Despite progress in both primary and secondary prevention, cardiovascular diseases (CVD) are still the largest group of ailments contributing to morbidity and mortality worldwide. Atherosclerotic changes, the primary pathological substrate for CVD, are closely related to hypercholesterolemia. Therefore, the treatment of hypercholesterolemia is a key therapeutic strategy for CVD management. Statins, as the gold standard in the treatment of hypercholesterolemia, have shown enhanced cardiac outcomes in many randomized clinical trials. However, often despite the maximum allowed and tolerated dosage of statins, we are not able to reach the target cholesterol levels, and thus patients persist at an increased cardiovascular risk. Recently, most of the large clinical studies in the field of preventive cardiology have focused on proprotein convertase subtilisin kexin type 9 (PCSK9) and its activity regulation. PCSK9 plays an essential role in the metabolism of LDL particles by inhibiting LDL receptor recirculation to the cell surface. Recent studies have shown that inhibition of PCSK9 by the administration of monoclonal antibodies is capable of significantly reducing LDL levels (up to an additional 60%) as well as reducing the incidence of CVD. However, this treatment procedure of administering the anti-PCSK9 antibodies, most frequently two times a month, has its limitations in terms of time, patient adherence, and nevertheless cost. Administration of active vaccination instead of passive immunization with anti-PCSK9 antibodies may be an effective way of controlling blood cholesterol levels. However, clinical data, as well as human testing, are still inadequate. This work aims to provide an overview of PCSK9 vaccines and their potential clinical benefit.

Keywords: Antibody; Antigen; Atherosclerosis; PCSK9; Vaccine
Methods of activation of immune system by vaccination to produce antibodies against self-antigens have been proven to be effective in several diseases such as cancer and hypertension.

Several preclinical studies have manifested successful induction of anti-PCSK9 antibodies production by special epitope/peptide vaccines, however clinical studies with sufficient results are still missing.

INTRODUCTION

Atherosclerosis is defined as a degenerative-inflammatory disease affecting the artery wall; however, it shows characteristics of an autoimmune reaction against apolipoprotein B particles, the best-known atherogenic agent. Many processes are involved in atherogenesis. One of the most significant factors in development is plasma cholesterol concentration. Several studies have been performed proving the direct correlation between the plasma cholesterol and LDL concentrations and the prevalence of major cardiovascular events and in general directly and indirectly cardiovascular disease-associated morbidity and mortality [1]. Other autoimmune characteristics of atherosclerosis include CD8 and CD4 helper cells accumulation in the atheroma as the reaction to oxidized LDL, ApoB, and other autoantigens [2]. The multiple factors involved in atherogenesis highlight the multiple possibilities of atherosclerotic process management.

Therapeutic strategies to reduce levels of LDL cholesterol (LDL-C) are the gold standard in the management of patients with cardiovascular diseases [3]. According to the World Health Organization can be up to 80% of cases of coronary artery disease (CAD) to prevent their occurrence, progression, and possible complications [4]. The results of several studies suggest that lipid-lowering statin (HMG-CoA reductase inhibitor) treatment is the most effective method. In the case of sufficiently indicated LDL-C lowering drugs and with proper adherence to the therapy, the risk of MACE could be reduced by up to 40%. However, statin therapy can be associated with negative pleiotropic effects such as myopathy or even rhabdomyolysis, and this can contribute on the fact that at least 20% of high-risk patients will not reach the target LDL-C levels with solo statin therapy either because of the lower dose as required or because of the statin therapy discontinuance [5].

The other key factor playing an important role could be bad adherence to statin therapy. Nowadays, most of the accessible lipid-lowering agents are administered once per day, and to reach the therapeutic approach of lowered CV risk, we need several years to manifest the desired effect. Those factors are highly participating in the adherence of the patients to statin therapy [6].

Persistent cardiovascular risk, as well as efforts to reach even lower target values of LDL and lower the CV risk, have further motivated the emergence and research of many other potential hypolipidemics.

The aim of this review article is to make an overview of PCSK9 inhibition with a focus on the possibilities of active vaccination inducing endogenous anti-PCSK9 antibodies production. This article is based on previously conducted studies and does not contain any studies with human participants or animals performed by any of the authors.

PCSK9 AND ITS INHIBITION

Proprotein convertase subtilisin/kexin type 9 (PCSK9) represents a new therapeutic target in the management of dyslipidemias. PCSK9 is a secretory protein, primarily produced by hepatocytes, but many other tissues and cell types have been proven to express the PCSK9 gene [7]. Mechanistically, after the initial secretion, PCSK9 circulates in the bloodstream and binds to the LDL-R predominantly on the surface of hepatocytes via binding to the growth factor-
like repeat A catalytic domain, which is responsible for the recirculation of the LDL-R to the cellular surface [8–10]). One LDL-R has the ability to transport 3000–6000 LDL particles from extracellular fluid typically, however after the binding of PCSK9 to the EGF-A domain, the normal recirculation of the LDL-R is blocked, and the lysosomal degradation is enhanced.

There are several naturally occurring vari-
cances in the PCSK9 activities. The most com-
mon variants are gain- or loss-of-function
PCSK9 gene mutations [11]. Several mendelian
randomizations and observational studies
[12, 13] have associated gain of function muta-
tions with autosomal dominant hypercholes-
terolemia and early onset of cardiovascular
diseases. However, loss-of-function mutations
were connected with the decrease of LDL-C by
up to 25% and a significant decrease of the
cardiovascular risk of carriers without signifi-
cant side effects [14].

Several preclinical and clinical studies have
focused on the inhibition of the PCSK9 activity
and validated safety of PCSK9 inhibition has
encouraged the development of novel PCSK9
inhibitors. There were three the most
notable large clinical studies with the clinical
impact changing the treatment of dyslipidemia
[15]. FOURIER (Further Cardiovascular Out-
comes Research With PCSK9 Inhibition in Sub-
jects With Elevated Risk) was the first completed
anti-PCSK9 double-blinded multicenter trial
with evolocumab, fully human antibody
including 27,564 high cardiovascular risk vol-
unteers [16]. Patients received either 140 mg of
evolocumab every 2 weeks or 420 mg every
4 weeks or placebo. Results showed that evolo-
cumab treatment decreased baseline LDL-C by
up to 59%. Relative risk reduction in compar-
ison with the placebo group was 15%. Risk of MI
was decreased by 27%, of stroke by 21%. From
the whole treated group, 0.3% of patients
exhibited the production of binding antibodies
against evolocumab, but not neutralizing.

The next study made with a fully humanized
antibody was an ODYSSEY Outcomes (Evalua-
tion of Cardiovascular Outcomes After an ACS
During Treatment With Alirocumab) trial that
enrolled 18,924 volunteers who had an acute
 coronary syndrome in last 12 months and who
did not reach the levels of LDL-C < 70 mg/dl
[17, 18]. The target LDL-C levels were between
25 and 50 mg/dl but not lower than 15 mg/dl.
In the case of low levels, therapy was discon-
tinued, which was the case in 8% of the vol-
unteers. Risk reduction in the ODYSSEY
Outcome trial was comparable to the FOURIER
trial; 15% reduction in primary endpoints (first
occurrence of CHD death, non-fatal MI, fatal/
nonfatal stroke, unstable angina pectoris
requiring the admission to the hospital). Con-
centrations of LDL-C decreased after 4 weeks by
up to 62% and was decreased by 44.7% after
4 years of the follow-up.

Despite great progress in the treatment of
dyslipidemias due to PCSK9 inhibitors, as well
as the low values that we managed to obtain
due to the combination of statins and PCSK9,
several disadvantages of this treatment persist.
A good example is a short half-life of antibodies
in vivo, which may reduce long-term adherence
to treatment (however, still higher as in the case
of statins): according to the studies, the number
of patients who discontinued or break the anti-
PCSK9 therapeutic scheme can be up to 8.5%. However, this number can be higher, because
most of the studies were done during the clini-
cal trials, which can mean that adherence can
be lower in real-life data and long-term follow-
ups [19]. The high cost of treatment may espe-
cially limit the full use of the clinical benefits of
these drugs. A typical regimen for PCSK9 anti-
obodies is twice a month, which, combined with
high monthly and annual costs, has limited the
widespread use of this category of drugs, despite
the recommendations in the guidelines.

Important competitor of passive, however
also active immunization are the new treatment
strategies with small interfering RNA, such as
inclisiran, which has significant results in phase
III clinical trials [20]. Inclisiran is a small
interfering RNA decreasing the hepatic synthesis
of PCSK9. Studies ORION-10 and ORION-11 have
enrolled patients with atherosclerotic cardio-
vascular disease, respectively patients with dis-
ease risk equivalent. These studies have shown
significant LDL cholesterol reduction by up to
50% with generally similar adverse effects in
comparison to the placebo arm. Considering
the two-times-per-year administration
scheme and lower cost than anti-PCSK9 antibodies, inclisiran could be a significantly competitive drug to active and passive immunization approaches.

The cheaper alternatives such as active vaccination inducing ideally permanent endogenous antibodies production could replace the methods of passive immunization with lower costs as well as the lower frequency of injections, and they could find a place in the management of dyslipidemia.

VACCINATION

In past decades, vaccination was the domain of infectious medicine as the tool to decrease the spread of infectious diseases, and in recent years, also viruses predisposing for cancer. The significant shift in the understanding of vaccines and active immunization happened in recent years. Recent clinical and preclinical studies aimed to target diseases such as Alzheimer’s [21], several types of cancer [22], hypertension [23], as well as diabetes mellitus [24], suggested the possibility to modify disease progression. Historically, the first vaccines (first generation) have been composed of fixed or live whole pathogens (measles, mumps, chickenpox, etc.). They contained weakened pathogens that can trigger long-term immunity response; however, in individuals with a weakened immunity, the vaccine can produce the actual disease. The inactivated vaccines, for example, are more stable by the inactivation create the need for additional booster vaccination to obtain the requested immunity. A further second generation of vaccines has used purified proteins obtained from the pathogenic organism (such as hepatitis B vaccine), and later the third generation has consisted of DNA plasmids/naked DNA (influenza, herpes; still in development) which are capable of providing by expression of the crucial protein of the pathogen. The latest generations, however, focus on short amino acid sequences—epitopes [25]. Experimental studies have also shown the possibility of displaying the antigens of HIV or rabies, measles on the attenuated bacteria.

The immune system consists of two big components empirically divided according to the memory aspects and the speed of the reaction: the innate system and the adaptive system. Both of the components interact and work together, although they represent a different approach of the body’s defenses against pathogens. The innate system (including component, lysozyme, collectins, interferons, etc.) is a first-line defense, though it is not specific to particular pathogenic agents. They are specific for generally occurring molecular patterns on the pathogens [26]. The important mechanism sensing of antigens and adjuvants by the innate immune system, which later properly activates the adaptive immune response. Under normal conditions, the innate immune system senses foreign antigens through pattern recognition receptors (PRR), for example the well-known Toll-like receptors (TLR), but also C-type lectin-like receptors, receptors sensing viruses as cytosolic RIG-I-like receptors and many others. These receptors sense various substances—pathogen-associated molecular patterns (PAMPs) as peptidoglycans, LPS (endotoxin), double-stranded DNA, and single-stranded RNA (viruses and many others) and they are not specific for a certain pathogen. They are located on the membrane of various cells such as dendritic cells (DC) but also other cells of the innate immune system [27]. After successful recognition of PAMPs, the activation of complement, phagocytes as well as cytokine release is triggered. During the infection, granulocytic cells containing neutrophils, eosinophils, and basophils are the first response. The innate immune system can eradicate the pathogen; however, it can also trigger an adaptive immune system consisting of B-cells (antibody-producing cells and antibodies) and T-cells. B-cells (produced in the bone marrow and later residing in lymph nodes, later maturing) can recognize the antigen without the antigen-presenting cells in their native form, and they can later present them to T-helper cells. Mostly those antigens are big polymeric molecules. Those T-independent antigens can activate B-cells directly, though the activation of B-cells without T-helper cells induces weaker immune response and memory. A long-lasting memory and
proper immune response are the main goals of immunization. By the binding of the antigen to the Fab region of the B cell receptor together with the cytokines produces by T-helper cells, this region starts the hypermutation and the maturation of B cells into the plasma cells which response by the production and corresponding antibodies to the recognized region of the antigen. By clonal expansion, the cells proliferate and differentiate to plasma cells, eventually to memory cells remaining in the lymph nodes. The adaptive immune response also consists of T-cells (two major types CD4, CD8), which function as cell-mediated immunity. They mature in the thymus and released to circulation. CD4 cells are essential in the activation of B-cells. Th1 CD4 cells help in cell-mediated immune response and Th2 cells trigger antibody-mediated immunity. CD8 cells are important for the cell-mediated immune response. In comparison with B-cells, T cells require processing and presentation of the antigen by APC to recognize it [28]. The processing of antigens (viral and tumor cells) by APC consists of attaching of antigen with MHC I protein to the surface. Extracellular antigens (bacteria, toxins, pathogens) are attached by MHC II proteins on the surface of APC. By APC activated T-cells later according to the major type CD4 or CD8 trigger B cells activation or contribute on cell-mediated immunity. The first step in the immunization process by vaccines is the recognition of epitopes on the antigens. The epitopes are the small regions on the antigens which are able to stimulate the immune system. The first defense line the innate immune system will bind or opsonize the agents, and later they will be processed by the APC and presented by MHC proteins on their surface for the activation of the adaptive immune response. APC cells will then activate the different defense response predominantly determined by MHC type [29]. To induce immune response either of regulatory as well as antibody inducing T cells the vaccines need to contain adjuvants which can be in the past widely used aluminum salts, TLR ligands, nanoparticle formations, squalene oils, or others [30].

However, in comparison with infectious diseases, the origin of the vaccine is the endogenous antigen. From infectious agents, derived vaccines target specific non-self-antigens. In contrast, the non-communicable disease vaccines target the self-protein antigens. For example, in the case of hypertension, the goal is to induce the adaptive immune response against angiotensin-II, or in the case of Alzheimer's disease epitopes of Aβ protein.

**PCSK9 ANTIBODY-INDUCING VACCINES**

The fundamental goal of PCSK9 vaccines is to induce a sufficient production of antibodies against PCSK9, which should neutralize the interaction between PCSK9 and LDL-R. Until this time, multiple mostly preclinical studies have proven that PCSK9 vaccines could have these properties. Several preclinical studies have been studying different types of PCSK9 vaccines [31]. Vaccination requires the proper activation of the immune system. However, stimulation of antibody responses directed against the antigen, in our case PCSK9, is limited by the mechanisms of B cell tolerance to the body's own antigens. Though this mechanism is often ineffective and antibody responses are readily elicited by vaccines, particularly those that activate T helper lymphocytes, which stimulates high activation of antibody production [32, 33]. In the case of the vaccination against self-proteins, several safety tests are required. A vaccine cannot induce the specific T cell response and to have cross-reactivity of antibodies with other endogenous proteins and antigens. During vaccine development, it is important to exclude peptide antigens which have the ability to lead to specific T cell responses. Anti-PCSK9 T-cell response can be accompanied by the lymphocyte infiltration to organs such as liver and destruction of PCSK9-expressing cells. However, to induce the high-affinity antibodies, there is a need for the follicular T helper cells during the B cells activation, maturation, B cells proliferation, as well as differentiation into plasma cells secreting antibodies as described in the previous section (Crotty, 2011).

The first approach in the development of an anti-PCSK9 vaccine was described by Fattori...
et al. [34]. This study focused on two immunization techniques in mice: DNA and protein-based vaccination. In the first set of experiments in the mentioned study, DNA plasmid expressing mouse PCSK9 under the human CMV promoter in conjugation with CpG oligonucleotide was injected and applied. However, in the first set, no antibody response was observed. In the second step, a xenogeneic approach with a plasmid-expressing human PCSK9 was planned. This approach was successful, and it has resulted in high titers of anti-hPCSK9 antibodies. In the third step, protein immunization was tested and compared with the previous DNA immunization protocol. Highly purified human PCSK9 together with CpG oligonucleotide was applied, resulting in strong anti-hPCSK9 antibody production. In both cases, high anti-hPCSK9 antibody titers resulted in a significant reduction of LDL by up to 40% after 2 weeks. After 42 days, the cholesterol reduction persisted on 28% [34].

EPITOPE VACCINES

An important method in the development of anti-PCSK9 vaccines is the epitope vaccines. They are the novel method in the development, where instead the whole cognate protein only short amino acid sequences of targeted proteins (epitopes) are used. They are characteristic with the more direct and potent immune response than traditional earlier generation vaccines [25]. Epitope strategy requires the exact knowledge of the sequence of amino acids in the immunogenic protein. Reverse vaccinology technique uses the knowledge coding DNA (gene) of the protein of interest, which is further processed and translated to the target protein. This protein is later presented to antigen-presenting cells (APC), which process it. Later, the T cell epitopes are proteolytically harvested and later by the MHC molecules they interact with the receptors of T cells. The epitopes can be identified by the cloning of the domain [35]. The AFFITOPE technology in detailed studied in Alzheimer’s vaccines aim to develop the non-self-epitopes, which are very similar to the original but not identical. They aim to overcome humoral and cellular autoimmunity. The limited length of peptides prevents T cell activation, and by this, the cellular autoimmune response and tissue damage [36].

One of the first studies that established the goal to test the peptide-based anti-PCSK9 vaccines was the study of Galabova et al. [37], which tested the AFFITOPE-based anti-PCSK9 active immunization. The goal of the study was to produce an active peptide-based vaccine and to demonstrate the possibilities of reducing total cholesterol as well as LDL levels. They used peptides (8–13 amino acids long) of the N-terminal PCSK9 region, which differed from the amino acids from the native sequence, but they were capable of inducing an immune response. The vaccines themselves consisted of PCSK9 conjugated to the keyhole limpet hemocyanin (KLH) as to the peptide carrier and of the aluminum adjuvant. Protein KLH is the most commonly used carrier for the conjugation of antigens, and it belongs to the group of proteins containing copper in non-heme hemocyanins [38]. After the immunization of Wistar rats in the mentioned study, significant generation of high-affine PCSK9 antibodies was found. Total cholesterol concentrations decreased by the mean value of 30%, and LDL by up to 50%. They have found that PCSK9 humoral immune response and antibody production persisted up to 1 year with the reduction of cholesterol concentrations, without the PCSK9-specific T cell induction, which emphasized that the selected peptides do not compose a T cell epitope [37]. Study [39] focused on the testing of the above-mentioned anti-PCSK9 vaccine AT04A on a humanized mouse model: ApoE*3 Leiden. CETP mice are a double transgenic and are ideal for preclinical tests because they have similar plasma lipid profiles as humans [40]. In preclinical testing of alirocumab on this mice model, a significant decrease of plasma lipids and atherosclerosis was observed [41]. Authors suggested the inhibition of PCSK9 by the vaccine produced antibodies could have the same effect. They have found out that AT04A vaccine induced the effective anti-PCK9 reaction and antibody production following by the significant plasma cholesterol, LDL reduction,
decreased vascular and overall inflammation and atherosclerosis [39].

OTHER APPROACHES

Several other teams have also studied the possibilities of anti-PCSK9 vaccines by other approaches and methods [42, 43]. Crossey et al. designed vaccines that use bacteriophage virus-like particles (QBeta-VLP) which exhibit on the surface PCSK9 peptides together with Freunds and aluminum adjuvant. The mechanism of displaying the self-antigen on the virus-like particle was tested in several studies focusing on vaccines in Alzheimer’s disease, hypertension, and cancer [32, 33, 44, 45]. This method has proved that it is capable of inducing a strong antibody response against the self-antigen. In this study, a significant reduction in total cholesterol up to 55% and other lipid parameters as well was observed. The results of the study corresponded with Alzheimer’s disease clinical trial with same QBetal-VLP vaccine, which showed the same antibody response and weak T cell response [46].

Another preclinical study of [47] testing anti-PCSK9 on apolipoprotein E deficient mouse used a similar approach as described by Galabaova et al. [37]. The vaccine itself consisted of short peptides consisting of 9–10 amino acids (C-terminal sequence of PCSK9 580–589 amino acids) conjugated with KLH as a carrier protein. The length of peptides allowed to prevent the binding to MHC 2 class and the activation PCSK9-specific T cell activation/response. The administration scheme was three times in the 2-week period. The peak of the antibody titer was reached after 6 weeks. Significant decrease in LDL-C and TC was observed.

A recent study by Montazi-Borojeni et al. [48] investigated the possibility of a nanoliposome-based delivery system. It was based on a similar method as an AFFiRiS group [37]. A peptide different from human or murine was used to be identified as foreign by the immune system and to overcome the self-tolerance. To increase the CD4+ T-cell response, epitope belonging to tetanus toxin was added and coupled with the PCSK9 fragment. The study has revealed that this approach initiated significantly lower response of IFNγ-producing CD8+ in comparison with the positive control group, while tetanus peptide can promote CD4+ T-cell response without safety concerns. The values of anti-PCSK9 antibodies were the highest in the eighth week and persisted up to the 48th week, however with almost five times lower values.

Nowadays, phase I. clinical study assessing safety, immunogenicity and LDLc -lowering activity of 2 PCSK9 targeting AFFITOPE vaccines in healthy subjects (AFF012), ClinicalTrials.gov Identifier: NCT02508896 is ongoing. However, the updates and the results had yet to be provided by the day of writing this article according to the marked schedule [49].

CONCLUSIONS

Currently, methods of active immunization directed against PCSK9 represent an interesting alternative to passive immunization by administering antibodies. Active immunization would have an interesting application in the presumed better adherence, significantly lower cost of treatment. However, there are currently no significant clinical studies investigating the possibility of active immunization against PCSK9 that could provide us with clinically suitable treatment in the near future. The possibilities of PCSK9 vaccinations look promising, however the monoclonal antibodies to PCSK9 were approved in Europe and in the US only in 2015 and there are needed a real-life data, how the long-term permanent PCSK9 inhibition is affecting other biological processes in the body and the safety of the therapy.

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Data Availability. Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

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