
CHAPTER 3

METHODOLOGY

3.1 INTRODUCTION

Limited information is available regarding the fibrinogen and fibrinogen γ' concentrations of black Africans and how these concentrations in this cohort are influenced by genetic polymorphisms and environmental factors. The main aim of the proposed study, therefore, was to determine the change in fibrinogen and fibrinogen γ' concentrations over a five-year period (from 2005 to 2010) in a black South African cohort (n≈2010), subdivided according to genotypes previously reported to associate with fibrinogen and fibrinogen γ' concentrations to ascertain whether the observed changes were influenced by genotype and whether they were modulated by environmental factors. Specific objectives were:

- To determine the baseline (2005) and follow-up (2010) concentrations of fibrinogen and fibrinogen γ' in a black South African population residing in the North West Province.
- To determine the genotype distributions of the FGA 2224 G>A [rs2070011], FGA 6534 A>G [rs6050], FGB 1038 G>A [rs1800791], FGB Arg448Lys [rs4220], FGB -148 C>T [rs1800787], FGB 1643 C>T [rs1800788], FGB 40 A>G [rs2227385], FGB 749 A>G [rs2227388], FGG 10034 C>T [rs2066865] and FGG 9340 T>C [rs1049636] SNPs in the black South African cohort.
- To determine the change in fibrinogen and fibrinogen γ' concentrations over the five-year period in participants harbouring the different genotypes, as mentioned above, of a black South African population in the North West Province.
- To assess the major lifestyle determinants, of fibrinogen and fibrinogen γ' concentrations in black South Africans.
- To determine whether the hypothesised changes in the fibrinogen and fibrinogen γ' in subjects harbouring the different genotypes were modulated by identified environmental factors.

This chapter will provide details regarding ethical approval and subject characteristics and recruitment. Details are also provided on experimental methods that were used to obtain data for the 2005 and 2010 analysis. The data collection and analysis of blood samples for the 2005 and 2010 analysis were done before this mini-dissertation was begun, except for the blood sample analysis of total fibrinogen and fibrinogen γ' of 2010, which was conducted by the student of the present study and qualified laboratory technicians from the Centre of Excellence for Nutrition at the North-West University, Potchefstroom Campus.

3.2 ETHICAL APPROVAL

The study was conducted according to the Declaration of Helsinki (World Medical Association, 1964). The Ethics Committee of the North-West University, Potchefstroom, South Africa, approved this study [for 2005 and 2010 the ethics numbers were 04M10 (Addendum A) and NWU-00016-10-A1 (Addendum B), respectively]. The purpose of the study and all procedures were explained to the subjects in their home language [Addenda C (2005) and D (2010)]. Subjects gave written informed consent before being recruited [Addenda E, F (2005) and G (2010)]. Participants had the option of withdrawing from the study at any time.

3.3 STUDY POPULATION

3.3.1 Recruitment

Screening of the study population for the South African arm of the International Prospective Urban and Rural Epidemiology (PURE) study was performed by a census which included 6000 black South African households, starting from a randomly selected address in rural and urban communities. The rural communities (Ganyesa and Thlakangeng) were still under tribal law and the urban community (Ikageng) resided in informal and formal settlements surrounding a city (Potchefstroom). The census was performed by trained fieldworkers, starting from a specific point in the community and then completing questionnaires regarding the number of people in the household, their age and health status (Addendum H).

The head of the household gave signed consent to complete the questionnaire and if a person refused or was not at home, the next household was visited, until 6000 households had been included. After the data had been obtained, a paper selection was made according to the inclusion and exclusion criteria (see below), and 4000 subjects (2000 participants from rural and urban settlements, respectively) were identified. An extensive questionnaire (Addendum I) regarding physical and psychological health, socio-economic background, lifestyle practices and support systems was completed for the 4000 subjects after they had given voluntary and informed consent. From the completed questionnaires, a total of 2792 subjects (which included 1444 rural and 1348 urban subjects) were identified as eligible, based on the inclusion and exclusion criteria (see below). Of these, 2010 apparently healthy black South African women (n=1261) and men (n=749) from both rural (n=1006) and urban (n=1004) settlements finally participated in the study when blood was collected in 2005 and were prospectively enrolled in this study.

In 2010, only 1288 (699 from rural and 589 from urban communities) participants, of which 435 were men and 853 were women, returned for the follow-up blood collection. The reasons for the decrease in the study population from 2005 to 2010 included the deaths of a number of participants (n=233), subjects withdrawing from the study, subjects failing to show up on the day of the follow-up blood collection, participants with acute illness on the day of blood collection and relocation of subjects.

In this mini-dissertation, baseline (n=2010) and follow-up data (n=1288) collected from the South African PURE population during 2005 and 2010 were used for the analyses.

3.3.1.1 Inclusion and exclusion criteria

Apparently healthy household members (men and women) between the ages of 35 and 65 years, from rural and urban households, were eligible. Exclusion criteria included failure to provide consent, usage of chronic medication, reported chronic diseases, tuberculosis or known HIV infection and any known acute diseases. Subjects who were planning to move away from the area in the foreseeable future were also excluded from the study, as this study was planned to run for 12 years.

3.4 STUDY DESIGN

The PURE study is a prospective cohort study that intends to track changing lifestyles, risk factors and chronic disease by using periodic standardised data collection in urban and rural areas of seventeen countries in transition, including South Africa.

3.5 ANTHROPOMETRICAL ASSESSMENT

The weight of participants, wearing minimal clothing and not wearing shoes, with arms hanging freely at their sides, was measured in kilograms using portable electronic scales (Precision Health Scale, A&D Company, Japan). Before each weight measurement was performed it was ensured that the scale was zeroed and that the scale was stable on a horizontal floor. The scales were calibrated on every measurement day before the measuring started. The height of participants, not wearing shoes and with arms hanging freely at their sides, was measured using a stadiometer (IP 1465, Invicta, London, United Kingdom). For the height measurement, subjects stood upright with their heads in the Frankfort plane (a reference plane passing horizontally through the inferior orbit of the eye to the tragon of the ear) and their heels together. In addition, subjects' heels, shoulders and buttocks had to be in contact with the stadiometer. The height was recorded to the nearest 0.1 cm. BMI was calculated by dividing weight by stature squared (kg/m^2).

3.6 BLOOD PRESSURE MEASUREMENTS

Before the blood pressure of participants was measured, participants had to rest and be calm for more than five minutes. The participants were required not to have climbed stairs, exercised, eaten or smoked 30 minutes prior to the measurements. The participants had to sit in an upright position feeling relaxed, with the right arm supported at heart level. The systolic blood pressure and diastolic blood pressure as well as the heart rate were recorded with the Omron automatic digital blood pressure monitor (Omron HEM-757).¹

¹ Blood pressure was determined by members of the Hypertension in Africa Research Team (HART) of the North-West University, Potchefstroom

3.7 ADULT QUESTIONNAIRE

General instructions regarding the way the questionnaire had to be handled and completed were given to the fieldworkers. This questionnaire (Addendum J) covered basic descriptive characteristics of the household, such as demographic information, information regarding alcohol and tobacco use, the use of medication and the health status of the participant and his or her household members. The following demographic information was collected from the participants: gender, age, ethnicity, marital status, education level, occupation and employment status. The fieldworkers were Setswana-speaking and were trained to use the English questionnaire. Face-to-face interviews were conducted with each participant.

3.8 DIETARY INTAKE ANALYSIS

For the determination of dietary intakes and habitual alcohol consumption of the participants, interviewer-based, culture-sensitive quantitative food frequency questionnaires (QFFQ) were completed (Addendum K). The type and the amount (volume) of beverage ingested was indicated by the participants and the total daily ingestion of alcoholic beverages was calculated by dividing the total amount of alcoholic beverage ingestion indicated in one month on the QFFQ by 30 (alcoholic beverage ingestion was reported for the previous month by each participant). The alcohol content of each beverage was also used to determine the alcohol consumption of each participant by determining the ethanol content of each alcoholic beverage. The dietary data were computerised by the *Foodfinder3*[®] program (Medical Research Council, Tygerberg, 2007) and nutrient analysis of the dietary data was done by the Medical Research Council of South Africa.

3.9 ASSESSMENT OF PHYSICAL ACTIVITY

Urban and rural-specific forms (Addendum L) developed by Vaz *et al.* (2005) were used to determine physical activity cost. The physical activity index, developed and tested in the Transition and Health during Urbanisation in South Africa (THUSA) study, was used (Kruger *et al.*, 2000).

3.10 BLOOD SAMPLING

Qualified nursing staff and sisters collected 90 ml fasting (at least 8 hours without any food or drink, excluding water) blood per participant from the antecubital vein in the right arm with a disposable needle, before 11:00 in the morning. After collection, the tubes (vacutainers) were inverted gently. Inversion of collection tubes ensured thorough mixing of all the contents in the tube. The collection tubes were filled to their maximum capacity.

For the analysis of lipids, Interleukin-6 (IL-6) and CRP concentrations, serum samples were prepared by collecting blood in tubes without additives and this was allowed to clot at room temperature for 30 minutes before being centrifuged at 2000 x g for 15 minutes at 10°C. For the analysis of glucose and glycated haemoglobin (HbA1C), plasma samples were collected from fluoride and ethylenediamine tetra acetic acid (EDTA) tubes which were centrifuged, within 30 minutes after collection, at 2000 x g for 15 minutes at 10°C. For the analysis of fibrinogen and fibrinogen γ' , plasma samples were prepared by collecting blood in citrate tubes which were kept on ice until centrifugation at 2000 x g for 15 minutes at 10°C. For both serum and plasma samples, the supernatant resulting after centrifugation was aliquoted and snap frozen before being stored in cryofreezers. For the determination of SNPs, samples were collected in blood collection tubes containing citrate. Centrifugation of tubes was done at 10°C for 15 minutes at 2000 x g. The resulting leukocyte layer (buffy coat) of the blood was transferred to storage tubes, which were snap frozen and stored at -82°C until deoxyribonucleic acid (DNA) isolation and further analysis. The blood collection procedure of the blood samples for the years 2005 and 2010 was the same.

3.11 TOTAL CHOLESTEROL, HDL-CHOLESTEROL AND TRIGLYCERIDES

The total cholesterol (TC), HDL-cholesterol and TG concentrations of the 2005 blood samples of the participants were measured using the Sequential Multiple Analyser Computer (SMAC). TC was analysed by oxidase-peroxidase and phenol aminoantipyrine reagents with a KonelabTM auto analyser (Thermo Fisher Scientific, Vantaa, Finland). HDL-cholesterol was enzymatically analysed by cholesterol oxidase and polyethylene glycol, also using the KonelabTM auto analyser (Thermo Fisher Scientific, Vantaa, Finland).

TG was also analysed by the KonelabTM auto analyser (Thermo Fisher Scientific, Vantaa, Finland).²

The TC, HDL-cholesterol and TG concentrations of the 2010 blood samples of the participants were measured using Cobas Integra 400 plus (Roche, Basel, Switzerland), where TC and TG were determined by enzymatic colorimetric methods, and HDL-cholesterol was determined by a homogeneous enzymatic colorimetric assay.³ The analyses of TC, HDL-cholesterol and TG for 2005 and 2010 were conducted using analysers from different companies, although the principle of the methods was the same.

3.12 LDL-CHOLESTEROL

For the determination of LDL-cholesterol of the participants the same calculation was used for the 2005 and 2010 samples, which is the Friedewald formula as indicated below.

$$C_{LDL} = \frac{C_{plasma} - C_{HDL} - C_{TG}}{5}$$

3.13 HIGH SENSITIVITY C-REACTIVE PROTEIN

High sensitivity CRP concentrations of the 2005 blood samples of the participants were measured by using SMAC and the KonelabTM auto analyser (Thermo Fisher Scientific, Vantaa, Finland). Anti-human CRP coating microparticles were added to buffered samples and the immunoprecipitation was measured at 540 nm when the reaction had reached its final step. The amount of CRP in the solution was relative to the change in absorbance.⁴

CRP concentrations of the 2010 blood samples of the participants were measured using the Cobas Integra 400 plus (Roche, Basel, Switzerland), where CRP was determined by a high-sensitivity particle-enhanced turbidimetric assay.⁵

² TC, HDL-cholesterol and TG analysis for 2005 samples was done at the Vaal University of Technology

³ TC, HDL-cholesterol and TG analysis for 2010 samples was done at the North-West University, Potchefstroom

⁴ CRP analysis for 2005 samples was done at the Vaal University of Technology

⁵ CRP analysis for 2010 samples was done at the North-West University, Potchefstroom

The analyses of CRP for 2005 and 2010 were conducted using analysers from different companies, although the principle of the methods was the same.

3.14 GLUCOSE

The participants had to be in a fasting state (at least 8 hours without anything to eat or drink, including water) and the glucose concentrations of the participants for the 2005 and 2010 blood samples were measured by the hexokinase method, using the Synchron[®] System (Beckman Coulter Co., Fullerton, CA, USA) and its reagents. The Synchron[®] System (Beckman Coulter Co., Fullerton, CA, USA) automatically divided the samples and reagents into cuvettes by the ratio of one part of the sample to 100 parts of the reagent. The change in absorbance was relative to the concentration of glucose reagent in the sample, which was monitored at 340 nm. The change in absorbance was used to calculate and express the glucose concentration.

3.15 GLYCATED HAEMOGLOBIN

The HbA_{1c} of the participants for the 2005 and 2010 blood samples was measured by the Bio-Rad D-10[™] Haemoglobin A_{1c} testing system (Hercules, CA, USA), which is an automated device using ion-exchange high-performance liquid chromatography.

3.16 INTERLEUKIN-6

The IL-6 concentrations of the 2005 and 2010 blood samples were measured by the use of an enzyme-linked immunosorbent assay (ELISA) on the Quantikine[®] HS ELISA apparatus (R&D Systems, Minneapolis, USA). Six hundred blood samples (300 were HIV positive) were analysed in 2005 and 306 blood samples were analysed in 2010.

3.17 FIBRINOGEN

The fibrinogen concentrations of the 2005 blood samples of the participants were measured by the use of a modified Clauss method with the Multifibrin U-test kit on the Dade Behring BCS coagulation analyser (Multifibrin U-test, Dade Behring, Deerfield, USA). This method is based on coagulation of citrated plasma, where a large amount of thrombin is added and the coagulation time per blood sample is determined by the amount of fibrinogen in the blood sample (Mackie *et al.*, 2003).

A calibration curve was calculated by a fibrinogen calibrator kit and the concentration of fibrinogen, determined from a reference pooled blood sample, was indicated in g/L.⁶ The fibrinogen concentrations of the 2010 blood samples of the participants were also measured by the use of a modified Clauss method, but an Automated Coagulation Laboratory (ACL), with products from Instrumentation Laboratory (Milan, Italy), was used. This method is based on the prothrombin time where there is rapid thrombin generation and fibrin formation (Mackie *et al.*, 2003).⁷

The analyses of fibrinogen for 2005 and 2010 were done using analysers from different companies, although the principle of the methods was the same. To test possible differences in fibrinogen concentration due to the use of the two different analysers, the analysis of fibrinogen concentration of 140 randomly selected 2005 blood samples was repeated on the ACL and the mean difference was 0.7 g/L. Thus the results were found to be comparable.

3.18 FIBRINOGEN γ'

Fibrinogen γ' of the 2005 and 2010 blood samples of the participants was measured by the use of an ELISA on a Thermo Scientific (Multiscan FC) spectrophotometer, according to the method of Uitte de Willige *et al.* (2005).⁸ See Addendum M for the detailed fibrinogen γ' analysis methodology. Fibrinogen γ' is not only reported as absolute concentration (g/L) but is also expressed as relative concentration by expressing it as percentage (%) of total fibrinogen concentration, reported as γ' ratio.

⁶ Fibrinogen analysis for 2005 samples was done at the Vaal University of Technology

⁷ Fibrinogen analysis for 2010 samples was done at the North-West University, Potchefstroom

⁸ Fibrinogen γ' analysis for the 2005 and 2010 samples were done at the North-West University, Potchefstroom

3.19 HIV TESTING

Pre-test HIV counselling was given to participants in groups of ten. Participants were given information about the pathophysiology of HIV and acquired immunodeficiency syndrome (AIDS), methods of transmission of the disease, test procedures, ensuring of confidentiality of test results and the use of anti-retroviral therapy. HIV testing was done on-site according to the protocol of the South African Department of Health and the UNAIDS/WHO Policy Statement on HIV testing (UNAIDS/WHO: 2004). HIV testing was done by the Rapid HIV test (PMC Medical, India) and if the results were found to be positive, another test, the Pareeshak test (BHAT Bio-tech India), was performed and was used to confirm the results.

If participants chose to know the results of their test, private post-test counselling was given. Depending on their test results, follow-up plans were made with the participants, such as referring participants who were found to test positive for HIV to the nearest clinic for a confirmation cluster of differentiation 4 (CD4) test, and counsellors were available to participants if necessary.

3.20 GENETICS

3.20.1 DNA isolation

DNA isolation of the 2005 blood samples was performed by the FlexiGene™ DNA extraction kit (QIAGEN® Valencia, CA), which is a manual isolation method. Lysis buffer was added to 400 µl buffy coat of each participant and, after protein digestion by protease, the DNA was precipitated by isopropanol. The DNA was then recovered by centrifugation, washed with 70% ethanol and dried. Lastly, the DNA was resuspended by the addition of hydration buffer.

DNA isolation of 350 samples that initially yielded low concentrations of DNA was repeated by using the Maxwell® 16 System (Promega, Madison, WI), which is an automated system. It uses paramagnetic particles or beads to purify samples by providing a mobile solid phase which optimises capture, washing and elution of the target DNA (Maxwell® 16 DNA purification kit technical manual, 2010).⁹

⁹ DNA isolation was done at the North-West University, Potchefstroom

3.20.2 DNA amplification

Specific primer sets were designed by the Primer-BLAST program (software program, provided by Integrated DNA Technologies) for the region of interest. These primers were used for amplification of the specific DNA sequences needed for genotyping of specific polymorphisms of the fibrinogen gene.

Amplification of DNA sequences was done by the real-time polymerase chain reaction (rtPCR), with specific protocols for each primer. The primers indicated in *Table 3.1* were used for the DNA amplification.¹⁰

Table 3.1: Sequencing primers for the β -fibrinogen gene

Primer	Sequence	Annealing temperature
FGB_01_F	5' - GGT CAG AAG GAG GAT GGA TT - 3'	59°C
FGB_01_R	5' - CGC CTA CTA TGT CTG TCT TGC - 3'	59°C
FGB_08_F	5' - GAA TTT GGG AGG ACA ACC TA - 3'	62°C
FGB_08_R	5' - AAA GAT GGC AGG TTA TCA GG - 3'	62°C
FGB_11_F	5' - TGG ATT ACT GAC TGG TGT TCC - 3'	59°C
FGB_11_R	5' - TGT TAT TTT AAC ACT GGT GAA GTC T - 3'	59°C
FGB_13_F	5' - TCG GAG CTT GTG TAG TTT CC - 3'	59°C
FGB_13_R	5' - TCA TAC AAC TAT TAT TCT TTG TTG GTC - 3'	59°C

A = Adenine; C = cytosine; F = Forward; FGB = Fibrinogen β ; G = guanine; R = Reverse; T = Thymine

3.20.3 DNA sequencing

Sequencing of the promoter region of the β -fibrinogen gene of 30 randomly selected subjects of the PURE study was performed at the Central Analytical Facility of Stellenbosch University (ABI 3130 XL Genetic Analysers) to determine which of the SNPs are prevalent in the African population. The DNA was sequenced by fluorescently labelled cycle sequencing, which is based on the principles of Sanger sequencing (Sanger *et al.*, 1977).¹¹

¹⁰ DNA amplification was done at the North-West University, Potchefstroom

¹¹ DNA sequencing was done at the Central Analytical Facility of the University of Stellenbosch

3.20.4 Haplotyping

Haplotype analysis [molecular genetic testing which identifies closely linked segments of DNA (Nussbaum *et al.*, 2004)] was used to identify tagging SNPs [minimal informative subset of SNPs linked to a limited number of haplotypes in a block (Lin & Altman, 2004)]. The haplotype block structure was determined by using Haploview and haplotype-tagging SNPs were selected, preferring potentially functional SNPs. The following SNPs in the fibrinogen β gene were selected: FGB 1038 G>A [rs1800791], FGB 1643 C>T [rs1800788], FGB 40 A>G [rs2227385] and FGB 749 A>G [rs2227388].¹²

3.20.5 Genotyping of fibrinogen gene single nucleotide polymorphisms

Genotyping of SNPs of the fibrinogen gene was done at the University of the Witwatersrand, Johannesburg. The genotyping was performed by using Illumina's VeraCode technology and was determined by using the BeadXpress™ Reader. Illumina's VeraCode technology is a robust detection method for multiplex assays requiring high precision, accuracy and speed. Illumina's VeraCode technology consists of cylindrical glass microbeads which measure 240 microns in length by 28 microns in diameter. This technology precisely implants digital holographic elements within each microbead, creating unique bead types carrying high-density codes. The microbeads are then activated by a laser, resulting in the release of a unique coded image which can be detected by the BeadXpress™ Reader by the use of a dual-colour detection system. The following SNPs in the fibrinogen gene were identified for genotyping: FGA 2224 G>A [rs2070011], FGA 6534 A>G [rs6050], FGB 1038 G>A [rs1800791], FGB Arg448Lys [rs4220], FGB -148 C>T [rs1800787], FGG 10034 C>T [rs2066865] and FGG 9340 T>C [rs1049636].¹³

¹² Haplotyping was done at the North-West University, Potchefstroom

¹³ Genotyping was done at the University of the Witwatersrand, Johannesburg

3.21 STATISTICAL ANALYSIS

The computer software packages IBM® SPSS® Statistics 21 version 21 (Statistical Package for Social Sciences, IBM, New York, USA) and Statistica® version 11 (Statsoft Inc., Tulsa Oklahoma, USA) were used for statistical analysis of the data. The analyses were regarded as statistically significant if the p-value was less than or equal to 0.05. Normality of variables was tested and if variables were determined to be non-parametric, they were log transformed to improve normality to allow parametric statistics but still reported as median (25th-75th percentiles). Independent t-tests were performed when two groups containing different individuals were compared (e.g. men vs women or rural vs urban, or two genotypes). Analysis of variance (ANOVA) with Tukey's honest significant difference *post-hoc* tests for unequal n was performed when more than two groups were compared. Analysis of covariance (ANCOVA) was performed when adjustments were required. Paired t-tests were performed when two groups containing the same individuals were compared (e.g. 2005 vs 2010 comparison). Change over time in the fibrinogen variables between groups were determined by calculating delta variables (2010 – 2005 data). Independent t-tests (comparison between two groups) and ANOVA with Tukey's honest significant difference *post-hoc* tests for unequal n (comparison between more than two groups) were conducted to compare the delta variables. Pearson correlations were performed on the log transformed data, for the 2005 and 2010 data separately to determine associations between continuous variables. Hardy-Weinberg equilibrium was determined for all the SNPs investigated by the Chi-square analysis, and if a p-value was less than or equal to 0.05, the SNP was determined to not adhere to the assumptions of Hardy-Weinberg equilibrium (Hardy, 1908). The 95% confidence intervals (CI) of the SNPs were calculated using the SAS® System version 9.3 TS level 1M0 (SAS Institute Inc., Cary, NC, USA). Genotype distribution and observed minor allele frequency (MAF) were also determined for all the SNPs. Pair-wise linkage disequilibrium (LD) was determined using the Haploview software version 4.2, by calculation of the standardised disequilibrium (D') and correlation coefficient squared (r^2) values. SNPs are said to be in strong LD if the D' is 1, and strong linkage equilibrium exists if the D' is 0 (Ardlie *et al.*, 2002). If the r^2 is 0, the SNPs are in complete linkage equilibrium, whereas if the r^2 is 1, the SNPs are in complete LD (Ardlie *et al.*, 2002).

In order to determine if gene–environment interactions affected fibrinogen and fibrinogen γ' cross-sectionally and also the change in concentrations over the five-year period, a mixed-models approach was used, incorporating both 2005 and 2010 data. Missing data for environmental factors were imputed using multiple-imputation methodology (10x). Models were created for total fibrinogen concentration as well as for the γ' ratio as this is considered to be a true reflection of the relative fibrinogen γ' concentration in plasma (Pieters *et al.*, 2013). Different models were created based on the different interaction terms entered as fixed effects in the models *i.e.* a model for gene–environment interaction (to determine whether gene–environment interactions influenced the fibrinogen variables cross-sectionally); environment–time interaction (to determine whether environmental factors influenced the change over time); gene–time interaction (to determine whether genetic factors influenced the change over time); and gene–environment–time interactions (to determine whether there were gene–environment interactions that influenced the change over time). Time was entered as a categorical variable indicating the two time points at which data was collected (2005 and 2010). Gender, tobacco history, HIV status, BMI category, urbanisation status, alcohol category, systolic blood pressure, age, LDL-cholesterol, HDL-cholesterol, HbA1c, CRP, IL-6 and weighted physical activity index (WPAI) were entered as covariates in each model. Each model was repeated three times. At first, all genotypes and environmental factors were entered as main effects. Additionally, interaction terms for all combinations of main effects were entered in order to determine which interactions existed (this data is not reported). From this initial step, the model was modified to include only the significant interactions in order to reduce background noise (variables not contributing to the model). Data for this second step is reported in this study. In order to further refine the model, we finally entered only the significant main effects and interaction terms in order to include only factors that contributed significantly to the model. This data is also reported.

The results of the statistical analyses will be reported in the ensuing chapter.