Approximately 33% of the U.S. population is diagnosed with high cholesterol due to high levels of low density lipoprotein (LDL) cholesterol in the body. LDL is the primary culprit in a type of cardiovascular disease (atherosclerosis, which literally means hardening of the arteries), because it initiates plaque deposition. Cardiovascular disease has remained the top cause of death in the United States for several decades primarily because of poor diet and unhealthy lifestyles. Currently, Statins are the most widely used form of treatment for high LDL and work by decreasing hepatocyte cholesterol levels. Unfortunately, there are many adverse effects associated with statins. These include, but are not limited to, muscle pain and weakness, which can further cause problems with digestion. Additionally, studies have shown that rhabdomyolysis, heart failure, ALS and Parkinson’s disease can be correlated to the use of statin medications. This can decrease the patient’s quality of life to where they wish to be removed from the treatment altogether. To overcome this limitation, this grant will create a device to measure the levels of LDL and disperse inhibitors systemically to lower LDL levels.

Specifically, the device will emit an antibody called Proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitor. The liver is responsible for the removal of LDL cholesterol through the use of LDL cell-surface receptors. The PCSK9 enzyme binds to the LDL receptors and inhibits the liver’s ability to sense LDL and as such to remove LDL; therefore, using an antibody to bind to PCSK9 could increase LDL removal through the liver. Not only will this device measure the patient's cholesterol, but it will also assess these levels and disperse the inhibitor accordingly.

**Aim 1:** Measure levels of LDL with low dose over time and compare to large, biweekly dose in mouse models.  
**Hypothesis:** Current methodology of PCSK9 delivery involves a single dose every 2 to 4 weeks. A more stable control of LDL content can be achieved with smaller and more incremental dosages of the antibody.  
**Method:** Using small dosages over time, measure the amount of LDL in the bloodstream and compare it to the current treatment method of PCSK9 dosing. Assess these two types of doses with Apolipoprotein E knockout (ApoeE KO) mice and determine which is more effective and healthy for the patient. Two groups of 10 mice will each be studied over the same 6-month trial period. Levels of LDL will be measured through NMR to assess which method is preferred. While NMR is not the easiest or cheapest way to measure LDL, it is the most selective and will afford highly accurate results.

**Aim 2:** Create a device with a piezoelectric biosensor that can accurately measure LDL levels produce from the body cells (hepatocytes responsible for 20%) and food consumption. Then automatically disperse the PCSK9 antibody upon recognition of elevated LDL levels as needed.  
**Hypothesis:** Piezoelectric biosensors will allow for a small, low cost, a small volume sample, high sensitivity, high specificity, rapid response, and reproducible device. Piezoelectric biosensors have been shown to accurately measure LDL levels to within 0.1 to 150 mg/dl, which will allow for accurate monitoring of LDL patient levels.  
**Method:** Piezoelectric biosensors work through the use of quartz crystals making an ultra-sensitive weighing device that utilizes the mechanical resonance of piezoelectric single-crystalline quartz. The device will be assessed through the accuracy of NMR measurements, an industry standard. The device will be safety attached to the user upper arm and an IV interface with the patient’s basilica vane and a subcutaneous inject needle will be interfaced with the user’s upper arm. The buffer and regeneration solution will need to be periodically replaced. Data from the device is stored and uploaded to the patient's primary care physician during monthly checkups.

**Aim 3:** Implement device in the use of LDL control of mouse models using smaller incremental dosages.  
**Hypothesis:** The device will stably maintain low levels of LDL in the mouse specimens via the delivery of small incremental dosages of the PCSK9 inhibitor.  
**Method:** Two groups of 10 mice each will be studied over the same 6-month trial period. Members of one group will each individually be connected to a piezoelectric device that will monitor the LDL levels of the mice and administer PCSK9 inhibitor accordingly. A second group of mice will be connected to a piezoelectric device that periodically measures LDL at the same time interval of the first device but delivers a larger dose of PCSK9 inhibitor of the mice at an interval of two weeks between doses. Following the trials, the LDL of the mice will be monitored for an additional three months to observe any changes in their natural LDL levels.

The long term goal of this proposal is the development of a consumer device that can continuously measure LDL levels and reduce them via the introduction of the PCSK9 inhibitor. The continuous measurement will allow for greater insight into the factors that impact an individual’s LDL levels. Additionally, we hypothesize that
this continuous incremental treatment will offer greater reduction of cardiovascular risk through the continuous maintenance of low LDL levels rather than through the larger pulse dosages typically delivered bi-weekly

**Significance**

The American Heart Association reported that cardiovascular diseases are the leading cause of death globally with greater than 17.3 million deaths a year. It is predicted that this mortality rate will increase to 23.6 million deaths a year by 2030. In the United States, over 2 million people will experience a cardiovascular medical event, such as chest pain, stroke, blocked arteries, etc. This is both an issue in terms of patient health and health cost. Cardiovascular health problems are responsible for 25% of inpatient hospital care costs. Research has shown that reducing low-density lipoprotein cholesterol decreases the risk for cardiovascular disease. This proposed device will be able to help a significant amount of people because about 33% of the population, in the United States alone, has LDL cholesterol over the recommended amount (130-150 mg/dL).

LDL is introduced to the body in two ways: secreted by the liver or through food intake. The liver synthesizes very low-density lipoproteins (VLDLs) with triglycerides, cholesterol, and apolipoproteins. The VLDLs are then secreted and become LDLs after undergoing lipolysis within the plasma. The LDL receptor in the liver breaks down the LDL molecule and therefore determines the amount of LDL cholesterol in the body by controlling the rate at which it is produced and broken down. LDL is important for the body because it plays roles in cholesterol delivery to cells and synthesizing hormones, however too much can be bad for the body. Regardless of diet, the amount of LDL produced from person to person can vary. Furthermore, there are genetic conditions that increase LDL production.

Currently there are three types of treatments for high LDL that work by decreasing hepatocyte cholesterol levels. The treatments are statins, bile acid sequestrants, and cholesterol absorption inhibitors where statins are the most widely used as of now and cholesterol absorption inhibitors are the most recent development for a high cholesterol treatment. Unfortunately, there are many adverse effects associated with statins. These include, but are not limited to, muscle pain and weakness, which can further cause problems with digestion. Additionally, studies have shown that rhabdomyolysis, heart failure, ALS and Parkinson’s disease can be correlated to use of statin medications.

**Figure 1:** Schematic of PCSK9 antibody (mAb) decreasing LDL cholesterol and inhibiting LDL receptor and PCSK9 interaction. The mAb (pink) prevents PCSK9 (dark yellow) binding, which increases the amount of LDL receptors coming out of the cell (dark blue) onto the cell surface and the amount of LDL cholesterol (light blue and yellow) taken up by the cell.

Statin treatments function through their inhibition of the enzyme, HMG-CoA reductase (HCR). This enzyme is responsible for cholesterol production in the liver. Statins serve as a competitive inhibitor of this enzyme by replacing the HCR that exists in the liver in order to slow the production of cholesterol. As a response to this slowed reduction, the liver cells increase production of LDL receptors. These receptors subsequently relocate to the liver cell membranes and bind to passing LDL, bringing it into the liver for digestion.
The two major side effects that result from statin treatments are liver failure and skeletal muscle damage. Liver failure likely results from statins completely inhibiting the liver's natural production of cholesterol or by fostering unsafe elevation in liver enzyme concentrations. This has caused motivation for the use of inhibitors like PCSK9. This treatment, Alirocumab (Praluent – Sanofi/Regeneron), was FDA approved and released in October 2015. Its side effects are flu symptoms, muscle pain, urinary tract infections, and diarrhea. Current treatment involves a self-injection of 75-150 mg every 2 weeks and can cause allergic reactions or irritation at the injection site.

Due to the nature of the PCSK9 antibody, the proposed device will be able to successfully increase LDL removal from the liver and prevent PCSK9 from binding to the hepatic LDL receptors. The proposed device will be able to eliminate doctoral visits by sending the LDL screen results to the patient's local healthcare physician on a weekly basis. We hypothesize that the continuous measurement function will allow further research on factors that impact individual's LDL levels, lower cardiovascular risk, and lower adverse effects with smaller dosages. This device will also increase their standard of life because it presents an automated solution to current methods of treatment.

For this grant proposal, the focus of experimentation will be the creation and functionality of the device, specifically for the purpose of releasing the PCSK9 antibody when LDL cholesterol levels are above 150 mg/dL. There will be a sequence of experiments that test the accuracy of the biosensors measuring LDL levels and the effectiveness of the necessary release of PCSK9 antibody.

INNOVATION

1. Continuous, small dosing schedule

Current method of treatment involves the dosing of the patient once every two or four weeks. Greater reduction in LDL levels is associated with more frequent and smaller dosing. Once every two-weeks with 150 mg results in a 67.9% decrease, while a once every four-week dose of 300 mg only reduced LDL levels by 42.53%. This study seeks to adjust this method of dosing to a continuously monitored level and administered drug such that the reduction can be controlled and adjusted more easily. Other forms of treatment, such as statins, are poorly tolerated. One study states that between 10% and 25% of people are not tolerant to statins in doses that would impact their LDL levels. Additionally, PCSK9 has been shown to be as effective to statins when attempting to reduce LDL levels. Statin reduction is usually between 30% and 50% and PCSK9 reduction is between 40% and 70%.

In line implementation of the proposed device will allow for the administration of small levels of PCSK9. The storage of such inhibitor is appropriate at room temperature for the time frame, about a month. After a month, patients would need to replace the inhibitor cartridge as a refill will be necessary. This method of administration has never been used before and allows the patient to avoid the need to go to the doctor’s office once or twice a month to get injections. If the patient needs to remove the device for any reason, this can easily be maneuvered by unscrewing the connector between the intravenous needle and the device.

2. Accurate real time assessment on LDL levels

Prior to this study, methods to measure LDL levels included calculation, NMR, ultracentrifugation, and testing with compounds. Accurately testing these levels is one of the most important aspects in assessing and administering treatment. Piezoelectric biosensors have been theorized as capable of measuring accurate LDL levels. Putting an actual device in application has yet to be explored thoroughly. Through the use of this piezoelectric device, the hypothesis is that we will be able to accurately measure and track LDL levels in the body.

**Figure 2:** A simplified visualization of the intended final device. The device (orange) would be strapped to the patient's upper arm. The device would interface with the patient's blood flow in order to monitor LDL levels via intravenous needles at sites (B) and (C) in this image. The subcutaneous release of PCSK9 inhibitor would occur at site (A) in this image in response to elevated LDL levels.
Piezoelectric devices are optimal due to their small size, high sensitivity and simple nature. Current methods are known to be time consuming and to require technical skills. With the correct implication of a piezoelectric device, anyone with high cholesterol could use the device and accurately track their own LDL levels.

**APPRAOCH**

**AIM 1: Measure levels of LDL with low dose over time and compare to large, biweekly dose in mouse models.**  
**Hypothesis:** Current methodology of PCSK9 delivery involves a single dose every 2 to 4 weeks. A more stable control of LDL content can be achieved with smaller and more incremental dosages of the antibody.

**1A. Eliminate natural response to LDL levels in mice via knockout (KO) of Apolipoprotein E (ApoE).**

ApoE KO mice have a deficiency in Apolipoprotein E (ApoE). Since ApoE is responsible for the packaging and thus maintenance of cholesterol levels, using mice deficient in this eliminates the body’s natural control of LDL. Thus, the use of PCSK9 will be an alternative method to produce HDL, and thus control LDL levels in the subjects (Figure 3).

**1B. Preliminary measurements upon KO treated mice, KO non-treated mice, and wild type mice to confirm manipulation of LDL levels via PCSK9.**

In order to fabricate antibodies specific for PCSK9, we will use Protean software to analyze the correct amino acid sequence. The desired amino acid sequence is 500-520 and 583-603 of the C57BL-6 mouse form. We are using recombinant adenovirus particles to deliver the PCSK9 to the mouse. The control group utilizes an empty adenovirus, therefore eliminating the PCSK9 delivery. The adenoviruses are delivered subcutaneously. Upon collection of blood samples, LDL is separated from non-LDL plasma via centrifugation, and then analyzed via fast protein liquid chromatography (FPLC). Depending on measurements of LDL and HDL, success of PCSK9 delivery will be verified. These results will be compared to wild mice.

**1C. Compare multiple low doses to biweekly doses of PCSK9 effects on HDL versus LDL levels.**

The group of mice receiving the low doses will be administered 0.43 μg of PCSK9 adenovirus in PBS every four hours. The dose of PCSK9 adenovirus for the incremental mice was calculated to take into consideration the human dose delivered biweekly and to spread an equivalent mg/kg mouse dosage over the course of two weeks. Biweekly groups will be administered 36.14 μg of the PCSK9 adenovirus as determined by the calculation previously detailed. Blood samples will be collected two hours following injections to compare LDL and HDL levels. This experiment will run for a total of 1 month. Other than blood tests, optical imaging with fluorescents can be used as a non-invasive form to quantify the presence of LDL cholesterol.
**AIM 2:** Create a device with a piezoelectric biosensor that can accurately measure LDL levels and disperse the PCSK9 antibody upon recognition of elevated LDL levels as needed.

**Hypothesis:** Piezoelectric biosensors will allow for a small, low cost, small volume sample, high sensitivity, high specificity, rapid response, and reproducible device. Piezoelectric biosensors have been shown to accurately measure LDL levels to within 0.1 to 150 mg/dl, which will allow for accurate monitoring of LDL patient levels.

**2A. Develop a piezoelectric biosensor to measure LDL levels.**

Monitoring cholesterol levels in the body can be a challenging endeavor due to the dynamic nature of the hormone in the body and the difficulty in distinguishing the classes of cholesterol in the blood stream. Cholesterol is found in four classes; chylomicrons, very low-density lipoproteins, low-density lipoproteins, and high-density lipoproteins, with low-density lipoproteins being the class most closely associated with heart disease. The problem in measuring the amount of LDL in the blood stream is that the other classes interfere with the measurement of the others; this often leads to indirect methods of calculation. Currently it’s know that LDL can be precipitated out of blood plasma with dextran sulfate (DS)\(^\text{16}\). Thus, aim 2 focuses on a dynamic measuring system that can accurately measure LDL (Figure 4).

Piezoelectric biosensors allow for a small, low cost, small volume sample, high sensitivity, high specificity, rapid response, and reproducible device. Therefore, they are the best option in designing a portable wearable device to monitor dynamic LDL levels. With the added treatment of DS, the sensors become highly specific for LDL.

![Figure 4: Schematic of a Portable LDL monitoring device based around piezoelectrics. A pump controls the flow of buffer and TSM regeneration solution to the flow cell. An IV connected to the user supplies the user's blood to the sample loop of 21.8 µL. The flow cell consists of 2.54 cm quartz disks oscillating at 5 MHz. The TSM sensor is connected to an electronic oscillator circuit with AGC capabilities. A processor chip records, monitors, and controls the device. Recorder data is store and sent to primary care physician.](image)

The device uses DS covalently bound to a gold coated thickness shear-mode sensor (TSM) as a hydrogel. The TSM is a quartz crystal microbalance (piezoelectric) that measures mass difference flown over the shear sensor. They work by measuring the shift in the resonant frequency of the particle adhering to the TSM, with an oscillator circuit using automatic gain control (AGC). The AGC output a voltage that is proportional to the gain need to keep the TSM at a pre-set oscillation disturbed by adhering particles, thus the voltage is proportional the total dissipation and as such the total frequency shift \(\Delta F_T\). Through calibration and careful measurements of blood flows, the flowing equation can be derived to determine the frequency shift from dissipative mechanisms.

\[
\Delta F_D: \quad \Delta F_D = \frac{K_1}{K_1} (\Delta AGC + 473) - 712
\]

**Equation 1.** With \(K\) – calibration constants
The frequency shift due to mass loading, $\Delta F_M$, can then be calculated by $\Delta F_M = \Delta F_T - \Delta F_D$ which corresponds to the mass of the film on the TSM or specifically with DS treatment LDL. This method has a sensitivity of 0.018 mg/Hz/cm² and can actually measure form lows of 0.1 to high of about 200 mg/dL. As the LDL particles adhere to the TSM, a regeneration solution needs to be flowed over periodically to remove the particles. This solution consisted of PBS buffer devoid of magnesium ions with 10% isopropyl alcohol.

Due to the sensitive nature of piezoelectrics careful care will also need to be conducted in response to normal activity of the user. The TSM will have to be secured so that outside noise and any physical disturbance of the device will not affect the measurement of LDL levels. This will be tested and calibrated against repeated robotically driven mechanical test to deal with these issues appropriately and most cost effectively. The solutions will be in the form of extra support for the TSM to cancel out noise and physical disturbance and programming smart protocols to deal and recognize disturbances and then neglect those effects.

2B. Calibrate device measurements against samples of known LDL concentration.

It is essential to ensure not only the precision but also the accuracy of the piezoelectric biosensor. This will be achieved by reading replicate samples, of known LDL concentration, with both the piezoelectric biosensor and conventional NMR spectroscopy. This will allow for an accurate calibration of the piezoelectric biosensor readings in the event there is an error. Sample concentrations will range from 0.1 to 200 mg/dL in order to ensure the device’s efficacy across the spectrum of concentrations encountered in vivo.

2C. Preliminary tests on the ergonomics of the arm fasted device

Due to the necessity of an IV needed for the device to function properly, care will be needed in the ergonomics of the device. The arm fastening will have to be secure enough to not cause pressure on the IV, while at the same time not causing discomfort to the arm of the user. Robotically driven mechanical tests will again be conducted to show the force stress on the IV. Current ergonomics of the arm fastened iPhone apparatuses will be used as a based design to then be modified based on preliminary results. Final design specs will not be conducted here, but will be accomplished when the project has moved into clinical trials.

Care will also be taken into account with the removal of the device from the user. As the IV is hard to remove and install, it would not be feasible for the user to remove the IV each time the device needs to be removed. Easily removable ports on the device’s end will be installed to aid in removal by the user.

AIM 3: Implement device in the use of LDL control of mouse models using smaller incremental dosages.

Hypothesis: The device will stably maintain low levels of LDL in the mice specimens via the delivery of small incremental dosages of the PCSK9 inhibitor.

3A. Device interface with animal.

The device will interface with blood flow through the use of two small needles. Both needles will have a small adhesive patch to hold them in place. One needle will be intravenous (IV) and will constantly collect blood for analysis of LDL levels with the piezoelectric biosensor. The second needle will release PCSK9 subcutaneously when the device indicates elevated levels of LDL. While PCSK9 acts locally at the liver, it will be delivered there by normal blood flow.

3B. Observe and quantify device performance.

In order to prove the viability of the device, it will be implemented to maintain low levels of LDL in the mice. One group of 10 ApoE KO mice will be connected to the device and receive 0.43 µg of the PCSK9 inhibitor when the LDL levels are indicated to be above 150 mg/dL by the piezoelectric device. The device will constantly monitor the LDL levels of the mice over time and record the response of the LDL levels after each dose. The device will be programmed to wait 4 hours prior to administering a second dose. If, at four hours, the LDL levels of the mice are observed to be higher than 150 mg/dL then another dose will be administered. This process will repeat over the course of a 6-month study. A second group of 10 mice will be studied in parallel
with the previous group, receiving 36.14 µg biweekly doses of the PCSK9 inhibitor. These doses will be administered subcutaneously and manually. These mice will also, however, be connected to a piezo electric device in order to monitor their LDL levels over time and in response to the large biweekly bulk dose of PCSK9 inhibitor.

3C. Evaluate device safety.

The safety of Praluent was evaluated in nine placebo-controlled trials that included 2135 patients exposed for 6 months and 1999 patients exposed for more than 1 year. The note-worthy side effects noted in the studies were an increase in viral infections of 0.2%, an increase in injection site reactions of 2.1%, and an increase in influenza of 1.1% \(^{16}\). In order to ensure patient safety and minimize the risks associated with the injection sites of our device, we will monitor the health of the rats used in this study. Additionally, the adhesive pads that hold the needles into the patient will be coated with acyclovir and hydrocortisone in order to minimize the risk of infection \(^{17}\).

Figure 5: (A) A visual representation of the experiment to be performed with two groups of mice. One group will receive smaller incremental doses of the PCSK9 inhibitor once every 4 hours and the other will receive the conventional biweekly bulk dose. Both groups of mice will have their LDL levels monitored by the piezoelectric device. (B) Provides a qualitative representation of the expectations and benefits of incremental delivery, with a more consistent control of low LDL levels.
References

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