

# The Emerging Monoclonal Antibodies (mAbs) targeting proprotein convertase subtilisin/kexin type 9 (PCSK9) levels and Hypercholesterolemia

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## Abstract

**Background:** The guidelines on reducing cardiovascular disease (CVDs) risk have increasingly focused on lowering low density lipoprotein (LDL) cholesterol levels. Statin have become the drugs most commonly prescribed to decrease LDL and combating dyslipidemia. Although statin class of drug is extensively used, more than one half of cardiovascular events are not being prevented by these drugs. Monoclonal antibodies (mAbs) targeting proprotein convertase subtilisin/kexin type 9 (PCSK9) are a novel lipid lowering approach that recently gained fame.

**Aim:** We aim to shed light on serum PCSK9 level and its role in hypercholesterolemia. We objectively delineate the association of PCSK9 level with risk of hypercholesterolemia and cardiovascular diseases.

**Methods:** We have retrieved literature about PCSK9 level and its role in hypercholesterolemia. PCSK9 level is assessed by using certain devices such as Enzyme-linked immunosorbent assay (ELISA) kit. The serum level of PCSK9 (Standard reference range is 168-190 ng/ml) can be assayed by human PCSK9 ELISA kit.

### Conclusions and clinical significance:

1. Estimating the prevalence of hypercholesterolemia in relation to PCSK9 levels in any population will contribute to the current race against hypercholesterolemia.
2. The estimated differences in mean PCSK9 levels indicate the associated CVDs risks and expected overt hypercholesterolemia.
3. PCSK9 levels has direct impact on preventive management of CV events and future prospects of novel PCSK9 inhibitors entity anticipated to be launched in few months.
4. Shift in the strategies concerning CV risk in light of PCSK9 levels in the population.
5. New insights for early detection of risk categories based on a new evolving tool of PCSK9 assay and CV risk assessment scale

**Keywords:** Hypercholesterolemia; Proprotein convertase subtilisin/kexin type 9-PCSK9; Dyslipidemia and PCSK9, Cardiovascular diseases (CVDs) and PCSK9, LDL-cholesterol and PCSK9, Statin and LDL cholesterol.

## Introduction

The global average age for heart attack is 65 year. The guidelines on reducing cardiovascular disease (CVDs) risk have increasingly focused on lowering low density lipoprotein (LDL) cholesterol levels. Statin have become the drugs most commonly

prescribed to decrease LDL and combating dyslipidemia. Although statin class of drug is widely used, more than one half of cardiovascular events are not being prevented by these drugs. Monoclonal antibodies (mAbs) targeting proprotein convertase subtilisin/kexin type 9 (PCSK9) are a novel lipid lowering approach that recently gained fame.

Large outcome trial has proven the importance of reducing LDL cholesterol levels which in turn can reduce major cardiovascular mortality and morbidity, [1]. Consequently, practice guidelines always focusing on reducing LDL cholesterol as a fundamental means of reducing cardiovascular events, [2]. Majority of CVDs are polygenic, with both hereditary and environmental contributions, [3]. Approaches to identify the CVDs by human genome were poorly understood.

### Discovery of PCSK9 gene

In early 1970, anonymous scientists identified mutations affecting the LDL receptor reported to cause familial hypercholesterolemia, but no importance was given to this, because at that period hypercholesterolemia is a rare disease, [4]. It became evident in 1987, that mutations in the gene that codes for apolipoprotein B 100 (ApoB) prevents the LDL particles to bind to LDL receptor, [5]. This genetically distinct causes coined the cause of inherited hypercholesterolemia was due to defective ApoB. In the year 1999, French scientists identified a locus on chromosome one that was linked to inherited hypercholesterolemia, [6] and in May 2003, they pinpointed chromosome one-p32, [7]. These scientists highlighted two mutations in the coding region of the "PCSK9" gene that segregated with a hypercholesterolemia phenotype. Since then PCSK9 was reported, a targeted sequencing in a population with extreme values of LDL cholesterol identified variants in PCSK9 that occur in up to 3% of black/white American population, [8]. Such variants are associated with a low LDL cholesterol levels and a decreased risk of incident coronary artery disease. These variants estimated to be 2.6% in African descents, and results in comparatively low lipid levels and a reduced susceptibility to myocardial infarction, [9]. This major breakthrough has opened doors for a new attractive drug target that is known as PCSK9 drug inhibitors, hitherto two were FDA approved in last June 2015.

### Relationship between statin and PCSK9

PCSK9 is a key regulator of serum LDL-cholesterol levels. Statin is primary therapeutic options with proven safety and efficacy profile for hypercholesterolemia and has shown to reduce CVDs risk across all risk factors categories. Although statin class is widely used, more than one half of cardiovascular events are not being prevented by these drugs in addition to increased residual cardiovascular risk. Recent evidence suggests using statin will increase the PCSK9 levels while lowering total cholesterol, triglycerides and LDL levels. This suggests that the relationship between PCSK9 and LDL-serum levels are disrupted during statin treatment. Interestingly to note that, doubling the normal statin dose does not further reduce LDL levels in a dose-dependent manner, [9,10]. In addition, statin-induced increase in PCSK9 levels may inhibit a dose-dependent decrease in serum LDL levels, giving insight into a new hypothesis of why increasing dose fails to achieve a proportional decrease in LDL serum levels.

### Cardiovascular diseases in Gulf Region

In 2008, the WHO estimated a total of 17.3 million deaths contributed with cardiovascular diseases (CVD) and recognized

as the leading cause of death and disability, [11]. Rapid industrialization and urbanization in Arabian Gulf countries, Bahrain, Kuwait, Qatar, Oman, Saudi Arabia and the United Arab Emirates (UAE) has directly and indirectly shown a concomitant rise in the prevalence of major CVDs, [11]. The WHO estimated to the total non-communicable disease resulting in death in Gulf region in 2008, CVD accounts almost half of the deaths in Oman (49%) and Kuwait (46%). Furthermore, the rate of CVD deaths was also higher in Saudi Arabia (42%), the UAE (38%), Bahrain (32%), and Qatar (23%), respectively, [12]. These may be due to change in lifestyle by adopting sedentary lifestyle such as increased consumption of poor quality foods. As a consequences the rate of CVD and associated factors like diabetes, obesity, stress and tobacco use were also more than double compared to past ten years, [13].

The Gulf Registry of Acute Coronary Events (Gulf RACE), a Gulf Heart Association project conducted in 64 hospitals across the Gulf region reported a high prevalence of hypertension (49%), diabetes (40%), smoking (38%), dyslipidemia (32%) and obesity (27%) among acute coronary syndrome (ACS) patients in Gulf countries. The highest rates of the "smoking" as a risk factor were identified in the UAE and Kuwait populations (males 47% versus females 5%). The prevalence of CVD risk factors was higher in females than males, including hypertension (70% vs. 43%), dyslipidemia (44% vs. 28%), and diabetes (55% vs. 36%), [14].

### Example of cardiovascular risks in the UAE population

The Dubai Health Authority (DHA), [15] conducted Dubai Household Health survey among 5000 Emiratis in 2011 to identify the prevalence of CVD risk factors. This survey highlighted that, one in four individuals in Dubai have CVD risk factors and strongly advocated to cut down unhealthy eating habits as well as to reduce tobacco use. The key finding of DHA survey in Emirates includes:

1. The prevalence of CVD risk factors among men is twice (28.3%) than women (14.5%).
2. The prevalence rate was significantly higher among non-educated (39%) compared with university and above education (21%).
3. The significant among of CVD risk factors at age 60 and above is 44%, is 7 times higher than of a young individuals (18-24 age group).
4. Individuals with higher income groups are 1.3 times more likely to develop CVD risk factors compared to middle-income groups.
5. 63% of Emirates males aged above 60 and 63.5% females are under CVD risks.

Similarly in 2013, the Health Authority of Abu Dhabi (HAAD) conducted a national screening program for various health risk factors program called "Weqaya" (Prevention). This identified 71% of the Emiratis had at least one CVD risk factor. High rates of chronic diseases related to lifestyle such as obesity, diabetes,

and CVDs were observed. The CVD deaths accounts for 36.7% of all death cases in the UAE, 2013 and these rates are set to be increased further as the younger ages. Further, one-fourth of the hospital visiting patients were diabetic patients (12% outpatients and 13% inpatients) and 18.6% (7.5% outpatients and 11.1% in patients) were CVDs are among the top most non-communicable disease n UAE, [16]. It is estimated that the number of CVD related hospital admission will be more than twice by 2022 (28%) than that of 2012 (12%).

## Rational

Many controversies arise in statin clinical use. There is an emerging high evidence for the role of PCSK9 levels in management of hypercholesterolemia. To join the race against high LDL levels as a source of increasing CVDs, we anticipated that revealing the levels of PCSK9 in any population will provide important data to the prevention and management of high CVDs risk population such as the Emiratis.

## The question

We have hypothesized that studying PCSK9 levels in any population will further reveal the levels of LDL-cholesterol in general population associated with PCSK9 levels. This will facilitate understanding the associated risk factors, behavioral risks and incidence of cardiovascular risks in our population in relation with PCSK9 levels. This can be the gate to identify the associations between PCSK9 and predisposing factors to hypercholesterolemia and contribute to further prevention of major CVDs risks.

## Aim

We aim to shed light on serum PCSK9 level and its role in hypercholesterolemia. We objectively delineate the association of PCSK9 level with risk of hypercholesterolemia and cardiovascular diseases.

## Methods and materials

### PCSK9 levels

PCSK9 level will be assessed by using **Enzyme-linked immuno sorbent assay (ELISA)** kit. The serum level of PCSK9 (reference range= 168-190 ng/ml) will be assayed by human PCSK9 ELISA kit in all subjects. High levels of PCSK9 lead to higher plasma levels of LDL cholesterol, whereas low levels of PCSK9 lead to lower plasma LDL cholesterol levels.

### Human PCSK9 ELISA Kit

Human PCSK9 ELISA Kit employs the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody specific for PCSK9 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and the immobilized antibody binds any PCSK9 present. After washing away any unbound substances, an HRP conjugated monoclonal antibody specific for PCSK9 will be added to the wells. Following a wash to remove any unbound antibody HRP conjugate, the remaining conjugate will be allowed to react with the substrate H<sub>2</sub>O<sub>2</sub>-tetramethylbenzidine. The reaction will stop by addition of

acidic solution and absorbance of the resulting yellow product is measured at 450 nm using microplate spectrophotometer. The absorbance is proportional to the concentration of PCSK9. A standard curve is constructed by plotting absorbance values versus PCSK9 concentrations of calibrators, and concentrations of unknown samples are determined using this standard curve.

The human PCSK9 ELISA kit constitutes of:

1. **One microplate with 96 wells** (12 strips of 8 wells) in a foil and wells are coated with anti-PCSK9 polyclonal antibody as a capture antibody.
2. **10X wash buffer** (100 mL bottle) containing 2% Tween -20.
3. **Dilution Buffer:** One bottle (60 mL) containing 60 mL of 1X buffer, use for reconstitution of PCSK9 standard and sample dilution.
4. **PCSK9 Standard:** One vial (50 ng) of lyophilized recombinant PCSK9.
5. **HRP conjugated Detection Antibody:** One vial containing 12 mL of HRP (horseradish peroxidase) conjugated anti-PCSK9 monoclonal antibody.
6. **Substrate Reagent:** One bottle containing 20 mL of the chromogenic substrate, tetra-methylbenzidine (TBN).
7. **Stop Solution:** One bottle containing 20mL of 1 N H<sub>2</sub>SO<sub>4</sub>.

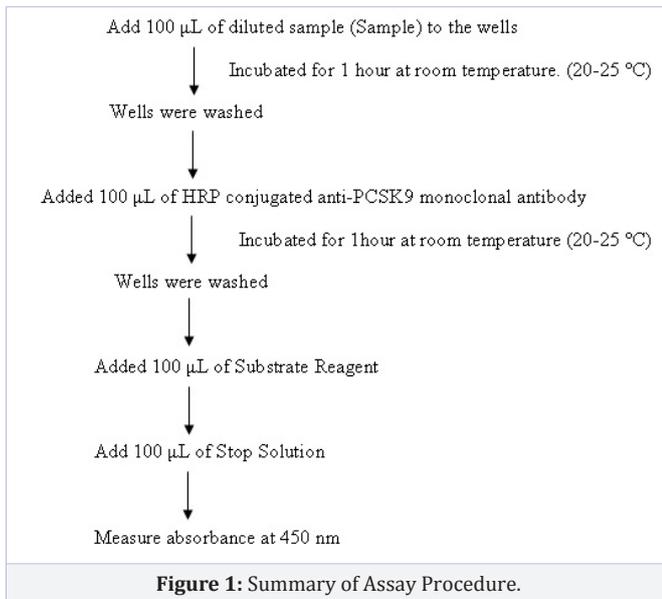
### Human PCSK9 Assay Procedure

**Sample Preparation:** The collected blood sample (5ml) will be allowed to clot for 60 ± 30 minutes in serum separation tube. The obtained sample will be centrifuged at 4°C for 10 minutes at 1000 x g. Separated serum will be immediately used for the assay or stored at -70°C for extended period. The obtained serum sample will be diluted to 100-fold dilution.

**Standard Preparation:** 100 mL of the 10X Wash Buffer will be diluted to 900 mL of deionized water and mixed well for 60 minutes. Reconstituted PCSK9 Stand with 0.5 mL of ddH<sub>2</sub>O to a concentration of 100 ng/mL. This will be referred and Labeled as "Master Standard of PCSK9" which acts as a Blank solution (0 ng/mL concentration). A 60 µL blank solution (standard 1) will be diluted with 540 µL Dilution Buffer to produce 10 ng/mL concentration. Similar methods with different volume of standards with different dilution using Dilution Buffer will be repeated up to 7 standard concentrations, Figure 1.

**Criteria of diagnosis:** Hypercholesterolemia is defined according to National Cholesterol Education Program (NCEP) Adult Treatment Panel III risk categories and Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III)

**Outcome measures:** The primary outcome will be development of hypercholesterolemia (increased PCSK9 levels). The secondary outcome will be to estimate the risk of CVD in relation with PCSK9 levels.



### How to perform prospective cohort design for PCSK9 estimation in a given population

In any cohort study the risk of disease (hypercholesterolemia), the incidence rate and/or relative risks are estimated. Non-cases should be enrolled from a well-defined population, current exposure status (at  $t_0$ ) will be determined, and the onset of disease will be observed in the subjects over time. Disease status at  $t_1$  will be compared to exposure status at  $t_0$ . The data will be displayed as Table 1.

The measures of disease frequency (hypercholesterolemia) and effect or association can be calculated from the above data as follows:

**1. Incidence Density (Incidence Rate):**

Among exposed:  $A/T_{Exposed}$

Among non-exposed:  $C/T_{Nonexposed}$

**2. Incidence Density Ratio (Risk Ratios; Relative Risk)**

$(A/T_{Exposed}) / (C/T_{Nonexposed})$

**3. Attributable Risk**

$(A/T_{Exposed}) - (C/T_{Nonexposed})$

### Cons and pros for prospective cohort study

**Cons:** Expensive, long-term study, need large sample size, more potential for loss-to-follow-up. **Pros:** calculates incidence rate, risk, and relative risk, good for rare exposure, good for multiple outcomes, less potential for recall bias, potentially more strong for causal investigations, possibly generalizable, allows examination of natural course of disease, survival

### The cohort design

The characteristic feature of cohort design is that it will be able to identify subjects at a point in time when they do not have

the outcome of interest (high PCSK9) and compares the incidence of this outcome among our groups of exposed and unexposed (or less exposed) subjects. Refer the groups being compared as exposure cohorts. Outcome status should be established at least twice. Ensure that cohort will not have the defined outcome at the beginning of the observation period and the cohort will be examined again to determine whether or not the outcome subsequently developed, i.e., the incidence in each of the exposure groups. Recruit subjects, and collect baseline exposure data on all subjects, before any of the subjects have developed any of the outcomes of interest. The subjects will be followed into the future in order to record the development of any of the defined outcomes. The follow up will be conducted in person with interviews, physical examinations, and laboratory-human PCSK9 ELISA kit (imaging tests if necessary). The data collected from the cohort over time will be used to answer many questions relevant to the PCSK9 levels (confounders/associations with other lipid profile levels) and will test many possible determinants, even factors that not considered in the protocol at the time protocol originally conceived, but with close relevancy and association to PCSK9 levels. Determine baseline exposure status before disease events occur which gives the prospective cohort an important advantage in reducing bias.

After collection of baseline information, subjects to be followed “longitudinally,” i.e. over a period of time, for 2 to 5years. This enables to know when follow up began, when subjects became diseased, when they became lost to follow up and whether their exposure status changed during follow up. Individual data based on details for each subject to be computed and compared in incidence rates for each of exposure groups. The incidence rate will be calculated by computing the disease free observation time for each subject, adding up the disease-free observation times for the entire group, and then dividing this into the number of events.

### Statistical analysis

Absolute risk, relative risk and odds ratio (95% Confidence interval) will be demonstrated. The differences between normal and abnormal mean levels of all variables will be compared by contingency chi squared test for discrete variables and student t test for continuous variables. The differences in PCSK9 mean values between cohort cases and controls will be assessed with student t test (paired sample).

### Conclusions and clinical significance

1. Estimating the prevalence of hypercholesterolemia in

**Table 1:** Estimation of incidence, relative risk and attributable risk in the cohort.

	Case (Number)	Non-cases (Unnecessary if T known)	Total Exposure (Person*Time)
Exposed	A	B	$T_{Exposed}$
Not-exposed	C	D	$T_{Non-exposed}$
	$T_{cases}$	$T_{non-cases}$	Total

relation to PCSK9 levels in any population will contribute to the current race against hypercholesterolemia.

2. The estimated differences in mean PCSK9 levels indicate the associated CVDs risks and expected overt hypercholesterolemia.
3. PCSK9 levels has direct impact on preventive management of CV events and future prospects of novel PCSK9 inhibitors entity anticipated to be launched in few months.
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