Chapter 4

Research Design and Methods
4.0 RESEARCH DESIGN AND METHODS

4.1 HUMAN SUBJECTS

For launching a systematic study to identify the genetic basis of type 2 diabetes in North Indians, several discussions with local clinicians including endocrinologists and diabetologists and medical experts at various medical institutes and hospitals were held. Based on the information gathered from pathologists and biochemists (running pathology laboratories) in different districts of Punjab, the “Khatri Sikh” community was selected as they have a higher degree of diabetes susceptibility compared to the other castes. We have selected to get the intact gene pool. The Khatri Sikhs, the most affluent among the Sikh caste groups are traders by profession and generally reside in cities or large towns and constitute about 6-7% of Sikh population in Punjab (Gupta, 1978). Most of this community migrated from Pakistan during partition in 1947 and settled in cities and small towns. They are of Aryan origin and are physically and culturally different from Jats and other caste groups (Gazitteer, 1917; Sharring, 1879). The Khatri Sikh community represent a genetically closed homogeneous population of northern India that is ideal for genetic studies.

4.1.1 Why Study Khatri Sikhs?

People of India do not constitute a single homogeneous community. The country of more than 1 billion people is the most diverse place in the world and constitutes about 4655 identifiable communities diverse in biological traits, languages, physical appearance, dress patterns, forms of worship, occupation, food habits and kinship patterns (Singh et al., 1994). People of India have a complex racial history complicated by the presence of caste system, which has prohibited inter-breeding to a greater degree. Although the ancient caste system in India was abolished legally in 1960, its practice still continues to determine the socio-economic and religious standing in the caste hierarchy and is very well governing the mating practices of nearly one sixth of world’s population. This has separated its people into numerous endogamous groups and interconnections, consequently interbreeding between various endogamous has remained at the minimal level due to social and cultural compulsions (Singh et al., 1994). People of different groups living side by side for hundreds or thousands of years are accustomed to retain
their separate identity by practicing endogamy (RoyChoudhury, 1979). Highly endogamous structure of this population is also evident from genetic patterns that are consistent with historic events in which invading Caucasoid males established the caste systems and placing the indigenous population who were more similar to Asians in the lower castes. DNA studies on Y chromosome and mitochondria also confirm that the people from the lower castes are more similar to Asians and the uppercastes are more European than Asian (Bamshad et al., 2001).

The Sikh religious moment originated in first half of 16th century started as a reaction towards the socio-economic conditions of India during the period between 15th and 16th century. Presently, 2% of Indian population follows this distinct and unique religion born 500 years ago (Gupta, 1978). Although the Sikh philosophy condemns the class or caste system, the division of community based on occupation-based castes remains deeply integrated even in Sikhs. The common Sikh castes groups are Khatri, Jat, Ramgarhia, Rajput, Lohar, Saini, Ramdasia etc. Khatri Sikh is one caste group of Sikhs that is most affluent among Sikhs. They are traders by profession and generally reside in cities or big towns of Punjab and adjoining areas. They have Aryan origin and physically and culturally different from Jats and other caste groups and have higher degree of diabetes susceptibility compared to other Sikh castes. Sikhs do not smoke because of religious and cultural compulsions and ~50% of them are life long vegetarians. Thus, this unique, highly endogamous population with high prevalence of the disease in the absence of traditional risk factors (smoking, BMI, meat-rich diet), may be helpful in identification of environmental and genetic factor responsible for the pathogenesis of type 2 diabetes in Khatri Sikh population of North India. We believe that the contribution of alleles at specific loci to T2DM susceptibility will likely to be different in North Indian Sikhs than among European Caucasians.

4.1.2 Recruitment of Study Subjects

The study sample included 600 human subjects (300 type 2 diabetics and 300 non-diabetic healthy controls) exclusively from Khatri Sikh community of North India. Khatri Sikh subjects originated from Punjab (Pakistan/India), Haryana, Delhi, Jammu and Kashmir were included in this study. We recruited only those who reported that all four
grandparents were Khatri Sikhs and only those with Khatri surnames. Excluded were the individuals of South, East and Central Indian origin, non Khatri Sikh, type-I diabetes (T1DM) or family member with T1DM, rare form of T2DM sub-type (MODY), and secondary diabetes (e.g. hemochromatosis, pancreatitis). The study was advertised region wise and type 2 diabetic patients and non-diabetic controls with no history of diabetes were invited to participate at the designated recruitment site. Potential participants identified at the referral centers/clinics or hospitals were invited to the designated clinical site and T2DM status assessed. The diagnosis of T2DM was based on clinical records and medication. After confirming eligibility, a full clinical examination was performed on each participant. For each subject, we confirmed a diagnosis of T2DM using recent criteria established by American Diabetes Association (2004) as follows: A medical record indicating either a fasting plasma glucose (FPG) levels ≥126 mg/dl or ≥7.0 mmol/l after a minimum 12-hour fast or 2-hour post glucose level (oral glucose tolerance test or 2-h OGTT) ≥200 mg/dl or ≥11.1 mmol/l on more than one occasion with symptoms of diabetes. The impaired glucose tolerance (IGT) was defined as the FPG levels 100-126 mg/dl (5.6-7.0 mmol/l) or 2-h OGTT of 140-180 mg/dl (7.8-11.1 mmol/l). In the absence of medical record information, we confirmed a self-reported T2DM case by establishing that there is regular treatment with hypoglycemic medication or by testing the self-reported T2DM cases by performing 2-h OGTT. Vast majority (97%) of the recruited patients had a confirmed diagnosis of T2DM prior to enrollment in this study. We performed 2-h OGTT on all those consenting subjects who either were borderline diabetic or previously reported hyperglycemic and were currently maintaining glycemic control with diet or exercise. Non-diabetic individuals were also given 2-h OGTT for ruling out the presence of IGT. Handicapped or elderly patients were also recruited from their homes after obtaining their consent.

4.1.3 Informed Consent

A model consent form was constructed to ensure the compliance to Indian, and International guidelines regarding the use of human subjects in research. All study protocols were approved by the research development committee of Panjab University, Chandigarh as well as the Human Subject Protection Committees of the participating hospitals in India. All researchers working in this study had completed the “Human
Participant Protection Education for Research Teams” an online course, sponsored by the National Institute of Health (NIH), USA.

4.1.4 Diabetes Questionnaire

A standard “Diabetes Questionnaire” was administered in which subjects provided information about age, sex, and disease status of living siblings, parents, adult biologic offspring and extended family members. Information on questionnaires also included smoking, alcohol, diet, physical activity, and migration history, household information, diabetic illness in family and medication. We also recorded information regarding the date and mode of diagnosis, clinical profile and medication use and associated diabetic complications.

Physical activity was scored as 1, 2, 3 based on the level of activity performed based on the forms designed for assessing the activity level. Activity score ‘1’ was given to those who performed 25-30 minute brisk walk or jogging or regular aerobic exercises or yoga for 5-7 days a week. Slow walkers or those performing occupational activities including, walk to work, house hold jobs, or regular light exercises were scored ‘2’ and very less active with sedentary life-style or critically sick individuals were scored ‘3’.

Alcohol consumption was specifically probed in the dietary interviews using detailed questionnaire and quantified by weekday and weekend intakes of beer, wine or whiskey. In dietary interviews, we also probed for the medium of cooking, (commonly butter-derived fat called ‘ghee’ or vegetable oils are used for Indian cooking). Vegetarian/non-vegetarian food habits and frequency of non-vegetarian food intake was also probed.

4.2 PHENOTYPIC EVALUATION OF STUDY SUBJECTS

Direct physical examination was performed to evaluate the severity and progression of diabetes-related complications reported in patient’s medical records as described by Voter and Peters, 2004. Information regarding age at the time of diagnosis, Coronary Heart Disease or stroke, coronary artery bypass graft (CABG) or angioplasty, and medication was obtained from patient records. Coronary artery disease (CAD) was diagnosed based on a history of documented myocardial infarction and/or drug treatment for CAD (aspirin or nitrates). Hypertension was defined as a self-reported history of
physician diagnosis or subjects who were receiving drug treatment for hypertension or a systolic blood pressure (SBP) of $\geq 140$ mm Hg and/or diastolic blood pressure (DBP) of $\geq 90$ mm Hg. Ocular complications including cataract, glaucoma, vision problems, ophthalmic nerve problems, retinal pigmentations, hemorrhages, and loss of sight was recorded based on medical records. Neuropathy included both peripheral and autonomic neuropathies. Peripheral neuropathy was recorded as tingling or burning of fingers or toes, numbness, or diminished sensation at lower extremities and/or sharp pains or cramps. Autonomic neuropathy included uncontrolled urinations, sexual dysfunction, perfused sweating at night or while eating, and hearing loss. Medical record indicating kidney complications included microalbuminuria, proteinuria, renal failure, transplant or dialysis.

4.3 ANTHROPOMETRIC MEASUREMENTS

Standard anthropometric measurements were performed including stature, weight, skin fold thickness (fore arm, sub scapular region, and abdomen), and waist and hip circumferences. Circumference was taken with a metal tape at the abdomen and hip. Waist circumference was measured at the narrowest point. Hip circumference was measured at the widest point of buttocks. Stature was measured with an anthropometer (Harpenden, UK), and weight with a portable balance beam scale. Skinfold measurements were taken with Holtain calipers (Holtain Ltd., Crymych, United Kingdom) at triceps, subscapular and abdomen regions to assess subcutaneous adipose distribution. Blood pressure was measured twice by Omron blood pressure machine (#HEM-705 CP) in sitting position from the left arm resting on the table, with legs uncrossed and feet flat (Mogensen et al., 2000). High blood pressure or hypertension was defined by systolic blood pressure $\geq 140$ mmHg and diastolic blood pressure $\geq 90$ mmHg or taking blood pressure medication. BMI was calculated according to Quetelet equation ($\text{BMI} = \frac{\text{weight in kg}}{\text{height in m}^2}$). Waist Hip Ratio (WHR) was calculated as ratio of abdomen to hip circumferences. BMI values $>24.9$ kg/m$^2$ and $\geq 30$ kg/m$^2$ are traditionally classified as over-weight and obesity respectively. However, recently, an expert group of WHO has recommended lower BMI thresholds for Asians (WHO Expert Consultation, 2004). According to these new guidelines, the BMI $<23$ kg/m$^2$ has been proposed for low risk, 23-27.5 kg/m$^2$ for increased risk and $\geq 27.5$ kg/m$^2$
for high risk for developing weight-related diseases in Asian populations. The abdominal obesity was measured according to the new cut-offs proposed for South Asian Indians (Snehalatha et al., 2003) (WHR > 0.89 for men and > 0.81 for women).

4.4 BIOCHEMICAL MEASUREMENTS

Fasting blood samples (overnight, 12 hours) were drawn in EDTA coated and plain vials and centrifuged to obtain plasma, serum and buffy coat that was stored at -80°C. Glucose measurements were carried out using the glucose oxidase method using Life scan’s OneTouch Ultra glucometer (Johnson & Johnson). Calibration of the glucometer was routinely verified using test strips provided by manufacturers. Serum was used for quantitation of all the biochemical estimations using standard enzymatic methods. Total Cholesterol, Triglycerides, High Density Lipoprotein (HDL)-cholesterol and Very Low-Density Lipoprotein (VLDL)-cholesterol were estimated by using Roche Diagnostics kits. Low-Density Lipoprotein (LDL)-cholesterol was calculated using Friedewald formula (1972) i.e. LDL-cholesterol = Total cholesterol – [HDL-cholesterol – (triglycerides in mmol/L/2.17 or triglycerides in mg/dL/5)]. Serum insulin was estimated by radioimmunoassay (Diagnostic Products Inc., USA). Homocysteine level in serum was measured by micro enzyme immunoassay (Biorad) and creatinine by kinetic colorimetric assay (Roche diagnostic kit). Glycosylated haemoglobin (HbAlc) was measured in the whole blood by turbidometric inhibition immunoassay (Tina quant). All the quantitative parameters were measured by following manufacture’s instructions using Hitachi 902 (Hitachi, Mannheim, Germany) and Selectra II (Merck) autoanalysers.

4.5 DERIVED MEASURES

4.5.1 Homeostasis Model Assessment (HOMA) Index

According to the homeostasis model assessment, the insulin resistance indices HOMA-IR and HOMA-BF were used to evaluate insulin resistance and beta-cell functions, respectively. The formulas used were as follows (Matthews et al., 1985):

HOMA-IR = [Fasting insulin (μU/ml) x Fasting blood glucose (mmol/L)] / 22.5; and
HOMA-BF = [20 x Fasting insulin (μU/ml)] / Fasting blood glucose (mmol/L).
4.5.2 Body Fat Content

Body fat content were calculated according to the method of Lean et al., (1996) using the following formula:

Body fat % for men = \[
(0.567 \times \text{waist circumference in cm}) + (0.101 \times \text{age in years}) \] - 31.8; and

Body fat % for women = \[
(0.438 \times \text{waist circumference in cm}) + (0.221 \times \text{age in years}) \] - 9.4.

4.6 REAGENTS USED

All the chemicals used in this study were molecular biology or biotechnology grades and are listed below:

<table>
<thead>
<tr>
<th>REAGENTS</th>
<th>SOURCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taq DNA polymerase, MgCl₂, and PCR buffer.</td>
<td>New England Biolabs, USA</td>
</tr>
<tr>
<td>Deoxynucleoside triphosphates (dNTPs)</td>
<td>Eppendorf AG, Germany</td>
</tr>
<tr>
<td>Restriction Enzymes (REs)</td>
<td>New England Biolabs, USA</td>
</tr>
<tr>
<td>Oligos sequences (Primers)</td>
<td>Integrated DNA Technologies Inc., USA</td>
</tr>
<tr>
<td>Agarose (Metaphor and Nuseive)</td>
<td>FMC Bioproducts, USA</td>
</tr>
<tr>
<td>DNA extraction kit</td>
<td>QIAGEN, USA</td>
</tr>
<tr>
<td>Tris-HCl, Sodium Dodecyl Sulphate TritonX-100, Ethanol (absolute), Isopropyl alcohol, Magnesium Chloride, Ammonium Chloride.</td>
<td>Amresco Reagents Inc., USA</td>
</tr>
<tr>
<td>Proteinase K, Sodium Chloride, EDTA-disodium salt, Sucrose, Tris base, Boric acid, Ethidium bromide, acetic acid etc.</td>
<td>Biogene Reagents Inc., USA</td>
</tr>
<tr>
<td>Roche Diagnostics kits</td>
<td>Roche Diagnostics, Germany</td>
</tr>
<tr>
<td>Insulin estimation kit</td>
<td>Diagnostic Products Inc., USA</td>
</tr>
</tbody>
</table>
4.7 PREPARATION OF SOLUTIONS

4.7.1 0.5M EDTA solution

186.1 g of disodium-EDTA•2H₂O was added to 800 ml of H₂O. The solution was vigorously stirred on a magnetic stirrer and its pH adjusted to 8.0 with 0.1N NaOH. The volume of the solution was adjusted to one litre to make final concentration of 0.5M EDTA solution. The solution was dispensed in aliquots and sterilized by autoclaving (15 psi, 121°C, 20 minutes).

4.7.2 Tris-Cl (pH 8.0) buffer

Tris base (121.1 g) was dissolved in 800 ml of H₂O. The pH of the solution was adjusted to 8.0 by adding concentrated HCl and the volume of the solution was adjusted to one litre to make final concentration of 1M Tris-Cl buffer.

4.7.3 10x TE (Tris - EDTA) solution

100 mM Tris-Cl (pH 8.0) and 10 mM EDTA (pH 8.0) were mixed, sterilized by autoclaving and stored at 4°C.

4.7.4 Proteinase K solution

Lyophilized powder was dissolved at a concentration of 20mg/ml in sterile 50 mM Tris (pH 8.0) containing 1.5 mM calcium acetate. The stock solution was divided into small aliquots and stored at -20°C. Aliquots were thawed and used for assay.

4.7.5 5X TBE buffer

Stock solution was prepared by dissolving 54.0 g of Tris base, 27.5 g of boric acid and 20.0 ml of 0.5 M EDTA (pH 8.0) in minimum quantity of water and the final volume was adjusted to one liter of H₂O. The pH of the concentrated stock buffer was 8.3. The buffer was passed through a 0.22-μm filter and diluted (1x) just before use.

4.7.6 50x TAE buffer

TAE stock solution was prepared by dissolving 242 g of Tris base, 57.1 ml of glacial acetic acid and 100 ml of 0.5 M EDTA (pH 8.0) in minimum quantity of water and the final volume was adjusted to one liter of H₂O. The buffer was diluted (1x) just before use from stock solution.
4.7.7 Gel-loading dye (6x)

Gel loading dye was prepared by dissolving 0.25% (w/v) bromophenol blue and 40% (w/v) sucrose in DDW.

4.7.8 Ethidium bromide solution

One gram of ethidium bromide was added to 100 ml of H₂O in a dark bottle. It was stirred on a magnetic stirrer for several hours to ensure that the dye was dissolved and stored at room temperature.

4.8 EXTRACTION OF GENOMIC DNA FROM HUMAN BLOOD SAMPLES

Genomic DNA was extracted from the blood samples (buffy coat) using modified salting out procedure as described by Miller et al., (1988) and also by using Qiagen kits (Qiagen, Valencia, California). Buffy coat is a leukocyte-enriched fraction of whole blood prepared by centrifuging whole blood at 2500 x g for 10 min at room temperature. After centrifugation, three different fractions are distinguishable: the upper clear layer is plasma; the intermediate layer containing concentrated leukocytes is buffy coat and the bottom layer contains concentrated erythrocytes.

4.8.1 Procedure:

1. Blood samples were collected in disodium-EDTA containers (5 ml). The buffy coat was separated and stored at -80°C.

2. After thawing, buffy coat was transferred to a sterile conical centrifuge tube and added 45 ml of cell lysis buffer [0.32 M sucrose, 10 mM Tris-HCl (pH 7.5), 5mM MgCl₂ and 1% Triton X-100 (v/v)] in a 50 ml conical tube. Cell lysis buffer was made fresh (same day) and stored at 4°C. The suspended cells were then set on ice for 30 min. the nuclei were pelleted by centrifugation at 3500 x g for 20 min at room temperature.

3. The supernatant was gently discarded to get the clean pellet. The pellet was then suspended in 3 ml of nuclei lysis buffer (10 mM Tris-HCl, 400mM NaCl and 2mM disodium-EDTA (pH 8.0) and gently broken with transfer pipette. The cell lysate solution was then transferred to 15 ml tube and digested with 0.2 ml of 10% SDS and 0.5 ml of Proteinase K solution (2 mg of Proteinase K in 1% SDS and 2mM EDTA) on a spin wheel at 37 °C.

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4. After the digestion was complete, 1 ml of saturated NaCl (approx. 6M) was added to each tube and shaken vigorously for 15 seconds, followed by centrifugation at 3500xg for 15 min. The precipitated protein was left at the bottom of the tube and the supernatant containing the DNA was transferred to another 15 ml polypropylene tube.

5. The upper aqueous phase (containing the DNA) was transferred into a clean and sterile conical centrifuge tube using a sterile pasteur pipette, followed by the addition of three volumes of chilled absolute alcohol.

6. DNA was precipitated by gentle swirling of the tube and observed visually as a white thread like strand.

7. The precipitated DNA strands were removed with a plastic spatula and placed in an eppendorf containing 1ml of 70% ethanol.

8. The DNA was then washed by inversion to clean it from any remaining salts and the tube centrifuged at 11000 x g for 5 minutes. The supernatant was discarded taking care not to discard the pellet. This step was repeated twice.

9. After discarding the supernatant, the pellet was dried from excess ethanol by leaving the tubes open and inverted in an oven at around 50-65 °C for an hour.

10. The dried pellet was resuspended in TE buffer and left overnight on a rotator.

11. DNA was then stored at -80°C. For routine storage the best condition was at 4°C. DNA precipitated in ethanol can be stored indefinitely at -20°C.

4.8.2 Determination of concentration, yield, and purity of DNA

DNA concentration was determined either by agarose gel electrophoresis or spectrophotometry and adjusted to the desired concentration by adding more TE buffer. DNA yield was measured by absorbance at 260 nm. Purity of DNA was checked by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm. Pure DNA has an $A_{260} / A_{280}$ ratio of 1.7–1.9.

$$\text{Amount of DNA} \ (\mu g/\mu l) = (50 \times \text{dilution factor} \times A_{260})/1000$$
4.9 GENETIC POLYMORPHISM OF CANDIDATE GENES IN T2DM

4.9.1 Genotyping of Calpain 10 gene (CAPN10) polymorphisms

Three SNPs of CAPN10 gene (SNP-43, -19, and -63) were genotyped in this study.

4.9.1.1 SNP-43 (CAPN10-g.4852G/A)

All subjects were genotyped for SNP-43 by a mutagenically separated PCR (MS-PCR) method, which uses a common forward primer and two allele-specific reverse primers of different lengths as described by Evans et al., (2001). Primer sequences used were as follow: forward primer, 5'-CAT CCA TAG CTT CCA CGC CTC-3'; reverse primer allele 1 (G), 5'-GCT TAG CCT CAC CTT CAA TC-3'; and reverse primer allele 2 (A), 5'-ATC CTC ACC AAG TCA AGC GTT AGC CTC ACC TTC AAG T-3'. PCR was performed in 25 μl volume containing 1x PCR buffer, 200 μM of each dNTP, 1.5 mm of MgCl2, 1 Unit of Taq DNA Polymerase and 50-100 ng of genomic DNA. Primer concentrations were 1,000 nmol of common primer/liter, 1,000 nmol of allele 1 primer/liter, and 67 nmol of allele 2 primer/liter, giving a 15:1 ratio of short primers to long primer. The cycling conditions were 96°C for 12 min; 35 cycles of 94°C for 30 sec, 57°C for 30 sec, and 72°C for 30 sec; and 72°C for 10 min. The PCR products were separated on a 3% NuSieve agarose gel and were visualized by staining with ethidium bromide: allele 1 (G) is 134 bp and, allele 2 (A) is 152 bp.

4.9.1.2 SNP-19 (CAPN10-g.7920indel32bp)

SNP-19 is a two-allele insertion/ deletion (indel) polymorphism consisting of two or three copies of a 32-bp repeat sequence. Genotypes of SNP-19 of CAPN10 gene were determined as described by Evans et al., (2001) with slight modifications. This polymorphism was typed using the forward and reverse primers 5'-GTT TGG TTC TCT TCA GCG TGG AG-3' and 5'-CAT GAA CCC TGG CAG GGT CTA AG-3' respectively. PCR was performed in 25μl volume containing 1xPCR buffer, 200 μM of each dNTP, 1.5 mM of MgCl2, 5% dimethyl sulfoxide (DMSO), 250 nM of each primer, 1U of Taq DNA Polymerase and 50-100 ng of genomic DNA. The cycling conditions were 94°C for 12 min; 35 cycles of 94°C for 30 sec, 57°C for 30 sec, and 72°C for 30 sec; and 72°C for 10 min. The PCR products were separated on a 3% NuSieve agarose gel and
were visualized by staining with ethidium bromide: allele 1 (two repeats of 32-bp sequence) is 155 bp, and allele 2 (three repeats) is 187 bp.

4.9.1.3 SNP-63 (CAPN10-g.16378C/T)

SNP-63 genotyping was carried out using a mismatch PCR method (Orho-Melander et al., 2002), which creates a \( HhaI \) site with the common C allele. The forward and reverse primers were 5’-AAG GGG GGC CAG GGC CTG ACG GGG GTG GCG-3’ and 5’-AGC ACT CCC AGC TCC TGA TC-3’, respectively. PCR was performed in 25 μl volume containing 1xPCR buffer, 200 μM of each dNTP, 1.5 mM of MgCl₂, 5% dimethyl sulfoxide (DMSO), 250 nM of each primer, 1 Unit of Taq DNA Polymerase and 50-100 ng of genomic DNA. The cycling conditions were 94°C for 12 min; 35 cycles of 94°C for 30 sec, 62°C for 30 sec, and 72°C for 30 sec; and 72°C for 10 min. The 192-bp PCR products was digested with 5 units of \( HhaI \) in 1x NE 4 buffer plus 1x BSA at 37°C for 16 hr to produce a 162 bp and 30 bp product in the presence of the C allele (allele 1). The T allele (allele 2) is not cleaved by \( HhaI \) and gives a product of 192 bp. The digested products were separated on 3% metaphor agarose gel and stained with ethidium bromide.

4.9.2 Genotyping of Pro12Ala polymorphism in exon B of \( PPAR-\gamma \) gene

The Pro12Ala polymorphism was detected by PCR-RFLP method of Hara et al. (2000). The sequences of the primers were 5’-TCT GGG AGA TTC TCC TAT TGG C-3’ (forward primer) and 5’-CTG GAA GAC AAC TAC AAG AG-3’ (reverse primer). The forward primer contained one nucleotide mismatch (underlined), which made it possible to use the restriction enzyme \( HhaI \) for the detection of the Pro12Ala polymorphism. PCR amplification was performed in 25 μl reaction mixture containing 50–500 ng of genomic DNA, 0.2 mM of each primer, 2.5 mM of MgCl₂, and 1.25 Units of Taq DNA Polymerase. The reaction mixtures were incubated at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec and extension at 72°C for 30 sec. PCR products were digested with 5 Units of \( HhaI \) at 37°C overnight, and resolved by 3% ethidium bromide-stained agarose gel electrophoresis. The Pro12 allele gives one 154 bp fragment whereas the Ala12 allele gives 132 bp and 22 bp fragments.
4.9.3 Genotyping of K121Q polymorphism in ENPP-1 gene

ENPP1 K121Q was detected by PCR-RFLP analysis using a PCR-amplified DNA fragment digested with Ava II restriction enzyme (Bacci et al., 2005). Exon 4 amplimers were obtained using the following primer pair: forward, 5'-GCA ATT CTG TGT TCA CTT TGG A-3' and reverse, 5'-GAG CAC CTG ACC TTG ACA CA- 3'. The PCR was carried out in a final volume of 25µl, containing 100 ng of genomic DNA, 1.5 mmol/l MgCl2, 0.2 mmol/l of each dNTP, 0.5 pmol of each primer, and 1.25 units of Taq DNA polymerase. After an initial denaturation of 5 min at 94°C, the samples were subjected to 30 cycles at 94°C for 1 min, 55°C for 40 sec, and 72°C for 40 sec, with a final extension of 10 min at 72°C. The 208 bp product was restricted with AvaII for 16h at 37°C. The unrestricted 208 bp product represents the K allele, while the Q allele was cut into 53 bp and 155 bp fragments. The three genotypes were scored after running on a 2.5% agarose gel and staining with ethidium bromide. To assure that the genotyping was of sufficient quality, we performed random duplicates in approx. 10% of the samples in each genotyping assay. The assays were performed in blind to the phenotype. No genotype errors were detected in the random duplicates.

4.9.4 Genotyping of insertion/deletion polymorphism in ACE gene

PCR amplification of ACE (I/D) polymorphism was done according to Matsumoto et al., (2000) using following primer sequences. Forward primer: 5'-CTG GAG ACC ACT CCC ATC CTT TCT-3' and reverse primer: 5'-GAT GTG GCC ATC ACA TTC GTC AGA T-3'. The PCR was carried out in a final volume of 25µl containing 100-500ng of genomic DNA, 25 pmol of each primer, 1.25 units Taq DNA polymerase and 5% of DMSO to avoid mistyping. The PCR cycle conditions were as follows: an initial denaturation at 94°C for 5 min, followed by 30 cycles of annealing at 55°C for 1 min, extension at 72°C for 1.5 min, denaturation at 94°C for 1 min, and final extension at 72°C for 10 min. PCR products were then separated by electrophoresis on a 2% NuSieve agarose gel. After electrophoresis, the gel was stained with ethidium bromide and bands were visualized by ultraviolet illumination and photographed. PCR amplified DNA from a DD homozygote yielded only the 190 bp D fragment. PCR product from a heterozygote
yielded both the 190 bp and 490 bp fragment. PCR products from an II homozygote yielded only the 490 bp fragment.

4.9.5 Genotyping of Gln192Ala polymorphism in PON1 gene

PON1 genotypes were determined by PCR amplification and restriction analysis as described by Gnasso et al., (2002). The following set of primers was used: forward 5'-TTG AAT GAT ATT GTT GCT GTG GGA CCT GAG-3' and reverse 5'-CGA CCA CGC TAA ACC CAA ATA CAT CTC CCA GgA-3'. The lower case base in PON1-192 reverse primer indicates a mismatch introducing a restriction site for HinfI (G/ANTC) in the DNA amplification product in presence of arginine (CGA) at codon 192 of PON1 gene. The 25µl PCR reaction contained 0.5 µg DNA template, 0.1 µM of each primer, 200 µM of four dNTP, 1 Unit of Taq DNA polymerase and 1.5 mM MgCl₂. 40 cycles of amplification (94 °C 1 min, 65 °C 45 s, 72 °C 45 s) with a final extension of 5 min at 72 °C. Restriction analysis with 5 units of HinfI for 3 h at 37 °C yielded one undigested 111-bp band in case of Gin and two digested products (77 and 34 bp) in case of Arg resolved on 3.5% agarose gel electrophoresis.

4.10 STATISTICAL ANALYSIS

Results were expressed as mean ± S.D. The genotypic distributions were tested for deviation from Hardy–Weinberg equilibrium. Chi-square analysis was applied to test the significance of differences in genotypic and allelic frequencies. Skewed variables were log-transformed before statistical comparisons were made. Comparisons between case subjects and control subjects were done using chi-square tests, and group comparisons were done using unpaired t-tests. One-way ANOVA test was used for comparison of normally distributed variables among genotypes. All the p-values <0.05 (two-tailed) were considered as significant difference. Logistic regression analysis was carried out to correlate various clinical parameters with genotypes. All statistical analyses were performed using the Statistical Package of Social Sciences (SPSS) for Windows, version 10.0 (SPSS Inc., 1999).