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REVIEW

Markers of Atherosclerosis: Part 2–Genetic and Imaging Markers

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This is Part 2 of a two-part review summarising current knowledge on biomarkers of atherosclerosis. Part 1 addressed serological biomarkers. Here, in part 2 we address genetic and imaging markers, and other developments in predicting risk. Further improvements in risk stratification are expected with the addition of genetic risk scores. In addition to single nucleotide polymorphisms (SNPs), recent advances in epigenetics offer DNA methylation profiles, histone chemical modifications, and micro-RNAs as other promising indicators of atherosclerosis. Imaging biomarkers are better studied and already have a higher degree of clinical applicability in cardiovascular (CV) event prediction and detection of preclinical atherosclerosis. With new methodologies, such as proteomics and metabolomics, discoveries of new clinically applicable biomarkers are expected.

Keywords Biomarkers • Atherosclerosis • Genetic polymorphism • Imaging biomarkers • Carotid intima media thickness • Micro RNA

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Introduction

This is Part 2 of a two-part narrative review of risk factors in the pathogenesis of atherosclerosis, Part 1 addressed serological biomarkers. Here, in part 2, we review knowledge of other biomarkers, including genetic and imaging markers.

Our literature search strategy was described in Part 1, and involved a PubMed search using the terms ''atherosclerosis'', ''markers'', ''biomarkers'', ''coronary artery disease'', ''serum'', ''inflammatory'', ''oxidative stress'', ''endothelial dysfunction'', ''novel'', ''genetic'', ''genomic-wide association studies'', ''GWAS'', ''epigenetics'', ''DNA methylation'', ''histone chemical modifications'', ''micro RNA'', ''miRNA'', ''ultrasonographic'', ''carotid intima-media thickness'', ''proteomics'', ''metabolomics'' in different combinations connected with Boolean operators AND and OR.

Appropriate articles were selected depending on abstract and reviewed in three different sections: serum biomarkers (Part 1); and genetic biomarkers and imaging biomarkers (Part 2).

Genetic Biomarkers

It is now widely accepted that genetics play an important role in the pathogenesis of atherosclerosis. In contrast to rare monogenic diseases, e.g. familial hypercholesterolaemia resulting in generalised atherosclerosis, coronary artery disease (CAD) is a multifactorial disease with variable genetic contribution of multiple loci.

Single Nucleotide Polymorphim (SNP)

Thus far, genomic-wide association studies (GWAS) have identified 58 independent loci associated with CAD (Table 1) together contributing approximately 13.3% to CAD heritability [1]. Most identified loci have low allele frequency (<5%) with minor contributions to CAD development. Their exact function is known only for some of them and is related to inflammatory response, oxidative stress regulation, lipid function, transportation, endothelial dysfunction and other pathogenic processes involved in atherosclerosis. Lp(a) single nucleotide polymorphism (SNP) locus is currently the most potent known contributor, but also one of the rarest. For many loci there is no known function, the most debated one being 9p21, which lies almost a 100 kb from the nearest known protein coding gene [2–4].

The individual SNP's contribution to risk stratification is negligible, despite being significantly associated with CAD risk. Therefore, many studies have investigated the influence of multiple SNPs testing in regard to risk stratification, but results were mostly still not satisfactory. Studies agree that, when more SNPs are included, better prediction is possible and that, with newly discovered SNPs, prediction is going to improve. A recent study [5] tested 49310 SNPs derived from CARDIoGRAMplusC4D Consortium meta-analysis [1] and reported the ability to reclassify subjects

Table 1 Coronary artery disease genomic-wide association studies identified loci. Adopted and modified from CARDIoGRAMplusC4D Consortium meta-analysis [100].

Chr	SNP	Allele	Allele freq	OR	Known locus
6	rs3798220	C/T	0,02	1.42	*SLC22A3-LPAL2-LPA*
9	rs4977574	G/A	0,49	1.21	*9p21*
22	rs180803	G/T	0,97	1.20	POM121L9P-ADORA2A
9	rs3217992	T/C	0,39	1.14	*9p21*
2	rs6725887	C/T	0,11	1.14	*WDR12*
7	rs3918226	T/C	0,06	1.14	NOS3
1	rs17114036	A/G	0,92	1.13	*PPAP2B*
12	rs11830157	G/T	0,36	1.12	KSR2
21	rs9982601	T/C	0,13	1.12	*KCNE2 (gene desert)*
1	rs646776	T/C	0,75	1.11	*SORT1*
6	rs12526453	C/G	0,71	1.10	*PHACTR1*
15	rs8042271	G/A	0,90	1.10	MFGE8-ABHD2
2	rs16986953	A/G	0,1	1.09	*AK097927*
19	rs445925	G/A	0,9	1.09	*APOE-APOC1*
10	rs501120	T/C	0,81	1.08	*CXCL12*
1	rs17465637	C/A	0,66	1.08	*MIA3*
15	rs7173743	T/C	0,56	1.08	*ADAMTS7*
17	rs7212798	C/T	0,15	1.08	BCAS3
1	rs11206510	T/C	0,85	1.08	*PCSK9*
10	rs12413409	G/A	0,89	1.08	*CYP17A1-CNNM2-NT5C2*
7	rs11556924	C/T	0,69	1.08	*ZC3HC1*
9	rs579459	C/T	0,21	1.08	*ABO*
19	rs1122608	G/T	0,77	1.08	*LDLR*
15	rs56062135	C/T	0,79	1.07	SMAD3
4	rs7692387	G/A	0,81	1.07	*GUCY1A3*
13	rs9515203	T/C	0,76	1.07	*COL4A1/A2*
10	rs1412444	T/C	0,37	1.07	*LIPA*
19	rs2075650	G/A	0,13	1.07	*APOE-APOC1*
2	rs515135	C/T	0,79	1.07	*APOB*
3	rs9818870	T/C	0,14	1.07	*MRAS*
11	rs974819	T/C	0,33	1.07	*PDGFD*
12	rs3184504	T/C	0,42	1.07	*SH2B3*
4	rs17087335	T/G	0,21	1.06	REST-NOA1
11	rs10840293	A/G	0,55	1.06	SWAP70
18	rs663129	A/G	0,26	1.06	PMAIP1-MC4R
4	rs1878406	T/C	0,16	1.06	*EDNRA*
2	rs1561198	T/C	0,46	1.06	*VAMP5-VAMP8-GGCX*
6	rs2048327	C/T	0,35	1.06	*SLC22A3-LPAL2-LPA*
10	rs2505083	C/T	0,4	1.06	*KIAA1462*
10	rs2047009	G/T	0,48	1.06	*CXCL12*
1	rs17464857	T/G	0,86	1.06	*MIA3*
5	rs273909	G/A	0,12	1.06	*SLC22A4-SLC22A5*
7	rs2023938	C/T	0,1	1.06	*HDAC9*
8	rs264	G/A	0,85	1.06	*LPL*
6	rs12190287	C/G	0,62	1.06	*TCF21*
6	rs10947789	T/C	0,78	1.05	*KCNK5*
7	rs10953541	C/T	0,78	1.05	*7q22*
11	rs964184	G/C	0,18	1.05	*ZNF259-APOA5-APOA1*
13	rs4773144	G/A	0,43	1.05	*COL4A1/A2*
1	rs4845625	T/C	0,45	1.05	*IL6R*
2	rs6544713	T/C	0,32	1.05	*ABCG5-ABCG8*

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Table 1. (continued).

Chr	SNP	Allele	Allele freq	OR	Known locus
15	rs17514846	A/C	0,44	1.05	*FURIN-FES*
17	rs216172	C/G	0,35	1.05	*SMG6*
8	rs2954029	A/T	0,55	1.04	*TRIB1*
10	rs11203042	T/C	0,45	1.04	*LIPA*
12	rs7136259	T/C	0,43	1.04	*ATP2B1*
13	rs9319428	A/G	0,31	1.04	*FLT1*
14	rs2895811	C/T	0,41	1.04	*HHIPL1*
17	rs46522	T/C	0,51	1.04	*UBE2Z*
6	rs4252120	T/C	0,74	1.03	*PLG*
2	rs2252641	C/T	0,48	1.03	*ZEB2-ACO74093.1*
17	rs12936587	G/A	0,61	1.03	*RAI1-PEMT-RASD1*
6	rs17609940	G/C	0,82	1.03	*ANKS1A*
6	rs6903956	A/G	0,35	1.00	*ADTRP-C6orf105*
19	rs12976411	T/A	0,09	0.67	ZNF507-LOC400684

from an intermediate risk group in the Framingham Risk Score (FRS) and American Cardiology College/American Heart Association 2013 (ACC/AHA13) score. Most recently, Wang et al. analysed whether the associations of SNPs with fasting lipoprotein subfractions in European-Americans are consistent across ethnicities with a non-European ancestry within the Multi-Ethnic Study of Atherosclerosis (MESA) study. Results showed that genetic associations with lipoprotein subfraction measures differ by ethnicity. Authors pointed to the importance of ethnicity in genetic risk for cardiovascular disease (CVD) and highlight the need to identify ethnicity-specific genetic variants associated with CVD risk [6].

Epigenetics

Another interesting field in genetics, regarding biomarkers of atherosclerosis, is epigenetics. A plethora of environmental risk factors may result in epigenetic modifications with abnormal phenotypic expression of genetic information. Exposure to various environmental pollutants induce epigenetic alterations of gene expression relevant to the onset or progression of CVD [7,8]. It is well described that CVDs, including atherosclerosis, can arise at the early stages of development and growth during pregnancy [9,10]. Fetal exposure to high-fat diet or dietary imbalance [11–13], gestational diabetes [14–16], maternal obesity [14,15], and smoking [17–22] are associated with increased risk and progression of atherosclerosis. For example, healthy offspring exposed to maternal diabetes during pregnancy demonstrated substantially increased levels of circulating cellular adhesion molecules, which are biomarkers of adverse endothelium perturbation and may be related to the earliest preclinical stages of atherosclerosis and also diabetes [16]. Epigenetics consists of three mechanisms that are interrelated [23]. They include DNA-based modifications, the histone modifications, and RNA-based mechanisms (Figure 1).

Several papers described altered DNA methylation and atherosclerosis [24]. Greißel et al. [136] summarised that DNA methylation and expression of some corresponding methyltransferases are significantly altered in atherosclerosis, suggesting a possible contribution of epigenetics in disease development. Ma et al. [26] aimed to identify an effective method for detecting early–phase atherosclerosis, as well as to provide useful DNA methylation profiles to serve as biomarkers for the detection of atherosclerosis. They found that the atherosclerosis-specific promoter methylation of TIMP metallopeptidase inhibitor 1 (TIMP1), ATP binding cassette subfamily A member 1 (ABCA1), and acetyl-CoA acetyltransferase 1 (ACAT1) may serve as a valuable biomarker for the early detection of atherosclerosis. Results of another clinical study suggested that altered aryl hydrocarbon receptor repressor methylation in monocytes, a cell type sensitive to cigarette smoking and involved in atherogenesis, represents a potential biomarker of subclinical atherosclerosis in smokers [27]. There are several other biofactors associated with atherosclerosis. Deficiency of folic acid has been epigenetically linked to endothelial dysfunction and different aspects of CVDs, including atherosclerosis [28]. Similarly, homocysteinuria has been linked with impaired endothelial function through compromised VEGF/Akt/endothelial nitric oxide synthase signalling [29]. Moreover, the findings of Kim et al. [30] provide evidence of epigenetic dysregulation of oestrogen receptor beta in atherosclerosis and vascular ageing.

Histone chemical modifications seem to play an important role in atherosclerosis. Recently, histone acetylations and methylations in the smooth muscle cells (SMCs) within atherosclerotic plaques from vessels at different stages of atherosclerosis were determined by Greißel et al. [31] Increased histone acetylation was observed on H3K9 and H3K27 in SMCs in advanced atherosclerotic lesions. H3K9 acetylations in SMCs and macrophages were associated with plaque severity of atherosclerosis. Methylations of H3K9 and H3K27 were reduced in SMCs and inflammatory cells and methylation on H3K4 was associated with the severity of atherosclerosis. Moreover, the expressions of some types of histone acetyltransferases and methyltransferases correlated with the severity of atherosclerosis. Data from another study showed a reduction in global levels of the H3K27Me3 modification in vessels with advanced atherosclerotic plaques [32]. One of the important endothelial genes relevant for angiogenesis and also atherosclerosis that is regulated by the histone chemical modifications is NOS3, coding endothelial nitric oxide synthase (eNOS). Based on novel data, it seems that histone deacetylase 5, which plays an important role in the Krüppel-like factor 2 activation and thus eNOS expression increase, could be a new biomarker and therapeutic target to prevent vascular endothelial dysfunction associated with atherosclerosis [33].

MicroRNAs (miRNAs) are a class of short, non-coding, regulatory RNA molecules which play an important role in intracellular communication and cell signalling and belong to the most important epigenetic risk factors for the

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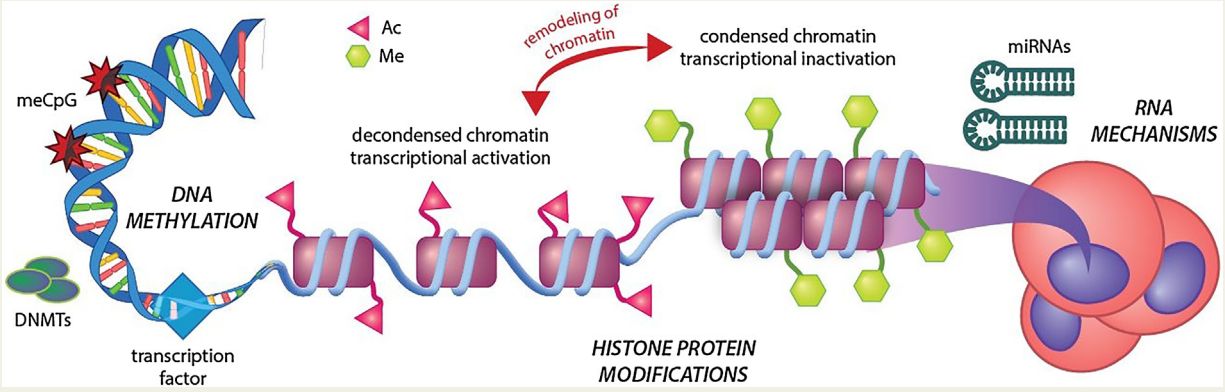

Figure 1 The relevance of epigenetics in the atherosclerosis.
Epigenetic biomarkers of atherosclerosis consist of three distinct processes: DNA methylation, histone protein methylation and acetylation, and RNA mechanisms including activity of mi-RNA.
Abbreviations: Ac, acetylation; DNMTs, methyltransferases; Me, methylation.

development of CVDs [34,35]. Among many other physiologic and pathophysiologic functions, miRNAs play an important role in the development and regression of atherosclerosis. miRNAs target genes involved in HDL metabolism, directly target macrophages, and promote expression of cell adhesion molecules, chemokines etc. [36]. Numerous studies have found different miRNAs to be associated with atherosclerosis. A recent review reported miR-1, miR-133a, miR-208a, miR-208b and miR-499 levels associated with acute myocardial infarction and miR-17, miR-92a, miR-126, miR-133, miR-140, miR-145, miR-155, miR-182 and miR-208a levels associated with chronic heart disease (CHD) [37]. Most current data from the MESA study revealed that lower circulating miR-221, miR-155, and miR-130a were potential risk factors for CHD. Results showed that miR-130a seems to be an independent predictor for atherosclerosis. In addition, associations between miR-221, miR-155 and miR-130a were evaluated. miR-130a and miR-155 showed positive association, moreover, the proportion of CHD attributable to the interaction between miR-130a and miR-155 was as high as 22%. miR-221 and miR-130a manifested a negative interaction [38]. Several other miRNAs have been described to modulate the function of endothelial cells (miR-221/222 and −126), vascular smooth muscle cells (miR-143/145) and macrophages (miR-33, −758, and −26), thereby regulating the initiation and progression of atherosclerosis [39]. In contrast to SNPs, epigenetic modifications and miRNA inhibition are reversible and, therefore, carry a great promise for targeted therapy. Nonetheless, each miRNA may control 100 mRNAs, thus rigorous connection of one miRNA to specific process as atherosclerosis yet seems impossible. Targeted therapy including anti-sense oligonucleotides as well as microRNA mimetics and inhibitors is increasingly being developed [37,36].

New promising serologic and genetic biomarkers that provide significant diagnostic and prognostic information about the cardiovascular risk prediction and atherosclerosis are summarised in Table 2.

Quantitative Imaging

Ultrasound has a variety of established clinical uses including detection of preclinical atherosclerosis by measuring carotid intima-media thickness (cIMT), carotid plaque presence and evaluating the brachial artery flow mediated dilatation and arterial stiffness (Figure 2). Moreover, coronary artery calcium score and ankle brachial index are proposed as valid markers of atherosclerosis risk evaluation. It has been proposed that these parameters might usefully predict vascular events. In this regard, the BioImage Study tested novel approaches in a typical health-plan population [66]. A total of 7687 men (55 to 80 years of age) and women (60 to 80 years of age) without evidence of atherothrombotic disease, but anticipated to be at risk for near-term atherothrombotic events, were included. Baseline scrutiny consists of evaluating the cardiovascular risk factors and screening for subclinical (asymptomatic) atherosclerosis. The quantification of selected biomarkers included coronary artery calcification (by computed tomography), intima-media thickness, carotid atherosclerotic plaques, and abdominal aortic aneurysm (by ultrasound), and ankle brachial index. Individuals with one or more abnormal screening test results underwent advanced imaging with contrast-enhanced magnetic resonance imaging for carotid and aortic plaques, contrast-enhanced coronary computed tomography (CT) angiography for luminal stenosis and noncalcified plaques, and 18F-fluorodeoxyglucose-positron emission tomography/CT for carotid and aortic plaque inflammation. Moreover, plasma, PAXgene RNA, and DNA samples were collected, frozen, and stored for future analyses. The purpose of The BioImage Study is based on the identification of patients with subclinical atherosclerosis who are at risk for near-term atherothrombotic events, consequently, this study can provide more personalised management for these individuals [66]. Recent outcomes of this study showed that the

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Table 2 **New promising biomarkers of cardiovascular risk prediction and atherosclerosis.**

Biomarker	Predictive ability	Ref.
High-sensitivity C-reactive protein	↑risk for CV events and mortality	[40,41]
Fibrinogen	↑risk of premature atherosclerosis	[42,43]
Apolipoprotein-associated phospholipase A2	correlates with the coronary HD and its severity	[44–46]
Matrix metalloproteinases	markers of plaque vulnerability and subclinical atherosclerosis, predictors of CVD and mortality	[47–51]
Myeloperoxidase	early detection of subclinical CAD, its severity, diagnosis of MI,	[52,53]
Endothelin-1	correlates with increased CAD and ACS risk and severity	[54–56]
Natriuretic peptides	↑risk for CV events and mortality	[57,58]
High-sensitivity assays for cardiac troponin	predictor of HF, mortality, and incident coronary HD	[59–62]
Pregnancy-associated plasma protein-A	marker of plaque vulnerability and predictor of CVD and mortality	[63]
Growth differentiation factor 15	predictor of CV and all-cause mortality, unstable AP	[64,65]
Micro-RNAs	association with the acute MI, predictor of atherosclerosis	[25,37]

Abbreviations: ACS, acute coronary syndrome; AP, angina pectoris; CAD, coronary artery disease; CV, cardiovascular; CVD, cardiovascular disease; HD, heart disease; HF, heart failure; MI, myocardial infarction.

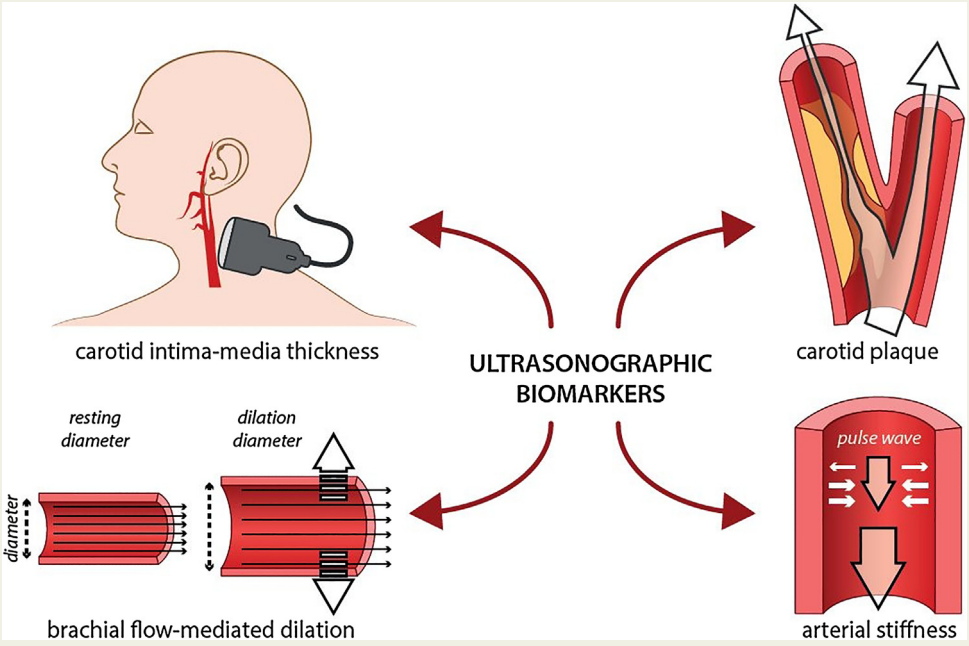

Figure 2 Ultrasonographic biomarkers of atherosclerosis.
Carotid intima-media thickness. The measurement of the common carotid artery intima and media combined layers is determined by high-resolution B-mode ultrasonography. This biomarker is an effective tool in the identification of subclinical and asymptomatic atherosclerotic vascular disease. *Carotid plaque.* Carotid plaques are evaluated by both qualitative (visual) and quantitative methods. Plaque may be characterised by its presence or absence, location, thickness, number, irregularity (smooth, irregular, or ulcerated), area, and echodensity (echolucent or echogenic). Quantification of carotid plaques is an important marker of increased cardiovascular event risk. *Flow-mediated dilatation* of the brachial artery is an appropriate method for non-invasive evaluation of systemic endothelial function. Endothelial dysfunction is one of several signs of atherogenesis and correlates with many cardiovascular risk factors. *Arterial stiffness.* Measurement of arterial stiffness is a sensitive biomarker of atherosclerosis and cardiovascular risk due to its principal pathophysiological mechanisms. Several factors, such as endothelial dysfunction, altered vascular smooth muscle cell function, vascular inflammation, or genetic determinants are clearly associated with the arterial stiffness.

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detection of subclinical carotid or coronary atherosclerosis improves risk predictions and reclassifications in comparison with the conventional risk factors, with comparable results for either modality. The definition of cost-effectiveness warrants future evaluations with the aim to find the optimal role of these complementary screening methods [67].

Carotid Intima-Media Thickness (cIMT)

Carotid intima-media thickness (cIMT) is an easily obtainable, noninvasive and reproducible measurement using B-mode ultrasound, although no global standard for its measurement exists. It is defined as the distance between lumen-intima and adventitia-media borders [68]. At first, many studies reported positive correlation with CAD and stroke risk, one of first meta-analysis even showed cIMT to be a strong predictor of future vascular events [69]. The Atherosclerosis Risk in Communities (ARIC) study [70] reported that an increase in cIMT for 1.9 mm increases the risk of myocardial infarction (MI) or sudden cardiac death by 36%. Another study reported nearly 10% net reclassification improvement of intermediate risk patients. cIMT has also been reported to decrease with statin therapy [68], while, on the contrary, a large meta-analysis showed its improvement did not predict CV events reduction [71]. Similar observations were made in the general population [72]. In contrast to the ARIC study, recent meta-analyses have shown no significant benefit if added to risk prediction [73]. Firstly, 2010 ACC/AHA guidelines recommended cIMT measurements for risk assessment, but, in 2013, ACC/AHA guidelines recommendation was withdrawn and, instead, stated that cIMT measurements are not recommended for routine clinical use in prediction of a first CV event [74]. Again, in contrast to the ACC/AHA, 2012 European guidelines recommend cIMT measurements in individuals with a 10-year risk of fatal cardiovascular disease between 1% and 5%—those at intermediate cardiovascular risk and in individuals with arterial hypertension as a sign of end-organ damage [74]. In a nutshell, cIMT is a strong predictor of CV events and preclinical atherosclerosis, but its clinical significance remains controversial. Unification of measurement methodology is needed.

Carotid Plaque

The definition of carotid plaque differs among studies, i.e. local thickening of the cIMT of >50% compared to the surrounding vessel wall, a cIMT >1.5 mm, or local thickening >0.5 mm [75]. Different studies used different modalities for carotid plaque determination as carotid total plaque area, total plaque volume, three-dimensional (3D) based ultrasound approach or just its presence [68,75]. Several studies have shown that the presence of carotid plaques is better than the cIMT for predicting CV events regardless of cIMT [73,76,77]. This could be because carotid plaque may represent a later stage of atherosclerosis. Moreover, it was reported that, among sonographic markers, carotid plaque burden is most strongly associated with coronary artery calcium score (a well established predictor of CV events) [75]. According to ESC guidelines, plaque presence is also a marker of target organ damage in hypertension [74].

Brachial Flow-Mediated Dilation

Endothelial dysfunction is a well-established initial step in the pathogenesis of atherosclerosis. One of the ways of its assessment is by measuring flow-mediated dilatation (FMD). This technique has been widely used on the brachial artery in a vast number of studies, as well as studies investigating its connection with preclinical atherosclerosis and risk assessment. Brachial FMD is defined as a change in brachial artery diameter in response to hyperaemia. Hyperaemia is achieved by first inflating an arm cuff to supra-systolic blood pressure level and then deflating it. As a result, blood flow burst increases shear stress and causes release of NO from endothelial cells with consequent dilatation of the brachial artery. Artery diameter is quantified using high-resolution ultrasound before and during hyperaemia and FMD is calculated [78,79]. Most prospective studies reported inverse association with brachial FMD, but not all studies were in agreement. Finally, two large meta-analyses reported consistent 13% [78] and 8% [79] overall risk reduction per 1% higher FMD, and meta-analysis also included asymptomatic subjects within diseased populations yielding stronger association. However, both studies mentioned low reproducibility of FMD measurements as the main disadvantage for its wide clinical use and as a reason for possible weakened association with CVD, therefore standardisation of the FMD technique is required.

Arterial Stiffness

Arterial stiffness is increasingly recognised to have a role in CV disease development, especially in arterial hypertension [80]. It can be measured by invasive methods and noninvasively using relatively reproducible ultrasound measurements. European Society of Cardiology 2013 guidelines [80] consider the carotid-femoral (aortic) pulse wave velocity (aPWV) to be the 'gold standard' for measuring aortic stiffness. An expert consensus suggested 10 m/s for the threshold value. A large number of small, prospective studies have shown a positive correlation of aPWV with cardiovascular risk. The latest meta-analysis [81] of 17,635 subjects has reported a 23–30% increased risk for CHD, stroke and CV events after adjustment for conventional risk factors and shown that the use of aPWV improved risk prediction by 13% in intermediate risk individuals. APWV may, therefore, serve as a useful biomarker in clinical settings but randomised controlled trials are still needed.

Coronary Artery Calcium Score

The coronary artery calcium score (CACS) is a measurement of the amount of calcium in the walls of the coronary arteries using a special CT. CACS is a well-established predictor of CV events [82]. A clinical study including more than 85,000 subjects has demonstrated that the absence of CACS is linked with a very low risk of cardiovascular events in the future [83]. The CACS plays an important role in

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cardiovascular risk stratification, demonstrating a significant linkage with the medium- or long-term occurrence of major cardiovascular events, such as all-cause mortality, cardiac mortality, and nonfatal myocardial infarction [84]. The CACS was analysed in association with other well-defined traditional risk score systems, mainly the Framingham Risk Score. The CACS provides an advantage regarding the independent added value in the prediction of all-cause mortality and mortality due to coronary disease in asymptomatic individuals [85]. In addition, CACS is useful in reclassification within the category of coronary artery disease risk (60% of atherosclerotic coronary events) occurring in subjects classified at low or medium risk according to the FRS. Well-established indications for the use of the CACS comprise the stratification of general CV risk for asymptomatic patients. It includes intermediate-risk individuals based on the FRS (class I); low risk based on a family history of early CAD (class IIa); and low-risk patients with diabetes (class IIa). The application of the CACS as the only method is limited in symptomatic patients. In these patients, it should serve as a proper tool to choose the best method to facilitate the diagnosis [84]. In diabetic patients, CACS allows the identification of the greatest risk in subjects, who could profit from screening for silent ischaemia and more aggressive therapy.

Computed Tomography Coronary Angiography

Computed tomography coronary angiography (CTCA) allows detection of noncalcified plaque coronary artery stenosis severity. It demonstrates excellent accuracy to identify and mainly exclude the presence of significant obstructive lesions [86]. Clinical data showed that CTCA provides incremental prognostic utility for prediction of mortality and non-fatal myocardial infarction for asymptomatic individuals with moderately high CACS, but not for lower or higher CACS [87]. Most recently, CTAC improved the prognosis of 6-year all-cause mortality beyond a set of conventional risk factors alone; however, no further incremental value was found by CCTA when CCTA data were added to a model incorporating conventional risk factors and CACS [88]. Min et al. [89] assessed whether CTCA-detected CAD is able to improve the risk assessment of asymptomatic diabetic individuals beyond clinical risk factors and CACS. CTCA manifested incremental risk prediction, discrimination, and reclassification on a per-patient, per-vessel, and per-segment basis. In another study, CACS has been found inadequate for the detection of obstructive and non-obstructive CAD compared with CTCA [90]. Moreover, Parson et al. [91] concluded that CTCA is a more efficacious approach to CAD evaluation when compared with CACS.

The clinical data discussed above demonstrated that CTCA provides incremental prognostic information to traditional risk factors and CACS. Thus, CTCA and CACS might serve as important complementary tools for CV risk stratification in asymptomatic patients [88].

Ankle Brachial Index

Evaluation of ankle brachial index (ABI) by ultrasound Doppler is the standard screening method for the detection of atherosclerosis in peripheral arterial disease patients. It is measured as the ratio of the blood pressure at the ankle to the blood pressure in the brachium. ABI is a significant indicator of atherosclerosis with the potential to improve the prediction of CVD events. Low ABI (<0.9) is associated with a higher risk of CHD, stroke, transient ischaemic attack, progressive renal insufficiency, and all-cause mortality [92]. The Ankle Brachial Index Collaboration Group [93] assessed if the ABI provides information on the risk of CV events and mortality independently of the FRS and if it can improve the risk prediction. The authors included 16 population cohort studies in the evaluation. A low ABI was linked with approximately twice the 10-year total mortality, cardiovascular mortality, and major coronary event rate in comparison with the overall rate in each FRS category. The authors found that the inclusion of the ABI in CV risk stratification using the FRS would result in reclassification of the risk category that is followed by treatment modifications; this was observed in 19% of men and 36% of women. Fowkes et al. [94] analysed the participant data from 18 cohorts where 24,375 men and 20,377 women were free of CHD with the ABI measurement. Subsequently, the participants were followed up for CV events. The authors concluded that the ABI risk model may improve prediction especially in individuals at intermediate risk and when performance of the base risk factor model is moderate. Based on data from Atherosclerosis Risk in Communities Study (11,594 individuals included), Murphy et al. [95] did not show clear evidence which supports the FRS modification after including the ABI as an independent predictor of atherosclerosis.

Biomarker Discovery And The "-OMICS"

Traditional techniques of protein biomarker discovery include immunological detection methods such as Western blots and ELISAs and detection of miRNAs currently depend on PCR [96–98]. These methods allow research on a relatively small number of molecules with a relatively small and insignificant contribution to risk stratification. Technological improvements in automated analytical methodologies allow analyses of the entire proteome, metabolome, lipidome or transcriptome. Omics assess the net biological effect of the investigated "-ome". Omics, although more expensive, have the potential to effectively detect more biomarkers or their combination (multi-markers panel) and thus improving their total contribution to risk stratification.

Transcriptomics approach utilises RNA identification using microarray analyses through the genome-wide study of RNA expression [96] of body fluids or histological specimens of atherosclerotic plaques, endothelial cells, smooth muscle cells, extracellular matrix [97], etc.

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Proteomics allow analysis of all proteins expressed in target tissue or blood, which can then be compared to non-diseased tissue e.g. atherosclerotic plaque compared to normal arterial wall. Nowadays, proteomic analyses are carried out with mass spectrometry approaches. Generally, two approaches are possible. The first is untargeted, which results in a list of present proteins, the second is a preselected panel of proteins which are measured with high precision (high sensitivity). The combination of both approaches seems to be the best strategy for biomarker discovery [96,98,99].

Metabolites are small molecule intermediates and products of cellular metabolism [99]. Metabolomics analyses the metabolite profile in plasma or tissues mainly using magnetic resonance spectroscopy (MRS) or nuclear magnetic resonance spectroscopy [98]. The association of some amino acids with atherosclerosis has already been shown [100]. On the other hand, lipidomics, a subset of metabolomics, analyses not only triglycerides, high density lipoprotein (HDL), low density lipoprotein (LDL), and total cholesterol, but also all other lipids–lipidome [98]. Knowing that lipids greatly contribute to process of atherosclerosis, lipidomics shows great promise in new biomarker identification.

Family History

Family history (FH) of premature CAD is a risk factor for the development of incident CV disease. However, based on a plethora of clinical data, the linkage between FH and clinical results in subjects with confirmed CAD is unclear. Recently, results from several trials have suggested that a genetic risk score (GRS) for CAD seems to be independent of FH in predicting of CV events in the future. Abdi-Ali et al. assessed the relation between FH of premature CAD and all-cause mortality using multivariable Cox proportional hazards regression[101]. FH of premature CAD was associated with reduced all-cause mortality over a median 5.6 years in follow-up (hazard ratio [HR] 0.77 [95% CI 0.73–0.80]). Results showed the silencing of the linkage with increasing age, but FH remained protective even in individuals aged older than 80 years (HR 0.86 [0.77–0.95]). The authors concluded that self-reported FH of premature CAD is linked with improved long-term survival rate, independent of clinical characteristics, mode of presentation, and extent of disease. Sivapalaratnam et al. concluded that, although FH of premature CHD was found to be an independent risk factor of future CHD, its application did not improve the classification of individuals into clinically relevant risk categories based on the FRS[102]. Hindieh et al. aimed to evaluate if the presence of CV risk factors, either individually or together, is linked with an elevated burden of angiographic CAD[103]. Researchers enrolled 763 patients (within the GENESIS-PRAXY study) with premature acute coronary syndrome (ACS) (median age, 50/46–53/years; 30.8% women) with at least one major epicardial vessel stenosis. Results demonstrated that the presence of either a high GRS or FH is related to greater severity of CAD in patients with premature ACS. According to the authors, it is questionable whether the preventive clinical strategies targeted to genetically predisposed individuals could decrease the risk of early ACS and the resolving of this query warrants further clinical evaluations.

Can Newer Risk Markers Improve Risk Prediction?

The important question for the preventative cardiologist is whether the newer risk markers for atherosclerosis could improve CHD risk prediction. There are several recent clinical studies evaluating the potential improvements in CHD risk prediction with the use of newer risk markers.

Regarding the urinary and serum biomarkers, Takahashi et al. [104] analysed the predictive ability of three CVD biomarkers: the urinary albumin-creatinine ratio (UACR), plasma B-type natriuretic peptide concentration (BNP), and serum high-sensitivity C-reactive protein concentration (hsCRP) for identifying the incidence of disability as future recipients of public long-term care (LTC) service. The risk predictive performance for the incidence of LTC as evaluated by an essential model (i.e. age- and sex-adjusted) was substantially improved by incorporating the UACR (net reclassification improvement = 0.084, $p < 0.01$; integrated discrimination improvement = 0.0018, $p < 0.01$). In two remaining biomarkers the hazard ratios were not significantly changed. In another study (the cohort included 5511 community-dwelling individuals), the significance of TIMP-1, MMP-9, and hsCRP levels was evaluated in CV risk prediction. The model of Cox proportional hazards regression was based on the FRS variables. TIMP-1 and hsCRP demonstrated the best continuous net reclassification improvement over the baseline model for 5-year survival [net reclassification index (NRI) 0.28 and 0.19, respectively, both $p < 0.0001$] and for 10-year survival (NRI 0.19 and 0.11, respectively, both statistically significant) [105]. In a prospective population-based study, Kavousi et al. [80 = 106] evaluated whether newer markers (NT-proBNP levels, von Willebrand factor antigen levels, fibrinogen levels, chronic kidney disease, leukocyte count, CRP levels, homocysteine levels, uric acid levels, CACS, CIMT, peripheral arterial disease, and pulse wave velocity) for CHD risk prediction and stratification improve the FRS predictions. Moreover, traditional CHD risk factors in the FRS such as age, sex, systolic blood pressure, treatment of hypertension, total and HDL-cholesterol levels, smoking, and diabetes were analysed in this study. The coronary artery calcium score added to the FRS improved the accuracy of risk predictions (c-statistic increase, 0.05 [95% CI, 0.02–0.06]; net reclassification index, 19.3% overall [39.3% in those at intermediate risk, by FRS]). Regarding the NT-proBNP measure, it also improved the risk predictions but to a lesser extent in comparison with the CACS (c-statistic increase, 0.02 [CI, 0.01–0.04]; net reclassification index, 7.6% overall [33.0% in those at intermediate risk, by FRS]). Using other, newer, markers, changes in risk

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predictions were insignificant. The authors pointed to certain limitations of the study, i.e. that these results may not be generalisable to younger or non-White individuals [106]. Moreover, the authors concluded that the use of CACS as a more routine screening method for CV risk assessment needs full consideration of the financial and clinical costs within the health systems. Another clinical study evaluated if the vascular and valvular calcification predicts incident major CHD, CVD, and all-cause mortality independent of Framingham risk factors in the community-based Framingham Heart Study. The coronary artery calcium score clearly improved the discriminatory value beyond risk factors for CHD (area under the curve 0.78–0.82; net reclassification index 32%, 95% CI 11–53) but not for CVD. CACS reclassified 85% of the 261 patients who were at intermediate (5–10%) 10-year risk for CHD based on Framingham risk factors to either low risk (n = 172; no events observed) or high risk (n = 53; observed event rate 8%). These results demonstrated that CACS improves the risk prediction and risk reclassification for major CHD and CVD beyond risk factors in an asymptomatic community and reclassifies 2/3 of the intermediate-risk subjects [107]. Amato determined the independence of carotid plaque thickness and mean common carotid intima-media thickness in plaque-free areas (PF CC-IMT_{mean}) in CV risk prediction and risk stratification[108]. $cIMT_{max}$ and PF CC-IMT_{mean} have been shown as independent predictors of VEs. The reclassification evaluation revealed that PF CC-IMT_{mean} significantly adds to a model including both Framingham risk factors and $cIMT_{max}$ (integrated discrimination improvement; IDI = 0.009; p = 0.0001) and vice-versa (IDI = 0.02; $p < 0.0001$). The meta-analysis of Ohkuma et al. revealed that the addition of brachial-ankle pulse wave velocity to a model incorporating the FRS significantly elevated the c statistics from 0.8026 to 0.8131 ($p < 0.001$), moreover, it improved the category-free net reclassification (0.247; $p < 0.001$[109]). Yeboah et al. analysed the reclassification value of FMD for incident CVD events in the MESA study [110]. The investigators summarised that the addition of FMD to the FRS did not improve discrimination of subjects at risk of CVD events, however, it significantly improved the classification of individuals at low, intermediate, and high CVD risk in comparison with the FRS alone.

Conclusion And Future Directions

As we stated in Part 1 of our review, atherosclerosis is a major contributor to morbidity and mortality worldwide. With therapeutic consequences in mind, several risk scores are being used to differentiate individuals with low, intermediate or high CV event risk. The most appropriate management of intermediate risk individuals is still not known, therefore novel biomarkers are being sought to help re-stratify them as low or high risk. Proposed biomarkers are intertwined in inflammation, oxidation, haemostatic and other processes involved in atherosclerosis. Among novel biomarkers, hsCRP has emerged as most promising in chronic situations, others need further clinical studies; however, it seems that a combination of serum biomarkers offers more to risk stratification than either biomarker alone. Significant improvements in risk stratification are expected with the further addition of genetic risk scores, obtained by genetic testing of risk SNP's presence. With more discovered loci, the extent of reclassifications may improve. In addition to SNPs, recent advances in epigenetics offer DNA methylation profiles, histone chemical modifications, and miRNAs as other promising indicators of atherosclerosis. A plethora of clinical studies have found an association between exposure of various environmental factors during the life or intrauterine period and increased occurrence of biomarkers for atherosclerosis. Better understanding of the long-term influence of the environment to individuals should provide more effective medical interventions to reduce the incidence of CVDs. The occurrence of apparent genetic biomarkers of atherosclerosis in high-risk individuals indicates an opportunity for early prevention programs.

Contrary to serum and genetic biomarkers, ultrasonography is better studied and already has a high degree of clinical applicability. Carotid intima-media thickness, carotid plaque detection, flow mediated dilatation and arterial stiffness measurements can all be used in CV event prediction and detection of preclinical atherosclerosis. All such methods can also be readily and inexpensively obtained, with a high degree of reproducibility.

The clinical value of any of these biomarkers may be hindered by many factors such as individual variability, lack of tissue specificity, differently used assays, sensitivity and specificity, age, weight, renal function, gender or ethnic differences [98], therefore large clinical studies with uniform patient characteristics on different ethnicities are needed. Moreover, with new methodologies (-omics) new panels of biomarkers are expected with potentially stronger and independent associations with atherosclerosis and contributions to risk stratification.

Conflict of Interest

Authors declare no conflict of interest.

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