



N Am J Med Sci. 2015 Oct; 7(10): 459–466.

doi: [10.4103/1947-2714.168673](https://doi.org/10.4103/1947-2714.168673)

PMCID: PMC4677471

PMID: [26713292](https://pubmed.ncbi.nlm.nih.gov/26713292/)

Cytokeratin 8 in Association with sdLDL and ELISA Development

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Abstract

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Background:

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Cardiovascular disease (CVD) remains the leading cause of morbidity and mortality worldwide. Cytokeratins (CKs) which may also be expressed in vascular smooth muscle cells (SMCs) are generally considered to be markers for the differentiation of epithelial cells. Small, dense, low-density lipoprotein (sdLDL) particles, also termed LDL-IV, independently predict risk of CVD.

Aims:

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The aims of this study were to develop an analytical method, apart from ultracentrifugation capable of isolating sdLDL in order to study any associated proteins.

Materials and Methods:

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Using modified gradient gel electrophoresis (GGE), de-identified sdLDL-enriched plasma was used to physically elute and isolate sdLDL particles. To validate the finding, additional plasma from 77 normal and 48 higher risk subjects were used to measure sdLDL particles and CK8. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting method were used to identify the characteristics of proteins associated with sdLDL. An enzyme-linked immunosorbent assay (ELISA) method was developed and validated for the measurement of CK8 in plasma.

Results:

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The validation of the CK8 ELISA method showed good analytical performance. The isolated sdLDL particles were verified with nondenaturing GGE with the apolipoprotein B component confirmed by Western immunoblotting. Confirmed by SDS-PAGE and Western immunoblotting, CK8 was associated with sdLDL. Two-tailed statistical analysis showed that CK8 and sdLDL particles were significantly higher in the high-risk CVD group compared to control group ($P < 0.01$ and $P < 0.01$, respectively).

Conclusion:

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This study reports a novel association between CK8 and sdLDL in individuals with CVD who have a predominance of sdLDL.

Keywords: Atherosclerosis, cardiovascular disease, cytokeratin 8, gradient gel electrophoresis, predominance of small dense LDL, small dense LDL

Introduction

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It is well-accepted that CVD remains the leading cause of morbidity and mortality worldwide. The cause of CVD believed is multifactorial and research is still looking into more details about the biomarkers associated with CVD. Of interest are the association between small, dense, low-density lipoprotein (sdLDL) and structural components of the cytoskeleton including intermediate filaments, actin filaments, and microtubules that provide cells with structural integrity against physical stress.[1,2] The size of intermediate filaments is between microfilaments and microtubules and they are assembled from several different proteins.[3,4,5,6] In this study, the focus will be on cytokeratin (CK), which when fully assembled are heteropolymers measuring between 10 and 12 nm in diameter,[7] and can be divided into acidic (28 members, including CK9-20) and basic or neutral (26 members, including CK1-8).[8,9,10] Intermediate filament proteins and lymphocytes show high dynamic adhesion at the site of contact,[11] which may explain the presence of CK along with macrophages and lymphocytes, in the atheroma. Moreover, cytoskeletal proteins are detected at the site of intimal proliferation, leading to the occlusion of the lumen of allograft vessels, although the immunopathogenesis of this process remains unclear.[12] Intermediate filaments are also reported to surround lipid droplets (forming cage-like structure) in macrophages and may, in some way, induce these cells to accumulate cholesteryl esters, leading to foam cell formation.[13,14,15,16] The investigation of smooth muscle cell (SMC) proliferation and changes in the cell's cytoskeleton may discover novel biochemical markers for the characterization of SMC during early and advanced phases of atheroma formation.[17,18] While CKs are generally considered markers of epithelial cell differentiation, in particular CK8 may significantly increase in patient sera with lung cancer, [19,20,21] colon cancer, and pancreatic cancer;[22] but not with prostate cancer.[23] In addition, CK8 may also occur in vascular SMC in response to stress, mitosis, or apoptosis; it cannot be detected in normal coronary arteries.[24] CK8 and CK18 are detected in human SMCs from atherosclerotic lesions from a variety of sites within the arterial vasculature,[25,26] and within the atheroma, the amounts of CK8 and CK18 correlate with lesion severity, ranging from intimal thickening to complicated lesions.[25]

sdLDL have been related to coronary atherosclerosis progression in many studies.[27] Methodologies that measure LDL subclasses are complicated, labor intensive, and require high scientific skills. We sought to link the presence of a relatively easily measured protein, specifically CK8, as an alternative to direct measurement of sdLDL and the objective of this research was development and validation of an enzyme-linked immunosorbent assay (ELISA) assay for the quantitation of serum levels of CK8 as a possible marker for cardiovascular disease (CVD).

Materials and Methods

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Plasma specimens

For the isolation of sdLDL and CK8; residual de-identified plasma specimens from subjects with predominance of sdLDL (defined as having LDL-IV subclass >10% of total LDL subclasses) were selected for physical isolation of small dense LDL-IV and CK8 by gel elution. For the control group, additional residual de-identified plasma specimens from subjects with undetected small dense LDL-IV were selected.

To validate the association of CK8 with sdLDL in a larger set of specimens in health and CVD subjects, a total of 77 volunteers (control group) from company employees (Health Diagnostic Lab, Inc., Richmond, VA) provided informed consent to donate blood to establish the assay reference range. The control group was presumed to be a healthy population with no evidence of CVD as determined from a self-report health questionnaire prior to blood withdrawal. For the diseased group, a total of 48 residual de-identified (the

samples were coded in a way that prevented us from tracing the sample back to a particular individual) samples from a recent clinical trial were utilized; consistent with the original study protocol and informed consent. The cohort represented a population without prior history of but at high risk for coronary artery disease (CAD), that is, patients will be eligible for this arm if they have documented CAD by an imaging modality. Such imaging modalities may include, but will not be limited to, a positive coronary artery calcium (CAC) score, a positive coronary computed tomography angiography (CTA), a positive cardiac catheterization, or a positive stress test (of any kind).

Isolation of small dense LDL and CK8

The following procedure was a modification of the 2-14% gradient polyacrylamide gel electrophoresis method as described by Rainwater *et al.*[28] to isolate small dense LDL-IV subclass directly from plasma. The Krauss and Burke method[30] for lipoprotein separation by GGE was adapted as well and modified as follows: To prepare a prestained lipoprotein standard, Sudan black 'Lipostain' (Beckman-Coulter, Fullerton, CA) was added to the lipoprotein standard to make a 4% (v/v) solution. This was then incubated at ~4°C overnight and was used within 1 week. Following preparation of four electrophoresis gels, a 10 µl aliquot of whole plasma was loaded in lanes 3 through 16, with a prestained lipoprotein standard loaded in lanes 1, 2, 17, and 18 on each gel. The electrophoresis was carried out at 40 V for 15 min followed by 80 V for 15 min, then ramped up to 125 V volts for approximately 18 h. Following the complete separation of lipoprotein subclasses based upon their size exclusion, a scalpel or razor blade and a ruler were used to physically excise a 0.5 cm-wide window in the gel, just below the LDL-IV bands (as guided by the prestained LDL-IV subclass standard band which is considered the smallest LDL subclass by size on the gel) to capture the lipoproteins flowing from lanes 3 through 16 into the so called elution window that filled with electrophoresis buffer [Figure 1]. The gel was reassembled in the holding cassette and reloaded into the electrophoresis chamber that filled with fresh buffer. Electrophoresis was continued at 250 V for 45 min to collect fraction one, and then for intervals of 1 h until all of the LDL subclasses had been separated and collected. After the elution of each fraction, the fraction was collected and the elution window was rinsed and filled with fresh buffer in preparation for the elution of following fraction. The volume of each fraction was approximately 750 µl. The collected fraction's volume were reduced to ~250 µl by centrifugation at 7,500 rpm for 30 min at a temperature of 4°C using the 2 mL concentrator Centricon-10 (cat # 4206, Amicon, Billerica, MA).

Particle size identification by nondenaturing GGE

The eluted fraction was applied into the nondenaturing polyacrylamide gradient gel electrophoresis (GGE) to qualitatively determine the present of small LDL-IV subclass in the eluted fraction.

Molecular weight determination of proteins by SDS-PAGE

Following the confirmation of the eluted fraction by GGE method, then the SDS-PAGE was performed according to the manufacturer's procedure (Cat. # EC60385, Invitrogen, Carlsbad, CA). Apolipoprotein B-100 (apoB-100) control material was isolated from plasma as described previously by Shen *et al.*[31] A CK8 positive control was purchased from Labvision (Cat. # RB-9095-PCL, Labvision, Fremont, California).

Western immunoblotting for protein identification

Following the separation of proteins by SDS-PAGE as described above, the proteins was transferred to a nitrocellulose membrane according to Bio-Rad laboratory's procedure (Cat. # 13849, Bio-Rad, Hercules, CA).

Nonspecific binding sites were blocked by incubating the nitrocellulose membrane with 3% bovine serum albumin (BSA) in phosphate buffer saline (PBS) containing 0.05% Tween-20. Following the blocking step,

the membrane was incubated either with primary CK8 antibody (Cat. # RB-9095-PO, Labvision, Fremont, CA) or with primary apoB-100 monoclonal antibody (Cat. # K90086P, Meridian Life Science, Inc., Saco, ME). The membrane was then washed and incubated with secondary anti-immunoglobulin G (IgG) horseradish peroxidase (HRP)-conjugated antibody (Cat. # 31410, Pierce, Rockford, IL). The membrane was then washed five times before development using the SuperSignal reagent (Cat. # 4096, Pierce, Rockford, IL). The membrane was exposed to X-ray and developed to visualize proteins of interest.

CK8 ELISA development and validation

Two different antibodies were custom developed by GenWay Biotech (GenWay Biotech Inc., San Diego, CA). The standard ELISA optimization steps were followed in order to select the most optimal conditions, as follows: The primary antibody was coated at 200 ng/well, detection antibody was optimized to 20 ng/well, the purified recombinant CK8 (Cat. # 30R-AC041, Fitzgerald Industries International, Acton, MA) was used at 250 ng/mL as the highest calibrator point. The standard, control, patient plasma, substrate, and stop solution volumes were optimized to 75 μ L per well and absorbance reading was performed at 450 nm.

Within-run and between-run precision was assessed at three different CK8 concentrations [Table 1]. The linearity was evaluated by testing intermixed levels of CK8 using two serum pools (level one was < 3.9 ng/mL and level five was ~260 ng/mL), to create five equally-spaced incremental levels. The observed concentration was plotted against relative dilutions and the degree to which the plotted curve conforms to a straight line is a measure of system linearity.

Sample diluent was assayed 20 times to establish the limit of blank (LOB) and this was calculated as mean + 2 standard deviations (SDs). Limit of detection (LOD) was calculated as LOB + 1.653*SDs of the LOB. [32] To establish limit of quantitation (LOQ), serum pool with a CK8 concentration of ~10 ng/mL was diluted with sample diluent to final concentrations of 25, 50, and 75% of the original, and each level was assayed 20 times. LOQ was defined as the lowest concentration resulting in a percent coefficient of variation (%CV) of ≤ 20 .

ASSURANCE™ Interference Test Kit (Cat. # INT-01, Sun Diagnostics, New Gloucester, ME) that contains triglyceride-rich lipoproteins (TRLP), hemoglobin, and conjugated/unconjugated bilirubin was used to evaluate the potential assay for presence of these interferences. The ELISA antibody specificity was tested against serum containing 125 ng/mL heterophilic antibodies (HA) and ~70 U/mL rheumatoid factor (RF; both obtained from ProMedDx, Norton, MA). The acceptable bias between the CK8 results obtained from the interference set compared to CK8 results obtained from the control set was defined as $\leq 15\%$.

Accuracy was tested by known amounts of CK8 recombinant protein (50, 100, 125, and 200 ng). Recovery was calculated using the following formula: (average measured/expected) \times 100. Acceptable recovery was set as 80-120% of the expected amount of CK8 protein.

Ion mobility

The sdLDL concentration was measured in the healthy and CVD subjects using the ion mobility analyzer as described by Caulfield *et al.*[37]

Statistical analysis

Two-tailed Student's *t*-test statistical analysis (two-sample assuming equal variances) was used to compare the different types of LDL subclasses concentration in cardiovascular group to the control group (level of significance, $P \leq 0.05$). In addition, the SD was calculated and presented as error bars. CK8 concentrations were not normally distributed in both groups, the value “one” was added to all CK8 values because some subjects return the zero values for CK8 concentration, then the data were log₂ transformed for

normalization purposes and then the two-tailed *t*-test statistical analysis (two-sample assuming unequal variances) was used to compare the concentration in the higher risk for cardiovascular group to the control group to examine for statistical differences between the two groups. The statistical significance between the two groups was based on $P \leq 0.05$.

Results

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Particle size identification by nondenaturing GGE

The nondenaturing polyacrylamide GGE verified the size of the eluted fraction as LDL-IV subclass [[Figure 2](#)].

Proteins associated with LDL-IV fraction as identified by SDS-PAGE

The SDS-PAGE gel [[Figure 3](#)] shows the high molecular weight standard ranging from 160 to 40 kDa (lane 1), apoB-100 standard (lane 2), and separated protein content of the LDL-IV fraction (lane 3 and 4) from two individual subjects. Three prominent bands were detected in the LDL-IV fractions (lanes 3 and 4). The highest molecular weight band was of similar molecular weight to the apoB-100 standard and a second band at approximately 66kDa was predicted to be albumin. The third, prominent band was of approximately 53 kDa. At this point, this band was not known, thorough examination of the literature revealed that this band might correspond with CK8 protein.[[19,20](#)]

Western immunoblotting

Individually eluted lipoprotein fractions from two individual subjects were immunoblotted. A specific monoclonal antibody for apoB-100, along with an apoB-100 standard, confirmed the presence of apoB-100 in the eluted fraction [[Figure 4](#)].

Since the eluted LDL-IV fraction contained a protein of similar molecular weight to CK8 [[Figure 3](#)], we next sought to confirm the association of CK8 with the sdLDL subclass eluted fraction. Therefore, immunoblot analysis was performed on an eluted fraction from five individual plasma samples, along with a CK8 plasma standard, and a specific CK8 antibody was used to confirm the presence of CK8 protein [[Figure 5](#)]. Indeed, a 53 kDa immunoreactive band corresponding to CK8 was present in the eluted samples from all five subjects and correlated well with the CK8 standard. The first two samples, which were recognized to have predominant sdLDL, showed a stronger immunoreactivity compared to the last three samples that had less sdLDL.

CK8 ELISA performance

Within-run and between-run variability of the three levels' mean, SDs, and %CV are summarized in [Table 1](#).

Acceptable linearity was observed up to 263 ng/mL through the straight line generated between the observed concentrations and expected values [[Figure 6](#)].

The LOB and LOD were 0.1 and 0.2 ng/mL, respectively. The LOQ was determined to be 3.82 ng/mL with % CV of 2.8%.

No significant interference was observed from spiked levels of up to 1,500 mg/dL TG, 500 mg/dL hemoglobin, or 25 mg/dL bilirubin. Also the CK8 ELISA found to have no cross reactivity with HA and RF.

The accuracy experiment confirmed that this ELISA achieved recovery of 97, 87, 87, and 94%, respectively, for the spiked materials; thereby confirming the accuracy of the method [[Table 2](#)].

CK8 and LDL subclasses distribution in disease

[Figure 7](#) (histogram) represents the concentration percentage frequency of CK8 in the control group.

[Figure 8](#) scatter graph depicts the CK8 distribution in control and subjects at high risk for CVD. The data showed that the CK8 was significantly higher in the group at high risk for CVD compared to control group ($P < 0.01$).

[Figure 9](#) scatter graph represents the distribution of sdLDL in healthy and in the group at high risk for CVD. The data showed that the total sdLDL subclass was significantly higher in CVD group compared to control group ($P < 0.01$).

Discussion

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A modified 2-14% GGE method was used to isolate small dense LDL-IV subclass directly from plasma. This procedure avoids exposing lipoproteins to a high salt concentration and prolonged ultracentrifugation, both of which promote the physical dissociation of lipoprotein particles, particularly loosely bound proteins.[29]

In this instance, CK8 was found to be associated with sdLDL in the plasma from subjects with predominance of sdLDL. Initially, upon SDS-PAGE of GGE-eluted plasma, three major bands were identified in the LDL-IV fraction: apoB-100, albumin, and a protein of ~53 kDa. An extensive literature search identified CK8 as a possible protein of interest, since it has a similar molecular weight to the 53 kDa band.[24,25] Indeed, a CK8-selective antibody reacted with the 53 kDa protein band blotted from the fraction that also contained sdLDL. Individuals with a predominantly high sdLDL concentration showed stronger CK8 signal intensity than those with less sdLDL-rich profiles [[Figure 5](#)].

The modified GGE method that led to the discovery of the association between CK8 and sdLDL is laborious and time consuming. An ELISA method for rapid measurement of CK8 in serum was developed allowing realistically high throughput for population screening and clinical studies, in addition to routine clinical applications. Two different immunoglobulin antibodies were raised and used to develop a CK8 ELISA successfully with excellent performance in terms of precision, linearity, LOQ, and lower susceptibility regarding interference with high chylomicron, hemolysis, or elevated bilirubin. HA and RF interferences were found to be negligible.

CK8 serves as a marker for atheroma as it is expressed in the SMC of human atherosclerotic lesions and correlates well with lesion severity.[17,18,24] The expression of CK8 and CK18 has been demonstrated in the SMC of atheroma.[17,18] Synthesis of CK is enhanced by cell-cell contacts,[33] and it is plausible that the induction of CK expression by endothelial cells plays a role in atherosclerosis, beginning with sdLDL entry and cellular deposition in the arterial wall. Synthesis of CK would increase in parallel with ongoing accumulation of cells and sdLDL further accelerating the atherogenesis. In this study, CK8 was found to be significantly higher in the de-identified subjects at higher risk for CVD compared to normal control group ($P < 0.01$) [[Figure 7](#)], which may support the use of CK8 for the assessment of patients at risk of developing CVD.

The evidence presented in the current study reveals a novel association between CK8 and sdLDL, and may implicate CK protein levels in the development and progression of CAD. This finding is supported by the significant higher concentration of sdLDL in subjects at higher risk for CVD compared to the control group ($P < 0.01$) [[Figure 8](#)].

Theoretically, the role of CK in the formation of lipoprotein-mediated atheroma may be a consequence of extending their normal, physiological function of providing mechanical support for cells to atheroma cells (e. g., vascular SMC, macrophages, and T lymphocytes), thereby protecting them from mechanical stress and stabilizing the plaque.[34,35,36] These conditions provide the necessary support for the arterial wall cells to produce CK, which would support the atheroma from mechanical stress. It is also possible that

CK8 may not initiate atheroma formation, but rather arise as a consequence of cellular proliferation (vascular SMC, macrophages, and T lymphocytes) and the presence of sdLDL at the site of atheroma. Their atherogenic role may involve the acceleration of atheroma build-up due to the increased synthesis of CK as a result of cellular deposition on the atheroma site. The presence of circulating CK8 in serum may indicate plaque instability, shedding, or rupture.

Although not evaluated in this study, the measurement of serum CK8 may be useful in subjects with unstable or ruptured plaque, restenotic coronary lesion after invasive intervention, heart transplantation-associated arteriosclerosis, and following coronary artery vein graft. Increased serum CK8 levels may correlate with the severity of the lesion from intimal thickening to complicated lesions. As CK8 may be detected in serum of patients with some cancers making it less specific to atherosclerosis, the CK8 value must be evaluated in conjunction with other clinical presentations. Further studies to examine the correlation of CK8 with other risk factors such as gender effect, age, weight (body mass index), lipids, diabetes, and blood pressure are required.

CK8 was isolated in association with sdLDL within the same eluted GGE fraction. A limitation of this study is that this association does not prove the physical association or the presence of any covalent bonds between CK8 and sdLDL and additional research is necessary to elucidate the nature of the relationship between these two proteins. Also other homology proteins to CK8 were not tested during this study, and future testing is warranted. The subjects at higher risk for CVD were de-identified and did not have any additional characteristics to examine the association of CK8 with any additional health abnormalities.

Conclusion

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CK8 has been found to be associated with sdLDL in the serum of subjects with a predominance of sdLDL. As CK8 and sdLDL are both risk factors for atherosclerosis, the ELISA method developed here, which measures CK8 in serum with high sensitivity and specificity, may provide information beyond that of current CVD biomarkers to better identify and treat subjects at risk for (primary prevention) or with atherosclerosis (secondary prevention).

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

Acknowledgment

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Thanks to professor Ronald Krauss and his colleagues from CHORI's the center for the Prevention of Obesity, Cardiovascular Disease and Diabetes for facilitating part of this research to be performed at his laboratory.

Thanks to Dawn Thiselton (PhD) and Yvette Edmonds (PhD) from Health Diagnostics laboratory (Richmond, Virginia) for their help in reviewing this article for error and grammar.

Thanks to Dr Thomas Dayspring MD, FACP, FNLA, NCMP, Director of Cardiovascular Education at the Foundation for Health Improvement and Technology (Richmond, VA) for the help in reviewing this article for error and grammar.

Thanks to James Pottala PhD, PStat from Health Diagnostics laboratory (Richmond, Virginia) for his statistical advice, Thanks to Alyne Abraham and Elizabeth Steger from Health Diagnostics laboratory for their technical help.

Thank you Joseph Andy Dyson from Health Diagnostics laboratory (Richmond, Virginia) for the pictures adjustment.

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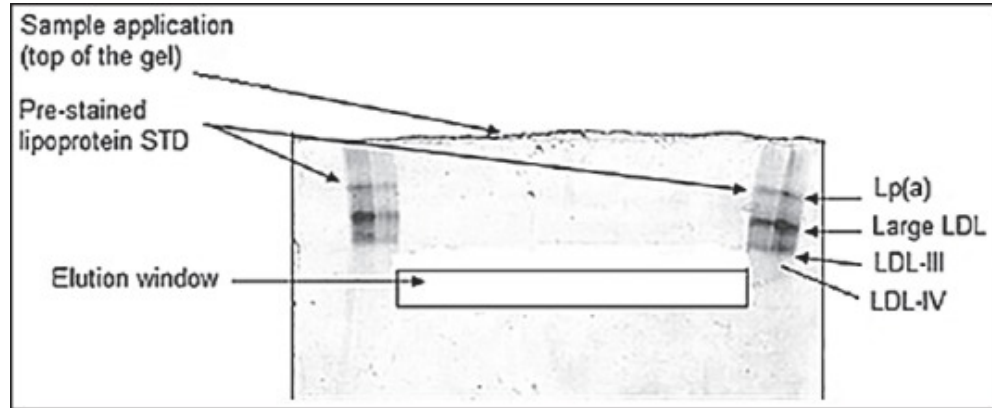
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Figures and Tables

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Figure 1



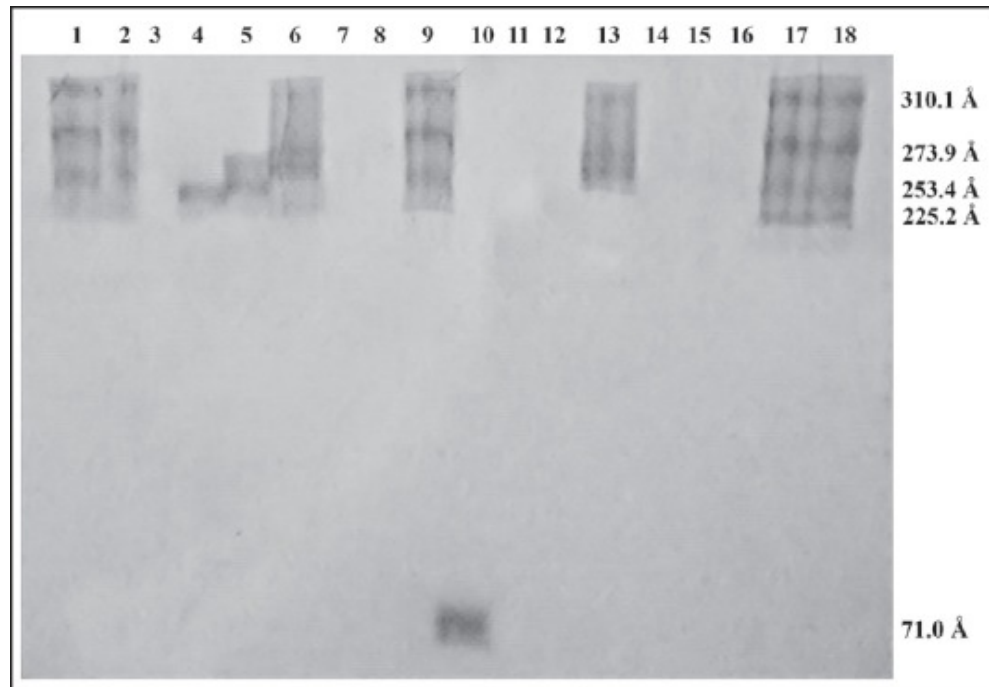
The 2-14% gradient gel showing lipoprotein standard and elution window

Table 1

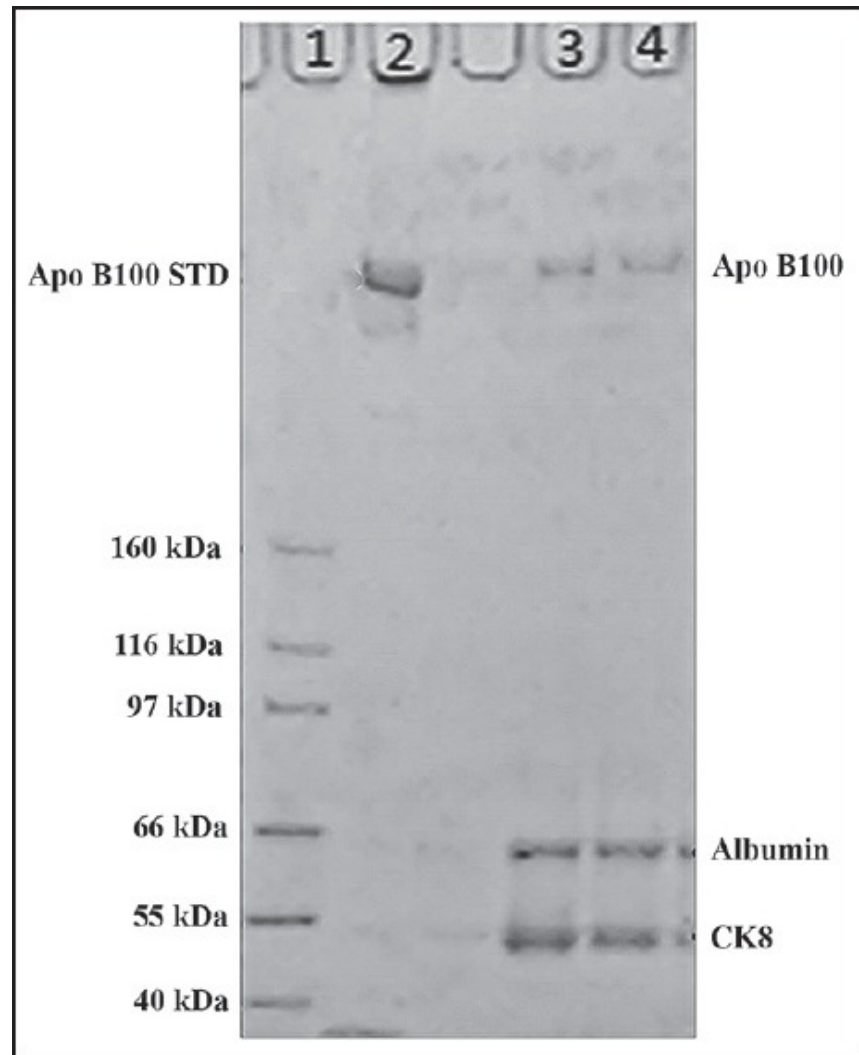
Intra- and inter-assay precision

Sample ID	Intra-assay precision			Inter-assay precision		
	Mean (ng/mL)	SD (ng/mL)	%CV	Mean (ng/mL)	SD (ng/mL)	%CV
Level 1	11.14	0.99	8.9	9.92	1.6	16.13
Level 2	23.83	1.14	4.8	18.57	1.5	8.24
Level 3	35.49	1.87	5.3	29.46	1.85	6.26

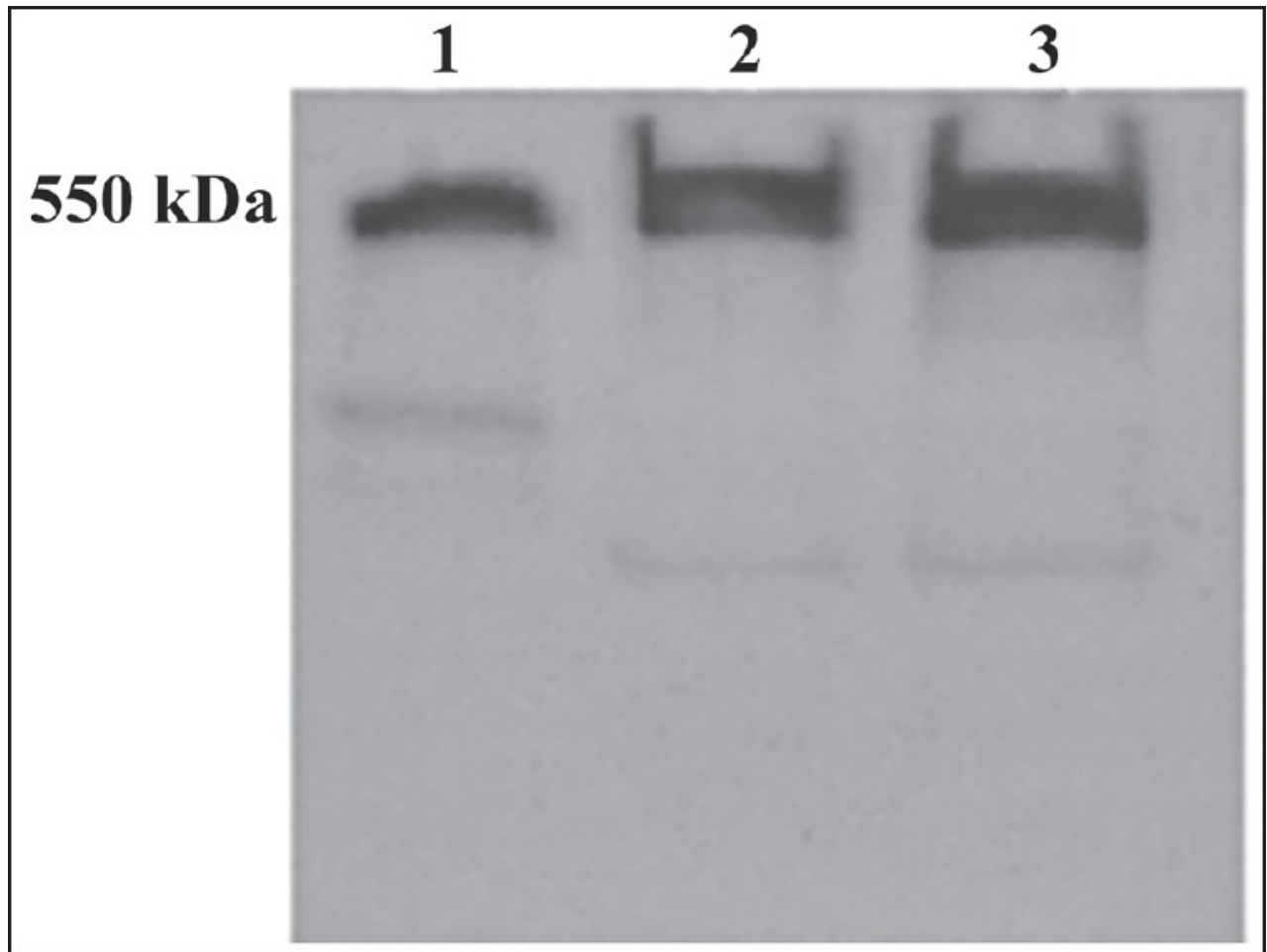
SD = Standard deviation, %CV = percent coefficient of variation

Figure 2

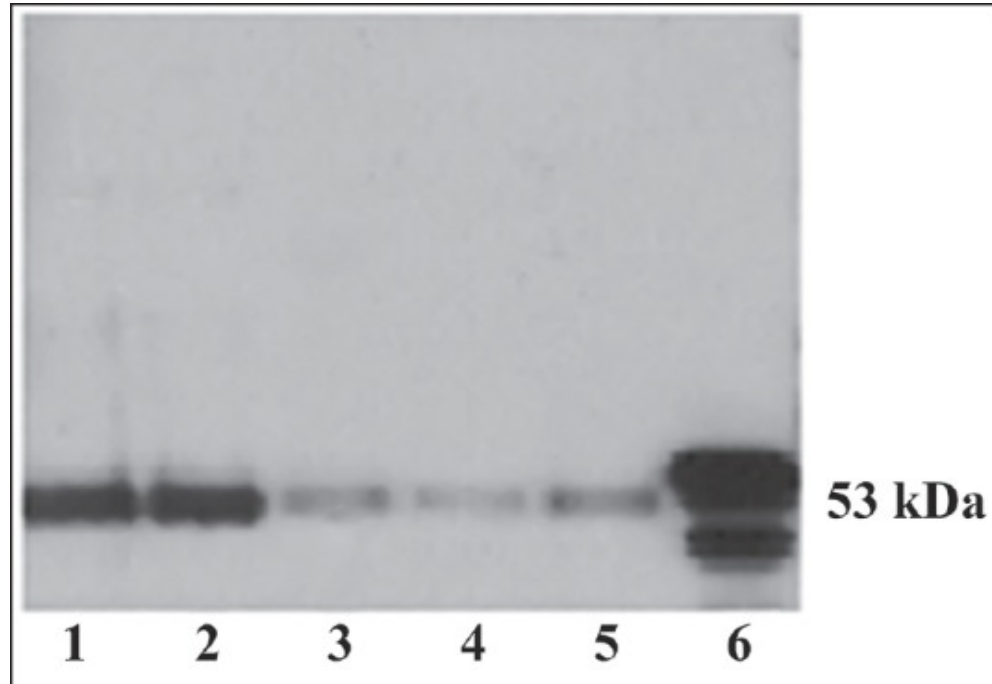
Isolated fractions' (lane 4, 5, and 6) particle size identification by nondenaturing 2-14% gradient gel electrophoresis (GGE). Lanes 1, 2, 9, 10, 17, and 18 are calibrator's lanes. Lane 13 is quality control. The particles size in Angstrom is showed on the right side of the gel image

Figure 3

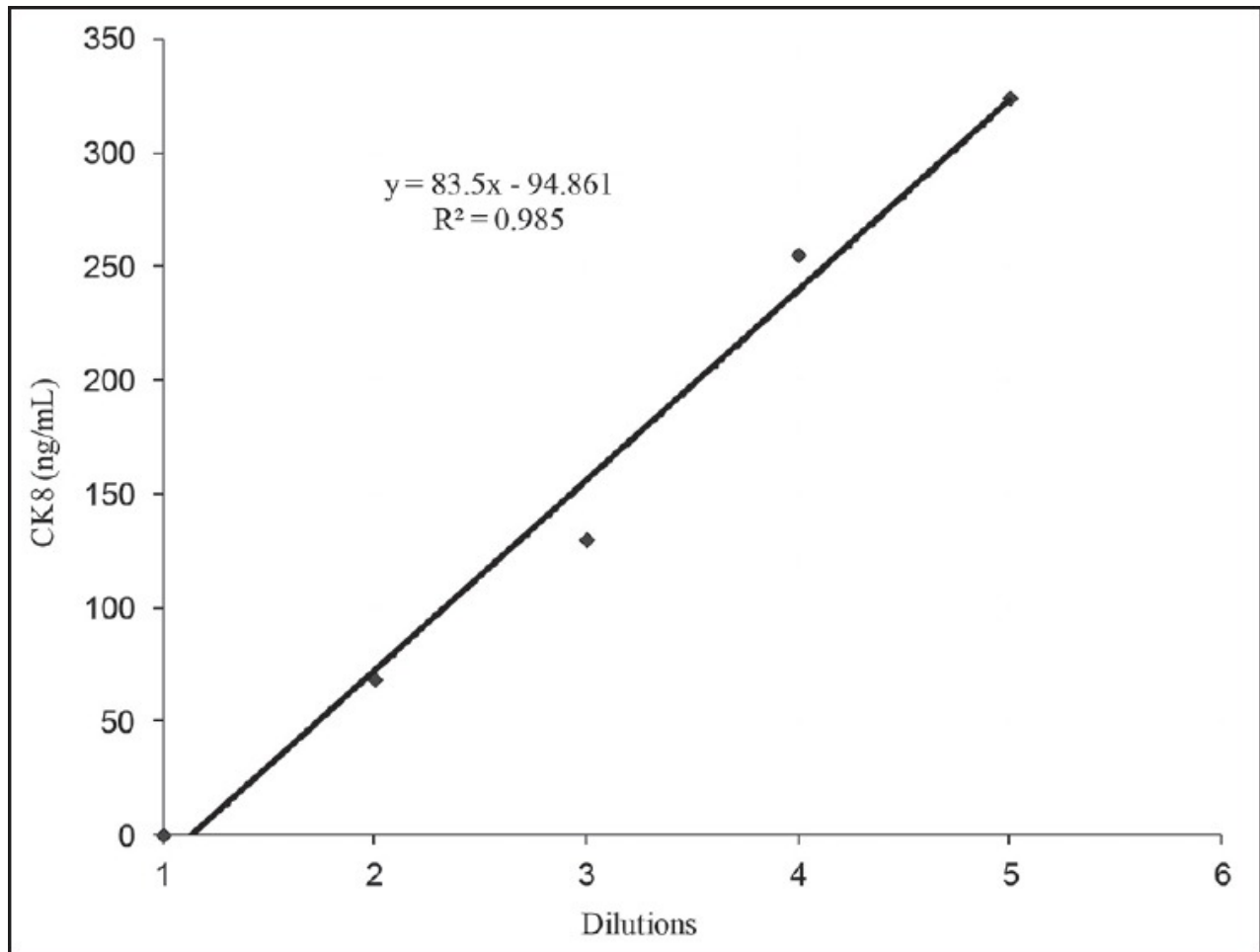
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showing the high molecular weight protein marker (lane 1), apoB-100 standard (STD) on lane 2, and the separated proteins (albumin and CK8) from the eluted fraction (lane 3 and 4)

Figure 4

ApoB-100 immunoblotting; from individually eluted fractions of two different subjects confirming the separation of intact low-density lipoprotein (LDL; lane 2 and 3). Lane 1 shows the apo B-100 standard (STD)

Figure 5

Cytokeratin 8 (CK8) protein is associated with small dense LDL. The first two subjects on the left side are recognized as having predominant small dense LDL (lane 1 and 2), and the small dense LDL was less predominant on the other three subjects (lanes 3, 4, and 5). On lane 6, a known CK8 protein used as control

Figure 6

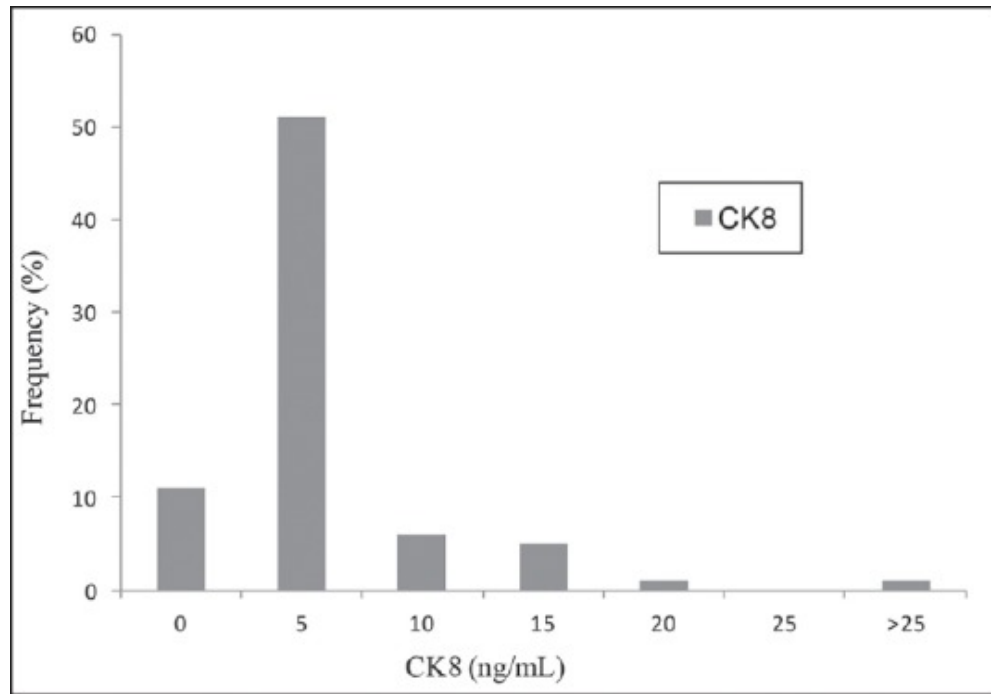
CK8 enzyme-linked immunosorbent assay (ELISA) assay linearity

Table 2

Recovery study

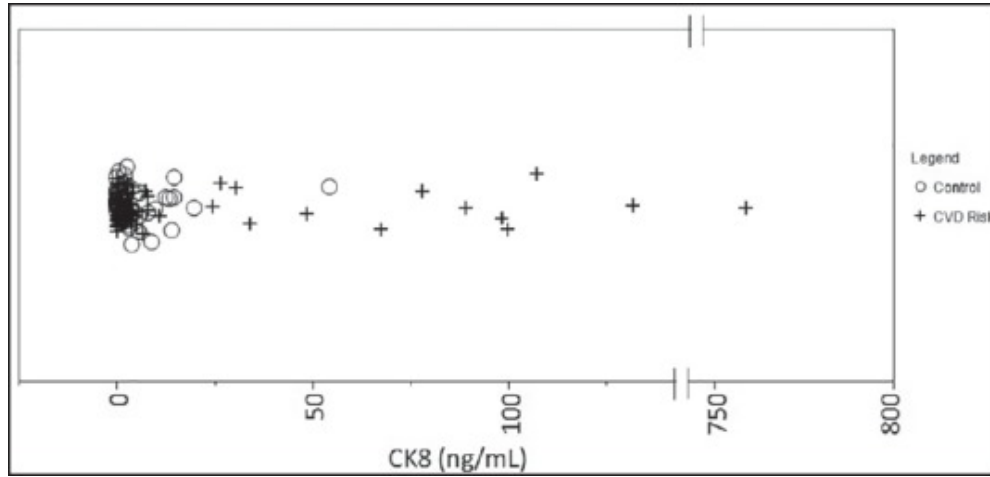
Specimens ID	Theoretical concentration (ng/mL)	Measured concentration (ng/mL)	% Recovery
Sample 1	50	48.65	97
Sample 2	100	87.20	87
Sample 3	125	109.26	87
Sample 4	200	187.76	94

Figure 7



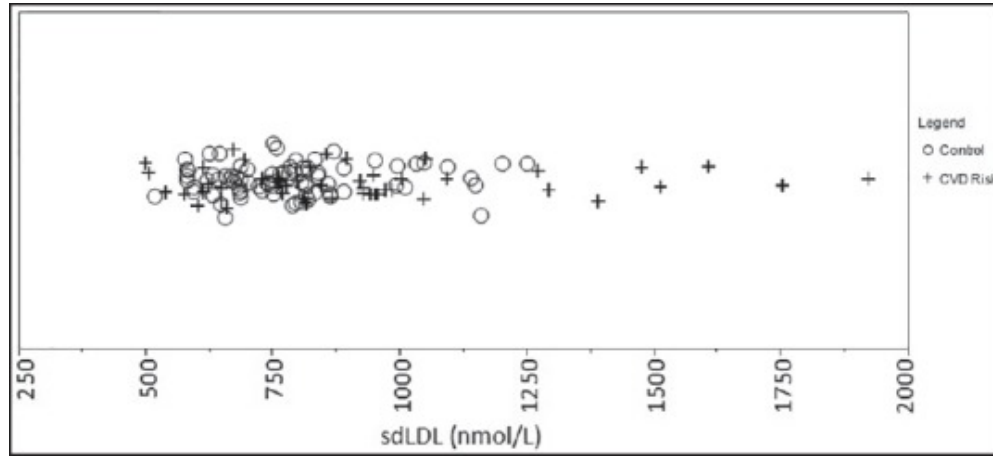
CK8 histogram with percentage distribution in control group

Figure 8



CK8 in control group and subjects at high risk for cardiovascular disease (CVD)

Figure 9



Small, dense, LDL (sdLDL) in control group and subjects at high risk for CVD

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