Supplementary Online Content

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eAppendix. Supplementary Methods

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Lipoprotein(a) KIV-2 genotyping

The lipoprotein(a) KIV-2 size polymorphism was genotyped by real-time PCR analysis using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) and 384 well formats. This assay was developed specifically for the present study. Genotyping resulted in an estimate of the total number (sum of repeats on both alleles) of KIV-2 repeats. The single-copy gene *albumin* was used to normalize for different concentrations of DNA in different samples. Reactions were performed in 10 μL final volume, using 1xTaqMan Universal PCR Master Mix (Applied Biosystems), 900 nmol/L primers, and 200 nmol/L probe. Primer sequences were as follows: KIV-2 forward 5'-ATCCAGATGCTGTGGCAGCT-3', KIV-2 reverse 5'-GCGACGGCAGTCCCTTCT-3', albumin forward 5'-ACACGCCTTTGGCACAATG-3', albumin reverse 5'-CCCTGGAATAAGCCGAGCTAA-3'. The sequence for the FAM labeled KIV-2 probe was 5'-CAACCTGACGCAATGC-3', while the sequence for the VIC labeled albumin probe was 5'- TGGGTAACCTTTATTTCCCTTC-3'. Our KIV-2 assay design targeted exon 2 of the KIV-2 repeat. All samples were run in duplicate for both the KIV-2 assay and the albumin assay. A discrepancy of more than 0.25 in Ct value (threshold cycle of the PCR) for duplicate samples for either the KIV-2 or the albumin assay resulted in a rerun of that sample. We prepared re-

runs twice, therefore >99.9% of all participants with available DNA were genotyped. In addition to unknown samples, each 384-well plate also included a calibrator sample and two control samples. To improve precision, all 40486 samples were analyzed by the same technician, using the same calibrator and control samples, and the same ABI PRISM 7900HT Sequence Detection System. We observed an interassay coefficient of variation of Ct values of 6% and 6%, at the level of 28 and 43 KIV-2 repeats, respectively. The calibrator and control samples were kindly supplied by Professor Gerd Utermann and coworkers (Institut für Medizinische Biologie und Humangenetik, Universität Innsbruck, Innsbruck, Austria)[1].

As stated above, the realtime PCR analysis resulted in an estimate of the total number (sum of repeats on both alleles) of KIV-2 repeats. The genotype estimate was obtained for all participants as follows:

1) the mean Ct value for the KIV-2 assay duplicate samples was subtracted from the mean Ct value of the *albumin* assay duplicate samples yielding a ΔCt value

$$Ctmean_{alb} - Ctmean_{KIV2} = \Delta Ct$$

2) the ΔCt value of the calibrator sample was then subtracted from the ΔCt value of the unknown sample yielding a ΔΔCt value

$$\Delta Ct_{unknown} - \Delta Ct_{cal} = \Delta\Delta Ct$$

3) the ΔΔCt value was exponentiated to the base 2 and multiplied by the KIV-2 calibrator value

$$2^{\Delta\Delta Ct} * KIV2_{calibrator}$$

In theory, the number of KIV-2 repeats in a sample could be deduced solely from the difference in Ct values between the KIV-2 and the albumin assay without the need of a calibrator. However, the use of a calibrator is recommended, as the use of different flourochromes to label the different probes may affect the direct comparibility of results from different assays (according to correspondence with Applied Biosystems).

A prerequisite for the above calculations to be valid is that the KIV-2 assay and the albumin assay run with comparable and preferably high PCR efficiencies. The PCR amplification efficiency is the rate at which a PCR amplicon is generated. Ideally, a doubling of product is seen at each cycle, equivalent to a PCR efficiency of 100%. If the KIV-2 and the albumin assays did not run with comparable efficiencies the difference in Ct values may not only reflect a copy number variation, but also the differing PCR efficiencies of the two assays. In the present study, the PCR efficiencies of the two assays were evaluated by running twofold dilution curves of a DNA sample (range 32 to 0.2 ng per well) for each assay. Each standardcurve point was run in 3 replicates for each assay. When Ct values vs. log input DNA are plotted, a slope of -3.3 is equivalent to a 100% PCR efficiency, as a 100% efficient reaction will yield a 10-

fold increase in PCR amplicon every 3.32 cycles ($2^{3.32} = 10$). We observed comparable slopes of approximately -3.6 for the KIV-2 and the *albumin* assays, indicating comparable and acceptable PCR efficiencies of ~ 90 % (efficiency = $(10^{-1/slope} -1) * 100$). Too low a PCR efficiency may result in poor sample replicate precision; we observed good precision, with $r^2>0.99$ for triplicate dilution curves for each assay.

Lipoprotein(a) measurements

In participants attending the 1991-1994 examination of The Copenhagen City Heart Study, lipoprotein(a) total mass was measured, as described previously[2], with an in-house turbidimetric assay using a Technicon Axon autoanalyser (Miles Inc., Diagnostics Division, Tarrytown, NY, USA), rabbit anti-human lipoprotein(a) polyclonal antibodies (Q023, DAKO A/S, Glostrup, Denmark), and a human serum lipoprotein(a) calibrator (DAKO A/S). The polyclonal antibodies did not recognize apolipoprotein B and plasminogen, and the assay was insensitive to differences in apolipoprotein(a) isoform size. At the 2001-2003 examination of the CCHS and for CGPS participants, lipoprotein(a) was measured again immediately after sampling, using a sensitive immunoturbidimetric assay from DiaSys (DiaSys Diagnostic Systems, Holzheim, Germany) containing goat anti-human lipoprotein(a) polyclonal antibodies purified by immunoadsorption against apolipoprotein B and plasminogen. For the 4609 individuals with a lipoprotein(a) measurement in

both 1991-1994 and 2001-2003, we observed a minimal bias between the two measurements of 1.6 mg/dL, and an r^2 value of 0.81 ($p<0.001$) when comparing the two measurements using linear regression. Finally, for the first 5543 participants in the CGPS we also used the DiaSys method to measure lipoprotein(a) levels.

REFERENCE LIST

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(2) Kamstrup PR, Benn M, Tybjærg-Hansen A, Nordestgaard BG. Extreme lipoprotein(a) levels and risk of myocardial infarction in the general population: the Copenhagen City Heart Study. *Circulation.* 2008;117(2):176-184.