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The Pakistan Risk of Myocardial Infarction Study: a resource for the study of genetic, lifestyle and other determinants of myocardial infarction in South Asia

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Abstract The burden of coronary heart disease (CHD) is increasing at a greater rate in South Asia than in any other region globally, but there is little direct evidence about its determinants. The Pakistan Risk of Myocardial Infarction Study (PROMIS) is an epidemiological resource to enable reliable study of genetic, lifestyle and other determinants of

CHD in South Asia. By March 2009, PROMIS had recruited over 5,000 cases of first-ever confirmed acute myocardial infarction (MI) and over 5,000 matched controls aged 30–80 years. For each participant, information has been recorded on demographic factors, lifestyle, medical and family history, anthropometry, and a 12-lead

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electrocardiogram. A range of biological samples has been collected and stored, including DNA, plasma, serum and whole blood. During its next stage, the study aims to expand recruitment to achieve a total of about 20,000 cases and about 20,000 controls, and, in subsets of participants, to enrich the resource by collection of monocytes, establishment of lymphoblastoid cell lines, and by resurveying participants. Measurements in progress include profiling of candidate biochemical factors, assay of 45,000 variants in 2,100 candidate genes, and a genomewide association scan of over 650,000 genetic markers. We have established a large epidemiological resource for CHD in South Asia. In parallel with its further expansion and enrichment, the PROMIS resource will be systematically harvested to help identify and evaluate genetic and other determinants of MI in South Asia. Findings from this study should advance scientific understanding and inform regionally appropriate disease prevention and control strategies.

Keywords Myocardial Infarction · Case-control study · South Asia · Pakistan · MI · Risk factors

Introduction

Coronary heart disease (CHD) is the single leading cause of death worldwide and a major cause of disability [1]. The projected increase in CHD is expected to be much greater in South Asia than in any other region worldwide [2], where it is expected to more than double over the next 20 years [3]. The burden of CHD in South Asians extends beyond regional concerns, as CHD mortality and morbidity remain higher in immigrant South Asians living in western regions compared with native western populations [4]. There is limited evidence, however, about the determinants of CHD in South Asia, although such evidence should contribute importantly to scientific understanding and to the development of regionally appropriate strategies to prevent and control CHD. In particular, it has been proposed that populations in South Asia are predisposed to cardiometabolic conditions owing to a combination of hereditary and lifestyle factors that promote metabolic dysfunction [5, 6], but direct evidence is sparse.

Until the initiation of the Pakistan Risk of Myocardial Infarction Study (PROMIS), fewer than 1,000 patients and a similar number of controls had been assessed in all available epidemiological studies of CHD in Pakistan, a country with a population of 175 million. The value of large case-control studies of CHD in relation to genetic and lifestyle factors has been demonstrated in non-Pakistani populations by ISIS (14,000 cases of acute myocardial infarction (MI), 16,000 controls: >95% white British) [7] and INTERHEART (15,000 acute MI cases, 15,000

controls: <5% Pakistani) [8]. An important challenge is, therefore, to establish epidemiological resources of considerable scale in a rigorous yet cost-effective manner in South Asian populations where there is unmet scientific and public health need.

The objectives of this report are to describe the methods used in the establishment of a epidemiological resource for the study of CHD in Pakistan and to outline the rationale for the study's plan of further expansion and sub-studies to enable scientific enrichment.

Methods

Study participants

PROMIS is a retrospective case-control study of acute MI. Following screening by medically-qualified research officers, patients aged 30–80 years admitted to the emergency rooms of collaborating hospitals (Fig. 1 lists recruitment centres active as of March 2009) have been eligible for inclusion as cases if they fulfil all of the following criteria: (1) sustained clinical symptoms suggestive of MI lasting longer than 20 min within the previous 24 h; (2) ECG changes of MI (i.e., new pathologic Q waves, at least 1 mm ST elevation in any 2 or more contiguous limb leads or a new left bundle branch block, or new persistent ST–T wave changes diagnostic of a non-Q wave MI); (3) confirmatory troponin-T measurements; and (4) no previous cardiovascular diseases, defined as self-reported history of angina, MI, coronary revascularisation, transient ischaemic attack, stroke or evidence of CHD on prior ECG or in other medical records.

Controls have been individuals without a self-reported history of cardiovascular disease (who had no ECG changes consistent with a previous MI) drawn from individuals concurrently identified in the same hospitals as index cases. Controls have been recruited in the following order of priority: (1) visitors of patients attending the out-patient department; (2) patients attending the out-patient department for routine non-cardiac complaints, or (3) non-blood related visitors of index MI cases. Controls were frequency-matched to cases on sex and age (in 5-year bands). Participants were not recruited into PROMIS if any of the following features were evident: (1) a previous history of cardiovascular disease (including self-reported MI, angina, coronary revascularization, stroke, transient ischaemic attack, or peripheral vascular disease, and, in cases, presence of cardiogenic shock); (2) a history of a viral or bacterial infection in the previous 2 weeks; (3) documented chronic conditions, such as malignancy, any chronic infection, leprosy, malaria or other bacterial/parasitic infections, chronic inflammatory disorders, hepatitis or

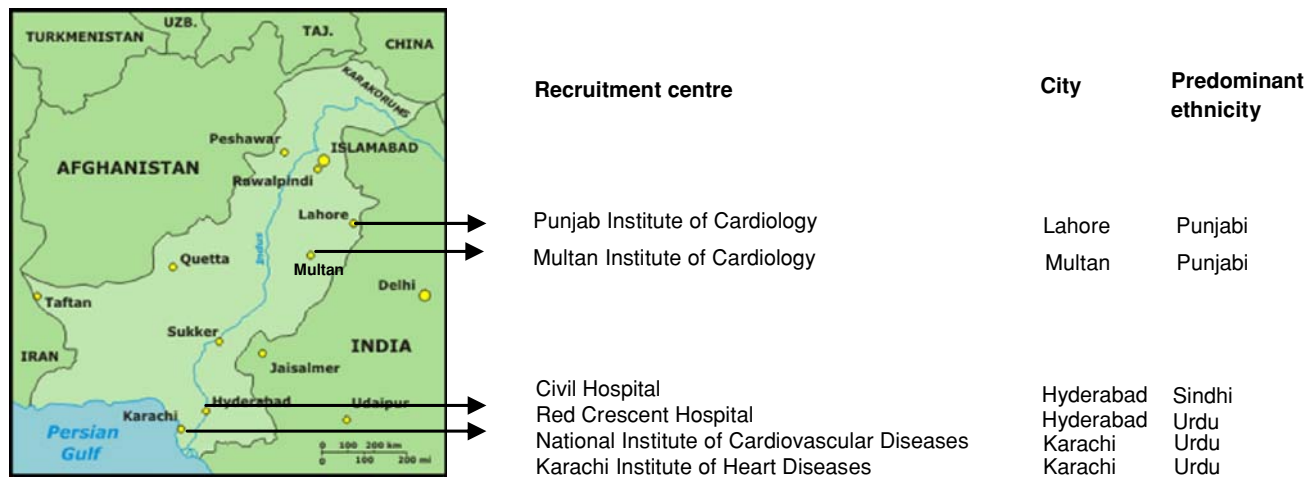


Fig. 1 Location and predominant ethnicities of PROMIS recruitment centres

renal failure on past medical history; (4) pregnancy; or (5) refusal to give consent.

Prior to adoption of the approach described above for the selection of controls, we carefully assessed several other options, such as choosing reference groups who had: an unrelated disease; population based community controls; controls from occupational settings or health-check clinics. Our chosen approach was considered desirable because it achieved a balance between feasibility and scientific rigor and because it was scalable in Pakistan. (By contrast, whereas use of population based community controls may be desirable in principle, it is considerably more labour-intensive and expensive and cannot guarantee that such controls will necessarily represent the catchment areas from which hospital-based cases are derived, particularly since referral patterns to hospitals are complex.) Furthermore, by analogy with the Wellcome Trust Case-Control Consortium (WTCCC) [9], PROMIS controls can be efficiently and validly used for patients with other cardiometabolic conditions owing to the broad geographical and ethnic scope in PROMIS.

PROMIS has received approval by the relevant research ethics committee of each of the institutions involved in participant recruitment. Informed consent has been obtained from each participant recruited into the study, including for use of samples in genetic, biochemical and other analyses.

Questionnaire administration and anthropometry

To maximise consistency and quality-control across recruitment centres, staff in each recruitment centre have been trained in the use of standard operating procedures. Research medical officers have administered pre-piloted epidemiological questionnaires to participants that seek a

total of >200 items of information in relation to: ethnicity (e.g. personal and paternal ethnicity, spoken language, place of birth and any known consanguinity); demographic characteristics; lifestyle factors (e.g., tobacco and alcohol consumption, dietary intake and physical activity); and personal and family history of cardiovascular disease; and medication usage (Table 1). As dietary habits and types of tobacco consumption, in particular, can be substantially different in South Asia than in western populations, it has been necessary to develop a locally relevant questionnaire that adequately records such information (Table 2). Development of the dietary component of this questionnaire involved completion of a 24 h dietary recall questionnaire and the completion of a 7-day food diary by 200 healthy adults, which enabled the identification of 160 different foods consumed that were categorised into 54 groups by a local nutritionist. A provisional food-frequency questionnaire was then further refined after its piloting among 100 additional healthy adults (e.g., the final version added further questions about foods typically consumed only during Ramadan, the month of fasting). For MI cases, questionnaires have been administered only after medical stabilization and relate to habits and characteristics during the time before the diagnosis of acute MI. Using standardized procedures and equipment, research officers have obtained measurements of height, weight, waist and hip circumference, systolic and diastolic blood pressure, and heart rate. Waist circumference has been assessed over the abdomen at the widest diameter between the costal margin and the iliac crest, and hip circumference has been assessed at the level of the greater trochanters. Research officers have also recorded a standard 12-lead ECG. Information extracted from questionnaires, physical measurements and ECG recordings has been entered by two different operators

Table 1 Summary of questionnaire-based information collected

Type of information	Detail recorded
Symptoms, arrival and management at the hospital	Date of MI, time of onset, time since last meal, review of symptoms, diagnosis and management at the hospital and any investigations ordered by the attending physician
Medication and medical history	Medication class and duration of use for each of anti-coagulant, blood pressure- and lipid-lowering, diabetes related, hormonal and, for women, contraceptive and HRT medications
Female reproductive history	Age at first menstrual period, age periods stopped, hormones for menopause treatment, oral contraceptive use
Family history	Approximate age of diagnosis/occurrence of hypertension, diabetes, angina, MI, stroke, cancer or sudden death, for each of mother, father, sister, brother, son and daughter
Ethnicity and other genetic related information	Place of birth, personal and parental ethnicity (e.g. Urdu, Punjabi, Pathan, Balooch, Sindhi, Memon, Gujrati), parental co-sanguinity
Socio-economic status	Occupation(s), monthly income, level and duration of education, marital status, no. of dependants, ownership/wealth
Physical activity	Type, frequency, duration and intensity of activity for each of occupational, work related commuting and leisure time
Tobacco consumption	See Table 2 for details
Psychosocial factors	Experience of traumatic events in the past year (e.g., loss of crop, family bereavement), perceived level of occupational, domestic and financial stress, perceived level of mental and physical health
Anthropometry	Height, weight, waist and hip circumference

HRT, hormone replacement therapy

Table 2 Information collected on dietary intake and tobacco consumption

Type of food	Routinely recorded information	Additional information recorded to reflect local habits
Cooking medium	Oil (recording type), butter, margarine	Ghee (type of fat)
Breads	N/A	Naan, chapatti (recording type of flour used), paratha (butter-coated chapatti)
Fruits and vegetables	Separately for cooked and raw, green leafy vegetables, yellow vegetables, cruciferous vegetables, salad items, fruits	Method of cooking (grilled, curried and fried)
Meats	Chicken, beef, mutton, lamb and fish	Method of cooking (grilled, curried and fried)
Legumes	Nuts and seeds	Pulses (including different daals)
Sweets	Bakery items	Kheer (dairy based dessert), halwa, mithai, jalabe (unrefined sugars), nimko (fried desserts)
Brewed beverages	Tea, coffee, herbal tea	Qahwa (local form of herbal tea)
Other beverages	Milk, carbonated and non-carbonated soft drinks, fruit juices	Alcohol (including local varieties), lassi (yoghurt based drink, recorded separately for sweet and salty)
Tobacco consumption	Usage status, quantity, frequency and lifetime. Information on sources of passive smoking also recorded	For each of cigarettes, beedies (rolled tobacco), huqqa (water pipe), paan (chewing tobacco), naswar (snuff), gutka and supari (preparations of crushed betel nut, tobacco, and sweet or savory flavourings).

onto the central database, which is securely held both in Karachi, Pakistan, and Cambridge, UK.

Collection of biological samples and initial biochemistry

Non-fasting blood samples (with the time since last meal recorded) have been drawn by phlebotomists from each participant and centrifuged within 45 min of venepuncture. In MI cases, blood sampling has been due to occur within

24 h of the onset of MI symptoms and prior to the administration of any thrombolytics, with the time since onset of symptoms recorded to the nearest hour. Because blood is collected from MI cases while they are in a recumbent position (e.g. at about 45°), blood samples have been drawn in the same manner from controls to limit the possibility of systematic differences (e.g. plasma volumes may be higher in the supine position than when sitting [10, 11]). A total of 29 ml of blood has been drawn from each participant in 2 × 6 ml serum tubes and 3 × 5 ml EDTA

tubes (a further citrate tube has been obtained only in suspected MI cases for troponin assay). Hence, a total of five blood tubes have been collected per participant, including serum, EDTA plasma and whole blood which have been stored in cryogenic vials. All samples have been stored temporarily at each recruitment centre at -20°C . A second sample (2 ml) has been drawn in cases 10 h after the onset of clinical symptoms for measurement of troponin. Serum, plasma and whole blood samples are transported daily to the local laboratory where they are stored at -80°C . The long-term -80°C sample repository is kept in both Karachi and Cambridge. Measurement of total cholesterol (using an enzymatic colorimetric method, Roche Diagnostics GmbH, USA) and high-density lipoprotein-cholesterol (using a homogeneous enzymatic colorimetric method, Roche Diagnostics GmbH, USA) has been completed in over 5,000 MI cases and over 5,000 controls thus far.

DNA quality, quantity and initial genotyping

Research technicians trained in accordance with standard operating procedures in laboratories in Karachi have extracted DNA from leucocytes using a reference phenol-chloroform protocol [12]. PCR-RFLP has been performed in all samples on the day of extraction to provide an initial quality check. DNA concentrations have been determined

by fluorescence measurement using PicoGreen molecular probes. The yield of DNA per participant has typically been between 600 and 800 ng/ μl in a total volume of about 750 μl . To minimise any systematic biases arising from plate- or batch-specific genotyping error and/or nonrandom missingness, stock plates have been used to generate genotyping plates which contain a mixture of cases and controls along with negative and positive controls designed to address genotyping quality control (QC), plate identification and orientation. As part of the QC, samples have been subject to molecular bar-coding and checked for degradation by running samples on agarose gels. Samples passing QC have been diluted in tris borate EDTA buffer to 50 ng/ μl and arrayed into 96-well master plates.

Further expansion and measurements

By March 2009, PROMIS had recruited over 5,000 confirmed first-ever MI cases and over 5,000 controls. The main objectives of the study's next stages are: to expand the study to 20,000 MI cases and 20,000 controls; to enrich the bioresource in various ways in order to increase its scientific value; and to accelerate harvesting of its biological resources. Assays have been completed in the first 2,000 MI cases and in the first 2,000 controls of 45,000 genetic markers in 2,100 candidate genes in the Illumina

Table 3 Biological measurements in progress in PROMIS

Approach	Analytical strategy	Potential value
Genomics		
Genomewide association study	Illumina 610 quad array + 60 K for CNV analysis	(1) discovery of genetic variants associated with MI; (2) investigation of genetic architecture in Pakistani population
IBC Illumina cardiochip	45,000 variants selected from 2,100 candidate genes	Comprehensive evaluation of candidate genes in MI
Sequencing	In-depth sequencing to investigate LD structure	Investigation of LD and haplotypes to identify causal genetic variants
Transcriptomics		
Genomewide expression study	Illumina high throughput beadchip platform (46,000 gene targets)	(1) assessment of transcript levels associated with cis-acting sequence variants; (2) comparison of resting and activated transcript levels in cases and controls; (3) functional consequences of established and novel variants
Lymphoblastoid cell lines	Transfection with Epstein-Barr virus	Functional genomics
Metabonomics		
Spectroscopy	^1H NMR and GC-MS	Metabolic determinants of MI
Biomarkers		
Circulating candidate analytes	Total cholesterol, HDL-C, LDL-C, triglycerides, apo-AI, apo-B, Lp(a), CRP, Lp-PLA ₂ , adiponectin	(1) associations of biomarkers with MI; (2) genetic determinants of the measured biomarkers in controls

CNV, copy number variants; LD, linkage disequilibrium; ^1H NMR, nuclear magnetic resonance; GC-MS, gas chromatography mass spectroscopy; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; apo-AI, apolipoprotein-A1; apo-B, apolipoprotein B100; Lp(a), lipoprotein (a); CRP, C-reactive protein; Lp-PLA₂, Lipoprotein-associated phospholipase A₂

IBC “cardiochip” array [13]. As part of WTCCC-2, a genomewide association scan (GWAS) has been conducted in the first 5,000 MI cases and in the first 5,000 controls using the Illumina 610-Quad array (including >610,000 SNPs plus 60,000 selected copy number variants) [14] (Table 3).

Serial epidemiological questionnaires, anthropometry, and blood sampling is planned in about 1,000 participants approximately 2–3 years after the initial visit, enabling quantification of and correction for regression dilution [15, 16]. Such resurveys should also help to validate further the food-frequency questionnaires developed for use in PROMIS. Pilot studies have already commenced to assess small metabolites and lipids by mass spectroscopy in plasma samples (Table 3) [17]. In a subset of participants who will be part of the GWAS, collection is planned of RNA samples from monocytes (e.g., Illumina High Throughput Beadchip platform with 46,000 gene targets) to enable study of the transcriptome and to elucidate mechanisms by which sequence variation causes change in gene expression [18, 19]. In a subset of participants who will have both GWAS and gene expression data, collection is also planned of peripheral lymphocytes (using EasySep Human Selection kits, Stem Cell Technologies, London, UK) that will be transformed by Epstein-Barr virus for the establishment of permanent lymphoblastoid cell lines to enable functional experiments.

Discussion

Although Pakistan is experiencing a substantial increase in the burden of cardiovascular disease [2, 3], risk factors for CHD in this population have been relatively little studied [3, 5, 6, 20, 21]. Before PROMIS, the previous largest study was INTERHEART-Pakistan, which comprised 650 MI cases and 650 controls [8]. Whereas INTERHEART has assessed risk factors common to over 50 countries, the complementary approach in PROMIS has been to focus on these factors plus a more detailed assessment of distinctive exposures in Pakistan, notably: indigenous smoking modes (e.g., beedies, huqqa, naswar); dietary intake (e.g., ghee consumption, a major source of fat); and a history of consanguinity. By March 2009, PROMIS had recruited over 5,000 first-ever confirmed MI cases and over 5,000 controls in whom locally relevant exposure information had been recorded and from whom various biological samples had been collected, processed and stored.

PROMIS is addressing two distinct but related strategic needs. First, the study is estimating the impact of various modifiable risk factors for MI that should be of considerable relevance to the prevention and control of cardiovascular disease in Pakistan, including, as noted above, tobacco

consumption, dietary intake, and physical activity [5, 6, 22, 23]. The second focus of PROMIS is to contribute to scientific (particularly genetic) discovery in an ethnic-specific context. Evidence is emerging of variation in the impact of risk factors in different racial groups, such as a two-fold higher odds ratios for MI with total cholesterol in South Asians than Europeans [24]; higher odds ratios for MI with the *LTA4H* haplotype in Africans than Europeans [25]; and relevance of *TCF7L2* to type 2 diabetes in West Africans but not East Asians [26, 27]. The value of large ethnic-specific studies is illustrated by discovery of the strongest common susceptibility locus (*KCNQ1*) yet for type 2 diabetes, identified in East Asians [28, 29] but not initially in Europeans because the allele frequency in East Asians is much higher (40% vs. 5%) despite similar odds ratios in both races [30]. This highlights how an ethnic-specific study like PROMIS should reveal entirely new loci, particularly for low-frequency causal alleles of more recent origin.

The potentially distinctive risk profile of South Asian populations [24], together with the apparently earlier onset of MI and greater familial clustering of CHD [6], reinforces the need to evaluate directly genetic and other risk factors in South Asia [1–4]. Despite this need, genetic investigation of CHD in South Asian populations has hitherto been limited. By early 2009 at least nine GWAS of clinical coronary outcomes had been reported, all of which had been conducted in populations of Northern European or Japanese ancestry (Table 4) [31–39]. None of these had adequate statistical power to detect reliably odds ratios with common variants of 1.5 or smaller. To our knowledge, at least three studies are currently conducting GWAS for CHD in South Asians: PROMIS (5,000 confirmed MI cases and 5,000 controls in urban Pakistan), LOLIPOP (3,500 cases of MI and/or coronary stenosis and 4,000 controls in London, UK) [40] and INTERHEART (400 MI cases and 400 controls in South Asia) [8]. These (and other) studies should yield novel insights into the genetic determinants of CHD in South Asians, particularly in potential eventual combined analyses of data. In addition to the GWAS, PROMIS is concomitantly attempting other discovery approaches (e.g., metabolomics employing ^1H -nuclear magnetic resonance spectroscopy and gas-chromatography mass spectroscopy), and functional studies. Data from these complementary methods are likely to enhance biological insights, particularly with the application of emerging statistical techniques for systems biology [41, 42].

The strengths and potential limitations of PROMIS merit consideration. Retrospective case-control studies of MI can usefully complement prospective studies because the former involve ascertainment of exposure information and blood sampling of people who have already developed

Table 4 Genomewide association studies (GWAS) of clinical CHD outcomes

Study	Geographical location	No. of cases/ controls	Case definition	Genotyping platform	No. of SNPs assessed	Significantly associated loci identified and validated
GWAS of CHD published by early-2009						
WTCCC [9, 31, 32]	UK	1,926/2,938	MI or coronary revascularisation	Affymetrix 500 K	469,557	9p21.3, 6q25.1, 6q26-q27
deCODE [33]	Iceland	1,607/6,728	MI	Illumina Hap300	305,953	9p21.3
German MI family study [31]	Germany	875/1,644	MI with family history of CAD	Affymetrix 500 K	272,602	9p21.3, 6q25.1 and 2q36.3
Celera34	USA	340/346	MI	Celera sequencing technology	11,053	4q32, 6q22, 12p13, q44 and 19p13.2
Ottawa heart study [35]	Canada	322/312	Coronary revascularisation	Oligonucleotide arrays	72,864	9p21.3
Framingham [36]	USA	118/1,227	Major CHD	Affymetrix 100 K	70,987	16q23, 2q32, 15q21, 17q24, 8q22, 4q22, 12p12 (not yet validated)
OACIS [37]	Japan	94/658	MI	PCR-Invader assay	65,671	6p21
German MI family study II [38]	Germany	1,222/1,298	MI	Affymetrix 600 K	567,119	3q22.3
MIGen [39]	USA, Sweden, Finland, Spain, Italy	2,967/3,075	Early onset MI	Affymetrix 600 K	727,496	21q22, 6p24, 2q33, 1p13, 10q11.1, 1q 41, 19p13, 1p32
GWAS of CHD in South Asians known to be in progress						
PROMIS	Pakistan	5,000/5,000	MI	Illumina 610 Quad(including 60,000 CNVs)	610,000	
LOLIPOP [40]	South Asians in London	3,500/3,500	MI or coronary stenosis	Illumina 610 Quad (including 60,000 CNVs)	610,000	
INTERHEART-South Asia [8]	South Asia	400/400	MI	Illumina 550 K	555,000	

MI and a comparable group of controls without MI, enabling rapid and cost-effective accrual of large numbers of relevant cases. By contrast, prospective studies need several hundred thousand people to be screened (with all of their blood samples kept in frozen storage) and followed for at least one or two decades in order to accrue about 20,000 MI cases. Furthermore, retrospective studies are often able to include large numbers of individuals who have developed the disease at younger ages, when associations with risk factors are often stronger, providing particularly sensitive tests of certain hypotheses. As demonstrated by the WTCCC [9], case-control studies can powerfully and efficiently facilitate genetic discovery and can use GWAS data to quantify and robustly correct for any population structure [43].

The ISIS and INTERHEART studies have, moreover, demonstrated that appropriately conducted, large case-control studies can usefully address some nongenetic hypotheses in MI, such as tobacco and alcohol consumption, and serological evidence of infection [7–9, 44–46]. Nevertheless, particularly in relation to nongenetic hypotheses, retrospective case-control studies may be liable to potential biases, such as recall bias (although there may be less scope for such bias in a study of acute MI than in a study of chronic stable CHD, as hours rather than months or years have elapsed since the index event) and selection biases. As noted earlier, selection of controls in case-control studies invariably involves trade-offs between scientific rigor and feasibility. Following the example of INTERHEART [8], we have opted to recruit controls drawn from attendants of people visiting out-patient clinics or (non-blood-related) attendants of cardiac patients; these groups will be compared in sensitivity analyses. Studies of plasma components may be affected by case-control studies of acute MI because certain circulating markers may be altered by sampling of blood within 24 h of MI symptoms. If as in PROMIS, time since the onset of symptoms has been recorded, any material bias can be quantified and at least partially corrected for. Similar considerations apply to fasting status and the recording of time since last meal [7, 44].

Conclusion

PROMIS is a large epidemiological resource for CHD in South Asia that will be systematically harvested to help identify and evaluate genetic and other determinants of MI in South Asia. It should help to hasten discovery of disease-causing pathways and to inform regionally appropriate strategies that optimise public health action in a country with limited resources.

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