From,
Dr Rajesh Kumar T.
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Date:16-09-2022

To,
Dr. Sadhana Srivastava
Scientist F, IPR Unit
Indian Council of Medical Research
Department of Health Research
(Ministry of Health & Family Welfare)
New Delhi-110 029

Subject: *Requesting for filing patent of invention emanated from Ph.D research work of ICMR SRF fellow, Ms Kavya Sugur (5/3/8/23/ITR-F/2019-ITR)*

Dear Dr Sadhana Srivastava,

The Ph.D. work carried out by my student Ms. Kavya Sugur, who is a recipient of the ICMR-SRF fellowship (5/3/8/23/ITR-F/2019-ITR) has resulted in a invention, which can be patented.

The present invention relates to a new method to monitor oxidative stress in type 2 diabetic patients by measuring patient's serum lipid peroxidation potential. In particular, this invention is very useful in assessing the risk of diabetic patients to develop cardiovascular disease such as coronary artery disease, stroke, peripheral artery disease and their management. The tentative title of the invention is **"Method for cardiovascular risk assessment in diabetic patients by performing oxidative stress test and uses thereof".**

As instructed by you, we have enclosed 'New invention Report' duly signed by Head of our institute. We request ICMR to file the patent related to this proposed invention. Please feel free to contact us for any additional information required pertaining to this invention and request.

Thank you,

Sincerely,

Associate Professor
Dept. of Biochemistry
JSS Medical College
MYSORE-570 015

HOD : Professor & Head Dept. of Biochemistry, J.S.S. MEDICAL COLLEGE Sri Shivarathreeshwara Nagar, Old Bannimantap, MYSORE-570 015

Principal: PRINCIPAL J.S.S. Medical College Mysore-570 015

Enclosures: i. New Invention Report; ii. Details of the invention; iii E-mail correspondence

NEW INVENTION REPORT

NAME OF THE LABORATORY: **Dept of Biochemistry, JSS Medical College, JSS Academy of Higher Education & Research,SS Nagar, Mysuru-570015**
Inventors: **Dr Rajesh Kumar Thimmulappa** and **Ms. Kavya Sugur (ICMR-SRF)**

SECTION – A

The proposal for patenting titled: _ **Method for cardiovascular risk assessment in diabetic patients by performing oxidative stress test and uses thereof.**

Sl. No.	Details	TICK	
1.	Is the invention new (Novel) and useful	**Yes**_x____	**No**_____
2.	Relates to a process, method or manner of manufacture	**Yes**_x____	**No**_____
3.	Relates to a machine, apparatus or other article.	**Yes**_____	**No**_x____
4.	Relates to a substance produced by manufacture	**Yes**_____	**No**_x____
5.	Is a new (Novel) & useful improvement of 2, 3 or 4 above	**Yes**_x____	**No**_____
6.	Relates to a substance intended for use or capable of being used as a food	**Yes**_____	**No**_x____
7.	Relates to a substance intended for use or capable of being used as medicine / drug / vaccine	**Yes**_____	**No**_x____
8.	Relates to a substance prepared by chemical process (including alloys, optical glass, semi-conductors & intermetallic compounds)	**Yes**_____	**No**_x____
9.	Relates to atomic energy	**Yes**_____	**No**_x____
10.	Is a mere discovery of a scientific principle or the formulation of an abstract theory.	**Yes**_____	**No**_x____
11.	Is a mere discovery of a new property of known substance	**Yes**_____	**No**_x____
12.	Is a new use for a known substance	**Yes**_____	**No**_x____
13.	Is a mere use of a known process/method	**Yes**_____	**No**_x____
14.	Is a mere use of a known machine/apparatus/device	**Yes**_____	**No**_x____
15.	Relates to a composition/formulation	**Yes**_____	**No**_x____
16.	Relates to a mere admixture of ingredients	**Yes**_____	**No**_x____
17.	Relates to a synergistic mixture of ingredients	**Yes**_____	**No**_x____
18.	Is a mere arrangement or rearrangement or duplication of known devices each functioning independently of one another in a known way.	**Yes**_____	**No**_x____
19.	Relates to a method or process of testing	**Yes**_____	**No**_x____
20	Relates to a method of agriculture or horticulture	**Yes**_____	**No**_x____
21.	Relates to a process for the medicinal, surgical, curative, prophylactic or other treatment of humans, animals, or plants.	**Yes**_x____	**No**_____

SECTION – B

Sl. No.	Details	TICK	
1.	Has the matter covered in the patent proposal been made public (including publication or presentation at seminar etc.)	Yes_____	No__x__
2.	Is the laboratory level work complete	Yes__x__	No_____
3.	Is a brief write up enclosed (with drawings, if any)	Yes__x__	No_____
4.	Has the patent proposal been checked for novelty and inventive steps(s), as compared the prior art knowledge available throughout the world.	Yes__x__	No_____
5.	Has research been done of prior art literature and information available in the public domain.	Yes__x___	No____
6.	Has a prior art patent search (covering Indian and Foreign patents) been done	Yes__x__	No_____
7.	Has the relevant prior-art details as obtained in items 5 & 6 above (reference, brief description and drawbacks) been included in the patent write up	Yes__x__	No_____
8.	Has the novelty & inventive step(s) of the invention been clearly brought out in the write-up for patent proposal.	Yes__x__	No_____
9.	Has the approval of the Director/ Competent Authority been taken for this patent proposal.	Yes__x__	No_____
10.	Is this patent proposal to be filed as a joint patent application (i.e. the joint assignees being ICMR and another organization).	Yes_____	No__x__

We hereby answered all the questions in both Section – A items 1 to 21 and Section – B items 1 to 10 after careful consideration.

We hereby confirm that to the best of our knowledge and belief this patent proposal is novel (i.e. having novelty on a worldwide basis) and is our original work.

NAME OF INVENTOR(S) **SIGNATURE(S)**

1. Rajesh Kumar Thimmulappa

2. Kavya Sugur

NAME & SIGNATURE OF HEAD OF DIVISION _____

Dr. M N SUMA

Professor & Head
Dept. of Biochemistry,
J.S.S. MEDICAL COLLEGE
JSS Academy of Higher Education & Research
MYSORE-570 015

LABORATORY NAME: Dept of Biochemistry, JSS Medical College, JSS Academy of Higher Education & Research, Mysuru- 570015

FORWARDING BY DIRECTOR/ OFFICER IN-CHARGE _____

PRINCIPAL
J.S.S. Medical College
Mysore-570 015.

Title: Method for cardiovascular risk assessment in diabeticpatients by performing oxidative stress test and uses thereof.

Inventors: Rajesh Kumar Thimmulappa., Ph.D and Kavya Sugur., M.Sc

Name of Laboratory: Dept of Biochemistry, JSS Medical College, JSS Academy of Higher Education & Research, SS Nagara, Mysore 570015

1. Abstract

The present invention relates to a new method to monitor resilience (resistance) to oxidative stress in patients by measuring the patient's serum lipid peroxidation potential. In particular, this invention is very useful in assessing the risk of diabetic patients developing cardiovascular diseases such as coronary artery disease (CAD), stroke, and peripheral artery disease and their management. The method may also be used for assessing the risk of microvascular complications such as retinopathy, nephropathy, and neuropathy among diabetic subjects. Furthermore, the method may also be useful in the management of preeclampsia and other prenatal complications in pregnant women with gestational Diabetes Mellitus. The method may also be used for assessing the antioxidant activity of therapeutics in patients and population-based studies.

2. Field of Invention:

The invention relates to a rapid, low-cost, point-of-care, sensitive, and faster method to evaluate or monitor oxidative stress status in patients by measuring serum lipid peroxidation potential for risk assessment and risk management. In particular, the method is very useful in the risk assessment of diabetic patients for future development of cardiovascular disease and microvascular complications and risk management by guiding effective preventive and treatment regimens.

3. Background of the invention:

Compared to non-diabetic general population, the risk of developing the first myocardial infarction (MI) in Type-2 diabetic patients is >20% within 10 years of developing diabetes (Haffner, Lehto et al. 1998). Death among diabetic patients is 2-times more than non- diabetics due to MI (Kannel and McGee 1979, Haffner, Lehto et al. 1998). Patients with Type 1 diabetes and Type 2 diabetes have 8-10 times and 2-4 times elevated risk of developing cardiovascular diseases (coronary artery disease, stroke, peripheral artery disease) respectively (Bertoluci and Rocha 2017). Around 8% of the world's population is affected by diabetes (Saeedi, Petersohn et al. 2019). Despite these alarming facts, there is no clinical laboratory test to identify high-risk diabetics who are more likely to develop CVD (Leon and Maddox 2015).

Currently, at clinic, the traditional risk factors such as high triglycerides, low HDL-cholesterol and high LDL-cholesterol, hypertension, and hyperglycemia are used for risk estimation and identifying high-risk diabetic patients for aggressive therapeutic management for the prevention of CVD development. Although hyperlipidemia elevates the CVD risk, several studies have reported around 50% of all future vascular events are occurring in patients with mild hyperlipidemia or normal LDL cholesterol (Nelson 2013). Hence, therapeutic management of diabetic patients to prevent or reduce CVD development is abig challenge in clinics (Bruemmer and Nissen 2020). There is an unmet need for independent biochemical biomarkers to characterize and identify high-risk diabetic subjects for developing CVD.

Oxidative stress state characterized by excess reactive oxygen species (ROS) and low antioxidant defenses plays a crucial role in the initiation and progression of atherosclerosis in the coronary artery and aorta, carotid artery, and peripheral arteries, which ultimately leads to coronary artery disease, stroke, and peripheral artery diseases respectively. Chronic systemic oxidative stress contributes to endothelial injury/ dysfunction and oxidation of LDL resulting in the generation of oxidized-LDL (Ox-LDL). Ox- LDL in turn propagates endothelial injury, inflammation, and formation of foamy macrophages. These initial pathogenic events driven by oxidative stress, if not halted or mitigated, eventually lead to the development of atherosclerosis. This paradigm was confirmed by following preclinical studies: i) ablation of NADPH oxidase 1 (NOX1) that produces superoxide either by genetic deletion or chemical inhibitor attenuated development of atherosclerosis in mice model (Gray, Marco et al. 2013); ii) over-expression of antioxidant enzymes, superoxide dismutase and catalase in mice mitigated atherosclerosis development (Yang, Roberts et al. 2004); iii) Neutralization of Ox-LDL by specific antibody mitigated the development of atherosclerosis in mice (Que, Hung et al. 2018).

Because Ox-LDL plays a causal role in the development of atherosclerosis, it is well characterized as a risk marker for CAD in patients (Taleb, Witztum et al. 2011). Circulatory levels of Ox-LDL are elevated in patients with CAD (Tsimikas, Brilakis et al. 2005, Tsimikas, Aikawa et al. 2007, Tsimikas, Willeit et al. 2012); correlate with the severity of the disease and predict future CVD events and mortality(Tsimikas, Kiechl et al. 2006). Systemic ROS and Ox-LDL are detoxified by plasma enzymatic and non- enzymatic antioxidants. The major non-enzymatic antioxidants in plasma include vitamin E,Vitamin C, glutathione, protein thiols, and albumin, which function as scavengers of ROS and neutralize its toxic effects. The key antioxidant enzymes in plasma that detoxify ROSand mitigate Ox-LDL generation includes superoxide dismutase, catalase, glutathione peroxidase, and paraoxonase. Elevated levels of circulatory ox-LDL in patients would suggest high ROS levels and poor antioxidant defenses.

Despite the robust evidence implicating Ox-LDL in CVD, to date, Ox-LDL measurements have not been implemented for routine clinical practice for screening and risk assessment in diabetic patients (Vasan 2006, Nayor, Brown et al. 2021). This is attributed to several reasons. First, circulatory Ox-LDL is measured by the enzyme-linked immunosorbent assay (ELISA) method that relies on using detecting antibodies. Currently, three monoclonal detector antibodies i.e 4E6, DLH3, or E06 are widely used by researchers and each one is directed against a different epitope of Ox-LDL (Nayor, Brown et al. 2021) . 4E6 binds to epitopes consisting of 60 modified lysine residues whereas DLH3 or E06 binds to specific oxidized phosphocholine species linked to LDL. There is no consensus on which antibody is most sensitive, specific, and suitable. Second, the oxidation of LDL produces many oxidized lipid species. ELISA method detects only single oxidized lipid species but not all the oxidized lipid species generated on LDL. Further, this method detects oxidized lipids species that are linked to LDL but not the detached fragment oxidized lipids released in circulation. Third, the half-life of Ox- LDL in the blood is short and removed from circulation by reticuloendothelial systems. Fourth, to date, most studies have reported elevated ox-LDL in patients with CAD compared to subjects with no disease. But its clinical value in identifying high-risk diabetic patients without clinical disease has not been well evaluated. Lastly, the assay method is not standardized for easy use at the clinic. Several parameters such as the method for capturing equal amounts of LDL particles onto microtiter plate, the method for normalization

of output values to LDL particle concentration, reference standard and data representation vary from study to study. Hence, there is an unmet need for newer methods/technology for assessing systemic oxidative stress levels at the clinic for risk assessment and risk management among diabetic patients before the development of CVD.

4. Summary of Invention

The invention herein is a method for risk assessment and risk management of diabeticpatients. The method involves performing an 'oxidative stress test' in the isolated serum ofadiabetic patient and monitoring serum lipid peroxidation (LPO) potential, which informshow resilient is the patient to oxidative stress.

5. Details of Invention

To overcome prior art problems described above, we have developed simple, sensitive, low-cost, faster, and easy for implementation and interpretation at the clinic for risk assessment and risk management among diabetic patients before the development of CVD. Our invention involves performing an 'oxidative stress test' in the isolated serum of diabetic patients to estimate the resilience of patients to combat/resist oxidative stress. The method requires three basic components- a) fasting serum or plasma isolated from the patient or healthy subjects, b) a free radical initiator, 2,2-Azobis(2-amidinopropane)dihydrochloride (AAPH), which generates peroxyl radicals (free radical) at a constant rate of $1.36x10^{-6}$/sec by thermal decomposition at 37^{o}C; c) a florescent lipid peroxidation reporter dye, C11-BODIPY 581/591. C11-BODIPY 581/591reacts with oxy-radical species generated during lipid peroxidation but not hydroperoxides per se (Drummen, Liebergen et al. 2002). BODIPY-C11 is also insensitive to superoxide and transition metals per se (Drummen, Liebergen et al. 2002). Oxidation of C11-BODIPY is accompanied by a decrease in red fluorescence at Ex 580/Em 595nm (C11-BODIPY^{red}) and a concomitantly increase in green fluorescence at Ex 500/Em 520nm (C11-BODIPY^{oxi}). The assay was performed by dispensing diluted serum into each well of a 96-well microtiter plate and then adding AAPH (final concentration, 25-100mM) or an equal volume of phosphate buffer saline (PBS) and C11- BODIPY (final concentration 10µM). The reaction was initiated by placing the assay plate in a microplate spectrofluorometer reader (Perkin Elmer) set at ex/em: 500/520, 37^{o}C. The fluorescence readings were recorded every 10 min for a total of 120 min. We assessed the serum lipid peroxidation (LPO) potential by calculating the area under the curve(AUC) and lag time. AUC was calculated for oxidized C11-BODIPY (C11-BODIPY^{oxi}) by applying the trapezoid rule AUC=((ΔX)*((Y_2+Y_1)/2)-Baseline) for 20-30min, 30-40min, 40- 50min, 50-60min, where X=time (min), Y=RFU. Further, the summation of all four time period readings of AUC was represented as Serum LPO (AUC from 20-60min). Lag time was calculated by using a GraphPad prism for C11-BODIPY^{oxi} oxidized readings of BODIPY-C11 of the reaction. Two tangent lines were drawn, one was straight to the induction phase and the other was straight to the propagation phase of the curve. The point of intersection of these tangent lines indicated the initiation point of the reaction and is termed the 'Lag time' of the reaction. The lag time corresponds to a delay in the time of serum LPO.

Stimulation of serum with AAPH produced a time- and concentration-dependent increase in serum LPO as indicated by an increase in C11- BODIPY^{oxi} (**Figure 1A**) and the same at 60min (**Figure 1B**). At 25mM of AAPH, the total serum LPO levels were moderately higher than baseline (no AAPH). However, at both 50mM and 100mM AAPH, there was a time- and dose-dependent increase in C11-BODIPYoxi AUC (**Figure 1C**).

The length of lag time was significantly lower in serum exposed to 100mM AAPH compared to 50mM AAPH, suggesting early induction of LPO (**Figure 1D**). Exogenous spiking of water-soluble vitamin E (Trolox) and Vitamin C to serum from healthy subjects before AAPH addition, significantly inhibited LPO and increased the lag time (**Figure 2A-D**). Similarly, treatment with various natural antioxidant molecules curcumin, quercetin, N-acetyl cysteine, and GSH and with a metal quencher, EDTA significantly lowered total serum LPO and increased lag time following 100 mM of AAPH incubation (**Figure 2E-F**).

Next, to evaluate if serum LPO potential assay stratifies high-risk diabetic subjects from normal healthy subjects, we recruited diabetic patients (n=69) with non-significant clinical CAD (DNSC) (gensini score <20) and non-diabetic subjects (n=69) with non-significant CAD from JSS hospital after the angiogram. Basal clinical characteristics are presented in **Table 1**. We observed that the C11-BODIPY^{oxi} AUC was significantly elevated in diabetic groups (DNSC) when compared to nondiabetic groups (NDNSC) (**Figure 3A**). Subsequently, a statistically significant decrease in the Lag time was detected in the DNSC group compared to the NDNSC group, which suggested early induction of LPO in diabetic subjects (**Figure 3B**). Correlation analysis showed a positive association between LDL-C and AUC (**Figure 3C**). We subsequently compared the LPO potential between subjects based on who was taking or not taking atorvastatin medication. Independent of statin consumption, we found a significant increase in C11-BODIPY^{oxi} AUC in diabetics compared to non-diabetic subjects (**Figure 3D).** We also correlated LPO potential with tobacco smoking status. The C11-BODIPY^{oxi} AUC levels were significantly elevated in smokers' NDNSC and smokers' DNSC compared to the respective non-smoker's group (**Figure 3E)**, which further validated and proved the utility of our assay.

To see the impact of glycemic control on LPO potential, we performed a Pearson correlation analysis between serum LPO (AUC) vs HbA1c in the DNSC group alone and NDNSC merged with the DNSC group. We observed a significant positive association between HbA1c vs serum LPO potential in combined groups (NDNSC+DNSC) (**Figure 4A-B**). This association between AUC vs HbA1c reveals the subjects with poor glycemic control have higher serum LPO potential. We also analyzed the serum LPO potential vs. fasting blood sugar levels. Serum LPO levels in DNSC subjects with fasting blood sugar of 100-200 and >200mg/dl were significantly higher compared to the NDNSC group (**Figure 4D**).

6. Direct area/areas of application and use for which the invention has been made.

a. The invention is useful in evaluating the risk of diabetic patients (type 1 and type 2) to developing CVD such as coronary artery disease (CAD), stroke, and peripheral artery disease. For example, If the patient is chronically associated with greater oxidative stress (above normal range), the risk of developing CVD increases.
b. The invention is useful for evaluating the risk of diabetic patients developing microvascular complications such as diabetic retinopathy, neuropathy, and nephropathy.

c. The invention is useful for guiding better therapeutic management of diabetic patients and their prognosis. For example, if the patient is associated with greater oxidative stress, the physician can prescribe lifestyle changes, medication, and other holistic approaches to lower oxidative stress and more frequent clinic visits for check-ups
d. The invention is useful to evaluate the antioxidant activity of potential medications in clinical trials.
e. The invention is useful to evaluate the risk of developing preeclampsia and other maternal and neonatal adverse complications in pregnant women, who develop gestational diabetes.
f. The invention is useful for determining oxidative stress by monitoring serum lipid peroxidation(LPO) for preclinical and clinical research

7. Problems of the existing technology that our invention proposes to solve

a. There are no sensitive, simple, and low-cost routine clinical laboratory tests to identify high-risk diabetics who are more likely to develop CVD. At the clinic, the traditional CVD risk factors such as dyslipidemia (total cholesterol, LDL-C, HDL-C, triglycerides), hypertension, and obesity are less powered to identify the high-risk diabetic subject, and hence therapeutic management of diabetic patients to reduce CVD development is a big challenge.
b. Both preclinical and clinical research has demonstrated that patients with CAD have elevated levels of baseline serum levels of oxidized low-density lipoprotein (Ox-LDL) by immunoassay. The levels of ox-LDL in patients with CAD correlated with the presence and severity of CAD and also predicted MI. However, the method is not sensitive to differentiate between diabetic patients with or without CAD disease. Although significant, the values of serum ox-LDL in a patient without disease were quite overlapping with patients with diseases.

8. How your invention proposes to overcome the above-noted problems?

Our new method is simple, cost-effective, and can be implemented as a point of care testto evaluate oxidative stress state in patients; second, this new method was sensitive to identifying high-risk diabetic patients without disease as compared to non-diabetics.

9. Brief description of drawings

1) Optimization of serum LPO potential assay using C11-BODIPY dye

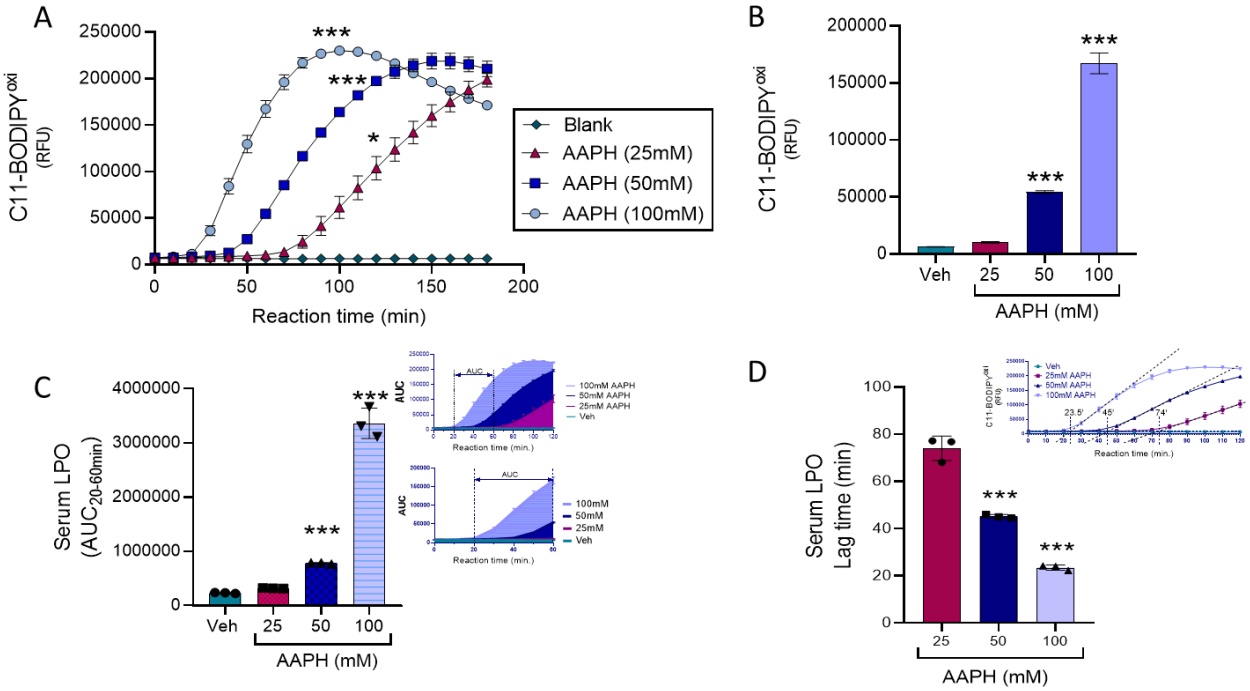

Figure 1. Optimization of LPO assay in healthy serum using C11-BODIPY probe. A) Fluorometric oxidation kinetics of C11-BODIPY in healthy serum treated with AAPH for 180 min. B) Levels of only C11-BODIPY^{oxi} in serum at 60 min of AAPH treatment. C) Area under the curve of oxidized readings from 20-60min post AAPH exposure is presented as Serum LPO ($AUC_{20-60min}$). (D) Differential levels of Lag time in the oxidized readings of AAPH-induced serum. (*=P<0.05,**=P<0.01 and ***=P<0.001).

2) Basal levels of patient demographic characteristics

Parameters	NDNSC (n=68)	DNSC (n=69)
Age (Year)	57.70 ± 12.94	55.85 ± 9.69
Gensini score	8.91 ± 9.51	10.92 ± 6.70
Sex (male/female)	44/24	40/29
Glucose (mg/dL)	101.45 ± 31.74	181.08 ± 82.60 ***
HbA1c (%)	5.61 ± 0.52	8.52 ± 2.22 ***
Lipid levels (mg/dL)		
Total cholesterol (mg/dL)	149.08 ± 45.27	158.83 ± 45.86
HDL-C (mg/dL)	37.92 ± 13.28	34.54 ± 11.12
LDL-C (mg/dL)	88.13 ± 37.58	94.34 ± 36.63
TGL-C (mg/dL)	121.98 ± 59.97	185.42 ± 128.2 ***
VLDL-C (mg/dL)	24.47 ± 12.17	37.03 ± 25.78 **
Lipoprotein(a) (mg/dL)	23.27 ± 22.81	22±20.73

Table 1. Baseline characteristics and lipid profile of the participants in the study group. Data for continuous variables as mean±SD. *P<0.05,**P<0.01,***P<0.001, compared with NDNSC group.

3) Exogenous antioxidants and metal quenchers abrogate serum LPO potential

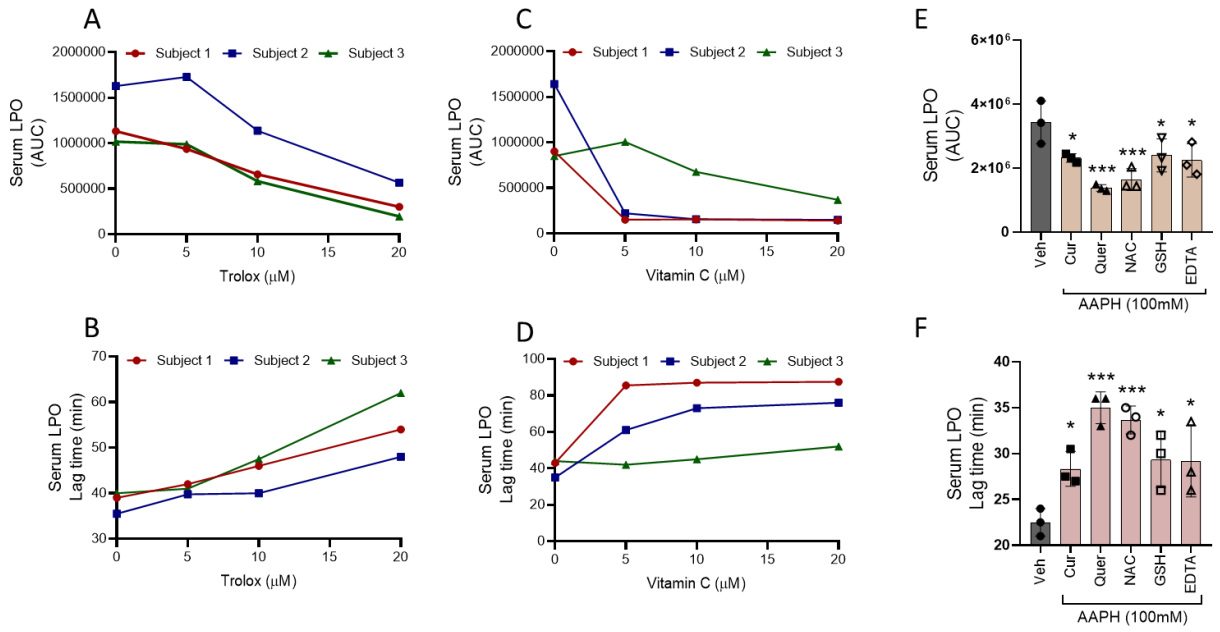

Figure 2. The reduction of serum LPO potential when spiked with vitamin E, Vitamin C, and, other antioxidants. Serum isolated from healthy subjects (n=3) were spiked with indicated antioxidants (vitamin E (Trolox), vitamin C, Curcumin (10μM), Quercetin (5μM), N-acetyl cysteine (50μM), Glutathione (100μM) and EDTA (100μM)) prior to adding AAPH and C11-BODIPY. Levels of serum LPO (AUC) (A, C & E) and Lag time (B, D & F) were calculated.
*P<0.05,**P<0.01 and ***P<0.001 compared to vehicle.

4) LPO potential in diabetic and non-diabetic patients with or without significant CAD

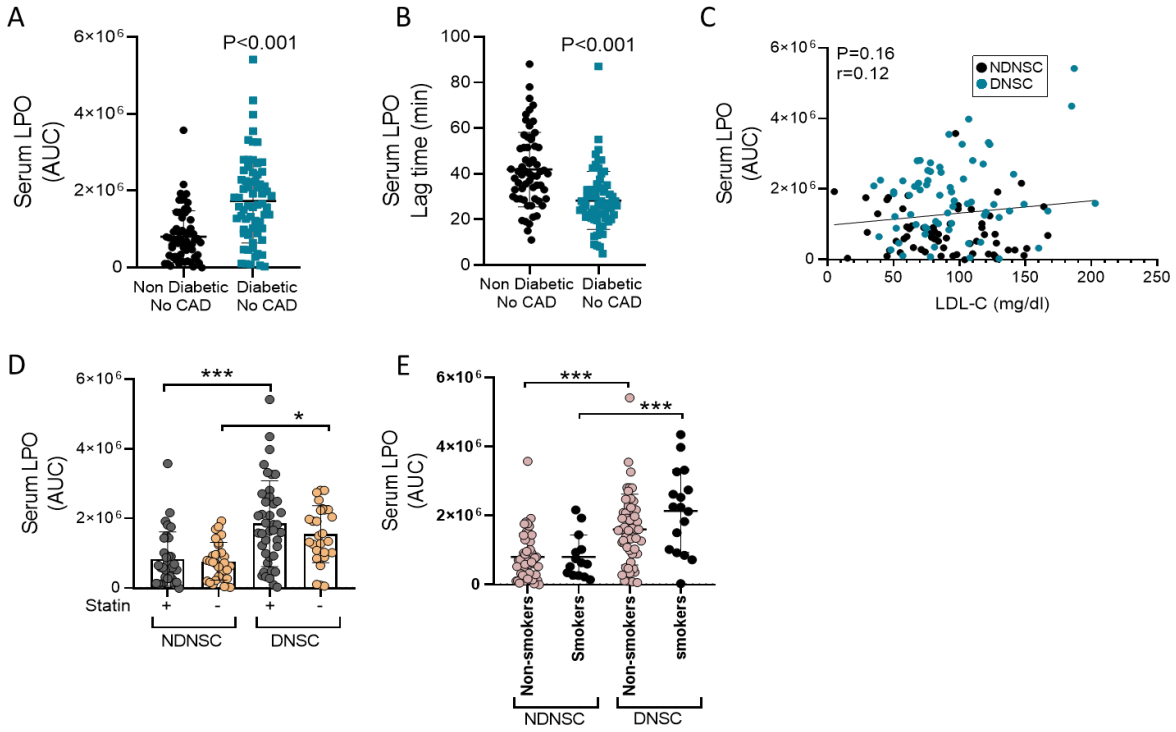

Figure 3. LPO potential in diabetic and non-diabetic patients with or without significant CAD. (A-B) Serum LPO Levels (AUC $_{20\text{-}60min}$) in Non-diabetic (NDNSC) and Diabetic subjects without CAD (DNSC). (B) Lag time in NDNSC and DNSC groups. (C) Pearson correlation in the levels of Serum LPO (AUC) vs. LDL-C levels in NDNSC and DNSC. (D) Comparison of serum LPO (AUC) levels between NDNSC and DNSC groups with subjects who were on a statin and without statin. (E) Comparison of Serum LPO (AUC) levels between smoker and non-smoker subjects within NDNSC and DNSC groups. *P<0.05,**P<0.01 and ***P<0.001.

5. Impact of glycemic control (poor or normal) on LPO potential in DNSC compared to NDNSC

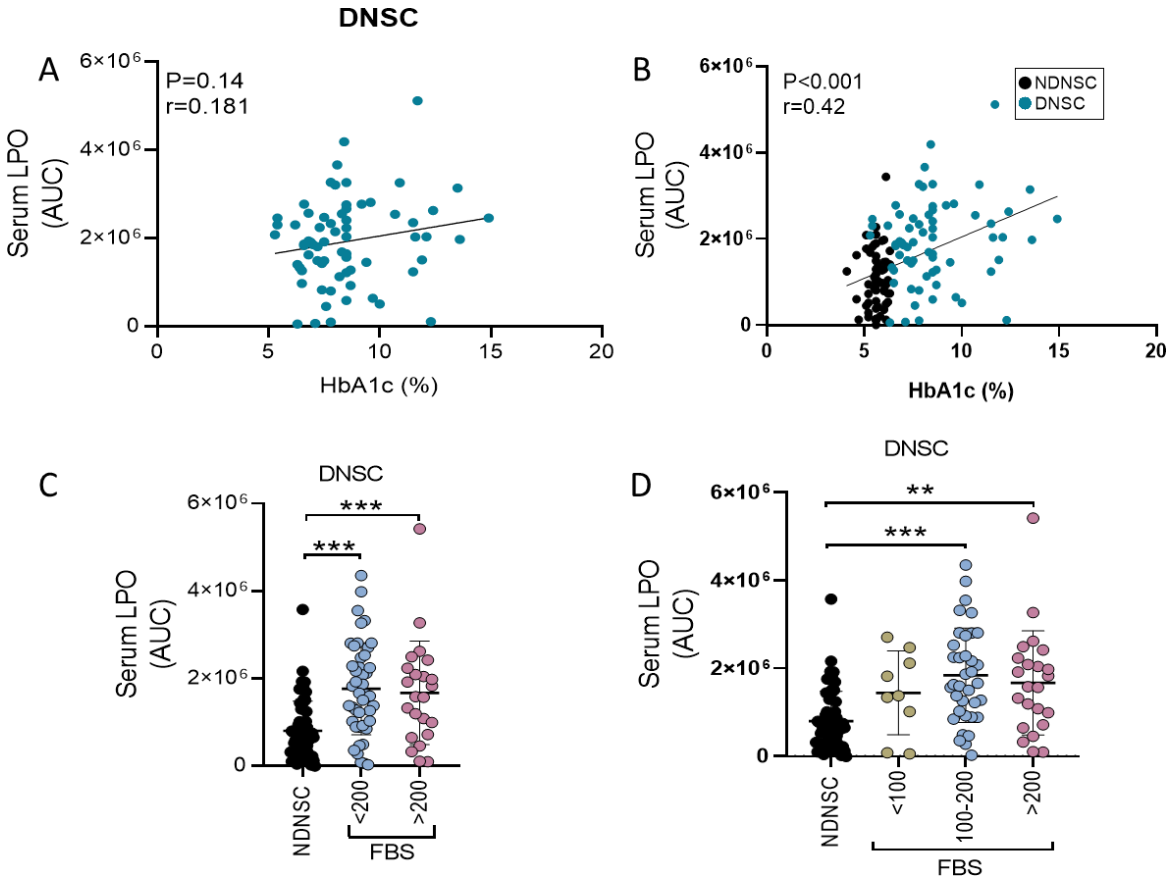

Figure 4. Impact of glycemic control (poor or normal) on serum LPO potential in DNSC compared to NDNSC. Pearson correlation analysis between Serum LPO(AUC) and HbA1c (Glycated hemoglobin) in DNSC group (A), and combination of NDNSC group with DNSC group (B). Levels of serum LPO in DNSC groups with increasing fasting blood sugar (FBS) levels (C&D). *P<0.05,**P<0.01 and ***P<0.001.

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