

Re-emerging Pathogens Associated With Atherosclerosis

E. Kozarov^{1,2} and L. Badimon¹

¹Institute for Cardiovascular Sciences (CSIC-ICCC), Hospital de la Santa Creu i Sant Pau and Autonomous University of Barcelona, St. Antoni M. Claret 167, 08025 Barcelona, Spain

²Section of Oral and Diagnostic Sciences, Columbia University Medical Center, 630 W 168 St, Box 20, New York, NY 10032, USA

Based on multiple epidemiological studies, molecular and cell biology investigations consistently implicated infectious agents with vascular inflammations that are the underlying cause of myocardial infarction and stroke. We have recently shown, using molecular, genetic and immunological approaches, the association of variety of pathogens with atheromatous tissues. The re-emergence of bacterial pathogens as potential initiators and/or exacerbators of atherosclerotic cardiovascular disease (CVD) pose new challenges to medical research and to an overwhelmed health care budget, however there is a silver lining to it. The availability of a new approach to CVD provides a much-needed opportunity to develop entirely novel theranostics and to introduce personalized medicine in the most critical area of human health.

Keywords: Atherosclerosis, infection

“Knowing is not enough; we must apply. Willing is not enough; we must do it.”—Johann Wolfgang von Goethe (1749 – 1832)

1. Introduction

Infectious diseases have been all along suffered by the human race during the entire history. Only during the last two centuries, significant advances have been made to address this health problem. The causative agents of many diseases have been identified, and during the last century, efficient pharmaceuticals and antimicrobial therapies have been designed. Specifically, the advent of the antibiotics led to a never seen before jump in the human life span. In fact, in 1970, the Surgeon-General of the United States of America, William Stewart indicated that it was "time to close the book on infectious diseases, declare the war against pestilence won, and shift national resources to such chronic problems as cancer and heart disease"[1].

However, due to overuse and misuse and to the quick evolution of the target microorganisms, the efficiency of the introduced antimicrobials is in severe decline. In addition, the “re-emergence” of pathogens, often well-known bacterial species, now poses a significant threat to hospital and emergency room patients, as well as to the healthcare providers.

Interestingly though, the widely-quoted statement of Dr. Stewart regarding heart disease is a foresight of a trend that only now starts getting attention: the involvement of infectious agents with this chronic disease.

Unlike infectious diseases associated with devastating epidemics, chronic non-communicable diseases (NCD) are not (noticeably) transmitted between individuals and progress slowly, taking decades to become symptomatic. Nevertheless, NCD are now becoming a primary concern for the public health officials, government budgets and international organizations. It is recognized that they are the leading cause of morbidity and mortality in the developed, and lately – in the developing countries. According to the World Health Organization (WHO), NCD affect disproportionately low- and middle-income countries where ~80% of the NCD deaths occur [2].

The main types of NCD are cardiovascular disease (CVD), a leading condition, and also include cancers, chronic obstructive pulmonary disease (COPD) and diabetes. It is currently known that ~30% of all global deaths are due to CVD, where an estimated 7.3 million were due to coronary heart disease and 6.2 million were due to stroke [3]. Significant advances in CVD are crucial, especially due to the increased prevalence with age. Expanded efforts in this field can thus significantly improve quality of life and lead to extended lifespan.

Despite the identification of risk factors for CVD and the introduction of highly effective treatment regimens, CVD continues to present major problem for public health globally. Myocardial infarction and stroke continue to occur in as many as two thirds of all patients [4], [5]. Entirely novel approaches for diagnosis and treatment are therefore needed.

The majority of the CVD deaths are caused by atherosclerotic vascular disease (atherosclerosis), characterized by a chronic focal inflammatory lipid-laden proliferative lesion (atherosclerotic plaque, or atheroma) at discrete arterial sites where they can obstruct blood flow. The progressive accumulation of lipids (LDL) in the intima and the endothelial activation (upregulation of expression of cytokines, chemokines and cell adhesion molecules) lead to permeability of the endothelial layer and to monocyte rolling on, adhesion, diapedesis and recruitment underneath the tunica intima. The monocytes differentiate into macrophages that secrete growth factors, engulf oxidized LDL particles turning in the process into foam cells, inducing smooth muscle cell proliferation and forming the lipid core of the lesion. The foam cells also secrete metalloproteinases and reactive oxygen species that can degrade extracellular matrix and compromise tissue's stability. For a recent detailed review, see [6].

When plaque destabilization and rupture of the plaque's fibrous cap occurs, the coagulation cascade is triggered by the exposure to thrombogenic collagen and other plaque components, initiating platelet aggregation with the resulting thrombus leading to partial (angina pectoris) or complete obstruction of blood flow. The latter is the mechanistic cause of acute ischemic events such as myocardial infarction (MI) or stroke [7].

2. Atherosclerosis is an inflammatory condition

It has convincingly emerged that inflammation is a hallmark of atherosclerosis, the main underlying pathology in CVD [8], [9]. Inflammation is often triggered by infectious agents, and when a localized infection has access to the circulation, the inflammation can metastasize, either directly – caused by disseminated infectious agents – or indirectly, via immunological “priming” where the proinflammatory mediator molecules access distant sites of the body. Either of those inflammation triggers, in presence of hyperlipidemia (increased plasma levels of LDL particles), can cause endothelial activation. The activation in turn initiates the atherogenic process as described above, leading to fatty streak formation and lipid accumulation in the arterial wall. In fact, a correlation has been shown between arterial deposition of cholesterol and the concentration of circulating plasma lipoproteins [10].

There are several targeted risk factors for atherogenesis, mainly hyperlipidemia, hyperglycemia, smoking and hypertension. Since the incidence of atherosclerosis not fully explained by conventional risk factors [11], it is accepted that other factors also exist, such as genetic predisposition [12], and even mental stress that has been shown to induce prolonged endothelial dysfunction [13]. Here we will focus on the microbial etiology of atherosclerosis and on the strategies to combat infections at systemic locations.

3. Evidence of microbial component of atherosclerosis

In addition to Robert Koch's postulates, which we will be considering, the criteria for causation (does disease A cause disorder B) established by Sir Bradford Hill are addressed here. His criteria include significance of epidemiological association, biological plausibility and experimental evidence of the impact of treatment of one disease on the other. The accumulated so far epidemiological, seroepidemiological and animal model evidence does suggest infections as a major risk factor for vascular inflammation.

Atherosclerosis does have many of the characteristics of a chronic inflammatory disease. There is a substantial epidemiological evidence implicating infections in atherogenesis [14]. Clinical data have indicated that infections with multiple pathogens result in chronic inflammation [15]. "Infectious burden," an aggregate measure of multiple chronic infectious exposures, has been associated with the risk of stroke and carotid atherosclerosis [16]. In one study of 572 patients, IgG or IgA antibodies to eight pathogens were measured, together with the extent of atherosclerosis. A significant association between infectious burden and the extent of atherosclerosis was demonstrated, and the risk for future death was shown to correlate with the number of infectious pathogens [17]. In particular, bacterial internalization in vascular host cells results in a “privileged niche”, where bacteria can persist in a dormant, non-replicating state, sheltered from humoral and cellular immune responses. The best characterizes example of dormant intracellular pathogen causing chronic inflammation is *Mycobacterium tuberculosis* [18].

The link between periodontal infections and vascular inflammations deserves specific attention. In a pilot study of long-term clarithromycin treatment of CVD patients, the treatment reduced recurrent cardiovascular events in subjects without periodontitis, but had no effect in patients with periodontitis [19] and in a random seroepidemiological population-based investigation of 8911 subjects aged 30-59 years, who participated in a CVD risk factor survey and were followed for 15 years, CVD-free subjects with systemic exposure to the major periodontal pathogen *Porphyromonas gingivalis* had the risk of stroke increased, compared to seronegative subjects [20]. Animal models of infection corroborate the link, including murine, rabbit and porcine models of atherosclerosis. Repeated systemic inoculations with *P. gingivalis* led to up to 9-fold greater progression of atherosclerosis in ApoE(+/-) mice [21] and mice challenged with *P. gingivalis* presented with increased macrophage infiltration, innate immune marker expression, and atheroma formation [22]. Larger animal studies supported the murine model, such as the rabbit model [23] and the porcine model. In the latter, *P. gingivalis* bacteremia induced coronary and aortic atherosclerosis in normocholesterolemic and hypercholesterolemic pigs [24]. Importantly considering the treatment options, pre-challenge immunization with heat-killed bacteria prevented *P. gingivalis*-accelerated atherosclerosis in ApoE knockout mice [25].

There is an acute need to introduce a new paradigm in the study of infections as potential causes of atherosclerosis. We hypothesize that atherosclerosis, a chronic vascular inflammation, is in many cases a disease with a polymicrobial etiology that is induced and aggravated by persistent intracellular infectious agents (the “atherosclerosis microbiome”). According to our model, large randomized controlled clinical trials of antibiotics in patients with atherosclerosis failed largely because of two reasons. First, many intracellular bacteria that have the ability to illicit low grade inflammation are shielded intracellularly, are dormant at any given time point and unsusceptible to antibiotics, therefore the timing of the trials has to be adjusted. There is a turnover period, “stochastic decay” during which dormant organisms become active therefore the patients need to be treated until the intracellular pool of dormant bacteria is depleted and dormant

atheroma-associated pathogens no longer are source of underlying recurring infection. We propose that previous trials might have been too short to allow for complete clearance of relevant infectious burden. Second, the antibiotics tested could lack activity against the dormant intracellular organisms present in the tissue of the particular patient. Therefore, the identification (individual diagnosis, personalized medicine) and the cultivation of these pathogens is the key to their identification, to investigation of their ability to initiate or exacerbate disease, of their genetics, antibiotic susceptibility and virulence factors, and to establishing a causal relationship.

For recent reviews on related subjects such as bacterial invasion of vascular cell types; the atherogenic sequelae of bacterial presence such as endothelial activation; and the identification of the species that are able to colonize this niche, see [26] and [27].

4. Identification of re-emerging pathogens

As a recent historical precedent, gastritis and stomach ulcers were assumed result of stress or unhealthy diet, until in the 1980s Marshall and Warren demonstrated curved bacilli in specimens from 58 patients. Almost all patients with active chronic gastritis, duodenal ulcer, or gastric ulcer contained gram-negative, flagellate, and microaerophilic bacteria that are now known as *Helicobacter pylori*, an important factor in the etiology of these diseases [28]. Experimenting on himself, Marshall fulfilled the Koch postulates and demonstrated the causative link of the newly found bacterium with peptic ulcers [29]. This allowed for application of antimicrobial "triple therapy" (proton pump inhibitors combined with antibiotics), essentially curing the disease, while previously, the only option has been symptom (acidity) control [30].

The plausibility of infectious agent localization in atheromatous tissues has now been examined at DNA, protein and cellular level. Firstly, there is significant evidence of systemic bacteremia, and not exclusively in septic patients. In one study of periodontitis patients undergoing teeth scaling and root planing, routine dental cleaning procedure, 80.9% of the patients presented positive cultures after the procedure and it occurred immediately after treatment [31]. Next, there is abundant evidence of the presence of bacterial DNA at the atherosclerotic lesion sites [32], [33], [34]. We have shown for the first time the presence of live invasive bacteria (of periodontal origin) in carotid plaque tissue [35] and moreover, we recently demonstrated the presence of a variety of live bacterial pathogens in atheromatous tissues from multiple patients [36-37], describing for the first time the atherosclerosis microbiome. We have also shown, using protein-nucleic acid (PNA) probes in fluorescence in situ hybridization (FISH) and also double fluorescence immunohistochemistry, the presence of bacterial DNA and protein antigens in atheromatous tissues, respectively (Kalachikov et al, submitted; [37]).

5. Implications for theranostic technologies in personalized medicine. Strategies for atherosclerosis prevention and control using antimicrobial approach

Theranostics (also theragnostics) is a combination diagnostic/treatment design and monitoring strategy for the individual patient. It is both a diagnostic test that identifies the patients most likely to benefit from a treatment, and identifies the specific drug therapy that would offer the best outcome. In addition to diagnosis, prognosis and treatment, a theranostic test would be used throughout the course of treatment to monitor the results and to inform the treating clinicians on the progress.

The introduction of theranostics in the critical area of CVD prevention and treatment will bring unsurpassed knowledge of the infectious component of CVD. Bacterial pathogens associated with atheromatous tissues have now been described by us (above) and others [34], [38]. The presence of microbial signatures in host cells has been shown [39]. The recent discovery of the atherosclerosis microbiome in arterial atherosclerotic lesions, coupled with advancements in high-throughput (HT) omic technologies allows for an expedited approach to CVD theranostics as a realistic plan of action. Since specific subset of species was found in each individual patient in our and other groups' patients, only precision ("personalized") medicine approach can successfully address the underlying infections. Thus, theranostic technology will be critical for introduction of correct treatment regimens, since prescribing antimicrobials to vascular inflammation patients must reflect legitimate targets, which vary with each patient.

The personalized medicine approach is indispensable if we are to improve the clinical outcomes. Several large-scale, randomized, controlled clinical trials from the last decade that were initiated after the cultivation of a single bacterial species, *Chlamydomphila pneumoniae* from atheromas, did not meet the expectations [40], [41]. Notwithstanding, now we have data demonstrating the existence of a variety of intracellular bacteria in clinical specimens, an important treatment target that can explain the failure of these clinical trials. This advancement can be considered as "good news: you have an infection" message, since existing drugs can be repurposed for this new setting, serving as a buffer until entirely novel treatment regimens are developed. If infectious burden does play a role in atherosclerosis or stroke, it is plausible that preventive anti-infective treatment, including vaccination, may reduce the risk of acute ischemic events [42]. In a similar manner, recent evidence suggests that bacterial components in blood could play an early role in events leading to diabetes. Using a bacterial marker (16S rDNA) in analysis of 3,280 participants without diabetes or obesity at

baseline, it was shown that 16S rDNA concentration was higher in those destined to have diabetes to predict the onset of diabetes in a general population [43].

Since drug treatment has not been tested yet on this specific set of organisms, at this new site setting and on this particular subset of patients (unlikely to have been treated with antimicrobials for this condition), drug resistance may not be encountered (soon) and a cautious optimism regarding positive outcome could be extended.

Specific strategies for chronic vascular infection control may include low (sub antimicrobial) dose antimicrobials, successful in oral inflammations [44] and for other chronic conditions, such as skin disorders [45]. Another, more developed vision for control of bacterial infections in a time of drug- and multi-drug resistance is the “third era of antimicrobial treatment” where active therapeutics must be complemented with enhancers of the host’s immune system [46]. This latest approach calls for stimulation (rather than suppression that only postpones a relapse) of the innate immune response in order to successfully destroy the invading pathogens [47], [48]. Immune enhancers have been also suggested for *Clostridium difficile* infection [49]. Further, treatment with vitamin E prior to microbial challenge in mouse model of wound infection caused by methicillin-resistant *Staphylococcus aureus* (MRSA) and subsequent antibiotic treatment were shown to act in synergy [50]. Most importantly, the bacterial messenger molecule cyclic dinucleotide c-di-GMP has been proposed for clinical use as an immunomodulator, immune enhancer, immunotherapeutic, immunoprophylactic, or vaccine adjuvant [51].

Another atheroprotective strategy is based on the anti-inflammatory properties of statins, the competitive 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors. During the decades since they have been introduced, statins have a relatively good adverse effect record and variety of statin therapy effects of on CVD – related morbidity and mortality have been investigated, including additional physiological effects. Consequently, clinical and epidemiological studies of their effect on atherosclerotic patients demonstrated cholesterol-independent pleiotropic effects such as improvement of endothelial function and of plaque stability, antioxidative properties, innate immunity modulation, reduced inflammation and thrombogenic response that might be due to anti-microbial activity [52], [53]. Further, statins enhance phagocytosis [54] and block the opsonized bacteria-triggered oxidative burst. Thus, an understanding that statin treatment may actually alleviate both hyperlipidemia and infections [55] is currently gaining hold. In concordance with the notion for enhancing the innate response, statins also upregulate the production of proinflammatory mediators elicited by FcγR stimulation [56].

The ultimate goal, identification of novel antimicrobial drug candidates for CVD and development of innovative approaches for diagnosis and treatment of atherosclerotic inflammations may thus be conceivable, making a significant step toward personalized medicine.

6. Conclusions

This brief outline of the microbial component of atherosclerosis and of the opportunities that arise with it underscores the importance of understanding all factors that affect whether the public health or clinical practice communities will adopt, successfully implement and sustain the lessons of this research, supported with medical literature references.

Each year, billions of tax dollars are spent on research and hundreds of billions are spent on service delivery and community health programs. However, relatively little is spent on how best to ensure that the lessons learned from research are relevant to, inform and improve the quality of health.

Consequently, the education of health care professionals has to cover the latest investigations concerning the link between infections and CVD. The success of treatment and even more importantly, of prevention of disease mandates relevant teaching curricula in the medical schools. The doctoral students, both in medical and dental degree programs, have to be knowledgeable regarding the impact of the infections on the systemic health. The formal training must include a discussion on the communication with the patient and how to reinforce the information so that a relevant compliance with the prescribed treatment is achieved. In particular, the link between oral infections and atherosclerotic inflammations deserves special attention.

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