

CHAPTER 3

METHODS

3.1 Introduction

This study formed part of the THUSA-survey, an epidemiological study done in the Northwest province of SA during 1996, 1997 and 1998. THUSA is an acronym for Transition and Health during Urbanization of South Africans. The survey was performed by a team of 36- 50 researchers and field workers on 1854 subjects. The study was approved by the Ethics Committee of the PU for CHE (Ethics no HHK 4M5-95). Additional approval to test anonymously for HIV was obtained from the same committee in 1998.

The major aim of the THUSA-study was to assess the impact of urbanisation and the consequent demographic transition on health determinants and status of Africans in the Northwest province, in order to provide information for the development of appropriate health interventions. The aim of this sub-study was to investigate the metabolic syndrome in the participating subjects. Because only “apparently healthy” subjects were recruited, the results of this study will not provide information on the incidence nor prevalence of diseases or risk factors in this population.

3.2 Study design

THUSA was a cross-sectional, comparative survey. The population studied consisted of a convenience sample of “apparently healthy” Africans in the Northwest province. A stratified sampling technique was used to recruit subjects. With the help of the Statistical Consultation Services of the PU for CHE, the Northwest province was divided into four quarters. In each quarter sites representing five levels of urbanisation were randomly chosen. Volunteers were recruited from 37 selected sites. Logistical considerations such as taking a blood sample before 12h00 from fasted subjects, a period of 6-10 hours needed for completion of questionnaires, and a culture of voluntary participation opposed to random selection participation, prevented the selection of a truly random sample. The five strata were: stratum 1: deep rural people; stratum

2: farm dwellers; stratum 3: squatter camp dwellers; stratum 4: urban “middle class” citizens; stratum 5: urban “upper class” citizens.

Although a proportional sample stratification was planned, the research team was forced to adopt a disproportional sample stratification of subjects in some regions due to the non-availability of reliable subjects. This could have introduced some bias in the sample population in the sense that unemployed women in certain strata were more “available” than employed men. Therefore, special attention was given to the recruitment of sufficient men and women of all levels of urbanisation for comparative purposes.

3.3 Subjects

A sample of 1854 apparently healthy subjects participated voluntarily in the study after they signed an informed consent form. A cross was accepted from those subjects who were illiterate. This informed consent form as well as all research procedures were explained to the subjects by a Setswana-speaking fieldworker.

Although all subjects were supposed to be fasted, it turned out that the majority had a drink with sugar or a fruit before coming to the venues. The way in which the subjects were interviewed to decide whether they were fasted or not, did not provide sufficient information for a confident decision regarding the fasting state of each subject. Unfortunately it was only discovered after the first 900 subjects were examined. Therefore, only the subjects studied during the last year of the study (1998) were suitable to include in this study. Those subjects who were definitely fasted were selected to investigate the characteristics of the metabolic syndrome. These were 193 men and 233 women, between the ages of 15 and 65 years.

3.4 Exclusion criteria

The following subjects were excluded:

- Pregnant and/or lactating women.
- Subjects with a temperature above 37,5°C.
- Acute or chronic ill subjects: all subjects suffering from infectious or **known** chronic diseases like DM, hypertension, CHD, CVD, epilepsy, TB, etc.

- Subjects younger than 15 years and older than 65 years.
- Users of chronic medication.
- Inebriated subjects.
- Visitors to the area.
- Subjects fasting for less than 8 hours.

3.5 Research methods

A variety of quantitative and qualitative research techniques were used by a multi-disciplinary team to collect data.

3.5.1 Questionnaires and interviews

Although dietary and psychological data were also obtained by questionnaires only the questionnaires relevant to this study will be discussed. The relevant questionnaires were designed and validated for this population group and are given in Addenda 1 to 7.

3.5.1.1 Recruitment of subjects and informed consent

Each research site was visited by the study leader or her representative one week prior to the arrival of the research team. During that visit a suitable venue was identified and arranged. Suitable community leaders who had the ability to inform the community about the THUSA-study were also identified and educated about the aim and conditions of the THUSA-study. These leaders had to notify potential participants on the THUSA-study, explain the conditions of the study such as “to be fasted”, to bring identification documents, and to inform them about the date, time and venue of the arrival of the team.

On arrival of the team, the study leader with a Setswana-speaking fieldworker recruited the subjects according to the exclusion criteria set in 3.4 after they explained the aim of the THUSA-study to the volunteers. They also explained the research procedures and the time that will be taken up by the research (normally 6 to 10 hours). After the subjects signed an informed consent form (Addendum 1) they were given a number and sent to the next experimental station.

3.5.1.2 Demographic and socioeconomic data

During an individual interview with each subject, a questionnaire in which demographic data as well as socioeconomic and lifestyle habits were reflected, was completed by researchers and/or a field worker. This questionnaire was developed and validated by the researcher, and is given in Addendum 5.

3.5.2 Physical examination

This included a clinical examination for the signs and symptoms of malnutrition, anthropometric measurements, blood pressure and oral temperature.

3.5.2.1 Clinical examination

Two trained nursing sisters (the researcher and another trained by her) examined each subject for signs and symptoms of malnutrition (under- and overnutrition). The examination included the thyroid, hair, skin, tongue, nails, glands, eyes, teeth, gums and lips. Oral temperatures were taken with a clinical thermometer. A person with a temperature above 37,5°C was excluded from the study. Blood pressure measurements were taken with 10 min intervals with a mercury sphygmomanometer (Tycoo ®, USA) with an adjustable cuff, while subjects were sedentary and calm (Addendum 4).

3.5.2.2 Anthropometric measurements

The Institute of Biokinetics, PU for CHE, was responsible for the training and standardisation of the researchers who did the anthropometric measurements of each subject (Addendum 6).

The following measurements were taken:

1. Height

Height was measured using an anthropometer (Invicta, 1465, UK). Measurements were taken with the head in the Frankfort Horizontal Plane. The subjects were asked to remove their outdoor clothes, shoes, caps, etc., and readings were taken avoiding parallax error.

2. Weight

Weight was measured with a calibrated electronic scale (Precision Health Scale, A+D Company, Japan). Subjects were weighed in their underclothes.

3. Waist circumference

The waist circumference was measured at the narrowest area below the rib cage and above the umbilicus as viewed from the front while the subject was standing in the anatomical position. An inelastic but flexible standard tape was used to measure the area at the end of a normal expiration.

4. Hip circumference

The hip circumference was measured at the point of greatest circumference around the buttocks with the subject standing in the anatomical position. The same inelastic, but flexible standard tape was used.

5. Skinfold measurements

The following skinfolds were measured in triplicate and the mean values were calculated and reported:

- triceps
- sub scapular
- supra-iliac
- abdominal
- thigh
- calf
- iliac-crest

Standardised skinfold callipers were used (John Bull, British indicators, Ltd).

6. Girth measurements

An inelastic, but flexible standard tape was used to measure the following girths:

- Relaxed upper arm
- tens upper arm
- forearm
- thigh - 1 cm below gluteal fold
- calf
- wrist

3.5.3 Calculation of indices

Additional variables were created by calculations from measured data.

3.5.3.1 Anthropometric indices

1. Waist-to-hip ratio

Waist-to-hip ratio was calculated from the measured waist circumference (cm) divided by the measured hip circumference (cm). This index provides knowledge on regional body fat distribution which is a valuable guide in assessing health risk for CHD and DM (Kaplan, 1989). According to Kaplan (1989) an android build will be indicated by an index $> 0,85$ and a gynoid build by an index < 0.85 .

2. Body mass index

BMI or the Quetelet index was obtained by dividing the measured weight (kg) by the square of measured height (m^2). Several researchers have shown that BMI is the preferred method to classify obesity as well as a convenient and reliable indicator of obesity in epidemiological studies (Bray, 1998 and Must *et al.*, 1991). An index of $<18.5 \text{ kg/m}^2$ is an indication of underweight, $18.5 - 24.99 \text{ kg/m}^2$ is the normal range, and an index of $25 - 29.99 \text{ kg/m}^2$ is an indication of grade 1 obesity, $30 - 39.99 \text{ kg/m}^2$ of grade 2 obesity and $> 40 \text{ kg/m}^2$ of grade 3 obesity (WHO, 1995:329).

3. Body density (BD)

The following formula was used to calculate body density:

BD of women = $1.0994921 - (0.0009929 \times \text{sum of skin folds: triceps, thigh, supra-iliac}) + (0.0000022 \times [\text{sum of skin folds: triceps, thigh, supra-iliac}]^2) - (0.0001392 \times \text{age})$ (Jackson *et al.*, 1980).

BD of men = $1.1043 - (0.001327 \times \text{thigh skin fold}) - (0.00131 \times \text{subscapular skin fold})$ (Sloan, 1967).

4. Fat percentage

% fat for women = $(5.03 / \text{BD}) - 4.59$

% fat for men = $(4.95 / \text{BD}) - 4.5$

5. Fat mass

Fat mass = (%fat / 100) x body mass

(Mc Ardle *et al.*, 1994).

6. Lean mass

lean mass = body mass - fat mass

(Mc Ardle *et al.*, 1994).

7. Percentage Body fat from girths (% fat)

Women:

% fat = (abdominal x 0.53) + (thigh measured 1 cm below gluteal fold x 0.82) - (forearm x 1.7).

Men:

% fat = (upper arm relaxed x 1,46) + (abdominal x 0.52) - (forearm x 2.14)

(Mc Ardle *et al.*, 1994).

3.5.3.2 Biochemical indices

Insulin sensitivity (IS)

Insulin sensitivity index(ISI) = 10 000 x the reciprocal of the (fasting insulin x fasting glucose).

(Donahue *et al.*, 1988).

For the purpose of this thesis the terminology “insulin resistance” will be used as alternative for a decrease in insulin sensitivity (Colagiuri and Brand Miller, 1997).

3.5.4 Sample collection

Blood and urine samples were collected. In this study only the blood samples were used and therefore the collection of blood samples will be described. Blood samples were collected to measure serum and plasma indicators/markers of risk factors for DM, hypertension and CHD.

3.5.4.1 Method of blood sample collection

The researcher, a trained registered professional nurse, was responsible for the blood sampling. There were always two additional registered professional nurses, trained by the researcher, to draw blood from each subject.

The baseline blood sampling was preferentially carried out between 08:00 and 11:00 to control for the effect of environmental temperature and circadian rhythms on the level of variables and to keep the period of fasting relatively constant.

A sterile disposable No. 21G Butterfly infusion needle was placed into the vena cephalica of each subject. A total of 75 ml blood (7 x 10 ml syringe and 1 x 5 ml syringe) was drawn at baseline from each subject with no (or the minimum) stasis. These blood samples were then immediately divided by the field laboratory staff to prepare:

- 2 x 5 ml citrated plasma for the analysis of fibrinogen and MPC (sodium citrate 0.11 mol/L; pH 4.5-4.8' 9:1).
- 1 x 5 ml fluoride serum for the analysis of fasting (t_0) glucose.
- 1 x 5 ml EDTA blood for the analysis of haematocrit and haemoglobin.
- 5 x 10 ml serum for the analysis of lipids, stress hormones, minerals, vitamins and other metabolites. These samples were left at room temperature to clot before centrifugation.
- 4 x 50 μ l blood pipetted as four drops onto blotting papers. These samples were dried and sealed in separate envelopes. The dry samples were sent to Wits University for the isolation of DNA.

The samples were centrifuged at 3000 rpm for 10 minutes and kept on ice until they were divided into prior marked Ependorff tubes (43/subject). In the field the aliquots were immediately placed in a standard freezer (-18 to -22 °C) and back in the laboratory, stored at -84 °C in a biofreezer until analysed.

The butterfly system was kept viable in the vein of all the subjects with a solution of 2½ U of 5000 μ U/ml Heparin in 5 ml 0,9% normal saline. The arm of each subject was

immobilised by making use of a splint.



Figure 3.1 The arms of the subjects were immobilised in a splint

3.5.4.2 Glucose Tolerance Test (GTT)

The researcher was also in charge of this station. The help of Ms Marelize van Staden and Ms Cecilia Snyman are acknowledged.

After the arm of the subject was immobilised with the splint, he/she received a 250 ml glucose drink (standard GTT procedure: 75g glucose dissolved in 250 ml water, which had to be drunk within a period of 5 minutes). The time was noted and after two hours another blood sample for preparation of 5 ml fluoride serum was taken. The butterfly system was removed after this sample was taken.

During the two hour waiting period, the subjects went from station to station to be interviewed by the different researchers to complete the different questionnaires. The subjects were kept as calm as possible and had nothing to eat or drink during that period.

To be able to give immediate feedback to the subjects on DM, blood glucose concentrations were measured from the initial 75 ml blood drawn as well as from the 5 ml blood drawn on time 120 minutes with a standardized glucometer (Ames® G_{x1} Miles,

USA) and glucostrips (Glucostix ®, Bayer Diagnostics, Ames, UK).

3.5.5 Biochemical analyses

A wide range of biochemical analyses were done to measure the risk factors/markers for DM, CHD, hypertension and nutritional status of the subjects. Due to the fact that this study focuses on the metabolic syndrome, only the biochemical analyses relevant to this will be reported. Some of the biochemical analyses were done by the researcher while others were done at larger institutions with standardised quality control laboratory protocols and methods.

3.5.5.1 Serum glucose (t_0 and t_{120})

Fasting serum glucose (t_0) concentrations were measured in the laboratory of the Department of Chemical Pathology; University of Pretoria as part of the DAX profile (DAX: discrete analyser, Technicon DAX 48) along with the Thistle and Murex quality control methods.

The serum glucose (t_{120}) concentrations were done by the researcher in the laboratory of the Department of Nutrition, PU for CHE with the Peridochrom ® glucose GOD-PAP method. This is an enzymatic colorimetric method based on the principles of Trinder, (1969). The Peridochrom ® kit (cat. No. 676543 and 676551; lot nr/ch.-B.: 69053501) of Boehringer Mannheim was used. Precinorm ® U was used as control and Precinorm ® UPX as precision control. The coefficient of variance of this method was 2.4%.

3.5.5.2 Serum insulin

The serum insulin concentrations were measured by the researcher and Dr. F.C. Eloff in the laboratory of the Department of Physiology, PU for CHE with the ¹²⁵I-Insulin-RIA kit (Cat. Nr.:IC 13021) of Immuno Biological Laboratories (IBL), Hamburg. In this procedure radio labelled insulin competes with unlabeled insulin for binding sites on anti-insulin immobilized to the inside wall of a tube. After a period of incubation, the contents of the tubes are decanted, washed and the radioactivity of the ¹²⁵I-Insulin bound to the anti-insulin on the solid phase measured in a gamma-counter (Packard

Cobra auto gamma counter, model B5003).

Standards of known insulin concentrations are run concurrently with the samples being assayed and a standard curve plotted. All samples and standards were done in duplicate. The unknown insulin concentration in each sample was calculated from this curve. The mean value of duplicate samples was calculated and given as pmol/L and the conversion factor used was: pmol/L x 0.138 = mU/L.

3.5.5.3 Serum lipogram

Total serum cholesterol(TC); HDL-C; LDL-C and triglycerides (TG) were analysed in the laboratory of the Department of Chemical Pathology of the University of Pretoria. (DAX; discrete analyser, Technicon DAX 48). The Thistle and Murex quality control methods were used.

3.5.5.4 Blood haematocrit

Haematocrit was determined in the field laboratory by Dr. F.J. Veldman using a haematocrit centrifuge with its tubes (Hettich Zentrifugen, Haematocrit 24D-78532, Tuttlingen).

3.5.5.5 Plasma fibrinogen

The plasma fibrinogen levels were measured with a functional method in the laboratory of the Department of Nutrition PU for CHE. The Automated Coagulation Laboratory Analyser (ACL 200™) and appropriate standard plasmas were used.

3.5.5.6 Additional biochemical analyses

- **Serum metabolites and liver enzymes**

Part of the DAX profile done by the University of Pretoria were serum urea, uric acid, creatinine, bilirubin and the liver enzymes ALP, GGT, ALT and AST.

- **Serum minerals and electrolytes**

Part of the DAX profile done by the Department of Chemical Pathology, University of Pretoria, were serum sodium, potassium, calcium, chloride and

magnesium.

- **Serum iron status**

Haemoglobin was determined in the field laboratory by Dr. F.J. Veldman using the colorimetric method from Boehringer Mannheim. Serum iron, ferritin, percentage iron saturation and TIBC were measured at the Medical Research Council (MRC) laboratories (National Nutrition Intervention Programme) in Tygerberg, Cape Town, with colorimetric methods from Boehringer Mannheim, Mannheim, Germany.

- **Human immunodeficiency virus (HIV) infection**

HIV status was determined with an enzyme-immunological method (Enzymun-Test ®, anti-HIV 1+2+subtype ϕ , Boehringer Mannheim, Mannheim, Germany) by Dr. F.J. Veldman.

3.5.6 Statistical analyses

The researcher, with the help of Prof. B. M. Margetts, University of Southampton, did the statistical analysis of the data with the SPSS 9.0 for windows- program of the SPSS Inc., 1989 - 1997. Prof. H.S. Steyn of the Statistical Consultation Service, PU for CHE assisted in the interpretation of results.

Non-parametric statistical tests were performed. Spearman's correlations were used to identify relationships between risk factors of chronic diseases of lifestyle and insulin sensitivity.

Frequency tests were used to calculate the number of subjects with risk factors for the diseases of lifestyle above normal ranges and expressed as a percentage of the studied subjects. These tests were also used to quantify the clustering effect of these risk factors.

To investigate conditional probabilities in the predictive value of certain variables for the development of insulin resistance and the metabolic syndrome, cross tab statistics (SPSS 9.0) were used to determine odds ratios. For the calculation of odds ratios, subjects who

were insulin resistant were compared per quartile of distribution per particular risk factor. Those in the bottom quartile were regarded as not exposed and those in the other quartiles as exposed to the risk factor.

Logistic regression models were used to estimate lifestyle risks in the development of insulin resistance and the metabolic syndrome. Stepwise linear regressions were used to describe the importance of all non-categorical variables which were associated with insulin sensitivity.

The GLM Multivariate procedure was used to investigate interactions between risk markers for the chronic diseases of lifestyle and insulin sensitivity, and to investigate the effects of some lifestyle factors on these interactions.