Optimization and validation of lipoprotein(a) KIV2 isoform measurement in patients with familial hypercholesterolemia

Objectives of the study:

1- Optimize and validate Lp(a) KIV2 repeat isoform measurement.
2- Measure Lp(a) isoforms of FH patients with confirmed mutations and mutation negative, and further investigate the correlation between serum Lp(a) concentration with KIV2 repeats.
3- Measure apo(a) isoform size phenotypically by SDS-PAGE and Western blot.
4- Present in IAS meeting findings of Lp(a) study

Results:

1) Study sample

We collected all patients with familial hypercholesterolemia (FH) and stratified them per occurrence of cardiovascular disease (CVD) events . Total of 32 patients had CVD and 116 did not have CVD. Blood samples were collected for Lp(a) measurement and isoform determination.

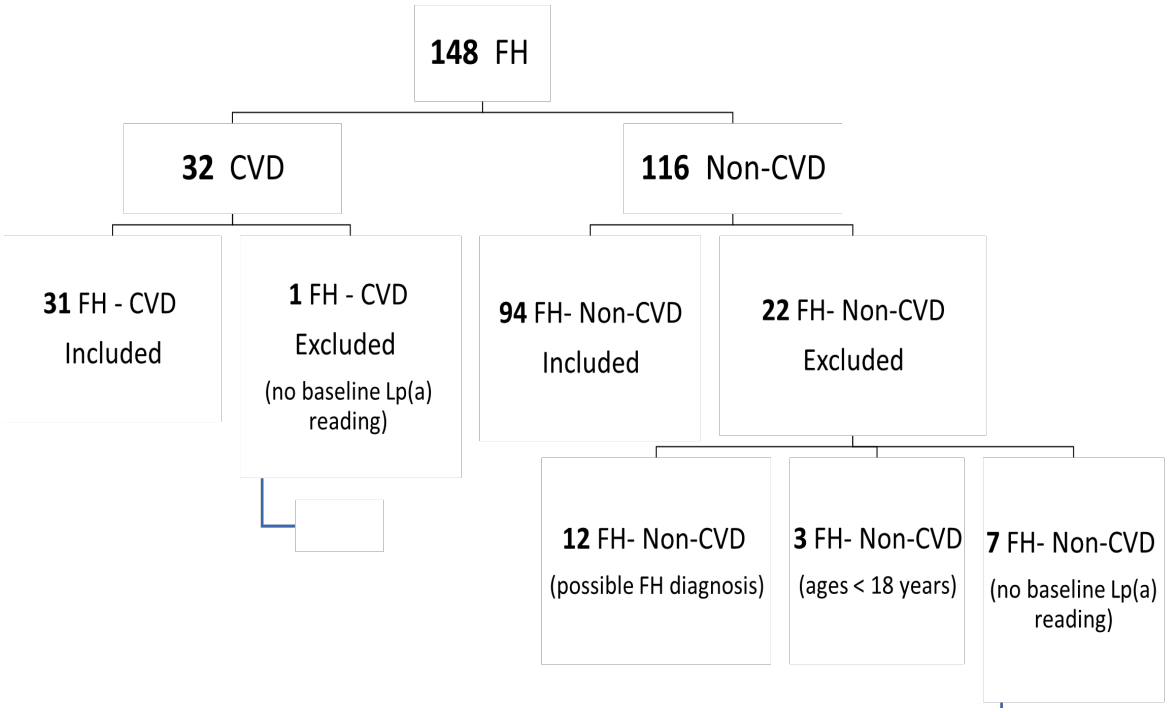


Figure 1: The Final number of FH patients with and without CVD

2) Lpa isoform determination by PCR technique

a. Efficiency of KIV-2 repeat genotyping PCR assay

To test the PCR efficiency of the both albumin and KIV-2 PCR assays, the mean Ct value for both albumin and KIV-2 genes were calculated for 7 DNA sample serial dilutions as shown below in Table (1).

Table 1: Log input DNA concentration with the corresponding mean Ct values for both albumin and KIV-2gene assays

DNA input (ng/µl)	Log (DNA)	Mean Ct (Albumin)	Mean Ct (KIV-2)
40	1.60	25.73	20.47
20	1.30	26.25	20.97
10	1.00	26.83	22.13
5	0.70	27.87	23.52
2.5	0.40	29.05	24.50
1.25	0.10	30.33	25.78
0.625	-0.20	31.53	27.22

Standard curves were made for both albumin and KIV-2 PCRs. Figure (2) shows the PCR efficiency of the albumin gene amplification, the slope was -3.2937 and coefficient of determination was excellent R^2 = 0.9763. The PCR efficiency calculated from the slope around 101.9%.

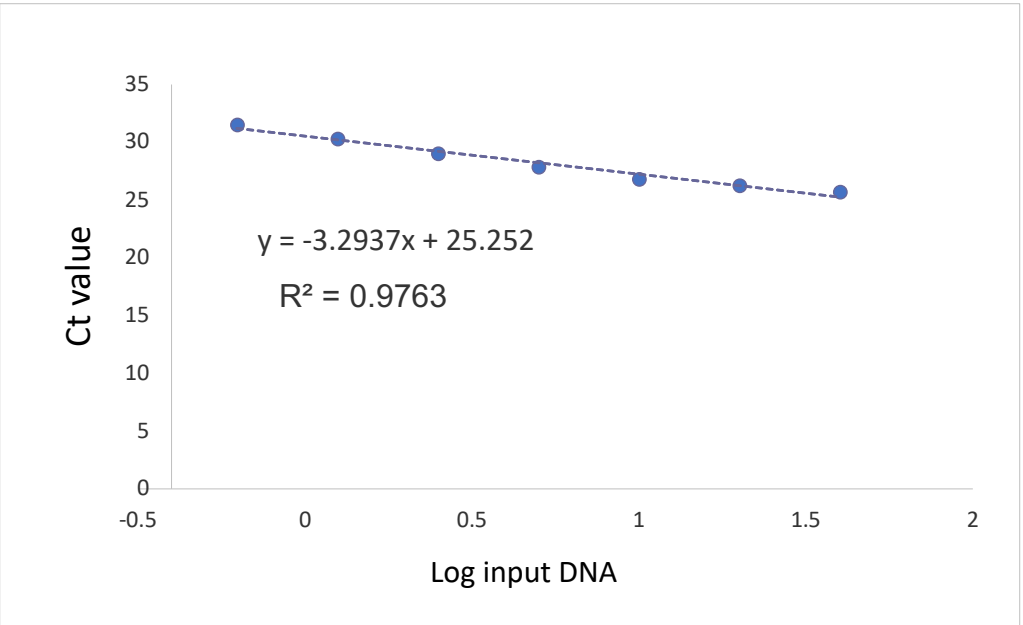


Figure 2. Standard curve of the albumin PCR assay, the slope = -3.2937 and coefficient of determination R^2 = 0.9763.

Figure (3) shows the PCR efficiency of the KIV-2 assay in which the slope was -3.8254 and coefficient of determination was excellent R^2 = 0.99. The efficiency of the PCR calculated around 82.6.

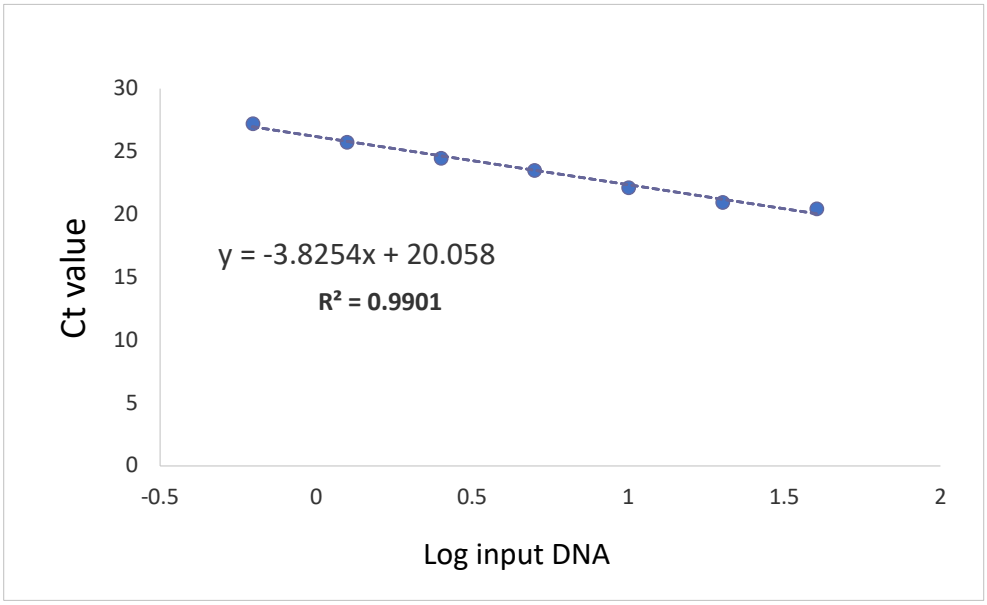


Figure 3. Standard curve of the KIV-2 PCR assay, the slope = -3.8254 and coefficient of determination R^2 = 0.99

b. Reliability of KIV-2 repeat genotyping PCR assay

By plotting the coefficients of variation of the mean Ct values for the two-fold sample dilutions and the calibrator in both the albumin and the KIV-2 gene assays, all samples in each assay showed very close variations that fluctuates the range of 0-2 % C.V. The mean coefficient of variation for each assay is represented by the 2- D column chart, the two assays established only less than 1 % coefficient of variation (0.96 % and 0.74 % for the albumin and KIV-2 assays respectively). This showed a very good reliability as coefficient of variation of less than 10 is considered acceptable.

Table 2: The input DNA concentration (ng/µl) and calibrator with the corresponding coefficient of variation for both albumin and KIV-2 genes

		Input DNA concentration (ng/µl)								
		40	20	10	5	2.5	1.25	0.625	Calibrator	mean
C.V %	Albumin	1.06	1.61	0.24	0.79	1.12	0.89	1.27	0.67	0.96
	KIV	0.24	0.31	0.78	1.06	0.06	0.39	1.16	1.91	0.74

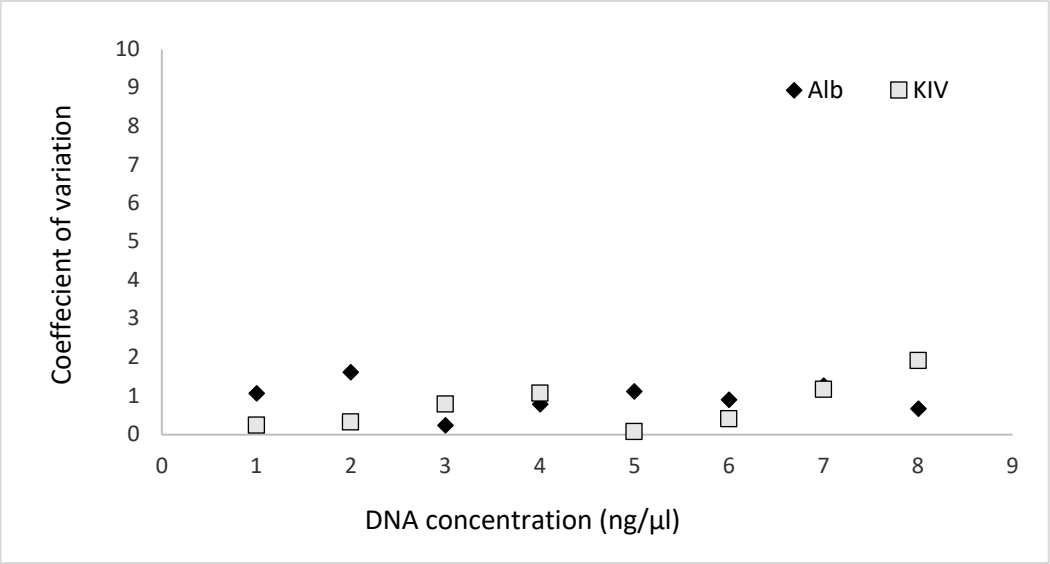


Figure 4. Coefficient of variation of the mean Ct values for the two-fold sample dilutions and the calibrator is plotted against its corresponding DNA sample concentration (ng/µl) for both the albumin and the KIV-2 gene assays.

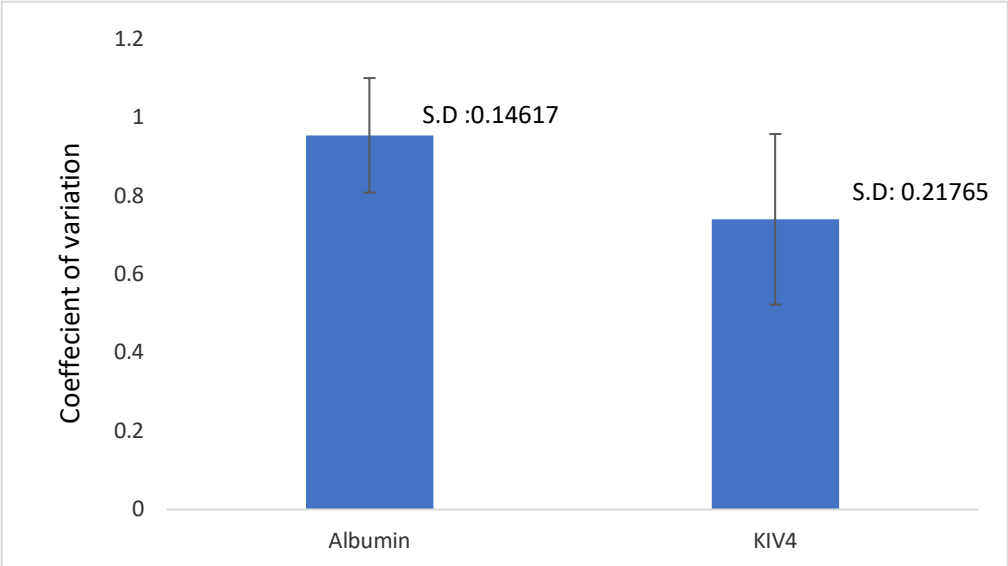


Figure 5. The mean Coefficient of variation for both the albumin and the KIV-II gene assays represented by a 2-D column chart supported by the standard deviation

c. KIV-2 repeats genotyping PCR assay

Table (3) showed KIV-2 repeats in the LPA gene determined by the RT-PCR. The overall mean of KIV-2 repeats was 49 ± 16. Additionally, there was no difference in the % of patients with (⩾16, ⩾20, ⩾48) KIV-2 repeats.

Table 3: KIV-2 repeats in the LPA gene stratified by CVD and non-CVD in patients with FH.

KIV-2 repeats in LPA	**Total** (n = 125)	**FH CVD** n = 31 (24.8%)	**FH non-CVD** n = 94 (75.2%)	***P*-value**
Overall, mean±SD	49 ± 16	49 ± 12	49 ± 18	0.881
% of patients with ⩾16 KIV-2 repeats		100	99	0.571
% of patients with ⩾20 KIV-2 repeats		100	95	0.197
% of patients with ⩾48 KIV-2 repeats		47	52	0.602

Figure (6) showed moderate negative correlation between serum Lp(a) concentration and the KIV-2 repeats for the total cohort with a spearman's correlation coefficient= - 0.425 (p< 0.001).

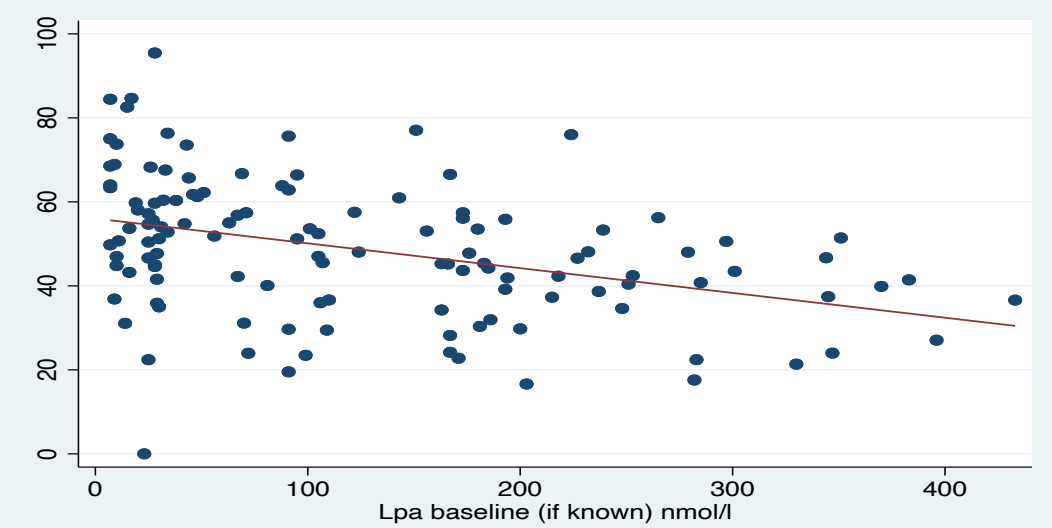


Figure 6. Negative correlation between lipoprotein(a) concentration and KIV-2 repeats in the LPA gene with correlation coefficient (r(s) = - 0.425, p<0.001).

3) Determination of Lp(a) isoform size via Western blot

Main goal of the project was determining Lp(a) isoform size using the following Western blot protocol:

Samples:

1- Thaw plasma samples at room temperature
2- In Eppendorf tubes add:
    a. Plasma according to table below
    b. 25ul of BBP4X
    c. Up to 100ul with dH2O

Lpa (nmol/L)	<15	15-30	30-100	100-200	>200
Plasma (uL)	10ul	8ul	6ul	4ul	2ul

3- Heat at 95C for 5 mins

3% Agarose gel:

1- In flask mix:
    a. 1.5g ultrapure agarose
    b. 5ml of 10X TBE
    c. 45ml of dH2O
2- Microwave 20-30 seconds → mix → repeat until agarose has dissolved
3- Immediately add 500ul of 10% SDS (immerse tip in solution to avoid bubbles)

4- Pour gel in center of cassette (remove any bubbles with tip) → Insert comb
5- Wait until gel solidifies (around 15 mins)

Migration:

1- Remove comb → Place gel in electrophoresis tank
2- Cover entire gel with cold migration buffer (do not reuse buffer)
3- Load 10ul of each sample and standards (aka ladder) ; make sure to wipe tip with tissue before loading to minimize chance of samples entering other wells
4- Set the 300V power bank at 60mA for 90 mins

Semi-dry transfer:

1- Cut nitrocellulose membrane according to size of gel → place in cold 1X transfer buffer
2- Use either 4 thin filter papers or 2 thick filter papers → place in cold 1X transfer buffer
3- After run is complete, take gel straight for transfer → wash briefly with 1X transfer buffer
4- Assemble transfer sandwich according to the following order (starting from bottom);
    a. Filter papers → roll → add buffer
    b. Membrane → add buffer
    c. Gel → roll well (make sure no bubbles are seen under) → add buffer
    d. Filter papers → roll → add buffer
5- Set the 200V power bank at 30mA for 70mins

Blocking:

1- Rinse membrane with 1X TBST
2- Incubate membrane in 5% blocking buffer for 1hr shaking at room temperature
3- Wash 3 times for 5 mins with 1X TBST

Primary antibody:

1- Incubate membrane in 1:15000 primary antibody for 1hr shaking at room temperature
    * Membrane can also be incubated overnight shaking at 4C
2- Wash 3 times for 5 mins with 1X TBST

Secondary antibody:

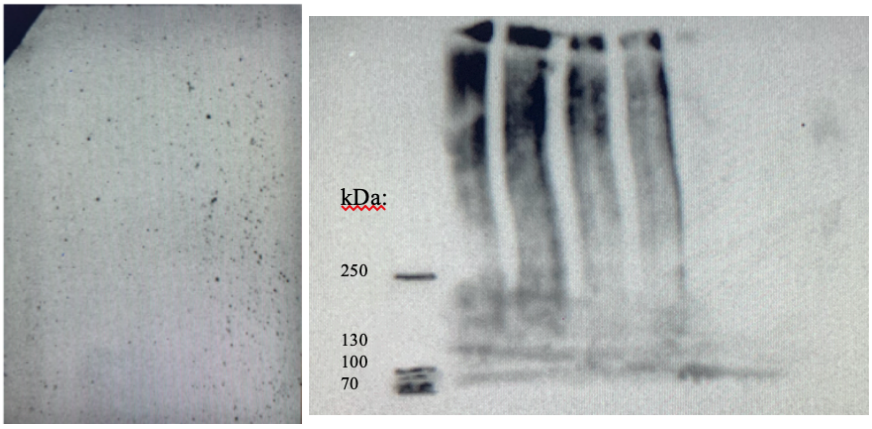
1- Incubate membrane in 1:10000 secondary antibody for 1hr shaking at room temperature
2- Wash 3 times for 5 mins with 1X TBST

Detection:

1- West pico detection kit: mix 500ul of each reagent
2- Select smart exposure; Increase exposure time if needed

Results:

Many Western blots have been run and troubleshooted to optimize the technique. As shown in Figures 7-8, the bands were not clear to be able to determine Lp(a) isoform size.



Figures 7-8

In March 2022, we have visited Professor Gilles Lambert at the University of Reunion (Reunion Island) to further troubleshoot the Western blot. After working in their labs and observing the optimized technique, we took aliquots of the antibody and standards to use in our labs at Sultan Qaboos University (Oman). As seen in Figure 9, the bands are clear and the Western blot will be repeated again using the same conditions but with more washing time to get clearer bands for analysis.

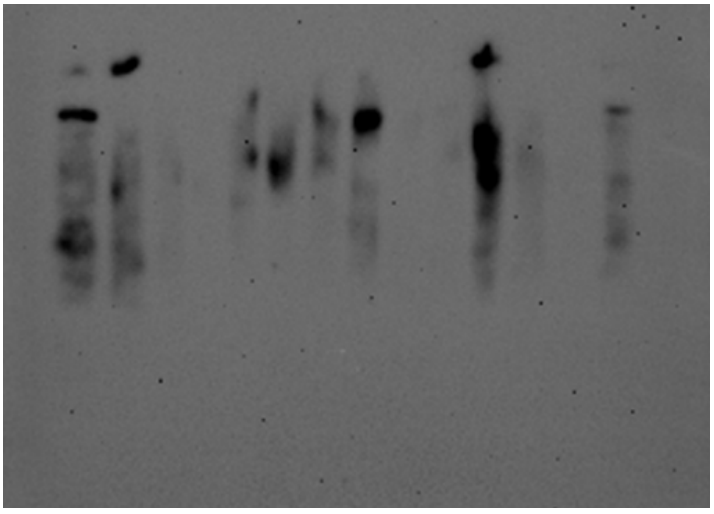


Figure 9.

4) Poster presentation at European Atherosclerosis Society congress 2022

I will be presenting this project as a poster at the 90th EAS Congress in Milan next month (Abstract #506).

Abstract

Lp(a) is a lipoprotein that resembles LDL lipoprotein with the exception of extra apoprotein (apo a) linked to apoB-100.[1] Apo(a) is highly polymorphic in size due to different numbers of a kringle 4 type 2 domain (KIV2). A positive correlation is shown between the concentration of Lp(a) and the severity of coronary artery disease (CAD). Elevated levels of Lp(a) have been associated with restenosis after percutaneous coronary intervention (PCI). The Copenhagen City Heart Study (CCHS) has found that extreme Lp(a) levels > 95th percentile predict a 3- to 4-fold increase in risk of myocardial infarction. In addition, the European Atherosclerosis Society (EAS) Consensus Panel recommends to screen patients for elevated Lp(a). The Lipid clinic in Oman measures Lp(a) in plasma but does not perform specific Lp(a) isoform measurement. The current proposal aims to optimize and validate the assay used to measure Lp(a) isoforms. We have evaluated PCR efficiency and coefficient of variation of the Lp(a) KIV-2 repeat genotyping assay, and correlated the repeat size in patients with familial hypercholesterolemia with confirmed mutation and mutation negative patients. We have further optimized a Western blot technique to measure apo(a) protein isoform size which will now be used for studies on patients with premature cardiovascular disease (CVD).

Outcome and Conclusion:

1) Protocol is optimized to measure lipoprotein (a) isoform size using molecular technique
2) Western blot technique was difficult to optimize and required training in a collaborating laboratory
3) The Western blot technique is now optimized and will be used for the study
4) Presentation of poster in EAS congress in Milan 2022

To Whom It May concern

Ms Razan AL Zadjali has worked in lipoprotein a isoform determination. She has spent excellent time in the laboratory and were able to present a poster in the upcoming European Society of Atherosclerosis meeting in Milan. She has also done short internship in an international lab. To Optimize the laboratory technique. **Ms Razan was working in this project from September 2021 April 2022.**

30th April 2022

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