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CONTRIBUTION OF THE POSITIONAL CANDIDATE GENE OXR1 TO PREMATURE CORONARY HEART DISEASE AND TYPE 2 DIABETES IN THE MAURITIAN POPULATION

Final Report

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Contribution of the positional candidate gene

OXR1

to premature coronary heart disease and

Type 2 Diabetes in the

Mauritian population

Final Report submitted by
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Abstract

Background: The burden of disease due to coronary heart disease (CHD) and type 2 diabetes (T2D) is important in Mauritius and is likely to increase in the future. Like most common chronic diseases, CHD and T2D are multifactorial, having both environmental and hereditary components contributing to their pathophysiology. A previous genome scan carried out in Mauritian families of North-Indian origin affected by CHD and/or T2D unveiled several chromosomal regions harbouring positional candidate genes for these complex traits. Chromosomal 8q23 region was an interesting region where microsatellite markers showed simultaneous co-segregation with premature CHD, T2D and HBP in the families studied. The strongest candidate gene within that region appeared to be the Oxidation Resistance 1 *(OXR1)* gene, given the increasing role attributed to oxidative stress in the patho-physiology of T2D or CHD and the putative role of the mitochondrial OXR1 protein in protection against oxidative stress.

The present study was carried out to evaluate the contribution of known variants in the positional candidate gene *OXR1* to increase susceptibility to premature CHD and/or to T2D in a Mauritian population of North Indian origin.

Method: We selected 6 tagging single nucleotide polymorphic (SNP) variants with minor allele frequency > 0.20, spanning the *OXR1* gene, which we genotyped in unrelated case-control cohorts from the North-Indian ethnic group using TaqMan SNP genotyping assays.

Results: We obtained reliable results for 5 out of the 6 *OXR1* SNPs that were genotyped. Haplotype analysis of the 5 SNPs showed that haplotype *H2* (TACGC) tend to be protective against CHD in women (p<0.025). Analysis of individual genotypes showed trends towards association between homozygous TT genotype for SNP rs776959 in intron 9 of the *OXR1* gene and several phenotypes such as premature CHD (p<0.039), T2D (p<0.02) and the metabolic syndrome (p<0.04) in women only, no association was seen with any phenotype in men. The T allele of rs776959 SNP was found to be associated with increased risk for CHD and T2D in women only (CHD: OR=1.63, 1.01<OR<2.64, p=0.045; T2D: OR=1.84, 1.05<OR<3.23, p=0.03). We found an interaction of gender on association between TT genotype and both CHD and T2D.

Discussion and conclusions: Our findings suggest an important gender-specific role of the *OXR1* rs776959 variant in the pathology of CHD and T2D. Replication studies are warranted to confirm association of this variant in independent case-control female groups of North Indian and South Indian origin. If results are confirmed, sequencing within intron 9 of the *OXR1* gene will be undertaken to look for new variants in linkage disequilibrium with rs776959 SNP that may be causal. We tried a replication study of a variant that showed consistent association with CHD in other populations and we did not find any association between CHD and this variant in our study population. This shows the importance of our initial strategy to look for positional candidate gene(s) or gene variant(s) within regions that showed linkage in our own genome scan.

Keywords: coronary heart disease, Type 2 diabetes, positional candidate gene, *OXR1* gene, SNP rs776959

Acknowledgements

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Abbreviations

ADAMTS9:	A disintegrin and metalloproteinase with thrombospondin motifs 9
AGTR2:	Angiotensin II Receptor, Type 2
ALOX5AP:	arachidonate 5-lipoxygenase-activating protein
ATP III:	Adult Treatment Panel III
BMI:	Body mass index
BP:	Blood Pressure
CAD:	Coronary Artery Disease
CAMK1D:	calcium/calmodulin-dependent protein kinase ID
CAPN10	Calpain 10
CASQ1:	calsequestrin 1
CDC123:	cell division cycle 123 homolog
CDKAL1:	cyclin-dependent kinase 5 regulatory subunit associated protein 1-like 1
CDKN2A/B:	cyclin-dependent kinase inhibitor 2A/B
CELSR2:	Cadherin, EGF LAG seven-pass G-type receptor 2
CEU:	Utah residents with Northern and Western European ancestry from the 'Centre d''Etude du polymorphisme humain' collection
CHB:	Chinese Han in Beijing
CHD:	Coronary Heart Disease
CI:	Confidence interval
cM:	centiMorgan
COIDF:	Central obesity as defined by IDF
CVD:	cardiovascular disease
CX37:	Connexin 37
CXCL12:	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)
DGI:	Diabetes Genetics Initiative
DNA:	Deoxyribonucleic Acid
dNTP:	deoxynucleoside triphosphate
EDTA:	Ethylenediaminetetraacetic acid
FFAs:	Free fatty acids
FOG2:	Friend of GATA2
FPG:	Fasting plasma glucose
FPGIDF:	Raised fasting plasma glucose as defined by IDF
FUSION:	Finnish United States Investigation of Non-insulin-dependent Diabetes Mellitus Genetics
GENNID:	Genetics of Non-insulin-dependent Diabetes Mellitus
GGT:	Gamma Glutamyl Transferase
GWAS:	Genome Wide Association Studies
HBP:	High Blood Pressure
HBPIDF:	High blood pressure as defined by IDF
HDL:	High Density Lipoprotien
HDLIDF:	Reduced HDL-cholesterol as defined by IDF

HHEX:	hematopoietically expressed homeobox
HNK4A:	Hepatocyte nuclear factor 4, alpha
2HPG:	2-hour plasma glucose
HWE:	Hardy Weinberg Equilibrium
IDE:	insulin-degrading enzyme
IDF:	International Diabetes Federation
IGF2BP2:	Insulin-like growth factor 2 mRNA binding protein 2
IGT:	Imparied Glucose Tolerance
IL1A:	Interleukin 1, alpha
IL1B:	Interleukin 1, beta
JAZF1:	Juxtaposed with another zinc finger protein 1
JPT:	Japanese in Tokyo
KCNJ11:	Potassium inwardly-rectifying channel, subfamily J, member 11
LBM:	Lean body mass
LD:	Linkage disequilibrium
LDL:	Low-density-lipoprotein
LGR5:	leucine-rich repeat-containing G protein-coupled receptor 5
MAF:	Minor allele frequency
MGB:	Minor groove binding
MgCl$_2$:	Magnesium chloride
MI:	Myocardial Infarction
mmHg:	Millimetres of Mercury
mmol/L:	Millimoles per litre
MOH & QL:	Ministry of Health and Quality of Life
MRAS:	Muscle RAS oncogene homolog
MSIDF:	Metabolic syndrome as defined by IDF
MSIRI:	Mauritius Sugar Industry Research Institute
mtDNA:	Mitochondria DNA
MTHFD1L:	Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like
NCD:	Non Communicable Diseases
NCEP:	National Cholesterol Education Progaram
NCOA7:	nuclear receptor coactivator 7
NI:	North Indian
NOTCH2:	Neurogenic locus notch homolog protein 2
OGGT:	Oral glucose tolerance test
OR:	Odds Ratio
OXPHOS:	Oxidative phosphorylation
OXR1:	Oxidation Resistance 1
PCR:	Polymerase chain reaction
PKLR:	liver pyruvate kinase
PPARG:	Peroxisome proliferator-activated receptor-gamma
PROC:	Protein C (inactivator of coagulation factors Va and VIIIa)
PSRC1:	Proline/serine-rich coiled-coil protein 1

PTPN1:   Protein tyrosine phosphatase 1B
RNA: Ribonucleic Acid
ROS: Reactive oxygen species
SAFDGS: San Antonio Family Diabetes/Gallbladder Study
SGOT: Serum Glutamic-Oxaloacetic Transaminase
SGPT: Serum Glutamic-Pyruvic Transaminase
SLC30A8:   Solute carrier family 30 (zinc transporter), member 8
SNP: Single Nucleotide Polymorphism
SORT1:   Sortilin 1
T2D: Type 2 diabetes
TBE: Tris Borate EDTA
TCF7L2:   Transcription factor 7-like 2
TGIDF: Hypertriglyceridaemia as defined by IDF
THADA: thyroid adenoma associated
TRH: Thyrotropin releasing hormone
TRHR: Thyrotropin releasing hormone receptor
TSPAN8:   Tetraspanin 8
UK: United Kingdom
UTR: Untranslated region
WTCCC: Wellcome Trust Case Control Consortium
YRI: Yoruba in Ibadan, Nigeria
ZFPM2:   Zinc finger protein multitype 2

Introduction

Coronary heart disease (CHD) mortality in Mauritius is among the highest in the World (Vos et al, 1998). In 2006, 21.7% of total deaths in the Mauritian population were attributed to cardiovascular diseases. According to epidemiological survey carried out in 2009, prevalence of Type 2 diabetes among adults aged 30-74 years in the Mauritian population was 26.9% (NCD report, 2009). Diabetic patients have a two to four times increased risk of developing cardiovascular diseases and a four-fold risk for CHD death (Haffner & Cassels, 2003).

Coronary heart disease and Type 2 diabetes are both complex diseases involving interactions between genetic and environmental factors. Previous genome scan carried out in around 100 Mauritian families of North Indian origin had revealed several chromosomal regions that showed evidence for linkage to CHD (Francke et al, 2001) and these regions overlapped with chromosomal regions that have been linked to type 2 diabetes in other populations. One particularly interesting chromosomal region was reported in the 8q23 region where microsatellite markers co-segregated with premature CHD, type 2 diabetes and hypertension in our studied population (Francke et al, 2001)

Within the 8q23 chromosomal region, there are several potential candidate genes for CHD and/or type 2 diabetes, which seem interesting, given the biological functions of their proteins. Among them is the oxidation resistance 1 gene, which is a nuclear gene whose protein product, when localized in the mitochondria, protects against oxidative damage. Given the growing body of evidence that links oxidative stress to the pathophysiology of CHD and type 2 diabetes, the *OXR1* gene appears as the best positional candidate gene in the 8q23 region. Previous study carried out in Mauritians of Asian Indian origin with impaired glucose metabolism showed that oxidative stress preceded endothelial dysfunction and insulin resistance (Gopaul et al, 2001). However, there is no published study yet, that describes an association between *OXR1* polymorphisms and CHD and/or type 2 diabetes or their associated traits.

In our present study, we investigated the contribution of variants of the *OXR1* gene to genetic susceptibility to premature coronary heart disease and/or type 2 diabetes in a subgroup of the Mauritian population of North Indian origin.

The development of high throughput cost-effective genotyping technologies, the discovery of a large number of variants in the human genome and the availability of large well-characterized sample sets have allowed an unbiased genome wide approach in the study of common variants associated with common diseases. Within the past ten years, different genome wide association studies have identified several genes or gene variants associated with myocardial infarction (MI) and/or type 2 diabetes, while confirming others as potential genetic markers. However, these genome wide association studies were carried out mainly in Caucasian populations and

these have to be replicated in various independent studies, including studies in different ethnic groups in order to validate positive association between susceptibility loci and diseases.

In this context, we also carried out a replication study in our North Indian population, of a SNP variant (rs1333049) on chromosome 9p21 that have shown association with coronary artery disease and MI in genome wide association studies and which have been widely replicated in different populations.

I. General Background

I.1 Overview of CHD and Type 2 diabetes

Type 2 diabetes (T2D) is the most common metabolic disease affecting about 150 million people worldwide and its prevalence is increasing such that the global burden of the disease is expected to double over the next 25 years (Zimmet et al, 2001). Coronary heart disease (CHD) is the leading cause of morbidity and mortality in developed countries and mostly in diabetic patients. The primary cause of CHD is atherosclerosis, which is frequently present at the time when diabetes is first diagnosed, suggesting that atherogenesis begins prior to the onset of overt diabetes. Insulin-resistant states without diabetes have also been associated with cardiovascular diseases (Ginsberg, 2000)

Cardiovascular diseases including coronary heart disease (CHD) are defined as complex diseases caused by a combination of genetic susceptibility, environmental factors and lifestyle. Atherosclerosis, the major cause of CHD, is characterized by lipid accumulation and an inflammatory response in medium-sized and large arteries. Epidemiological studies have revealed numerous risk factors that influence the development and severity of atherosclerosis including diabetes, hypertension, elevated plasma levels of low-density lipoprotein cholesterol, and low levels of plasma high-density lipoprotein cholesterol (ATP III, 2002). CHD combines symptoms from angina pectoris to sudden death due to myocardial ischaemia. CHD typically manifests itself by chest pain, although the disease can also be symptomless. Angina pectoris is a stable form of CHD characterized by chest pain caused by an increased oxygen demand which cannot be met because of an imbalance between oxygen demand and supply in the myocardium, leading to myocardial ischaemia.

Type 2 diabetes, which is a complex heterogeneous group of metabolic conditions, is characterized by chronic hyperglycaemia, primarily caused by insulin resistance in liver, skeletal muscle and adipose tissue and impaired insulin secretion in the pancreatic β-cell (Das & Elbein, 2006). Moreover, type 2 diabetes clusters with dyslipidaemia and hypertension, which together with insulin resistance, glucose intolerance and central obesity are hallmarks of the metabolic syndrome. Type 2 diabetes is often associated with severe complications of the cardiovascular system, leading to increased morbidity and mortality from cardiovascular diseases. Indeed, epidemiological studies have shown that patients with type 2 diabetes have a two to four times greater risk of CVD mortality than those without diabetes (Wannamethee et al, 2004). Several studies have also reported that the risk of CHD mortality among diabetic patients without prior MI was similar to non-diabetic patients with prior MI (Haffner et al, 1998; Mukamal et al, 2001), which had led to the recommendation by the National Cholesterol Education Program Adult Treatment Panel III that diabetes without CHD should be treated as a

CHD risk equivalent (NCEP report, 2001). In contrast, other studies have found a higher risk of CHD death associated with a history of CHD rather than with diabetes alone (Saely et al, 2010). However, in almost all studies, diabetic patients who have CHD were found to have a worse prognosis for survival than non-diabetic patients with CHD (Haffner et al, 1998; Mukamal et al, 2001; Saely et al, 2010). In addition, women were found to have a 50% greater risk of death due to coronary heart disease associated with diabetes than men and this may be a consequence of diabetes inducing a more unfavorable cardiovascular risk profile in women (Huxley et al, 2006).

It is now accepted that genetic factors contribute significantly to the epidemiology of CHD and Type 2 diabetes. A family history of CHD is considered to be a major independent risk factor for CHD, especially for those with an early age of onset. This was demonstrated in twin studies where monozygotic twins had 8-15 times more risk of dying from CHD when one of them had already died from this disease before the age of 55 (Marenberg et al, 1994). Genetic factors also contribute significantly to most of the major risk factors for CHD. In Type 2 diabetes, strong evidence from twin and family studies support the important role of genetics in the pathogenesis of the disease: Twin studies have reported a high concordance in monozygotic twins (over 80%) and a 50% fall in dizygotic twins (Poulsen, 1999; Medici, 1999). In family studies, a nearly 4-fold increased risk for Type 2 diabetes was observed in siblings of a diabetic proband compared with the general population, the odds ratio being 3.5 with only a single affected parent, which increases to 6.1 if both parents are affected (Meigs, 2000).

I.1.1 CHD and Type 2 diabetes in Asian Indian populations

Type 2 diabetes mellitus and premature CHD have a high prevalence among urban and migrant Asian Indians, despite lower rates of conventional risk factors compared to other populations (McKeigue et al, 1989; McKeigue & Sevak, 1994; Barnett et al, 2006; Gupta, 2008). Evidence exists that Asian Indians are more insulin resistant than Caucasians and that insulin resistance may play an important role in the pathogenesis of these diseases (Bajaj & Banerji, 2004). Indeed, insulin resistance has been proposed as a central feature of the metabolic syndrome, leading to type 2 diabetes, atherosclerotic vascular disease and CHD (Barnett et al, 2006).

Several studies have reported excess risk of CHD in Asian Indians to be greater at younger ages (Enas & Senthilkumar, 2002). For example, in UK, the relative risk of CHD mortality in Asian Indians as compared with Caucasians is 3.3 in the age group 20-29 (Balarajan, 1996). About 25% of acute myocardial infarction (MI) in India occurs under the age of 40 and 50% under the age of 50. In general, MI develops 5-10 years earlier in Asian Indian than in other populations and is 5-10 fold higher in patients under 40 (Enas & Senthilkumar, 2002).

I.1.2 Prevalence of CHD and T2D in Mauritius

According to statistics from the Ministry of Health, cardiovascular diseases and diabetes mellitus were the two principal causes of mortality in 2006, accounting for 21.7% and 22.6% of total deaths (Health Statistics 2006, MOH&QL). Overall CHD mortality rate in Mauritius is among the highest in the World (Vos et al, 1998; Khaw, 2006) (Figure 1).
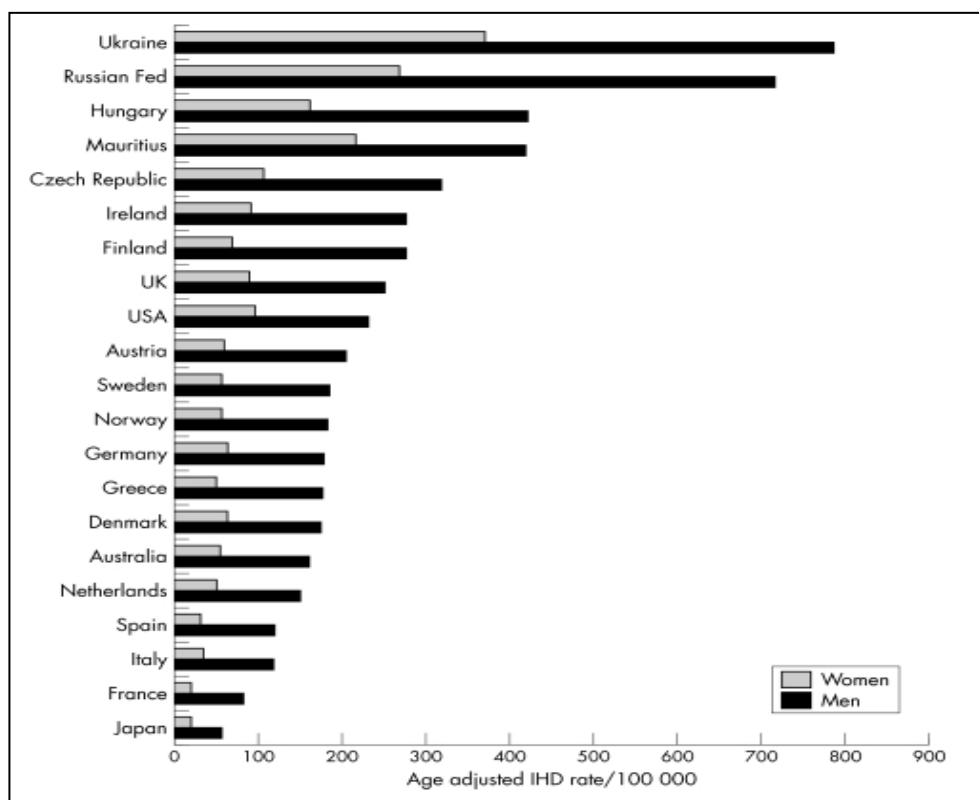
Figure 1: Age adjusted coronary heart disease rates for men and women aged 35–74 years in
Mauritius as compared with other selected countries, 1999–2000
(Adapted from Khaw KT, 2006)

Mortality trends over the last 3 decades show a slow but constant increase in the % of death due to heart diseases, from 17.2% in 1975 to 21.7% in 2006 (Health Statistics 2006, MOH&QL). This trend is not expected to decline in spite of improvement in the management of CHD since the prevalence of cardiovascular diseases and mortality is generally increased among diabetic patients and Type 2 diabetes has reached epidemic proportions in our country.

Data from epidemiological surveys carried out in 1987, 1992, 1998, 2004 and 2009 showed that prevalence of diabetes and impaired glucose tolerance (a risk factor for both type 2 diabetes and cardiovascular diseases) was high in Mauritius. (NCD report 2004, NCD report 2009). Since 1987, the prevalence of diabetes in adults of age group 30 and above has increased from 14.3% to 16.9% in 1992 and 19.5% in 1998 to stabilize to 19.3% in 2004. However, data from recently published 2009 survey showed an increase to 26.9% in the prevalence rate of diabetes. This prevalence rate would place Mauritius as the second country with the highest prevalence

of diabetes in the world, according to the International Diabetes Federation's Diabetes Atlas (Sicree et al, 2009). Moreover, components of the metabolic syndrome such as hypertension, obesity and dyslipidaemia, which are risk factors for cardiovascular disease, are also very high in the Mauritian population (Table 1).

Table 1: NCD survey results showing trends in prevalence of Type 2 diabetes and associated risk factors in the Mauritian population
Year 1987 to 2009

	1987	1992	1998	2004	2009
Age-standardized prevalence of diabetes (WHO criteria)[1]	14.3	16.9	19.5	19.3	26.9
Age-standardized prevalence of IGT (WHO criteria) [1]	19.3	17.3	16.6	12.1	15.6[3]
Age-standardized prevalence of hypertension[1] (BP≥140/90 mmHg)	30.2	26.2	29.6	29.8	37.9
Age-standardized prevalence of obesity [2] (WHO criteria - BMI>30kg/m^3)	6.3	10.2	11.5	10.3	16.0
Age-standardized prevalence of overweight [2] (WHO criteria - 30≥BMI>25 kg/ m^3)	24.2	29.8	29.1	25.4	34.9
Crude prevalence of dyslipidaemia 6.5< Cholesterol <5.2 mmol/l	30.7	26.7	32.3	36.6	34.7[4]
Crude prevalence of dyslipidaemia: HDL-chol <1.0 mmol/l	18.3	13.8	52.1	47.5	17.5[4]
Crude prevalence of dyslipidaemia: Triglycerides ≥2 mmol/l	21.0	18.2	31.7	21.9	16.9[4]

Data for years 1987- 2004 – Source NCD report 2004- *www.gov.mu/portal/site/mohsite* [Accessed 4 April 2010)
Data for year 2009 – Source: NCD report 2009 - *www.gov.mu/portal/site/mohsite* [Accessed 18 May 2010)
[1] Age-standardized prevalence rate in adults aged 30 years and above
[2] Age-standardized prevalence rate in adults aged 20-74 years
[3] Age-standardized prevalence rate in adults aged 25-74 years
[4] Age-standardized prevalence rate in adults aged 25-74 years

I.2 Strategies to identify susceptibility gene variants associated with CHD and Type 2 diabetes

CHD and Type 2 diabetes are both complex diseases involving interactions between multiple genetic and environmental factors. The genetic etiology of complex diseases has been increasingly emphasized as a means to better understand the pathogenesis of the diseases and ultimately to facilitate the development of new diagnostic tool and therapeutics as well as to improve preventive strategies (Risch, 2000). However, the search for genetic factors in complex diseases is hampered by their complexity and heterogeneity. Indeed, it is now apparent that several dozen of genes may contribute to the genetic predisposition to complex diseases such as type 2 diabetes and cardiovascular disease. In fact, the genetic etiology of complex diseases is thought to be based on a combination of multiple rare and common susceptibility loci (Panoutsopoulou & Zeggini, 2009).

I.2.1 Genome wide studies

The current strategy to look for novel genes or gene variants associated with complex diseases is through a genome wide approach. The development of improved strategies has been guided by advances in genotyping and sequencing techniques, first with the availability of microsatellite markers for linkage analysis and nowadays of high-density SNP maps for genome wide association studies, and also by the assembly of large cohorts suitable for genetic studies. Genome-wide studies can be linkage studies based on whole-genome screens or association studies involving genetic variations across the whole genome (Figure 2).
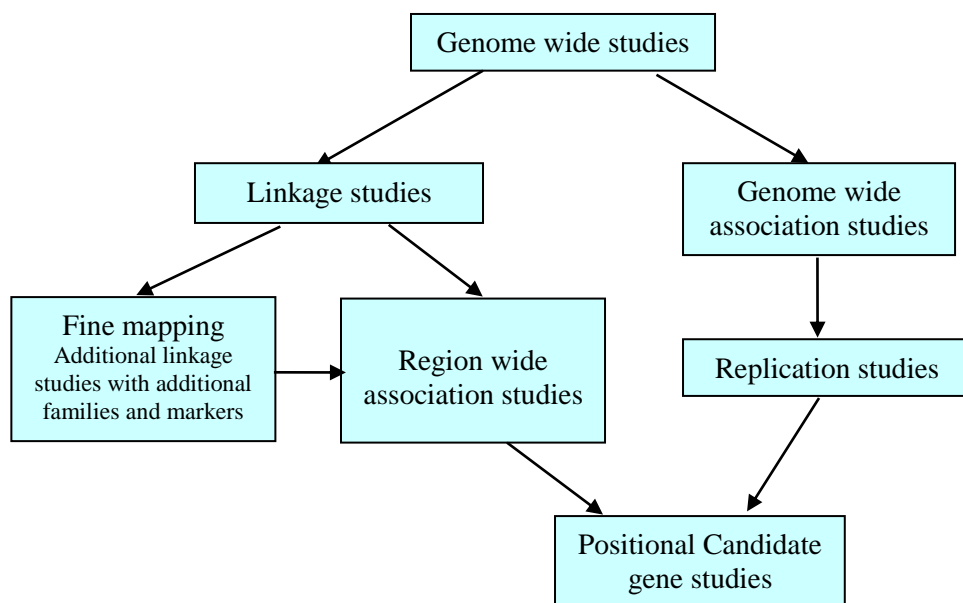
Figure 2: Genome wide strategies to search for susceptibility genes or gene variants in complex diseases

I.2.2 Linkage studies

Linkage studies used to be a common way to look for novel disease loci. They were the first stage in the genetic investigation of a trait or disease to identify chromosomal regions that might contain disease genes. Linkage analysis methods attempt to identify regions of the genome that is transmitted within families along with the disease phenotype of interest. Non-parametric linkage analysis is used to study complex diseases and has the advantage of being an unbiased approach. It therefore has the potential of identifying new chromosomal regions or loci that previously would have remained undetected on the basis of *a priori* knowledge of disease mechanisms. Linkage studies are performed by using polymorphic DNA markers, usually microsatellite markers evenly spaced across the genome (Topol, 2006). Microsatellite markers are short tandem repeat DNA sequences that are found throughout the genome and they are highly polymorphic. Once a chromosomal region is identified through linkage analysis, the region of interest has to be further studied through fine-mapping in an attempt to identify the causal gene or variant.

Linkage analysis has been extremely useful in the identification of genes responsible for diseases with simple Mendelian inheritance, such as cystic fibrosis (Bolstein & Risch, 2003). The application of linkage analysis to complex disorders without obvious Mendelian inheritance was only partially successful because complex diseases are most likely influenced by genetic heterogeneity, environmental factors, incomplete penetrance, gene-environment interactions, and multiple locus effects (Silverman & Palmer, 2000). This method has also limited power to detect loci with modest or small effects. Beside the lack of statistical power, a major obstacle in linkage studies is that even if evidence of linkage is observed, the genome region linked to disease is often very large, ~20 cM representing 20 million bases and harboring 200 or more genes. This region will contain a very large number of common variants (mostly single nucleotide polymorphisms (SNPs)) within the coding sequence as well as in sequences potentially relevant to gene function and regulation (McCarthy, 2002). Thus identifying the causal gene or variant within a disease-linked region can be a very difficult task.

Another limitation in linkage studies is the availability of a sufficiently large collection of families with the disease of interest in order to achieve optimal power to detect linkage. Recruitment of families can be a limiting factor in the study of diseases with high mortality rate like premature CHD or in late onset diseases like Type 2 diabetes.

Genome Wide Linkage studies for cardiovascular diseases

Several genome wide linkage studies for myocardial infarction and coronary artery disease have been reported (Table 2), each identifying particular loci or linkage peaks of interest. One

of the genome wide scans for coronary heart disease and/or type 2 diabetes was carried out in the Mauritian population and this has revealed several chromosomal regions (16p13, 10q23 and 3q27), which showed suggestive linkage to CHD (Francke et al, 2001).

However, results from the different genome wide scans have not been replicated and this may be due to genetic heterogeneity of the disease and/or lack of power in individual scans (Altmuller et al, 2001). In order to circumvent problems with lack of statistical power, meta-analysis has increasingly been applied. Combination of results from different studies can confirm evidence of regions identified in at least one scan or reveal new loci where weak but consistent linkage is seen across studies (Chiodini et al, 2003). Chiodini et al (2003) performed a meta-analysis of 4 published coronary heart disease genome-wide scans, including genome wide scan in the Mauritian population and confirmed that the strongest evidence for linkage was for loci on chromosomes 3q26-27 and 2q34-37. Another meta-analysis conducted by Zintzaras & Kitsios (2006) involved five genome searches in Caucasians and they reported four chromosomal regions, namely 6p22.3-6p21.1, 14p13-14q13.1, 8q13.2-8q22.2, 8q24.21-8q24.3 that confer susceptibility to MI. However, the specific genes or genetic variants responsible for these linkages have yet to be identified.

Table 2: Genetic linkage studies for myocardial infarction and coronary artery disease
(Adapted from Kullo JI & Ding K, 2007)

Study	*Population*	*Study sample (No of samples)*	*Locus or loci*	*Candidate gene(s)*
Pajukanta (2000)	Finnish	156 sibships (526)	2q21-22 Xq23-26	*AGTR2*
Francke (2001)	Indo-Mauritian	99 sibships (525)	3q27 8q23 16p13.3-pter	*Unknown*
Harrap (2002)	Australian	61 sibships	2q36-37	*Unknown*
Broeckel (2002)	German	513 sibships (1,406)	14q32	*Unknown*
Helgadottir (2004)	Icelandic	296 multiplex families (713 cases; 1,741 first-degree relatives)	13q12-13	*ALOX5AP*
Hauser (2004)	Predominantly European-American	438 sibships (1,168)	3q13 5q31	*Unknown*
Wang (2004)	American	426 multiplex families (1,613)	1p34-36	*CX37*
Samani (2005)	British	1,933 sibships (4,175)	2q14.3-21.2	*IL1A, IL1B, PROC*
Engert (2008)	French Canadian	50 families (320)	8p22	*Unknown*

Genome Wide Linkage Studies for Type 2 diabetes

More than 27 genome wide scans for linkage with Type 2 diabetes have been reported in populations of different ethnic groups including the Pima Indians, the Caucasians, the Mexican-Americans, the Africans, the Chinese, the Japanese and the Indians (Table 3). Results from the different genome wide scans reveal that there is no single locus that contributes to T2D but certain susceptibility regions showed some evidence of replication and these regions were most likely to harbour susceptibility genes to T2D. The most promising replicated signals were on chromosomes 1, 2, 12 and 20 (Ehm et al, 2000):

- Chromosome 1q21-23 is the best replicated region of T2D linkage (Table 3) and two genes have been reported to be associated with T2D in this region, liver pyruvate kinase (*PKLR*) and calsequestrin 1 (*CASQ1*) [Das & Elbein, 2006].

- Chromosome 2q region (2q33-q37) was the first locus associated to T2D in Mexican American sibpairs (Hanis et al, 1996). Within the region of linkage on this chromosome, 3 common intronic variants of the gene *calpain 10* were found to be associated with T2D in several populations (Horikawa et al, 2000; Cox et al, 2004).

- A T2D susceptibility loci was first described on chromosome 12q in subset analysis of families from Western Finland (Mahtani et al, 1996) and the finding was subsequently replicated in other populations including Caucasians (Ehm et al, 2000) and Western Africans (Rotimi et al, 2004). However, no susceptibility gene has yet been identified within this region.

- Evidence for linkage was observed on chromosome 20q in multiple studies including the FUSION study (Ghosh et al, 1999), studies in Ashkenazy Jewish (Permutt et al, 2001), Han Chinese (Luo et al, 2001), Japanese (Mori et al, 2002) and West African families (Rotimi et al, 2004). 2 candidate genes, protein tyrosine phosphatase 1B (PTPN1) and hepatocyte nuclear factor 4, alpha (HNK4A), showed evidence for association with T2D within the 20q region, however replication studies have yielded inconsistent results (Das & Elbein, 2006).

Table 3: Summary of published genome wide scans for Type 2 diabetes
(Source: Das & Elbein, 2006)

Study	Population	Study sample	Loci (LOD≥2)
Hanis (1996)	Mexican American	330 affected sibpairs	2q; 5
Mahtani (1996)	Botanian Finn	26 extended families	12q
Hanson (1998)	Pima Indian	264 nuclear families	1q; 6; 11q
Duggirala (1999)	Mexican American	32 extended families	3p; 4q; 9p; 10q
Elbein (1999)	US Caucasian	42 extended families	1q; 2q; 18p
Ghosh (1999, 2000)	Finnish Caucasian	477 families (716 sibpairs)	11q; 20p; 20q
Ehm (2000) GENNID	US Caucasian African American Mexican American	77 families 65 African-American families 53 Mexican American families	5, 12, X 10p (African American) 3p (Mexican American)
Vionnet (2000)	French Caucasian	143 families	1q21-24; 3q
Permutt (2001)	Ashkenazi Caucasian	267 families	4q; 20q
Parker (2001)	Finnish Swedish Caucasian	353 families	18p11
Wiltshire (2001)	UK Caucasian	573 families (743 sibpairs)	1q24-25; 8p21-22; 10q23.3
Francke (2001)	Indo Mauritian	99 families	2q37; 3q22
Luo (2001)	Chinese Han	102 families	2q36.1; 9p21.3; 9q21.13; 20q
Meigs (2002)	Framingham Caucasian	330 families	1q21 (HbA1c level) 1q42.2 (Mean glucose)
Mori (2002)	Japanese	224 affected sibpairs	2q; 7p; 11p13; 15q13; 20q12
Lindgren (2002)	Botnian Finn	58 families	9q21
Bushfield (2002)	Indigenous Australian	1 extended family (232 members)	2q24; 8p22; 3q27
Hsueh (2003)	Amish Caucasian	1 extended family (691 members)	1q21; 1q31; 14q11
Van Tilburg (2003)	Dutch Caucasian	178 families	12q12-14; 18p11
Aulchenko (2003)	Dutch Caucasian	1 extended pedigree (79 nuclear families)	18p11
Reynisdottir (2003)	Iceland Caucasian	227 families	5q34-35.2
Iwasaki (2003)	Japanese	256 affected sibpairs	15q; 21q; 9q : in ordered subset analysis
Xiang (2004)	Chinese Han	257 families	1q21; 6q21
Ng (2004)	Chinese Hong Kong	64 families (126 sibpairs)	1q21-q25; 4q
Silander (2004)	Finnish Caucasian (FUSION II)	242 affected sibpairs	6q16-22; 14q23
Sale (2004)	African American	247 families	6q25.3
Rotimi (2004)	West Africa	343 sibpairs	20q13.3; 19; 12q24
Nawata (2004)	Japanese	102 affected sibpairs	11p13-p12; 6q15-16
Hunt (2005)	Mexican American (SAFDGS)	39 extended families	3p
Zhao (2005)	Chinese Han	34 multiplex nuclear families	1p34.2; 1q24.3

I.2.3   Genome wide association studies

For many years, linkage studies have constituted the only means of screening the entire genome for disease-susceptibility loci. Numerous associated chromosomal regions have been identified through these linkage studies but were rarely reproduced in independent studies and very few linkage studies had eventually led to the identification of causal disease variants (Panoutsopoulou & Zeggini, 2009).

Over the last decade, advances in high-throughput genotyping technologies, the availability of high-density SNP maps from the HapMap project coupled with the availability of large-scale sample sets have allowed genetic studies to evolve from genome-wide linkage scans to genome-wide association studies (Panoutsopoulou & Zeggini, 2009).   The genome wide association study approach enables the genotyping of hundreds of thousands of SNPs, usually 300,000 – 1,000,000 SNPs, across the entire genome to assess association for each SNP with disease status.   Genome wide association studies (GWAS) have more statistical power than linkage studies to detect small gene effects.   In contrast with traditional candidate-gene studies, GWAS involve an unbiased scan of genomic sequence variants independent of any biologic assumptions.   Over the past few years, GWAS have succeeded in identifying common variants associated with numerous common complex diseases and many of the discoveries have implicated previously unsuspected loci or genes, highlighting the strength of unbiased genome wide approach to uncover novel biology of disease (McCarthy & Hirschhorn, 2008).

However, the most significant disadvantage is the statistical problem of multiple comparisons inherent when simultaneous association tests are carried out on thousands of markers.   This may generate large number of false-positive results. Replication of results in several different study samples is thus essential to increase the probability of a true association.   Replication of genetic associations among subjects of different ethnicities will also address variability in SNP allele inheritance patterns among ethnic groups that could influence the way a particular variant affects disease risk (Hunter & Kraft, 2007).

GWAS can detect common susceptibility variants of modest effect size (allelic odds ratios ORs between 1.1 and 1.5) but are limited by the need for stringent statistical thresholds (P=5 x. 10^{-8}) (Panoutsopoulou & Zeggini, 2009).   They are often underpowered to identify variants with modest effects because of inadequate sample size.   These susceptibility variants may have low odd ratios even when they are be statistically significant, but once they are established as genuine, they may reveal novel causal mechanisms of disease.

Meta analysis of GWAS

One way to overcome sample size restrictions is to carry out meta-analyses by combining data from various individual GWAS. Results of additional replication studies on the most promising variants from initial GWAS may also be included in meta-analytic calculations (Ioannidis et al, 2007). Meta analyses thus provide a low-cost approach to augment power to detect novel loci with modest effects. They can also confirm association of variants previously identified in original GWAS and information for SNP selection for subsequent replication studies. However, studies included in meta analyses should be homogenous in study designs, target populations, exposure and outcome measurements to exclude between-study heterogeneity. Publication bias and selective reporting towards highly statistical significant results may threaten the validity of meta-analyses. This problem can be overcome by performing meta-analyses on primary individual patient data from the different studies. In fact, the trend today is towards the setting up of consortia and sharing of individual, de-identified data for meta-analyses (Chanock et al, 2007).

GWAS for cardiovascular diseases

Recently, several independent GWAS have revealed a common region on chromosome 9, which was consistently associated with coronary artery disease (CAD) and MI in several white cohorts. Mc Pherson et al (2007) identified a locus spanning a 58kb region extending from 22,062,301 to 22,120,389 on chromosome 9. Two single nucleotide polymorphisms (SNPs), namely rs10757274 and rs23833206 located within 20 kb on chromosome 9p21 define an allele which is associated with a 15-20% increase risk for CHD in 50% of individuals with heterozygosity for the allele and a 30-40% increase risk CHD in 25% Caucasians homozygous for the allele. The risk allele was not found to be associated with any of major risk factors for atherosclerosis. Helgadottir et al (2007) reported an association between two other SNPs (rs10757278 and rs23833207), located in the same 9p21 chromosomal region and myocardial infarction in an Icelandic population. The same genetic locus was found to be associated with CAD in a genome wide association study carried out by the Wellcome Trust Case-control Consortium (WTCCC, 2007) and the associated SNP in this study (rs1333049) is in strong linkage disequilibrium with the 4 above SNPs.

Replication case-control association studies carried out in several populations have confirmed chromosome 9p21 as an important susceptibility locus that confers high risk for the development of coronary artery disease across different populations (Shen et al, 2008, Hinohara et al, 2008; Asssimes et al, 2008).

Interestingly, the same 9p21 chromosomal region has been associated with increased susceptibility to type 2 diabetes in several independent population-based case-control studies (Zeggini et al, 2007; Scott et al, 2007; Saxena et al, 2007). However, the SNPs most strongly associated with CAD were not in linkage disequilibrium with those most strongly associated with T2D, though secondary signals for both phenotypes were found to be in LD with each other (Mohlke et al, 2008). This raises the possibility of a shared rather than a single mechanism causing both coronary heart disease and T2D.

One GWAS conducted by Samani et al (2007) identified, in addition to the 9p21 locus, additional loci (1p13.3, 6q25.1 and 10q11.2) associated with CAD that reached genome-wide significance level ($p<5x10^{-8}$). These loci include variants in *PSRC1* gene (1p13.3), in *MTHFD1L* gene (6q25.1) and *CXCL12* gene (10q11.2) (Table 4). Another study involving a three-stage analysis of GWAS data followed by a replication study in 25 000 individuals identified a risk locus on chromosome 3q22.3 [$p=7 \times 10^{-13}$] (Erdmann et al, 2009).

GWAS for Type 2 diabetes

Until recently, only a handful of loci have been associated with increased Type 2 diabetes risk through linkage fine-mapping and candidate gene studies and have been replicated in different populations. These include *PPARG*, peroxisome proliferators-activated receptor-gamma gene (Altshuler et al, 2000), *KCNJ11*, potassium inwardly-rectifying channel, subfamily J, member 11 gene (Gloyn et al, 2003), *TCF77L2*, transcription factor 7-like 2, hepatic gene (Grant et al, 2006) and *CAPN10,* calpain 10 gene (Horikawa et al, 2000).

With the advent of GWAS, there has been rapid progress in the field of genetics of Type 2 diabetes. The first GWAS was conducted on a French case-control cohort (Sladek et al, 2007) and the four subsequent studies were performed in an Icelandic population (Steinthorsdottir et al, 2007), in the UK (Wellcome Trust Case Control Consortium (WTCCC), 2007) and in two Finnish/Swedish populations (Diabetes Genetics Initiative (DGI) - Saxena et al, 2007 and FUSION - Scott et al, 2007). These studies confirmed association with the 3 previously established T2D loci (*PPARG, KCNJ11, TCF7L2*) and have led to the identification of novel T2D susceptibility loci (Table 3). The new loci include variant in zinc transporter *SLC30A8* gene*,* variants in a linkage disequilibrium block containing genes encoding the homeodomain protein (*HHEX)* and the insulin-degrading enzyme (*IDE);* variants in an intron of insulin growth factor binding protein 2 (*IGF2BP2*), in an intron of cyclin-dependent kinase 5-regulatory subunit associated protein 1-like 1 (*CDKAL1*), in non-coding regions near the genes coding for cyclin-dependent kinase-inhibitor A and B (*CDKN2A/B*) and variants in fat mass and obesity associated gene (*FTO*) (Sambuy, 2007; Zeggini, 2007; Vionnet & Hager, 2007). Most of the associated loci have been shown to have an impact pancreatic beta-cell function

(Perry & Frayling, 2008) except for the *PPARG* P12A variant which has been associated with insulin resistance and variants in *FTO* with increased obesity (Frayling et al, 2007).

Across the five GWAS, *TCF7L2* showed the largest effect size with an odds ratio of 1.37. The other confirmed loci displayed more modest effect size (odds ratio between 1.1 and 1.25), stressing on the need for very large sample sizes to increase power of the study.

Meta-analysis of three of the above T2D GWAS (DGI, FUSION, and WTCCC; involving a total of 4,549 cases and 5,579 controls), followed by replication testing in a large independent sample (comprising of 79,792 additional individuals of European origin) revealed six novel loci that reached statistically significant levels of association with T2D ($P<5.0 \times 10^{-8}$), odds ratios (OR) ranged from 1.09 to 1.15 (Zeggini et al, 2008). These novel loci were found to be in intron 1 of *JAZF1*, between *CDC123* and *CAMK1D*, between *TSPAN8* and *LGR5*, in exon 24 of *THADA*, near *ADAMTS9* and in intron 5 of *NOTCH2* (Table 4). Regardless of their modest effect sizes, these novel T2D-associated loci, through the genes to which they are associated, provide additional important clues as to the process involved in the maintenance of normal glucose homeostasis and in the pathogenesis of T2D. The first three loci have been associated with measures of insulin release, providing more evidence as to the crucial role of defects in pancreatic beta-cell function in the pathogenesis of T2D (Grarup et al, 2008).

I.2.4   Positional candidate gene approach

Once chromosomal loci associated with disease have been identified, subsequent analysis to resolve the signal is typically through a candidate gene approach. Based on the growing body of genome resources, the positional candidate gene approach allows researchers to combine information about a chromosomal location with increasingly detailed genetic and physical maps, allowing for easier identification of a potential causative genetic variant.

After localization of disease-associated chromosomal loci, genomic databases are searched for candidate gene(s) within that region. The linked chromosomal locus may harbour several potential candidate genes for the disease. Interesting candidate genes are selected on the basis of their biological functions and their possible involvement in the pathophysiology of the disease. The candidate gene(s) is then tested for disease-causing mutations.

The candidate gene approach involves association studies that compare allele frequencies in cases and controls to assess the contribution of genetic variants to phenotypes of interest. Association analysis can be used in population-based case-control studies or in family-based

association studies where samples consist of affected offsprings and unaffected parents (Panoutsopoulou & Zeggini, 2009).

Table 4: Loci associated with CAD and T2D, identified or confirmed by GWAS
(Source: Hindorff et al, 2010)

Phenotype	SNP	Chr	Gene/Nearest gene	OR (95% CI)	P- VALUE
CAD	rs1333049	9p21.3	*CDKN2A/CDKN2B*	1.36 (1.27-1.46)	2.9×10^{-19}
CAD	rs9818870	3q22.3	*MRAS*	1.15 (1.11-1.19)	7×10^{-13}
CAD	rs501120	10q11.2	*CXCL12*	1.33 (1.20-1.48)	9×10^{-8}
CAD	rs599839	1p13.3	*CELSR2-PSRC1-SORT1*	1.29 (1.18-1.40)	4.1×10^{-9}
CAD	rs6922269	6q25.1	*MTHFD1L*	1.23(1.15-1.33)	2.9×10^{-8}
T2D	rs7903146	10q25	*TCF7L2#*	1.37 (1.31-1.43)	1.0×10^{-18}
T2D	rs10811661	9 p21.3	*CDKN2A/B*	1.20 (1.14-1.25)	7.8×10^{-15}
T2D	rs8050136	16q12	*FTO*	1.17 (1.12-1.22)	1.5×10^{-12}
T2D	rs7578597	2p21	*THADA*	1.15 (1.10-1.20)	1.1×10^{-9}
T2D	rs4402960	3q27	*IGF2BP2*	1.14(1.11-1.18)	8.9×10^{-15}
T2D	rs5219	11p15	*KCNJ11#*	1.14 (1.10-1.19)	6.7×10^{-11}
T2D	rs1801282	3p25	*PPARG#*	1.14 (1.08-1.20)	1.7×10^{-8}
T2D	rs1111875	10q23	*HHEX/IDE*	1.13 (1.08-1.17)	5.7×10^{-10}
T2D	rs10923931	1p13	*NOTCH2*	1.13 (1.08-1.17)	4.1×10^{-8}
T2D	rs7754840	6 p22	*CDKAL1*	1.12 (1.08-1.16)	4.1×10^{-11}
T2D	rs13266634	8 q24	*SLC30A8*	1.12 (1.07-1.16)	5.3×10^{-8}
T2D	rs12779790	10p13	*CDC123/CAMK1D*	1.11 (1.07-1.14)	1.2×10^{-10}
T2D	rs864745	7p15	*JAZF1*	1.10 (1.07-1.13)	5.0×10^{-14}
T2D	rs7961581	12q14	*TSPAN8/LGR5*	1.09 (1.06-1.12)	1.1×10^{-9}
T2D	rs4607103	3p14	*ADAMTS9*	1.09 (1.06-1.12)	1.2×10^{-8}

The table includes those loci that reach GWAS significance level p= 5x10⁻⁸ , with only a single SNP reported in the given locus. Odds ratio and p-value are based on currently available data from catalogue of GWAS (Hindorff et al, www.genome.gov/gwastudies).

CAD, coronary artery disease; T2D, type 2 diabetes

* T2D loci identified in GWAS meta-analysis

 # T2D loci identified in linkage or candidate gene analysis and confirmed in GWAS

I.3.   Search for candidate gene(s) for CHD and Type 2 diabetes in the Mauritian population

Our strategy for a better understanding of the molecular basis in the pathogenesis of premature CHD and Type 2 diabetes was to use non-parametric linkage analysis through a genome wide scan to define chromosomal regions associated with the diseases. Few genome wide scans have been reported to look for regions harboring susceptibility genes to CHD. This was probably due to the difficulty in recruiting sufficient number of families to achieve optimal power to detect linkage regions, in a disease with high mortality rate.

The genome-wide scan was carried out in 1999 and it involved 99 independent Mauritian families of North Indian origin. Several chromosomal regions, namely 16p13, 10q23 and 3q27 showed nominal or suggestive linkage to CHD in our study population and after fine mapping with additional markers, these regions were found to overlap with chromosomal regions that have shown linkage with Type 2 diabetes in other populations (Francke et al, 2001). A particularly interesting chromosomal region was reported in the 8q23 region where microsatellite markers were found to co-segregate with premature CHD, Type 2 diabetes and hypertension (Franck et al, 2001) (Figure 3). Further ordered-subsets analyses confirmed the contribution of this region to susceptibility to both CHD and Type 2 diabetes. The 8q23 chromosomal region has been reported to show linkage to low HDL-Cholesterol trait in Finnish families which were ascertained for familial low HDL-cholesterol and premature CHD (Soro et al, 2002).
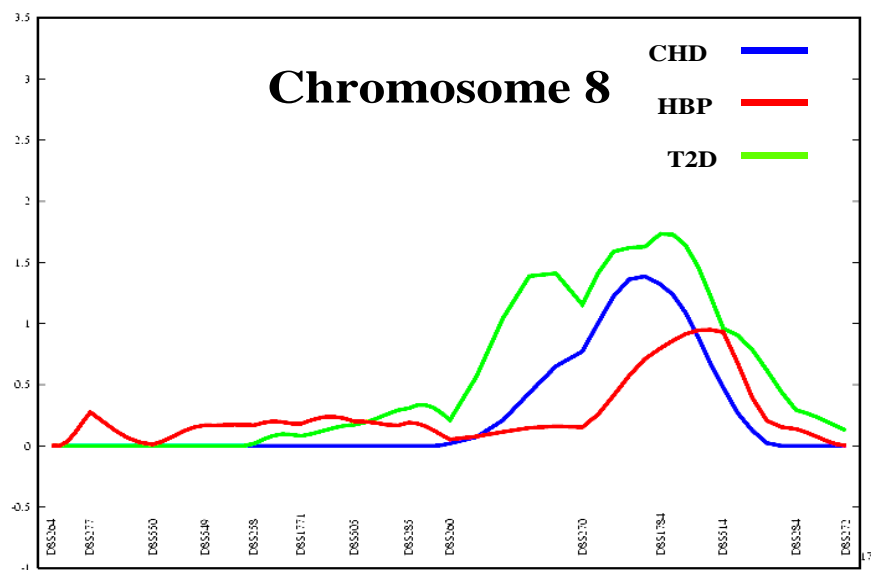
Figure 3: Multipoint analysis results for chromosome 8 showing overlapping between
qualitative traits CHD, T2D and HBP in the Mauritian genome scan
(Source: Manraj, 2002)

I.3.1. Candidate genes in the 8q23 chromosomal region

Once a chromosomal region has been linked to a disease or phenotypic trait of interest, the next step is to identify the candidate genes within the genetic interval and to select the best candidate gene(s). Given the interesting results in the Chr 8q23 region, we proposed to look for susceptibility gene or gene variants to premature CHD and/or Type 2 diabetes within this specific region. One common approach in the selection of candidate genes within a particular chromosomal region is to use biological information relating to the molecular pathology of the disease (Risch, 1997). Using publicly available databases, we therefore identified several potential candidate genes within the 8q23 chromosomal region based on the activities of their proteins (Figure 4), which we hypothesized to be most relevant in the pathogenesis of CHD and/or T2D and among these candidate genes were:

- The *Fog2* (Friend of GATA2) gene also known as *ZFPM2* (Zinc finger protein multitype 2) gene codes for a multi-zinc finger transcriptional corepressor protein (FOG2) that binds specifically to transcription factor GATA4. Studies using knockout mice suggested a role of FOG2 in heart morphogenesis and development of coronary vessels (Crispino et al, 2001). In addition, cardiomyocyte expression of *Fog2* was shown to be essential for maintenance of myocardial and coronary vessel function in the adult heart (Bin Zhou et al, 2009).

- *TRHR* gene encodes the thyrotropin releasing hormone receptor (TRHR), which belongs to the G-protein coupled receptor family. Binding of Thyrotropin releasing hormone (TRH) to TRHR activates the inosito-phospholipid-calcium protein kinase C transduction pathway. The TRHR gene is associated with lean body mass (LBM) variations (Xiao-Gang Liu et al, 2009). LBM accounts for 60% or more of body weight and thus contributes significantly to variations in body mass index (BMI), an index commonly used for obesity. Previous linkage studies have linked the 8q23 locus to BMI (Chagnon et al, 2001; Platte et al, 2003).

- The oxidation resistance 1 (*OXR1*) gene codes for the oxidation resistance 1 protein. . The expression of this gene is found to be induced by oxidative stress and the protein is involved in the protection against oxidative damage when localized in the mitochondria (Volkert et al, 2000). Oxidative stress is increasingly being recognized as an important contributor to the pathogenesis of Type 2 diabetes and cardiovascular diseases including CHD (Ceriello & Moetz, 2004). Oxidative stress is also believed to precede the development of endothelial dysfunction and insulin resistance in individuals with impaired glucose metabolism in the Mauritian population (Gopaul et al, 2001).
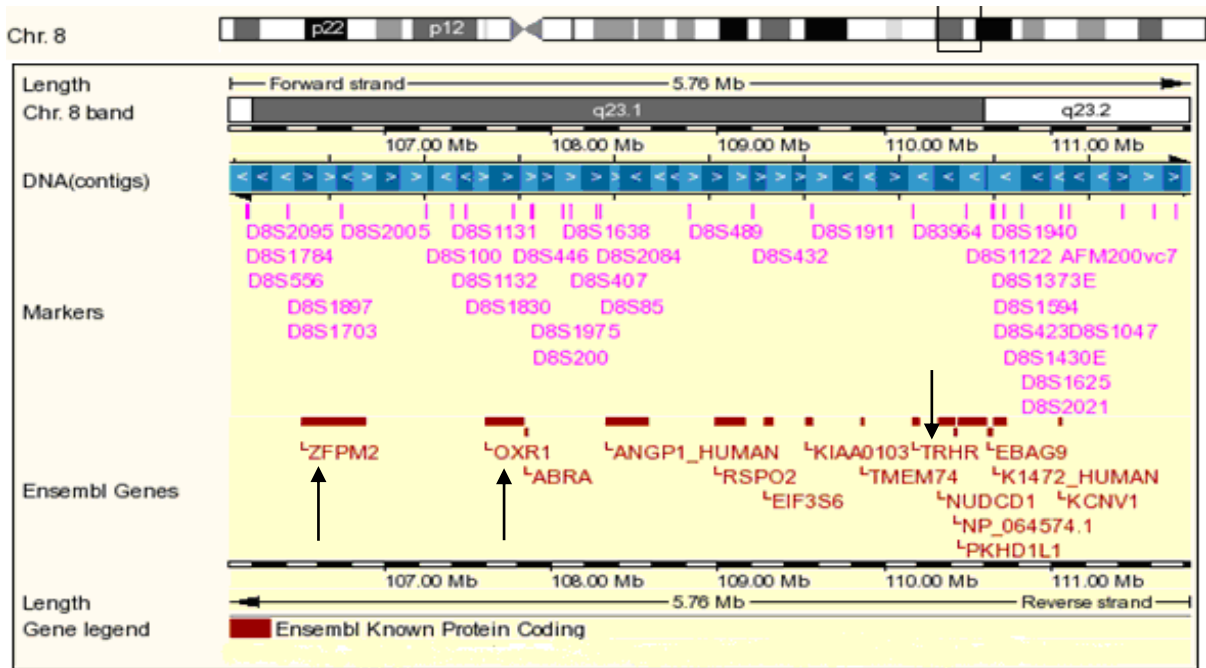
Figure 4: Genes within the 8q23 chromosomal region
(Ensembl Genome Browser, Version 39)

Potential candidate genes for CHD and/or T2D within this region are ZFPM2 (zinc finger protein multitype 2), OXR1 (oxidation resistance 1) and TRHR (thyrotropin releasing hormone receptor) [indicated by arrows]

I.3.2. *OXR1* gene as the best candidate gene.

We chose the Oxidation Resistance 1 (*OXR1*) gene as our best candidate gene in the 8q23 chromosomal region, given the increasing role attributed to oxidative stress in the pathophysiology of CHD (Ceriello & Moetz, 2004) and T2D (Gopaul et al, 2001).

The human *OXR1* gene was discovered by Volkert et al (2000) in their search for human genes that provide protection against oxidative damage. This gene belongs to a family of well-conserved oxidation protection genes (the *OXR* family genes) present in a variety of eukaryotes, but not in prokaryotes. The eukaryotic *OXR1* genes encode proteins of various sizes, although they all contain a well-conserved 300-amino-acid C-terminal domain.

The Oxr1 protein is thought to play an important role in maintaining normal levels of resistance to oxidative damage in the eukaryotic cell and this oxidation protection function is contained within the conserved domain of the protein (Volkert et al, 2000). Mitochondrial localization is required for the hOxr1 protein to protect against ROS molecules derived from peroxides and expression of the protein is induced by multiple stress conditions such as heat and oxidative stress.

Expression of the *OXR1* gene has been characterized in multiple human tissues: Two transcripts of 2.9 and 4.9 kb have been identified in nearly all human tissues except in the brain. In fact, the brain was found to express a unique 5.1 kb transcript in addition to the 2.9 kb transcript. The relative abundances of the two transcripts however differ in the different tissues with the 4.9 kb transcript being more abundant in the lung, liver, kidney, pancreas and placenta. In the heart and skeletal muscles, the two transcripts are found in equal amounts. The *hOXR1* (human *OXR1*) mRNA appears to be abundant in tissues with a relatively high respiration capacity (heart, skeletal muscle, brain), where it would be advantageous to counteract mitochondrial ROS production (Elliot and Volkert, 2004).

Experiments carried out in mutant yeast strains showed that the Oxr1 protein provided protection from oxidative damage associated with hydrogen peroxide and acted on these molecules specifically rather than simply shielding DNA or other cellular components from their damaging effects. It is thought to protect the cells by suppressing the production or reactivity of reactive oxygen molecules derived from hydrogen peroxide (Elliot and Volkert, 2004). However, the exact mechanism by which this occurs is still unclear.

I.3.3.   Mitochondrial oxidative dysfunction

In most mammalian cells, the mitochondria are recognized as the most important cellular source of energy as well as the major generator of intracellular ROS. ROS generation in the mitochondria is influenced by several factors which include the efficiency of the oxidative phosphorylation (OXPHOS) system itself, the oxygen concentration, and the activity of the antioxidant defenses among others. The mitochondria itself is a target of the ROS generated and numerous proteins have evolved to protect against or repair oxidative damage within the mitochondria. Under normal conditions, the mitochondria are able to counteract the production of ROS with its antioxidant defense system which can detoxify the amount of ROS produced. In case of minor oxidative stress, the cells may increase the number of mitochondria to produce more energy (Wang et al, 2009). However, excessive release of ROS from the OXPHOS system due to an overproduction or a deficiency in the defense mechanism can lead to an accumulation of mutations within the mitochondrial DNA (mtDNA). Indeed, mtDNA is the most sensitive cellular target of ROS as it is found in close proximity to the sites of ROS production within the mitochondria itself. In addition, it is not protected by histone proteins as is the case for nuclear DNA (Richter et al, 1988). The mtDNA consists of 37 genes coding for 13 proteins that function as subunits for the respiratory complexes I, III, IV and V. Damage to the mitochondrial genome eventually leads to inefficient oxidative phosphorylation, causing the production of more ROS which will further damaged the mtDNA, and eventually disrupting the mitochondrial function.

Increased production of reactive oxygen species in mitochondria, accumulation of mtDNA damage, and progressive mitochondrial dysfunction are associated with atherosclerosis or Type 2 diabetes in human studies and in animal models of type 2 diabetes (Mulder et al, 2009; Lu et al, 2010). Moreover, major precursors of atherosclerosis such as hypercholesterolemia, hyperglycemia, hypertriglyceridaemia, all induce mitochondrial dysfunction, with an accumulation of oxidative mtDNA mutations (Puddu et al, 2009). Chronic overproduction of mitochondrial reactive oxygen species leads to destruction of pancreatic beta-cells, increased oxidation of low-density lipoprotein and dysfunction of endothelial cells and these factors promote atherosclerosis. In addition, mitochondrial dysfunction can result in apoptosis, favoring plaque rupture.

I.3.4. Oxidative stress as the pathogenic mechanism underlying CHD and T2D

Oxidative stress represents the imbalance between the production of reactive oxygen species and the antioxidant defense system (Betteridge, 2000). Reactive oxygen species (ROS) are compounds that are produced during normal cellular metabolism. They are also involved as signaling molecules and in defense mechanisms such as phagocytosis. However, they are highly reactive molecules that can interact with cellular components, resulting in lipid, protein and DNA damage. It is therefore essential for cells to control the production and activity of these molecules. Intact cells thus have pro-oxidant/anti-oxidant systems that continuously generate and detoxify oxidants during normal aerobic metabolism. When there is an overproduction of ROS or a deficiency of enzymatic or non-enzymatic antioxidants, this leads to a state of oxidative stress whereby oxidative damage to lipids, proteins, carbohydrates, and nucleic acids may occur with impairment in the cellular structure and function. Severe oxidative stress may ultimately lead to apoptosis.

Oxidative stress may be the leading mechanism underlying insulin resistance, diabetes and cardiovascular diseases (Figure 5) (Ceriello & Moetz, 2004). Inflammatory changes are considered a common pathogenic step in all of these conditions (Lusis, 2000; Hu & Stampfer, 2003) and it is well established that inflammation is induced by oxidative stress through the activation of pathways that generate inflammatory mediators such as interleukins and adhesion molecules, leading to atherosclerosis (Roebuck, 1999). Several studies have associated oxidative stress with Type 2 diabetes (Gopaul et al, 2001). Indeed, reactive oxygen species can induce inactivation of the signaling pathway between the insulin receptor and the glucose transporter system, leading to insulin resistance (Puddu et al, 2009).
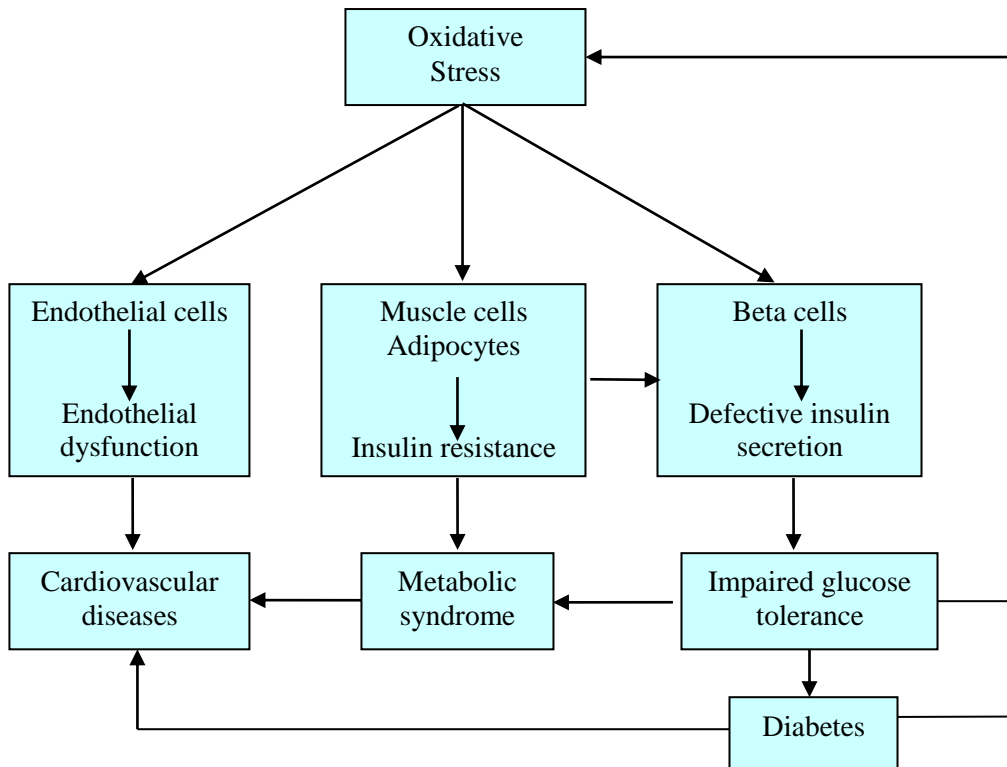
Figure 5: Oxidative stress as the common denominator of insulin resistance, T2D and CVD (Modified from Ceriello & Moetz, 2004).

Increased free radicals generation leads to state of oxidative stress within cells. In endothelial cells and pancreatic beta cells, oxidative stress induces dysfunction: Endothelial dysfunction may lead to the development of cardiovascular diseases and beta cells dysfunction is characterized by defective insulin secretion, resulting in impaired glucose tolerance and eventually to overt diabetes. In presence of glucose and free fatty acids (FFAs) overload, muscle cells and adipocytes protect themselves against the resulting oxidative stress, producing resistance to insulin action, aiming at reducing the entry of glucose and FFAs within the cells. Insulin resistance is very often accompanied by a cluster of risk factors, the metabolic syndrome. The metabolic syndrome as well as diabetes also contributes to the development of cardiovascular diseases.

I.4 Aim of study

I.4.1 *OXR1* study

The main objective of this project was to investigate the contribution of the positional candidate gene *OXR1* to susceptibility to premature coronary heart disease and/or to Type 2 diabetes in the Mauritian population through a population-based association study (Study 1).

I.4.2 Replication study of Chromosome 9p21 variant

In addition, based on recent GWA findings, we carried out a replication study to explore association between variant rs1333049 on chromosome 9p21 and premature CHD in our population (Study 2).

II.   Methodology

We used a population-based case control design to test for association between SNP variants in the *OXR1* gene and premature CHD and /or T2D and to conduct replication study of the Chr9p21 locus in our population. Ethical clearance for the *OXR1* study was granted by the National Ethics Committee of the Ministry of Health & Quality of Life (Ref: MHS 458/33) in 2005.

II.1   Study samples

We used DNA samples from pre-existing resources that were collected between 1995 and 1997 for the study of premature CHD and the metabolic syndrome at the SSR Centre. CHD patients were recruited from two cardiac outpatient departments of the Ministry of Health, namely at the Victoria Hospital and SSR National Hospital with the permission of cardiologists-in-charge. Most of the controls were recruited among workers of several sugar estates. Biochemical measurements performed in the fasting state included plasma glucose, total cholesterol, HDL-cholesterol, triglycerides, uric acid, urea, creatinine, liver enzymes (SGOT, SGPT, GGT), haemoglobin and insulin levels. Oral glucose tolerance test (OGTT) was carried out for individuals who were not known to be diabetics and who did not present glucosuria in morning urine.   All participants underwent a clinical examination with blood pressure and anthropometric measurements. Data on past personal history, smoking, alcohol intake, physical activity, medication and family history was collected using a standard questionnaire.

II.2   Selection of North-Indian case-control groups

The North Indian (NI) case-control groups were selected from the original phenotypic databases. The North Indian groups included Muslims and Hindus from the Indo-Mauritian community: their ancestors shared the same geographic origin, having migrated from the port of Calcutta in the North of India. This ensured a homogenous genetic background in our study population. Population stratification, which may lead to false gene-disease associations, was less likely to be present.

We used Epi 2000™, a Database and Statistics application software (www.cdc.gov/epiinfo), to analyze data from the existing databases so as to select our case-control group.

The patients group included 368 unrelated NI patients (294 men, 74 women) affected by premature coronary heart disease (CHD) with or without type 2 diabetes (T2D).

Coronary heart disease status was characterized in previous study (Manraj, 2002) as being positive, based on the following criteria:

- Previous diagnosis of myocardial infarction
- History of revascularization procedures such as angioplasty or coronary artery bypass
- Following treatment for angina pectoris

Glucose metabolism status was defined, based on fasting plasma glucose (FPG) and 2-hour plasma glucose values (2HPG), and on past history of type 2 diabetes, using the ADA 1997/WHO 1999 criteria (Gabir et al, 2000)

- Normal glucose metabolism: FPG < 6.1 mmol/l and 2HPG < 7.8 mmol/l .
- Impaired fasting glucose metabolism (IFG): 6.1 < FPG < 7.0 mmol/l and 2HPG < 7.8 mmol/l
- Impaired Glucose Tolerance (IGT): FPG < 7 mmol/l and 7.8 < 2HPG < 11.1 mmol/l
- Type 2 Diabetes (T2D) if having a previous history of diabetes or FPG $\geq$ 7.0 mmol/l or 2HPG$\geq$ 11.1mmol/l

The control group included 252 unrelated healthy NI individuals (160 men, 92 women) who do not show signs and symptoms of CHD and have a normal glucose metabolism (i.e. without T2D or IGT), confirmed by an oral glucose tolerance test.
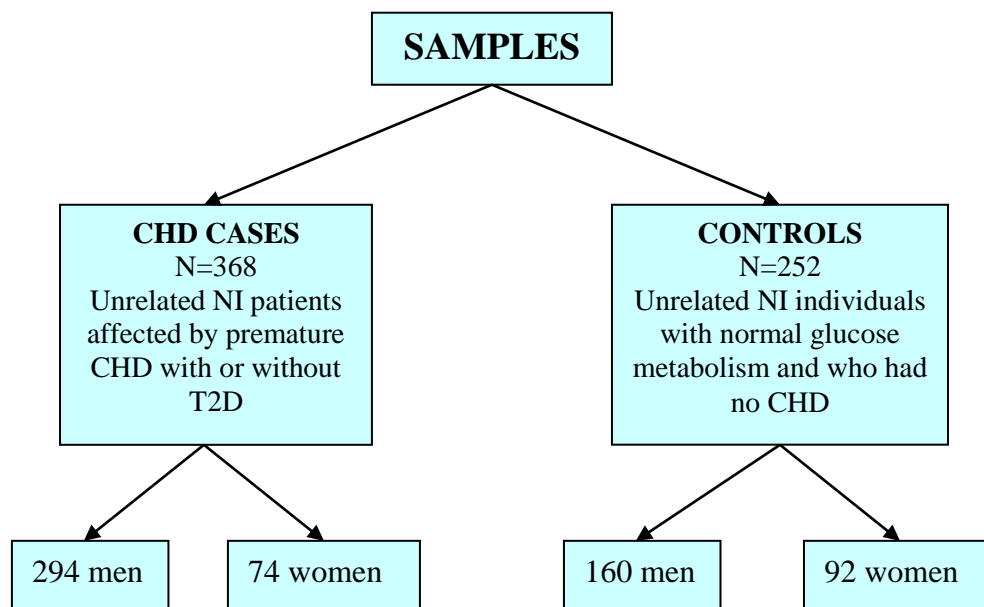
II.3   DNA samples

In the previous study, whole blood EDTA samples for DNA extraction were collected and DNA was extracted from white blood cells using the phenol-chloroform method. The stock DNA samples were stored at -20°C and diluted samples were kept at 4 °C.

II.3.1 Quantification

The concentrations of the stock DNA samples were re-assessed by spectrophotometry, using a Genequant *pro* DNA/RNA calculator: 10 µl of genomic DNA was diluted in 1 ml of distilled water and its absorbance was read at 260 nm using a quartz cell of 1 cm light path. For greater accuracy, absorbance readings should be between 0.1 and 1.0. An absorbance of 1 unit at 260 nm (light path 1 cm) corresponds to 50 µg/ml of genomic DNA at pH 7.0 ($A_{260} = 1 \Rightarrow 50$ µg/ml) and the concentration of the DNA sample was calculated using the following formula:
Concentration of DNA (in µg/ml) $= A_{260}$ x 50 µg/ml x dilution factor (101).

II.3.2 Quality control

High molecular weight DNA templates (> 50 kb) are required for good PCR performance. The integrity and size of our DNA samples were assessed by agarose gel electrophoresis (Figure 1): 2 µl of stock DNA sample was diluted with 8 µl sterile distilled water. The diluted DNA sample was mixed with 3 µl of loading buffer (Ficoll blue containing bromophenol blue and xylene cyanol as dyes) and was loaded onto a 0.8% agarose gel in Tris-Borate-EDTA (TBE) 1X buffer (Figure 6). Electrophoresis was carried out at 100 V for 15-20 minutes.
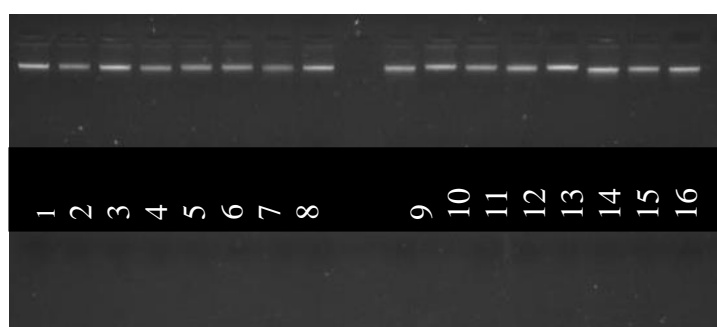
Figure 6: Quality control of diluted DNA samples by electrophoresis on 0.8% agarose gel

II.3.3. Dilution of the samples

After ensuring that the DNA samples were of good quality, the DNA stock solutions were diluted with sterile distilled water to a working concentration of 10 ng/µl in a final volume of 500 µl. The diluted samples were kept at 4ºC in microrack system of 96 tubes.

II.4. Selection of SNPS

II.4.1 Study 1: Selection of *OXR1* SNPs

Selection of single nucleotide polymorphisms (SNPs) is a problem of primary importance in association studies. Several approaches have been proposed but none provides a satisfying answer to the problem of how many SNPs should be selected, and how this should depend on the pattern of linkage disequilibrium (LD) in the region under consideration.   Moreover, SNP selection is usually considered as independent from deciding the sample size of the study. However, when resources are limited there is a tradeoff between the study size and the number of SNPs to genotype (Pardi et al, 2005).

SNPs occurring within regions of functional significance such as coding regions, splice junctions, promoter regions are of particular interest because changes in these genic regions have been shown to be the most common causes of Mendelian diseases and they are hypothesized to contribute to common, complex diseases as well (Bolstein and Risch, 2003). However, SNPs within untranslated regions, intronic sequence and upstream sequence also need to be considered during SNP selection since variants involved in complex trait susceptibility very often act through effects on transcriptional regulation, and /or RNA stability and those variants are found in the non-exonic regions (McCarthy, 2002).

Given the vast number of SNPs now available for association studies, it is essential to have software tools that enable the selection of cost effective SNP panels that provide high power of study. *Ensembl* genome browser and SNPbrowser are the two softwares used in our SNP selection procedure.

6 SNPs were chosen so as to span the whole *OXR1* gene from the 5'upstream-untranslated region to the 3' downstream-untranslated region. Initially, we used publicly available Ensembl Genome Browser (Version 39 – *www.ensembl.org*) to search for SNPs in the *OXR1* gene (Figure 7) and we shortlisted 33 SNPs.
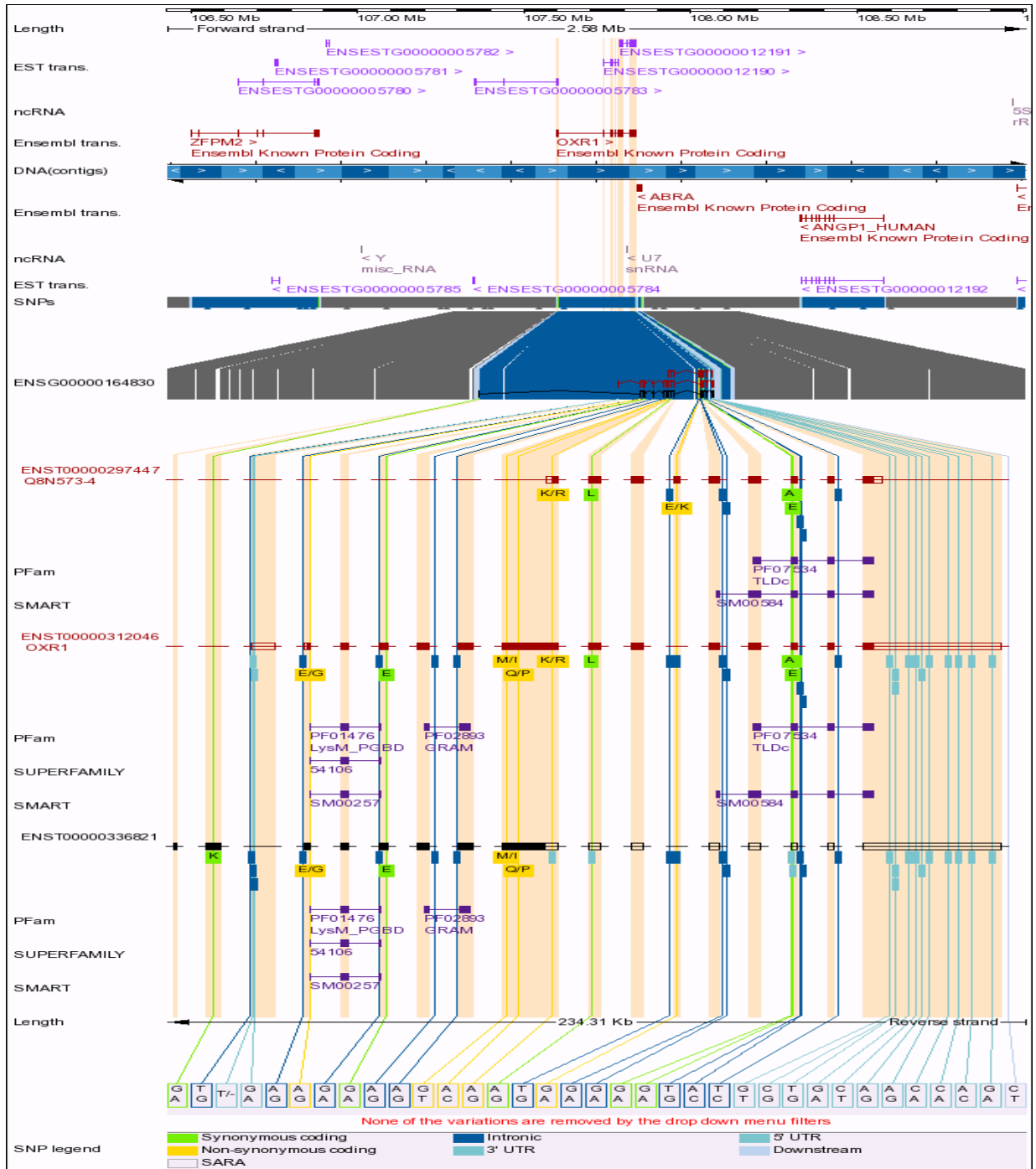
Figure 7: SNPs and variations in the region of *OXR1* gene
(*www.ensembl.org*)

Using another browser, the Applied Biosystems SNPbrowser™, we looked for validated SNPs from the *Ensembl* list that met our selection criteria. The Applied Biosystems SNPbrowser™ software enables a knowledge-driven selection of optimal sets of SNPs for association and fine mapping studies from over 6 million SNPs associated with TaqMan® System assays (Issac and De La Vega, 2003). SNPs can be visualized in their genomic context along with linkage disequilibrium maps and putative haplotype blocks derived from the analysis of over 3 million SNPs genotyped in several populations [CEPH-Utah population of European ancestry (CEU), Chinese Hans (CHB), Japanese (JPT) and African Yoruba (YRI)] by either the International HapMap project or Applied Biosystems. Since no LD data was available for Asian Indian

population, our SNPs selection was based on HapMap data in CEU population, which we hypothesized to be the least divergent from our study population. We favored SNPs with higher MAF (minor allele frequency ≥ 20%), based on the a priori scientific belief that these SNPs are more likely to be associated with the disease or that the statistical power to detect an existing association is higher with these SNPs (Jorgensen et al, 2009).

Criteria used for SNP selection in SNPbrowser™:

- HapMap MAF in CEU population >=20%
- Haplotype R^2 threshold = 100%
- LDUs: single= 0.5, cumulative=1.0
- Validated in Hapmap dataset

Out of the 33 SNPs from *Ensembl* list, only 5 were validated in Hapmap dataset and available in TaqMan Assays (Applied Biosystems SNPbrowser™). From these 5 validated SNPs, we selected 2 that met our selected criteria for minor allele frequency i.e. (MAF) ≥ 20 in the CEU population. The remaining 4 SNPs were selected from a list of HapMap validated tagging SNPs obtained from SNPbrowser™, so as to span the whole *OXR1* gene. The 4 SNP selected from SNPbrowser™ were all tagging SNPs i.e. they can be used to predict the allelic status of other SNPs (tagged SNPs).

The 6 selected SNPs include (Table 5):

- **rs2282509** in the 5'upstream UTR at location 8:107,739,104. The 5' UTR is important as it may harbour sequences, which code for signaling peptides.
- **rs1681886** in intron 2 at location 8: 107761056
- **rs2126579** in intron 6 at location 8:107785629
- **rs776959** in intron 9 at location 8:107799609. One SNP was selected in intron 9 since it is the longest intronic region of the gene.
- **rs1681904** (synonymous coding in exon 12) and
- **rs6983111** (3'downstream)

Figure 8 shows the genomic structure of *OXR1* gene and the location of the selected SNPs within the gene.

Table 5: Selected SNPs within the *OXR1* gene

SNP ID	Type/Location	Position: Chr:bp	Alleles	HapMap Minor Allele Frequency			
				CEU	CHB	JPT	YRI
rs2282509	5' UTR	8:107739104	T/C	0.42	0.36	0.41	0.20
rs1681886	Intronic (intron 2)	8:107761056	G/A	0.39	0.34	0.42	0.22
rs2126579	Intronic (intron 6)	8:107785629	C/T	0.24	0.16	0.23	0.37
rs776959	Intronic (intron 9)	8:107799609	T/C	0.37	0.47	0.31	0.07
rs1681904	Synonymous_coding (exon 12)	8:107823649	G/A	0.40	0.34	0.41	0.22
rs6983111	3' Downstream	8:107834190	C/T	0.24	0.16	0.23	0.37
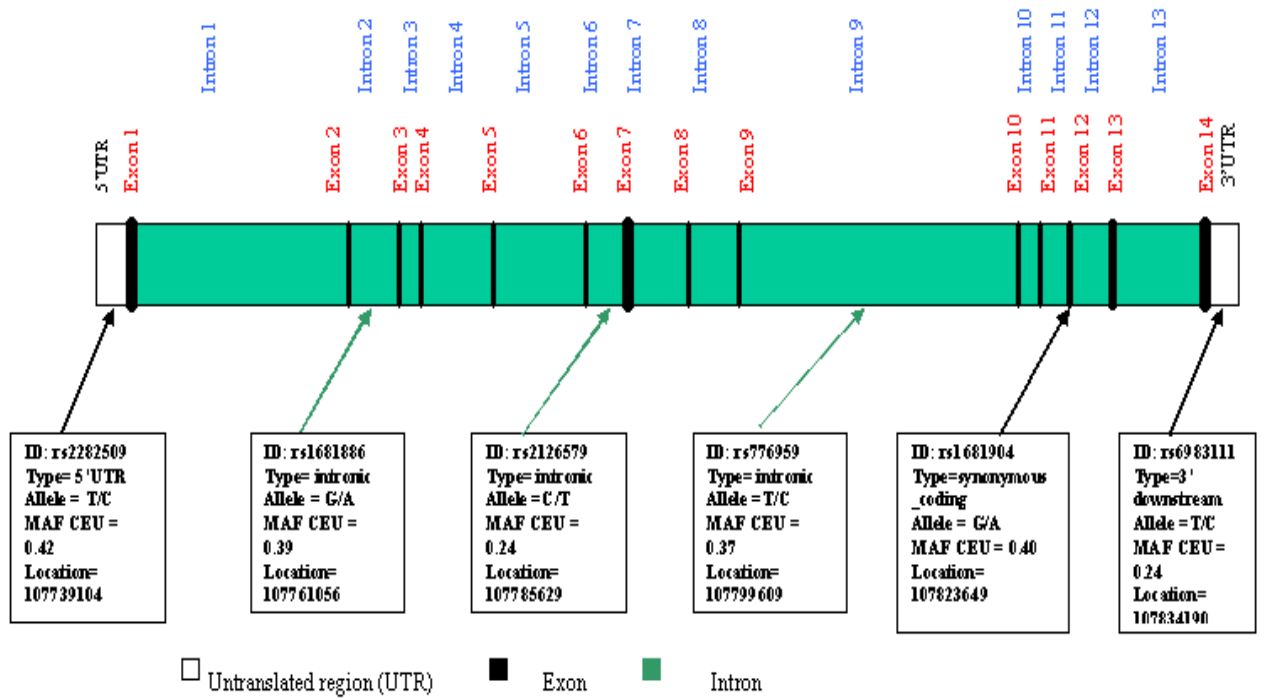
Figure 8: Genomic structure of the *OXR1* gene and location of selected SNPs

II.4.2 Study 2: SNP in 9p21 chromosomal region

Within the 9p21 chromosomal region, more than 19 SNPs have been associated with coronary artery disease (CAD) in the WTCCC study (WTCCC, 2007) and these SNPs were in strong linkage disequilibrium (LD) with each other. Two haplotypes blocks were identified, with very strong LD within each block (average |D'| 0.90) and moderate LD between blocks (average |D'| ~ 0.60). Three SNPs in block 1 (rs7044859, rs1292136, rs7865618) and one SNP in block 2 (rs1333049) were sufficient to tag the region (Figure 9).

SNP rs1333049 was found to be strongly associated with CAD (p=2.91 x10^{-19}). The risk-associated allele, defined by the C allele is common (allele frequency ~ 50%) and has shown an increased risk for CAD of 20-30% per copy of allele (Samani et al, 2007).
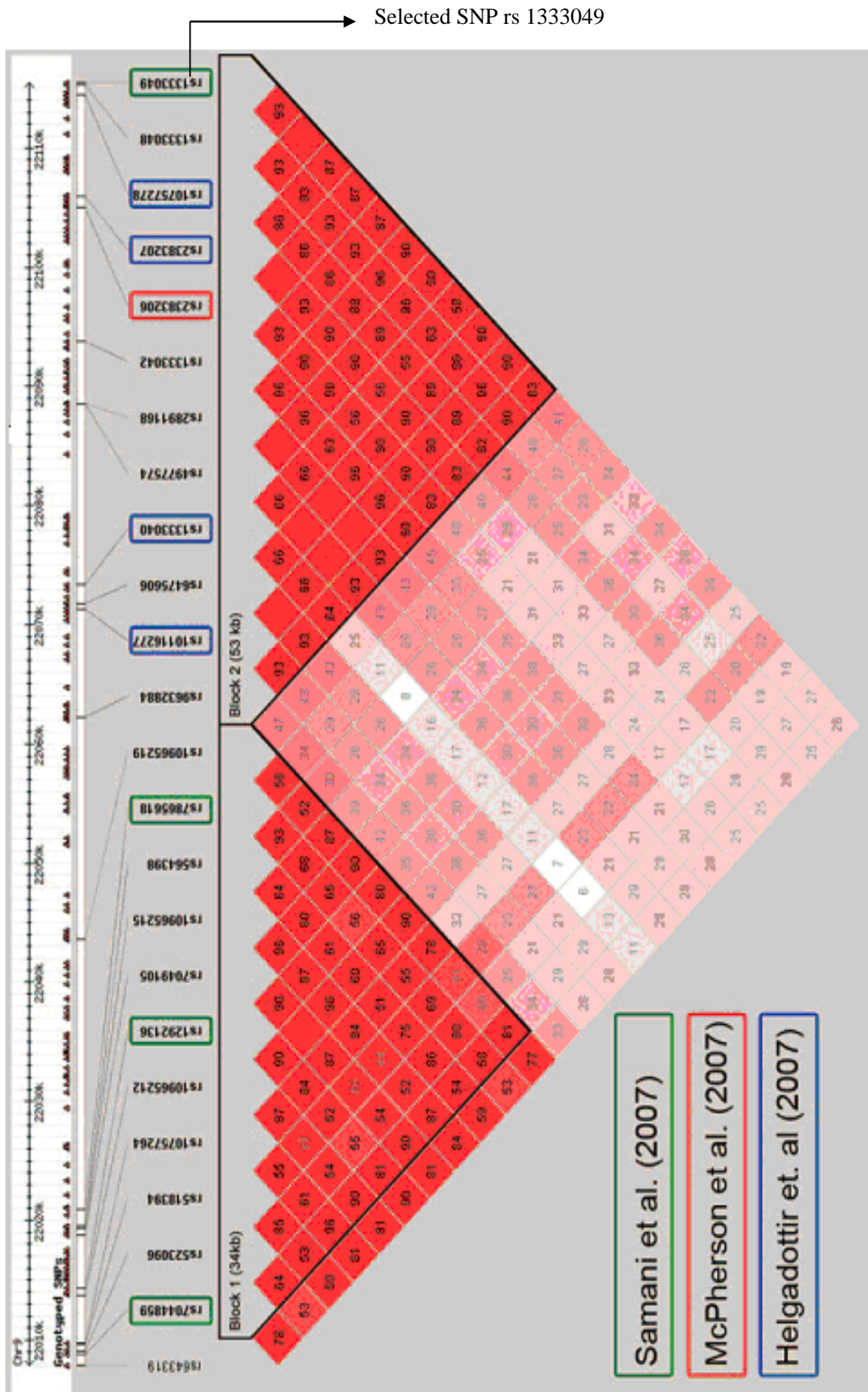
Figure 9: LD diagram of HapMap data (r^2) showing SNPs of Chromosome 9p21 region described in Samani et al, McPherson et al and Helgadottir et al studies. The lead SNPs in each study are marked in different colours. (Source: Schunkert et al, 2008). Two haplotype blocks have been identified and our selected SNP rs1333049 is found in haplotype block 2.

Given the strong association reported between SNP rs1333049 and CHD, we chose to genotype this tagging SNP in our North Indian study population.

II.5 SNP Genotyping

Genotyping was performed by Taqman^{TM} assay in 96-well plates after optimization of each set of primers. The Taqman^{TM} assay (also known as the 5' nuclease allelic discrimination assay) combines both PCR amplification and detection of alleles into one single step. It uses two allelic-specific probes, which bind only to the specific complementary sequence. Each probe contains a different fluorophore dye at the 5' end and the fluorescent activity of the dye is suppressed by the close proximity of a non-fluorescent 'quencher' at the 3' end of the probe.

During PCR, the probe hybridizes specifically to its complementary sequence at the SNP site between the two primer sites. The 5' exonuclease activity of the *Taq* DNA polymerase cleaves the bound probe as it replicates the template. Cleavage of the probe separates the fluorophore from the quencher, resulting in an increase in fluorescence. Depending on the type of fluorescence generated, this will indicate which alleles are present in the DNA sample.

Pre-designed TaqMan SNP genotyping assay kits from Applied Biosystems (Foster City, CA) were used (Table 6). The assay kit included both the target-specific PCR primers (forward and reverse) and theTaqMan MGB probes labelled with FAM and VIC fluorescent dyes.

Table 6: Selected genotyping SNPs and their Assay ID No (Applied Biosystems)

SNP	Assay ID No (Applied
rs2282509	C_11849826_1_
rs1681886	C_8900393_10
rs2126579	C_11849885_10
rs776959	C_1317286_10
rs1681904	C_1317300_20
rs6983111	C_29131445_20
rs1333049	C_1754666_10

All PCR reactions for SNP genotyping were carried out in the Department of Biotechnology at the Mauritius Sugar Industry Research Institute (MSIRI) using a real-time PCR system (MJ

Research PTC 200, Chromo4 System). PCR was carried out in a 10-µl volume containing: 2 µl of template DNA (10 ng /µl) and 8 µl of the following reaction mix, (0.5µl of 20X TaqMan MGB Assay Mix, 1 µl of Vivantis PCR Buffer (10X), 0.5 µl of $MgCl_2$ (50mM), 0.08 µl of dNTPs (25 mM), 0.05 µl of Vivantis Taq DNA polymerase (10 U/ µl) and 5.87 µl of H_2O).

The PCR conditions included an initial denaturation of 10 minutes at 95^0C, followed by 40 cycles of (denaturation at 95^0C for 15 seconds and primer annealing/extension at 60^0C for 1 minute) and a final extension of 5 minutes at 60^0C.

For quality control purposes, one positive and one negative control were included in each 96-tube macroplate. The same positive control was used across macroplates, thus providing duplicate samples for comparison. In addition, 10% of the total no of samples genotyped were replicated and the results of quality control were 99.5% in agreement with the initial genotyping results. To ensure genotyping quality, we have genotyped 90 trio families of mother, father and child and the Mendelian inheritance of each SNP was checked.

Genotyping results were analysed using the MJ Opticon Monitor Analysis software (Version 3.1) at the MSIRI. The software sorted the data by creating scatter plots of $C_{T(FAM)}$ v/s $C_{T(VIC)}$ or endpoint-fluorescence of FAM v/s endpoint-fluorescence of VIC (Figure 10). An increase in VIC dye only indicated homozyogosity for allele 1. Similarly, an increase in FAM dye only indicated homozygosity for allele 2 and when there was increase in both VIC and FAM dyes, this indicated heterozygosity for allele 1 and 2.
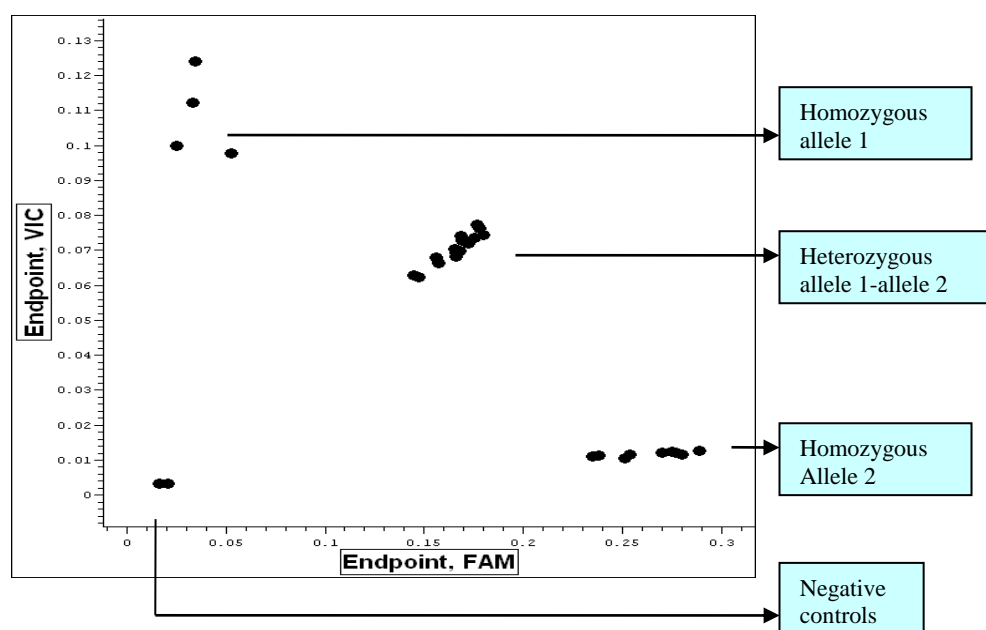
Figure 10: Scatter plot showing endpoint fluorescence of VIC v/s endpoint fluorescence of FAM
Note that each dot on the plot represents an individual sample

II.6   Statistical analysis

Among the 6 SNPs selected for genoptyping in *OXR1* gene, SNP rs2126579 yielded unreliable genotyping results and was thus excluded from our statistical analysis. Statistical analyses were carried out using Epi Info™ (Version 3.4.1, CDC, Atlanta, July 2007, *www.cdc.gov/epiinfo*).

We used the allele counting method to calculate the expected frequencies for polymorphisms in affected and unaffected individuals and we tested for deviation from Hardy-Weinberg Equilibrium (HWE) using the Pearson's Chi-Square goodness of fit test to check for genotyping errors or population stratification.

Two-by-two contingency tables were used to explore associations between allele frequencies and phenotypes by comparing affected and unaffected groups. Two-by-three tables were used to compare genotypes in affected and unaffected groups. Allele frequencies and genotypes were compared using the Yates corrected Chi-Square test. Odds ratios (OR) and Cornfield 95 % confidence limits (95% CI) for OR were given for 2 x 2 contingency tables. When expected values in contingency tables were less than 5, Fisher Exact test results and Exact confidence limits were used. The tables were stratified by sex so as to detect possible differences between genders in association between genotypes and phenotypes. Gene-gender interaction was searched for, by comparing 2x2 tables, using Mantel-Haezel Chi-square tests.

- In study 1, where several SNPs were tested, Bonferroni correction was applied and α was set at 0.01. (α =0.05/5=0.01 where 5 is the number of SNPs being analysed). We thus considered a p-value of <0.01 to be statistically significant for multiple SNP testing. The different SNPs were first analysed in haplotypes for association with premature CHD and/or T2D using Haploview v3.32 software (Broad Institute, Cambridge, USA; June 2006, *http://www.broad.mit.edu/mpg/haploview/*). Individual SNP analysis was then carried out for each of the 5 *OXR1* SNPs, to look for association with premature CHD, T2D. When association or trend of association was observed, we further studied the associated SNP(s) in relation with risk factors for CHD and T2D which included the different components of the metabolic syndrome, namely hypertriglyceridaemia, high blood pressure, central obesity, reduced HDL cholesterol.
- In Study 2, the α significance value was set at 0.05 for an individual SNP analysis.

III. Results

III.1 Characteristics of the North Indian case-control group

The North-Indian case-control group was composed of 620 individuals, including 368 CHD patients and 252 healthy controls. A summary of some of their characteristics is given in table 7.

Table 7: Comparison of some characteristics in NI case-control group

Characteristic	CHD	Controls	p-value
	Mean (±SD)		
No of individuals	368	252	-
Male	294 (79.9%)	160 (63.5%)	-
Female	74 (20.1%)	92(36.5%)	-
Age (years)	48.87 (6.77)	45.54 (6.09)	$p<10^{-4}$
BMI (kg/m^2)	24.30 (3.33)	23.38 (3.89)	$p=0.0008$
Waist	88.49 (9.04)	82.90 (10.34)	$p<10^{-4}$
Fasting plasma glucose (mmol/L)	6.84 (3.0)	4.98 (0.54)	$p<10^{-4}$
Triglycerides (mmol/L)	2.20 (2.41)	1.39 (1.33)	$p<10^{-4}$
Total Cholesterol (mmol/L)	5.61 (1.36)	4.94 (1.03)	$p<10^{-4}$
HDL- Cholesterol (mmol/L)	0.96 (0.25)	1.18 (0.34)	$p<10^{-4}$
LDL-Cholesterol (mmol/L)	3.71 (1.02)	3.15 (0.88)	$p<10^{-4}$

III.2 Prevalence of the different traits of the metabolic syndrome in study population

We used the International Diabetes Federation definition (IDF, 2002) to classify metabolic syndrome in our North Indian population.

According to IDF definition, a person is said to have the metabolic syndrome when he/she has central obesity, which is a prerequisite risk factor and any two of the four following traits below:

Central Obesity – Measured by waist circumference and it is gender and ethnic specific.

For South Asians which include Chinese, Malay and Asian Indian populations:
Waist circumference Male ≥ 90 cm and Female ≥ 80 cm

Any two of the four following traits:

- *Hypertryglyceridemia* with a triglyceride level ≥ 1.7 mmol/L or specific treatment for this abnormality.

- *Reduced HDL-cholesterol* with an HDL-cholesterol level <1.03 mmol/L in males and <1.29 mmol/L in females or specific treatment for this lipid abnormality.

- *High blood pressure* with a blood pressure of systolic BP ≥ 130 mmHg or diastolic BP ≥ 85 mmHg or treatment of previously diagnosed hypertension.
- *Raised fasting plasma glucose* with a fasting plasma glucose level ≥ 5.6 mmol/L or treatment of previously diagnosed Type 2 diabetes.

Table 8 summarizes the distribution of the different traits of the metabolic syndrome as defined by IDF in our study population. Prevalence of the metabolic syndrome was similar in both male and female groups. Central obesity and low-HDL traits were more prevalent in women whereas prevalence of hypertriglyceridaemia was higher in men.

Table 8: Distribution of qualitative traits of the metabolic syndrome in study population

Phenotype	Male n (%)	Female n (%)	OR [95%CI]	p-value
MSIDF	153(33.7)	57(34.3)	0.97	0.96
No MSIDF	301(66.3)	109(65.7)	[0.67-1.41]	
COIDF	203(44.7)	91(54.8)	0.67	0.03
No COIDF	251(55.3)	75(45.2)	[0.47-0.95]	
TGIDF	209(46)	43(25.9)	2.44	0.00001
No TGIDF	245(54)	123(74.1)	[1.65-3.62]	
HDLIDF	283(62.3)	126(75.9)	0.53	0.002
No HDLIDF	171(37.7)	40(24.1)	[0.35-0.79]	
HBPIDF	234(51.5)	78(47)	1.20	0.36
No HBPIDF	220(48.5)	88(53)	[0.84-1.71]	
FPGIDF	157(34.6)	56(33.7)	0.96	0.92
No FPGIDF	297(65.4)	110(66.3)	[0.66-1.40]	

MSIDF: metabolic syndrome, COIDF: central obesity; TGIDF: hypertriglyceridemia; HDLIDF: reduced HDL-cholesterol; HBPIDF: high blood pressure, FPGIDF: raised fasting plasma glucose.

All qualitative traits are defined according to the International Diabetes Federation (IDF) 2002 definition.

III.3 Study 1: Association of variants of *OXR1* gene with CHD and T2D

We analysed genotyping results from 5 of the 6 SNPs genotyped: one SNP rs2126579 yielded unreliable genotyping results and was thus excluded from our statistical analysis.

III.3.1 Haplotype analysis

Haplotypes represent a combination of alleles that are located closely together on the same chromosome and that tend to be inherited together. One advantage of studying haplotypes is that they are usually more polymorphic than single marker loci: if the SNPs from which haplotypes are constructed are closely linked, then it may be easier to demonstrate association between a particular region of the genome with disease, than by using single marker loci. We therefore analysed the 5 SNPs in haplotypes first, to look for association with premature CHD and /or T2D in our study population.

Haplotype analysis was carried out using Haploview v3.32 software (Broad Institute, Cambridge, USA; 21 June 2006, *http://www.broad.mit.edu/mpg/haploview/*). The Haploview is a freely available software package designed to compute linkage disequilibrium statistics and haplotypes patterns from genotyped data. Analysis of linkage disequilibrium (LD) patterns derived from our genotyped data showed that the 5 SNPs are in strong linkage disequilibrium (Figure 11).

Among the five different SNPs analysed, we identified 7 haplotypes in men and 5 haplotypes in women with frequency >0.01, which account for more than 97.4% of the observed haplotypes (Table 9). Haplotype analysis showed that the most frequent haplotype in men was haplotype *H1* - CGTAC (0.3815) as compared in women where the most frequent one was haplotype *H2* - TACGC (0.366). In addition, the haplotype *H2* was associated with a decreased risk of CHD in women (OR: 0.60, 0.3<OR<0.96, p=0.025). Comparing the elements of the protective haplotype *H2* with the other haplotypes in women, the C allele of SNP rs776959 seemed to be the protective determinant to CHD.

A. Male   B. Female
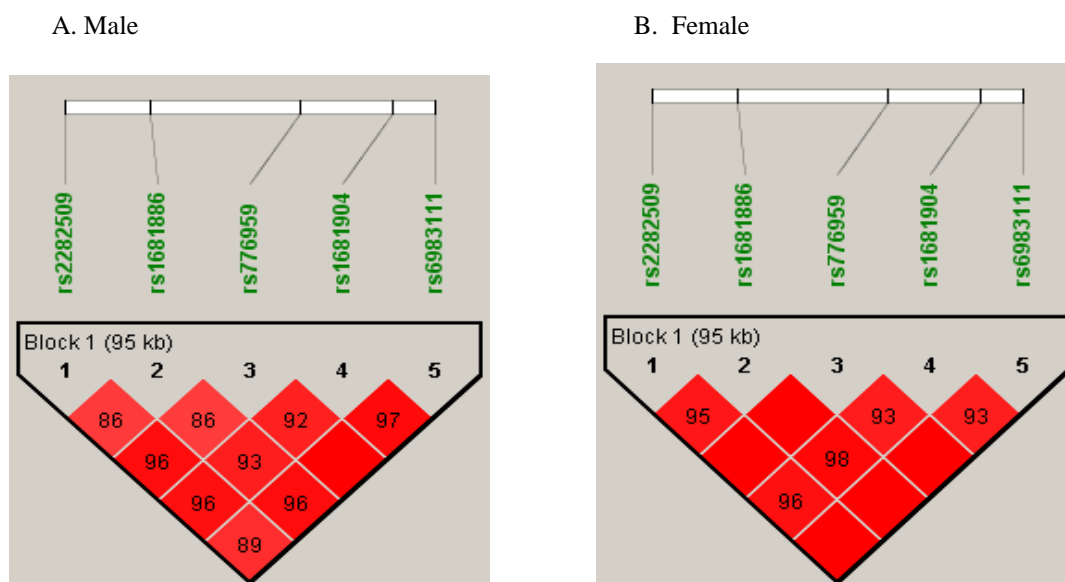
Figure 11: Pairwise LD diagram for the 5 SNPs in the *OXR1* gene.

LD patterns were derived from genotyping data of NI male and female case control cohorts. The pairwise correlation between the SNPs was measured as D' and shown (x100) in each diamond.

Table 9: Haplotypes in *OXR1* gene with frequency >0.01 in NI case-control group

Haplotype	SNPs 12345*	Male Frequency		χ^2	OR (95%CI)	P value	Female Frequency		χ^2	OR (95%CI)	P value
		CHD	CT				CHD	CT			
H1	CGTAC	0.387	0.376	0.101	1.05 (0.79-1.40)	0.751	0.340	0.346	0.012	0.97 (0.60-1.57)	0.913
H2	**TACGC**	0.320	0.347	0.704	0.88 (0.66-1.18)	0.401	**0.307**	**0.425**	**5.02**	**0.60 (0.37-0.96)**	**0.025**
H3	TATGT	0.139	0.152	0.29	0.90 (0.61-1.34)	0.590	0.187	0.122	2.69	1.65 (0.87-3.13)	0.101
H4	CATAC	0.083	0.073	0.341	1.18 (0.70-2.02)	0.559	0.120	0,064	3.255	2.0 (0.88-4.59)	0.071
H5	TGTAC	0.019	0.009	1.362	2.22 (0.59-12.34)	0.243	-	-		-	-
H6	TATGC	0.008	0.021	2.721	0.39 (0.10-1.43)	0.099	0.020	0.011	0.502	1.90 (0.21-2.96)	0.479
H7	TGCGC	0.016	0.004	2.665	5.56 (0.78-242.2)	0.103	-	-	-	-	-

* SNP 1: rs2282509   SNP 2: rs1681886   SNP 3: rs776959   SNP 4: rs1681904   SNP 5: rs6983111

III.3.2 Individual SNP analysis

One of the criteria that we used for SNP selection in SNPbrowser[TM] (Applied Biosystems, *www.appliedbiosystems.com*) was HapMap MAF Minor Allele Frequency (MAF) in CEU population >=20% (we assumed our studied population to be closest to the CEU population among the 4 populations studied in the HapMap project, *www.hapmap.org*).   We therefore compared allelic frequencies observed in our case-control groups with HapMap data for the different SNPs studied (Table 10).   Observed MAFs in our North Indian population did not differ much from the CEU HapMap data except for SNP rs698311 where our observed MAF was more similar to that of the Hans Chinese population.   Our data also showed that our population was different from the African Yoruba population.

Allelic Frequency analysis in relation with CHD and T2D

Allelic frequencies in CHD and controls for the different SNPs studied were compared and are summarized in Table 11.   No difference in allelic frequencies was observed in both male and female groups except for SNP rs776959.   While allelic frequencies for this SNP were similar in male CHD and controls (OR=1.1, 0.80<OR<1.47), p=0.57), the frequency of the common T allele was significantly higher in female CHD compared to female controls (CHD 69% v/s CT 58%; OR=1.63, 1.01<OR<2.64, p=0.045).

Similar trends were found when allele frequencies were compared in Type 2 diabetic patients and controls (Table 12). Allelic frequencies for SNPs rs2282509, rs1681886, rs1681904, rs6983111 were similar in both men and women. For SNP rs776959, allele T was more frequent in diabetic women compared to women controls (OR=1.84, 1.05<OR<3.23, p=0.03) whereas in men the allelic frequencies were similar in diabetics and controls (OR=1.17, 0.80<OR<1.70, p=0.45).

Table 10: Observed minor allele frequencies for genotyped SNPs in our case-controls group compared to HapMap data

SNP	Minor allele (MA)	Observed MAF		HapMap MAF Data			
		CHD	CT	CEU	CHB	JPT	YRI
rs2282509	C	0.48	0.45	0.42	0.36	0.41	0.20
rs1681886	G	0.41	0.38	0.39	0.34	0.42	0.22
rs776959	C	0.34	0.39	0.37	0.47	0.31	0.07
rs1681904	A	0.50	0.46	0.40	0.34	0.41	0.22
rs6983111	T (except for YRI, MA= C)	0.16	0.14	0.24	0.16	0.23	0.37

Table 11: Comparison of allelic frequencies for the different *OXR1* SNPs between CHD and control groups in male and female groups

SNP	Allele	NI Male (M)				NI Female (F)			
		CHD n=294	CT* n=160	OR (95% CI)	P value	CHD n=74	CT* n=92	OR (95% CI)	P value
		Allele frequency				Allele frequency			
rs2282509	C T	0.48 0.52	0.46 0.54	1.09 (0.82-1.45)	0.57	0.47 0.53	0.42 0.58	1.25 (0.79-1.97)	0.38
rs1681886	G A	0.43 0.57	0.40 0.60	1.15 (0.86-1.53)	0.37	0.34 0.66	0.36 0.64	0.92 (0.57-1.48)	0.8
rs776959	T C	0.65 0.35	0.63 0.37	1.1 (0.80 -1.47)	0.57	**0.69 0.31**	**0.58 0.42**	**1.63 (1.01-2.64)**	**0.045**
rs1681904	G A	0.49 0.51	0.53 0.48	0.88 (0.66-1.17)	0.4	0.53 0.47	0.56 0.44	0.9 (0.57-1.42)	0.72
rs6983111	T C	0.14 0.86	0.15 0.85	0.98 (0.66-1.47)	1.0	0.20 0.80	0.14 0.86	1.62 (0.87-3.01)	0.14

Controls were individuals with no signs and symptoms of CHD and with a normal glucose metabolism (ie. without impaired fasting glucose or impaired glucose tolerance)

Table 12: Comparison of allelic frequencies for the different *OXR1* SNPs between T2D and controls in male and female groups

		NI Male (M)				NI Female (F)			
		T2D n=110	CT* n=160	OR (95% CI)	P value	T2D n=49	CT* n=92	OR (95% CI)	P value
SNP	Allele	Allele frequency				Allele frequency			
rs2282509	C	0.49	0.46	1.12	0.57	0.50	0.42	1.39	0.24
	T	0.51	0.54	(0.78-1.60)		0.50	0.58	(0.82-2.34)	
rs1681886	G	0.44	0.40	1.18	0.41	0.35	0.36	0.93	0.86
	A	0.56	0.60	(0.82-1.69)		0.65	0.64	(0.54-1.60)	
rs776959	T	0.66	0.63	1.17	0.45	**0.71**	**0.58**	**1.84**	**0.03**
	C	0.34	0.37	(0.80 -1.70)		**0.29**	**0.42**	**(1.05-3.23)**	
rs1681904	G	0.48	0.52	0.83	0.32	0.51	0.56	0.82	0.50
	A	0.52	0.48	(0.58-1.18)		0.49	0.44	(0.49-1.38)	
rs6983111	T	0.15	0.15	1.03	0.98	0.20	0.14	1.63	0.19
	C	0.85	0.85	(0.62-1.71)		0.80	0.86	(0.81-3.27)	

Controls were individuals with no signs and symptoms of CHD and with a normal glucose metabolism (ie. without impaired fasting glucose or impaired glucose tolerance)

Genotype analysis:

Study of *OXR1* SNPs in relation to CHD

Genotypic proportions for the 5 SNPs did not deviate significantly from HWE (P >0.50 in both case and control groups). We analysed each SNP individually for genotypic association with CHD. We found no association between the 5 SNPs and CHD in men (Table 13). In women, we found no association between 4 SNPs (rs2282509, rs1681886, rs1681904, rs6983111) and association between one SNP (rs776959) and CHD (Chi-square=6.50, p=0.039) (Table 13).

Study of *OXR1* SNPs in relation to T2D

Individuals were classified as T2D when fasting plasma glucose level was ≥ 7.0 mmol/l or 2-hr oral glucose tolerance test ≥ 11.1 mmol/l or on treatment of previously diagnosed Type 2 diabetes. Controls included healthy individuals without signs and symptoms of CHD with a normal glucose metabolism (i.e without impaired fasting plasma glucose or impaired glucose tolerance). We observed similar trends of association with Type 2 diabetes as with CHD. There was no association between the different *OXR1* SNPs and T2D except for SNP rs776959 where we found an association with T2D in women only (Chi-square=7.69, p=0.02) (Table 14).

Given the significant association of SNP rs776959 observed in women, we carried out additional genotype analysis for this specific SNP (Table 15). We found that NI women with homozygous TT genotype had an increased risk of CHD (OR=2.28, 1.20<OR<4.32; p-value=0.017). Moreover, the heterozygous CT genotype tended to have a protective effect to CHD risk in the same female group (OR=0.52, 0.28<OR<0.98; p=0.06). When the male and female groups were compared, an interaction was found between gender and homozygous TT genotype (Chi-square=3.8, p=0.05) and heterozygous CT genotype (Chi-square=3.2, p=0.07) on CHD.

Homozygous TT genotype was also found to confer a higher risk for type 2 diabetes in women (OR: 2.72, 1.33<OR<5.58; p=0.0097) (Table 15). Moreover, a trend towards a protective effect of the heterozygous CT genotype on Type 2 diabetes was found in the female group (OR: 0.45, 0.22<OR<0.91; p=0.04). In addition, interactions were observed between gender and TT genotype (Chi-square=4.0, p=0.046) and CT genotype (Chi-square= 3.93, p=0.047) on T2D.

Table 13: Study of *OXR1* SNPs genotypes in relation to CHD

		NI Male			NI Female		
SNP	Genotype	CHD n=294	CT * n=160	p-value	CHD n=74	CT* n=92	p-value
rs2282509	CC CT TT	21.8% 53.4% 24.8%	23.1% 46.3% 30.6%	0.30	20.3% 54.1% 25.7%	18.5% 46.7% 34.8%	0.45
rs1681886	GG GA AA	18% 50% 32%	15% 49.4% 35.6%	0.61	12.2% 44.6% 43.2%	13% 46.7% 40.2%	0.92
rs776959	TT TC CC	41.8% 46.3% 11.9%	40% 45.6% 14.4%	0.74	**48.6%** **40.5%** **10.8%**	**29.3%** **56.5%** **14.1%**	**0.039**
rs1681904	GG GA AA	23.8% 51% 25.2%	28.8% 47.5% 23.8%	0.51	27% 52.7% 20.3%	32.6% 46.7% 20.7%	0.70
rs6983111	TT TC CC	2.4% 24.1% 73.5%	1.9% 25.6% 72.5%	0.89	1.4% 37.8% 60.8%	0% 27.2% 72.8%	0.17

* Controls were unrelated healthy individiuals with no CHD and having a normal glucose metabolism (i.e without T2D or IGT) confirmed by an oral glucose tolerance test.

Table 14: Study of *OXR1* SNPs genotypes in relation to Type 2 diabetes

SNP	Genotype	Male			Female		
		T2D n=110	CT* n=160	p-value	T2D n=49	CT* n=92	p-value
rs2282509	CC	23.6%	23.1%	0.63	24.5%	18.5%	0.41
	CT	50.9%	46.3%		51.0%	46.7%	
	TT	25.5%	30.6%		24.5%	34.8%	
rs1681886	GG	19.1%	15.0%	0.62	14.3%	13.0%	0.80
	GA	49.1%	49.4%		40.8%	46.7%	
	AA	31.8%	35.6%		44.9%	40.2%	
rs776959	TT	42.7%	40.0%	0.56	**53.1%**	**29.3%**	**0.02**
	TC	47.3%	45.6%		**36.7%**	**56.5%**	
	CC	10.0%	14.4%		**10.2%**	**14.1%**	
rs1681904	GG	23.6%	28.8%	0.56	26.5%	32.6%	0.73
	GA	48.2%	47.5%		49.0%	46.7%	
	AA	28.2%	23.8%		24.5%	20.7%	
rs6983111	TT	2.7%	1.9%	0.88	2.0%	0%	0.18
	TC	24.5%	25.6%		36.7%	27.2%	
	CC	72.7%	72.5%		61.2%	72.8%	

Controls were unrelated healthy individuals with no CHD and having a normal glucose metabolism (i.e without T2D or IGT) confirmed by an oral glucose tolerance test.

Table 15: Genotype association between SNP rs776959 and either CHD or T2D

CHD	Genotype	NI Male			NI Female		
		CHD n=294	CT n=160	p-value	CHD n=74	CT n=92	p-value
rs776959	TT v/s (CT+CC)	OR:1.08 0.73<OR<1.60		0.78	**OR:2.28 1.20<OR<4.32**		**0.017**
	CC v/s (CT+TT)	OR:0.80 0.46<OR<1.42		0.54	OR:0.74 0.29<OR<1.88		0.68
	CT v/s (CC+TT)	OR:1.03 0.70<OR<1.51		0.97	**OR:0.52 0.28<OR<0.98**		**0.06**

Interaction between gender and TT genotype on CHD, Chi-square=3.8, p=0.05
Interaction between gender and CT genotype on CHD, Chi-square=3.2, p=0.07

T2D		T2D n=110	CT n=160	p-value	T2D n=49	CT n=92	p-value
rs776959	TT v/s (CT+CC)	OR:1.12 0.68<OR<1.83		0.75	**OR:2.72 1.33<OR<5.58**		**0.0097**
	CC v/s (CT+TT)	OR:0.66 0.31<OR<1.42		0.38	OR:0.69 0.23<OR<2.06		0.069
	CT v/s (CC+TT)	OR:1.07 0.66<OR<1.74		0.87	**OR:0.45 0.22<OR<0.91**		**0.04**

Interaction between gender and TT genotype on T2D, Chi-square=4.0, p=0.046
Interaction between gender and CT genotype on T2D, Chi-square=3.93, p=0.047

We also studied the rs776959 SNP in association with risk factors for CHD and T2D, which included the metabolic syndrome and its different components, in women (Table 16). We observed trends towards association between metabolic syndrome and SNP rs776959 (p=0.04). Women with the TT genotype carried a higher risk for metabolic syndrome (OR=2.28, 1.18<OR<4.41; p=0.02).

When we studied SNP rs776959 in relation to the different components of metabolic syndrome, no association was observed between rs776959 polymorphism and either low HDL or central obesity (Table 16).

A trend was noted towards association with hypertriglyceridaemia in women (p=0.05). As shown in Table 16, women with the homozygous TT genotype were more affected by hypertriglyceridaemia than those with CT or CC genotype (OR: 2.39, 1.17<OR<4.84; p=0.02). Moreover, an interaction was found between homozygous TT genotype and gender (Chi-square for interaction=6.21; p=0.01). A trend was also apparent between the same TT genotype and hypertension in women (OR: 1.94, 1.03<OR<3.65; p=0.059). Gender-genotype interaction on hypertension was also present (Chi-square= 4.76; p=0.03).

Table 16: Study of SNP rs776959 in relation with metabolic syndrome and associated traits in women

Phenotype	SNP rs776959 Genotype	NI Female		
MSIDF		MSIDF n=57	No MSIDF n=109	p-value
	TT TC CC	50.9% 40.4% 8.8%	31.2% 54.1% 14.7%	0.04
COIDF		COIDF n=91	No COIDF n=73	
	TT TC CC	39.6% 46.2% 14.3%	37.0% 53.4% 9.6%	0.54
TGIDF		TGIDF n=43	No TGIDF n=123	
	TT TC CC	53.5% 37.2% 9.3%	32.5% 53.7% 13.8%	0.05
HDLIDF		HDLIDF n=126	No HDLIDF n=39	
	TT TC CC	40.5% 48.4% 11.1%	28.2% 53.8% 17.9%	0.29
HBPIDF		HBPIDF n=78	No HBPIDF n=85	
	TT TC CC	46.2% 46.2% 7.8%	30.6% 52.9% 16.5%	0.06

MSIDF=metabolic syndrome , COIDF= central obesity, TGIDF= hypertriglyceridaemia, HDLIDF= low HDL cholesterolaemia, and HBPIDF= hypertension, as defined by the International Diabetes Federation, IDF (2002).

IV.4   Study 2: Replication study of SNP rs1333049

The allele frequencies of SNP rs1333049 in our study population were in Hardy Weinberg Equilibrium (CHD cases: p>0.13 and controls: p>0.99).

We compared the allelic frequencies in our studied population with HapMap data (http://www.hapmap.org) for the genotyped SNP (Table 17). Observed frequency of rs1333049-C allele was 0.51 in our controls, showing that allelic frequencies in our NI population did not differ much from 0.49 in Caucasians to 0.48 in Han Chinese to 0.51 in the Japanese population.

The frequency of the risk-associated allele C in CHD cases was not different from that observed in the control group (CHD/controls: 0.52/0.51; OR: 1.04, 0.83<OR<1.31; p=0.75) (Table 18). When stratified by sex, no difference were observed in allelic frequencies between cases and controls in both males and females (P=0.57 and 0.65 respectively).

We also found no difference in allelic frequencies of SNP rs1333049 between diabetic patients and controls (allele C in T2D/ controls: 0.49/0.51)

Table 17: Observed allele frequencies and HapMap data for SNP rs1333049

SNP	Allele	Observed data		HapMap MAF Data			
		CHD	CT	CEU	CHB	JPT	YRI
rs1333049	C	0.52	0.51	0.49	0.48	0.51	0.17

Table 18: Comparison of allelic frequencies of SNP rs1333049 between controls and either CHD or T2D patients

	CHD				T2D			
Allele	Frequency		OR (95% CI)	P value	Frequency		OR (95% CI)	P value
	CHD N=381	CT N=260			T2D N=164	CT N=260		
C	395(0.52)	264(0.51)	1.04 (0.83-1.31)	0.75	162(0.49)	264(0.51)	0.95 (0.71-1.26)	0.75
G	367(0.48)	256(0.49)			166(0.51)	256(0.49)		

No association was observed between SNP rs1333049 genotypes and either CHD or T2D in both male and female subgroups (Table 19).

Table 19: Association of SNP rs1333049 with CHD and T2D

		Male			Female		
Phenotype	SNP rs1333049 Genotype	CHD (N=305) n (%)	CT * (N=166) n (%)	p-value	CHD (N=76) n (%)	CT * (N=94) n (%)	p-value
CHD	CC CG GG	78 (25.6) 166 (54.4) 61 (20.0)	44 (26.5) 80 (48.2) 42 (25.3)	0.33	17 (22.4) 39 (51.3) 20 (26.3)	23 (24.5) 50 (53.2) 21 (22.3)	0.83
		T2D (N=114) n (%)	CT* (N=166) n (%)	p-value	T2D (N=50) n (%)	CT* (N=94) n (%)	p-value
T2D	CC CG GG	23 (20.2) 65 (57.0) 26 (22.8)	44 (26.5) 80 (48.2) 42 (25.3)	0.31	15 (30.0) 21 (42.0) 14 (28.0)	23 (24.5) 50 (53.2) 21 (22.3)	0.44

Controls were healthy individuals with no CHD and having a normal glucose metabolism (i.e. without T2D or IGT) confirmed by an oral glucose tolerance test

IV. Discussion

CHD and Type 2 diabetes are complex diseases involving interaction between multiple genetic and environmental factors. Despite considerable advances in the detection and treatment of CHD during the past 2 decades, this complex disease remains the leading cause of mortality and morbidity in developed countries. The prevalence of Type 2 diabetes is increasing at an alarming rate, very often reaching epidemic proportions in developing nations, which are ill-equipped to face the public health burden associated with these complex diseases. *"While it is important to make public education focusing on risk factors (such as obesity, diet, sedentary lifestyle, cigarette smoking) a high priority in managing these conditions, it is also important to attempt to uncover the basic causes of these conditions through the application of genomics."* (WHO Report, 2002).

The Oxidation resistance 1 gene belongs to a family of well conserved oxidation protection genes in eukaryotes. The role of the Oxr1 protein is to provide protection from oxidative damage by reactive oxygen molecules, especially hydrogen peroxide in the mitochondria (Elliot & Volkert , 2004). The exact mechanism by which this occurs is still unclear, but it is thought that the Oxr1 protein acts on these reactive molecules specifically by suppressing the production or reactivity rather that simply shielding DNA or other cellular components from their damaging effects. The importance of this gene and gene product in the protection against oxidative stress is highlighted by the relative abundance of *OXR1* mRNA in tissues with high respiration capacity such as the heart, skeletal muscle and brain where it would be advantageous to counteract mitochondrial ROS production (Elliot & Volkert, 2004).

In our present study, we examined the association of 5 known variants in the *OXR1* gene with premature coronary heart disease and/or Type 2 diabetes in the Mauritian population of North Indian origin, given the increasing role attributed to oxidative stress in the pathogenesis of these conditions. We genotyped 6 known SNPs, of which 5 yielded reliable genotyping data for analysis. When first analysed the 5 SNPs in haplotypes, one specific haplotype, H2, was found to be associated with a decreased risk of CHD in women but not in men. When comparing the different alleles within the *H2* haplotype to the other haplotypes in women, the C-allele of SNP rs776959 was found to be the protective element. When the different SNPs were studied individually, we found no association between 4 SNPs (rs2282509, rs1681886, rs1681904 and rs6983111) and association between one SNP (rs776959) with premature CHD or Type 2 diabetes in women. This association confirmed the importance of SNP rs776959 which already showed association with both CHD and T2D when included within a specific haplotype in women. We found no association between CHD and T2D and any of the 5 SNPs studied individually or within a haplotype in our male study population. We even found an interaction

of gender on the relation between SNP rs776959 TT genotype and both CHD and T2D phenotypes, confirming a positive gender-specific association between that SNP and the two phenotypes.

Differences in cardiovascular disease incidence, complications and burden are shown to exist between men and women. Premenopausal women are relatively protected against CHD when compared with men of same age and this protective effect is attributed to the physiological effects of estrogen (Masding, 2003). In contrast, in diabetic women, the relative risk for fatal CHD is 50% higher than in diabetic men (Huxley, 2006). This excess risk for CHD has been partly explained by a more adverse cardiovascular risk profile induced by diabetes in women. Women with diabetes tend to have significantly higher levels of blood pressure and lipids than men with diabetes (Masding, 2003; Huxley, 2006).

Gender specific genetic risk factors for cardiovascular disease have been reported in several studies (Yamada et al, 2002; McCarthy et al, 2003; Silander et al, 2008). Genetic variants in several genes (carboxypeptidase B2, upstream stimulatory factor 1, coagulation factor XIII A1 polypeptide, lipin 1 genes) have been identified as contributing to female specific risk for CHD and/or cardiovascular disease (Silander, 2008). Our own results showing a gender-gene variant interaction therefore emphasize the need to study the two gender groups separately in order not to miss a possible gender-specific influence of gene variants on CHD and Type 2 diabetes.

Gender specific susceptibilities to disease can be partly explained by the influence of sex hormones including estrogen. In cardiovascular disease, sex steroid hormones and receptors interact with and activate other proteins or genes that are involved in the pathogenesis of the disease in endothelial and smooth muscle cells (Mendelsohn & Karas, 2005). A decreased estrogen receptor expression in atherosclerotic arteries has been observed in women with premature atherosclerosis (Gouva & Tsatsoulis, 2004). An estrogen receptor-associated protein coding gene, highly similar to *OXR1* gene, has been characterized and located in Chr 6q22.23 (Durand et al, 2007). Genomic analysis of the *NCOA7* gene indicated a similar gene structure to *OXR1* and it was thought to have arisen from a duplication event. The functions of the 2 gene products are very similar in that they both protect against oxidative damage, but unlike the mitochondrial and inducible Oxr1 protein, the NCOA7 is constitutively expressed and appears to localize to the nucleus following estrogen stimulation. The NCOA7 protein has also been associated with the estrogen receptor and it functions as an activator of estrogen receptor mediated transcription. Oxidative DNA damage has been shown to occur as a result of estrogen metabolism (Seacat et al, 1997) and NCOA7 protein provides protection against oxidative damage resulting from estrogen metabolism in the nucleus. Taking into account the high similarity between the *NCOA7* gene and the *OXR1* gene, we could have expected a similar

association of *OXR1* gene with estrogen metabolism, which would explain partly the gender-gene interaction observed in our study. However, comparison of the 2 genes revealed that the estrogen receptor binding site present in exon 8 of the *NCOA7* gene is lacking in the *OXR1* gene (Durand et al, 2007).

Up to now, no study has reported an involvement of the *OXR1* gene in the pathogenesis of any disease. Haplotype analysis and individual SNP analysis in our study provided evidence of a gender-specific contribution of the *OXR1* gene variant rs776959 to susceptibility to CHD, Type 2 diabetes and metabolic syndrome. Odds ratio of carrying TT genotype was 2.28 (95% CI=1.2-4.32) for CHD and 2.72 (95% CI = 1.33-5.58) for T2D in women, suggesting a significant effect size of SNP rs776959 in our female population, which might explain the nominal linkage signal obtained for both CHD and T2D in chromosome 8q23 in previous genome scan (Francke et al, 2001). Because of our small female sample size, we cannot exclude the possibility that odds ratio may have been overestimated (Nemes et al, 2009). We must therefore replicate our study in a larger female case control group of same ethnic origin (i.e. North Indians). Replication studies in other case control groups of different ethnic origin (namely South Indians) should also be carried out in order to confirm our findings. Even if association is validated in the replication studies, at this stage, we cannot speculate whether this SNP is the causal variant or whether it is in strong linkage disequilibrium with another unidentified variant located near. Further studies such as resequencing within region of the associated variant would then be needed to shed more light on the involvement of this *OXR1* gene variant in the pathogenesis of CHD and Type 2 diabetes in women.

Given that recent GWAS have found the 9p21 chromosomal loci to be strongly associated with CAD and MI (McPherson et al, 2007; Helgadottir et al, 2007; WTCCC, 2007; Samani, 2007), we tried a replication study of one of the most interesting variant (rs1333049) in 9p21 chromosomal region for premature CHD and T2D in our Mauritian population of North Indian origin. This association of the 9p21 locus has been confirmed and consistently replicated in several Caucasian and non-Caucasian populations, making it the most widely replicated locus for CAD and MI to date (Assimes et al, 2008; Broadbent et al, 2008, Hinohara et al, 2008; Shen et al, 2008; Schunkert et al, 2008). However, we did not find any association between SNP rs1333049 and CHD in our study population, despite the fact that allelic frequency in our population was similar to the Caucasian populations (Frequency of allele C in our control: 0.51 compared to CEU: 0.49).

The 9p21 chromosomal region has also been associated with T2D (DGI, 2007; Scott et al, 2007; Zeggini et al, 2007). However, we did not find any association between rs776959 SNP and T2D in our study population. This was not unexpected since SNPs associated with CHD and T2D are found in two separate LD regions and the effects of the variants are independent

i.e CHD risk variant does not confer increased risk for T2D and similarly for T2D risk variant (DGI, 2007; Scott et al, 2007; Zeggini et al, 2007).

One possible explanation for the lack of association of SNP rs1333049 with premature CHD in our case-control group could be that our study lacked power to detect modest effect size association (i.e. OR < 1.5). However, there are studies involving sample size similar to ours that have been able to detect association between SNP rs1333049 and CAD and/or MI: the Atherogene study involving 370 cases and 345 controls of European origin has reported association with CAD [p-value: 0.0175] (Schunkert et al, 2008). A UK-based study involving 9,776 Asian Indians and 4,407 European Caucasians has reported an association between SNP rs1333049 and CHD in both populations but with an odds ratio lower in Asian Indians compared to Europeans (Chambers et al, 2008). The subpopulations from which the Asian Indians originate were not stated. Previous studies have shown allelic differences among Asian Indian subpopulations (Manraj, 2002). Varying differences in LD patterns across populations can explain why positive associations may be found in some populations but not in others.

Within the 9p21 chromosomal region, several SNPs have been associated with CAD and MI and these SNPs were in strong linkage disequilibrium with each other. Looking closer at the risk interval, 2 haplotype blocks have been described to be in moderate LD and rs1333049 SNP, which we have genotyped, has been identified as the lead SNP for the main haplotype block while 3 other SNPs (rs7044859, rs1292136, rs7865618) were sufficient to characterize the second haplotype block (Samani et al, 2007). Haplotype association analysis carried out by Schunkert et al (2008) showed that the rs1333049 SNP alone may not be sufficient to explain association with CAD. Thus other associated SNPs within the chromosome 9p21 locus may have to be investigated before excluding any association between CAD and the 9p21 chromosomal locus in our North Indian population.

Genome-wide association studies have successfully identified numerous loci associated with common polygenic diseases including CHD and T2D, however most of them involved Caucasian populations. No GWAS involving the Asian Indian population has been reported to date. Since disease susceptibility risks vary among different ethnic groups, additional GWAS in multiple populations of different ethnic origins may lead to the discovery of new loci, which have not yet been identified in Caucasian populations. Conducting such a study in our disease-prone population would be most interesting.

On the other hand, replication studies of associated loci in populations of different ethnicities may also help in validating positive associations and provide additional information as to the contribution of these loci in disease susceptibility across populations. Our multiethnic population provides the possibility of conducting association studies in several well-defined ethnic groups. Since women and men were found to differ in various aspects relating to disease

risks and this aspect has not been given enough importance in most of the GWAS (Silander et al, 2008), we propose to conduct further gender-specific case-control studies of CHD and T2D associated loci in different ethnic groups (North Indian, South Indian and General population) within our population, first by studying the 2 gender groups separately and then in combination if the 2 groups do not show significant differences. One strategy to increase our chances of obtaining interesting results in future replication studies would be to crosslink linkage signals from genome scan in our own population with best loci from GWAS in other populations. When selecting SNPs for replication in association studies, we shall consider in priority those SNPs that were associated to CHD or T2D in GWAS from other populations if they are located in regions that showed at least nominal linkage with either phenotype in our own population.

Our case-control study of a "positional" candidate gene yielded more interesting result than our first replication study of a variant that had shown consistent association with CHD in several GWAS from other populations. These results support our present strategy to look for susceptibility variants to premature CHD and/or T2D through a positional candidate gene approach, based on results obtained from previous genome scan in our own population. Several chromosomal regions such as 16p13, 10q23 and 3q27 showed suggestive linkage with CHD (Manraj, 2002). In addition to the 8q23 chromosomal region which we studied, two other chromosomal regions, the 1q44 and 16q12 regions showed nominal linkage with both CHD and T2D in ordered subset analyses (Manraj, 2002). These chromosomal regions probably harbour genes with more that modest effect size (OR>2).

Our strategy is to carry out gender specific case-control association studies where SNPs will be selected if they satisfy all of the following criteria:

- Located in regions showing linkage with CHD and/or T2D in genome scan in our own population.
- Located in regions that showed association with CHD and/or T2D in several GWAS.
- located close to or within candidate genes that are in linkage disequilibrium with those in GWAS.

V. Conclusions

Coronary heart disease and Type 2 diabetes are complex diseases in which multiple genes and environmental factors are believed to influence disease susceptibility. Based on results from previous genome wide scan in the North Indian Mauritian population showing nominal linkage between chromosome 8q23 and both CHD and T2D, we selected the *OXR1* gene as positional candidate gene in a population-based case control study given the important role of oxidative stress in the pathogenesis of these diseases. We found a trend towards gender-specific association between SNP rs776959 in intron 9 of the *OXR1* gene and several phenotypes such as premature coronary heart disease, type 2 diabetes and the metabolic syndrome. Whereas no association was seen with any phenotype in men, homozygous TT genotype for SNP rs776959 was found to confer a higher risk for CHD, T2D and metabolic syndrome in our female group. Odds ratio (OR) was relatively higher than those described in GWAS (highest OR observed<1.5), showing a larger effect size of the rs776959 SNP on CHD and T2D risk in our female population (CHD: OR=2.28, 1.2<OR<4.32; T2D: OR=2.72, 1.33<OR<5.58). An interaction between gender and TT genotype of rs776959 variant emphasized the need to consider the effect of this genetic variant on CHD and T2D in men and women separately.

We were not able to find any association between CHD in our study population of North Indian origin and a variant in the 9p21 chromosomal region that was widely replicated in several GWAS which did not include any Asian Indian population. Since disease susceptibility may differ among ethnic groups, availability of GWAS for CHD and T2D in a population similar to ours would be an advantage for replication studies.

Given the interesting results from study of *OXR1* gene variants which we chose as a positional candidate gene for CHD and T2D, we propose to continue to look for susceptibility variants in regions that showed suggestive linkage with CHD or T2D in our population, taking into account signals arising from GWAS in other populations, for the same phenotypes and in the same region.

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