Biomarkers in Cardiovascular Disease

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Atherosclerosis and its consequences are the leading cause of mortality in the world. For this reason, we have reviewed atherosclerosis biomarkers and selected the most promising ones. We focused mainly on biomarkers related to inflammation and oxidative stress, such as the highly sensitive C-reactive protein (hs-CRP), interleukin 6 (IL-6), and lipoprotein-associated phospholipase A2 (Lp-PLA2). The microRNA (miRNA) and the usefulness of the bone mineralization, glucose, and lipid metabolism marker osteocalcin (OC) were also reviewed.

Keywords: atherosclerosis ; inflammation ; oxidative stress ; biomarkers ; cardiovascular disease ; hsCRP ; Lp-PAL2 ; IL-6 ; miRNA ; osteocalcin ; angiogenin

1. Introduction

It is well-known that cardiovascular diseases (CVD) are the leading cause of death globally since the beginning of the 20th century. According to WHO data in 2017 only, coronary artery disease (CAD) was a cause of almost one in three (17.79 out of 56 million) deaths worldwide.

Although atherosclerosis is the main cause of CVD, the mechanisms behind atherosclerotic plaque formation are still not fully understood. We are now aware that this is a consequence of complex, often interdependent processes, among which inflammation, oxidative stress, and the body's immune response play a key role [1]. Atherosclerotic plaque formation occurs in four main successive stages: (1) damage to endothelial cells with their dysfunction; (2) lipoprotein deposition and oxidation; (3) inflammatory process; (4) formation of a fibrous cap. It should be remembered that there are individual differences in the dynamics of plaque development, see [2][3], which are at least partly due to aging and comorbidities [4]. As a result of the complexity of this phenomenon, there is widespread agreement among researchers that determining the parameters of lipid metabolism is not enough to predict what is happening in the arterial subendothelial layer. Sometimes, we can even draw the wrong conclusions if we rely on them. For example, consider the JUPITER study, which revealed that men and women with low low-density lipoprotein cholesterol (LDL-C) but increased markers of local low-grade inflammation (high sensitive C-reactive protein (hs-CRP)) showed a significant cardiovascular risk. Participants who achieved hs-CRP less than 1 mg/L had a 79% reduction in vascular events and achieved hsCRP concentrations that were predictive of event rates irrespective of the lipid endpoint [5].

Keeping in mind that the development of atherosclerotic plaque formation stages is at least partly reversible, we reviewed some of the atherosclerosis biomarkers to discuss their ability to predict the development of CAD, giving patients and their doctors time to react.

The main limitation in identifying a universal biomarker of the inflammatory response, a key player process in atherosclerosis, is the variability of the metabolic stress response among patients and the multifaceted nature of the complex disorder itself.

Therefore, we recommend using more than one biomarker because there is still no one that fits all the patients and gives reliable results in every case. Our study reviewed biomarkers from different related processes to emphasize the complexity of forming atherosclerotic plaque and show future work directions to translate their role into clinical use. We included well-recognized biomarkers: (1) CRP, one of the acute-phase proteins produced mainly by the liver in response to low-grade inflammation underlying atherosclerosis; (2) interleukin 6 (IL-6); a pro-inflammatory cytokine secreted, among others, by tunica medias' smooth muscle cells, whose leading role consists in activating the inflammatory and autoimmune processes; (3) Lp-PLA2, an enzyme associated with both traditional (cholesterol-linked) and novel (inflammatory) pathways of atherosclerosis, which is synthesized by inflammatory cells and bonded mainly to LDL-C, with a small fraction linked to high-density lipoprotein cholesterol (HDL-C). We have also included potentially new biomarkers, such as (4) miRNA regulating gene expression by silencing complementary to itself mRNA pieces, with overexpression related to the development of vascular changes and CVD; (5) osteocalcin (OC), which is a multifunctional hormone

produced by osteoblasts whose action regulates mineralization, glucose, and lipid metabolism with a role in the process of vascular calcification and atherosclerosis; and (6) angiogenin, which is a protein involved in forming new blood vessels that interact with endothelium and smooth muscles, which possibly plays a role in destabilizing coronary plaque.

There is still debate about the need to include markers of atherosclerosis and inflammation in traditional cardiovascular risk assessment. The pathogenesis of atherosclerosis is a complex process involving so many molecules and pathways that it is impossible to present them all. In addition, not every particle meets the criteria of being a potential biomarker. A biomarker is a term that describes different types of objective indicators of health or disease. As technology advances, these indicators are becoming more and more precise. On the other hand, the detection of new biomarkers turns out to be a time-consuming and costly operation due to the complexity of the structure of potential markers, most often proteins, and difficulties in the reproducibility of the methods used for their determination.

2. Biomarkers

2.1. Recognized Biomarkers

2.1.1. High Sensitivity C-Reactive Protein

C-reactive protein (CRP) is one of the essential positive acute-phase proteins usually present at negligible levels in the plasma. Its concentration increases after 24–48 h after acute inflammatory trauma up to 1000-fold at sites of infection or inflammation, reaching a maximum of 24–48 h. CRP is synthesized predominantly as a native pentameric CRP (pCRP) in the liver [6]. This isoform can irreversibly dissociate at sites of inflammation into five identical non-covalently linked subunits—monomeric CRP isoforms (mCRP). This dissociation is promoted by the pCRP binding to phosphocholine residues of lysophosphatidylcholines on the cell surface exposed by phospholipase A2 (PLA2), which is a biomarker of vascular inflammation [7]. IL-6, promoting CRP de novo synthesis, appears to be CRP primary regulator. In addition, IL-6 signaling can be enhanced by interleukin-1β (IL-1β) and, to a lesser extent, by the tumor necrosis factor-alpha (TNF-α) [6][7][8][9].

A growing body of research indicates that pCRP has both pro-inflammatory and anti-inflammatory effects in a context-dependent manner. In contrast, mCRP has a strong pro-inflammatory effect on endothelial cells and their progenitor cells, leukocytes and platelets. The latter CRP may even exacerbate the inflammatory response. The existence of two protein conformations may explain conflicting data on CRP properties; see for a review [8].

Until recently, the only known physiological function of CRP was to label cells to initiate their phagocytosis via activation of complement and elimination. However, it is known that vital cells with reduced energy supply are also marked, which is helpful for a classic wound. However, it turns out that such an action has the opposite effect on internal injuries, e.g., during a myocardial infarct or stroke. This mechanism is disadvantageous, as CRP levels have been established to correlate with prognosis in these indications. In addition, it has recently been shown that CRP can directly affect blood pressure in rabbits [10]. It seems to justify the concept of essential hypertension as a complex immune-inflammatory disorder [11].

2.1.2. Interleukin 6

IL-6 is a pleiotropic cytokine synthesized by lymphocytes, activated macrophages, astrocytes, ischemic myocytes, and endothelial cells. It acts via a hexameric complex composed of IL-6, IL-6 receptor (IL-6R), and glycoprotein 130 (IL-6/IL-6R/gp130). IL-6 and its receptor system structure are essential because of the many activities that this cytokine can exert. This glycoprotein is present in picogram per milliliter (pg/mL) amounts in the serum in physiology. The physiological processes in which IL-6 is involved are diverse and include primarily: (1) aging; (2) menstruation; (3) spermatogenesis; (4) liver regeneration; (5) skin proliferation; (6) participation in brain development; (7) bone-strengthening; (8) role in hematopoiesis; (9) function in regulating metabolism; (10) role in postprandial glucose levels; (11) regulating appetite and body weight control; (12) taking part in immune modulation/host defense (acute phase reaction, B lymphocyte differentiation, T helper activation, and T regulatory lymphocyte inhibition); (13) playing the critical role in the balance Th17-Treg cells in gut-associated lymphoid tissue (GALT) [12].

IL-6 levels can rise in virtually any inflammation, even during exercise by releasing IL-6 from skeletal muscle, or even after multiple traumas (proportional trauma), reaching micrograms per milliliter (µg/mL) values under severe conditions such as septic shock. In pathology, IL-6 basal function focuses on initiating the acute-phase response after injury or trauma, leading to inflammation or infection to remove infectious agents [13]. IL-6 mediates pro-inflammatory effects through trans-signaling, while through classical signaling, it is responsible for anti-inflammatory and regenerative effects [14]. Although classical IL-6 signaling occurs through membrane-bound IL-6 receptors, trans-signaling IL-6 is driven by systemic and

localized increases in extracellular soluble IL-6 receptor (sIL6R) generated by proteolytic cleavage receptor “shedding” from the cell surface. sIL-6R can be activated by IL-6 and activate IL-6 signaling cascades via a constitutively expressed gp130 coreceptor. Thus, IL-6 trans-signaling enables the activation of IL-6 signaling pathways in cells that do not express IL-6R.

IL-6 signaling is involved in CAD and has recently become a focus of attention due to the global COVID-19 pandemic. This pro-atherogenic cytokine reached elevated serum levels during the cytokine storm generated by SARS-CoV-2 and was also associated with smoking or classical cardiovascular risk factors that promote inflammation and obesity. IL-6 levels were found to be associated with dyslipidemia, hypertension, and glucose dysregulation and were associated with poor outcomes in patients with unstable angina or AMI [14].

2.1.3. Lipoprotein-Associated Phospholipase A2

Lipoprotein-associated phospholipase A2 (Lp-PLA2) is a calcium-independent lipase that hydrolyzes the acetyl group of platelet-activating factor (PAF) as well as oxidizes phospholipids in LDL [15]. Compared with other members of the phospholipase A2 superfamily, it can catalyze hydrolysis at the sn-2 position of phospholipids [16][17][18][19].

Plasma levels of Lp-PLA2 have been identified as a biomarker of vascular inflammation and atherosclerotic vulnerability, which help predict future cardiovascular events [20]. The enzyme is mainly produced by monocytes and macrophages and circulates in plasma, being associated with LDL and HDL. The Lp-PLA2 expression is activated by apolipoprotein CIII (apo CIII, a protein that is found in triglyceride-rich lipoproteins such as chylomicrons, very-low-density lipoprotein (VLDL), and remnant cholesterol, whose function is inhibition of lipoprotein lipase and hepatic lipase), oxidized LDL (oxLDL, lipoproteins formed under the influence of reactive oxygen species, which are not recognized by receptors for primary LDL), serum amyloid A and leukocytes. In contrast, nitro-oleic acid downregulates Lp-PLA2 expression [21].

Lp-PLA2 has a dual role in the inflammatory process, depending on the type of lipoprotein with which the enzyme is associated [22]. HDL-Lp-PLA2 has an anti-inflammatory, anti-oxidative, and anti-atherogenic role, while the LDL-Lp-PLA2 expresses pro-inflammatory and pro-atherogenic effects. It should be underlined here that Lp-PLA2 is carried bound mainly to LDL in the circulation. The hydrolysis of oxLDL lead to the release of lysophosphatidylcholine (lyso-PC) and oxidized free fatty acids (OxFFA), which are the triggers of inflammatory cascade by induction of chemotaxis of monocytes and leukocytes and promotion of their entry in the sub-intimal space of the artery wall [18]. What is more, these substrates attach to activated macrophages through scavenger receptors and are phagocytized, leading to foam cells’ formation [17]. These cells play an essential role in atherosclerosis. They induce the accumulation of lipids, which lead to fatty streak formation in the vascular wall [19]. The muscle cells also migrate to the intima, where these cells start to produce collagen and elastin, which are involved in stabilizing the atherosclerotic plaque [17]. Lyso-PC is also engaged in the production of reactive oxygen species (ROS). When lyso-PC activates the endothelial nicotinamide adenine dinucleotide phosphate (NADP), it oxidizes and induces the endothelial nitric oxide synthase (eNOS). Those pro-inflammatory and pro-oxidative effects of Lp-PLA2 are involved in the pathogenesis of atherosclerosis [18].

On the other hand, enzyme activity associated with HDL can play the opposite role. Studies have shown that HDL-Lp-PLA2 decreases endothelial adhesiveness and macrophage recruitment to prone lesion sites. It is confirmed by the dual action of Lp-PLA2, which depends on the lipoprotein to which the enzyme is associated. Lipase associated with HDL shows an anti-atherogenic role, while LDL-Lp-PLA2 stimulates the process of atherosclerosis [17]. Lipase can also be associated with Lp(a), and this complex may play a role similar to that observed for the LDL-Lp-PLA2 in the artery wall. HDL-Lp-PLA2 level testing has shown that it is reduced in patients with combined hyperlipidemia, primary hypertriglyceridemia, pre-diabetes, and metabolic syndrome, while LDL-Lp-PLA2 level is elevated in these patients [22]. What is more, research has shown that Lp-PLA2 was increased in the subjects with the incidence of CVD; also, the patients with heart failure have an elevation of Lp-PLA2 levels [17]. This is why Lp-PLA2 is one of the most promising atherosclerosis biomarkers that can be useful in assessing cardiovascular risk in asymptomatic patients [18]. That inflammatory biomarker was also approved by the United States Food and Drugs Administration (FAD) as a predictor of ischemic stroke. Lp-PLA2 is considered to be a more specific marker of cardiovascular risk. However, many epidemiological studies have found inconsistent results regarding whether this lipase can be used to predict atherosclerosis. That is why it may be helpful to use a combination of hs-CRP and Lp-PLA2 in the prediction of the risk of CVD, including CAD and stroke [23].

2.2. Potentially New Biomarkers

2.2.1. MicroRNA

MicroRNAs (also known as miRNAs or miRs) represent a group of about 17–25 nucleotides of long, non-coding RNAs that have been shown to modulate gene expression at the translational level by interfering with the 3' untranslated region (UTR) of messenger RNA [24]. Circulating miRNAs are highly stable and considered to be novel biomarkers for the diagnosis and/or prognosis of CVD. Many studies concerning miRNA as a potential biomarker in atherosclerosis were published in the last few years, but new miRNAs are still emerging as possible clinical biomarkers in diagnosing atherosclerosis.

MiRNAs by interactions with mRNAs impact protein synthesis; hence, they play a significant role in developing numerous diseases. The effect of harmful stimuli and the influence of miRNA may be the cause of atherogenesis [25]. It has been found that miRNAs are regulated in different stages of atherosclerosis, from activation and proliferation to cellular senescence. For instance, miR-21 expression was increased in peripheral blood mononuclear cells (PBMCs) in patients with severe vascular disease and AMI in the medical history and continues rising along with the severity of atherosclerosis [26][27]. miR-21 high abundance was observed in macrophages [28] and was revealed to affect foam cell formation [29]. Berkan et al. found that the formation of atherosclerotic plaque was associated with miR-486-5p downregulation [30].

2.2.2. Osteocalcin

Osteocalcin (OC), also known as bone glutamic acid protein (BGLAP), is a non-collagenous synthetic protein secreted mainly by osteoblasts. OC regulates the bone extracellular matrix by binding to calcium ions and hydroxyapatite crystals; thus, it is considered a traditionally bone formation marker [31][32].

The maturation of OC is quite complicated and not fully understood. OC is initially synthesized as a proprotein by osteoblasts, chondrocytes, and osteoblast-like VSMCs. Next, OC proprotein experiences signal peptide removal and is converted into an uncarboxylated isoform (uOC). The active form of vitamin D ($1,25(OH)_2D_3$) increases the OC expression in humans and rats, as opposed to mice, where it decreases OC expression [33]. Animal experiments have demonstrated that only the uOC isoform exhibits hormonal activity; however, data from clinical observational trials are conflicting [34]. Then, uOC, thanks to the γ-glutamyl carboxylase (GGCX) action and its coenzyme—vitamin K, is converted into carboxylated OC (cOC). About 20% of cOC enters the bloodstream, and the rest enters the bone and binds to calcium deposits in the bone matrix. cOC inhibits the bone resorption activity of osteoclasts. During active bone resorption, cOC may be transformed into uOC and an undercarboxylated OC isoform (ucOC) again following decarboxylation. cOC and uOC may enter the blood circulation. The ucOC cannot be released into the blood in the vitamin K environment due to its reduced binding affinity to bone minerals [34]. The mechanism of cOC entry into the blood circulation and whether it promotes or inhibits the calcification of blood vessels is not currently precise [33]. The ucOC transference into cOC could be enhanced by vitamin K [35], and the level of both OC isoforms could be dependent on diet [36]. Some studies exposed that cOC and ucOC were linked with energy metabolism and atherosclerosis [37]. Low ucOC was associated with abdominal aortic calcification in the male cohort [38][39].

OC has several features of the hormone and has recently been linked to increasing extra-bone biological roles. These extra-bone functions include the following: (1) influencing brain development and function, which sheds new light on the cause of cognitive decline with age [40]; (2) stimulating the expression of cyclin D1 and insulin in pancreatic β cells and adiponectin (an insulin-sensitizing adipokine) in adipocytes and improving glucose tolerance [41]; (3) linking the pathway between central obesity and insulin resistance [42]; and (4) promoting male fertility by increasing testosterone production [43][44].

2.2.3. Angiogenin

Angiogenin (ANG) is a 14 kDa molecular weight extracellular protein, a member of the ribonuclease (RNase) superfamily of enzymes (also known as RNase 5); it was initially identified in a medium conditioned with tumor cells. ANG was detected in human tissues and fluids (plasma, amniotic, tumor microenvironment, and cerebrospinal fluid). It was found localized to different cellular compartments under different conditions, such as growth (nuclear) and stress (cytoplasmic). Due to ANG properties, is involved in many processes, such as (1) tumorigenesis; (2) neuroprotection; (3) inflammation; (4) innate immunity; (5) reproduction; and (6) regeneration of damaged tissues [45].

ANG, one of the strongest angiogenic factors, interacts with endothelial cells and induces a cellular response, initiating the process of new blood vessel formation [45].

Atherosclerosis is a complex process [46][47][48], which was lately linked to angiogenesis [49][50]; some studies show the importance of ANG, mainly in the development of microvessels inside the core of the atherosclerotic plaque. These vessels are thin-walled and lined with a discontinuous endothelium without supporting the VSMCs, making the vessel wall very sensitive and prone to cracking. Hemorrhage within the plaque destabilizes it, leading to occlusive thrombosis and

clinical symptoms of ACS. ANG is required for vascular endothelial factor (VEGF) to stimulate angiogenesis [51]. In addition, it plays a vital role in the interaction of proteases that activate wound healing, such as the metalloproteinase family, and in the stimulation of tissue plasminogen activator (tPA) to produce plasmin [52][53]. These proteases are also associated with the destabilization of atherosclerotic plaque. Hence, high levels of angiogenic factors could be potential markers of unstable plaque in ACS and could be a risk marker of future ACS [51].

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