes differentiate in macrophages and enhance their scavenger receptor expression (Figure 1, 4). The macrophages take up oxidized LDL particles by their scavenger receptors and the ongoing cholesterol accumulation eventually turns the macrophages in foam cells (Figure 1, 5). These foam cells will die and form the necrotic core of the lesion (Figure 1, 7). As response to cytokines produced by the damaged endothelial cells, smooth muscle cells proliferate and migrate from the media to the intima. Ultimately, smooth muscle cells will form a fibrous cap composed mostly of collagen that covers the fatty streak (Figure 1, 6). (7)

Through this multi-factorial nature of atherosclerotic plaque formation, there are multiple targets that could function as a biomarker for cardiovascular risk assessment. In the next part the process of atherosclerotic plaque formation will be further discussed. During this discussion the focus will be on the possibility of these targets to monitor atherosclerotic plaque development and thereby the possibility to estimate a persons risk for a cardiovascular event.

Biomarker candidates for monitoring atherosclerotic plaque development

Apolipoproteins

As mentioned before, the initial event that causes atherosclerotic plaque development is oxidative stress followed by endothelial cell activation which enhances an inflammatory response (Figure 2). The initiation of vascular inflammation is caused by low density lipoproteins (LDL). The LDL particles contain esterified cholesterol and triglycerides surrounded by a shell of phospholipids, free cholesterol and Apolipoprotein B100 (Apo B100). (7) The circulating LDL particles can accumulate in the intima where Apo B100 binds to proteoglycans of the extracellular matrix. Within the intima the LDL particles are prone to oxidative modifications. Modified phospholipids can initiate innate immune responses and thereby facilitate atherosclerotic plaque formation. (7)

Because LDL and cholesterol play a major role in atherosclerotic plaque formation, either the total cholesterol concentration in plasma or the LDL/HDL ratio in plasma is used as one of the main variables in CVD risk prediction models. (1) However, with one molecule of Apolipoprotein B (Apo B) in each atherogenic particle, levels of Apo B are a direct measurement of the number of potentially atherogenic particles. (8, 9) Plasma Apo B levels proved to be a better marker of risk for vascular disease and a better guide to the adequacy of statin treatment than any cholesterol index. Moreover, as Apolipoprotein A-I (Apo A-I) reflects anti-atherogenic HDL particles the ratio of Apo B / Apo A-I seems superior to the ratio of total LDL/HDL cholesterol as an overall index of the risk of vascular disease. (8, 9) Both Apo A-I as Apo B have high potential for cardiovascular disease prediction and could therefore be used in novel CVD risk assessment models.

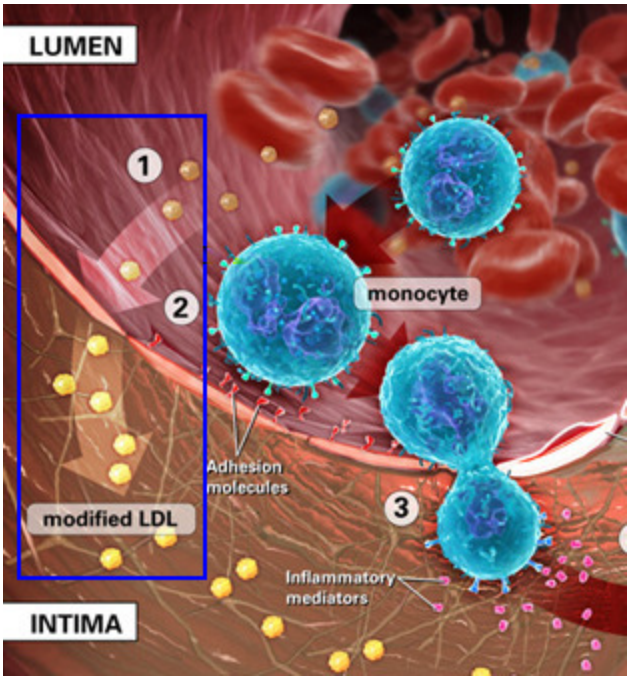

Figure 2 The process of atherosclerotic plaque formation. (1) Oxidized LDL particles cause endothelial cell activation.

Adhesion molecules

As the circulating LDL particles accumulate in the intima and become oxidatively modified, the modified phospholipids initiate an inflammatory response by activating arterial endothelial cells to produce adhesion molecules and chemokines (Figure 3). The adhesion molecules ICAM-1, E-selectin and VCAM-1 lead to the rolling, activation, and firm adhesion of leukocytes to the endothelium. (7) Levels of the soluble adhesion molecules sVCAM-1, sICAM-1, and sE-selectin have been, independent of classic risk factors and clinical features, significantly related to future death from cardiovascular causes in patients with documented coronary artery disease (CAD). (10) In addition to the classic risk factors and hs-CRP, sVCAM-1 significantly improved determining the risk of future cardiovascular death. (10)

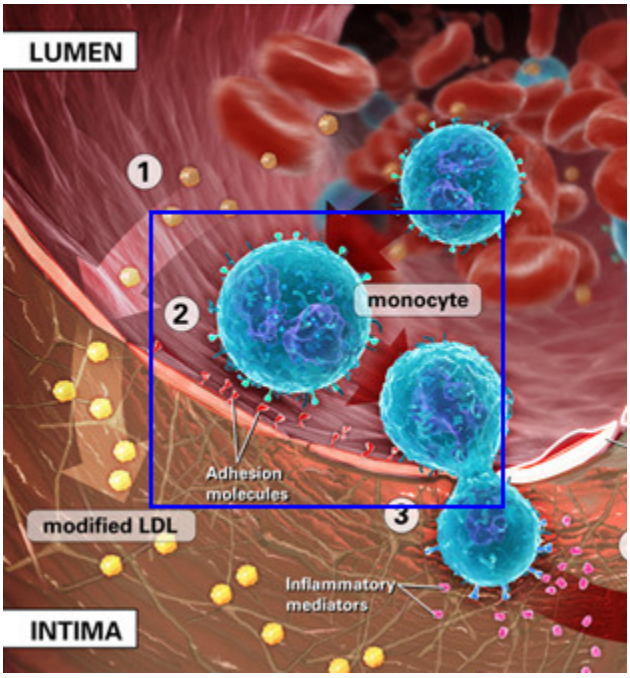

Figure 3 The process of atherosclerotic plaque formation. (2) Activated endothelial cells express adhesion molecules and chemokines that promote monocyte migration.

Chemokines

During this initial stage of atherosclerotic plaque formation, activated endothelial cells also produce chemokines as CCL2 (MCP-1), CCL5 (RANTES), CXCL10 (IP-10), CX3CL1 (fractalkine) (Figure 4). (7) MCP-1 and interleukin (IL)-8, chemoattractants for monocytes and neutrophils, have been associated with coronary heart disease. (11) However, after correcting for cardiovascular and immunological risk factors this observed association became non-significant. Therefore, they might not represent novel independent cardiovascular disease risk factors and are thus unlikely to significantly improve disease prediction by established cardiovascular risk factors. (11) In the PRIME study neither the level of RANTES, IP-10, MCP-1 or eotaxin-1 could be associated with future coronary heart disease. (12) However, RANTES and IP-10 were independently of traditional cardiovascular

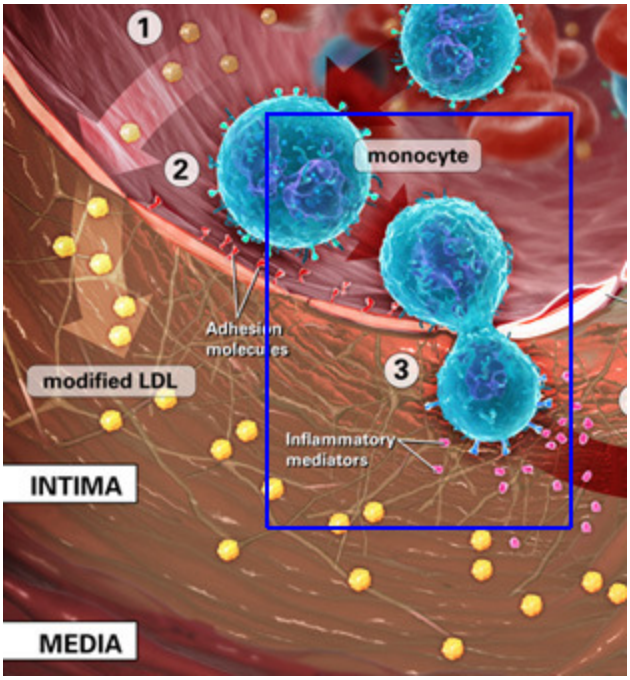

Figure 4 The process of atherosclerotic plaque formation. (3) Monocytes adhere to endothelial cells and migrate into the intima.

risk factors, hs-CRP, and fibrinogen associated with ischemic stroke. Their addition to a traditional risk factor model predicting ischemic stroke substantially improved the C-statistic and thereby RANTES and IP-10 may improve the accuracy of ischemic stroke risk prediction over traditional risk factors. (12)

When monocytes under the influence of chemokines have migrated into the intima, they are stimulated by macrophage colony-stimulating factor (MCSF) to differentiate into macrophages. (7) The differentiation into macrophages is essential for the development of atherosclerosis and therefore MCSF is a possible candidate marker for CAD. An association between MCSF and CAD has been observed in patients with chronic CAD. (13) The prognostic value of MCSF was independent and complementary to that of CRP. (13)

Scavenger receptors

As the monocytes, now located in the intima, have differentiated into macrophages they up-regulate their scavenger receptor expression and start taking up modified LDL. When the macrophages are loaded with cholesterol they become foam cells, which are characteristic of atherosclerotic plaques (Figure 5). (7) Scavenger receptors that can internalize modified LDL particles are: SRA-1, SRA-2, MARCO, CD36, SR-B1, LOX-1 and PSOX21. (7)

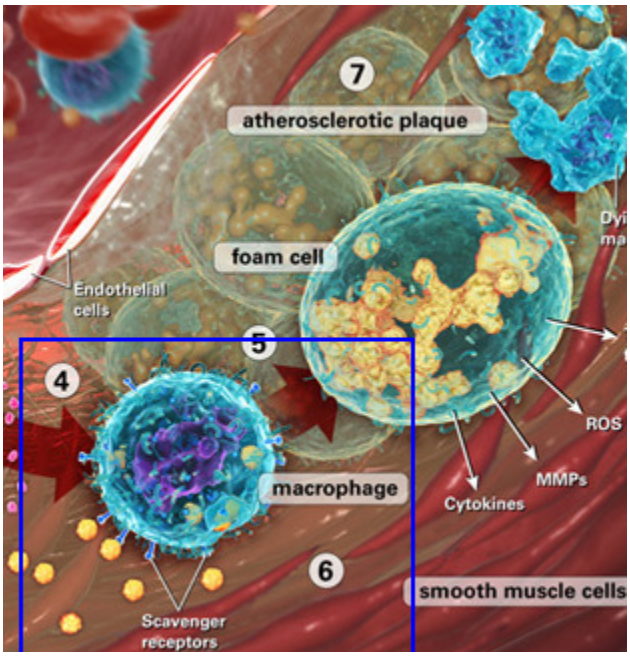

Figure 5 The process of atherosclerotic plaque formation. (4) Monocytes differentiate in macrophages and enhance their scavenger receptor expression.

Among the array of macrophage scavenger receptors CD36 is regarded as one of the most important in the uptake of oxLDL. (14) Monocytes in patients with familial hypercholesterolemia (FH) show over-expression of the CD36 scavenger receptor. (15) Blocking of the CD36 receptor with an antibody to prevent the uptake of oxLDL resulted in a reduced uptake of oxLDL by more than 50%. The CD36 scavenger receptor was therefore identified as the major receptor for oxLDL in circulating monocytes from patients with FH. (15) CD36 is hereby an interesting target for CVD risk assessment.

Soluble lectin-like oxidized low-density lipoprotein receptor-1 (sLOX-1) is the scavenger receptor which has most of all been associated with CAD. Serum sLOX-1 levels have been correlated with coronary lesion complexity in patients with CAD. (16) sLOX-1 levels in serum from stable CAD patients with complex coronary lesions were significantly higher than those with simple coronary lesions. The sLOX-1 levels were independent predictors of the presence of complex coronary lesions in patients with stable CAD. (16) Furthermore, patients with acute coronary syndrome (ACS) had significantly higher circulating sLOX-1 levels than patients with stable CAD. sLOX-1 levels did also correlate with the number of complex coronary lesions. (16)

When the diagnostic value of sLOX-1 in patients with ACS was compared to those of troponin T (TnT) and heart-type fatty acid binding protein (H-FABP), the circulating sLOX-1 levels were a more and specific biomarker for ACS than TnT and H-FABP, and provide additional diagnostic values when measured in combination with TnT. (17)

Cholesterol exporters

Cholesterol that has accumulated within the foam cells can also be mobilized to high-density lipoproteins by ABC-type cassette transporters for export through the liver and bile system (Figure 6). (7) ABCA1, which is part of the ABC-type cassette transporter family, is critical for the cellular cholesterol efflux. Mutations in the gene encoding ABCA1 result in reduced cellular cholesterol efflux and lower HDL levels and are associated with increased CVD risk. (18) Furthermore, ABCA1 may also influence inflammatory signaling pathways. This is either accomplished indirectly by modifying cell surface lipid domains or directly by inducing signaling through the JAK2/STAT3 pathway in response to binding lipid poor Apo A-I. (18) In patients with FH an impaired ABCA1 gene expression and a down-regulation of ABCA1 protein expression in monocytes has been observed. (15) This can contribute to an increased foam cell formation and thereby increased CVD progression. The scavenger receptor / ABCA1 ratio, which should represent the kinetics of cholesterol uptake into the monocytes or macrophages and thereby reflect foam cell formation, could therefore be an interesting marker for CVD risk prediction.

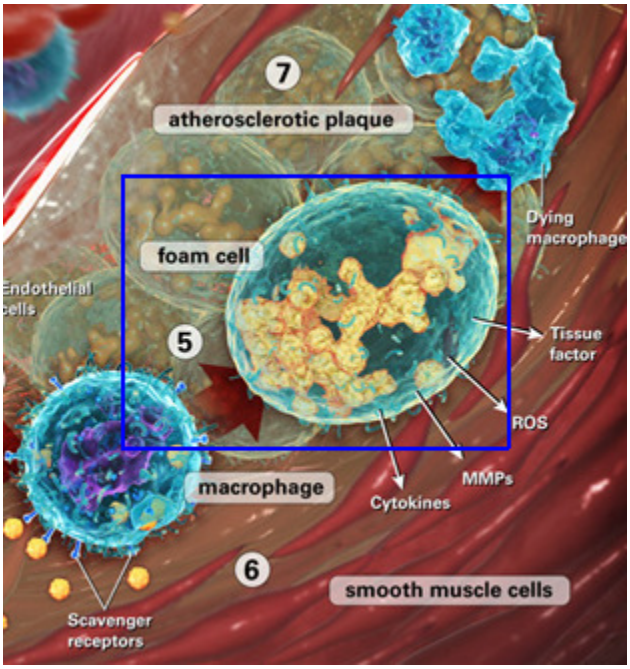

Figure 6 The process of atherosclerotic plaque formation. (5) Cholesterol accumulation turns the macrophages in foam cells.

Monocyte subsets

The cholesterol transporters also modulate the differentiation of hematopoietic stem cells and thereby control the number of circulating monocytes. (7) Monocytes are the main cellular component of atherosclerotic plaques and are therefore important in atherogenesis (Figure 7). (14) Monocytes are a relatively heterogeneous population that is phenotypically polarized by their microenvironment. (14) Human monocytes are identified through their large amount of CD14 expression. By the differential expression of CD16 multiple monocytic subsets can be identified. The $CD14^{++}CD16^{-}$ monocytes comprise the major monocyte population (~85-95%) and are also called the “classical monocytes”. (14, 19) The remaining monocytes express CD16

and are divided in two subtypes according to their different expression levels of CD14: $CD14^{+}CD16^{+}$ and $CD14^{++}CD16^{+}$. (19)

Changes in monocyte subsets and phenotypes have been associated with the clinical course after acute stroke. After an acute stroke an increase of the $CD14^{++}CD16^{+}$ monocyte subset and a decrease of the $CD14^{+}CD16^{+}$ monocyte subset has been observed. No significant change within the classical subtype, $CD14^{++}CD16^{-}$ monocytes, has been observed. (19) An increased proportion of $CD14^{++}CD16^{-}$ monocytes were associated with poor outcome, increased mortality, and early clinical worsening after stroke. On the contrary, mortality was inversely related to $CD14^{++}CD16^{+}$ and poor outcome and infarct size were inversely related to $CD14^{+}CD16^{+}$. (19)

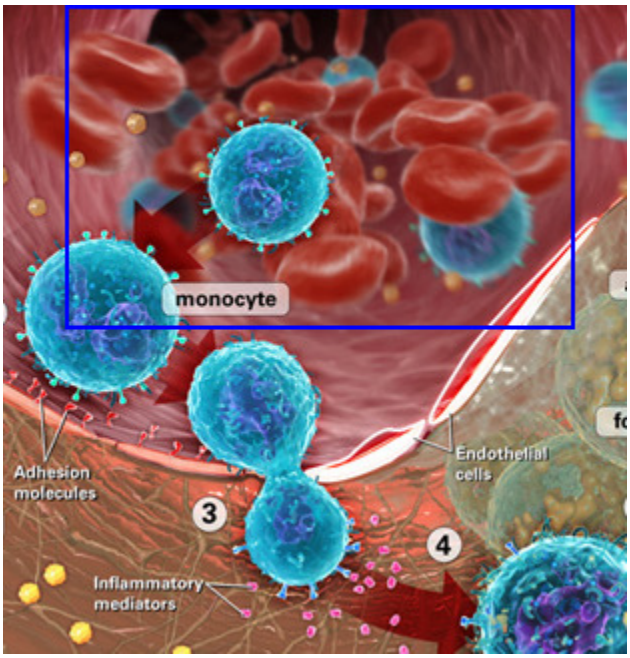

Figure 7 The process of atherosclerotic plaque formation. The composition of the monocyte population contributes to atherosclerotic plaque formation.

In a cohort of healthy individuals a significant association between counts of $CD16^{+}$ monocytes, but not for total monocytes or $CD16^{-}$ monocytes, and both obesity as well as subclinical atherosclerosis has been found. (20) However, in a linear multiple regression analysis the association of $CD16^{+}$ monocyte counts and BMI with subclinical atherosclerosis failed to achieve statistical significance. This is probably caused by the effect of obesity on the traditional risk factors. (20)

In another study involving a severely obese population undergoing gastric surgery and a moderately obese population on a weight loss program a significant increase of both $CD14^{+}CD16^{+}$ and $CD14^{++}CD16^{+}$ monocytes compared to lean healthy controls has been observed. Diabetes was also associated with an increased frequency of $CD14^{+}CD16^{+}$ cells. (21) In parallel with reductions in bodyweight the percentages of $CD16^{+}$ subsets decreased, which strengthens the association between bodyweight and $CD16^{+}$ monocytes. However, no relationship between $CD16^{+}$ monocyte subsets and subclinical atherosclerosis, evaluated by intima-media thickness (IMT) measurement, could be found after adjustment with other risk factors. (21)

In patients with chronic kidney disease undergoing renal transplantation the frequency of circulating $CD16^{+}$ monocytes is independently associated with subclinical atherosclerosis. (22)

In patients with non-dialysis chronic kidney disease (CKD) has been demonstrated that $CD14^{++}CD16^{+}$ monocytes are independently associated with CV events. (23) The $CD14^{++}CD16^{+}$ monocyte counts were higher in patients who experienced a CV event during follow-up compared with patients without an event, whereas counts of total monocytes, $CD14^{++}CD16^{-}$, and $CD14^{+}CD16^{+}$ monocytes did not differ significantly. Patients with the highest $CD14^{++}CD16^{+}$ monocyte counts had the shortest event-free survival and the shortest overall survival.(23)

In patients with CAD increased levels of CD14+CD16+ monocytes compared to healthy controls have been observed. Furthermore, serum TNF-α concentrations correlated with the number of $CD14^+CD16^+$ monocytes. (24) The association between coronary atherosclerosis and TNF-α was independent of potential confounders. (24)

The role of each monocyte subset in recruitment at early stages of atherogenesis and the development of the vulnerable plaque is yet to be established. Although there are contradicting results, monocyte subsets possibly provide additional variables for new CVD risk prediction models.

Monocyte membrane markers

Beside CD14 and CD16, monocytes also express a broad variety of other membrane bound proteins (Figure 8). One family of these membrane proteins comprises the pattern-recognition receptors (PRR). PRR identify pathogen-associated molecular patters (PAMPs). Among the family of PRR, toll-like receptors (TLR) are the most associated with atherosclerotic plaque development. (25)

In patients with stable CAD the levels of TLR2 and TLR4 expression on $CD14^+$ monocytes positively correlated with vessel score and weakly correlated with the Gensini score independent of hs-CRP, which suggests that TLR2 and TLR4 expression correlates with the extent and severity of coronary artery disease. (26)

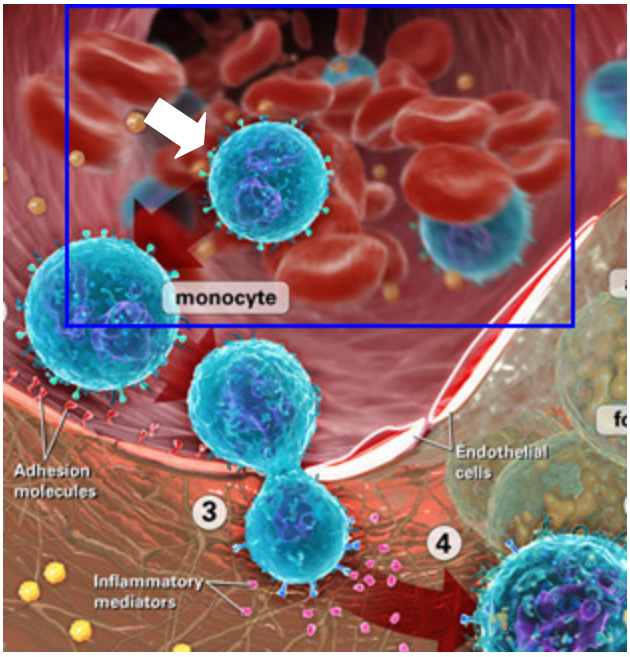

Figure 8 The process of atherosclerotic plaque formation. The composition of the monocyte membrane markers contributes to atherosclerotic plaque formation.

A relation between TLRs and the severity of coronary artery disease was also observed in patients with stable coronary artery disease scheduled for a percutaneous coronary intervention (PCI). Both the TLR2 as the TLR4 response in this cohort was associated with percentage diameter stenosis, multi-vessel disease and free flow rate (FFR) outcome. (27) In this study the TLR response was expressed as tumor necrosis factor-α (TNF-α) levels corrected for $CD14^+$ monocyte count. Whether the observed increased expression is caused by an increased TLR responsiveness or by a shift towards more $CD14^+CD16^+$ monocytes, which have been associated with increased serum TNF-α levels (24), remains unknown, however TLRs have been associated with atherosclerotic plaque development and are therefore either direct or indirect good candidate markers for new atherosclerotic risk assessment models.

Another monocytic membrane marker that could be related to CVD is the angiotensin converting enzyme (ACE). Patients with chronic renal disease have an extraordinarily high risk for cardiovascular disease which is partly explained by increased inflammation and oxidative stress mediated by the activated renin-

angiotensin system. (28) Activation of the renin-angiotensin system involves the angiotensin converting enzyme (ACE) which is expressed on monocyte cell membranes. (28) Monocytic ACE expression, which is increased in dialysis patients compared to individuals with intact renal function, is significantly and independently associated with prevalent CVD. (28) Although, monocytic ACE expression has been independently associated with CVD, this association was only observed in patients with chronic renal disease. Whether this association can be made in patients who suffer from CVD without further renal complication should be evaluated before ACE can be used as a potential marker in CVD risk assessment models.

Matrix metalloproteinases

When the macrophages have turned into foam cells, the foam cells release matrix metalloproteinases (MMPs) and thereby contribute to plaque destabilization (Figure 9). (29) MMPs are part of a family of endopeptidase enzymes involved in the degradation and reorganization of the extracellular matrix. (29) Extracellular matrix degradation is regulated by the balance between MMPs and their specific tissue inhibitors (tissue inhibitor of metalloproteinases, TIMPs). An unbalance between the activation and inhibition of MMPs results in the proteolysis of the vascular extracellular matrix and is crucial for atherosclerotic plaque formation and plaque destabilization. (30)

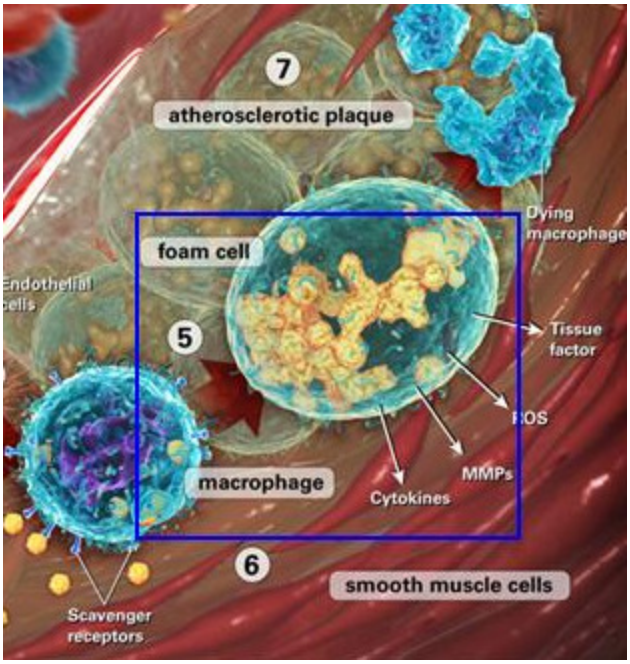

Figure 9 The process of atherosclerotic plaque formation. Matrix metalloproteinases (MMPs) contribute to plaque destabilization.

Serum MMP-10 levels have been associated with subclinical atherosclerosis. After multiple regression analysis controlling for traditional atherosclerotic risk factors and inflammatory markers, MMP-10 and not MMP-1 and MMP-9, remained significantly associated with IMT. (31)

In patients with chronic kidney disease (CKD) concentrations of MMP-8, -10, and TIMP-1 were significantly higher in the group of patients with more severe atherosclerosis when compared with those with no or mild atherosclerosis. (30) After multivariate analysis, being on dialysis, age, C-reactive protein, and the concentration of MMP-10 were significantly associated with the risk of severe atherosclerosis. Only circulating MMP-10 concentrations remained thus as a possible independent risk factor for atherosclerosis in CKD patients. (30)

In patients with either stable CAD or unstable angina pectoris (UAP) plasma MMP-8 levels were higher compared to age- and gender-matched controls. Furthermore, MMP-8 levels in patients with UAP were even higher than MMP-8 levels in patients with stable CAD. (32) The plasma MMP-8 level was an independent

factor for UAP in multivariate analysis and may thus reflect coronary plaque instability. (32) Hereby, MMPs could provide additional information for new CVD risk assessment models.

Cytokines and other soluble proteins

During the whole process from endothelial cell activation, plaque formation and finally atherosclerotic plaque destabilization, acute phase proteins are increased in the circulation (Figure 10). These acute phase proteins could also serve as markers of plaque progression. Among the multiple circulating markers for atherosclerotic disease, CRP is the most extensively studied. CRP is associated with unstable CAD and acute coronary syndrome and its predictive role for future cardiovascular events is widely accepted. (33) CRP is not only associated with the incidence of CAD, but also a strong univariate association of CRP with the prevalence of angiographically assessed CAD has been observed. (33) Although in a multivariate analysis CRP could not be associated with the severity of CAD, the association with the prevalence of CAD remained significant and independent. (33)

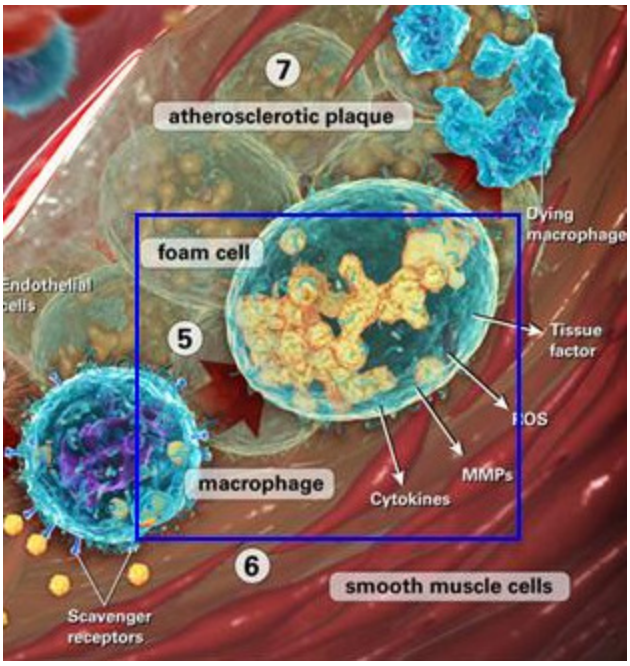

Figure 10 The process of atherosclerotic plaque formation. Acute phase proteins contribute to plaque progression.

Furthermore, increased high sensitivity-CRP (hs-CRP) levels before coronary stent implantation have been associated with risk of death or MI, but were not related to target vessel revascularization or stent thrombosis. (34) This indicates that hs-CRP levels do not predict the course of development of local atherosclerotic plaques but reflect the atherosclerotic burden within large parts of the vascular tree.

Beside CRP multiple cytokines have also been associated with CVD. Increased concentrations of IL-6 (35), IL-8 (36), and TNF-α (37) have been independently associated with coronary heart disease. On the contrary in a group of patients that were admitted with acute chest pain and underwent coronary angiography, no association could be made between inflammatory markers (IL-6, hs-CRP, and TNF-α) and major adverse cardiac events or angiographic severity of CAD. (38) While in another study only IL-8 could independently predict cardiovascular events, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, TNF-α, granulocyte-macrophage colony stimulating factor (GM-CSF) and γ-interferon (IFN-γ) could only in a univariate model be related to CVD. (39) Cytokines, thus, have proved to possess predictive power in cardiovascular disease risk estimation; however the results are very controversial.

Beside the acute phase proteins also other circulating proteins have been associated with CVD. Osteopontin (OPN), a secreted calcium-binding glycophosphoprotein, has also been independently associated with future

adverse cardiac events in patients with chronic stable angina. (40) Furthermore, pre-procedural OPN levels of patients with CAD undergoing PCI have been associated with coronary plaque progression and in-stent restenosis. (41) OPN is thus related to atherosclerotic plaque progression and can possible contribute in short-term CVD risk prediction models.

Possible analytical tool to measure new CVD related biomarkers

As previously discussed, CVD caused by atherosclerosis is a multi-factorial continuous disease. This implies that measurement of a single biomarker is not sufficient to gain a realistic impression of the status of a progressing atherosclerotic plaque. The meaning of a certain biomarker concentration should always be considered in the context of other biomarker concentrations. Therefore, current predictive and diagnostic models do not meet the requirements for individual based short term CVD risk estimation and personalized treatment. Thus, new predictive and diagnostic models are needed that meet the requirements of modern medicine. To develop these new CVD risk prediction models not only new biomarkers related to CVD are necessary, but also an analytic platform that can measure multiple analytes in as less sample possible. Furthermore, a diverse spectrum of markers should be able to be analyzed with one single tool. And last but not least, new biomarkers should be cost effective and preferentially not require the purchase of expensive equipment and training of specialized personal. What analytical tool can be used to measure multiple markers, in as less sample as possible, is present in modern clinics, is easy to use, is reliable, has a high reproducibility, can be further developed, and has low running costs? A technique that meets all these requirements and has already widely been used in the clinical setting is: flow cytometry.

Flow cytometry, originally established as an automated method for measuring optical or fluorescence characteristics of cells or particles in suspension, has become an increasingly important technique for both basic research and clinical application. (42) A flow cytometer is an instrument designed to detect and enumerate corpuscular (mostly cellular) elements in a suspension. Cells pass an interrogation point one by one in a precise and rapid way, which is accomplished by generating a pressurized stream of sheath fluid into which cells are injected. Pressurized sheath fluid causes hydrodynamic focusing of the cells and ensures that the cells pass the interrogation point single file. At the interrogation point a laser is directed on the stream of cells. The features of a cell are determined by their ability to scatter this incident laser light. (43) The scattering of the laser light determines the physical properties of the cells. Cell size is defined by the forward scatter, and cell granularity is defined by the sideward scatter. Furthermore, labeling the cells by fluorescently-tagged reagents enables further characterization of cells. Multiple cellular components can be tagged by fluorescent dyes: specific proteins on the cell membrane or inside the cell, RNA, or DNA. (44)

New advances in flow cytometry also enable the quantification of soluble proteins. In this technique a combination of conventional immunoassays with microbeads is used to analyze and quantify specific proteins. The surfaces of the fluorescent microbeads constitute the platform for specific molecular reactions, and the presence of bead-bound analyte is detected with a conjugate coupled to a reporter fluorochrome. Each

microbead set contains a unique ratio of fluorochrome labels, which enables the detection of multiple analytes in a single sample. Modern multiplex systems can detect up to 100-500 analytes simultaneously. (42)

Flow cytometry is a well established technology and in the clinic it is used for the testing of benign and malignant hematopoietic disorders or predicting, staging, and monitoring disease progression and response to treatment in HIV-infected individuals. (44) Although flow cytometry is already used in the clinic, it has not found its way to be used for diagnosing or monitoring cardiovascular disease. However, it has been shown that there is increasing evidence that suggests cross-talk between the diseased vascular tree and subsets of circulating cells and that functional tests could serve as a surrogate measure of disease progression. (45) For example, it has been shown that obesity, a risk factor for cardiovascular disease, is associated with an enhanced TLR response on circulating cells in patients suffering from established atherosclerotic disease. (46)

Recent findings and current ongoing studies could thus open the doors for implementation of flow cytometry in cardiovascular diagnosis and disease monitoring. Furthermore, new technical developments result in a decrease of instrument size, lower costs and an increase in the number of detectable analytes. The combination of standard flow cytometry and multiplex analysis makes flow cytometry thereby an important technique in basic research and a potential analytical tool in multi-marker screening for CVD risk assessment after successful implementation in the clinic.

Discussion

A large effort has been put in the search for new biomarkers for cardiovascular disease. Many markers related to atherosclerosis were found to be independently, significantly associated with cardiovascular disease. However, addition of the new biomarkers to the classical CVD risk prediction models only resulted in a little increment of risk prediction. (4, 5) Furthermore, the new risk prediction models are only useful for long-term risk estimation. (1, 3) Thus, monitoring disease progression and thereby short-term risk prediction is still not possible.

How is it possible that after years of effort and many large population studies only slow progression is achieved with CVD risk prediction? Are we still looking in the right direction or is it time to let the current paradigm go and focus on the development of new models which fit the requirements of modern medicine?

Classical CVD risk assessment models

The foundation of current CVD risk assessment is based on the findings of the Framingham Heart Study and several other comparable studies which showed that CVD is usually the product of multiple interacting risk factors. The identified risk factors are used in multivariate models to estimate the 10-years risk of CVD (Figure 11). (1) Current research is focused on the addition of new biomarkers to the established models to improve CVD risk estimation. Addition of new biomarkers does however only marginally increase the predictive power of these models. (4, 5) To find out how this is possible we first need to have a look at the classical model.

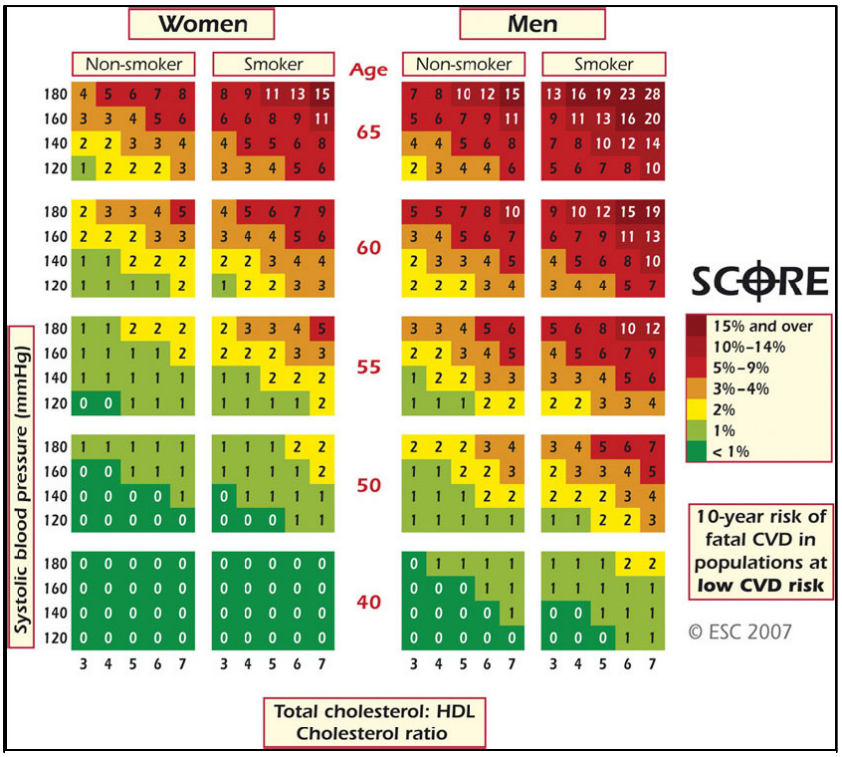

Figure 11 SCORE chart: 10-years risk of fatal CVD in populations at low CVD risk based on the following risk factors: age, gender, smoking, systolic blood pressure, and total cholesterol: HDL cholesterol ratio. Adapted from (1)

The most common variables in the current models are: age, gender, blood pressure, smoking and cholesterol with the occasional addition of diabetes, family history or BMI. (1) By long-term follow up, these risk factors have been identified as potent contributors to CVD development. To identify their power in short-term risk assessment we should take a closer look at these variables. To start with, we divide them into two groups: dichotomous variables (gender,

smoking, diabetes, and family history) and continuous variables (age, blood pressure, cholesterol levels, and BMI).

The dichotomous variable, gender is mostly a static variable which ones it has been set it will not change during a lifetime and therefore stays the same for both short-term as long-term risk assessment. The other three dichotomous variables are a little more complicated because they can change during a lifetime. Diabetes and family history can be prevalent at birth or have their onset during aging; however once they are positive they become a static variable that cannot be returned to its original value. Smoking can be a more changing feature. People start smoking, keep on smoking, quit smoking, start again with smoking or do not smoke at all. Although this seems to be a more fluctuating variable, the effects of long-term smoking can remain even after quitting for a long time; therefore smoking can be seen more as a yes or no feature than a possible changing variable. The before mentioned variables are, thus, either static or change only once during a lifetime. From this observation we can conclude that the dichotomous variables are useful for a strong foundation of risk prediction models, but they can not be used as variables that significantly contribute to a change in risk over a defined short period of time because of their static appearance. For example, we could compare a non-smoking woman, age 65, systolic blood pressure 180 mmHg and total cholesterol: HDL cholesterol ratio of 7 with a smoking woman with the same age, blood pressure, and total cholesterol: HDL cholesterol ratio. According to the SCORE chart, the 10-years risk of fatal CVD almost doubles from 8% to 15% (Figure 11). However, the effect on her short-term risk for a CVD related event remains largely unknown. Prediction of acute CVD related events is thus not possible with the dichotomous variables of classical risk prediction models probably because they are little dynamical.

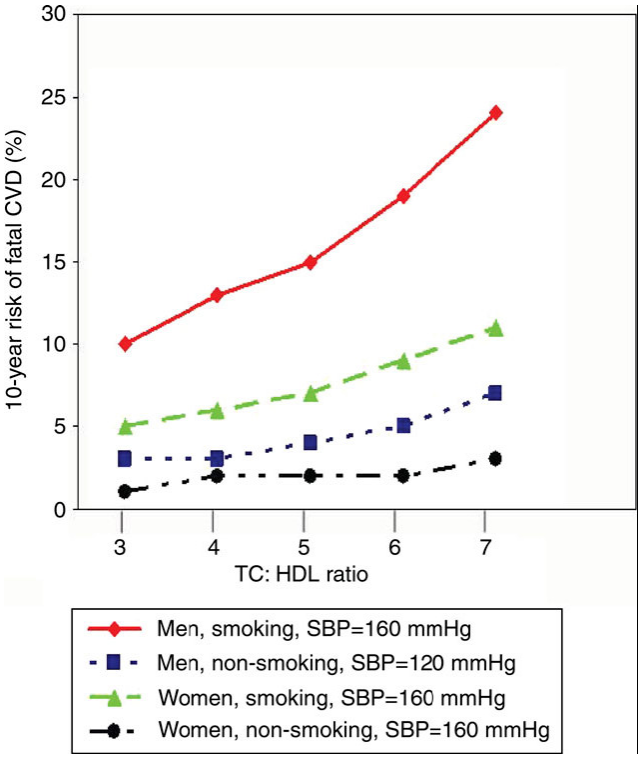

Figure 12 The relationship of total cholesterol (TC):HDL cholesterol ratio to 10-years fatal CVD events in men and women aged 60 years with and without risk factors, based on a risk function derived from the SCORE project. SBP = systolic blood pressure. Adapted from (1)

On the other hand, current risk prediction models also contain continuous variables (age, blood pressure, cholesterol levels, and BMI). These variables can assume a value over a broad range and can strongly fluctuate during a lifetime. Therefore, these variables which are more dynamical seem to provide information about the risk of CVD over a defined period of time. But do they?

When we first take a look at the physiologically or pathologically changing parameters (blood pressure, cholesterol levels and BMI) we can conclude that these parameters can change over a defined period of time. Blood pressure can change in a matter of seconds, cholesterol levels can fluctuate during the day and BMI

can significantly change over a period of several weeks. As these changes can occur over a defined short period of time, why can they not predict the course of CVD within a short period of time? This can mostly be explained by the effect these parameters have on atherosclerotic plaques. High blood pressure, high cholesterol levels, and a high BMI are thought to contribute to the initiation and propagation of atherosclerotic plaque formation, but with the exception of blood pressure a sudden change in these parameters has not been associated with acute CVD related events. (1) The effect of these parameters on CVD is more reflected by their long term accumulative contribution to plaque development than by their immediate or short-term effects on CVD.

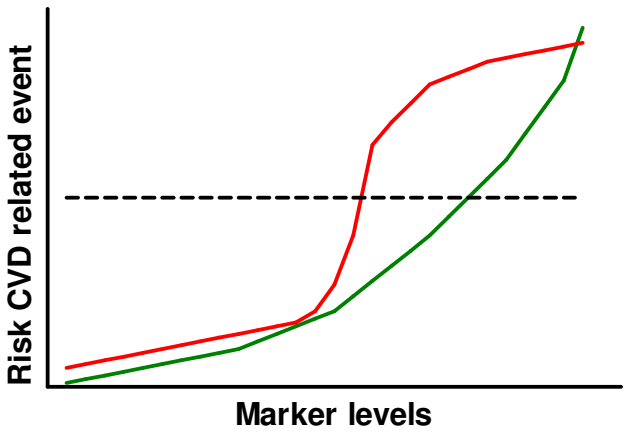

Figure 13 A long-term risk prediction model that could be suitable for short-term risk prediction. The red line indicates a short but strong increase of risk that could happen in advance of an adverse event. The green line indicates an exponentially increasing risk. The dotted line indicates a threshold that could predict an adverse event.

When we take a look at age, age can be assumed as a dynamical element in current risk prediction models. But can we truly assume age as being a dynamical element that could enhance short-term CVD risk prediction? First, a simple conclusion can be drawn: everybody ages. The second obvious conclusion which we can draw is: everybody dies. By keeping the average human lifespan in mind, the chance of dying thus increases as someone ages. Age should thus be assumed, although it sounds paradoxically, to be a continuously increasing constant. In other words, age is a continuous variable, but because of its continuously increasing property it does, in the case of short-term risk prediction models, not behave as a dynamical variable. Thus, age can not, by for example reaching a certain threshold, be independently associated with acute disease progression.

The previously mentioned observations that both the dichotomous variables as the continuous variables in classical CVD risk assessment models are little dynamical, can for example be seen in Figure 12. When we look at the 10-years risk of fatal CVD related event according to a model from the SCORE project; for a smoking woman aged 60 years and a SBP of 160 mmHg, the risk continuously increases in an almost linear manner as the total cholesterol: HDL cholesterol ratio increases (green line Figure 12). Although cholesterol levels are one of the most dynamical variables in this model the risk increases in an almost linear manner. According to the same model, even high risk populations (smoking men aged 60 years and a SBP of 160 mmHg) show an almost linear increase of risk as the total cholesterol: HDL cholesterol ratio increases (red line Figure 12) (1). The fact that the risk increases in a steady manner and the lack of a sudden short but strong increment of risk reflects that this model needs a certain threshold at which an adverse event nearly always happens otherwise it is not suitable for short term risk assessment. In models where a short but strong increase of risk (red line Figure 13) or exponentially increasing risk (green line Figure 13) predict the long-term risk of a CVD related event a certain threshold (the dotted line Figure 13) could be the indication that an

adverse event is about to happen on the short-term and therefore fast intervention could be advisable. However, because there is no such a threshold known, the current models, without the addition of other variables, can not be used for short-term risk assessment.

Taken these observations together, current CVD risk assessment models are strong models to define the possible pathological burden at one single moment in time and estimate the chance of adverse events on the long-term, but lack the power of short-term risk assessment. This could be explained by the effect these parameters have on CVD, which is more reflected by their long term accumulative contribution to plaque development than by their immediate or short-term effects on CVD. Thus, one of the shortcomings of classical CVD risk assessment models is: they are little dynamical and by lacking any fast changing variables that could affect short-term outcome of CVD or a threshold that could predict an adverse event, they do not enable short-term CVD risk prediction.

Current CVD risk assessment models

Classical CVD risk assessment models lack the power of short-term risk assessment. Therefore, current research focuses on the discovery of new CVD related biomarkers to improve the classical risk prediction models. The question that arises is whether these biomarkers have the power to make such a contribution to the classical long-term CVD risk assessment models so that they make short-term CVD risk assessment possible. To answer this question we should take a look at the process of biomarker research. Conventionally, biomarkers are mostly searched in prospective or retrospective studies with large biobanks. During these studies a baseline measurement is taken and the patients are followed during a certain follow up period. The baseline measurement is either taken pre-event, when subclinical atherosclerosis has been diagnosed, or post-event, after clinical symptoms occurred. By comparing the target biomarker levels in patients with an event during follow up and patients without an event during follow up, investigators tested whether a candidate biomarker could be associated with a clinical event. A candidate marker that was independently associated with a clinical manifestation was mostly added to classical models and the increment of this marker to the classical models was tested. These are strong study designs; because the sample size is mostly large and therefore high numbers of (primary or secondary) events can be compared with a large non-event population. Unfortunately, many candidate markers that were independently associated with a clinical manifestation did not significantly improve the risk prediction after addition to the classical models. (4, 5)

To find out how it could be that although a potential biomarker was related to cardiovascular events it could not increase the predictive power of the classical models, we should take a look at how the results of biobank studies mostly look. In Figure 14 an example of a possible result from a biobank study is shown. In Figure 14.A the levels of a supposed biomarker (baseline measurement) are plotted against the age of the persons during their first CVD related event. The persons who developed a second CVD related event are stained red. In Figure 14.B the biomarker levels are plotted against persons who either do or do nor develop a secondary

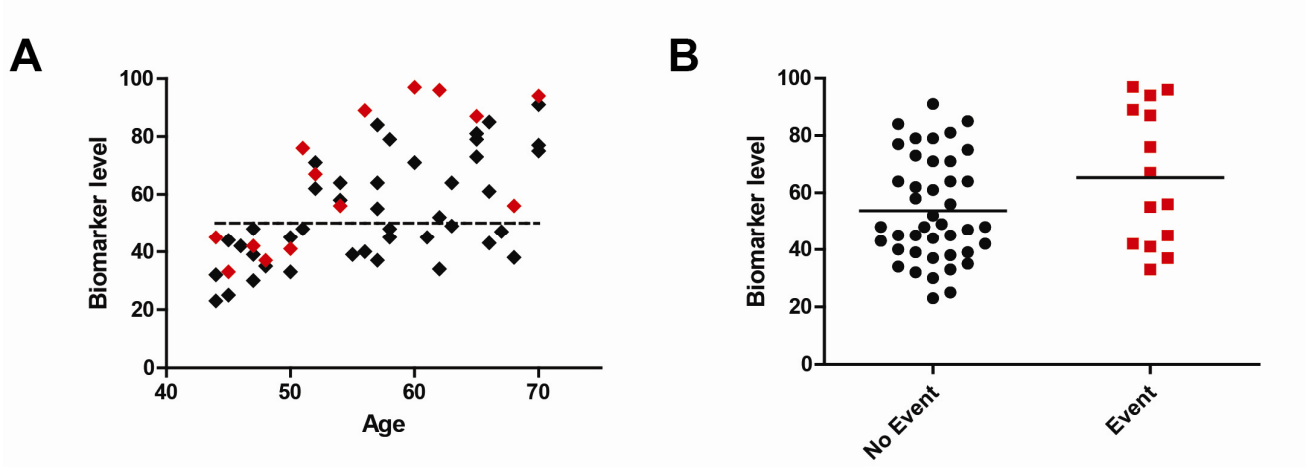

Figure 14 Examples of current CVD research biobank studies. A) The levels of a possible biomarker are plotted against the age of the persons during their first CVD related event. The persons who develop a second CVD related event are stained red. B) The population is separated in a group who develops no secondary event and a group who develops a secondary event. The individual biomarker levels are shown.

CVD related event. According to Figure 14.A there is no clear relation between the biomarker levels, a secondary cardiovascular event, or age. However, when the event group and the non-event group are separated, there seems to be an association between high biomarker levels and secondary events. The association is unfortunately not very strong. There a lot of patients who have raised biomarker levels but do not develop a secondary event. Even more important: there are patients who have relatively low biomarker levels but still develop a secondary event (Figure 14.B).

Because atherosclerosis is a multi-factorial disease other factors could have influenced the final outcome of a patient and a single biomarker level does not necessarily be an indication of a good or bad outcome. It was expected that adding more biomarker to these models should narrow down the chance of false positive or false negative results and thereby improve the classical risk prediction models. However, adding more biomarkers to the classical model did not significantly improve the risk prediction. (4, 5)

One possible explanation for the phenomenon that independently associated markers not significantly improve the risk prediction after addition to the classical models could be: the kinetics of most biomarkers is unknown and most ongoing or completed studies only include a single baseline measurement and thereby exclude kinetic variances. What this means is: do the biomarker levels that were measured during a baseline measurement represent the levels of this single biomarker during the ongoing process of atherosclerotic plaque progression? While the kinetics of biomarkers is mostly unknown many questions remain unanswered. Could biomarker levels fluctuate very strongly and thereby could a high or low biomarker level be a pathological or physiological pulse that was coincidently associated with the outcome of a patient? Or, are the biomarker levels relatively constant and differ between individuals? Or, do they increase or decrease over time when atherosclerotic plaques develop?

The major effects that biomarker kinetics could have on disease prediction are illustrated in Figure 15. In Figure 15 biomarker levels of a large population are plotted against time. For the ease of understanding, the

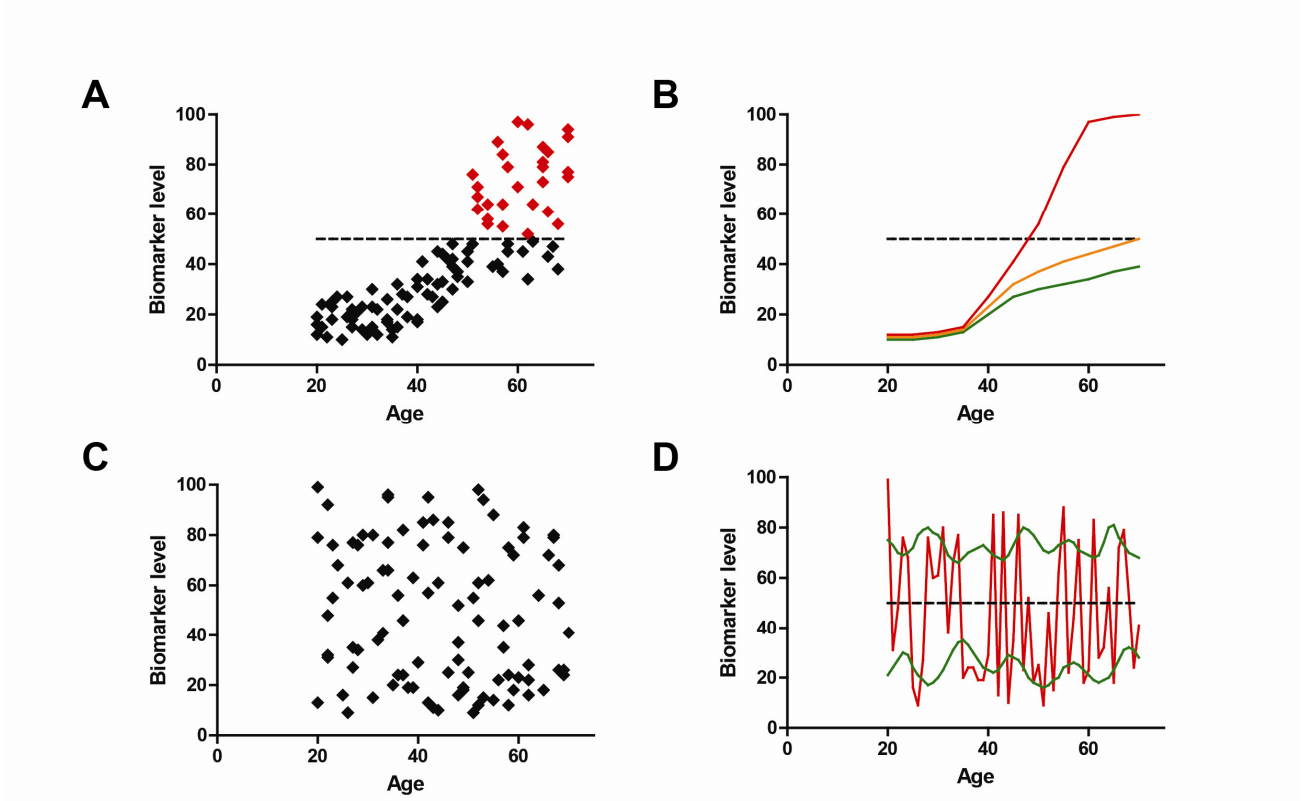

Figure 15 Good versus bad biomarkers. The dotted lines represent a possible threshold for an increased risk for CVD related events. A) A relatively good biomarker is represented. The biomarker levels of an aging population increase. The high-risk population is highlighted by red markers. B) The results of a possible good biomarker are transformed in a suggested model of aging individuals that develop high risk (Red line), intermediate risk (orange line), and low risk (green line) atherosclerotic plaques. C) A more difficult to interpret biomarker is shown. No clear pattern can be observed. D) No clear translation to individuals is possible. The biomarker could be highly fluctuating during aging which is relatively bad for event prediction (red line) or could be little fluctuating but highly varying between individuals which is relatively good for event prediction (green lines).

biomarker levels are shown on a 0-100 scale and time is represented as age because it is known that atherosclerotic plaques develop as people age (1). However, the variable age in Figure 15 could be any unit (days, weeks, months etc) from the quantity time. In Figure 15.A an almost ideal biomarker is shown. Each dot represents the biomarker level of one individual at his or her respective age. Starting from an age of 20 the biomarker levels slowly increase as the first significant atherosclerotic plaques develop. Without any severe symptoms the biomarker levels remain very low. As soon as atherosclerotic plaques start to cause pathological effects the biomarker levels rapidly increase. At a relatively high age the biomarkers levels have diverged in this population between high levels in people with severe atherosclerosis and low levels in people with only mild atherosclerosis (Figure 15.A). With the biomarker levels from the large group of individuals from this population possible kinetics of a biomarker within an individual could be extrapolated. A model for this biomarker could thus be generated which is shown in Figure 15.B. In this figure the red line represents people with severe atherosclerosis and the green line with mild atherosclerosis. The dotted line could be a threshold at which a clinician could decide to start an intervention.

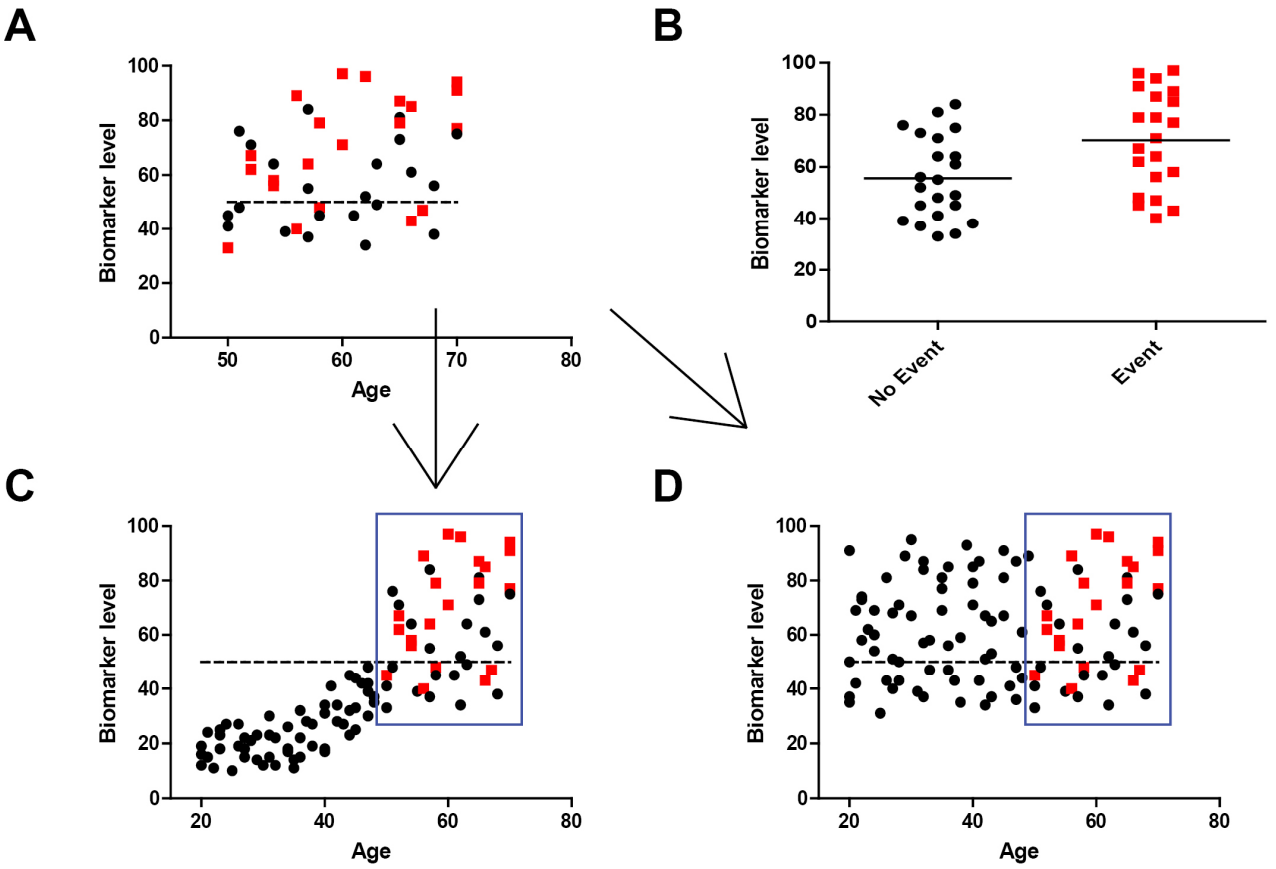

Figure 16 Examples of current CVD research biobank studies. A) The levels of a possible biomarker are plotted against the age of the persons during their first CVD related event. The persons who develop a second CVD related event are stained red. B) The population is separated in a group who develops no secondary event and a group who develops a secondary event. The individual biomarker levels are shown. C) A relatively good biomarker is represented. The possible values from the example biobank study are highlighted in the blue rectangle in the relatively good biomarker model. D) A relatively weak biomarker is shown. The possible values from the example biobank study are highlighted in the blue rectangle in the relatively good biomarker model.

Unfortunately, assuming that adding a biomarker that is significantly associated with CVD to the classical CVD risk prediction models biomarkers did not result in better prediction, biomarkers are not as ideal as represented in Figure 15.A. Therefore, Figure 15.C could be more representative of the kinetics of any single biomarker. In Figure 15.C no clear pattern between age (and thereby possibly atherosclerotic burden) and biomarker levels could be observed in this population. The varying biomarker levels within the population could mean that the biomarker levels within an individual could fluctuate very strongly (Figure 15.D red line). An individual could be diagnosed with severe atherosclerosis and a high biomarker level on one day and thus has a potential high risk for an event, but on another day the biomarker levels could be strongly reduced while the individual could still have a high risk for an event. Understanding the true kinetics of a strong fluctuating biomarker is thus very important. A strong fluctuating biomarker should be measured at multiple time points in order to understand its true value. For example blood pressure can vary greatly during the day. A single measurement is insufficient to make a good estimation of someone's daily blood pressure, because it could be incidentally high or low. When the data from multiple measurements is combined, a better estimation of the

regular blood pressure of this person can be made. In the second case (the green lines in Figure 15.D), the biomarker levels in an individual have a more constant pattern and the variance between multiple individuals causes the variation observed in the population from Figure 15.C. A constant pattern would be favorable above strong fluctuation because high or low biomarker levels could again be associated with CVD. When the kinetics of such a biomarker is known, a single or just a few measurements could be sufficient to estimate its true value. In practice different biomarkers behave as ideal as described by Figure 15.A (47) and as bad as the red line in Figure 15.D and every possibility within (46). Understanding the kinetics of a biomarker is thus very important.

The lack of power for short-term risk estimation by the current risk assessment models and the strength of biomarker kinetics is clearly demonstrated by the inflammatory marker CRP. As stated before, CRP reflects the vulnerability of a patient for CVD instead of the vulnerability of a single plaque (34). By covering the whole vascular tree, CRP could be a good candidate for short-term CVD risk assessment. However, CRP is associated with CVD both during plaque development as well as when a cardiovascular incident occurs. (33) This suggests that single time point CRP measurements can be associated with either plaque development or plaque rupture / thrombus formation, which makes it thereby hard to distinguish immediate stress signs for possible near future plaque ruptures from ongoing and thereby less urgent plaque progression. Furthermore, in the study from Albert *et al.*, in a multivariate analysis CRP could independent of established cardiovascular risk factors still be associated with the prevalence of CAD but not with the severity of CAD. This was explained by the fact that conventional risk factors correlate with CRP levels and CRP thereby thus loses it independent association with the severity of coronary artery disease. As the conventional risk factors are mostly unchangeable variables, as explained above, an abnormal change in CRP levels within a well defined period of time could possible be more valuable than one increased CRP level at a single time point. The kinetics of CRP could thus provide the additional information that is currently lacking for short-term risk prediction.

Future possibilities for CVD risk assessment models

There are thus biomarkers which have useful kinetics for CVD related event prediction and biomarkers that have less useful kinetics for event prediction. What does biomarker kinetics mean for the current biobank studies? As we take another look at the example in Figure 14, the cloud of individuals and their represented biomarker levels could either be compared with the upper right part of the example population in Figure 15.A or the right part (starting at an age of 45 years) of Figure 15.C this is summarized in Figure 16. When the biomarker from Figure 14 behaves as the biomarker represented by the population in Figure 16.C there is a high chance that the biomarker could be associated with CVD and could predict CVD related events in a model. However, CVD risk prediction becomes more challenging when the biomarker from Figure 14 behaves more like Figure 16.D. While statistical models do the same extrapolation as described above, how do we know what the true kinetics are for this biomarker? There are two problems that make the answer for this question challenging: first, we only have information from a diseased population and not a sample from the

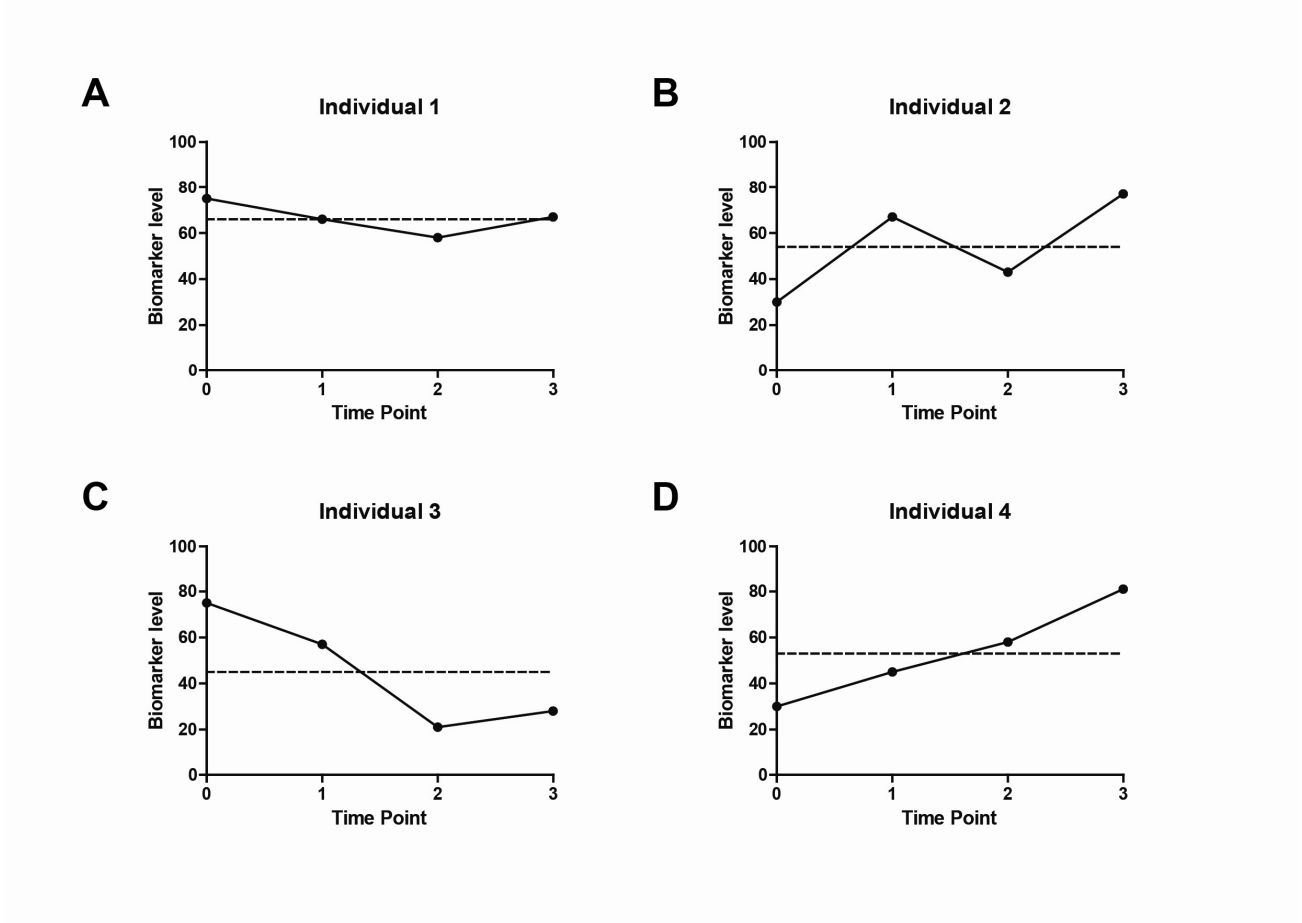

Figure 17 Examples of multiple time point measurements of a biomarker in diseased individuals. A through D show different scenarios for multiple time point measurements of a single biomarker in diseased individuals. The dotted line represents the average value of four different measurements.

whole population. Second, with only one measurement per individual the question rises whether this measurement is representative for the fluctuating biomarker levels within this individual. First, by measuring biomarker levels in a cohort of both healthy and diseased individuals, a feeling of the biomarker kinetics can be acquired. As demonstrated in Figure 15, models for biomarker kinetics could possibly be extrapolated from this population and could serve as a backbone for biomarker kinetics in diseased individuals. This population study does not necessarily need to contain a very large cohort of individuals. A group of about 10 volunteers in every 5 years of lifetime between the age of 25 and 70 (about 100 in total) should be sufficient to get a biobank that could provide the material for determining biomarker kinetics. As previously described, technologies like luminex or flow cytometry could be used the for high throughput analysis of this biobank while using as less sample as possible.

Secondly, to get a better understanding of the meaning of the baseline measurement in the diseased population, the baseline measurement of each individual could be extended with two or more follow up measurements. The addition of more measurements could be of great value for understanding the biomarker kinetics and prediction of a future CVD related event. For example in Figure 17 different scenarios for multiple time point measurements of a biomarker in diseased individuals are shown. In each scenario the biomarker

levels at baseline (t=0) and three subsequent follow up measurements are shown. The average value of the four different measurements from one individual is represented by the dotted line (Figure 17). As we take a look at individual 1 and 2 in Figure 17, the baseline values are respectively 75 and 30. When we assume that an increased level of this example biomarker is associated with an increased risk for CVD related events, individual 1 should have a high risk for a CVD related event and individual 2 has a relatively low risk for an event on the short-term. This assumption however includes that the biomarker levels are merely behaving in a steady state. However, the subsequent measurements show that individual 2 has a strong fluctuating biomarker level and the baseline measurement is not representative for his average biomarker level. The average biomarker levels of the individuals 1 and 2 are respectively 66 and 54 (Figure 17). The risk estimation for a CVD related event in individual 2 is thus higher after additional measurements than with one single baseline measurement. The more measurements are taken the better the estimation of the true biomarker levels within one individual probably is.

Individuals 3 and 4 in Figure 17show another scenario that could occur. Here, the biomarkers do not behave in a steady state but increase or decrease over time. According to the baseline measurements individual 3 should have a high risk for a CVD related event and individual 4 a low risk for a CVD related event on the short-term. However, subsequent measurements show a decrease in biomarker levels in individual 3 and an increase in biomarker levels in individual 4 (Figure 17). According to these results individual 3 could have a low risk for a CVD related event and individual 4 an increased risk for a CVD related event on the short-term.

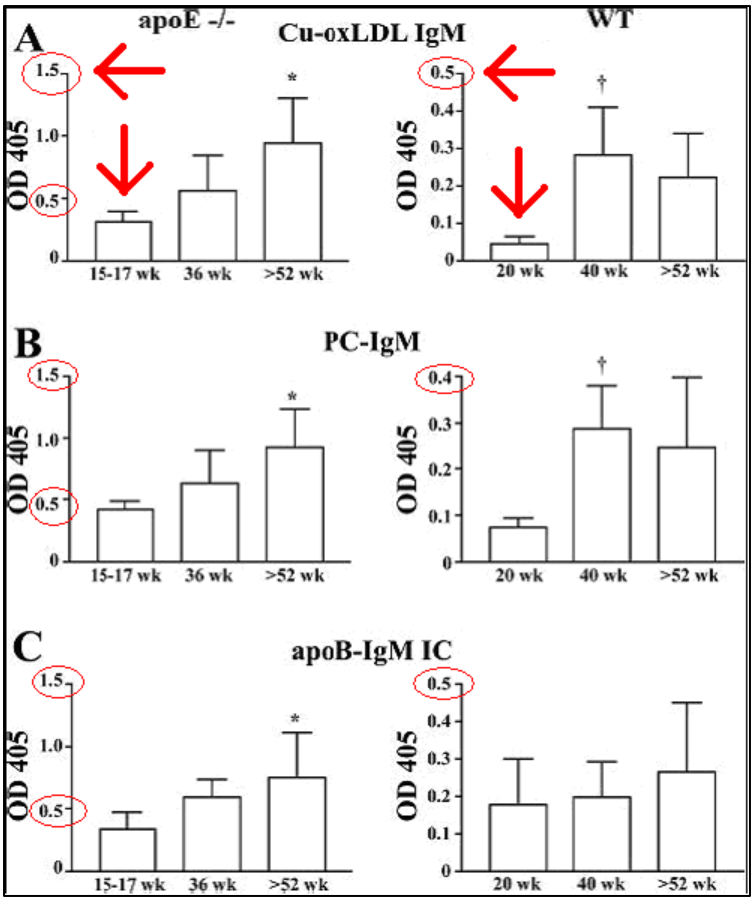

Figure 18 Copper-oxidized LDL (Cu-oxLDL) IgM (A) and phosphorylcholine (PC)-IgM (B) in apoE(-/-) mice gradually increased over time, reaching statistical significance at >52 wk. In the WT mice, the increase was more abrubt at the 40-wk time point and leveled off. Circulating apoB-IgM immune complexes in apoE(-/-) mice gradually increased over time, doubling by >52 wk (C). No differences were opbserved in WT mice. *P < 0.05 vs. 15-12 wk. † P < 0.05 vs. 20 wk.

Multiple mouse models have already shown that biomarker kinetics can reflect atherosclerotic plaque progression (47, 49). As a model of atherosclerotic plaque development Apo $E^{-/-}$ mice were used in these studies (47). Apo $E^{-/-}$ mice lack the glycoprotein apolipoprotein E which is essential for lipid transport and metabolism. The mice are healthy born, but develop severe

atherosclerotic lesions when placed on a high-fat diet. (14) During a study which investigated the TLR expression in aging atherosclerotic Apo $E^{-/-}$ mice, TLR2 and TLR4 expression significantly increased after 40 weeks. On the contrary, control mice aging under non-atherosclerotic conditions lead to a decreased surface expression of TLR2 and TLR4. (47) This suggests that during atherosclerotic plaque progression immunological changes can be observed.

In another study the auto-antibody titers against modified LDL were monitored in aging Apo $E^{-/-}$ mice. (48) Increasing age was associated with increasing extent of atherosclerotic lesions. Cu-oxLDL IgM antibody titers in Apo $E^{-/-}$ mice gradually increased with age (Figure 18). There was also a progressive increase in serum Apo B-IgM immune complexes in Apo $E^{-/-}$ mice. The same results were also observed for Cu-oxLDL IgG and Apo B-IgG immune complexes in Apo $E^{-/-}$ mice (Figure 18). (48) The control group showed more fluctuations during aging, while the Apo $E^{-/-}$ mice showed a constant increase of antibody titers during aging (Figure 18). This was explained by the fact that Apo $E^{-/-}$ mice were under constant immunologic pressure from hypercholesterolemia which caused an increasing immune response during aging. However, not mentioned by the authors, the variations of the control group during aging had the same range as the variation within young Apo $E^{-/-}$ mice (Figure 18). Assuming that the performed assays where quantitative assays, this suggests that variations during aging in the wild type mice could be a baseline level, wherein small fluctuations do occur. The significance of some fluctuations could then be explained by the small titer values of the control mice. When the values are small; small absolute changes are relatively large and become more easily significant. By comparing the antibody titers at every age point between the Apo $E^{-/-}$ mice and the control mice, a strong increase in the antibody titers in the Apo $E^{-/-}$ mice can be observed. In combination with the observed increasing atherosclerotic plaque size in Apo $E^{-/-}$ mice during aging, we can conclude that Cu-oxLDL antibodies and Apo B-IgG immune complexes serve as surrogate markers for atherosclerotic disease progression.

This study perfectly reflects the importance of understanding the dynamics of the process that is investigated. While according to the authors of the paper the wild type mice showed “different kinetics compared with the apo$E^{-/-}$ mice during the aging process” (48), in my opinion the wild type mice show a baseline level of the antibody titers which strongly increase as atherosclerotic plaques progress (see aging apo$E^{-/-}$ mice in Figure 18).

Thus, adding measurements during follow up provides more information about the baseline level of a biomarker when it stays in a stead state (fluctuations, average and amplitude) and could provide information about the future levels of a biomarker when it moves in a certain direction (increase or decrease). However, the results from more measurements added to the baseline measurement should also be used carefully. The kinetics should be well interpreted as the increasing or decreasing biomarker levels in individuals 3 and 4 renders the average of these biomarker levels useless for risk prediction (Figure 17 C and D have nearly the same average) but the information is stored in the direct of the biomarker kinetics. While using the average from the measurements from the individuals 1 and 2 probably reduces the false negative predictions and increases the number of true positive predictions.

Conclusion

Although the last decades many biomarkers associated with CVD related events have been discovered, current CVD risk assessment models fail to monitor disease progression and thereby make assessment of the short-term risk of an individual patient impossible.
CVD caused by atherosclerosis is a multi-factorial continuous disease. This implies that measurement of a single biomarker at a single point is not sufficient to gain a realistic impression of the status of a progressing atherosclerotic plaque. Furthermore, current CVD risk prediction models lack the power for short-term risk prediction because of their relatively static design. The effect of the classical parameters on CVD is more reflected by their long term accumulative contribution to plaque development than by their immediate or short-term effects on CVD. Thus, current CVD predictive and diagnostic models do not meet the requirements for individual based short term CVD risk estimation and personalized treatment. New predictive and diagnostic models are needed that meet the requirements of modern medicine.

Generating new models in which biomarker kinetics are taken into consideration could possibly make short-term risk prediction possible. New candidate biomarkers for cardiovascular disease should be searched on a rational base and the variability of candidate markers should be measured in a large population within a specific time window to find out how these markers behave during different phases of atherosclerotic disease progression. Models for biomarker kinetics could possibly be extrapolated from this population and could serve as a backbone for biomarker kinetics in diseased individuals.
A better understanding of the kinetics of potential CVD related biomarkers could identify a time frame within the markers could be measured, how many follow up measurements are necessary, and what magnitude of changes in biomarker levels could be associated with CVD related events. Biomarker dynamics have thus the potential to be the crucial element that is currently lacking from CVD risk assessment models. Multi-marker screening and biomarker dynamics could make monitoring disease progression, short term risk assessment and personalized treatment possible.
Furthermore, biomarkers with a strong predictive power should be combined in a single assay which can be easily applied in the clinic. With new advances in flow cytometry and multiplex technology, reducing costs and wider application possibilities, flow cytometry and multiplex have developed to high potential techniques that can analyze multiple biomarkers at many biological levels. Therefore, flow cytometry and multiplexing are the most preferred techniques for multi-biomarker screening and future cardiovascular risk assessment.

The development of new CVD risk assessment models will be challenging, however new analytical methods and tools can reduce both the costs and labor of large population studies. Once a better understanding of biomarker dynamics and multi-marker interactions is established this knowledge can be used for the development of new CVD risk assessment models that can meet the requirements of current medicine.

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