AN INVESTIGATION ON THE CIRCULATING TRANSCRIPTOME SIGNATURES IN PERIPHERAL ARTERY DISEASE AND THE HOMOCYSTEINE PATHWAY GENOMIC SIGNATURES IN CORONARY ARTERY DISEASE

BY

RIZWAN MASUD

DEPARTMENT OF BIOLOGICAL SCIENCES
QUAID–I–AZAM UNIVERSITY
ISLAMABAD, PAKISTAN
2011

DECLARATION

The material contained in this thesis is my original work and I have not presented any part of this thesis/work elsewhere for any other degree. During the write up of thesis, I made all possible effort to avoid plagiarism as much as possible.

Rizwan Masud


بسم الله الرحمن الرحيم

DEDICATED

TO

MY LOVING PARENTS,

MY BELOVED WIFE,

MY BLESSED CHILDREN,

MY SUPPORTING BROTHER &

MY DEAR NEPHEW

CERTIFICATE

This thesis, submitted by **Mr. Rizwan Masud** is accepted in its present form by the Department of Biological Sciences, Quaid-i-Azam University, Islamabad, as satisfying the thesis requirement for the award of degree of Doctor of Philosophy in Physiology

SUPERVISOR:

(Dr. Irfan Zia Qureshi)

EXTERNAL EXAMINER:

(Prof. Dr. Muhammad Aslam)

EXTERNAL EXAMINER:

(Prof. Dr. A. R. Shakoori)

CHAIRMAN:

(Prof. Dr. M. Shahab)

Dated: 09 / 12 / 2011

CONTENTS

LIST OF ABBREVIATIONS

CVD	Cardiovascular disease
CAD	Coronary artery disease
PAD	Peripheral artery disease
EC	Endothelial cells
PSGL-1	P selection glycoprotein ligand 1
Th1	T helper cell type 1
Ig	Immunoglobulin
CAM	Cell adhesion molecules
ICAM1	inter-cellular adhesion molecule 1
VCAM1	Vascular cell adhesion molecule 1
PCAM1	Platelet cell adhesion molecule 1
PECAM1	Platelet endothelial cellular adhesion molecule 1
IFNγ	Interferon gamma
TNFα	Tumor necrosis factor alpha
IL	Interleukin
LFA–1	Leukocyte function associated molecule 1
VLA–4	Very late antigen 4
TIA	Transient ischemic attack
IC	Intermittent claudication
MI	Myocardial infarction
ACS	Acute coronary syndrome
ApoE	Apolipoprotein E
Ldlr	Low density lipoprotein receptor
LDL	Low density lipoprotein
VLDL	Very low density lipoprotein
HMDM	Human monocyte derived macrophages
ROS	Reactive oxygen species
Ox–LDL	Oxidized low density lipoprotein
M–CSF	Monocyte colony stimulating factor
PRR	Pattern recognition receptors
TLR	Toll-like receptor
HSP60	Heat shock protein 60
PAMP	Pathogen accociated molecular patterns
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
APC	Antigen presenting cells
MHC	Major histocompatibility complex
SR	Scavenger receptors
T_{reg}	Regulatory T cells
NK T	Natural killer T cells
Th2	T helper cell type 2
DC	Dendritic cells
CRP	C-reactive protein
MMP	Matrix metalloproteinase
MCP	Monocyte chemoattractive protein
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
TGFβ	Transforming growth factor beta

PPAR (α/γ)	Peroxisome proliferator activator receptors (alpha/gamma)
TZD	Thiazolidinediones
HDL	high density lipoprotein
VSMC	Vascular smooth muscle cells
TF	Tissue factor
tPA	tissue plasminogen activator
Lp A	Lipoprotein A
vWF	von Willebrand Factor
RFC	Red cell folate
ADM	Adrenomedullin
BNP	Brain natriuretic peptide
ANP	Atrial Natriuretic peptide
GWAS	Genome wide association study
NO	Nitric oxide
NOS	Nitric oxide synthase
IMT	Intima media thickness
PVD	Peripheral vascular disease
PAOD	Peripheral artery occlusive disease
ASO	Arteriosclerosis obliterans
ABI/ABPI	Ankle brachial index/Ankle brachial pressure index
TBI/TBPI	Toe brachial index/Toe brachial pressure index
NIRS	Near infra red spectroscopy
PSV	Peak systolic velocity
CTA	Computed tomography angiography
MRA	Magnetic resonance angiography
DSA	Digital subtraction angiography
QTL	Quantitative trait loci
Hcy	Homocysteine
MTR	Methyl tetrahydrofolate homocysteine methyltransferase
MTHFR	Methylene tetrahydrofolate reductase
SAM	S adenosyl methionine
AdoMet	S adenosyl methionine
SAH	S adenosyl homocysteine
AdoHcy	S Adenosyl homocysteine
CBS	Cystathionine β synthase
PON1	Paraoxonase 1
Hcy-LDL	Homocysteinylated low density lipoprotein
nNOS	Neuronal nitric oxide synthase
iNOS	Inducible nitric oxide synthase
eNOS	Endothelial nitric oxide synthase
SOD	Superoxide dismutase
DDAH	Dimethyl arginine dimethyl aminohydrolase enzyme
ADMA	Asymmetric dimethyl arginine
NMDA	N methyl D aspartate
GABA	Gamma amino butyric acid
PBMC	Peripheral blood mononuclear cells
RT-PCR	Real time polymerase chain reaction
SNP	Single nucleotide polymorphism
LD	Linkage disequilibrium
IVT	In vitro transcription

Cy	Cyanine dye
PBS	Phosphate buffered saline
RIN	RNA integrity number
GCOS	Gene chip operating software
RMA	Robust multichip analysis
FDR	False discovery rate
GO	Gene ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
ER	Emergency department
VEGF	Vascular endothelial growth factor
SRA	Steroid receptor RNA activator
SNARE	SNAP (soluble NSF attachment protein) receptor
ACE	Angiotensin converting enzyme
BMI	Body mass index
RFLP	Restriction fragment length polymorphism
DHPLC	Denaturing high pressure liquid chromatography
dNTP	Deoxynucleotide triphosphate
ddNTP	Dideoxynucleotide triphosphate
FRET	Fluorescence resonance energy transfer
qRT.PCR	Quantitative real time polymerase chain reaction
SSCP	Single strand confirmation polymorphism
MALDI	Matrix assisted laser desorption ionization
TOF-MS	Time of flight mass spectroscopy
CNV	Copy number variants
AS-PCR	Allele specific polymerase chain reaction
T-ARMS	Tetra primer allele refractory mutation system
ECG	Electrocardiography
TE	Tris EDTA buffer
NCBI	National center for biotechnology information
dbEST	NCBI Expressed sequence tags database
UCSC	University of California, Santa Cruz

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

All praises be to Allah, the most beneficent, the most merciful. His prophet Muhammad (P.B.U.H), the most perfect of human beings ever born, is the source of guidance and knowledge for humanity, forever.

I initiate this acknowledgement with profound appreciation to Dr. Irfan Zia Qureshi, Associate Professor, Biological Sciences, Quaid-i-Azam University, Islamabad Pakistan, who extended complete support during preparation, implementation of this work, and the writing of thesis. He was always willing to help and advise apposite remedies. I thank him for all the support and motivation.

I deeply appreciate the warm guidance and wholehearted support of Prof. Dr. Muhammad Shahab, the Chairman, Biological Sciences, Quaid-i-Azam University, Islamabad, during various stages of my Ph.D. course work and research.

I would really wish to thank Prof. Dr. I. J. Kullo, Head Cardiovascular Biomarker Research clinic and laboratory, Mayo Clinic, Rochester Minnesota USA, for inviting me on a six month research rotation in his clinic and laboratory. He was extremely professional, helpful, highly inspiring and open. A major portion of the work would not have been possible without his guidance, help and mentoring. My lab fellows and great friends at Kullo Lab and Mayo Clinic; Allison, Angela, Al-Omari Malik, Abdel Rehman, Keyue Ding, Jouni Hayan, Aparna Dhar, Saleem Umer, Liu Guanghui, and Sadek Ibrahim. They all made my stay in the beautiful town of Rochester, indeed, the most memorable experience of my life.

I must acknowledge the immense and huge support of the Higher Education Commission (HEC) Pakistan. The previous chairman, the current chairman, and the Executive Director envisioned and made this great program possible. I acknowledge that without the Indigenous 5000 PhD Scholarship Program and the helpful guidance of Ms Saima Naureen, Mr Baqar Husnain, Mr Babar Rasheed and Mr Shahid Saleem, I would not have progressed much. Regarding the finalization of my PhD thesis, were it not for the International Research Support Initiative Program (IRSIP) and the personal care of Mr Jehanzaib Khan (project director IRSIP), major chunk of the thesis would be incomplete. I owe my PhD also to the HEC and the guidance, help, care, and guidance of the mentioned (and to those not mentioned by name here) associates at HEC.

The colleagues and co-workers at Physiology Laboratory need to be appreciated for their whole-hearted cooperation. My lab fellows Tariq, Zeeshan Kashif, Faiqah, Dr Samina, Qamar, Fareeha and rest all provided strength in work support, sustained environment, and moral and material support.

I wish to thank my friends Masroor, Khalid, Inam, Amir, Shakeel, Naseer, Tariq, Jawad, Salman Chishti, Latafat, Fazal, Salman, Musharraf, Dr Sababa, Dr Arozia, and Zahid, colleagues at Quaid-i-Azam University, whose prayers, care, and well-wishes were always handy and forth-coming.

I would be failing my duty if I do not acknowledge the moral, material and spiritual support of my loving parents, I pray for their health and I need their blessings always. I am indebted to my brother Salman who bore with me during testing times.

Last but not the least I must acknowledge the patience, dedication, devotion, and care of my wife. I am thankful to my adorable and loving children; sons Zulkifl Rizwan, Zaafir Rizwan, and dear daughter Zarnish Rizwan, who despite my preoccupation, filled my life with thrill, happiness and joy as the most beautiful gifts of my life.

RIZWAN MASUD

ABSTRACT

ABSTRACT

It is now well recognized that in vascular disease patients, coronary artery disease and peripheral artery disease often coexist. Due to polygenic inheritance pattern, many genetic and environmental effects have cumulative effects in a particular vascular disease phenotype. The aims of the present studies were to delineate all the genes, and the variants in homocysteine pathway, that associate with the peripheral artery disease and coronary artery disease respectively. Despite the fact that both are subtypes of cardiovascular disease yet the underlying genetic and environmental effects are different and result in varying phenotypes.

The first part of the study was carried out at Mayo Clinic Rochester Minnesota, USA and focused on insight into the genetics of peripheral artery disease. Whole transcript microarray technology was employed for the genetic analysis in two different groups of peripheral artery disease cases and controls without disease. In the first group nine cases and nine control subjects, whereas in the second group ten cases and nine control subjects were studied. This study reports for the first time the use of peripheral blood mononuclear cells for the expression profiling in peripheral artery disease. The RNA was extracted from mononuclear cells and was hybridized to Affymetrix expression arrays. The analysis of results, following stringent statistical analysis, revealed a grouping of thirty differentially regulated gene transcripts in both patient groups. Out of the thirty differentially regulated transcripts, twenty two transcripts were upregulated, seven transcripts were downregulated and one transcript was unannotated, respectively. Excluding the gene transcripts with known associations, this study provides with a novel set of fourteen upregulated genes and six downregulated genes with no previous known association with the peripheral artery disease. The modulated pathways and the disease pathology mediated by the implicated genes include; immunity and inflammation, gene transcription, cell growth, cellular metabolism and signalling, and cell death/apoptosis. The highlighted genes and the pathways modulated by these genes may enhance the understanding of disease causation and serve as targets for early disease stratification in high risk individuals.

The second part of the study examined association of homocysteine pathway gene variants with coronary artery disease through the use of tetra primer ARMS–PCR genotyping. Elevated blood homocysteine levels is a known coronary artery

disease risk factor, and polymorphisms in homocysteine genes result in elevated homocysteine levels and high risk of vascular disease. The study was performed in the Physiology laboratory, Department of Biological Sciences Quaid-i-Azam University, Islamabad. Five single nucleotide polymorphisms, in four homocysteine pathway genes and one polymorphism in *ACE* gene were studied because of their prior, known, association with CAD in world populations. A total of 230 participants were recruited from the tertiary care hospitals in Rawalpindi and Islamabad. These included 129 participants with coronary artery disease and 101 subjects without disease. Peripheral blood was obtained for DNA isolation and for analyses of serum parameters. Tetra primer ARMS–PCR was used for allelic discrimination as it is a relatively new, rapid, reliable method and is more time, effort and cost effective allele discrimination method as compared to conventional approaches. The results of the study revealed that two out of five single nucleotide polymorphisms in homocysteine pathway genes and the variant in *ACE* gene were associated with risk of coronary artery disease in the Pakistani population. Another significant finding was that gene-gene interaction networks, in the studied polymorphisms, were additional modulators of coronary artery disease. This study is the first to report the use of tetra primer ARMS–PCR for allele discrimination in homocysteine pathway genes, and to report the importance of these genes as risk factors for coronary artery disease in local population.

The peripheral artery disease associated genes and implicated pathways, and the allelic variants in homocysteine pathway may provide with insights into the progression of cardiovascular disease, add to the diagnostic tools and aid in designing management strategies.

CHAPTER 1: GENERAL INTRODUCTION

GENERAL INTRODUCTION

The human genome sequencing project was completed in February 2001 and reported 31,000 genes in human genome (Lander et al., 2001; Venter et al., 2001). The revision of the human genome has brought the number of genes down to between 20–25,000 (International Human Genome Sequencing Consortium, 2004). A complete knowledge of the genes bears significance for disease management. The Mendelian genetic disorders and polygenic disorders are both subdivisions of genetic diseases. In simple Mendelian or monogenic disorders a single gene aberration is associated with a particular disease, whereas in 'complex' polygenic disorders a myriad of genetic as well as environmental effects associate with a particular phenotype (Givelber et al., 1998; Kumar, 2008; Tayo et al., 2009). Recent technological advancements have aided comprehensive analyses of the human genome, the transcriptome (RNA), and the proteome (translated proteins). Genome alterations may lead to changes in the transcribed and translated products; the RNA and proteins in turn can determine the frequency and manner in which genes are transcribed and translated (Nibbe et al., 2010).

Cardiovascular disease (CVD) and coronary artery disease (CAD) are chronic "complex" diseases. Although lifestyles in the developed and developing countries are much diverse, yet these and genetic components augment each other to produce similar disease frequencies (Yusuf et al., 2001a; Santos et al., 2008). Number of individuals affected with CVD is increasing, and because of increasing number of CVD cases presenting in health care centers, CVD is projected to be the leading cause of death in future (Mathers and Loncar, 2006).

The vascular disease is caused by atherosclerosis, the process that results from alteration in the immune system and inflammation (Mandal et al., 2004; Touyz, 2004; Segel et al., 2011; Touyz and Briones, 2011). Worldwide, cardiovascular disease is one of the leading causes of morbidity and mortality with a greater predominance in South Asian countries (Yusuf et al., 2002; Joshi et al., 2007; Jafar et al., 2008), yet compared to developed nations, the genetic studies carried out in this region are sparse. Little is therefore known about the genetic diversity related to CAD in Pakistani population. In developed nations, specialized genetic studies are

performed for vascular phenotypes, such as peripheral artery disease (Evans et al., 2008; Fu et al., 2008), while these phenotypes are rarely examined in South Asian countries. Extensive efforts are underway to determine and validate the genetic causes of CVD in general and in particular the CAD and peripheral artery disease (PAD) (Wyler von Ballmoos et al., 2006; Dahl et al., 2007; Evans et al., 2008; Fu et al., 2008). Consequently the proteins, enzymes, and the genes associated with initiation, progression, and development of immune responses and vascular disease are main targets for cardiovascular risk stratification and disease management.

1.1 Cardiovascular system

Cardiovascular system comprises the integrated complex of heart, blood vessels, and blood itself. Virtually each and every cell of the body is supplied by the blood vessels. Being itself a vital organ, the heart receives its nutrition through the coronary blood vessels. Under normal physiologic conditions, blood vessels maintain homeostasis whereas disease states affecting the vessel walls disrupt homeostasis and result in pathological consequences. The atherosclerotic, inflammatory, and occlusive involvement of the blood vessels manifests clinically as specific cardiovascular disease such as CAD, cerebrovascular disease, or PAD (Krishnaswamy, 2010).

1.1.1 Blood vasculature and transport through the vessel wall

Blood vessels do not serve merely as conduits of blood but are highly organized and comprise continuous array of living cells. The blood vessels carry nutrients to the body tissues and carry the toxins and wastes to the excretory organs. Additionally, being part of the immune system of the body, they carry the inflammatory cells, biomolecules, and antibodies as well (Mandal et al., 2004; Hansson, 2005; Roodink et al., 2005).

1.1.2 Components of vessel wall

Blood vessels have three main layers; the tunica intima, tunica media, and tunica adventitia. In normal blood vessels, the tunica intima is comprised of simple squamous endothelial cells, the subendothelial connective tissue with collagen fibers and proteoglycans, while the internal elastic membrane separates the intima from the media. Tunica media consists of elastin fibers, collagen, the connective tissue, and the concentrically arranged smooth muscle cells. The external elastic lamina separates tunica media from tunica adventitia. The adventitia consists of collagen, elastic fibers, the macrophages, and the connective tissue fibers which blend with the surrounding

connective tissue. The intimal and sub-endothelial retention of low density lipoproteins, during vascular disease, is the initial step for atherosclerotic plaques development (Skalen et al., 2002).

1.1.3 Selectin mediated homing of immune cells

Atherosclerosis results from uncontrolled and enhanced activation of normal immune mechanisms in the body. The immune cells, the inflammatory and the metabolic responses all play an active role in vascular disease progression (Hansson, 2005). The activated immune cells favor an enhanced expression of adhesion molecules from the inflamed endothelium and this in turn favors migration of greater number of inflammatory cells to the developing atherosclerotic plaque.

As a first step for atherosclerosis, the circulating immune cells must be loosely tethered to and roll over the intimal endothelial cells (EC), a process mediated by adhesion molecules called "selectins" (McEver and Cummings, 1997; Huo and Xia, 2009). Selectins are adhesion molecules and include E selectin (endothelial cells), P selectin (platelets and endothelial cells), and L selectin (leukocyte neutrophils) (McEver and Cummings, 1997; Huo and Xia, 2009). The most distinctive selectin ligand is P selectin glycoprotein ligand 1 (PSGL–1) and is expressed on virtually all the leukocytes. Leukocytes, activated endothelial cells, and platelets express the respective selectins and/or the specific selectin ligands. The selectins and PGSL-1 interactions modulate the tethering and rolling of inflammatory cells over endothelium which is the first phase for cellular adhesion and migration (McEver and Cummings, 1997; Huo and Xia, 2009). E selectin and P selectin mediate acquired immunity as they favor type 1 helper T cells (Th1) recruitment; as Th1 migration through the endothelium is shown to be inhibited by E and P selectin specific antibodies (Austrup et al., 1997). L selectins are present on neutrophils and facilitate their tethering and therefore migration to the inflamed vascular tissues. These prospective migrating neutrophils then shed the L selectin and replace them with integrins which subsequently facilitate neutrophil migration by binding with endothelial E selectin, thereby initiating diapedesis of neutrophils (Delves and Roitt, 2000).

1.1.4 Diapedesis / migration of cells across endothelial cells

The endothelial cell immunoglobulin (Ig) family of cell adhesion molecules (CAMs) such as inter-cellular adhesion molecule 1 (ICAM1), vascular cell adhesion

molecule 1 (VCAM1), platelet cell adhesion molecule 1 (PCAM1), and platelet endothelial-cellular adhesion molecule 1 (PECAM1), are necessary for transmigration of the immune cells across the endothelial cell barrier. The immune cells of vascular intima release several cytokines such as interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα) and interleukin–1 (IL–1). The cytokines then stimulate endothelial cells to release selectins, as well as the ICAM1 and VCAM1 (Blankenberg et al., 2003). The leukocyte cell integrins act as ligands for CAMs and include the leukocyte function associated molecule 1 (LFA–1) and very late antigen 4 (VLA–4) that bind with endothelial ICAM1 and VCAM1 respectively (Blankenberg et al., 2003). PECAM1 is expressed not only by the circulating platelets but also by endothelial cells, monocytes, neutrophils and a subset of activated T cells; the various isoforms of PECAM1 modulate transmigration (Wang and Sheibani, 2002). The PECAM1–PECAM1 interactions among adjacent cells and immune–endothelial cells results in elevated integrin expression and leukocyte transmigration/diapedesis. The leukocytes become static and firmly adhere to the endothelial cells after leukocytes bind to ICAM and VCAM. The bound immune cells take either the 'paracellular transmigration' or 'transcellular transmigration' pathway. The very same route is utilized by the immune cells migrating to the atherosclerotic lesions. As vascular disease progresses, more and more of the transmigrated immune cells move to the atherosclerotic plaque and participate in and amplify the inflammatory response (Carman and Springer, 2004; Wittchen, 2009). The schematics of adhesion molecules and atherosclerosis are shown in Fig. 1.1.

1.2 The risk factors and inflammatory basis of disease

Atherosclerosis is an inflammatory disease characterized by the enhanced accumulation of lipids, immune cells, and also by apoptosis and fibrosis. The earliest atherosclerotic lesion is a fatty streak and consists of lipid rich macrophages called "foam cells". Fatty streaks develop early in life and either resolve spontaneously or undergo pathophysiologic transformation to form atherosclerotic plaques. (Hansson, 2005). Atherosclerotic plaque is the main lesion in atherosclerosis and denotes asymmetrical focal thickening of the arterial intima. The plaques are rich in vascular smooth muscle cells, endothelial cells, the lipids, the mononuclear monocytes and the 'T' lymphocytes (Jonasson et al., 1986). The affected blood vessels exhibit narrowing of vascular lumen and diminished blood flow through the affected vessels.

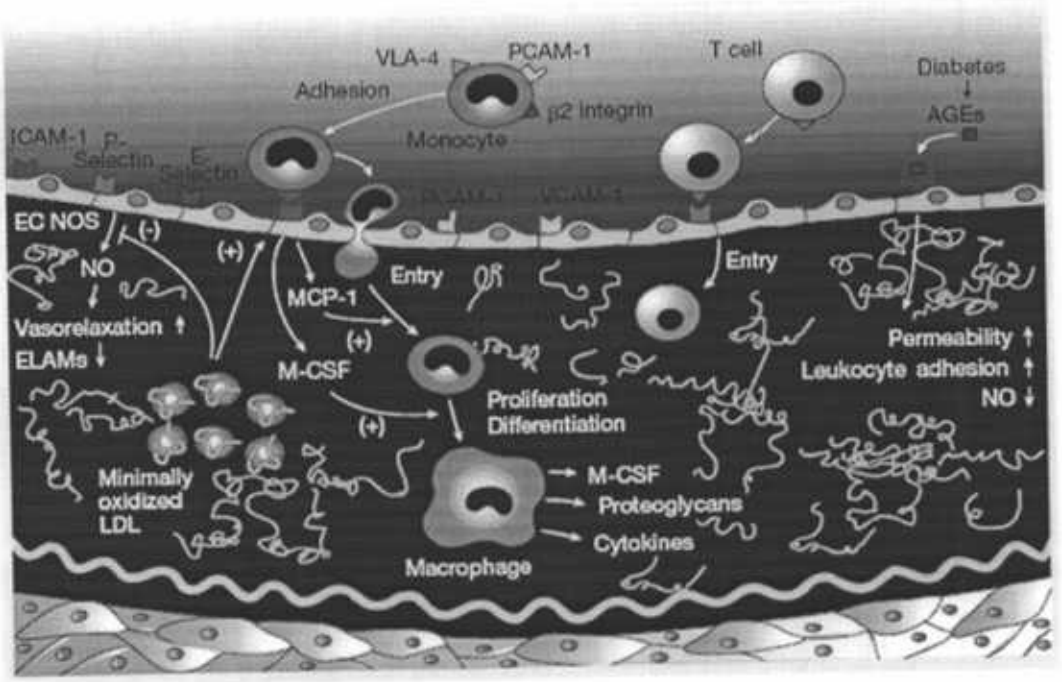

Fig. 1.1. Oxidized LDL stimulates endothelial cells to express selectins and adhesion molecules. Monocytes and T cells enter into the vascular intima and produce inflammatory cytokines to recruit and to activate more cells and cause plaque formation. (Lusis, 2000).

The athetrosclerotic plaques, due to restricted perfusion in the coronary, cerebral and peripheral vasculature, can result in coronary ischemia, transient ischemic attacks (TIA) or intermittent claudication (IC) (Tsang et al., 2005). Alternately, plaques rupture and thrombosis in the coronary artery causes myocardial infarction (MI) or acute coronary syndrome (ACS), while in cerebral vessels it causes stroke. In the aorta, vascular thrombosis or rupture leads to the abdominal aortic aneurysm or dissection of aorta while in the limb vasculature, it leads to critical limb ischemia. The occluding thrombus is formed either on the plaque surface (following endothelial denudation) or within the intima and in vascular lumen after the blood enters the ruptured atherosclerotic plaques. Either way there is vascular occlusion, disruption of blood flow to the target tissues and resultant tissue infarction (Davies, 1996).

1.2.1 Cholesterol theory for atherosclerosis

The cholesterol theory of atherosclerosis was first established by the classical experiments of Ignatowsky in 1908, Starokadomski in 1909, and of Anitschkow and Chalatow in 1913 (cited by Anitschkow and Chalatow, 1983). Ignatowsky and

Starokadomski demonstrated massive aortic lesions, fatty infiltration, and intimal thickening in rabbits fed with egg yolk. Anitschkow and Chalatow (1983) fed rabbits and guinea pigs pure cholesterol and observed similar results. These findings clearly indicated that 'cholesterol' holds the key for progression of vascular disease. Subsequently, with the use of mutant technology, apolipoprotein E deficient ($apoE^{-/-}$) and low density lipoprotein receptor negative ($Ldlr^{-/-}$) mice lines were created. These animals developed hypercholesterolemia, elevated LDL, and atherosclerotic plaques after they were fed high cholesterol diet (Plump et al., 1992; Ishibashi et al., 1994). These set of experiments provided evidence that cholesterol was an integral component for vascular lesions. Although there is unequivocal and overwhelming evidence in favor of cholesterol and LDL as major players, a substantial number of coronary events occur in individuals with normal cholesterol and normal LDL levels (Sniderman et al., 1980; Braunwald, 1997). The importance of cholesterol as a risk factor is also diminished in ischemic stroke (Shahar et al., 2003), suggesting that elevated cholesterol level by itself cannot explain the complex etiology of cardiovascular disease.

1.2.2 Inflammatory theory for atherosclerosis

Atherosclerosis is a complex inflammatory process characterized by an unchecked build up of immune cells and alteration in normal immune mechanisms of the body. The process involves the leukocytes that are activated in lieu of tissue injury for which the stimulus might be elevated cholesterol, excess toxic radicals, or oxidative damage (Libby and Aikawa, 2002; Hansson, 2005). Fatty streaks and foam cells are the hallmark of atherosclerotic lesions (Hansson, 2005). For initiation of atherosclerosis, macrophage uptake of apoB rich low density lipoproteins (LDL) and very low density lipoproteins (VLDL) is the basic step of a prolonged, complicated, and highly integrated process (Hurt et al., 1990; Costandi et al., 2011).

1.2.3 LDL, formation and significance of modified LDL

Hypercholesterolemia leads to excessive subendothelial retention of LDL molecules. The interaction between LDL and chondroitin rich proteoglycans in the subendothelial layer causes a conformational change in the LDL molecules; these modified LDL molecules are preferentially uptaken by human monocyte derived macrophages (HMDM) transforming them into foam cells (Hurt et al., 1990). LDL and proteoglycan interaction is a vital requirement for atherosclerosis, as animal

models with defective proteoglycan biding sites on LDL molecules, characteristically show greatly decreased atherosclerosis (Williams and Tabas, 1995; Skalen et al., 2002). The LDL molecules can also be readily oxidized by reactive oxygen species (ROS) produced by many cell types such as smooth muscle cells, endothelial cells, and macrophages, as well as by food products, by smoking, and by homocysteine (Perna et al., 2003; Niki, 2004). Furthermore, ROS can oxidize LDL to yield oxidized LDL (ox–LDL). Ox–LDL has the capacity to activate endothelial cells to release leukocyte adhesion molecules. The formation of foam cells brings into motion a highly integrated set of events that ultimately lead to plaque rupture and thrombosis (Steinberg, 2002; Perna et al., 2003; Niki, 2004; Hansson, 2005).

1.2.4 Macrophages, foam cells, and innate immunity

Monocytes in the blood stream interact with endothelial selectins and are tethered to and roll over the endothelium. The monocytes then firmly adhere to the endothelium through ICAM1 and VCAM1 and thereafter enter subendothelial space. Vascular smooth muscle cells and endothelial cells release molecules such as monocyte colony stimulating factor (M–CSF) that activate monocytes and T cells as well. The release of M–CSF dictates the binding, migration and differentiation of monocytes and their conversion to macrophages, a necessary prerequisite for atherosclerosis (Rajavashisth et al., 1990; Smith et al., 1995). The macrophages in the atherosclerotic plaque influence and enhance the processes which are a necessary for the plaque buildup. The activated macrophages express different pattern recognition receptors (PRRs); the scavenger receptors and toll–like receptors (TLRs), these are of extreme importance for initiation and progression of different aspects of normal innate immunity and for the pathogenesis of atherosclerosis as well (Martin-Fuentes et al., 2007).

1.2.5 Toll like receptors

The toll–like receptors (TLRs) are expressed by macrophages, dendritic cells, mast cells, and endothelial cells. They control both the innate and adaptive immune responses. TLR expressing cells can directly induce inflammatory responses (Janeway and Medzhitov, 2002). The TLR–ligand binding activates innate immunity; the activated macrophages directly enhance atherosclerosis by releasing inflammatory cytokines, toxic radicals, and proteases. The toll receptors additionally mediate

adaptive immune responses for they are also present on macrophages that act as antigen presenting cells (APCs).

The ligands for TLRs include human heat shock protein 60 (HSP60) and ox-LDL, highlighting the importance of these receptors and their expressing cells in modulation of atherosclerosis (Ohashi et al., 2000; Miller et al., 2003). TLRs also suggest role of microbes in CAD as they recognize unmethylated CpG motifs in bacterial DNA, as well as the pathogen associated molecular patterns (PAMP) including the microbial lipopolysaccharides, the peptidoglycans, and the lipotechoic acids (Medzhitov, 2001). In atherosclerotic lesions the macrophages and endothelial cells exhibit enhanced expression of numerous TLRs. The toll receptors colocalize with nuclear factor kappa light chain enhancer of activated B cells (NF-κB), the NF-κB not only induces TLR production but also induces downstream effectors and inflammatory genes of TLR activated cells (Edfeldt et al., 2002; Janeway and Medzhitov, 2002). The APCs with major histocompatibility complex (MHC) class II are activated by ox-LDL or PAMP; these activated APCs then present the processed antigen to T cells, specifically to the Th1 cells. This innate immunity mediated activation of acquired immune response has been shown to be dependent on toll mediated signaling and is necessary for the plaque buildup (Schnare et al., 2001).

1.2.6 Scavanger receptors

Scavenger receptors (SR) are cell surface protein receptors present on macrophages and dendritic cells. The macrophage SR include SRA, SRB-1, CD36, CD68, CXCL–16, and lectin type oxidized low density lipoprotein receptor 1 (LOX-1) (Steinberg, 1997; Pluddemann et al., 2007). SRs are also important mediators of atherosclerosis and vascular disease. With hypercholesterolemia, it is not the native LDL but the modified/oxidized LDL that is taken up by the macrophages expressing the SRs (Steinberg, 1997). SRs are also known to bind heat shock proteins released in response to stressful stimuli (Pluddemann et al., 2007). SRs mediate first the internalization, and later the lysosomal degradation of ox–LDL, PAMP, fragments of malarial parasite, and apoptotic bodies (Peiser et al., 2002). The macrophages are then converted to the prototype cells of atherosclerosis, the foam cells. Though the foam cells act as antigen presenting cells, yet they do not directly release the inflammatory cytokines. The foam cells and the dendritic cells (containing SR) degrade the internalized molecules, process and transfer the antigenic epitopes through the MHC

class II molecules to $CD4^+$ Th1 cells; the activated foam cells facilitate linkage of innate immunity and adaptive immune responses and result in progression of atherosclerosis and vascular disease (Pearson, 1996; Nicoletti et al., 1999; Platt and Gordon, 2001; Pluddemann et al., 2007).

1.2.7 T lymphocytes–Subtypes, activation, and acquired immunity

T lymphocytes are present in several varieties and include $CD4^+$ helper T cells (activated by APC such as foam cells and dendritic cells), $CD8^+$ cytotoxic T cells (activated by MHC class I containing APC), memory T cells, regulatory T (T_{reg}) cells, natural killer T (NKT) cells, and gamma delta T cells.

In atherosclerotic plaques, the T cells are mostly the $CD4^+$ T helper cells; these 'Th' cells are activated after interaction with APCs that present antigens through MHC class II (Hansson, 2005). The antigens presented by the APCs to 'Th' cells include ox–LDL (Stemme et al., 1995), HSP60 (Xu, 2002; Benagiano et al., 2005), and *Chlamydia pneumonia*. The *C. pneumonia*–HSP molecules and responsive T cells have been isolated from atherosclerotic plaques (de Boer et al., 2000; Campbell and Kuo, 2004). T cells interact through the T cell receptors (TCR) to the APC. Th cells may differentiate into subtype 1 or 2 depending on activation signals. Th1 cells activate macrophages and promote inflammatory processes whereas Th2 cells promote allergic reactions (Hansson, 2005).

1.2.8 Pro-inflammatory cells and responses for adaptive immunity

The Th1 cell induction is dependent on IL-12, and the principal cytokine produced by activated Th1 cells is IFNγ. Conversely Th2 cells are induced by IL-10, and the activated Th2 cells in turn release IL-4. The human atherosclerotic lesions reveal presence of the elevated levels of IL-12 mRNA and protein, IFNγ, IL-10, and low levels of IL-4 (Uyemura et al., 1996; Daugherty and Rateri, 2002). This indicates that the atherosclerotic process is Th1 and not Th2 mediated process. In humans and mice, the activated monocytes release excess mRNA and proteins for IL-12 with elevated Th1 release of pro-inflammatory cytokine IFNγ, while IL-4 levels are relatively less (Uyemura et al., 1996; Lee et al., 1999). IFNγ activates the macrophages, Th1 cells, vascular smooth muscle cells, endothelial cells, enhances release of adhesion molecules and proteins favoring atherosclerosis, and induces production of potent pro-inflammatory cytokines IL-1 and TNFα (Huber et al., 2001; Szabo et al., 2003; Hansson, 2005). The observation that atherosclerosis is dependent on

Th1 responses is reinforced by experiments in animal models that lack T-bet (transcription factor inducing Th1 cell differentiation) (Buono et al., 2005), IL-12 (Davenport and Tipping, 2003), IFNγ (Gupta et al., 1997), and the receptors for IFNγ (Buono et al., 2003).

Another pro-atherogenic system comprises of CD40 and its ligand (CD40L) (Mach et al., 1998). CD40 is a protein present on APC including macrophages, dendritic cells (DC), as well as on the B cells, vascular smooth muscle cells, and endothelial cells. It was previously known that CD40L was expressed only by $CD4^+$ T cells, but it is now known that macrophages, endothelial cells, as well as vascular smooth muscle cells co-express the receptor as well as the ligand (CD40L). Thus paracrine or autocrine CD40 and CD40L interactions activate adjacent inflammatory cells to produce proatherogenic molecules and cytokines. This ligand receptor complex favors disease progression as evidenced by experimental disruption of CD40L or of CD40, with resulting diminished atherosclerotic lesions (Mach et al., 1998).

Ongoing studies have added to the list of novel biomolecules, chemokines, and the cytokines implicated with atherosclerosis, thrombosis, and vascular disorders. These include homocysteine; C-reactive protein (CRP including the highly sensitive variety: hsCRP); D–dimer; fibrinogen; selectins; integrins; CAMs; serum amyloid-A; TNFα; matrix metalloproteinases (MMPs); proteases; pro-inflammatory interleukins (IL-1, 6, 8, 15, 18, and 33) and IFNγ (Koenig et al., 2001; Perna et al., 2003; Hansson, 2005; Empana et al., 2008; Matsuda et al., 2011). All these risk factors and risk markers are associated with cardiovascular disorders.

1.2.9 Anti-inflammatory adaptive T immune cells and cytokines

Atherosclerotic plaques display elevated levels of IL–10, a Th2 cell inducer but not for IL–4 (a marker of Th2 cellular response) (Uyemura et al., 1996; Lee et al., 1999), this signifies that Th2 activation is not the characteristic feature of augmented atherosclerotic lesions. In the atherosclerotic lesions, IL–4 does not colocalize with activated macrophages, and additionally IL–4 limits Th1 responses (Huber et al., 2001). Animal models with higher Th2 responses exhibit decreased atherosclerotic lesions (Huber et al., 2001), while in some other instances, IL–4 deficiency may result in reduced atherosclerosis (Davenport and Tipping, 2003). These findings favor

conflicting role of Th2 cells and IL–4 as inhibitors of inflammation and atherosclerosis.

The regulatory T cells (T_{reg}), like Th2 cells are related to anti-inflammatory responses. $CD4^{+}CD25^{high}T_{regs}$ are immunosuppressive, reduce adaptive and innate immune responses, and reduce atherosclerosis as well as vascular inflammation (Yang et al., 2008). Disruption of regulatory T cell axis leads to enhanced atherosclerosis, providing strong evidence for the anti-inflammatory effects of T_{reg} (Mallat et al., 2007; Xiong et al., 2009).

1.2.10 B cells and adaptive immune responses

The immune responses also include the B cells that modulate humoral arm of adaptive immunity. The role of B cells in progression of atherosclerosis is conflicting. The B cells (like T cells) are implicated at times with enhancing atherosclerosis (Daugherty et al., 1997; Song et al., 2001). In contrast, in other instances, B cells and the humoral immunity are known to decrease atherosclerotic lesions (Major et al., 2002; Andersson et al., 2010). The B cells have atheroprotective role; as spleenectomy in humans as well as in experimental animals (with resultant depletion of B cell pool) enhanced atherosclerotic lesions (Robinette and Fraumeni, 1977; Caligiuri et al., 2002;). Similarly, the transfer of B cells in non-spleenectomized as well as in the spleenetomized *apoE*$^{-/-}$ mice generates antibodies to oxidized LDL (ox–LDL) and as a result reduced atherosclerosis (Caligiuri et al., 2002).

1.2.11 Pro-inflammatory cytokines and Anti-inflammatory cytokines

In the developing atherosclerotic plaques, activated macrophages and vascular cells release IL–12, IL–18, and IFNγ, all are potent Th1 cell activators. Th1 cells induce TNFα, IFNγ, and IL–1 and these in turn can further activate macrophages, vascular smooth muscle cells, and endothelial cells creating amplified immune responses. Macrophages and endothelial cells in the plaque additionally release IL–8, monocyte chemoattractant protein (MCP), M–CSF, granulocyte colony stimulating factor (G–CSF), and granulocyte macrophage colony stimulating factor (GM–CSF) to induct, and activate more immune cells (Rajavashisth et al., 1990; Frostegard et al., 1999). In plaques, another feature of IFNγ is inhibition of vascular smooth muscle cells proliferation (by inhibiting α-Actin), and the inhibition of endothelial cells proliferation (Friesel et al., 1987; Hansson et al., 1989). Simultaneously, TNFα induces transcription factor NF–κB with resultant transcription

of proteolytic enzymes (that digest extracellular matrix), and the production of nitrogen reactive species, reactive oxygen species (ROS), and tissue factor (pro–thrombotic agent) (van Hinsbergh et al., 1990; Lee et al., 1996; Saren et al., 1996). The inflamed vascular tissues and adipose tissues also release TNFα and IL–6. These cytokines act on hepatic tissue to promote secretion of fibrinogen, of serum amyloid–A, and of CRP, and these compounds augment the inflammatory/immune responses (Hansson, 2005).

The anti–inflammatory cellular component includes the Th2 cells, T_{reg}, and the B cells. Th2 and T_{reg} cells, like the Th1 cells, require activation through antigen presentation. The fact that pro-inflammatory molecules and processes mediate atherosclerosis, indicate that the anti-inflammatory molecules may in fact lower the progression of atherosclerosis and the development of vascular lesions. Few immune cells in the plaques release IL–4, which helps differentiation of Th2 cells from $CD4^+$ T cells. Th2 cells release anti-inflammatory cytokines IL–4, IL–10, IL–5, and IL–13 and through B cell activation additionally promote humoral immune responses (Binder et al., 2002). Macrophages, endothelial cells, vascular smooth muscle cells, platelets, Th2, and $CD4^+CD25^+$T cells are said to release IL–10 and transforming growth factor beta (TGFβ). IL–10 and TGFβ are two cytokines with consistent anti-athrogenic effects. An excess of IL–10 and TGFβ strongly inhibit atherosclerosis and plaque lesions (Grainger et al., 1995; Pinderski et al., 2002). TGFβ diminishes atherosclerotic complications and stabilizes the plaque by increasing collagen production. Th2 cytokine IL–5 relates innate with adaptive immunity, it inhibits atherosclerosis by activating B cells, and the deficiency of IL–5 enhances atherosclerosis (Binder et al., 2004).

An alternate anti-inflammatory mechanism favoring the inhibition of atherosclerotic disease progression is peroxisome proliferator activator receptor system. Activation of peroxisome proliferator activator receptors 'alpha' and 'gamma' (PPARα and PPARγ) diminishes innate immunity, acquired immunity, as well as atherosclerosis progression. Ligands and agonists for PPAR (for example PPARα–activating acid derivatives, TZD) decrease activation of T cells and vascular smooth muscle cells, and reduce proteins and mRNA for IFNγ, TNFα, and IL–2 (Marx et al., 2002). Estrogen is an additional modulator of atherosclerotic processes as it decreases LDL levels (and therefore decreases production of LDL oxidation products), diminishes cellular adhesion, migration, and activation; increases HDL levels, and

promotes vascular dilatation. All these processes serve to inhibit atherosclerosis progression (Nathan and Chaudhuri, 1997; Nathan et al., 1999). At the same time, aromatase dependent conversion of testosterone to estradiol, also inhibits atherosclerotic process by inhibiting the adhesion molecules and cellular migration (Mukherjee et al., 2002). The complex interplay of pro–atherogenic and anti–atherogenic stimuli guides the disease progression and vascular complications.

1.2.12 Athero-thrombosis, vascular occlusion and tissue infarction

Normal coagulation processes are necessary to prevent blood loss when a vessel is severed, but thrombosis in a major blood vessel following rupture of an atherosclerotic plaque can result in grievous circumstances. The basic underlying pathophysiology of the cardiovascular disorders, stroke cases, and peripheral artery disease is atherosclerosis and athero–thrombosis. Fully developed atherosclerotic plaques comprise of a 'lipid rich core' surrounded by a fibrous cap. The lipid rich core comprises of foam cells, dendritic cells (DC), mast cells, B cells, and the T cells (Lusis, 2000; Hansson, 2005). The surrounding 'fibrous cap' is formed by smooth muscle cells, collagen, and extracellular matrix proteins derived from smooth muscle cells. The initial perception that disproportionate smooth muscle cell growth narrows and completely occludes the vascular lumen, and therefore causes tissue infarction, has been revised (Hansson, 2005). In myocardial infarction/acute coronary syndrome (MI/ACS), stroke, or critical limb ischemia, there is moderate stenosis whereas the main lesion is thrombosis within the vascular lumen. Athero–thrombosis refers to 'disruption' of an unstable atherosclerotic plaque with platelet activation and 'thrombus' formation within the vessel lumen. Plaque rupture precedes vascular thrombosis because in ACS, disruption of the plaques followed by formation of a thrombus is evident in three fourths of the cases (Falk et al., 1995).

Normally the endothelial cells and the fibrous cap function as barriers between blood and plaque components and inhibit thrombosis. Proteolytic enzymes and inflammatory molecules transform the plaques into 'susceptible unsteady structures' that can rupture with ensuing thrombosis leading to vascular occlusion. The interstitial collagen gives strength to the fibrous cap and stability to plaque. Numerous mechanisms and molecular patterns destabilize the plaque and result in plaque rupture. TGFβ increases interstitial collagen whereas IFNγ decreases proliferation of vascular smooth muscle cells and destabilizes plaque architecture by

decreasing the production of collagen by vascular smooth muscle cells (Amento et al., 1991). Macrophages, vascular endothelial cells, vascular smooth muscle cells, and T cells (with CD40/CD40L and TLRs) on activation, produce numerous proteolytic enzymes, inflammatory mediators, and coagulants. Matrix metalloproteinases (MMPs), released by endothelial cells and immune cells within plaques, in addition weaken the plaques, aid in cellular migration, cause the digestion of collagen and other matrix proteins and increase likelihood of plaque rupture (Galis et al., 1994; Dollery and Libby, 2006). TLR4 modulates activation of NF–κB which in turn enhances release of TNFα, and MMP9. There is increased production of these plaque destabilizers in ACS patients as compared to normal individuals, these pro-inflammatory mechanisms also destabilize the plaques and lead to plaque ruptures (Xie et al., 2010).

Mechanism of athero-thrombosis

Thrombosis is the formation of blood clot by the aggregated platelets and fibrin meshwork at the site of vascular injury. When a vessel is cut, the resulting clotting and thrombosis is beneficial as it prevents blood loss, whereas the clot formation secondary to an atherosclerotic plaque rupture is detrimental for the tissue perfused by the injured vessels. 'Tissue factor' (TF) is a highly pro-thrombotic constituent and is central for the formation of blood clots and for the thrombotic processes following plaque rupture. Macrophages in the plaques are known for long to produce this highly pro–atherogenic factor (Libby and Aikawa, 2002). T cells expressing CD40L can activate macrophages (rich in CD40) to release MMPs and tissue factor to weaken the plaque and to increase the propensity for thrombosis (Mach et al., 1997).

In vascular atherosclerotic disease, the coagulation proteins in the circulating blood are kept isolated from their potent activators (such as tissue factor) by the fibrous cap and intact endothelium. Following plaque disruptions, however, the blood coagulation proteins come in contact with tissue factor and undergo differentiation and activation. Tissue factor ultimately leads to the rate limiting step of coagulation pathway; production of thrombin from pro-thrombin. Thrombin produced at the inflamed vascular area not only induces MMP production to destroy collagen and cause plaque destabilization, but also augments the coagulation cascade and thrombosis. Thrombin catalyzes conversion of inactive fibrinogen to its active

proform 'fibrin'; the fibrin deposition and platelet aggregation are the final steps in thrombus formation (Galis et al., 1997).

Occluding thrombosis within the plaque itself and in the vessel lumen, can results in acute coronary syndrome, stroke, or critical limb ischemia (Davies, 1996; Lusis, 2000; Hansson, 2005). It is worth mentioning that not all thrombotic events take place with plaque rupture; thrombi also form when vessels are severed (therefore prevent blood loss), and few thrombi form on the surface of denuded plaques (Davies, 1996).

Patients with ACS show evidence of 'multiple disrupted plaques' but only one ruptured plaque is the reason for acute coronary event, and also that complete vessel occluding thrombosis cannot occur solely through plaque related TF (Cimmino et al., 2011). Circulating blood holds the key, as many cells types in circulating blood are known to express TF in its inactive as well as active forms. Circulating and cellular sources of TF include the circulating monocytes; microparticles originating from apoptotic macrophages, smooth muscle cells, and endothelial cells; platelets; and circulating neutrophils (Maugeri et al., 2010; Cimmino et al., 2011). In CAD patients, homocysteine is an additional source of circulatory TF, as higher plasma levels of homocysteine result in higher plasma TF levels (Marcucci et al., 2000).

CRP is not just a novel cardiovascular biomarker but is also a potent inducer of MMPs, MCP1, and TF mRNA/protein (Matsuda et al., 2011). In ACS patients, there is also enhanced concentration of MMP2 and MMP9, platelet factor–4, soluble CD40L, and phospholipase A2, all these are either markers of inflammation or are sustained activators of platelet activation and aggregation (Gresele et al., 2011). The finding that soluble form of CD40L (sCD40L), MMPs, TF, and soluble forms of CAMs (sICAM1, sVCAM1) are present in circulating blood and are elevated in CAD, provide additional mechanisms for the vascular disease progression (Blankenberg et al., 2003; Gresele et al., 2011). Therefore the old paradigm has been revised and additional mechanisms are now known to contribute to and lead to vascular thrombosis and tissue infarction (Fig. 1.2).

1.3 The Risk Factors For Vascular Disease

1.3.1 Established metabolic and endocrine risk factors of vascular disease

Physiological as well as pathological risk factors are associated with cardiovascular diseases in general, these include increasing age, male gender (females

after menopause tend to have similar event rates as males), obesity, sedentary life styles, African American population group, smoking status, oral contraceptive usage (with and without tobacco usage) low socioeconomic status, positive family history, and type A personality. Some pathological factors including high LDL levels, hypertension, diabetes, and metabolic syndrome are also positively associated with the progression and complications of vascular disorders (Akinkugbe, 1990; Kannel, 1990; Thorneycroft, 1990; Sebregts et al., 2000; Blum and Blum, 2009).

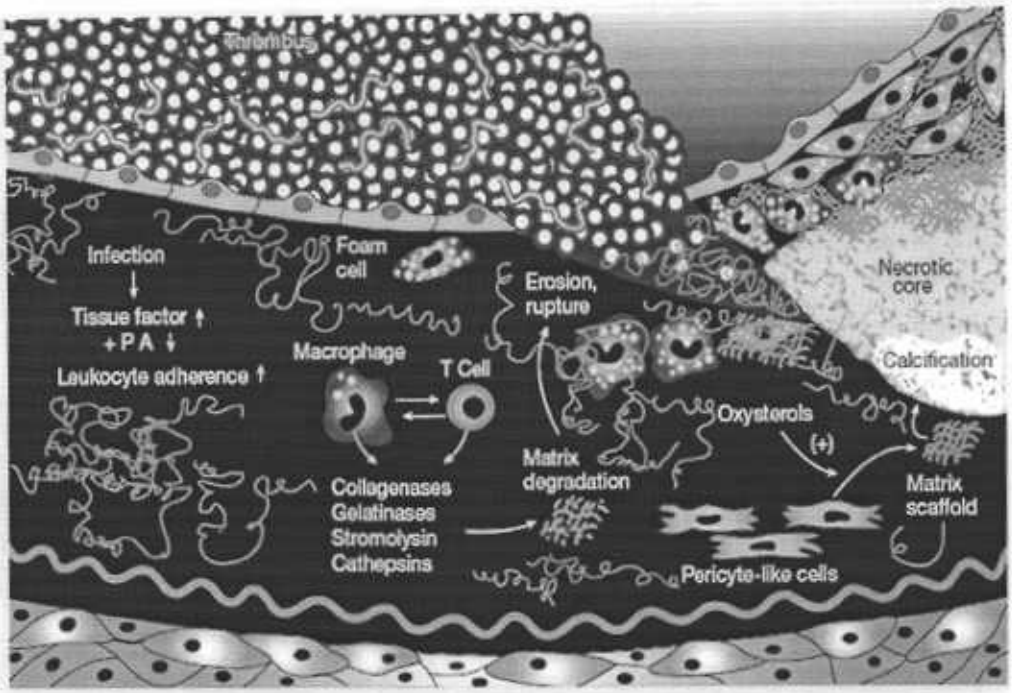

Fig. 1.2. The plaques are destabilized by production of different matrix degradation enzymes, plasminogen activators, and the Tissue factor (TF). The plaque rupture is quickly followed by activation of coagulation cascade through TF and occluding thrombus formation composed of activated platelets, red cells, and fibrin mesh–work. (Lusis, 2000).

1.3.2 The novel risk factors for atherosclerosis and vascular disease

Numerous studies for evaluation of novel risk factors have been carried out and they result in an ever growing list of biomolecules and biomarkers. These molecules are implicated as risk factors for hypertension, atherosclerosis, and vascular disorders. Many of these proposed risk modulators serve important functions in the body, yet errors of metabolisms in their involved pathways result in elevated levels and associated pathological consequences. These novel risk factors include homocysteine, CRP, fibrinogen, plasmin and tissue plasminogen activator (tPA), lipoprotein A (Lp A), von Willebrand Factor (vWF), and red cell folate (RFC)

(Maldonado et al., 2010; Wagner et al., 2010; Khandanpour et al., 2011). The proform of adrenomedullin (MR–pro–ADM) (Chan and Ng, 2010), and natriuretic peptides are such additional risk factors. The natriuretic peptides include brain natriuretic peptide (BNP) and its precursors (such as the N terminal proform the 'NT pro BNP') (Zeng et al., 2010); atrial natriuretic peptide (ANP) and its precursors (the mid regional proform the 'MR pro ANP) (Chan and Ng, 2010); and C terminal pro-vasopressin (Copeptin) (Chan and Ng, 2010).

Novel genetic studies aim at defining quantitative trait loci (QTLs), specific candidate genes and the gene variants associated with genetic disorders. The genetic risk factors are important in disease stratification, and quite a few genomic loci have been identified by genome wide association studies (GWAS). Such GWAS relate CVD to the 'highly reproducible' 9p21 genetic locus (Helgadottir et al., 2007; Samani et al., 2007; Welcome Trust Case Control Consortium, 2007; Helgadottir et al., 2008) as well as to the 6q25, 2q36, 1p13, 1q41, 10q11, and 15q22 genetic loci (Samani et al., 2007). In addition to genetic loci, the polymorphisms in adrenergic receptor and receptor genes (*B2AR*, *ADRB1*, *ADRB2*) (Pacanowski et al., 2008; Piscione et al., 2008), in the endothelin gene (Petidis et al., 2008; Casey et al., 2010), as well as nitric oxide (NO) and the polymorphisms in nitric oxide synthase enzymes (NOS) (Morray et al., 2007; Ko et al., 2008; Dafni et al., 2010) are also associated with CVD. Nitric oxide is an effective vasodilator and diminished NO production by the vascular endothelial cells, alongwith disproportionately high levels of angiotensin, endothelin–1, and ROS causes endothelial dysfunction and favors atheromas (Verma and Anderson, 2002). Vessel related risk factors assessment includes the carotid intima media thickness (IMT) scoring, vascular (such as coronary vasculature) calcium scoring, and the vascular angiography. The vascular 'calcium load' increases as plaque develops, and although it is not certain, still the vessel calcium load may serve to stabilize the plaque and decrease chances of plaque rupture (Prati et al., 2010).

1.4 Peripheral Artery Disease

Peripheral vascular disease (PVD) refers to the disease of vascular beds other than the coronary and cerebral vessels. This may include pathology affecting the carotid vessels, the mesenteric vessels, the renal vessels, and the upper and lower limb vessels. The term peripheral artery disease (PAD) by convention is designated to vascular phenotypes affecting the lower limbs only. Other names for peripheral artery

disease include peripheral artery occlusive disease (PAOD), arteriosclerosis obliterans (ASO–affecting abdominal aorta and lower limb vessels), lower extremity artery disease, and lower extremity peripheral artery disease (Hennrikus et al., 2010; Lawall et al., 2011; McDermott et al., 2011).

1.4.1 Peripheral artery/occlusive disease (PAD/PAOD) and classification

The earliest symptom of PAD is 'intermittent claudication' (IC) and it signifies the leg pain and discomfort that is aggravated by exercise and is relieved by rest. In peripheral artery disease, fatty tissue and the plaque buildup affects the iliac, femoral, and popliteal vessels and may lead to insufficient perfusion of lower limbs, with critical limb ischemia, gangrene, and/or leg ulcerations. Thrombi form in limb vessels (as in coronary vessels) and the late complications of limb thrombosis may necessitate surgical intervention. Severe gangrenous leg may require amputation, and the thrombi can release emboli, resulting in life threatening emergencies. PAD is strongly associated with routine risk factors that aggravate atherosclerosis, including tobacco use, elevated blood sugar levels, high blood pressure, and elevated homocysteine levels (Hirsch et al., 2006; Norgren et al., 2007). Although PAD can coexist with CAD, it can however develop independent of the conventional cardiovascular risk factors (independent of coronary artery calcium load and IL-6) (Aboyans et al., 2011). As PAD affects the quality of life, an early diagnosis and prompt palliative measures can improve the quality of life and life expectancies in the affected, high risk individuals.

PAD is classified into five stages or groups (based on guidelines by Leriche-Fontaine), termed Fontaine's classification (Hirsch et al., 2006; Castro-Sanchez et al., 2009), and include: Stage I in which the plaque and obstruction produce no symptoms; Stage II where IC is present, this stage is further divided into two sub-stages (IIa/IIb) depending on whether IC is present after walking more than or less than 150 meters; Stage III is where pain is present at rest; and Stage IV/V when ulcers, limb necrosis and critical limb ischemia develop. Stage III, IV, and V necessitate surgical management (Hirsch et al., 2006; Castro-Sanchez et al., 2009).

1.4.2 Diagnosis of PAD

The diagnosis of peripheral artery disease is dependant on the history of the patients and symptoms, general physical examination, and the specific diagnostic tests.

1.4.2.1 History/Symptoms

The most common and distinctive symptom of PAD is intermittent claudication (IC) (Cunningham et al., 2010), symbolized by the history of leg pain, discomfort or severe leg cramps (most noticeable in calf area). This symptom increases in intensity with physical exertion and is relieved by rest. As disease progresses the pain appears earlier with lesser exertion and takes longer to settle after rest. A severe manifestation of disease is that the leg pain and intermittent claudication may appear at rest. There is also history of accompanying disorders such as hypertension, diabetes, and elevated lipid levels. The presence of IC in patients is associated with generalized vascular abnormality and higher mortality rates due to coupled stroke, and the CAD (Cunningham et al., 2010).

1.4.2.2 General physical examination

For the patients referred for PAD assessment and diagnosis, the best approach is the examination of entire cardiovascular system because PAD is often accompanied with generalized atherosclerotic disease. The general physical examination in PAD includes examination of peripheral pulses (femoral, popliteal, dorsalis pedis, carotid, brachial, and radial), their presence, volume (absent/weak/normal), and their pattern (regular or irregular). The systolic and diastolic blood pressure readings are recorded, any sores or ulcers present in the limbs are examined; temperature differences between the limbs are monitored as temperature difference between adjacent limbs is another indication for PAD (Hirsch et al., 2006; Norgren et al., 2007).

1.4.2.3 Diagostic Tests

The diagnostic tests used for diagnosis of PAD can be grouped into two major categories; non angiography based diagnostic tests and angiography based diagnostic tests, the latter group absolutely requires tertiary care medical settings.

Ankle brachial index

The estimation of ankle brachial index (ABI) or ankle brachial pressure index (ABPI) is the most common and routinely used non angiography based clinical measure of PAD. ABI is measure of ratio of the systolic blood pressure at ankle to the systolic blood pressure at arm (systolic dorsalis pedis or posterior tibial artery pressure/systolic brachial pressure). Its range in healthy normal subjects is in between

0.9 to 1.3. ABPI measurements require a sphygmomanometer as well as a hand held Doppler apparatus. ABPI can diagnose PAD in patients with more than 95 % accuracy (Fowkes et al., 1988).

Segmental pressure measurement

The modification of ABPI, the 'segmental pressure measurement' can be undertaken by placing the sphygmomanometer cuff at different locations in the limbs to determine the exact site of vascular stenosis. ABPI > 1.3 denotes non–compressible vessels; arterial calcification, and high risk of CVD, CAD, and PAD (Potier et al., 2011). The ABPI measurement holds less accuracy in these cases; and toe brachial pressure index (TBI or TBPI) should be measured in these individuals to reliably detect PAD (Hirsch et al., 2006; Potier et al., 2011).

Post exercise ABI or treadmill ABI

Individuals with normal ABPI values often have lower levels after exercise (Hirsch et al., 2006), therefore in individuals with borderline ABPI, both resting and post exercise or treadmill measurements have greater accuracy for diagnosis. Post exercise ABPI can be used to differentiate between intermittent claudication due to PAD, from pseudo–claudications (lower limb pain due to vertebral disorders, osteoarthritis, muscular disorders, cysts, and tumors) (Hirsch et al., 2006; Norgren et al., 2007).

Near infra red spectroscopy

Near infra red spectroscopy (NIRS) can be used to detect the peak oxygen saturation in muscles and tissues, as the infra red light easily passes through body membranes and skeletal tissues, and is retained in tissues depending on oxygen content. The oxygen saturation gives different values before and after exercise (due to different tissue oxygen content before and after exercise) (Comerota et al., 2003). Tissue oxygen saturation (StO_2) is significantly lower in PAD cases after exertion as compared to the subjects without PAD, therefore NIRS can be used for vascular disease identification (Comerota et al., 2003).

Duplex ultrasound

The highly specialized 'imaging and angiography techniques' for PAD assessment have greater utility in diagnosing stenosis/obstruction in advanced PAD conditions (grade III onwards) and are therefore of less importance in diagnosis of the

asymptomatic PAD (Hirsch et al., 2006). Duplex ultrasound is a very sensitive ultrasound procedure and can identify PAD with great accuracy although it is a highly operator dependent procedure. It can accurately identify the site of stenosis as well as determine the need for revascularization or surgery. With duplex ultrasound the most reproducible endpoint measurement is peak systolic velocity (PSV) at the site of vascular obstruction. A PSV value ≥ 2 is most predictive and is concordant with ≥ 50 % vascular obstruction (Sensier et al., 1996; Winter-Warnars et al., 1996). Duplex ultrasound has significance as well to identify the sites for bypass and vascular anastomosis procedures (Hirsch et al., 2006).

Computed tomography assisted angiography

Computed tomography assisted angiography (CTA) is a non–invasive, highly informative test for PAD assessment. This test can be used to visualize the arterial tree and the site or sites of stenosis (Hirsch et al., 2006). Technical advancement of this technique is the multi-detector CTA (MD–CTA) with 64 channels and can take as many images simultaneously. MD–CTA is therefore a faster test for PAD diagnosis, less toxic, and more economical. It can also be used safely in individuals with cardiac pacemakers and defibrillators (Hirsch et al., 2006; Norgren et al., 2007). The greatest disadvantage of CTA is that arterial calcification (either due to diabetes or CRF) results in the 'blooming artifact' and can greatly diminish accurate diagnosis of PAD (Norgren et al., 2007; Chan et al., 2010; Meyer et al., 2010).

Magnetic resonance angiography

The next technique 'Magnetic resonance imaging assisted angiography (or magnetic resonance angiography 'MRA')' is a revolutionary technique that allows complete analysis of vascular tree and better visualization of occlusive vascular pathology. MRA, in contrast to CTA can be used safely for analysis of calcified plaques, but MRA is not suitable for patients with cardiac stents, defibrillators, and pacemakers (Hirsch et al., 2006; Norgren et al., 2007). Another edge of MRA over CTA and contrast angiography is the exclusion of ionizing radiation in MRA analysis. The latest modification of MRA, the whole body MRA (WB–MRA) can analyze, excepting coronary vessels, the blood vessels of the entire body. As PAD is associated with atherosclerosis in other arterial beds, WB–MRA procedure can detect the entire body atherosclerotic burden (Nielsen et al., 2009; Nielsen, 2010).

X–ray contrast angiography

X–ray contrast angiography is a 2–D 'invasive' angiography technique and is a "gold standard" diagnostic tool for lower limb PAD. Contrast angiography is most useful in situations where patients have severe phenotypes and when there is indication of revascularization, percutaneous or surgical management. Modified form of contrast angiography, the digital subtraction angiography (DSA) has superior imaging utility as compared to previous non–subtracted variant (Hirsch et al., 2006). To increase the safety profile of this test, contrast mediums like CO_2 and gadolinium (which is an MRA specific contrast) can be used instead of traditionally used (more invasive, nephrotoxic) iodinated contrast medium (Norgren et al., 2007). The numerous tests for PAD assessment gain their significance after their results are compared with DSA, as DSA is still considered the benchmark and gold standard for diagnosis of vascular disorders (Nielsen et al., 2009; Eiberg et al., 2010; Nielsen, 2010; Wang et al., 2010).

1.5 Genetics of Peripheral Artery Disease

An exhaustive list of quantitative trait (QTL) loci, various genes, and gene clusters that associate with peripheral vascular disease moieties are now known. The associations of these loci and genes have been correlated by various association studies. Many of the tabulated disease variants and genetic determinants associated with PAD represent findings in rare familial cases and need validation. The genetic determinants along with genomic positions and MIM IDs are provided in Table 1.1.

1.6 Homocysteine, Homocysteinemia and Vascular Disease

Homocysteine (Hcy) is a non–essential, non protein forming, sulphur ported amino acid that is generated from the amino acid 'methionine', after methionine loses its methyl group (to ultimately produces homocysteine). Homocysteine has gained considerable importance as the new cardiovascular disease biomarker, as well as a causative agent for various vascular disorders, cancers, neurologic and metabolic disorders (Hazra et al., 2009).

1.6.1 Homocysteine theory of atherosclerosis

Homocysteine was first discovered in 1932, when Butz and de Vigneaud isolated, identified and named homocysteine (a di-sulfide compound), after experimentation on methionine (Butz and de Vigneaud, 1932). Later, homocysteine was termed the perpetrator responsible for the third alternate theory of atherosclerosis.

Table 1.1. The tentative list of the gene variants, proteins, and diseases (with MIM ID) related to PAD.

MIM ID	Gene map loci	PAD related diseases/gene variants
107741	19q13.2	Apolipoprotein E (*APOE*)
107680	11q23	Apolipoprotein A
147720	2q14	Interleukin 1 beta (*IL1B*)
147760	2q14	Interleukin 1 alpha (*IL1A*)
60030	9q32-q33	Toll like receptor 4 (*TLR4*)
603031	1q41–q42	Toll like receptor 5 (*TLR5*)
192240	6p12	Vascular endothelial growth factor A (*VEGFA*)
300386	Xp26	CD–40 Ligand (CD–40L)
600835	10q11.1	Chemokine CXC motif ligand 12 (*CXCL12*)
173610	1q23-q25	Selectin P (*SELP*)
147570	12q14	Interferon gamma (IFNγ)
190182	3q22	Transforming growth factor beta receptor II
107269	11pter-p13	CD44 antigen (CD44)
186940	12pter-p12	CD4 Antigen (CD4)
131210	1q23-q25	Selectin E (*SELE*)
163731	12q24.2-q24.31	Nitric oxide synthase 1 (*NOS1*)
147545	2q36	Insulin receptor substrate 1 (*IRS 1*)
601487	3q25	Peroxisome proliferator–activated receptor gamma
606787	1p31	Peripheral arterial occlusive disease 1
162200	17q11.2	Neurofibromatosis 1
232500	3p12	Glycogen storage disease IV
205400	9q22–q31	Tangier disease
161200	9q34.1	Nail patella syndrome: NPS
211900	13q12, 12p13.3	Tumor calcinosis, hyperphosphatemic, familial
264800	17q21.3–q22, 16p13.1	Psuedoxanthoma elasticum (PXE)
151660	1q21.2	Familial partial lipodystrophy type 2
208000	6q22–q23	Generalized arterial calcification of infancy 1

Contd...

MIM ID	Gene map loci	PAD related diseases/gene variants
133100	19p13.3–p13.2	Familial erythrocytosis 1
192430	22q11.2	Velocardiofacial Syndrome
245150	12p13.1-p12.3	Keutel syndrome
269700	11q13	Congenital generalized lipodystrophy 2 (CGL2)
277450	2p12	Vitamin K dependent clotting factors combined deficiency 1 (*VKCFD1*)
227500	13q34	Factor VII deficiency
130160	7q11.2	Elastin: ELN
05441	3q27	Adipose most abundant gene transcript 1 (*APMI*)
173470	17q21.32	Integrin beta 3 (*ITGB3*)
190198	9q34.3	Drosophila homologue of Notch1 (NOTCH1)
6012	20p12	Jagged 1
612052	15q25.1	Smoking as a quantitative trait locus 3 (SQTL3)
188890	(9q22.1,5p15.3,20q13.2-q13.3,19q13.2)	Susceptibility to tobacco addiction
158120	5q31.1	Monocyte differentiation antigen CD14
134830	4q28	Fibrinogen B beta polypeptide
165070	13q12	FMS related tyrosine kinase 1 (FLT1)
602048	Not specified	Ras related botulinum toxin substrate 1 (RAC1)
605747	1p36-p35	LDL receptor adaptor protein 1 (*LDLRAP1*)
600160	9p21	Cyclin dependent kinase inhibitor 2A (*CDKN2A*)
182138	7q11.1–q12	Solute carrier family 6 member 4 (*SLC6A4*)
120180	2q31	Collagen type III alpha 1 (*COL3A1*)
147679	2q14.2	Interleukin 1 receptor antagonist
123260	1q21–q23	C reactive protein, pentraxin related; (*CRP*)
607093	1p36.3	5'10' methylenetetrahydrofolate reductase (*MTHFR*)
236200	21q22.3	Homocystinuria
607314	-	Homocysteinemia

McCully (1969) reported two patients with extremely high homocysteine levels; the affected individuals had arterial lesions in larger, medium, and minute sized arteries, and the arterial lesions affected numerous arterial beds. One patient was only two months old and other patient was eight years old, both patients had different enzyme defects but the underlying causative molecule was homocysteine in both the patients (McCully, 1969). The patient characteristics and the implicated causative agent changed the previous concept, and homocysteine theory of atherosclerosis was finally postulated.

In an experimental series, rabbits developed aortic and arterial plaques, emboli, and tissue infarctions after they were injected or fed homocysteine and methionine (McCully and Wilson, 1975). Homocysteine has been extensively applied to explain the pathophysiological aspects of atherosclerosis. The Hcy theory gained more support, after homocysteine and its derivatives were confirmed as autonomous causative agents in large cohorts of coronary artery disease, stroke, and artery disease patients (Wilcken and Wilcken, 1976; Refsum et al., 1998).

1.6.2 Homocysteine metabolism

Homocysteine is involved in two metabolic pathways, the reversible transmethylation (remethylation) pathway and the irreversible transsulfuration pathway. In the transmethylation pathway homocysteine flips over with methionine, whereas in transsulfuration pathway homocysteine is irreversibly converted to cystathionine (Welch and Loscalzo, 1998). Methionine can be obtained from diet, from the body proteins, as well as from homocysteine and betaine (Finkelstein, 1998; Welch and Loscalzo, 1998). In transmethylation pathway, homocysteine acquires a methyl group from 'N^5 methyl tetrahydrofolate' and is converted to methionine, through a reaction catalyzed by vitamin B12 dependent 'methionine synthase' (*MTR*) enzyme. The methyl donor for the reaction; N^5 methyl tetrahydrofolate is formed from N^5, N^{10} methylene tetrahydrofolate through another enzyme, 'N^5, N^{10} methylene tetrahydrofolate reductase' (*MTHFR*). The methionine in turn can lead either to formation of body proteins, or it can be demethylated to form homocysteine through S adenosyl methionine (SAM, also called AdoMet) and S adenosyl homocysteine (SAH, also called AdoHcy) (Jakubowski, 2008; Trabetti, 2008). The only recognized source of homocysteine in the human body is S adenosyl homocysteine (Trabetti, 2008).

When methionine is in excess or when the body requires cysteine, then *MTR* is inhibited and homocysteine turns to the transsulfuration pathway, where homocysteine is irreversibly converted to cystathionine through vitamin B6 dependent 'cystathionine beta synthase' (*CBS*) enzyme (Welch and Loscalzo, 1998; Trabetti, 2008). Cystathionine is converted by another vitamin B6 dependant enzyme γ cystathionase (cystathionine γ lyase) to form cysteine and α–ketobutyrate (Trabetti, 2008). Hcy can also be obtained when 'homocysteine thiolactone' is converted to Hcy through the thiolactinase action of the vital enzyme termed paraoxonase 1 (*PON1*) (Perla-Kajan and Jakubowski, 2010). The cycles of homocysteine are shown in Fig. 1.3.

1.6.3 Factors modulating homocysteine levels

Many factors, physiological as well as pathological, modulate the blood homocysteine levels. Age, gender, and nutrition are some important physiologic modalities that control the blood homocysteine levels. Normally, men have higher homocysteine levels than women, and individuals have higher levels on empty stomach as compared to the levels after meals. Normal homocysteine levels also change with age, the average level is 10.8 µmol/L during middle age group (40–45 yrs), and the level increases to 12.8 µmol/L during old age (65–67 yrs) (Nurk et al., 2001). Hyperhomocysteinemia, which is the elevated Hcy levels beyond the cut off range, is categorized into three subtypes depending on the blood homocysteine concentration, these include: moderate hyperhomocysteinemia (between 12–30 µmol/L); intermediate (31–100 µmol/L); and severe (>100 µmol/L) (Weiss et al., 2002). Most of the homocysteine in plasma is transported in a 'protein bound' form, as disulfide of 'homocysteine–cysteine' or as 'homocystine' (disulfide of homocysteine–homocysteine), the remaining minute amount is present as free or reduced form (Beltowski, 2005).

1.6.4 Genetic causes/inborn metabolism errors

The case study reported by McCully (1969), was the classical example of the inborn errors of homocysteine metabolism (severe hyperhomocysteinemia–homocystinuria). One patient had *CBS* enzyme deficiency and the other patient suffered from the *MTR* enzyme deficiency (McCully, 1969). Both the patients had multivessel disease, generalized endothelial dysfunction and generalized atherosclerotic disease. In addition to the above mentioned enzyme defects,

homocystinuria can result secondary to *MTHFR* enzyme deficiency as well (Kanwar et al., 1976; Welch and Loscalzo, 1998). The inborn errors of homocysteine metabolism have autosomal recessive mode of inheritance, with affected individuals having very high levels of plasma homocysteine (>100 μmol/L) and detectable levels in urine samples (Welch and Loscalzo, 1998).

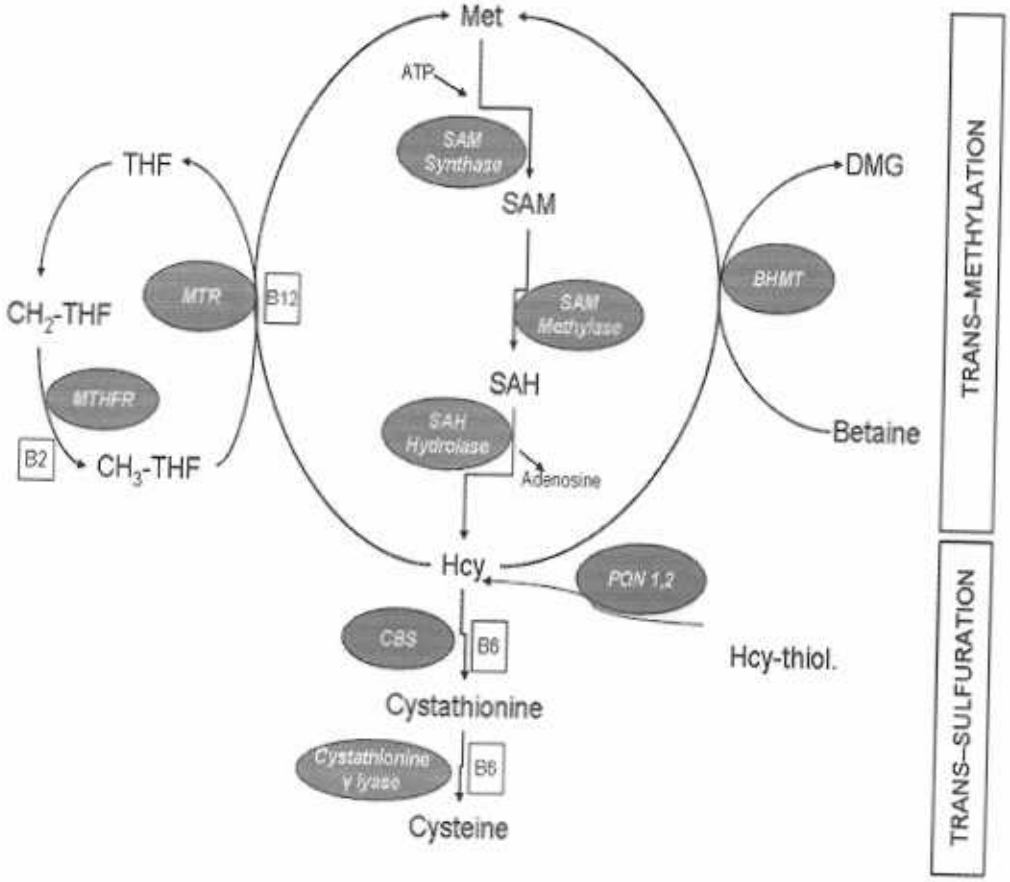

Fig. 1.3. The important molecules and enzymes in homocysteine pathway. THF (tetra hydrofolate), CH_2–THF (N^5, N^{10} Methylene–tetrahydrofolate), CH_3–THF (N^5, Methyl–tetrahydrofolate), Met (Methionine), Hcy (Homocysteine), SAM (S Adenosyl methionine), SAH (S Adenosyl homocysteine), Hcy–thiol (homocysteine thiolactone). The enzymes modulating the transmethylation homocysteine pathway include Vit. B2 dependant *MTHFR* (N^5, N^{10} Methylene tetrahydrofolate reductase), Vit. B12 dependent *MTR* (Methionine synthase), *BHMT* (Betaine homocysteine methyltransferase) and SAM Methylase (including SAM methyltransferase and nicotinamide N methyltransferase). The enzymes modulating transsulfuration pathway includes Vit. B6 dependent *CBS* (Cystathionine β synthase) and cystathionine γ lyase. *MTHFR* converts CH_2–THF to CH_3–THF, Met converts to Hcy through two intermediates, SAM and SAH. Conversion of Hcy to Met is catalyzed by *MTR* and *BHMT*. Formation of cystathionine and cysteine is catalyzed by Vit. B6 dependent enzymes *CBS* and cystathionine γ lyase.

Severe hyperhomocysteinemia is associated with several systemic findings, including the eye abnormalities (ectopia lentis or displaced optical lens), skeletal abnormalities (deformity and/or osteoporosis), vascular disorders (premature athero–thrombotic disease), and mental retardation. The homozygous mutations affecting *MTHFR* and *CBS* enzymes are associated with severe hyperhomocysteinemia, with adverse cardiovascular disease before individuals are 20 years of age and mortality occurs in affected individuals by the age of 30 (Mudd et al., 1985; Welch and Loscalzo, 1998).

The individuals with heterozygous enzyme deficiency and point mutations in the homocysteine pathway genes exhibit less remarkable disease and mild to intermediate hyperhomocysteinemia. However these individuals still are at an increased risk to develop cardiovascular disease and complications (Welch and Loscalzo, 1998).

1.6.5 Significance of Vitamins

Several B vitamins act as cofactors for enzymes in the homocysteine pathway, therefore the vitamin B category is of considerable importance in hyperhomocysteinemia. As vitamin B6 is a necessary cofactor for CBS and cystathionine γ lyase, it is therefore required for activity of the enzymes in the transsulfuration pathway. Reduced intake or deficiency of vitamin B6 are related to higher homocysteine levels and enhanced risk of diseases like CAD (Morris et al., 2008).

Folate (or vitamin B9) is an integral constituent of homocysteine remethylation pathway; diminished folate levels lead to inadequate formation of methyltetrahydrofolate (the methyl donor for methionine formation). Inadequate intake or deficiency of folate is related to hyperhomocysteinemia, especially in the context of genetic abnormalities. High folate intake reduces the disease status and reduces the homocysteine levels in affected individuals (MacMahon et al., 2000; de Bree et al., 2001). Higher blood levels of folic acid and vitamin B6 or higher dietary intake of folic acid and vitamin B6 diminish the homocysteine levels, and result in a decrease in incidence of cardiovascular disorders (Selhub et al., 2000).

Vitamins B12 and B2 are the cofactors for MTR and MTHFR enzymes respectively. Both vitamins catalyze different reactions of homocysteine transmethylation cycle and modulate the 'methylation' of homocysteine to form

methionine. Lower blood levels and lower dietary intake of folic acid, vitamins B2, B6 and B12 individually, are associated with higher homocysteine levels (Chen et al., 2005). Deficiency of vitamin B12 and folate synergistically elevate homocysteine, whereas increased dietary intake of the B vitamins decreases the elevated homocysteine concentration (MacMahon et al., 2000; Chen et al., 2005).

1.6.6 Hyperhomocysteinemia, Mechanism of Action and Atherosclerosis

1.6.6.1 Homocysteine and protein homocysteinylation

Hyperhomocysteinemia results either due to high protein or methionine intake, due to nutritional deficiency of essential vitamin cofactors, or due to genetic abnormalities in the enzymes of homocysteine pathway. Excess homocysteine is toxic to the body, and causes atherosclerosis and vascular disease, however there is ongoing debate as to the mechanism of harmful effects. An important proposed mechanism is the formation of Hcy-thiolactone (Hcy-thiol) and resultant protein *N*–homocysteinylation (Beltowski, 2005; Jakubowski, 2006). In hyperhomocysteinemia, homocysteine (instead of methionine) incorporates with Met-RS, during translation. The Met-RS bound homocysteine (homocysteinyl adenate) leads to formation of the Hcy-thiol. Hcy-thiol reacts with amino groups of lysine residues in the proteins, and results in *N*–homocysteinylation of the proteins through amide bonding (Jakubowski, 2006).

The *N*–homocysteinylated protein (*N*–Hcy protein) results in damaging consequences in a number of ways. *N*–Hcy proteins have aberrant protein folding tendency, and undergo spontaneous denaturation and aggregation ultimately leading to cell death (Jakubowski, 2006; Jakubowski, 2008). Homocysteinylation can result in protein inactivation; for example *PON1* is inactivated, with resultant elevated Hcy–thiol levels (and therefore amplification of protein homocysteinylation). Homocysteinylation not only inactivates albumin and hemoglobin, but also increases their destruction by oxidation. *N*–homocysteinylation of fibrinogen results in the formation of blood clots with higher resistance to clot lysis. The homocysteinylation of fibrin also involves those lysine residues where tissue plasminogen activator (tPA) and plasmin bind, and this may explain resistant clots, and increased risk for CAD in hyperhomocysteinemic individuals (Jakubowski, 2008). Protein homocysteinylation also severely affects the body by autoimmune mechanism. Autoantibodies (of IgG variety) directed against Hcy–lysine epitopes accumulate in the body particularly in

patients with CAD and cerebrovascular disease. The Hcy–thiol mediated damage therefore results in reduced cellular viability, activated proinflammatory processes, autoimmune mechanisms as well as enhanced thrombosis (Jakubowski, 2006; Jakubowski, 2008).

1.6.6.2 Homocysteine and clotting cascade

Homocysteine is associated with higher levels of circulating tissue factor and it also induces tissue factor release from the endothelial cells (Fryer et al., 1993; Marcucci et al., 2000). Tissue factor is a strong activator of coagulation factor VII. Activated factor VII then results in activation of the downstream factor X and along with activated factor V, activation of factor II (prothrombin) to thrombin. Homocysteine also directly results in the activation of factor V through an endothelial activator (Rodgers and Kane, 1986), favoring enhanced thrombin formation. Thrombin also exerts positive feedback by enhancing activation of factors V and VII. Elevated thrombin levels result in the cleavage of fibrinogen to fibrin. Fibrin is the most critical protein for formation of blood clots, thrombosis, and resultant tissue infarction; hyperhomocysteinemia therefore activates coagulation proteins and favors thrombogenesis (Rodgers and Kane, 1986; Masuda et al., 2008).

Homocysteine also favors thrombogenesis by inhibiting the antithrombotic agents and by inhibiting fibrinolysis. Hyperhomocysteinemia reduces anticoagulation by inhibiting potent anticoagulant, 'antithrombin III' (Palareti et al., 1986). Thrombin normally activates fibrin and favors thrombosis and clotting. Interestingly however, through an alternate mechanism, thrombin can modulate anticoagulation as well. This alternate effect is accomplished via activation of thrombomodulin and protein C. Thrombin on binding with thrombomodulin, leads to conversion of protein C to activated protein C (APC) which inhibits coagulant protein V and VIII, thereby disrupting the coagulation and clot formation (Rodgers and Conn, 1990; Lentz and Sadler, 1991; Hayashi et al., 1992). Homocysteine favors thrombosis and clotting as it also inhibits both thrombomodulin and APC (Rodgers and Conn, 1990; Lentz and Sadler, 1991; Hayashi et al., 1992). The Hcy-thiol modified fibrin clots are relatively firm with reduced permeability, which decreases the prospect of fibrinolysis by plasmin and such clots are also resistant to lysis by aspirin (Undas et al., 2006). Plasminogen and tissue plasminogen activator bind to fibrinogen through its lysine residues, and since Hcy–thiol also targets lysine residues of fibrin; fibrinolysis is

inhibited (Jakubowski, 2006). Furthermore, homocysteine reduces lipoprotein A (Lp A), reduced Lp A has very high affinity for fibrin and therefore inhibits fibrinolysis by competitive inhibition of fibrin and plasmin interaction (Harpel et al., 1992). Hcy–thiol induced fibrinogen modification results in enhanced thrombosis and vascular damage (Undas et al., 2006; Jakubowski, 2008).

1.6.6.3 Homocysteine and LDL

Hyperhomocysteinemia causes homocysteinylation of LDL to yield modified 'Hcy–LDL'. Homocysteine at the same time can undergo autooxidation resulting in the production of enhanced ROS; including hydrogen peroxide and superoxide; lipid oxidation, and formation of 'ox–LDL'. Such modified LDL molecules (Hcy–LDL/ox–LDL) are readily incorporated into macrophages favoring formation of foam cells (which marks the initiation and progression of atherosclerosis) (Perna et al., 2003; Ferretti et al., 2004). In addition to ROS induced indirect damage, Hcy–LDL directly induces cell damage by peroxidation of lipids and through oxidation mediated damage to endothelial cells (Welch and Loscalzo, 1998; Perna et al., 2003; Ferretti et al., 2004). Modified LDL molecules mediate direct and indirect damage resulting in atherosclerosis, plaque destabilization, and the formation of foam cells. Homocysteine is also a source of circulating tissue factor providing an additional mechanism for plaque instability (Marcucci et al., 2000).

1.6.6.4 Production and modulation of NO

One component of Hcy mediated endothelial dysfunction is dependent on nitric oxide (NO), a derivative of amino acid 'L–arginine'. L–arginine administration (oral or parentral) is linked to the vasodilatory responses and decline in endothelial dysfunction in patients with hypercholesterolemia, hypertension, and patients with atheromatous coronary vessels (Creager et al., 1992; Lekakis et al., 2002; Siasos et al., 2007). Three different enzymes are correlated with NO synthesis and are termed NOS (the nitric oxide synthases). These are named according to the tissues that express them as: neuronal NOS or nNOS (NOS1), inducible NOS or iNOS (NOS2), and endothelial NOS or eNOS (NOS3). NOS1 is expressed in the neuronal tissue, NOS2 is induced in a wide array of cells and tissues, while NOS3 is expressed by the endothelial cells (Alderton et al., 2001).

NOS enzymes have differences in their effects and in their downstream effectors. Cells rich in NOS can produce not only NO but also other nitrogen reactive

species such as nitroxyl compounds (NO^-/HNO) and peroxynitrite compounds ($ONOO^-$/ONOOH) (Alderton et al., 2001). The stimulation of eNOS results in the spontaneous production of NO and inhibition of atherosclerosis. In contrast, iNOS on stimulation releases peroxynitrite resulting into enhanced atherosclerosis (Alderton et al., 2001; Loscalzo, 2003). The atherogenic potential of iNOS activation and expression is revealed by the fact that $apoE^{-/-}$/$iNOS^{-/-}$ animal models have attenuated atherosclerosis (Loscalzo, 2003). nNOS/iNOS on activation support the production of nitroxyl compounds (instead of NO), and the nNOS/iNOS enzymes synthesize NO only in the presence of another enzyme the superoxide dismutase (SOD). SOD mediates rapid oxidation of the NO^- generated by NOS to NO, and it also removes the 'superoxide' that would otherwise convert the available NO to peroxynitrite. This means that SOD inhibits the atherogenic tendencies of NOS (Murphy and Sies, 1991; Hobbs et al., 1994; Schmidt et al., 1996). Hypercholesterolemia is associated with increase in endogenous production of the enzyme 'asymmetric dimethyl arginine' (ADMA), which is a potent inhibitor of NOS and of NO production, providing an additional mechanism for vascular disorders (Leiper and Vallance, 1999).

1.6.6.5 Homocysteine mediated NO metabolism and endothelial dysfunction

Homocysteine can directly activate NF–κB and can additionally induce cytokine based activation of NF–κB. NF–κB in turn modulates elevated iNOS mRNA level, and elevated iNOS enzyme production and activation. Elevated production of iNOS by NF–κB results in oxidative trauma, severe inflammation and atherogenesis (Welch et al., 1998). Hcy also inactivates enzymes that have antioxidant properties like SOD and glutathione peroxidase (Weiss et al., 2001). These reactions favor formation of ROS and peroxynitrites, and cause endothelial dysfunction (Weiss et al., 2001; Weiss, 2005). L–arginine is also a substrate for creatine production. Creatine is formed after L–arginine catalyzed formation of guanidino–acetate, the latter compound then amplifies homocysteine generation by catalyzing conversion of S adenosyl methionine (SAM) to S adenosyl homocysteine (SAH) (Loscalzo, 2003). This hypothesis is strengthened as L–arginine administration results in increased production of creatine/guanidino–acetate/Hcy; and in enhanced 'Hcy mediated release' of ROS and markers of inflammation/vascular disease (Weiss et al., 2001; Loscalzo, 2003).

eNOS–induced NO acts on endothelium and depresses cellular migration (resulting in dampening of immune responses), whereas peroxynitrite results in ancilliary cellular migration by altering the endothelial gap junctions (Goligorsky, 2000). Homocysteine, decreased intermediates of oxygen, and asymmetric dimethyl–arginine (ADMA) are potent inhibitors of eNOS mediated NO production and favor peroxynitrite generation from other NOS enzymes (Goligorsky, 2000). Homocysteine also inhibits dimethyl–arginine dimethyl–aminohydrolase enzyme (DDAH), a potent inhibitor of ADMA. Hcy mediated inhibition of DDAH results in higher ADMA levels, and associated decreased production of NO and higher peroxynitrite production (Stuhlinger et al., 2001). Hcy-thiol by generating peroxynitrite results in the modification of LDL molecules, in the peroxidation of lipids, and production of the 'ox-LDL'. Ox-LDL/modified LDL in turn inhibit the expression and activity of eNOS and stimulate extended production of detrimental superoxide and ROS by eNOS itself (Rosenkranz-Weiss et al., 1994; Pritchard et al., 1995; Leeuwenburgh et al., 1997). All these mechanisms counter the protection and the anti atherogenic effects mediated by NO, and shift to the situation where atherosclerosis, thrombosis and endothelial dysfunction develop and result in vascular disease (Rosenkranz-Weiss et al., 1994; Pritchard et al., 1995; Leeuwenburgh et al., 1997).

1.7 Homocysteine and its putative receptor

N–methyl–D–aspartate (NMDA) receptor is also implicated with mediation of downstream effects of Hcy. The Hcy–NMDA receptor complex leads to ROS mediated vascular damage, cell death, enhanced cellular calcium entry, and disrupted endothelial function. Homocysteine causes vascular disease and plaque instability by autoimmune and ox–LDL mediated mechanisms, and additionally by inhibiting cellular ATP levels through the NMDA mediated calcium entry, loss of cellular respiration, and resultant cellular apoptosis (McCully, 2009). Recent reports indicate that homocysteine is the ligand for other receptors in addition to NMDA. These include gamma amino butyric acid (GABA) and PPAR. Homocysteine acts on and represses these receptors to result in disrupted endothelium and MMP–mediated destruction of vascular matrix. The therapeutic agonists of the GABA/NMDA/PPAR receptors reverse the adverse manifestations of elevated Hcy levels, corroborating the significance of these receptors in homocysteine driven cardiovascular disorder (Steed and Tyagi, 2010).

AIMS / HYPOTHESIS OF THE STUDY

Aims and objectives of the Study conducted in USA

The aim of the study was to isolate peripheral blood mononuclear cells for determination of transcriptomic signatures, to ascertain the gene expression differences and genetic variants associated with peripheral vessel disease, by microarray technology.

Aims and objectives of the Study conducted in Pakistan

The aim of the study was to identify the allele variants in the homocysteine pathway associated with CAD, and to determine gene-gene interactions or epistasis, using tetra primer allele refractory mutation system (ARMS) polymerase chain reaction (PCR).

HYPOTHESIS OF THE STUDY

Hypothesis of Study conducted in USA

The hypothesis was that RNA isolated from PBMC can be employed for hybridization to microarrays and can provide a list of differentially expressed genes between PAD cases and controls. It was also hypothesized that the results can be validated by subsequent microarray study and analogous gene variants can serve as biomarkers for PAD diagnosis and disease association.

Hypothesis of Study conducted in Pakistan

The hypothesis for this study was that tetra primer ARMS PCR can effectively and reliably detect the polymorphisms studied. Additional hypothesis was that a subset of the polymorphisms might be positively associated with coronary artery disease and might individually or through gene – gene interactions, predict risk for CAD.

SECTION I
CHAPTER 2: GENE EXPRESSION ANALYSIS FOR PERIPHERAL ARTERY DISEASE

SUMMARY

Circulating mononuclear cells are in contact with the blood vessel wall and can serve as reporters of vascular pathology. Gene expression analysis was carried out in patients with peripheral arterial disease (PAD) and control subjects, through peripheral blood mononuclear cells (PBMC). This was a two-step microarray study: with a "discovery set" comprising of nine PAD patients and nine control subjects (without PAD), and a "validation set" comprising of ten patients and nine subjects without PAD. The diagnosis for PAD was based on an ankle brachial index (ABI), individuals with ABI ≤ 0.9 were diagnosed as cases while individuals with ABI > 1.0 were diagnosed as control subjects. PBMC were isolated from whole blood using the density gradient centrifugation. Total RNA extracted from samples was hybridized to Affymetrix HG U133 Plus 2.0 microarrays. Genes having a fold change ≥ 1.5 were considered differentially expressed. The resultant gene list was subjected to unpaired Mann-Whitney test ($P<0.05$) and further correction for multiple testing by Benjamini Hochberg false discovery rate.

At fold change ≥ 1.5, the differentially expressed genes, simultaneously in both the sets consisted of a total of 30 genes/transcripts with 29 genes and one non annotated transcript. Of total 29 genes, 22 genes had higher expression (were upregulated) and 7 genes had lower expression (were downregulated) respectively, in cases as compared to the controls. Functional analysis of the differentially expressed genes, through gene ontology, revealed that these genes regulate a wide array of important metabolic and molecular functions. The genes and their functions include *C5orf41* (modulation of gene transcription); *FCAR* (modulation of immunity); *CFLAR* (cell death); *KLF6, DUSP1,* and *IL8* (activation of MAPK signalling cascade, activation of cytokines, chemokines, and immune responses); *NAMPT* (insulin metabolism and insulin sensitivity); *TRAF3IP3* (cell growth); and *CFH* (modulation of complement/coagulation cascade). Gene expression profiling of PBMC identified a set of 30 differentially expressed genes/transcripts. These differentially expressed genes modulate inflammatory pathways including gene transcription, immunity, cellular signalling pathway, and cell death or apoptosis. The genes and pathways highlighted by the present study may add to the insights into the genetic architecture, and mechanisms associated with progression of PAD.

INTRODUCTION

The studies that scrutinized microarray analysis of peripheral blood mononuclear cells (PBMC) and peripheral blood cells in healthy asymptomatic individuals revealed individual genetic variation, differential gene expression, and highlighted the use of these cells for genetic analyses (Whitney et al., 2003; Eady et al., 2005). Previously, circulating peripheral blood cells have been studied in relation to various cardiovascular disorders. Microarray analysis of coronary and peripheral vessels, and human coronary endothelial cells, has provided new insights into the genes and pathways involved in coronary artery disease and peripheral artery disease (Blaschke et al., 2004; Wyler von Ballmoos et al., 2006; Dahl et al., 2007; Evans et al., 2008; Fu et al., 2008). The significance of PBMC is illustrated by the finding that PBMC have been effectively used for differential genetic expression and pathway analysis in CVD (Aziz et al., 2007), elevated blood pressure (Timofeeva et al., 2006), CAD (Wingrove et al., 2008; Rosenberg et al., 2010) and ischemic stroke (Waehre et al., 2004; Patino et al., 2005; Wingrove et al., 2008; Meier et al., 2009; Stamova et al., 2010).

Peripheral artery disease (PAD) serves as a model of diffuse atherosclerotic vascular disease as it is frequently concomitant with atherosclerosis of coronary and cerebral vessels. Risk factors for atherosclerosis include increasing age, male gender, physical inactivity, high BMI, high blood pressure, elevated cholesterol, diabetes, and smoking, as well as novel risk factors such as elevated homocysteine levels (hyperhomocysteinemia), C–reactive protein, and fibrinogen. Of the conventional risk factors, diabetes and cigarette smoking are more strongly associated with PAD as compared to coronary artery disease (CAD) (Hirsch et al., 2006; Rosamond et al., 2007). The phenotypes, findings, and progression of these multigenic distinct entities are diverse, suggesting that differences exist in their causative pathways.

The genes and the transcript variants related to PAD and responsible for the vascular disease phenotypes have been elusive and as yet are unidentified. It is expected that PAD affects 8 – 12 million adults in United States, and is associated with severe complications, morbidity, and mortality (Hirsch et al., 2001). With addition of prevalence of PAD in European population, the combined disease prevalence rises to as high as 27 million (Belch et al., 2003). Individuals with PAD are at increased risk to develop MI and stroke, with the mortality factor increased two to three fold in PAD

cases compared to those without the disease (Dormandy, 1995). PAD is the surrogate for diffuse atherosclerosis and since the peripheral blood cells are in contact with diseased tissue, these can be used for gaining insight into the mechanisms and genetics of disease processes and disease progression (Hansson, 2005; Ardigo et al., 2007).

2.1 Genetic Studies for identification of PAD

There is an enormous list of disease conditions, loci, and genes associated with the peripheral artery disease (Table 1.1). Many of the designated loci and genes have not been validated. Genomic studies can be designed to merit greater emphasis on the validation of the associated loci. Two relatively new technical advances have been added to genetic studies, the 'gene expression analysis' and 'the genome wide association studies' (GWAS). These techniques can be reliably used for re-evaluation of the tentative genetic information. GWAS has tremendous potential to discern the QTL across the whole genome and the expression arrays can detect expression signal differences between cases and controls. Both techniques gain utility as the data generated have also been validated by RT–PCR or subsequent microarrays (Bull et al., 2004; McPherson et al., 2007; Samani et al., 2007; Welcome Trust Case Control Consortium, 2007; Kooperberg et al., 2010; Risbano et al., 2010).

2.1.1 Expression studies

The gene expression studies for PAD have mostly focused on transcriptome analyses of the limb vessels and have identified genetic variants associated with the peripheral arterial disease (Evans et al., 2008; Fu et al., 2008). The expression studies indicate a number of genes to be associated with the carotid artery disease and abdominal aortic aneurysm (Dahl et al., 2007; Rossi et al., 2010). The gene expression studies have used circulating cells, not for the peripheral arterial occlusive disease, but for analyses of alternate phenotypes such as CAD, Kawasaki disease, arterial and pulmonary hypertension (Bull et al., 2004; Timofeeva et al., 2006; Furuno et al., 2007; Wingrove et al., 2008; Meier et al., 2009; Risbano et al., 2010).

2.1.2 Genome–wide association studies

The genome–wide association studies (GWAS) have been made feasible through the discovery of millions of single nucleotide polymorphisms (SNPs) following completion of the Human Genome Project (Lander et al., 2001; Venter et

al., 2001). It is estimated that there exist 10 million SNPs in the human genome and these constitute the individual variations in the human population (Lai, 2001; The International HapMap Project, 2003). The estimation of linkage disequilibrium (LD) of the human genome, through the HapMap Project (The International HapMap Project, 2003; International HapMap Consortium, 2005), and the newer, cost effective, human SNP genotyping microarrays and analytical programs have made possible the analyses of causative loci and SNPs associated with the disease status (International HapMap Consortium, 2005; Syvanen, 2005; Hu et al., 2006; Xiao et al., 2007; Beaudet and Belmont, 2008).

The initial GWAS for PAD were undertaken in 2008 and two SNPs rs10757278 (also associated with CAD, and intracranial aneurysm) and rs1051730 (also associated with smoking and lung cancer) were found associated with abdominal aortic aneurysms/PAD (Helgadottir et al., 2008; Thorgeirsson et al., 2008). The former SNP (rs10757278), at locus 9p21 highlighted gene variants *CDKN2A* and *CDKN2B* with PAD; whereas the latter SNP (rs1051730) at locus 15q24 associated *CHRNA3* gene with PAD (Helgadottir et al., 2008; Thorgeirsson et al., 2008). In a GWAS for PAD in Japanese PAD cases four SNPs at chromosomal location 3p22.3 and involving *OSBPL10* gene were found strongly associated with PAD (Koriyama et al., 2010). GWAS through 'analysis of electronic medical record' has recently been applied for red blood cell (RBC) traits in PAD patients and has revealed four genomic loci (including three previously associated loci) as strongly associated with these traits (Kullo et al., 2010).

2.2 Microarray Based Gene Expression Platforms

Microarrays are latest genomic technologies that have the capacity to analyze the entire human genome and gene expression simultaneously. Microarray technologies have been applied for detection of the whole human genetic differences between cases and controls; and for the detection of genetic differences in cells and tissues in response to medications (Archacki and Wang, 2004; Bemmo et al., 2010; Lu et al., 2010; Peng et al., 2010; Shack, 2011).

2.2.1 Affymetrix microarray system

The human expression analysis microarrays by Affymetrix® (http://www.affymetrix.com) include the "3' IVT (in vitro transcription) based expression GeneChips" and the "Whole Transcription Expression GeneChips". The

3'IVT expression arrays include the HG U133 series (HG U133 Plus 2.0, HG U133A 2.0, HG U133 set–A/B arrays, and HG focus); and the HG U95 series (HG U95Av2, HG U95B, HG U95C, HG U95D, and HG U95E). Of these HG U133 Plus 2.0 GeneChip® targets the maximum number of genes/transcripts. Affymetrix HG U133 Plus 2.0 arrays have 54675 probesets to cover the entire annotated and a subset of the unannotated genes (http://www.affymetrix.com), (Higo et al., 2006; Bjork and Kafadar, 2007). The annotations for HG_U133 Plus 2.0 microarrays are derived from multiple sources, the NCBI resources include the: GeneBank database (http://www.ncbi.nlm.nih.gov/genbank); the dbEST NCBI database (http://www.ncbi.nlm.nih.gov/projects/dbEST); UniGene Build 131 and Build 159, (http://www.ncbi.nlm.nih.gov/unigene); and NCBI human genome assembly Build 31. The clusters thus obtained have been refined and confirmed by the two non NCBI sources: the Washington University EST trace repository, and 2001 release of University of California, Santa Cruz human genome database (www.genome.ucsc.edu/cgi-bin/hgGateway).

The whole transcription expression arrays include 'Human Gene 1.0 ST arrays' and 'Human Exon 1.0 ST arrays'. The Human Exon 1.0 ST arrays cover more probes per exon, and more probes per gene as compared to the Human Gene 1.0 ST arrays. It also targets more genes (annotated as well as un–annotated) as compared to Gene 1.0 ST, has better and enhanced coverage of gene expression, and is therefore considered highly sensitive for expression analysis of genes with alternately inititiated, terminated, and spliced gene variants (http://www.affymetrix.com), (Bemmo et al., 2008; Ha et al., 2009). Human Gene 1.0 ST microarray has probes for coverage of the entire length of the, specifically, well annotated genes (28,869 genes), and like the Human Exon 1.0 ST arrays covers the alternate spliced transcripts of the studied genes (http://www.affymetrix.com), (Ha et al., 2009; Kabakchiev et al., 2010).

The whole transcript expression arrays are used for gene expression analyses, and to study the effects of various isoforms and alternate spliced gene variants on disease status as well as on response to medication (Gardina et al., 2006; Kohli et al., 2009; Wiederholt et al., 2009; Bemmo et al., 2010; Hindle et al., 2010; Kabakchiev et al., 2010; Saghir et al., 2010). The 3'IVT expression arrays are used when association of global gene expression (annotated as well as un–annotated genes), is required to be ascertained with the disease status or with effects of medication (Zhang et al., 2004; Cardinal et al., 2007; Lu et al., 2010; Schwientek et al., 2010; Yang et al., 2010a; Lee

et al., 2011). Despite the individual differences between the study designs, there are highly comparable results of expression analysis between the HG U133 Plus 2.0 arrays with the whole transcript Human Gene 1.0 ST and Human Exon 1.0 ST arrays. Highly analogous results between these arrays accentuate their utility for expression analyses (Okoniewski et al., 2007; Bemmo et al., 2008; Pradervand et al., 2008; Linton et al., 2009).

2.2.2 Agilent microarray system

Agilent® provides a vast array of human gene expression microarrays and human exon arrays. The Agilent human gene expression arrays include three array types: (i) SurePrint G3 Human Gene Expression 8x60 K arrays; (ii) Human Gene Expression 4x44 K v2; and (iii) Whole Genome Microarrays 4x44 K. The SuperPrint G3 arrays have 27,958 probesets for detection of Entrez genes and 7,419 probes for detecting lincRNAs (long, interginic, non coding RNAs). Human Gene Expression 4x44 K arrays query 34,127 Entrez genes, and the Whole Genome 4x44 K Microarrays have probesets for coverage of 43,376 genes (http://www.genomics.agilent.com). The human exon arrays (for gene expression and alternate spliced variants analyses) include two array subtypes: (i) SurePrint G3 Human Exon 2x400 K arrays; and (ii) SurePrint G3 Human Exon 4x180 K arrays. The former microarrays target 27,696 genes and have additionally 233,164 probes for exons, whereas the latter exon arrays have probesets targeting 20,411 genes and 174,458 probes for exonic sequences (http://www.genomics.agilent.com), for the coverage of alternate spliced transcripts.

The Agilent arrays are single color as well as dual color arrays, whereas Affymetrix and Illumina arrays are single color arrays. The control or patient samples are alternately stained, with Cy3 or Cy5 (fluorescent water soluble cyanine dyes) and vice versa. The Agilent expression microarrays have been extensively used for gene expression analyses and for analyses of differentially regulated genes in context of the disease status and response to medication (Verstraelen et al., 2009; Cai et al., 2010; Gruber and Holtz, 2010; Muggerud et al., 2010; Riis et al., 2010; Khoo et al., 2011).

2.2.3 Illumina microarray system

For the gene expression analysis, the Illumina genome scale Beadarrays® synthesized by Illumina include the Sentry® HumanWG-6, HumanWG-6 v2, HumanWG-6 v3, HumanRef-8, HumanRef-8 v2, HumanRef-8 v3, HumanHt-12 v3,

and HumanHt-12 v4. The numbers 6, 8, and 12 signify the number of samples that can be assayed simultaneously by the individual Beadchip (http://www.illumina.com). Each of the Beadchip has individual number of probesets for the gene expression: Human-6 Expression Beadchips have in excess of 46,000 probes (for each one of the 6 samples in a single Beadchip); Human-6 v2 Beadchip has excess of 48,000 probes; HumanRef-8 Expression Beadchip has coverage for the genome with more than 24,000 probes (for each of the 8 samples in the Beadarray); HumanRef-8 v2 targets 22,000 probes for the genes and the alternate spliced gene variants; HumanHt-12 v3 and v4 Beadchips can analyze 12 samples on a single Beadchip with 48,000 and 47,000 probesets, respectively (http://www.illumina.com). The HumanWG-6 v3, HumanRef-8 v3, HumanHt-12 v3, and HumanHt-12 v4 are relatively new and up to date additions in the Illumina microarrays. HumanWG-6 v3 and HumanRef-8 v3 have been redesigned from their respective v2.0 counterparts with additional sequences from UniGene database (http://www.ncbi.nlm.nih.gov/unigene), as well as from the dbEST database (http://www.ncbi.nlm.nih.gov/projects/dbEST) for more comprehensive genetic coverage.

Illumina Beadarrays® have utility in global gene expression analysis, delineating the genetic differences between cases and controls, and uncovering the effects of medications on genetic expression (Rozanov et al., 2008; LaBonte et al., 2009; Mhawech-Fauceglia et al., 2010; Niedoszytko et al., 2011; Rudkowska et al., 2011). The high concordance between the Illumina Beadarrays® and Affymetrix HG U133 Plus 2.0 arrays bear significance to and highlight the comparable gene expression analysis by these disparate microarray platforms (Barnes et al., 2005; Pradervand et al., 2008; Du et al., 2009).

The first objective of the present study was to establish the feasibility of using PBMC for the gene expression analysis in 'PAD'. The second objective was to ascertain the differentially expressed genetic variants in PAD cases and controls, for better insights into the pathophysiological aspects of vascular disease of the lower extremities.

MATERIALS AND METHODS

2.3.1 Study design

The present is a dual step expression study, with the first group termed "discovery set", and the following group termed "validation set", of the PAD cases and controls. The genes were designated as differentially regulated or expressed if they had fold change difference equal to or greater than specified cut off; genes were present in both the sets (discovery set as well as validation set); and the genes/transcripts had analogous expression profiling (either upregulated or downregulated in both studies, respectively), as shown in Fig. 2.1.

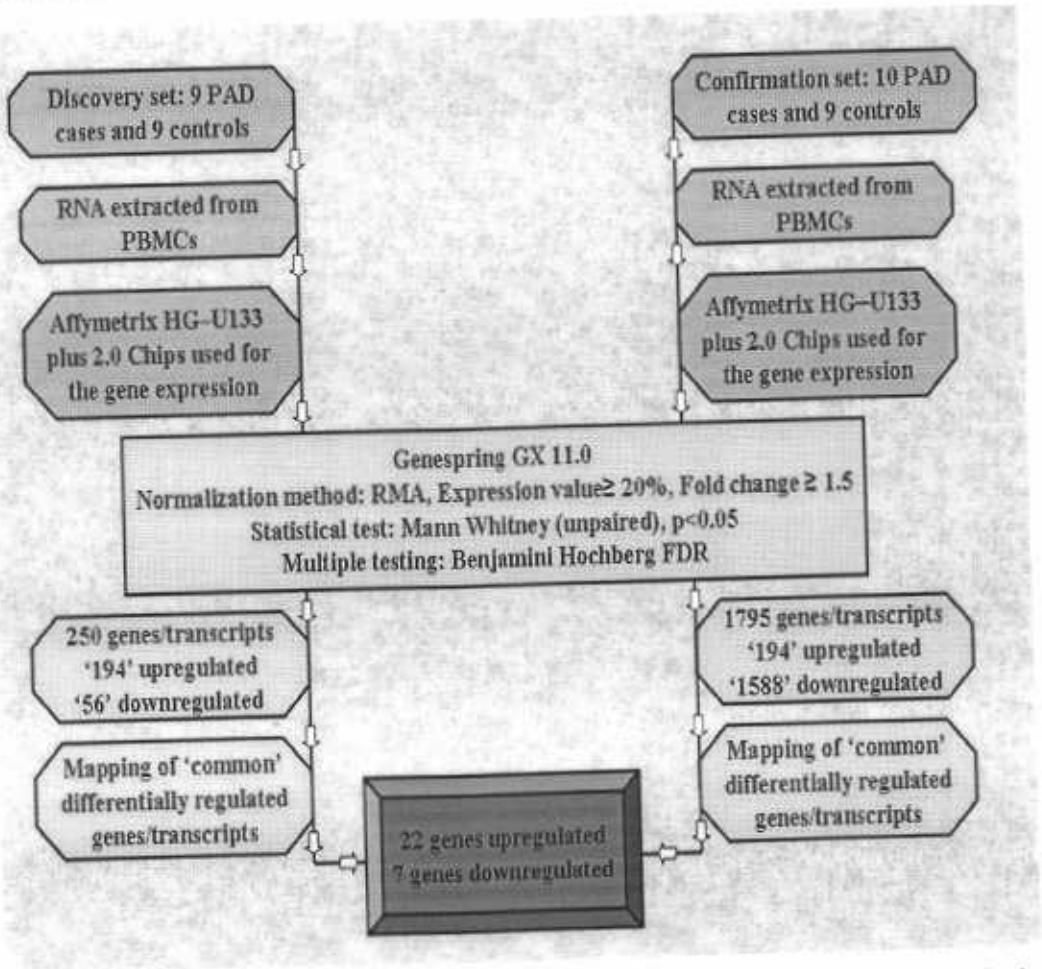

Fig. 2.1. Study design for the 'discovery' and 'validation' set of expression analysis.

2.3.2 Participant recruitment and sample characteristics

The study, as part of the PhD training, was completed at Mayo Clinic Rochester Minnesota USA, under the supervision of Prof. I. J. Kullo. All participants

were of non Hispanic white ethnicity. The studies were initiated after they were approved by Mayo Institution Review Board. All participants provided written and informed consents. The cases and controls for both the studies signed up and participated in the study after presentation to the Mayo non–invasive vascular laboratory. PAD cases were defined as individuals with an ABI ≤ 0.9. Age and gender matched participants that presented to the non–invasive vascular laboratory with symptoms of leg pain but ABI > 1.0 (normal ABI) were recruited as controls. The controls presented to the vascular lab for screening purposes. They had symptoms of leg pain but their resting as well as post exercise ABI was normal. The measurement of ABI was performed in both the lower limbs and the lower of the two readings was recorded for PAD diagnosis (Kullo et al., 2003). Participants with aneurysms and non compressible/poorly compressible vessels were removed from the study.

2.3.3 Isolation of PBMC and total RNA

The peripheral blood mononuclear cells were used for the isolation of RNA and these were isolated using the differential density gradient centrifugation method using Histopaque (Sigma-Aldrich, St. Louis, MO) (Ali et al., 1982; Bielecki et al., 2009). PBMC include only the lymphocytes and monocytes/macrophages and are exclusive of the red blood cells, platelets, and the polymorphs. Briefly, 18–20 ml whole blood was mixed with equal volume of phosphate buffered saline 'PBS' (Bio-Rad, Hercules, CA). To this mix were added 12 ml of Histopaque 1077 (Sigma-Aldrich, St. Louis, MO) for the gradient based separation of cells. The PBMC layer thus acquired was removed, washed and suspended in complete RPMI–10 medium. The suspended cells were counted using the hemocytometer and the PBMC were processed for isolation of the total RNA using the commercially available RNEasy Plus Mini Kit (Qiagen, Valencia, CA). The PBMC, processed with the RNEasy kit, were disrupted and homogenized by the preformed RLT buffer (supplied with RNEasy kit, Qiagen, Valencia, CA). The kit has a specialized genomic DNA eliminator (gDNA–eliminator) spin column which removes the genomic DNA and therefore allows isolation of purified RNA from the sample after downstream processing. Ethanol was mixed with the flow through from the gDNA–eliminator column and the sample was introduced to the RNEasy spin column. The RNEasy 'RNA' spin column because of the silica membrane based isolation, allows greatly accurate and effective RNA separation and purification. RNA was eluted with sterile

RNase free water. The quality and quantity of the total RNA isolated was assessed by NanoDrop 1000 (Thermo Scientific, Wilminnton, DE) and sample stored at –80°C until the microarray analysis.

2.3.4 Microarray analysis

The final assessment of RNA quantity and quality was performed through the Agilent 2100 Bioanalyzer system (Agilent, Santa Clara, CA). The samples with RNA integrity number (RIN) ≥ 9, were used for hybridization to the Affymetrix HG-U133_Plus 2.0 GeneChips. Affymetrix Two–cycle cDNA synthesis kit (Affymetrix, Santa Clara, CA) was used; biotin labeled cRNA was generated from 100 ng of the RNA sample. In vitro transcription based amplification of cRNA was done using the MEGAscript T7 IVT kit (Applied Biosystems / Ambion, Austin TX), for the first cycle. After the second cycle cDNA synthesis, Affymetrix IVT labeling kit was used to generate the biotin labeled cRNA. Final preparation of cRNA prior to microarray hybridization involved cleaning, quantification, fragmentation, and finally the hybridization of processed cRNA sample to Affymetrix HG–U133_Plus2.0 GeneChip. The hybridized chips were stained with streptavidin phycoerythrin and biotinylated antibody. The GeneChips were read on GeneChip scanner 3000 (Affymetrix, Santa Clara, CA), and washed at the Affymetrix Fluidics station 450. The data were extracted from the scanned arrays through GeneChip scanner 3000 (Affymetrix, Santa Clara, CA). The Affymetrix® GeneChip operating software (GCOS) was used to generate the raw data formats used for further analysis and gene expression profiling.

2.3.5 Data processing and statistical analysis

GeneSpringGx 11.0 software (Agilent® Technologies) was used for the gene expression analysis of microarray raw data. The discovery and validation sets were read using the software and analyzed individually. The initial analysis involved data normalization and summarization by the Robust Multichip Analysis (RMA) normalization (Irizarry et al., 2003). For the individual analysis, the baseline level was positioned at the median level for all samples, whereas the median log–transformed values from each probe and for all samples were calculated and the values were then deducted from all samples. The genes and transcripts with less than 20% expression levels were removed from the analyses. The 'fold change' cut off value was set at ≥1.5 for both the discovery and validation sets. The genes/transcripts that fulfilled the

criteria underwent analysis by unpaired Mann Whitney test. The results were corrected by the false discovery rate correction by the Benjamini Hochberg FDR (Benjamini and Hochberg, 1995). The genes differentially expressed in both the discovery and validation sets were clustered through K–means clustering algorithm, and results were displayed through the Cluster 3.0 and Treeview 1.1.0 software (Eisen et al., 1998; Henikoff et al., 2009).

2.3.6 Differentially regulated transcripts and their functional annotation

Gene ontology (GO) analysis was carried out for comprehension of the major biological processes and molecular functions modulated by the identified genes. The association of the differentially expressed genes and their mediated pathways were analyzed by SubPathway Miner (Li et al., 2009) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2010). Ingenuity Pathway Analysis 'IPA' (Ingenuity® Systems, www.ingenuity.com), BioGPS (Wu et al., 2009), and the STRING database (Jensen et al., 2009) were additionally used for analysis of functional pathways. The literature for the PAD genetic studies was collected through Gene Related InFormation (GeneRIF) and Pubmed databases (http://www.ncbi.nlm.nih.gov).

2.3.7 Informatics based Pathway analysis

Bioinformatics based pathway analysis was used for the pathways and networks involved in the differentially expressed genes in the discovery and validation sets of experiments. The output of presumed complex of interactions was acquired through Cytoscape (Shannon et al., 2003), after generation of expressed and localized genetic data from GeneMania (Warde-Farley et al., 2010). The significant GO terms, and associated genes were analyzed through 'Database for Annotation, Visualization and Integrated Discovery' (DAVID) (Dennis et al., 2003; Huang da et al., 2009). The existing GO terms and annotation data was set as background, and the significant genes from the current study (upregulated and downregulated genes separately), were used for analyzing the enrichment for the GO terms.

RESULTS

Participant characteristics, the traditional and new risk factors investigated in the cases and controls are shown in Table 2.1. The studied parameters including age, gender, height weight, BMI, systolic BP, diastolic BP, and hypertension did not differ markedly between cases and controls. The two significantly different factors between PAD cases and controls were the smoking status (high in cases (p<0.046) and ABI (low in cases p<0.0001). The present study, irrespective of individual genetic differences, facilitated the recognition of common set of genes associated with PAD. The functional annotations associated with a set of 29 differentially regulated genes demonstrated association with the immune, inflammatory, cell death and signaling pathways.

2.4.1 Expression difference between cases and controls

The evaluation of differentially expressed genes between cases and controls revealed 250 and 1795 transcripts in the 'discovery set' and the 'validation set' respectively. The upregulated genes in both the sets were 194, whereas the downregulated genes in discovery and validation sets were 56 and 1588 respectively. The differences in the number of downregulated genes between the discovery and validation sets can be the result of sample variations within and between the sets, differences in time of sampling for two studies, different microarray batches used, and operator differences during hybridizations. A few transcripts identified in the dual sets were not annotated in Affymetrix data, a query regarding these probes was submitted to BioMart (Smedley et al., 2009) and GATExplorer (Risueno et al., 2010) for the final mapping. Additionally UCSC genome browser database (Rhead et al., 2010) and NCBI 'Gene' database (http://www.ncbi.nlm.nih.gov/gene) were used for transcript descriptions. The details regarding the genes/transcripts, their absolute fold change values (FC), and the course of expression (upregulated or downregulated) is given in Table 2.2 for both the 'discovery set' and the 'validation set' respectively. Three genes in the genetic datasets *ARHGEF7*, *MLL3*, and *PDS5B*, were downregulated in both sets but had different probe mappings in the microarrays. An additional downregulated probe '241838_at' was not annotated in the Affymetrix data. With the use of Ensembl v58 (Fernandez-Suarez and Schuster, 2010), we could map this probe

to RP1–167A14.2, a non coding transcript. Since this transcript had no annotation and no functional description, this transcript was removed from further analysis. Figs. 2.2 – 2.3 represent the cluster diagrams for the differentially regulated genes and the participants.

2.4.2 IPA® based functional analysis

The differentially expressed genes from the two studies were analyzed by Ingenuity Pathway Analysis® (http://www.ingenuity.com), for categorization of the related biological functions and canonical pathways. The three important biological functional pathways, modulated by the differentially regulated genes in the current study included: (i) Diseases and disorders, (ii) Molecular and cellular functions, and (iii) Physiological system development and function. The important canonical pathways modulated by the genes in the current study included the genetic disorders, cancer, cellular death, cellular development, growth and proliferation, and connective tissue development. The networks defined by IPA are shown in Figs. 2.4 – 2.6, while the functions and pathways involved are given in Table 2.3.

2.4.3 Informatics based pathway analysis

The genetic data was generated from GeneMania (Warde-Farley et al., 2010) and the results of the genetic interactions were illustrated through Cytoscape (Shannon et al., 2003), (Fig. 2.7).

2.4.4 Gene ontology based gene enrichment study

The significant gene ontology (GO) terms were evaluated through Database for Annotation, Visualization and Integrated Discovery (DAVID) (Dennis et al., 2003; Huang da et al., 2009). The significant genes from the current study were used for analyzing the enrichment for the GO terms. The respective *p*-values represent the particular GO term in comparison to all the GO terms in the data. For the upregulated genes, GO analysis revealed two important processes, (i) the biological processes, and (ii) the molecular function ($P \leq 0.05$). For the downregulated genes the significant GO process was the biological processes ($P \leq 0.05$). As regards the upregulated genes the significant GO terms linked with biological functions involved inflammatory responses, like response to protein stimulus, response to organic substance, regulation of cell proliferation, and apoptosis. The molecular functions included cytokines and transcription regulation. The results are consistent with previous studies which signify that the genetic component of PBMC in PAD is enriched with immune mechanisms,

cytokines, inflammatory, and cell death mediated pathways. The GO results for upregulated genes are shown in Table 2.4. GO terms for downregulated genes were enriched for molecular functions and the responses related with apoptosis (Table 2.5). Thus the *CFH, FCAR, FFAR2, IL8, CFLAR, DUSP1, NAMPT* (inflammation) and the *ATF3, G0S2, KLF6, PTP4A1, CFLAR, PDS5B* (apoptosis) genes were found differentially expressed and differentially regulated in PBMC of PAD patients as compared to controls in the study.

Table 2.1. Sample characteristics. Participants were of non Hispanic white ethnicity.

Characteristic	Discovery Set		Validation Set	
	Cases	Controls	Cases	Controls
Participants	9	9	10	9
Age, years	71±10.2	69.6±7.6	68.3±8.5	65.9±5.5
Gender	6 males (67%)	6 males (67%)	7 males (70%)	7 males (78%)
Smoking Status	8 (89%)	4 (44%)	9 (90%)	8 (89%)
Height (*cm*)	169.9±11.6	167.6±8	172.3±8	173.9±7.6
Weight (*kg*)	80.2±18	86.8±22.3	87.3±20.3	92.3±14.7
BMI ($kg.m^{-2}$)	27.5±3.8	31.4±6	29.4±6.3	30.7±5.5
Systolic BP (*mm Hg*)	137.8±12.7	142.8±15.1	132.9±22.8	124.1±18.2
Diastolic BP (*mm Hg*)	64.8±6.9	78.7±14.2	70.9±11.6	72.6±12.6
HDL cholesterol (*mg/dl*)	43.8±10.3	49.5±15.8	44.5±7.1	46.4±11.3
Hypertension	8 (89%)	8 (89%)	9 (90%)	6 (67%)
Diabetes	2 (22%)	1 (11%)	3 (30%)	3 (33%)
Anti-hypertensive drugs	8 (89%)	8 (89%)	9 (90%)	6 (67%)
Anti-diabetic medication	1 (11%)	1 (11%)	3 (30%)	2 (22%)
Lipid lowering drugs	6 (67%)	6 (67%)	7 (70%)	7 (78%)
Ankle-brachial index	0.4	1.1	0.3	1.1
Family history of IHD	4 (44%)	2 (22%)	0	3 (33%)
CRP (*mg/l*)	22.5±2.9	0.7±0.4	3.1±2.7	4.3±3.4
Fibrinogen (*g/l*)	493.5±2.7	378±0.0	460±12.6	396.3±16.8
Homocysteine (*μmol/l*)	13±0.0	9±0.5	8±1	9.3±0.58

Values are expressed as either 'mean ± standard deviation' or as 'n (%)'.

Table 2.2. Differentially expressed genes from the two-step gene expression analysis. Arrows show upregulation and downregulation of genes, respectively.

Gene Symbol	Discovery Set		Validation Set		Regulation
	Probe ID	*P-value*	*Probe ID*	*P-value*	
ATF3	202672_s_at	0.013	202672_s_at	0.004	↑
C5orf41	1554229_at	0.038	1554229_at	0.004	↑
CDKN1A	202284_s_at	0.013	202284_s_at	0.021	↑
CDV3	213548_s_at	0.024	213548_s_at 228746_s_at	0.004 0.005	↑
CFH	215388_s_at	0.038	215388_s_at	0.021	↑
DNAJB6	208811_s_at	0.003	208811_s_at	0.004	↑
DUSP1	201044_x_at	0.028	201041_s_at 201044_x_at	0.009 0.004	↑
FCAR	207674_at 211307_s_at 211816_x_at	0.012 0.028 0.033	207674_at 211307_s_at	0.007 0.012	↑
FFAR2	221345_at	0.012	221345_at	0.012	↑
G0S2	213524_s_at	0.012	213524_s_at	0.031	↑
HIST1H2BC	214455_at	0.006	214455_at	0.045	↑
ID1	208937_s_at	0.028	208937_s_at	0.012	↑
IL8	211506_s_at	0.038	202859_x_at 211506_s_at	0.018 0.007	↑
KLF6	208960_s_at 208961_s_at	0.006 0.006	208960_s_at	0.006	↑

Contd.....

Gene Symbol	Discovery Set		Validation Set		Regulation
	Probe ID	*P-value*	*Probe ID*	*P-value*	
NAMPT	243296_at	0.007	243296_at 217738_at 217739_s_at 1555167_s_at	0.008 0.01 0.03 0.008	↑
NR4A2	204621_s_at 204622_x_at 216248_s_at	0.024 0.038 0.028	204622_x_at 216248_s_at	0.015 0.045	↑
OSM	230170_at	0.037	230170_at	0.046	↑
PTP4A1	200730_s_at	0.012	200730_s_at	0.004	↑
SAMSN1	1569599_at	0.033	1569599_at	0.032	↑
SLC2A3	202497_x_at 202498_s_at 202499_s_at	0.005 0.021 0.006	202498_s_at	0.031	↑
STX11	210190_at	0.017	210190_at	0.003	↑
TPR	215220_s_at	0.024	215220_s_at	0.005	↑
ARHGEF7	235412_at	0.024	229642_at	0.004	↓
C5orf28	238635_at	0.006	238635_at	0.004	↓
CFLAR	239629_at	0.033	239629_at	0.004	↓
MLL3	222413_s_at	0.010	244010_at	0.012	↓
OGT	229787_s_at	0.001	209240_at	0.001	↓
PDS5B	229704_at	0.017	215888_at	0.007	↓
TRAF3IP3	240265_at	0.033	240265_at	0.006	↓

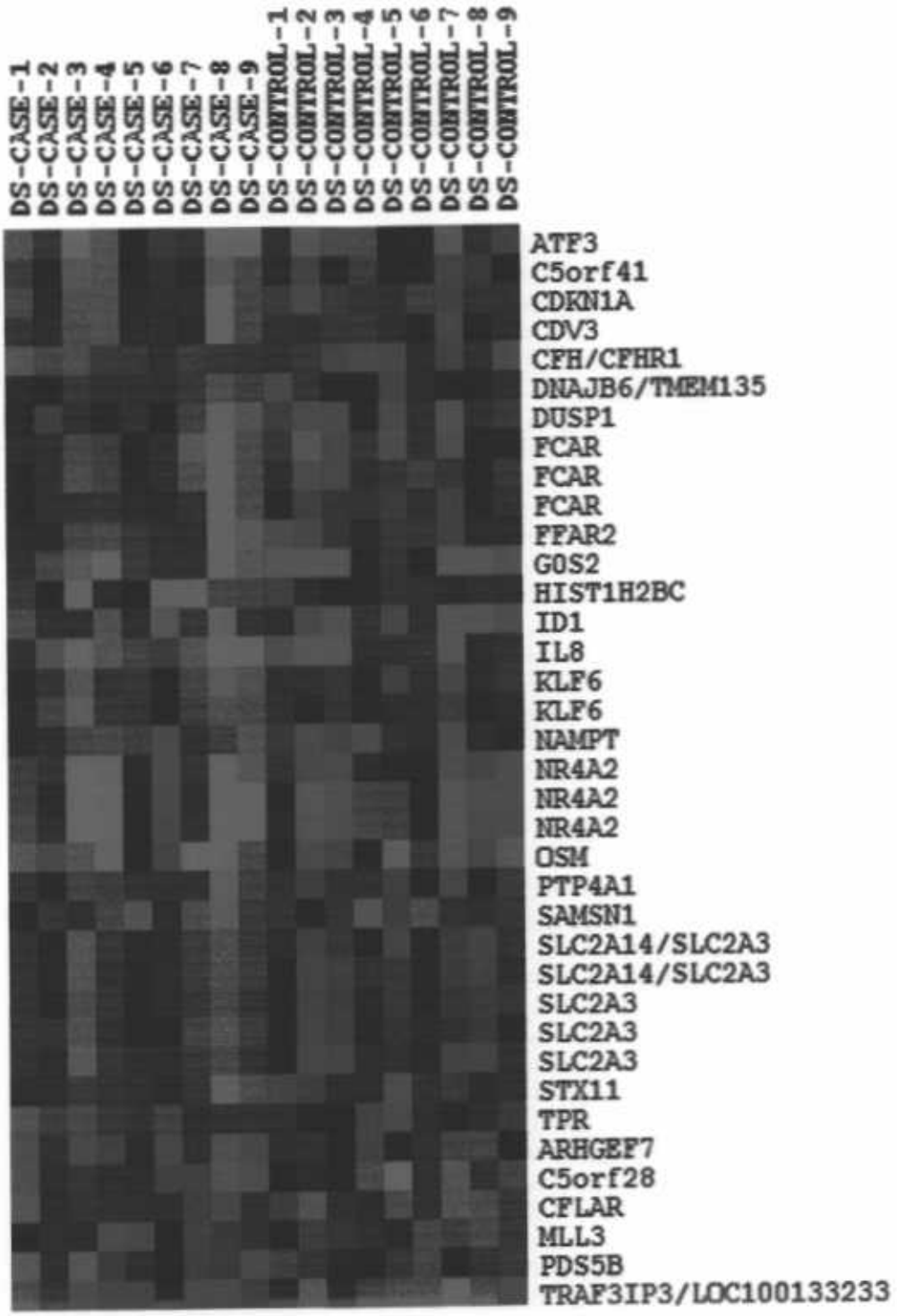

Fig. 2.2. Cluster diagram FDR corrected K–means clustering of PAD cases and controls in the 'discovery' set. At the top, from left to right, nine controls and nine PAD cases are represented. The genes are represented to the right of the figure. Red color represents upregulated genes, whereas green color represents downregulated genes. A clear demarcation of the participants as well as demarcation of the upregulated and downregulated genes is noticeable.

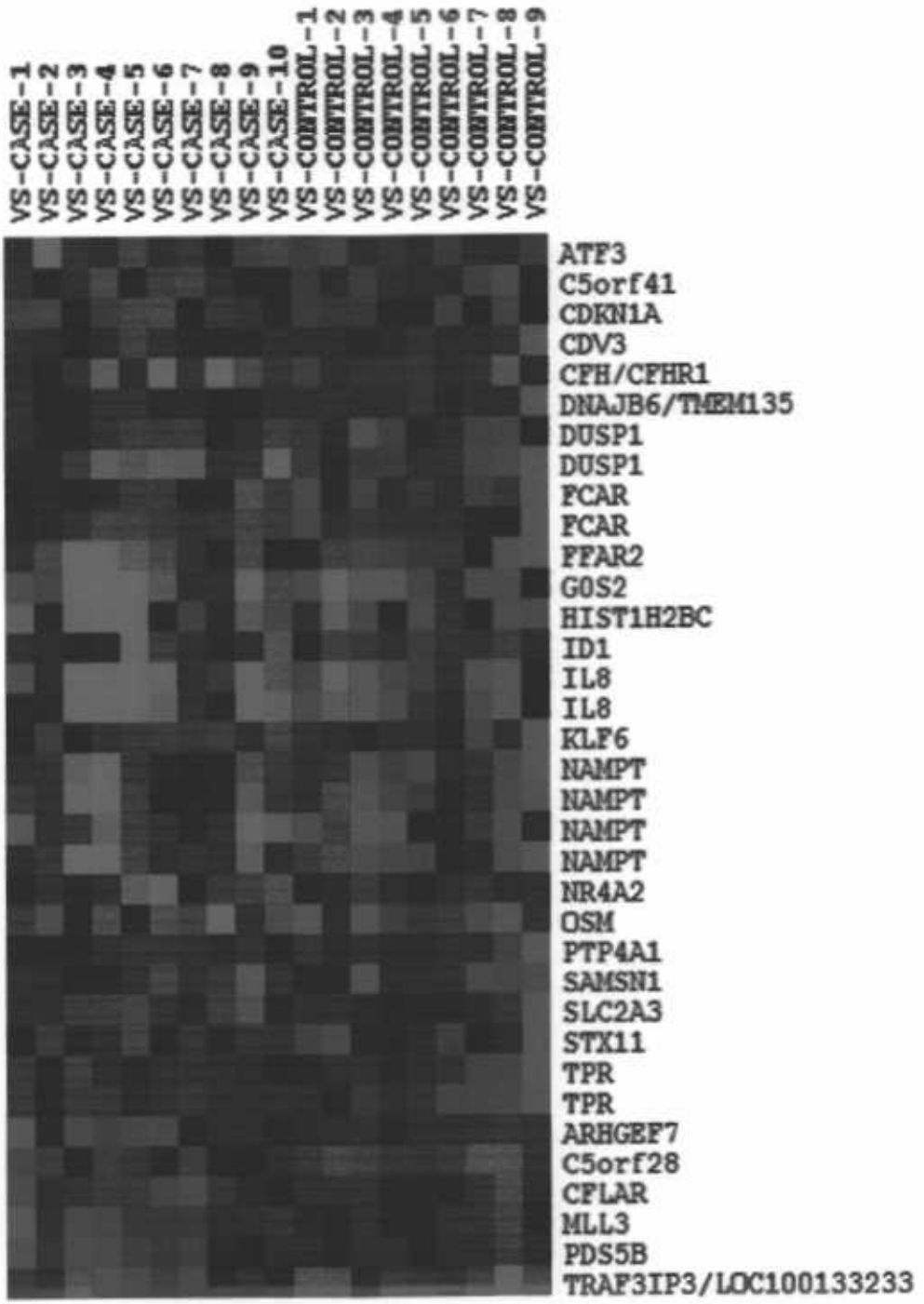

Fig. 2.3. Cluster diagram FDR corrected K–means clustering of PAD cases and controls in the 'validation' set. At the top, from left to right, ten PAD cases and nine control samples are represented. The genes are represented to the right. Red color represents upregulated, whereas green color represents downregulated genes. The figure shows clear demarcation of participants as well as demarcation of the upregulated and downregulated genes.

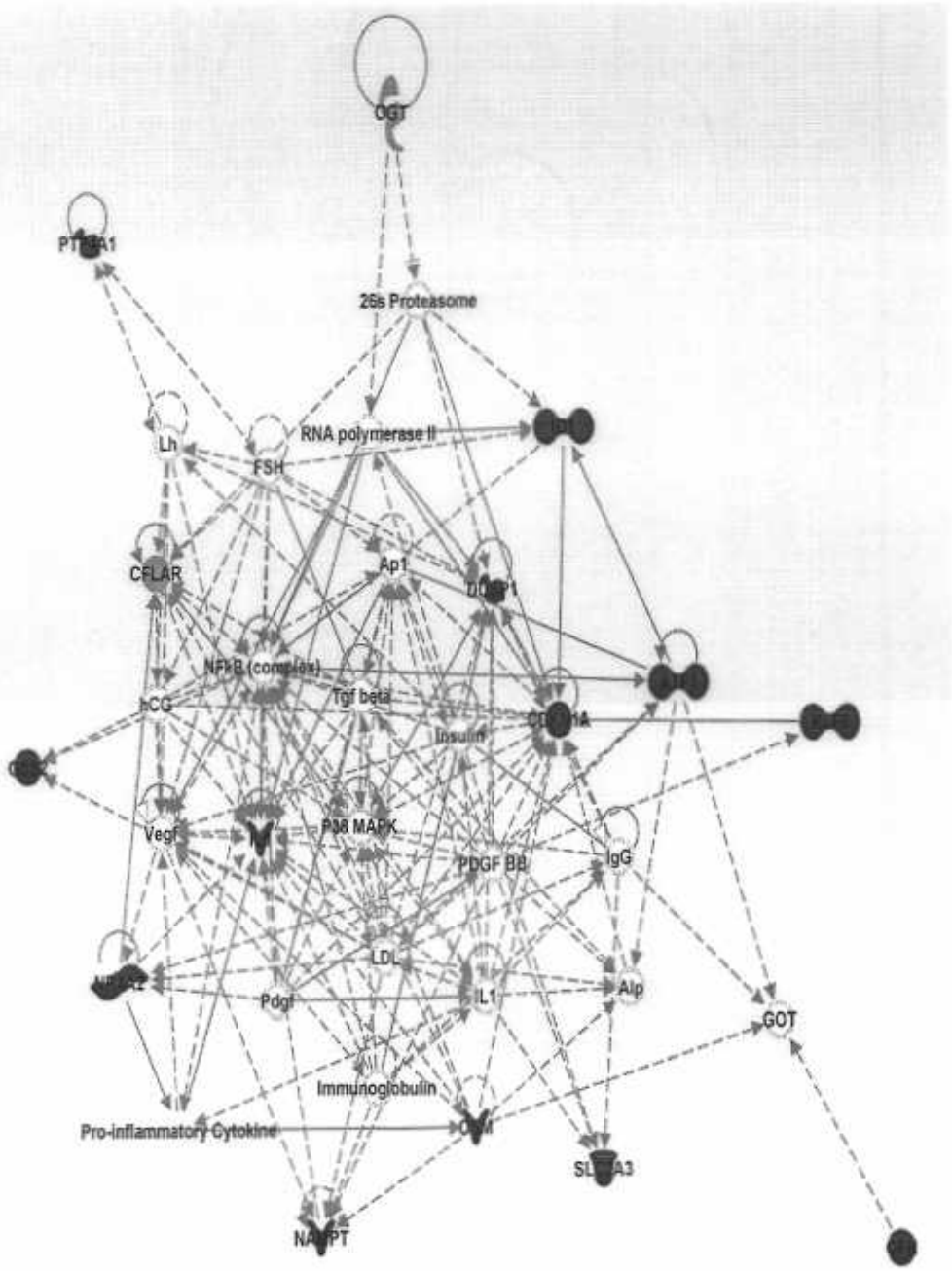

Fig. 2.4. The genes and the transcribed protein interactions in the IPA pathway: "Cell Death, Dermatological Disease and Conditions, Genetic Disorder". The filled symbols represent the differentially regulated genes in the present study.

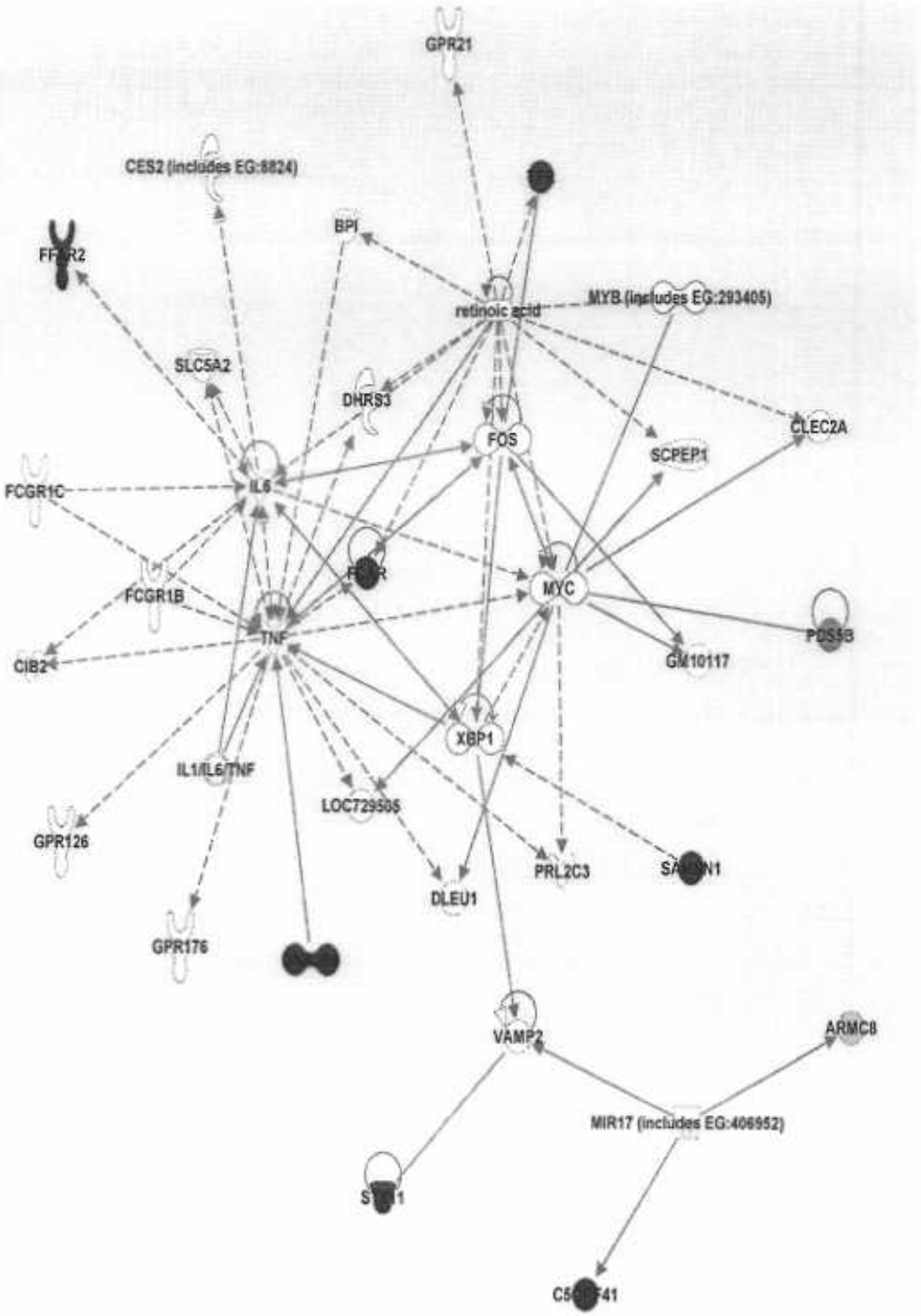

Fig. 2.5. The genes and the transcribed protein interactions in the IPA pathway: "Gene Expression, Cellular Development, Hematological System Development and Function". The filled symbols represent the differentially regulated genes in the present study.

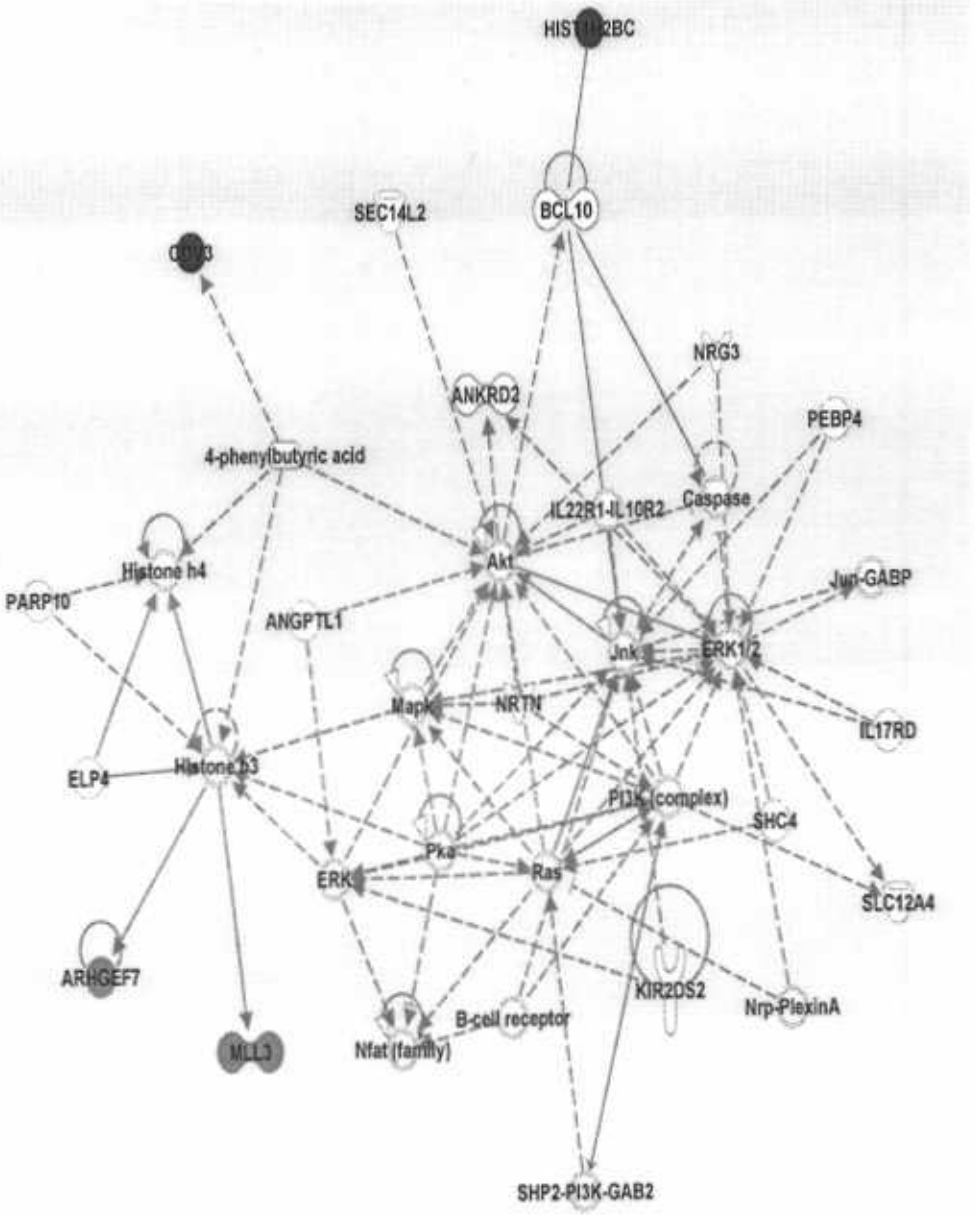

Fig. 2.6. The genes and the transcribed protein interactions in the IPA pathway: "Behavior, Drug Metabolism, Inflammatory Disease". The filled symbols represent the differentially regulated genes in the present study.

Table 2.3. IPA based Functional Pathway Analysis. 1st column represents the functions and pathways; 2nd column represents the genes associated with the functions/pathways and the third column represents *P* value.

Biological Functions Pathways	Associated Genes in the Study	*P* value
1. Disease and Disorders		
Genetic disorders	*ARHGEF7, ATF3, CDKN1A, CFH, CFLAR, DNAJB6, DUSP1, ID1, IL8, KLF6, MLL3, NAMPT, NR4A2, OGT, OSM, PDS5B, SAMSN1, SLC2A3, STX11*	0.006
2. Molecular and Cellular Functions		
Cell Death of blood cells	*ATF3, CDKN1A, CFLAR, FCAR, IL8, NAMPT, OSM*	0.0009
Cell Death of eukaryotic cells	*ATF3, CDKN1A, CFH, CFLAR, DUSP1, FCAR, GOS2, ID1, IL8, KLF6, NAMPT, NR4A2, OSM*	0.00003
Apoptosis of tumor cell lines	*ATF3, CDKN1A, CFH, CFLAR, DUSP1, GOS2, , ID1, IL8, KLF6, NR4A2, OSM*	0.00005
Cell growth and proliferation	*ATF3, CDKN1A, CFLAR, DUSP1, ID1, IL8, KLF6, NAMPT, OSM, PDS5B, PTP4A1, SAMSN1, TPR*	0.00004
Growth of eukaryotic cells	*ATF3, CDKN1A, DNAJB6, DUSP1, IL8, NR4A2, OSM, TPR*	0.0001
Cell–Cell Signalling and Interactions (eukaryotic cells)	*CDKN1A, CFH, DUSP1, FCAR, FFAR2, ID1, IL8, OSM*	0.00003
Cell–Cell Signalling and Interaction (normal cells)	*CDKN1A, CFH, DUSP1, FCAR, FFAR2, IL8, OSM*	0.0001

Contd.....

Biological Functions Pathways	Associated Genes in the Study	P value
Cell–Cell Signalling and Interaction (leukocytes)	*CDKN1A, CFH, DUSP1, FCAR, IL8, OSM*	0.0002
Cellular Development and Differentiation of Cells	*ATF3, CDKN1A, DUSP1, ID1, IL8, NAMPT, NR4A2, OSM*	0.003
3. Physiological System Development, Function		
Growth of Fibroblasts	*ATF3, CDKN1A, DUSP1, OSM, TPR*	0.000004
Hematological system (quantity of leukocytes)	*ATF3, CDKN1A, CFH, CFLAR, ID1, IL8, OSM, SAMSN1*	0.000002
Hematological system (quantity of mononuclear cells)	*CDKN1A, CFLAR, ID1, IL8, OSM, SAMSN1*	0.00004
Hematological system (quantity of lymphocytes)	*CDKN1A, CFLAR, ID1, OSM, SAMSN1*	0.0003
Infilteration of leukocytes	*CFH, DUSP1, FCAR, IL8, OSM*	0.0001
Infilteration of the granulocytes	*CFH, DUSP1, IL8, OSM*	0.0001
Activation of leukocytes	*CDKN1A, CFH, DUSP1, FCAR, IL8, OSM*	0.0002
Activation of lymphocytes	*CDKN1A, DUSP1, IL8, OSM*	0.001

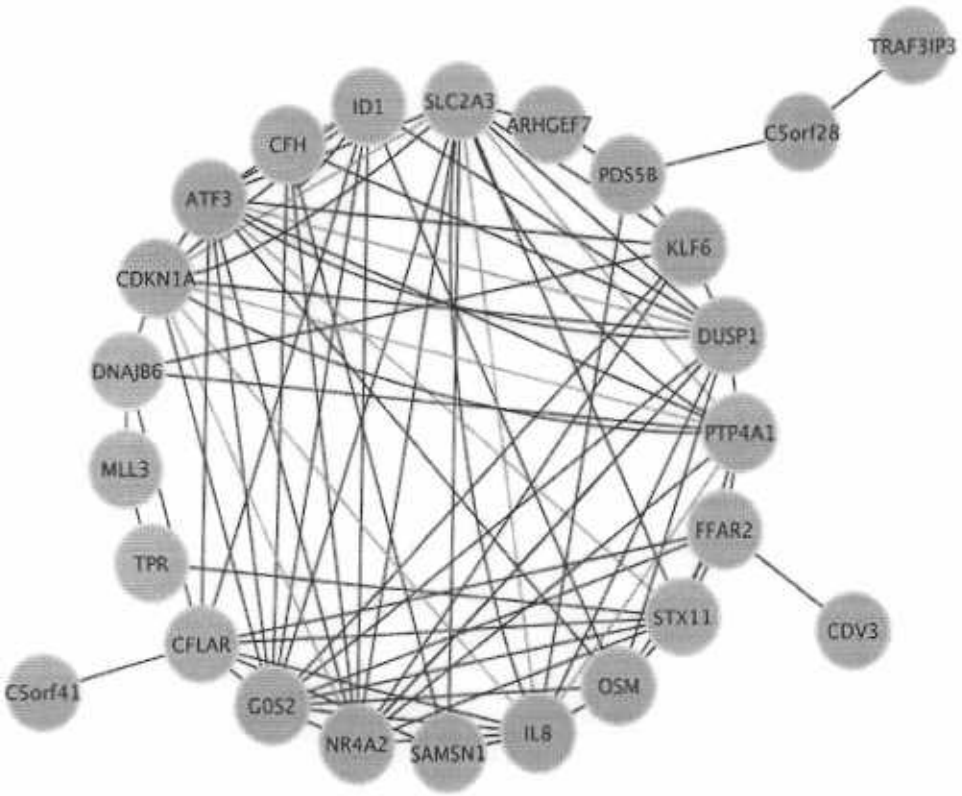

Fig. 2.7. Network of putative interactions between the genes identified from the current study. These genes form interactive network associated with disease.

Table 2.4. Significantly enriched GO terms of upregulated genes. 1st column represents the GO ID, GO term and the processes. 2nd column represents the *P* value.

GO ID – Term	*P-value*
GO Category: Biological Process	
GO:0051789 - response to protein stimulus	0.009
GO:0010033 - response to organic substance	0.016
GO:0008284 - positive regulation of cell proliferation	0.019
GO:0007049 - cell cycle	0.020
GO:0042127 – regulation of cell proliferation	0.021
GO:0006355 – regulation of transcription, DNA-dependent	0.029
GO:0010035 - response to inorganic substance	0.032
GO:0051252 – regulation of RNA metabolic process	0.033
GO:0010948 - negative regulation of cell cycle process	0.034
GO:0009991 - response to extracellular stimulus	0.037
GO:0045768 - positive regulation of anti-apoptosis	0.041
GO:0048584 - positive regulation of response to stimulus	0.042
GO Category: Molecular Function	
GO:0005125 - cytokine activity	0.026
GO:0030528 - transcription regulator activity	0.039

Table 2.5. Significantly enriched GO terms of downregulated genes. 1st column represents the GO ID, GO term and the processes. 2nd column represents the *P* value.

GO ID – Term	*P-value*
GO Category: Biological Process	
GO:0008624 - induction of apoptosis by extracellular signals	0.024
GO:0006917 - induction of apoptosis	0.069
GO:0012502 - induction of programmed cell death	0.069
GO:0043065 - positive regulation of apoptosis	0.092
GO:0043068 - positive regulation of programmed cell death	0.092

2.4.5 Pathway Analysis using KEGG

SubPathwayMiner, the pathway analysis package based on BioConductor (Gentleman et al., 2004) was additionally used for the significance analysis of the pathways related with the significant genes. In 32 KEGG pathways, 13 genes from the current study (out of 29) were robustly enriched. *IL8* was directly involved in 13 pathways, and CDKN1A in 10 other pathways. The results of KEGG analysis suggest that alterations in numerous cellular and signaling pathways are involved in the development of PAD. Some important pathways and genes implicated in PAD are shown in Table 2.6.

Table 2.6. Result of KEGG pathway enrichment analysis. 1st column represents the KEGG ID; 2nd column represents the involved disease pathways; 3rd column is for the associated genes; and the 4th column is for the associated *P* value. (KEGG path 05219 represents bladder cancer pathway and includes *CDKN1A* and *IL8* genes with significant *p* value = 0.001).

KEGGID	PATHWAY	GENES	*P-value*
path:05219	Bladder cancer	*CDKN1A, IL8*	0.001
path:05150	Staphylococcus aureus infection	*CFH, FCAR*	0.002
path:05200	Pathways in cancer	*CDKN1A, IL8, TPR*	0.005
path:05142	Chagas disease	*CFLAR, IL8*	0.005
path:05160	Hepatitis C	*CDKN1A, IL8*	0.009
path:00760	Nicotinate and nicotinamide metabolism	*NAMPT*	0.025
path:05216	Thyroid cancer	*TPR*	0.03
path:04060	Cytokine-cytokine receptor interaction	*IL8, OSM*	0.034
path:04130	SNARE interactions in vesicular transport	*STX11*	0.037
path:05144	Malaria	*IL8*	0.053
path:04621	NOD-like receptor signaling pathway	*IL8*	0.063
path:05131	Shigellosis	*IL8*	0.065
path:05214	Glioma	*CDKN1A*	0.066
path:05120	Epithelial cell signaling in H. Pylori infection	*IL8*	0.069
path:04115	P53 signaling pathway	*CDKN1A*	0.07
path:04610	Complement and coagulation cascades	*CFH*	0.07
path:05218	Melanoma	*CDKN1A*	0.072
path:04622	RIG-I-like receptor signaling pathway	*IL8*	0.072
path:04350	TGF-beta signaling pathway	*ID1*	0.086
path:04012	ErbB signaling pathway	*CDKN1A*	0.088
path:04210	Apoptosis	*CFLAR*	0.09
path:04620	Toll-like receptor signaling pathway	*IL8*	0.102
path:04062	Chemokine signaling pathway	*IL8*	0.183

DISCUSSION

The current study reports, for the first time, the use of transcriptome for the identification of genes differentially expressed in peripheral artery disease patients. A non invasive test 'ABI' was used for the stratification of participants into cases and controls. Gene set enrichment analyses through GO, KEGG, and IPA provide better understanding of the molecular and genetic mechanisms of PAD. The significant finding was that the common upregulated genes in the two sets were linked with vascular pathophysiology, for example: *DNAJB6, DUSP1* (atherosclerotic disease), *NAMPT* (vascular inflammation), *FCAR* (MI, IHD), *NR4A2* (restenosis), *ATF3, IL8* (vascular remodelling) and *FFAR2, OSM.* (lipid metabolism).

Presently, most of the differentially expressed genes between PAD cases and control subjects are involved in the immune and inflammatory processes, chemotaxis and apoptosis. The previous microarray based genetic studies of coronary vessels, peripheral vessels, and human coronary endothelial cells have reported that the genes that modulate immunity and inflammation are differentially expressed between patients and normal subjects (Blaschke et al., 2004; Wyler von Ballmoos et al., 2006; Dahl et al., 2007; Evans et al., 2008; Fu et al., 2008). The microarray based genetic analysis of femoral arteries in a cohort of PAD patients revealed enrichment for immune and inflammatory pathways (Fu et al., 2008). In another microarray study by Evans et al (2008), additional vessels (tibial, peroneal, and femoral vessels) were used to categorize the involved genes in PAD cases. Whole genome expression analysis of PBMC in CAD patients and controls, highlighted more than 500 genes at a fold change ≥ 1.3 ($p<0.05$). Subsequent real time PCR analysis of a subset of these genes recognized 11 differentially regulated genes for CAD (Wingrove et al., 2008). However, the differentially expressed genes isolated from PBMC and associated with PAD in the present study did not overlap with CAD associated genes identified by Wingrove et al. (2008). The reasons may be dissimilar methods of study, dissimilar sample profiles and discrete atherosclerotic disease expression in CAD and PAD. The relatively strict experimental design of the current study, validation experiment set to confirm results of discovery experiment set, accounts for a relatively small number of significant genes. Nevertheless, the study appears to be a significant step in

elucidation of the genetic signatures of PAD. The differentially expressed genes in the present study have diverse effects and are involved in modulation of vascular disease and have positive associations with vascular disease phenotypes. The following discussion considers the mechanisms of action, and downstream effects of the differentially regulated genes.

The upregulated genes in the current study and vascular disease

The expression of '*ATF3*' was upregulated in both microarray sets in the present study. The gene for activating transcription factor 3 (*ATF3*) is located at chromosomal location 1q32.3. *ATF3* was also upregulated in a previous microarray study for PAD indicating strong association with PAD (Fu et al., 2008). *ATF3* is an endoplasmic reticulum (ER) stress marker, and is involved in apoptosis of vascular endothelial cells as well as of macrophages (Zhang et al., 2001; Seimon et al., 2010). The release of *ATF3* is induced by the proinflammatory cytokines, TNF-α, and ox–LDL, enhanced *ATF3* results in enhanced cell death and rapid progression of atherosclerotic disease (Nawa et al., 2002). Hypoxia induced vascular damage mediated by endothelial cells is also dependent on high *ATF3* expression levels (Chen et al., 2008).

The next upregulated transcript, chromosome 5 open reading frame 41 (*C5orf41*) is located at chromosomal location 5q35.1. Also known as luman/*CREB3* recruiting factor (*LRF*), it has high affinity for the stress responsive nuclear protein '*luman*'. *ATF3* and *luman* are components of ER stress related nuclear transcription proteins and mediate lipid homeostasis, protein transcription, and apoptosis (Audas et al., 2008; Puskas et al., 2010). Additionally, a recent GWAS study indicated linkage of *C5orf41* with cardiac arrhythmia favoring the CVD modulation by this protein (Pfeufer et al., 2010). The upregulation of *C5orf41* through the protein–protein interactions, lipid homeostasis, apoptotic pathways, and nuclear transcription can result in vascular disease phenotypes.

The third upregulated gene in the current study, the cyclin–dependant kinase inhibitor 1A (*CDKN1A*) is highly expressed in endothelial cells in atherosclerotic lesions (Borradaile and Pickering, 2010). The strong association of this gene is evidenced as previous microarray analysis of atherosclerotic endothelial cells and earlier gene expression study for PAD reveal that *CDKN1A* has differential gene expression and is upregulated in atherosclerotic vessels (Rodriguez et al., 2007; Fu et

al., 2008; Borradaile and Pickering, 2010). The upregulation of *CDKN1A* and *ATF3* in previous expression study for PAD and in the current study highlight these variant as potential biomarkers for the PAD.

The fourth upregulated gene, in the current PAD cases, '*CDV3*' is located at chromosomal location 3q22.1. It was shown that *CDV3* was robustly upregulated in activated platelets (by the platelet activators thrombin, collagen, and more so by arachidonic acid), conferring involvement of this protein in signal transduction and platelet activation (Majek et al., 2010). It also regulates insulin transcription by interacting with the insulin transcription activator '*NEUROD1*' (Zhang et al., 2009b). As *CDV3* was upregulated in both microarray sets, the present study stresses this gene as an important regulator of atherosclerotic peripheral vascular disease.

The complementary factor H (*CFH*) was found upregulated in both microarray sets, and like *ATF3* and *CDKN1A*, its upregulation in a previous microarray gene expression study for PAD has been observed (Fu et al., 2008). *CFH* has strong interaction with novel cardiovascular biomarker, C-reactive protein (CRP) (Jylhava et al., 2009; Ferreira et al., 2010). *CFH* is a member of complement system and has significant role in the regulation of complement activation. The mutations and polymorphisms in either *CFH* or CRP can result in uncontrolled complement activation, enhanced atherosclerotic disease and severe phenotype of the vascular disease (Jylhava et al., 2009; Ferreira et al., 2010).

The sixth upregulated gene in the current study, *DNAJB6* is encoded by the gene at locus 7q36.3. It is involved in cell–cell interactions, adhesion, and protein breakdown through its interaction with specific plasminogen activator receptor (De Bock et al., 2010). Previously, known as *MRJ*, it maintains the mature T cells in dormant stage, and enables activation of a subgroup of antigen specific T cells (Zhang et al., 2008b). Gargalovic et al (2006), in their study demonstrated that oxidized phospholipids induce *IL8* and *DNAJB6*, signifying the inflammatory and atherosclerotic role of *DNAJB6*. As both the *IL8* and *DNAJB6*, were upregulated in the current study, this indicates that atherosclerosis mediated by oxidized phospholipids may be the underlying mechanisms for the PAD.

The gene for dual specificity phosphatase 1 '*DUSP1*', also called MAP kinase phosphatase 1 (*MKP1*) is located at chromosomal location 5q34, and was upregulated in current study. In EC, *DUSP1* is induced by thrombin, epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF); it also activates

the transcription factors (such as JNK) and mediates endothelial cell migration (Kinney et al., 2008; Chandrasekharan et al., 2010). *DUSP1* was also upregulated in a previous microarray study for PAD (Fu et al., 2008). This protein has additionally high cardiac expression in patients undergoing cardiac bypass surgery (Voisine et al., 2004). In contrast to other proteins in the current study, this protein has known anti-inflammatory role as well (Zakkar et al., 2008). Atrial natriuretic peptide '*ANP*' enhances *DUSP1* expression through ROS mediated mechanisms, and therefore exerts its antiinflammatory effects (Furst et al., 2005). High induction of *DUSP1* in response to ANP and ROS, indicates that, although not a causative agent for vascular disease, yet it can serve as a biomarker for vascular disease, particularly PAD.

The eighth upregulated gene, *FCAR* is a receptor protein and is highly expressed by activated monocytes in autoimmune disease states (van der Heul-Nieuwenhuijsen et al., 2010). *FCAR* is associated with increased risk of MI and CAD, mediates immunologic response, and is involved in activation of monocytes and macrophages in atherosclerotic lesions (Iakoubova et al., 2006; van der Heul-Nieuwenhuijsen et al., 2010). *FCAR*, therefore has proven contributory influence in atherosclerosis progression and vascular diseases.

FFAR2 gene is also a receptor protein, encoded by the gene at chromosomal location 19q13.1. It is activated by short chain free fatty acids, and is present in the adipose tissue in addition to gut (Miyauchi et al., 2010), with involvement in malignant transformation of gut carcinomas (Hatanaka et al., 2010). The upregulation of *FFAR2* and disease association, in the current study, is comparable to the previous studies. It is associated with endothelial dysfunction, inflammation, modulation of lipid and insulin levels and with metabolic syndrome indicating strong causative influences in vascular disease (Hong et al., 2005; Lee et al., 2008b; Swaminath, 2008; Miyauchi et al., 2010).

The tenth upregulated gene, G0/G1 switch regulatory protein 2 '*G0S2*' is located at chromosomal location 1q32.2. *G0S2* is a mitochondrial protein and has prominent role in apoptosis. The apoptotic effects of TNFα are mediated by NF-κB, which in turn activates its downstream apoptotic effector *G0S2* (Welch et al., 2009). *G0S2* (like *FFAR2*) is present in adipose tissue, controls lipid metabolism, and is associated with vascular disease (Welch et al., 2009; Yang et al., 2010c). The upregulation of *G0S2* in the current PAD study highlights that lipid metabolism and apoptotic pathways are actively involved in peripheral vascular disease.

The histone family member, *HIST1H2BC* had enhanced expression in the current PAD study. Histone family members exert indirect effects in CVD, as in a recent GWAS, histone gene polymorphisms were associated with the high serum billirubin levels, and billirubin levels have influence on CVD risk (Djousse et al., 2001; Johnson et al., 2009). The serum billirubin levels were not measured in the current study, therefore the indirect modulation of CVD by *HIST1H2BC* (through metabolism of billirubin), could not be ascertained.

Increased activity of the gene, 'inhibitor of DNA binding 1' *ID1*, in the PAD cases relative to subjects without PAD mirrored the strong atherogenic effects of this protein. Its gene is located at chromosomal location 20q11. It modulates VSMC proliferation and is associated with peripheral arterial hypertension (Yang et al., 2010b). *ID1* (through NF-κB) enhances inflammation, endothelial cell migration, angiogenesis, and inhibits apoptosis (Klein et al., 2002; Nishiyama et al., 2005; Chen et al., 2010).

Interleukin 8 (*IL8*), also known as CXC motif ligand 8 (*CXCL8*) is a member of CXC chemokine family cluster, and is located at chromosomal location 4q13–q21. *IL8* and *DNAJB6*, both were upregulated in the current study, and the enhanced release of these two proteins during atherosclerosis, is dependent on oxidized phospholipids, affirming the inflammatory and atherosclerotic role of *IL8* (Gargalovic et al., 2006). It is excreted by a variety of cells, is a strong chemoattractant for the granulocytes and T cells, and is involved in apoptosis, vascular remodeling, vascular disease and atherosclerosis (Li et al., 2003; Rajappa et al., 2009; Vogiatzi et al., 2010). The presence of elevated *IL8* in CAD and vascular stenosis also emphasize the significance of this protein in relation to the CVD (Vogiatzi et al., 2010). The upregulation of *IL8* in the current microarray PAD study highlights, that the granulocytes and T cell involvement in apoptotic pathways and atherosclerosis are necessary regulators of PAD.

The fourteenth upregulated gene in the present study, Kruppel-like factor 6 (*KLF6*) is a member of the Kruppel-like family of transcription factors and is located at position 10p15. It has diversified functions, it is a tumor suppressor gene and regulates cellular proliferation and apoptosis, whereas its splice variant (KLF6–SV1) is a strong oncogene (DiFeo et al., 2009; Andreoli et al., 2010). It is involved in signaling reactions and regulates gene expression through interaction with c-Jun (Andreoli et al., 2010). *KLF6* regulates endothelial cell motility and induces MMP9

secretion (Das et al., 2006; Atkins and Jain, 2007). The higher expression of KLF6 in the PAD cases as compared to subjects without PAD, indicates that gene regulation and matrix metalloproteinase mediated plaque destabilization are associated with PAD. *KLF6* can therefore serve as an additional biomarker for PAD.

The next upregulated gene, adipocytokine 'nicotinamide phosphoribosyl transferase' (*NAMPT*), also called visfatin or pre B cell colony enhancing factor 1, is located at 7q22.3. *NAMPT* is released by adipose cells as well as by monocytes and granulocytes (Laudes et al., 2010). It has a direct role in maturation of VSMC, it inhibits neutrophil apoptosis, and is associated with insulin metabolism and pathogenesis of diabetes (Romacho et al., 2009; Kadoglou et al., 2010; Laudes et al., 2010). *NAMPT* exerts its proatherosclerotic effect by activation of extracellular signal regulated kinases 1 and 2 (*ERK1/2*), NF–κB and by enhanced inducible NOS (iNOS) expression (Romacho et al., 2009). *NAMPT* additionally modulates cerebrovascular disease, hypertensive disease, and IHD (Wang et al., 2011). In a previous microarray study for peripheral vascular disease, *NAMPT* was shown strongly associated with atherosclerosis and enhanced the unstability of atherosclerotic plaques (Dahl et al., 2007). The study by Dahl et al. (2007), and the current study both assert the significant role of NAMPT in peripheral vascular disease.

The gene for nuclear receptor subfamily 4, group A member 2 '*NR4A2* or *Nurr1*' is located at chromosomal location 2q22–q23 and was upregulated in the current study. It is also a receptor protein and mediates glucose and lipid metabolism, T cell apoptosis and vascular disease (Pols et al., 2007; Zhao and Bruemmer, 2010). Furthermore, it is associated with vascular stenosis and has elevated expression in the human restenosis lesions (Bonta et al., 2010). The upregulation of *NR4A2* in the present study as well as in two previous microarray studies for PAD indicates that it has strong association with the peripheral artery disease (Evans et al., 2008; Fu et al., 2008), and can therefore serve as an important PAD biomarker.

The seventeenth upregulated gene, oncostatin M '*OSM*' is a member of the cytokine family. *OSM* induces production of VEGF in cultured human smooth muscle cells and in atherosclerotic plaques. It is associated with atherosclerotic lesion in coronary and peripheral vessels, and is involved in destabilizing atherosclerotic plaques (Demyanets et al., 2011). It is also involved in the progression of plaques, lowering the plasma triglycerides, thrombotic complications and rupture of atherosclerotic plaques (Mirshahi et al., 2002; Demyanets et al., 2007; Zhou et al.,

2007). In a previous microarray study for arterial hypertension, *OSM* was downregulated, in cases as compared to controls (Timofeeva et al., 2006). The differences in patient selection, and different genomic signatures and involved pathways in PAD, as compared to arterial hypertension, may account for the disparate results in the two studies.

The next upregulated gene, protein tyrosine phosphatase type IVA, member 1 '*PTP4A1*', known as *PRL1*, is located at 6q12. It downregulates the expression of p53, and effects development of tumors (Min et al., 2009). It is associated with cellular differentiation, and regulates reduction oxidation (redox) reactions as well (Skinner et al., 2009). It additionally increases the expression of actin and modulates the adhering, migrating and invasive potential of cells (Nakashima and Lazo, 2010), thereby making this tyrosine phosphate a key molecule in cellular dysfunction and disease processes. The upregulation of this protein in PAD cases maintains that it is a potential biomarker for PAD.

SAM domain SH3 domain nuclear localization signal 1 '*SAMSN1*', or the hematopoietic adaptor containing SH3 and SAM domains 1 (*HACS1*) is located at chromosomal location 21q11. It is a cytoplasmic protein, mediates signal transduction and is highly elevated in activated B cells. It effects activation and differentiation of the B cells, and is involved in metabolic disorders as well (Claudio et al., 2001; Zhu et al., 2004). It also undergoes nuclear translocation, and controls gene expression by interacting with *HDAC1* (Brandt et al., 2010). The upregulation of *SAMSN1* in the current study, its proatherosclerotic effects, and its upregulation in a previous PAD microarray study strongly indicate that it is a strong modulator of PAD (Fu et al., 2008).

The high expression of the glucose transporter 3 gene '*GLUT3*' also called solute carrier family 2 member 3 '*SLC2A3*' in this PAD study indicates the importance of glucose transport and metabolism in PAD. It is critical not only for glucose transport but also for the removal of endogenous small organic cations and toxins (Tregouet et al., 2009; Sallinen et al., 2010). It is highly expressed in the adipose tissue, skeletal muscles and the peripheral blood lymphocytes, and maintains insulin sensitivity in the lymphocytes (Piatkiewicz et al., 2010). It is a downstream mediator of steroid receptor RNA activator (*SRA*), which increases biosynthesis of steroids and of the muscular tissue, and modulates cellular division and apoptosis (Foulds et al., 2010). This protein has high expression levels in the cardiac tissue of

diabetic and non diabetic patients undergoing coronary artery bypass grafts (Voisine et al., 2004). In the microarray study by Fu et al (2008), this protein had higher expression in PAD patients (as in the current study), giving strong association of this glucose transporter with the peripheral vascular disease.

The upregulation of syntaxin 11 '*STX11*' gene in PAD cases in the current study signifies that T cell mediated atherosclerotic lesions mediate the pathogenesis of PAD. Being a member of SNARE family of trafficking proteins, Syntaxin 11 has specific enrichment in the lymphoid immune tissues and regulates transport across the membranes. It facilitates cellular trafficking, as well as targeting and fusion of intracellular transport vesicles (Valdez et al., 1999). The mutation in this gene is associated with a rare (potentially fatal) immune disease termed familial hemophagocytic lymphohistiocytosis. This disorder is characterized by enhanced phagocytic activity and defective T cells (zur Stadt et al., 2005). It has high expression levels in macrophages, cytotoxic T cells, and natural killer T cells (Prekeris et al., 2000; Zhang et al., 2008a), and is localized at high levels in macrophages which are actively involved in apoptosis, bearing key role of *STX11* in progression of inflammatory disorders (Zhang et al., 2008a).

The final upregulated gene in the present study, translocated promoter region to activated MET oncogene '*TPR*', is located at chromosomal location 1q25. It is an evolutionary conserved protein and directly interacts with several components of the nuclear pore complexes and is required for the nuclear export of specific mRNA molecules and proteins (Skaggs et al., 2007; Krull et al., 2010). The stress related nuclear transport of HSP is dependent on nuclear transport basket formed by *TPR* (Skaggs et al., 2007), whereas HSP is an important component of atherosclerotic disease (Benagiano et al., 2005). *TPR* protein is also involved in chromosomal stability and regulation of cell cycles as it directly interacts with mitotic arrest deficient protein complex (Lee et al., 2008a). The transport facility and cell cycle regulatory role of TPR assign pathological role to this protein.

The downregulated genes in the current study

The first downregulated gene in the current study, Rho guanine nucleotide exchange factor (GEF) 7 '*ARHGEF7*' is located at chromosome location 13q34. It interacts with the cell polarity (tumor suppressor) protein *Scribble*, and the loss or mislocalization of *Scribble* is associated with inhibition of apoptosis and enhanced

cellular transformations (Zhan et al., 2008). *ARHGEF7* modulates alternate splice transcription as well as cytoskeletal rearrangement, and cytoskeleton signalling processes (Locasale and Wolf-Yadlin, 2009). The downregulation of *ARHGEF7* in the current study maintains that alteration in cytoskeletal rearrangement and signaling are related to progression of PAD. The second downregulated transcript variant, chromosome 5 open reading frame 28 '*C5orf28*' is located at chromosomal location 5p12. The cardiovascular effects of this gene variant are as yet, unidentified.

The third downregulated gene in present study, CASP8 and FADD-like apoptosis regulator '*CFLAR*', also called caspase related inducer of apoptosis or cellular FLICE-like inhibitory protein 'c–FLIP' is located at chromosomal location 2q33–q34. It regulates apoptosis and modulates cell survival and cell death (Chen et al., 2009a). Homocysteine induces oxidative damage and apoptosis of endothelial cells; homocysteine mediates these vascular effects by inhibition of *CFLAR*. The effects of IL18 and IL2 on NK T cells are also mediated by inhibiting *CFLAR* (Huang et al., 2010). Downregulation of this gene (as in the current study) may be therefore associated with progression of vascular disease.

The gene for the myeloid/lymphoid or mixed-lineage leukemia 3 '*MLL3*' is located at chromosomal location 7q36.1. It is a member of myeloid/lymphoid or mixed-lineage leukemia (MLL) family, histone methyltransferase, and plays a key role in transcriptional coactivation (Lee et al., 2009). The histone methylases have critical roles in activating and regulating gene expression, and they interact with nuclear receptors and result in steroid hormone mediated gene activation as well (Ansari and Mandal, 2010). The *MLL3* modulated actions on steroid hormone receptor and nuclear receptors can result in cardiovascular disease (Ansari and Mandal, 2010).

The fifth downregulated gene in the current study, O-linked N-acetylglucosamine (GlcNAc) transferase '*OGT*' gene is present on X chromosome, at chromosomal location Xq13. *OGT* mediates O-GlcNAc mediated modification of proteins (O'Donnell et al., 2004; Bowe et al., 2006; Lima et al., 2010). Mutations in OGT are associated with increased apoptosis of T cells, altered expression of transcription factors, and arrest of fibroblast growth (O'Donnell et al., 2004) whereas enhanced expression of *OGT* results in increased leptin levels and insulin resistance (McClain et al., 2002). Analogous to the current study, this protein was

downregulated in previous microarray study for PAD (Fu et al., 2008). Inhibition of OGT expression is therefore positively associated with PAD progression.

The next downregulated gene, PDS5 regulator of cohesion maintenance homolog B (S. cerevisiae) '*PDS5B*' is located at chromosomal location 13q12.3. The high expression of *PDS5B* in embryonic cardiac tissue emphasizes its critical role in cardiac development during embryogenesis, and its deficiency results in cardiac failure (Zhang et al., 2009a). This protein is associated with resolution of chromatids, normal chromatid separation during mitosis, and therefore regulation of cell cycles (Shintomi and Hirano, 2009). It additionally has negative regulatory role for cell proliferation and may act as a tumor-suppressor (Denes et al., 2010).

The gene for TRAF3 interacting protein 3 '*TRAF3IP3*', also called TRAF3 interacting Jun N terminal kinase (JNK) activating modulator, is located at 1q32. This protein is an adapter molecule that regulates TRAF3-mediated JNK activation and cell growth by modulating the c-Jun N-terminal kinase (JNK) pathway (Ma et al., 2007). The inhibition of cellular proliferation, secondary to silencing of this gene, indicated significant role of this protein in cellular activation and proliferation (Ma et al., 2007).

Peripheral artery disease progression is dependent on plaque buildup in lower limb vasculature and is therefore influenced by the circulating blood cells. Studies report that progression of PAD and linked complications are associated with lower ABI and elevated red blood cell width as well as higher number of circulating white blood cells and monocytes (Violi et al., 1996; Nasir et al., 2005; Papazafiropoulou et al., 2010; Ye et al., 2011). The current study utilized peripheral blood mononuclear cells isolated from blood as blood sampling is distinctively easy in clinical settings. The literature cites that there are differences in gene expression, dependent on the cell types used for microarray analysis (Whitney et al., 2003), (Eady et al., 2005). Microarray analysis of RNA isolated from PBMC as opposed to whole blood (enriched mostly with neutrophils) reveals significant differences in gene expression patterns (Whitney et al., 2003). There are significant variations in gene expression, also, when there are differences in number of cells and types of cells used for expression studies (Whitney et al., 2003). Eady et al (2005) used only the PBMC for their study and concluded that these cells provided least variability in inter-sample expression analysis. As already mentioned many studies report that PBMC have been

reliably used for discerning gene expression differences between healthy and diseased individuals, allowing the use of PBMC for the current study.

The present study detected a large number of genes differentially expressed between PAD cases and controls. Our observation is that these genes relate to peripheral artery disease and can be used as genetic biomarkers for the diagnosis of lower extremity artery disease. Microarray studies, however, cannot readily determine whether the expression differences are the cause of the disease phenotype or simply the effect of disease. In order to assert that these genes are causing the phenotype, timeline experiments can be devised that check the expression levels of these genes in normal asymptomatic high risk individuals and correlate if levels differ when eventually the symptoms appear. Alternately the expression of upstream regulators of the differentially expressed genes in current study can provide clues whether the differences in gene expression represent the cause or just the effect of disease.

All the differentially regulated genes in the present investigation modulate biological functions as well as molecular functions. Literature search and GO annotation analysis for the significant genes in the study demonstrated relationship of genes with cellular metabolism, growth and proliferation, cell death/apoptosis, immune and inflammatory mechanisms, and insulin metabolism. A subset of differentially regulated genes between PAD cases and controls in the present study were also differentially regulated between PAD cases and controls in previous microarray studies (Evans et al., 2008; Fu et al., 2008). It emphasizes that the PAD has specific genomic signatures and that PBMC can be used for analyses of these signatures instead of using atherosclerotic vessels themselves. None of the genes in the present study were differentially regulated as shown in previous CAD microarray studies (Wingrove et al., 2008; Meier et al., 2009), to emphasize gene expression differences between PAD and CAD cases. Data inquiry of the altered gene expression in PAD cases and controls: including the *ATF3, C5orf41, ID1, KLF6, NR4A2* and *TPR* genes, and the genes/transcripts activated by these genes can be useful for gaining further insight into the molecular and genetic basis of PAD. The genes identified by the dual microarray sets can also be used as probable biomarkers of PAD and for consideration for genetic therapies.

SECTION II
CHAPTER 3: THE HOMOCYSTEINE PATHWAY
GENOMIC SIGNATURES IN CAD

SUMMARY

Cardiovascular disorders (CVD) and coronary artery disease (CAD) have significant contribution to the morbidity and mortality of heart patients. In this connection, the genes of the folate and homocysteine pathway link to the vascular disease. Presently, the relationship of homocysteine pathway gene polymorphisms with myocardial infarction/CAD was investigated by means of the relatively recent method, tetra primer allele refractory mutation system (ARMS) PCR. A total of 230 participants were recruited in the current study (129 CAD cases, 101 control subjects). Two SNPs in 5' 10' methylenetetrahydrofolate reductase (*MTHFR*), rs1801133 and rs1801131; one SNP in 5' methyltetrahydrofolate homocysteine methyltransferase (*MTR*), rs1805087; one SNP in paroxansel (*PON1*), rs662; and one in cystathionine beta synthase (*CBS*), rs5742905 were analyzed. Additionally, the role of insertion/deletion polymorphism (rs4646994) in the non-homocysteine pathway gene, angiotensin converting enzyme (*ACE*) was assessed through simple PCR. The covariates considered in the study included: blood pressure, fasting blood sugar, serum cholesterol and creatinine concentrations. The results demonstrated that the *MTHFR*, *MTR*, and the *ACE* gene alleles differed between cases and controls. The results of the logistic regression, after covariate adjustment, revealed significant relationship of the rs1801133 SNP, and the rs1805087 SNP with CAD in the additive, the dominant, and the genotype models, respectively. In recessive model however, logistic regression revealed that only *ACE* I/D polymorphism (rs4646994) was associated with CAD. Gene–gene interaction for CAD was revealed for two, three genetic polymorphisms: rs1801133 *MTHFR* SNP, rs662 PON1 SNP, and rs1805087 *MTR* SNP; and the rs1801131 *MTHFR* SNP, rs662 PON1 SNP and the *ACE* rs4646994. The latter interacting genetic polymorphisms had persistent significance with CAD after adjustment of covariates. Tetra primer ARMS–PCR effectively detected the alleles in the patient and control samples, it is a relatively faster and efficient technique that can provide better results as compared to the conventional approach. Currently, the results concluded that homocysteine pathway gene polymorphisms have significant contribution to CAD and may therefore enhance vulnerability to develop vascular disease.

INTRODUCTION

In year 2003, cardiovascular disorders accounted for over 16 million deaths worldwide and this number is expected to rise to 23 million by 2030, making CVD the leading cause of mortality (Mathers and Loncar, 2006). People in developing countries are at an increased risk to develop CAD (Yusuf et al., 2001a). Indigenous Asians, as well as Asian immigrants to other developed countries, have a higher risk of CAD, and a higher rate of CVD complications (McKeigue, 1992; Yusuf et al., 2001b; Jafar et al., 2008). Amongst the Asians, the South Asian population, particularly the residents of Indo-Pak region show evidence of elevated coronary events and greater susceptibility to CAD (Jafar et al., 2008).

Homocysteine is a sulfur containing amino acid and is the central molecule in methionine and cysteine metabolism (Welch and Loscalzo, 1998; Trabetti, 2008). Dietary methionine is the source of homocysteine and excess of methionine shifts the homocysteine from one carbon transmethylation cycle to the transsulfuration pathway. Hyperhomocysteinemia results either due to deficiency of the vitamin cofactors, or due to enzyme defects in the homocysteine cycles. Irrespective of underlying causes, hyperhomocysteinemia is associated with adverse cardiovascular effects (Chen et al., 2005; Morris et al., 2008). Some studies mark elevated homocysteine levels as a risk marker for CAD while others put emphasis on homocysteine as a risk factor and a causative agent for vascular diseases (Welch and Loscalzo, 1998; Ferretti et al., 2004; Ridker et al., 2004; Jakubowski, 2006; Helfand et al., 2009). Elevated homocysteine level is the predictor and an independent risk factor for CAD as well as PAD (Boushey et al., 1995; Graham et al., 1997; Lima et al., 2007). Various environmental factors and individual lifestyles modulate homocysteine levels. These include age, vitamin deficiency, the nutritional status of individual, CVD, body mass index (BMI), smoking and tobacco usage, hypertension, hypercholesterolemia, and the intake of coffee (Grubben et al., 2000; Urgert et al., 2000; Christensen et al., 2001; Nurk et al., 2004).

3.1 Gene variants in the homocysteine pathway

The various steps in the homocysteine metabolism are modulated by genes in homocysteine/folate pathways and their necessary cofactors. Some of the genes are

noticeably important as their activity (or loss of activity) has profound impact on homocysteine concentration as well as on downstream effects.

3.1.1 Cystathionine β synthase

It was recognized, by 1964, that elevated homocysteine levels, secondary to enzymatic defects and errors in homocysteine metabolism, resulted in early thrombo–embolic disease (Mudd et al., 1964). Thereafter deficiency of *CBS* enzyme and associated mutation was held responsible for the generalized atherosclerotic disease and demise (in 1933) of an eight year old first – documented homocystinuria patient (McCully, 1969). McCully (1969) concluded that CBS enzyme deficiency, and the resulting elevated homocysteine levels were directly responsible for the complications.

The *CBS* gene (NM_000071) is located at position 21q22.3, and has 17 exons. The cofactor for *CBS* enzyme is vitamin B6; *CBS* catalyzes conversion of homocysteine to cystathionine in an irreversible reaction in the transsulfuration homocysteine pathway. There are more than 150 mutations and polymorphism known for the *CBS* gene (Kozich et al., 2010). The important polymorphism in the gene includes T833C (rs5742905) in exon 8; this SNP lies in cis with an insertion polymorphism, 844ins68 also in exon 8. Both these SNPs are associated with diminished enzyme activity, hyperhomocysteinemia, and are related to various disease phenotypes (Franco et al., 1998a; Tsai et al., 1999; Dutta et al., 2005; Golimbet et al., 2009) and the T833C polymorphism has also been studied for association with the vascular disease (Tsai et al., 1996).

3.1.2 Methylene tetrahydrofolate reductase

The methylene tetrahydrofolate reductase '*MTHFR*' gene (NM_005957) is present at chromosomal position 1p36.3 and has 12 exons. This gene plays a vital role in the remethylation homocysteine pathway. The latest list of mutations comprises of more than 30 harmful mutations as well 9 polymorphisms in the *MTHFR* gene (http://www.ncbi.nlm.nih.gov/books/NBK6561/). Of mention, the polymorphisms and SNPs in *MTHFR* include the C677T (rs1801133, in exon 4) and A1298C (rs1801131, in exon 7); [MIM id 607093] (Frosst et al., 1995; Hanson et al., 2001; Falchi et al., 2005). These SNPs are associated with various disease phenotypes including the CAD, PAD, lipid metabolism, and ischemic stroke (Frosst et al., 1995; Szczeklik et al., 2001; Falchi et al., 2005; Laraqui et al., 2007; Al-Allawi et al., 2009; Zhang et al.,

2010a). Different genotypes at rs1801133 locus associate with differences in severity of the disease and the phenotype (Frosst et al., 1995; Kluijtmans and Whitehead, 2001; Bathum et al., 2007).

3.1.3 Methyltetrahydrofolate homocysteine methyltransferase

The methyltetrahydrofolate homocysteine methyltransferase '*MTR*' (NM_000254) is located at chromosomal position 1q43 and has 33 exons. It catalyzes the conversion of homocysteine to methionine. The most characterized SNP for this gene is A2756G (rs1805087, in exon 26); the SNP is responsible for hyperhomocysteinemia yet the causal effect of this SNP on CAD is not well characterized (Bathum et al., 2007; Laraqui et al., 2007; Vinukonda et al., 2009).

3.1.4 Paraoxonase gene

The paraoxonase gene cluster consists of three genes namely, *PON1*, *PON2*, and *PON3* at chromosomal location 7q21.3. The '*PON1*' (NM_000466) gene has 9 coding exons. Of various known SNPs in *PON1*, the A192G SNP (rs662) and M55L (rs854560) are well characterized and are associated with pro-inflammatory markers (Acampa et al., 2011). The rs662 SNP and rs854560 SNP variants in *PON1* gene are additionally significant modulators of the vascular phenotype (Mendonca et al., 2009; Mohamed et al., 2010).

3.1.5 Angiotensin converting enzyme

Angiotensin converting enzyme '*ACE*' (NM_000789) is located at chromosome 17 (17q23.3) and has 25 exons. *ACE* gene insertion/deletion polymorphism (rs4646994) is a significant modulator of CAD (Szperl et al., 2008; Zintzaras et al., 2008).The insertion allele is representative of an *alu* repetitive sequence with 287 bp difference between the 'I' allele as compared to the 'D' allele.

3.2 Methods for detection of SNPs

On the basis of the predicted SNP frequency of 1 SNP / kb of the human genome, initially, it was shown that human genome comprised of 3 million SNPs, however recent data suggests that more than 10 million SNPs exist in the human genome (Lai, 2001). According to NCBI human dbSNP data (build 131), human genome has 10.5 million submitted and 2.3 million reference SNPs. There is an extensive list of genotyping methods and techniques available for detection of SNP variants. Different research laboratories and clinical setups use specific methods for

SNP detection. A brief description of some of the widely used and important techniques is as follows:

3.2.1 Restriction fragment length polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) is the traditional approach for SNP detection. This technique is based on the foundation that different restriction enzymes (RE) recognize specific DNA sequences, cleave the target sites, and ultimately fragment the DNA. The size of the fragments depends upon whether or not the cleavage site for RE is present in the analyzed sequence. This technique allows determination of one base pair difference between the corresponding alleles. The restriction digestion analysis was initially used for detection of mutations in viruses such as human adenoma virus, and later, RFLP was used for building human genetic maps (Grodzicker et al., 1974; Botstein et al., 1980). The technique is widely used for detection of alleles for known SNPs, for SNP genotyping in various diseases, and for investigating the disease phenotypes involving hyperhomocysteinemia and homocysteine pathway gene variants (Guerzoni et al., 2009; Pasalic et al., 2009; Vinukonda et al., 2009; Lakshmy et al., 2010; Vijaya Lakshmi et al., 2011).

Certain drawbacks associated with RFLP analysis however include expensive kits, and a limited number of reactions catalyzed per kit. For RFLP analysis the SNP must either create or abolish a restriction site otherwise SNP cannot be analyzed. Additionally the reactions are lengthy, time consuming and require further processing of the samples after PCR, and are difficult to manage if the sample turnover is high (Ye et al., 2001; Okayama et al., 2004; Stefan et al., 2009; Scharrer et al., 2010).

3.2.2 Denaturing high performance liquid chromatography (DHPLC)

The method is relatively new, automated and can be used to detect sequence variants within minutes (Oefner and Underhill, 1995). In this technique, template is subjected to reverse phase HPLC with non porous alkyl column, and insertions, deletions, and single base pair mismatches are quickly and accurately detected (Oefner and Underhill, 1995; Liu et al., 1998; Yu et al., 2006). DHPLC can also be used for studies aimed at discerning the human evolution (Underhill et al., 1997; Underhill et al., 2000). The amplified PCR product is modified to yield heteroduplexes and homoduplexes; the mutated or mismatched fragments form hetroduplex, and have lower column retention as compared to the wild type or

homoduplex fragment (Oefner and Underhill, 1995; Liu et al., 1998; Yu et al., 2006; Scharrer et al., 2010).

DHPLC has been used with full accuracy for the detection of SNPs in genes associated with hypertension and CAD (Su et al., 2002; Su et al., 2003; Zhang et al., 2005). The limitation of this technique is high cost of the column, chemicals, HPLC equipment, and expertise required for operating these reactions. It is therefore less feasible for routine use especially in the less well equipped laboratories (Baris et al., 2010).

3.2.3 Direct DNA sequencing

Direct DNA sequencing is fast automated technique that can be used with great accuracy to detect the DNA sequences. It can be used for detection of known as well as unknown SNPs (Stefanius et al., 2011). Automated DNA sequencing is the modified form of Sanger dideoxy sequencing; primer is attached to the template DNA, and the DNA polymerase adds the deoxynucleoside triphosphate (dNTPs) and dideoxynucleoside triphphosphate (ddNTPs) complementary to target DNA. The strands elongate with addition of dNTP whereas incorporation of ddNTP results in termination of the reaction: termed 'single extension/dye termination sequencing' (Lee et al., 1992). The ddNTPs are fluorescently labeled and the analysis of reaction products involves capillary electrophoresis. The DNA sequences are read through laser enhanced fluorescence recognition technique, and can accurately identify the DNA sequences and variations/mutations in the DNA sequences (Rieder et al., 1998; Guttman et al., 2003). The modification of this procedure can be used for multiplex SNP typing (Blazej et al., 2003; Zhou et al., 2005).

Direct sequencing has been reliably applied to SNP genotyping in cancer, essential hypertension and CAD (Jiang et al., 2001; Li et al., 2004; Villanueva et al., 2008; Zhang et al., 2010b). The method is highly accurate for SNP detection in comparison to the traditional RFLP analysis (Davis et al., 2007). The disadvantage with this technique is the complex and expensive instrumentation; and high expertise required for these reactions (Lee et al., 1992; Zhou et al., 2005; Baris et al., 2010).

3.2.4 Real time analysis (RT–PCR)

Real time PCR analysis is based on detection of the fluorescence emitted by the fluorescent probes. For real time analysis, the fluorescent assays are of two main subtypes; one fluorescence method uses dye for double stranded DNA (Sybr Green®)

whereas the other method uses fluorescent dye for single stranded DNA annealing (Taqman®, and Molecular Beacon®). The Sybr Green method is simple fluorescence detection real time analysis, it (Sybr Green) emits fluorescence after intercalating the double stranded DNA. The disadvantage is that it gives fluorescence for specific as well as non specific DNA hybridization. This drawback can be overcome by the correction for PCR melting temperature (specific hybridization occurs at higher temperature than non specific hybridization) (Morrison et al., 1998). The specific melting curve based analysis greatly enhances the specificity of Sybr Green based real time analysis (Ririe et al., 1997; Nicolas et al., 2002).

The Taqman® and Molecular Beacon® are single strand DNA detection techniques and both employ fluorescence resonance energy transfer (FRET). The basic 'primer' used in both of these methods has two reporter proteins; a 'reporter' fluorophore (R) at 5' end and a 'quencher' (Q) at 3' end. For Taqman analyses, the unlabelled primer binds DNA upstream, whereas the fluorophore primer binds specific DNA, downstream (Holland et al., 1991; Heid et al., 1996; Arya et al., 2005). The polymerase extends the (unlabelled) upstream primer and the polymerase's 5' – 3' exonuclease activity disrupts the (downstream) fluorophore primer, the reporter fluorophore therefore emits fluorescence which is easily detectable. If the template sequence is non–specific, the primer remains unbound and there is no detectable fluorescence as the quencher and the reporter are in close proximity (Holland et al., 1991; Heid et al., 1996; Arya et al., 2005). Molecular Beacon analysis similarly uses dual fluorophore primer but it is designed so as to form a hair pin structure. When the primer is not hybridized to its specific template DNA, the reporter and quencher are in close proximity and there is no fluorescence, whereas when the primer binds specific template DNA and the hairpin opens, the two fluorophores separate and the reporter emits fluorescence proportional to the specific target hybridizations (Tyagi and Kramer, 1996; Vet et al., 2002).

Taqman probe based and molecular beacon based qRT. PCR, owing to their specific primer design and differences in annealing temperatures (for perfect matched and mismatched primer), have been accurately applied to SNP genotypings (Shi, 2001; Shi, 2002; Vet et al., 2002; Niederstatter et al., 2006). Real time analysis can be employed for gene expression, protein analysis, and microbial analysis (Vet et al.,

2002; Deutsch et al., 2005; Yesilkaya et al., 2006; Jones et al., 2009; Riches et al., 2010).

3.2.5 Single strand confirmation polymorphism (SSCP) genotyping

The gel electrophoresis of double stranded DNA is dependent on the length of the DNA product and has no difference in migration based on single base pair changes. The single stranded DNA products however form highly sensitive and specific loops and folds depending on their sequences, so that one base pair substitution results in alternate structure of single stranded DNA. This difference in confirmation can be detected by gel mobility shift observed by subjecting the restriction digested, single stranded segment to polyacrylamide gel electrophoresis (Orita et al., 1989; Walsh et al., 1995). The drawback with this method is that DNA has to be digested into segments prior to SSCP analysis (Orita et al., 1989). Originally applied for the detection of sequences up to 150 bp in length, with further modifications, SSCP could accurately detect single base pair sequence differences in 400 to 500 bp primers (Markoff et al., 1997).

The SSCP analysis and slight improvements in this method (such as multicolor fluorescent labeling of primers) have been widely and accurately used for SNP analysis in genes and for various disease conditions (Kozlowski and Krzyzosiak, 2001; Mitui et al., 2003; Hata et al., 2006; Chen et al., 2009b).

3.2.6 Matrix assisted laser desorption ionization (MALDI) time of flight (TOF) mass spectrometry technique

The MALDI–TOF MS technique is based on ultraviolet laser desorption of organic compounds and the high sensitivity to detect single and double ionic molecular compounds (Karas and Hillenkamp, 1988). In this method, the protein or nucleic acid (to be detected) is added to the solution in matrix, and the matrix crystal is then subjected to laser irradiation. The proteins or nucleic acids are ionized and desorbed into gas phase and are therefore rapidly and easily detected, depending on ionization differences and molecular weight (mass/charge ratio) (Karas and Hillenkamp, 1988; Griffin et al., 1999; Griffin and Smith, 2000b).

Several modifications in MALDI–TOF MS analysis have been reported that have enhanced the SNP detection potential of this technique (Sun et al., 2000; Mengel-Jorgensen et al., 2004; Boontha et al., 2008; Millis, 2011). The MALDI–TOF MS approach has been reliably applied for SNP genotyping for sequence

determination as well as for the disease stratification (Griffin and Smith, 2000a; Hung et al., 2002; Mengel-Jorgensen et al., 2004; Pinto et al., 2010; Yu et al., 2011). The disadvantages of MALDI TOF MS include expensive instruments and chemicals used, and the expertise required for this method (Baris et al., 2010).

3.2.7 Microarray based SNP typing

The microarray based 'Genome Wide Association Study' (GWAS) SNP typing is the only method for detecting hundreds of thousands of SNPs in the human genome simultaneously. The foundation for GWAS rests on identification of millions of SNPs in human genome, through the Human Genome Project (Lai, 2001; Lander et al., 2001; Venter et al., 2001; The International HapMap Project, 2003).

The microarray platforms for SNP analysis include the Affymetrix®, Illumina®, and Agilent® microarrays. The Affymetrix 'SNP' and 'copy number variant' (CNV) analyzing microarrays include: Human Mapping 10K 2.0 Array (10,000 SNPs); Human Mapping 100 K set (116,204 SNPs); Human Mapping 500 K set (2 arrays combined with more than 500,000 SNPs); Human SNP array 5.0 (500,568 SNPs and 420,000 probes for CNV analysis); and Human SNP array 6.0 (queries 1.8 million markers including 906,600 SNPs and probes for 946,000 CNV) (http://www.affymetrix.com), (Schosser et al., 2010). The Illumina multiple sample per Beadarray® variety of SNP microarrays include: Human cytoSNP-12 (300,000 SNPs); Human 660W-Quad (more than 658,000 SNPs); Human OmniExpress (more than 700,000 SNPs); Human 1M-Duo (greater than 1.0 million SNPs); Human Omni1-Quad (greater than 1.0 million SNPs and probes); and Human Omni1S-8 and Human Omni2.5-Quad (more than 1.25 million and 2.5 million SNPs and the probes respectively) (http://www.illumina.com). The Agilent genomewide SNP analysis arrays include: Human Genome CGH Microarray 4 x 44 K (42,494 target features); Human Genome CGH Microarray 105A (99,026 target features); Human Genome CGH Microarray 244A (236,381 target features); SurePrint G3 Human CGH Microarray 8x60 K (55,077 target features); SurePrint G3 Human CGH Microarray 4x180 K (170,334 target features); SurePrint G3 Human CGH Microarray 2x400 K (411,056 target features); and the SurePrint G3 Human CGH Microarray 1x1M (963,029 target features) (http://www.genomics.agilent.com). The Affymetrix and Illumina microarrays are by far the most common SNP genotyping platforms and

have been used for universal SNP analyses (Arking et al., 2010; Lind et al., 2010; Muglia et al., 2010; Vogler et al., 2010; Kim et al., 2011).

The DNA pooling can greatly reduce the GWAS cost as the samples are pooled based on defined characteristics and pooled samples are applied communally in a single chip thereby greatly reducing the cost as compared to individual hybridizations (Melquist et al., 2007; Homer et al., 2008; Schosser et al., 2010; Szelinger et al., 2011). The disadvantages with microarrays include the considerably expensive microarray scanners, reagents, and cost of microarrays themselves, limiting the universal use of this technique for SNP analysis (Szelinger et al., 2011).

3.2.8 Amplification/Allele refractory mutation system (ARMS/AS–PCR)

This technique is a simple allele detection technique that circumvents the restriction digestion of the amplified PCR product by the use of alternate primers for the allelic discrimination. Unlike the RFLP analysis (that requires site recognition by restriction enzymes), this technique can detect any mutation in the entire genome, and reliably detect hetrozygotes from homozygotes (of either allele) by simple inspection of the gel images (Newton et al., 1989a). There are two primers designed for the alleles, one primer has the normal nucleotide and the other the polymorphic nucleotide at the 3' end; with a deliberate base pair change near the 3' end to further enhance specificity of the primers. With complementary base pairing of specific primer, the PCR continues; with non-complementary base pairing, the primer is refractory to PCR amplification and the amplification product is not seen (Newton et al., 1989a; Okayama et al., 1989). The reaction for single allele discrimination is performed in two tubes; the common primer is added in both the tubes, whereas the normal and polymorphic primers are added separately in the two tubes. The gel image analysis showing the amplification band for either tube represents homozygosity, whereas the band for both tubes represents hetrozygosity.

ARMS–PCR has been associated with SNP analysis and has been successfully applied for detecting allelic differences in a variety of disease phenotypes (Newton et al., 1989b; Littlejohn et al., 2008; Liu et al., 2010; Ghandri et al., 2011). Despite the fact that allele discrimination by the allele refractory mutation analysis is reliable and fast, yet the significant complexity is the primer designing and use of two separate reaction tubes for single allele discrimination.

3.2.9 Tetra primer allele refractory mutation system (T–ARMS) PCR

Tetra primer ARMS–PCR is a relatively new technique and (unlike RFLP), can be used to genotype practically any SNP in the entire human genome (Ye et al., 2001; Okayama et al., 2004). This procedure, for detecting alleles at a locus, is dependent on two pairs of primers; one pair consists of the external (outer) primers that amplify a larger outer DNA amplicon and a second pair consisting of internal (inner) allele specific primers. The inner/internal primers are designed in a way that they amplify DNA fragments of different sizes with their respective external primer; the size differences between the inner bands therefore correspond to the two different alleles (Ye et al., 2001; Okayama et al., 2004).

Advantages of this technique are that it is fast, accurate, high throughput technique and a single PCR reaction can resolve the genotype (Ye et al., 2001; Galmozzi et al., 2010; Kim et al., 2010; Peruzzi et al., 2010) and in multiplex analysis more than one locus can be analyzed simultaneously in one PCR reaction (Piccioli et al., 2006; Yang et al., 2007). Tetra primer ARMS–PCR can reliably detect polymorphism where RFLP analysis may occasionally give erroneous results (Ward et al., 2006). This method can reliably detect the genotypes in a relatively less equipped laboratory, and in the laboratory setups with high sample turnover. The disadvantage of this technique is that it may require great deal of troubleshooting. The internal primers have lesser amplification efficacy due to the mismatches; and the internal primers yield non–specific bands. These drawbacks can be overcome and specific priming is achieved by increasing the concentration of internal as compared to outer primers; using gradient thermal cyclers; touchdown reactions where initial temperature for tetra primer ARMS–PCR is kept high and then the temperature is gradually reduced to optimal annealing temperature; or by using hotstart polymerase for the tetra primer ARMS reaction (Ye et al., 2001; Okayama et al., 2004). To the best of author's knowledge this technique has not been used for detection of alleles in the homocysteine pathway.

In the present study the individual alleles of SNPs in homocysteine pathway genes were analyzed by tetra primer ARMS–PCR. The aim of the study was to determine the effects of the specific alleles with CAD, individually as well as through the gene–gene interactions (epistasis).

MATERIALS AND METHODS

3.3.1 Study Design and participant recruitment

The study was a case control association study. Joint approval was acquired from the "Institutional Review Board", Quaid-i-Azam University Islamabad and from the "Pakistan Medical Research Council", Constitution Avenue, Islamabad. Written, informed consents were obtained from all participants. The study comprised of 129 CAD/MI patients (males 107) recruited from the medical unit at Rawalpindi General Hospital, Rawalpindi and from the cardiology units in Poly-technique Hospital, Islamabad, and the Postgraduate Institute of Medical Sciences, Islamabad. The control group was recruited from medical out patients department of the Rawalpindi General Hospital and incorporated 101 (males 57) hypertensive subjects. The mean age (± SD) for cases (patients of coronary artery disease) was 55.03 ±6.29 years and for the controls (normal subjects), the mean age was 56.36 ±8.16 years respectively. The enrolled CAD patients and controls were from similar socioeconomic background and belonged to the same geographical region. The demographic data of participants is provided as Table 3.1.

3.3.2 Participant case histories and the presenting complaints

The patient group complained of severe chest pain, breathlessness, sense of heaviness in the chest, and limitation in bodily activity. The symptoms were persistent and were present for greater than one hour. The past history was positive for chest discomfort, occasional breathlessness and a few patients had previous history of abdominal pain as well. In the patient and control group, the risk factor associated with vascular disease such as cigarette smoking, high BMI, hypercholesterolemia and hypertension were present. The participants were on different antihypertensive medications including the calcium channel blockers, beta blockers, diuretics, and aspirin. The cases presented to the emergency departments of the hospitals within three hours of the initiation of symptoms.

3.3.3 Participant baseline parameters and diagnosis

On presentation the general physical examination of the cases and controls was carried out for pulse, blood pressure, temperature and respiratory rate. Blood pressure (mmHg) was measured with a conventional mercury sphygmomanometer.

Table 3.1. Anthropomorphic and serum parameters of cases and control

Sample Characteristics	Controls	Cases	P value
Participants	101	129	-
Age, years	56.36 ±8.16	55.03 ±6.29	0.16
Gender	57 (56)	107 (82)	<0.001
Systolic B.P. (mmHg)	136±20.2	128±16.0	0.001
Diastolic B.P. (mmHg)	90±11.3	84±11.5	<0.001
BMI	31.1±5.9	31.3± 5.1	0.75
Smoking status	56 (55)	79 (61)	N.S.
S. Cholesterol (mg/dl)	210±44	219±54	0.17
Fasting blood sugar (mg/dl)	115±30	106±28.3	0.04
S. Creatinine (mg/dl)	1.27±0.42	1.40±0.50	0.03
β / Ca++ channel blockers(%)	59 / 35	67 / 33	N.S.

Values are expressed as either 'mean ± standard deviation' or as 'n (%)'. The row for gender represents values for males. The p-values for variables computed by ttest.

Serial electrocardiography (ECG) of the patients was performed at presentation to emergency department (ER) and patients with positive ECG changes were included as cases. The participants in the control group had no symptoms of ischemia and had no signs of ischemia or infarction on their respective ECG tracings. The study participant ages, as well as the tabulated blood pressure readings and serum fasting glucose, cholesterol and creatinine levels for the cases and the controls respectively, represent values at the time of blood sampling.

3.3.4 Blood Sampling, serum preparation, and analyses of biochemical parameters

Fasting blood samples were obtained from participants in plain tubes for serum preparation, and in EDTA vacutainers for extraction of genomic DNA. The serum parameters included blood sugar (mg/dl), cholesterol (mg/dl), and creatinine (mg/dl), (covariates). Serum samples were stored at –20°C until analyzed by commercially available kits (AMP Diagnostics, AMEDA Labordiagnostik GmbH).

3.3.5 Extraction of genomic DNA

DNA was extracted by standard phenol chloroform extraction protocol. Briefly 750 µl of whole blood was added to 1.5 ml eppendorf tubes (Eppendorf Hamburg Germany). An equal volume (750 µl) of solution A was added to the blood in tube. Solution A comprised of sucrose (0.32 M, Sigma, St. Louis, MO); Tris (10 mM, Sigma); $MgCl_2$ (5 mM, BDH Chemicals UK); and Triton X 100 (1%, Sigma, St. Louis, MO). The contents were mixed and left at room temperature for 15 minutes. The sample was run at 13,000 rpm for 1 minute. The supernatant was discarded and the pellet was resuspended in 400 µl of solution A. The sample was again centrifuged at 13,000 rpm for 1 minute, the supernatant was discarded and the pellet was resuspended in 400 µl of solution B that contained Tris (10 mM, Sigma, St. Louis, MO); NaCl (400 mM, BDH Chemicals UK); and EDTA (2 mM, Sigma). To the tube mix, after resuspending the pellet, 12 µl of 20% SDS (BDH Chemicals UK) and 6 µl Proteinase K (Fermentas, Glen Burnie, Maryland) were added and the sample was kept overnight at 37°C.

The following day equal volumes of Solution C (Phenol, BDH Chemicals, UK) and freshly made solutions D, containing 24:1 volumes Chloroform: Isoamyl alcohol (BDH, Chemicals UK), were mixed and 500 µl of this mix was added to the sample and centrifuged at 13,000 rpm for 10 minutes. The supernatant was carefully

collected in a new eppendorf tube, 500 µl of solution D was added and the sample was centrifuged at 13,000 rpm for 10 minutes. The supernatant was collected in a new eppendorf tube and 55 µl of 3M sodium acetate solution (Sigma, St. Louis, MO), and 500 µl refrigerated Isopropanol were added, tube was shaken several times, and sample was centrifuged at 13,000 rpm for 10 minutes. The supernatant was removed and the DNA pellet was washed with 70% chilled ethanol and dried in a DNA concentrator (Eppendorf DNA Concentrator 5301, Eppendorf, Hamburg, Germany). The dried DNA was mixed with 200 µl of TE buffer (Tris-EDTA buffer, Invitrogen, Carlsbad, CA). The DNA was diluted to 50 ng/ µl for the PCR based DNA amplifications and stored at 4°C.

3.4 PCR based SNP analysis

The current study protocol comprised of the primer design for PCR based amplification of the genomic DNA segments, restriction digestion of a limited number of samples to determine the genotypes, tetra primer ARMS–PCR analysis for all the SNPs studied in all the samples, and identification of the SNPs that are significantly associated with CAD.

3.4.1 Primer design for SNP detection

The genomic DNA sequences for all the genes in the study were accessed at the University of California Santa Cruz (UCSC) Genome Browser Database (http://genome.ucsc.edu/cgi-bin/hgGateway). For the design of allele specific and outer primers (4 primers/each SNP) for the tetra primer ARMS–PCR, the genomic sequence was submitted to the webpage at http://cedar.genetics.soton.ac.uk. The designed primers were slightly modified to get the optimal lengths of the allele specific bands for unambiguous visualization. The primers were ordered from Integrated DNA Technologies (IDT Inc. Coralville, Iowa).

3.4.2 Restriction digestion analysis of the SNPs

For the RFLP analysis, the outer primers designed (for tetra primer ARMS analysis) for each of the 5 SNPs, were used for amplification of genomic DNA segment containing the SNP. For the analysis, samples were randomly selected from the CAD and normal subject categories. The reaction for each SNP was carried in a total volume of 25 µl. The template DNA was 50–100 ng, 2.5 µl of the 10 x buffer, 2.0 µl of 25 mM $MgCl_2$, 50 ng of each of the two primers and 1.5U Taq polymerase (Fermentas Inc. Glen Burnie, MD). The PCR reaction had following steps: the

denaturation at 95°C for 7 min; followed by '36 cycles' of 95°C for 45 sec, the annealing steps at the annealing temperatures for individual SNPs (58°C for rs1801133, rs1801131, and rs5742905; 57°C for rs662; and 50°C for rs1805087) for 45 sec and extension step at 72°C for 1 min; followed by the final extension at 72°C for 7 min. The PCR reactions were run in a T1 thermal cycler (Biometra GmbH, Goettingen Germany). After the PCR, 5 µl of the amplified products were mixed with 3 µl of bromophenol blue and run on a 1% agarose gel.

The restriction digestion was performed for the five SNPs individually with different parameters and temperature specifications for each enzyme. For all the 5 SNPs, 10 µl of PCR amplified products were mixed with 18 µl of PCR grade water, 2 µl of specific buffer (supplied with the restriction enzymes) and 2 µl of specific restriction enzyme. The restriction enzymes included: HinfI for rs1801133; MboII for rs1801131; BsrI for rs5742905; AlwI for rs662; and HaeIII for rs1805087. The buffers included: Buffer R for HinfI (10 mM Tris-HCL (pH 8.5), 10 mM $MgCl_2$, 100 mM KCL, and 0.1 mg/ml BSA); Buffer B for MboII (10 mM Tris-HCL (pH 7.5), 10 mM $MgCl_2$, 0.1 mg/ml BSA); Buffer B for BsrI (10 mM Tris-HCL (pH 7.5), 10 mM $MgCl_2$, 0.1 mg/ml BSA); the Tango® buffer for AlwI (33 mM Tris-acetate (pH 7.9), 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/ml BSA); and Buffer R for HaeIII (10 mM Tris-HCL (pH 8.5), 10 mM $MgCl_2$, 100 mM KCL, and 0.1 mg/ml BSA). The products were digested overnight with the restriction enzymes, and incubated at individual temperatures. The temperatures for the overnight incubations for the individual SNPs were: 37°C for rs1801133, and rs1801131 respectively; 65°C for rs5742905; 55°C for rs662; and 37°C for rs1805087. After overnight digestion, the digested products were visualized on a 1% agarose gel and visualized using the Gel Doc XR System (Bio-Rad, Hercules CA 94547).

3.4.3 Primer Sequences for *MTHFR*

MTHFR rs1801133 SNP

The primers include internal forward: 5'–aggagaaggtgtctgcgggcgt–3', internal reverse: 5'–aagaaaagctgcgtgatgatgaaatagg–3', the external forward primer was: 5'– aagcatatcagtcatgagcccagcc–3', and external reverse: 5'–gggaagaactcagcgaactcagcac–3'. The underlined bases represent the deliberate mismatches. The *T*m for the specified primers were 70°C, and 67°C for internal forward and reverse primers; 68°C and 68°C for the two external primers respectively.

MTHFR rs1801131 SNP

The primers include internal forward: 5'–ggggaggagctgaccagtgagga–3', internal reverse: 5'–aaagaacgaagacttcaaagacacctg–3', external forward and external reverse primers were 5'–gagtcaggggcagaatttacaggaatg–3' and 5'–ttctccctttgccatgtccacag–3' respectively. The underlined bases represent the deliberate mismatches. The *T*m for the specified primers were 70°C, 64°C, 67°C and 67°C for the primers respectively.

3.4.4 Primer Sequences for *MTR*

MTR rs1805087 SNP

The primers include internal forward primer: 5'–tggaagaatatgaagatattagacatga–3, internal reverse: 5'–acttaccttgagagactcataattgc–3', external forward: 5'–gtgttatcagcattgaccattactac–3', and external reverse: 5'–gaagacctctgatttgaactagaaga–3' respectively. The underlined bases represent the deliberate mismatches. The *T*m for the specified primers was 58°C for all four primers.

3.4.5 Primer Sequences for *CBS*

CBS rs5742905 SNP

The primers were internal forward primer: 5'–agccgcgccctctgcagataat–3', internal reverse primer: 5'–gacccttcgggatccacccaag–3', external forward primer: 5'–cgccctgcactgaacatttaggtcat–3', and the external reverse: 5'–cagtgtgagggtgagttacaggctgc–3'. The underlined bases represent the deliberate mismatches. The primer *T*m was 69°C for the four primers.

3.4.6 Primer Sequences for *PON1*

PON1 rs662 SNP

The primers include internal forward primer: 5'–tcactattttcttgacccctacttccg–3', the internal reverse primer: 5'–taaacccaaatacatctcccaggctt–3', external forward and external reverse primers: 5'–tgttccattatagctagcacgaaggc–3', and 5'–tcagagagttcacatacttgccatcg–3' respectively. The underlined bases represent deliberate mismatches. The *T*m for the specified primers for this SNP was 65°C for all four primers respectively.

3.4.7 Primer Sequences for *ACE* variant amplification

ACE insertion/deletion polymorphism

For the ACE gene rs4646994, the primers used were forward primer: 5'–ctggagaccactcccatcctttct–3', and the reverse primer: 5'–gatgtggccatcacattcgtcagat–3'. The primer *T*m was 64°C for the forward and 66°C for the reverse primer respectively. As this polymorphism is insertion/deletion and not single nucleotide polymorphism, simple PCR instead of the T–ARMS–PCR was undertaken for analysis of this allele.

3.4.8 Tetraprimer ARMS–PCR analysis

The reaction was performed in a total volume of 25 µl containing 50–100 ng of template DNA, 50–100 ng of the outer primers (concentration dependent on best visualizations of bands), 100–150 ng of allele specific primers (concentration dependent on best visualizations of bands), 2.5 µl of 10 x buffer, 2.0 µl of 25 mM $MgCl_2$, 0.2 mM dNTPs, and Taq polymerase 1.5U (Fermentas Inc. Glen Burnie, MD). Touchdown reactions were performed for the *MTHFR* and *CBS* SNPs (due to individual differences in the *T*m of the primers). The first annealing touchdown step was at 67°C, the temperature was decreased 1° every two cycles to final 58°C for rs1801133 and rs5742905 SNPs, and to final 56°C for rs1801131. The subsequent steps for rs1801133, rs5742905, and rs1801131 were carried at these annealing temperatures for the remaining cycles. For rs662, annealing temperature was 57°C, and for rs1805087 it was 50°C (both without touchdown). The PCR reaction had the following steps: the denaturation at 95°C for 7 min; followed by 41–45 cycles of 95°C for 45 sec, the annealing steps at the annealing temperatures (as mentioned above) for 45 sec and extension steps at 72°C for 1 min; followed by the final extension at 72°C for 7 min. The method by Rigat et al. (1992) was used for analyzing the *ACE* I/D polymorphism. For the visualization of tetra primer ARMS–PCR products, 15 µl of the amplified PCR products were either run on a 2 % agarose gel or 8 % non denaturating polyacrylamide gel (PAGE). The images from the gels were captured with a Gel Doc XR System (Bio-Rad, Hercules CA 94547).

3.4.9 Statistical analysis

Analyses were carried out for all the SNPs under four genetic models; the additive, dominant, genotype, and the recessive genetic models. All the SNPs were subsequently analyzed by unconditional logistic regression. The results for genotype analysis and logistic regression analysis were adjusted for the covariates (age, gender, systolic blood pressure, diastolic blood pressures, fasting blood sugar, serum

cholesterol and creatinine). The best fitting genetic model was the one that exhibited the highest odds/likelihood with respect to the disease. Gene–gene interaction (epistasis) analysis was reviewed as well, with addition of all SNPs in analysis and after adjustment for the covariates. A probability value $p < 0.05$ was considered statistically significant difference. For a more strict statistical scrutiny, the results were also subjected to Bonferroni multiple correction and a $p < 0.0083$ was considered statistically significant difference. The R statistical analysis package R.2.11.1 was used for the SNP analysis.

RESULTS

3.5.1 Baseline profile of the covariates

Mean systolic blood pressures (mean±SD) were 136±20.2 mmHg and 128±16.0 mmHg (p=0.001) for controls and cases respectively. Diastolic blood pressures for controls and cases were 90±11.3 mmHg and 84±11.5 mmHg (p<0.001) respectively. Fasting blood sugar levels in controls and cases were 115±30 mg/dl and 106±28.3 (p=0.04), respectively. Mean values of serum cholesterol were 210±44 mg/dl and 219±54 mg/dl in controls and cases (p=0.17) respectively; whereas mean serum creatinine levels for controls and cases were 1.27±0.42 mg/dl and 1.40±0.50 mg/dl (p=0.03) respectively. The average systolic blood pressure of cases at the time of presentation was 134±21 mmHg and the diastolic blood pressure was 83±17 mmHg. The lower systolic and diastolic blood pressure and fasting blood glucose levels at the time of sampling are representative of critical care in the coronary care units. The rate of anti-hypertensive medication, for β blockers was 67% and 59%, for Ca^{++} channel blockers was 33% and 35%, and usage for diuretics was 50% and 55% for the cases and controls respectively. In the current study, although the studied covariates were not included as outcome variables, still logistic regression analysis of the covariates, without the polymorphisms, was carried out to find association with artery disease. There was significant association with gender (p<0.001), systolic and diastolic blood pressures (p<0.01), and serum creatinine (p<0.006), respectively.

3.5.2 Results from restriction digestion and tetra primer ARMS–PCR

For determination of the genotypes, restriction digestion was carried out in a subset of samples to verify the genotypes, and to confirm tetra primer results. The PCR amplified, restriction digested, and tetra primer ARMS–PCR visualized images are illustrated in Figs. 3.1 – 3.11. The bands after restriction digestion for rs1801133 and rs1801131 *MTHFR* polymorphisms are shown in Figs. 3.1 – 3.2, and tetra primer ARMS–PCR bands for the *MTHFR* polymorphisms are shown in Figs. 3.3 – 3.4. The results for rs5742905 *CBS* and rs662 *PON1* restriction digestion and tetra primer ARMS–PCR are shown in Figs. 3.5 – 3.6, and Figs. 3.7 – 3.8 respectively. Figs. 3.9 – 3.10 illustrate the restriction digestion analysis and tetra primer ARMS–PCR for *MTR*

rs1805087. The final figure, Fig. 3.11 (a, b) shows gel images for the amplified *ACE* gene insertion deletion polymorphism.

3.5.3 Correlation of studied SNPs with the Disease Status

Three polymorphisms; rs1801133, rs1805087, and rs4646994 had significant association with CAD under the additive and dominant models after adjusting for the covariates. Three SNPs; rs1801133, rs1801131, and rs1805087 had significant association with CAD under the genotype model. Under the recessive model, the ACE gene variant was found associated with CAD (Table 3.2).

3.5.4 Unconditional logistic regression SNP modeling for CAD

On logistic regression analysis, the *MTHFR* rs1801133 and *MTR* rs1805087 SNPs were found associated with CAD under additive, dominant, and genotype models after adjusting for covariates (Table 3.3). Under recessive model, again only the *ACE* rs4646994 was associated with CAD.

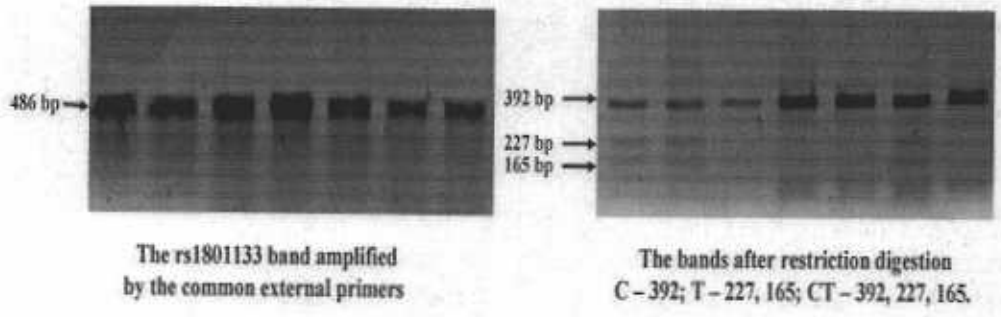

Fig. 3.1. Restriction digestion analysis of SNP rs1801133 *MTHFR* gene. (a) PCR products amplified by the outer primers [same as for the tetra-primer ARMS PCR analysis]. (b) Products after restriction digestion with Hinf1.

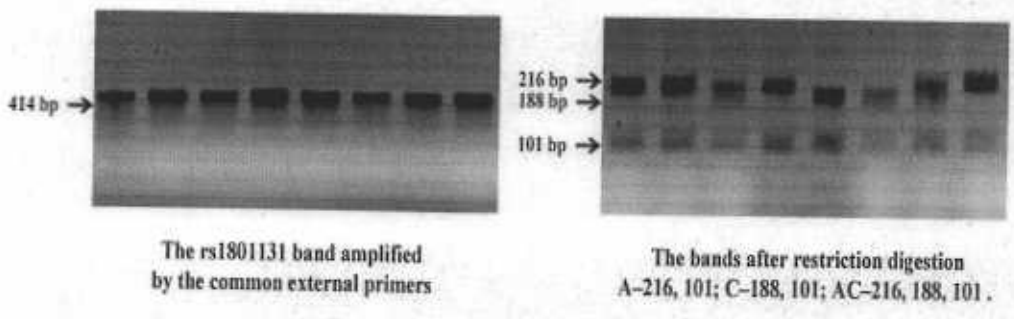

Fig. 3.2. Restriction digestion analysis of SNP rs1801131 *MTHFR* gene. (a) PCR products amplified by the outer primers [same as for the tetra primer ARMS – PCR analysis]. (b) Products after restriction digestion with MboII.

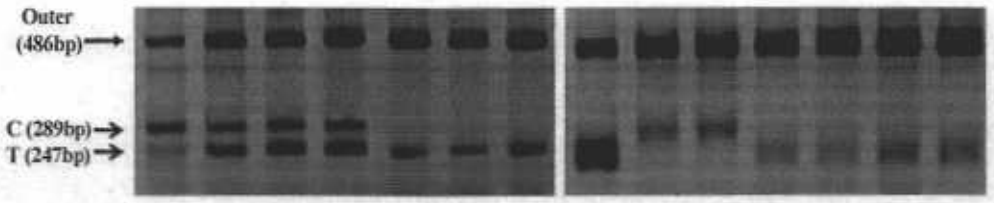

Fig. 3.3. Tetra primer ARMS–PCR SNP genotyping for rs1801133 *MTHFR* gene. The PCR products amplified by the two outer primers are represented as 486 bp, whereas the C and T allele specific PCR products are represented as the 289 bp and 247 bp respectively.

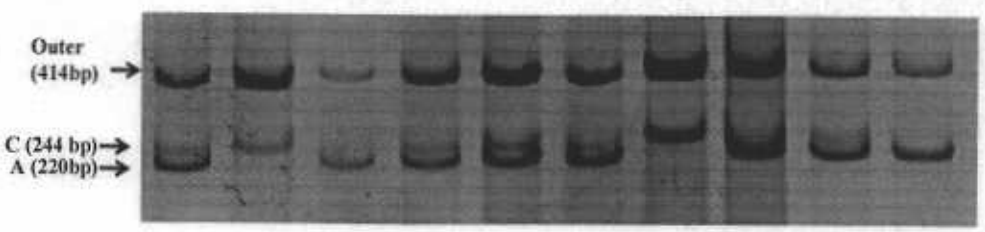

Fig. 3.4. Tetra primer ARMS–PCR SNP genotyping for rs1801131 *MTHFR* gene. The PCR products amplified by the two outer primers are represented as 414 bp, whereas the C and A allele specific PCR products are represented as the 244 bp and 220 bp respectively.

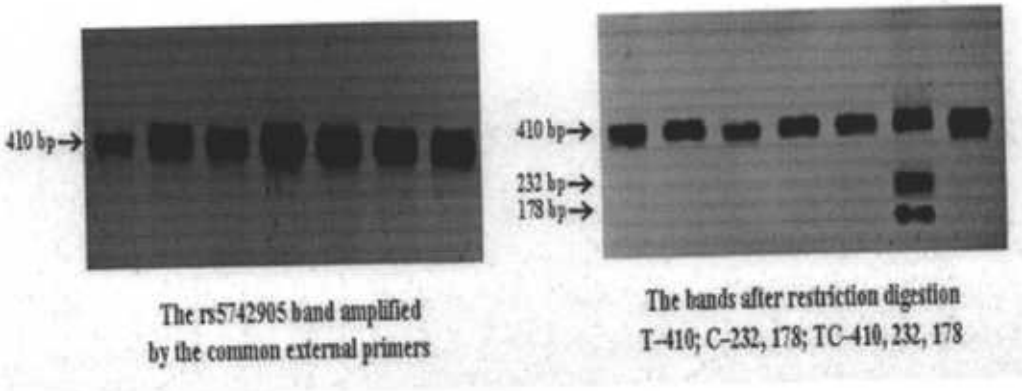

Fig. 3.5. Restriction digestion analysis of SNP rs5742905 *CBS* gene. (a) PCR products amplified by the outer primers [same as for the tetra primer ARMS–PCR analysis]. (b) Products after restriction digestion with BsrI.

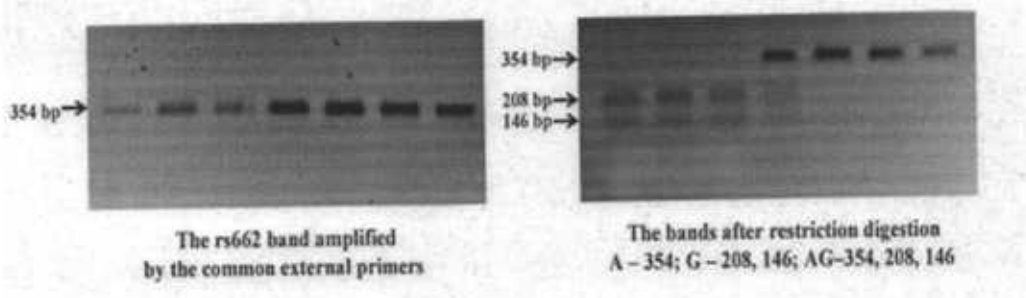

Fig. 3.6. Restriction digestion analysis of SNP rs662 *PON1* gene. (a) PCR products amplified by the outer primers [same as for the tetra primer ARMS–PCR analysis] (b) Products after restriction digestion with AlwI.

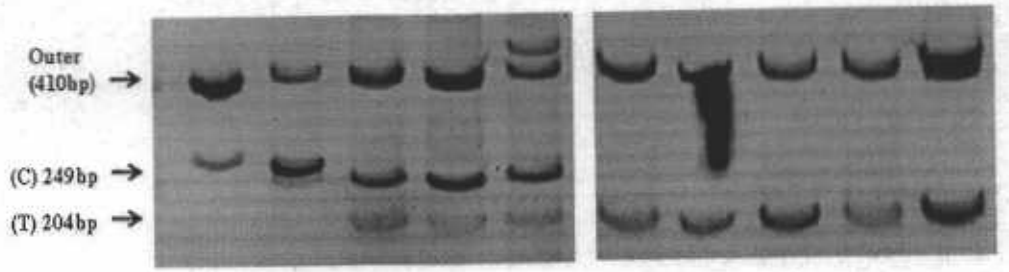

Fig. 3.7. Tetra primer ARMS–PCR SNP genotyping for rs5742905 *CBS* gene. The PCR products amplified by the two outer primers are represented as 410 bp, whereas the C and T allele specific PCR products are represented as the 249 bp and 204 bp respectively.

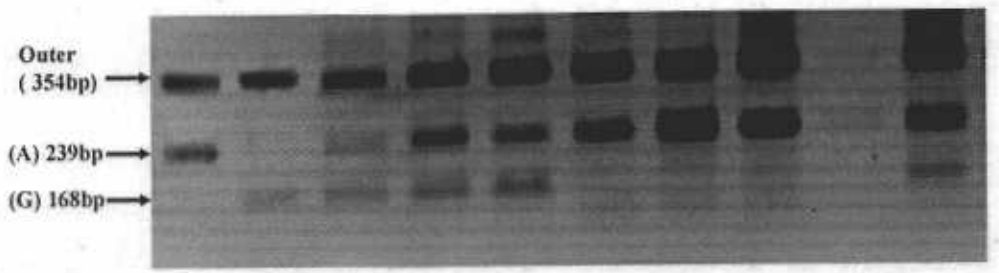

Fig. 3.8. Tetra primer ARMS–PCR SNP genotyping for rs662 *PON1* gene. The PCR products amplified by the two outer primers are represented by 354 bp, whereas the A and G allele specific PCR products are represented by the 239 bp and 168 bp respectively.

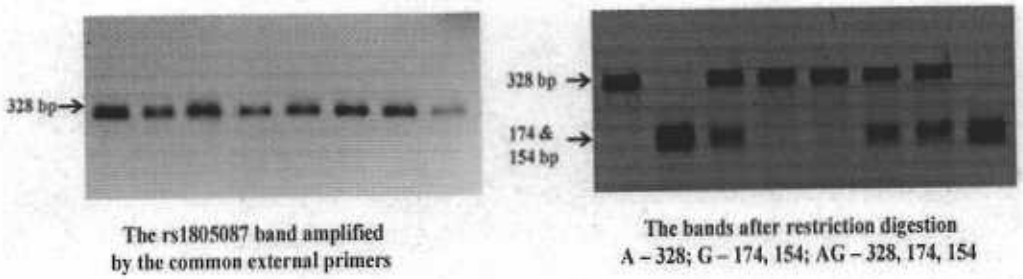

Fig. 3.9. Restriction digestion analysis of SNP rs1805087 *MTR* gene. (a) PCR products amplified by the outer primers [same as for the Tetra primer ARMS–PCR analysis] (b) Products after restriction digestion with HaeIII.

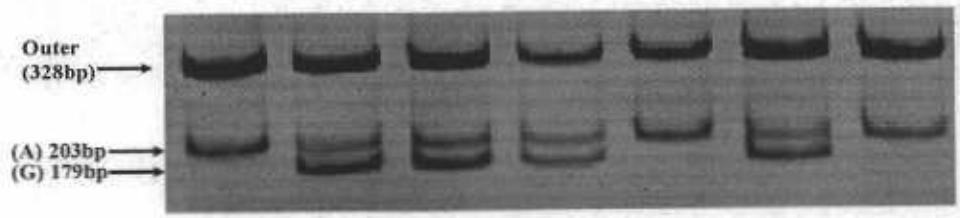

Fig. 3.10. Tetra primer ARMS–PCR SNP genotyping for rs1805087 *MTR* gene. The PCR products amplified by the two outer primers are represented by 328 bp, whereas the A and G allele specific PCR products are represented by the 203 bp and 179 bp respectively.

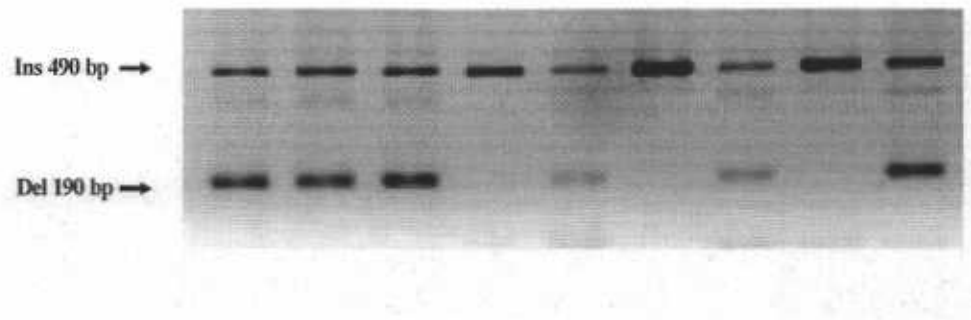

Fig. 3.11a. The amplified products for the *ACE* gene rs4646994 alleles. The insertion alleles represent 287 bp alu repetitive sequence and are represented as 490 bp PCR products whereas the deletion alleles are represented as the 190 bp PCR products.

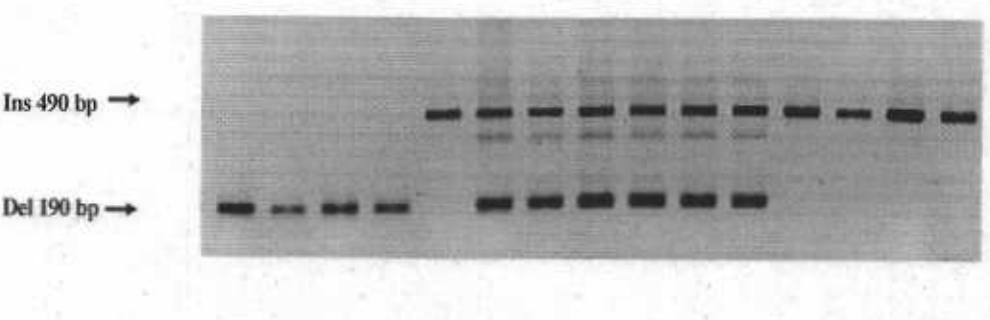

Fig. 3.11b. The amplified products for the *ACE* gene rs4646994 alleles. The insertion alleles represent 287 bp alu repetitive sequence and are represented as 490 bp PCR products whereas the deletion alleles are represented as the 190 bp PCR products.

Table 3.2. The correlation of SNPs with CAD under additive, dominant, genotype, and recessive models. The results represent values after adjusting for the covariates in the study.

GENE	SNP/Polymorphism	*p* value	OR	95% CI
Additive Model				
MTHFR	rs1801133 (C-T)	0.024*	1.14	1.04-1.27
MTHFR	rs1801131 (A-C)	0.442	1.02	0.94-1.11
CBS	rs5742905 (T-C)	0.288	1.25	0.79-1.98
PON1	rs662 (A-G)	0.951	0.99	0.91-1.08
MTR	rs1805087 (A-G)	0.010*	1.15	1.04-1.27
ACE	Insertion/Deletion	0.008**	1.08	0.99-1.20
Dominant Model				
MTHFR	rs1801133 (C-T)	0.016*	1.20	1.07-1.36
MTHFR	rs1801131 (A-C)	0.065	1.09	0.96-1.23
CBS	rs5742905 (T-C)	0.312	1.27	0.81-2.0
PON1	rs662 (A-G)	0.932	1.00	0.89-1.12
MTR	rs1805087 (A-G)	0.001**	1.22	1.08-1.37
ACE	Insertion/Deletion	0.023*	1.10	0.99-1.28
Genotype Model				
MTHFR	rs1801133 (C-T)	0.032*	1.02	1.0-1.03
MTHFR	rs1801131 (A-C)	0.013*	1.01	1.0-1.02
CBS	rs5742905 (T-C)	0.418	1.03	0.98-1.08
PON1	rs662 (A-G)	0.781	1.00	0.99-1.01
MTR	rs1805087 (A-G)	0.002**	1.02	1.01-1.03
ACE	Insertion/Deletion	0.905	1.00	0.99-1.01
Recessive Model				
MTHFR	rs1801133 (C-T)	0.41	1.12	0.86-1.48
MTHFR	rs1801131 (A-C)	0.21	0.89	0.75-1.07
CBS	rs5742905 (T-C)	NS.	NS.	NS.
PON1	rs662 (A-G)	0.94	0.99	0.82-1.19
MTR	rs1805087 (A-G)	0.91	1.07	0.85-1.36
ACE	Insertion/Deletion	0.01*	1.17	0.99-1.38

* $p < 0.05$ and ** $p < 0.008$ (Bonferroni multiple correction threshold), OR: Odds Ratio; CI: Confidence Interval; NS.: non significant.

Table 3.3. Logistic regression analysis of SNPs with the disease under additive, dominant, genotype, and recessive models, (results adjusted for the covariates).

GENE	SNP/Polymorphism	*p* value	OR	95% CI
Additive Model				
MTHFR	rs1801133 (C-T)	0.012*	2.07	1.20-3.73
MTHFR	rs1801131 (A-C)	0.545	1.15	0.73-1.82
CBS	rs5742905 (T-C)	0.272	4.33	0.35-106
PON1	rs662 (A-G)	0.876	0.96	0.61-1.52
MTR	rs1805087 (A-G)	0.003**	2.24	1.33-3.87
ACE	Insertion/Deletion	0.100	1.57	0.92-2.73
Dominant Model				
MTHFR	rs1801133 (C-T)	0.005**	2.66	1.36-5.37
MTHFR	rs1801131 (A-C)	0.190	1.54	0.81-2.95
CBS	rs5742905 (T-C)	0.240	4.84	0.38-119
PON1	rs662 (A-G)	0.978	1.01	0.53-1.91
MTR	rs1805087 (A-G)	0.001**	2.97	1.57-5.76
ACE	Insertion/Deletion	0.246	1.61	0.72-3.67
Genotype Model				
MTHFR	rs1801133 (C-T)	0.032*	1.08	1.01-1.16
MTHFR	rs1801131 (A-C)	0.159	1.05	0.98-1.12
CBS	rs5742905 (T-C)	0.204	1.20	0.92-1.67
PON1	rs662 (A-G)	0.885	1.00	0.94-1.07
MTR	rs1805087 (A-G)	0.002**	1.11	1.04-1.18
ACE	Insertion/Deletion	0.638	0.98	0.92-1.05
Recessive Model				
MTHFR	rs1801133 (C-T)	0.45	1.69	0.45-7.47
MTHFR	rs1801131 (A-C)	0.20	0.56	0.23-7.47
CBS	rs5742905 (T-C)	NS.	NS.	NS.
PON1	rs662 (A-G)	0.85	0.91	0.35-2.39
MTR	rs1805087 (A-G)	0.48	1.54	0.46-5.42
ACE	Insertion/Deletion	0.042*	2.49	1.06-6.26

* $p < 0.05$ *and* ** $p < 0.008$ (Bonferroni multiple correction threshold), OR: Odds Ratio; CI: Confidence Interval; NS.: non significant.

3.5.5 Epistasis analysis (gene –gene interaction) of the SNPs

Under the additive model significant interaction amongst three SNPs, *MTHFR* rs1801133, *PON1* rs662, and *MTR* rs1805087, with CAD, were revealed. When the epistasis result was adjusted for the confounders (age, gender, systolic and diastolic blood pressure, fasting blood sugar, cholesterol and creatinine levels), the interaction could not maintain statistical significance with CAD. Under the genotype model, the three genes that had gene–gene interactions included: the *MTHFR* rs1801131, *PON1* rs662, and *ACE* I/D. This genetic interaction remained significantly associated with CAD after adjusting for covariates (Table 3.4).

3.5.6 Results after Bonferroni correction

Following Bonferroni corrections, under the dominant and genetic models, the *MTR* rs1805087 maintained significance with CAD; under the additive model *ACE* I/D had significant association. With logistic regression analysis, with Bonferroni correction, *MTR* rs1805087 maintained association under the additive, genotype, and dominant models, whereas *MTHFR* rs1801133 maintained association with CAD under the additive model (Tables 3.2 – 3.3). In the current study, the epistatic clusters could not maintain significant association with CAD under Bonferroni multiple adjustment threshold.

3.5.7 Genotypes at the SNPs and Allele Frequencies

Figs. 3.1 – 3.11 represent the images of SNPs analyzed in the current study and show the restriction digestion based and tetra primer ARMS–PCR based assessment. The genotypes for the individual SNPs, separately for the cases and controls are represented in: Fig. 3.12 (rs1801133); Fig. 3.13 (rs1801131); Fig. 3.14 (rs1805087); Fig. 3.15 (*ACE* In/Del); Fig. 3.16 (rs662); and Fig. 3.17 (rs5742905), respectively. Allele frequencies were also computed for the cases and controls, the results are presented in Table 3.5.

Table 3.4. Gene-gene epistasis of SNPs with the disease status without and with adjustment (of results) for covariates.

SNP Cluster	Adjusted for covariates	*p* value
Additive Model		
Rs1801133:rs662:rs1805087	Not Adjusted	0.046*
	Adjusted	0.10
Rs1801131:rs662:ACEindel	Not Adjusted	0.73
	Adjusted	0.40
Genotype Model		
Rs1801131:rs662:ACEindel	Not Adjusted	0.038*
	Adjusted	0.048*
Rs1801133:rs662:rs1805087	Not Adjusted	0.99
	Adjusted	0.99

* $p < 0.05$.

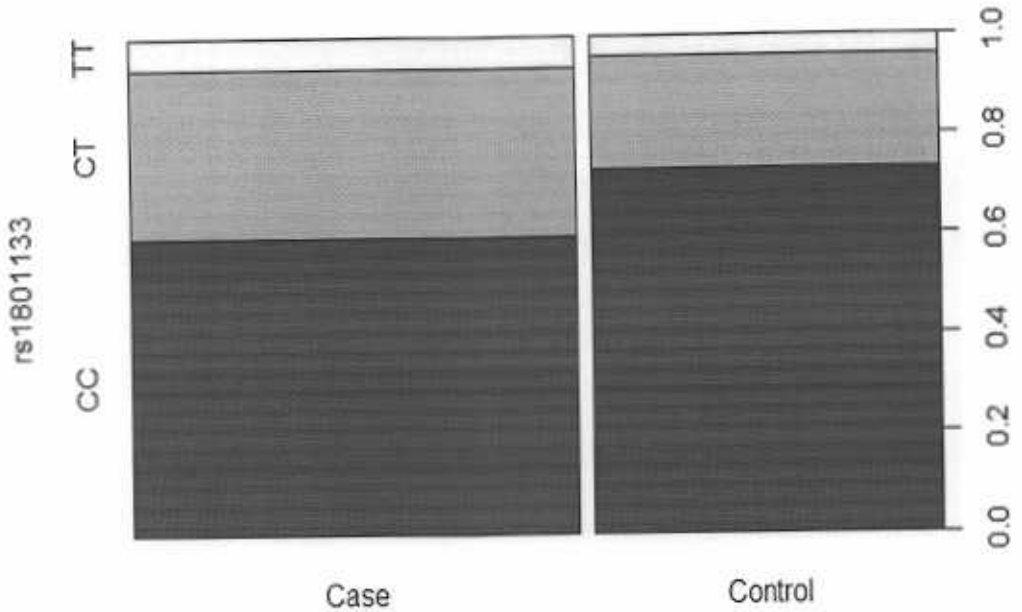

Fig. 3.12. Genotype frequency of SNP rs1801133 with disease status.

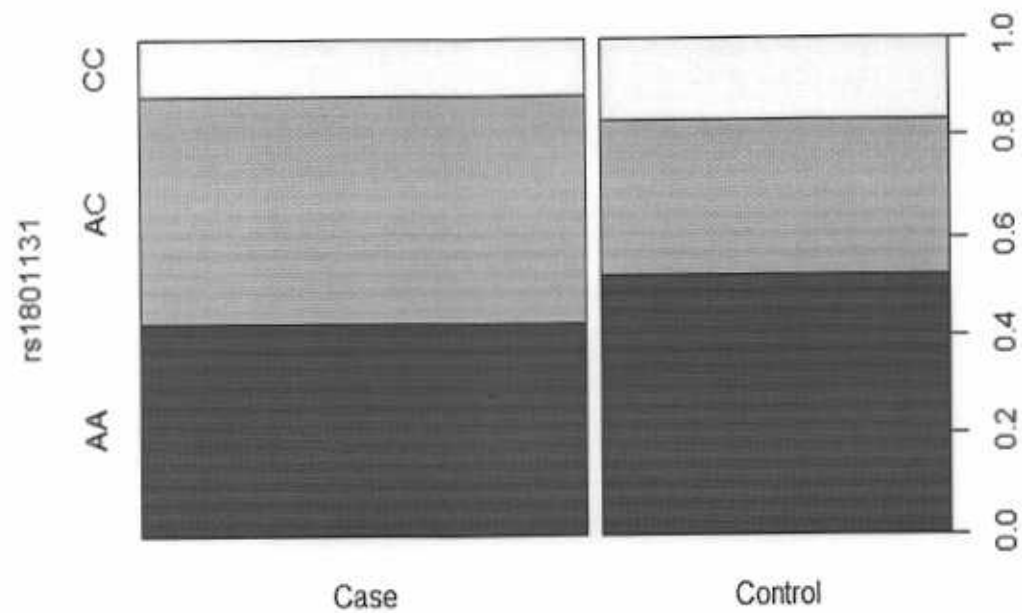

Fig. 3.13. Genotype frequency of SNP rs1801131 with disease status.

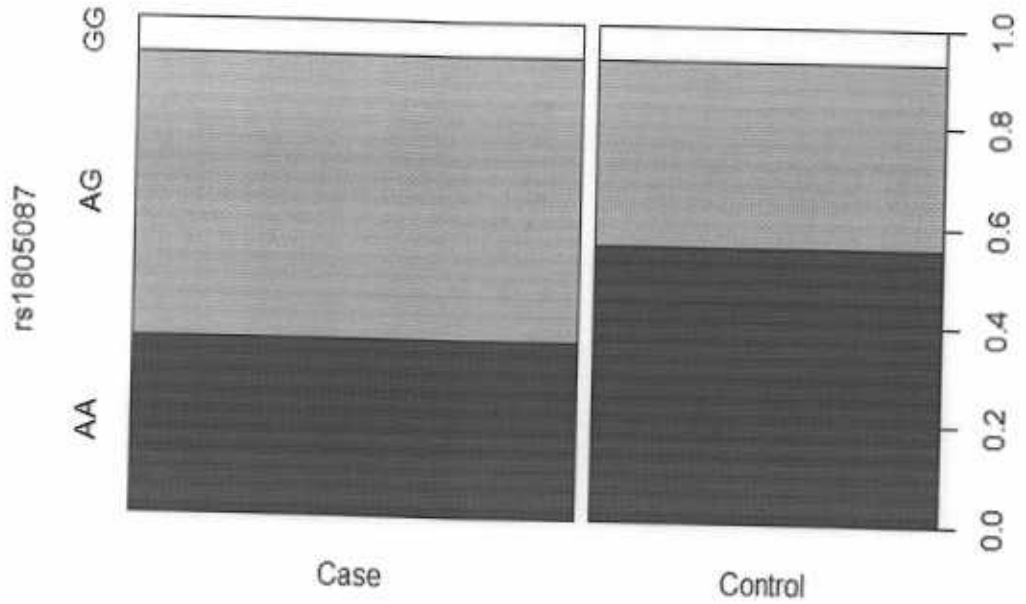

Fig. 3.14. Genotype frequency of SNP rs1805087 with disease status.

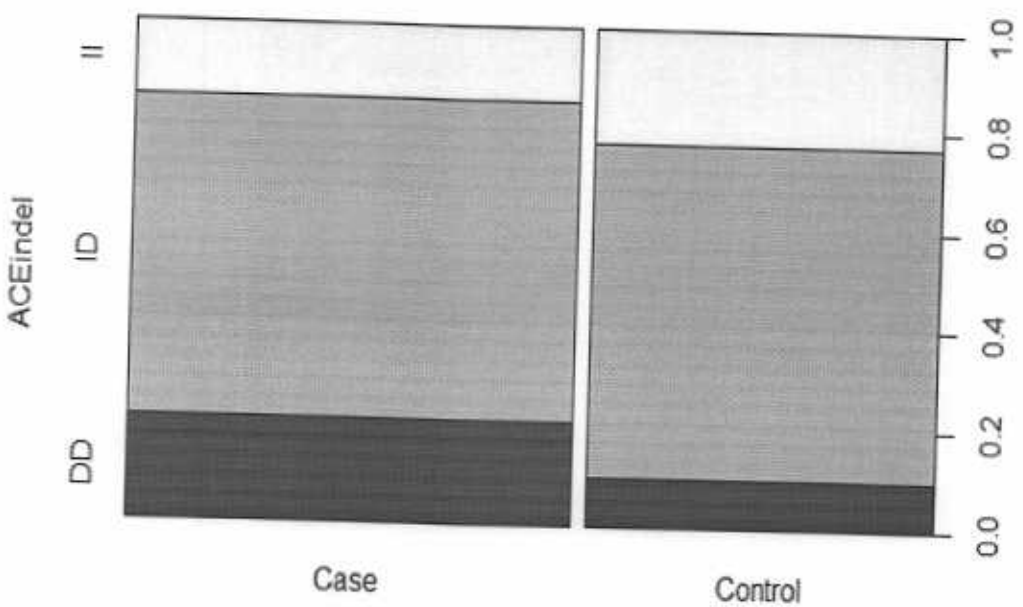

Fig. 3.15. Genotype frequency of *ACE* I/D with disease status.

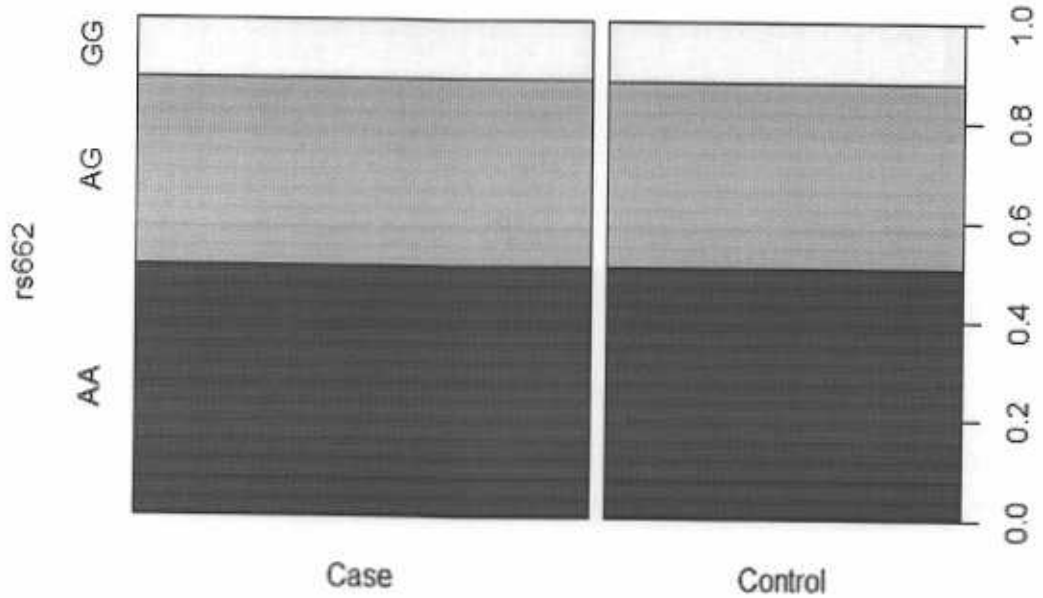

Fig. 3.16. Genotype frequency of SNP rs662 with disease status.

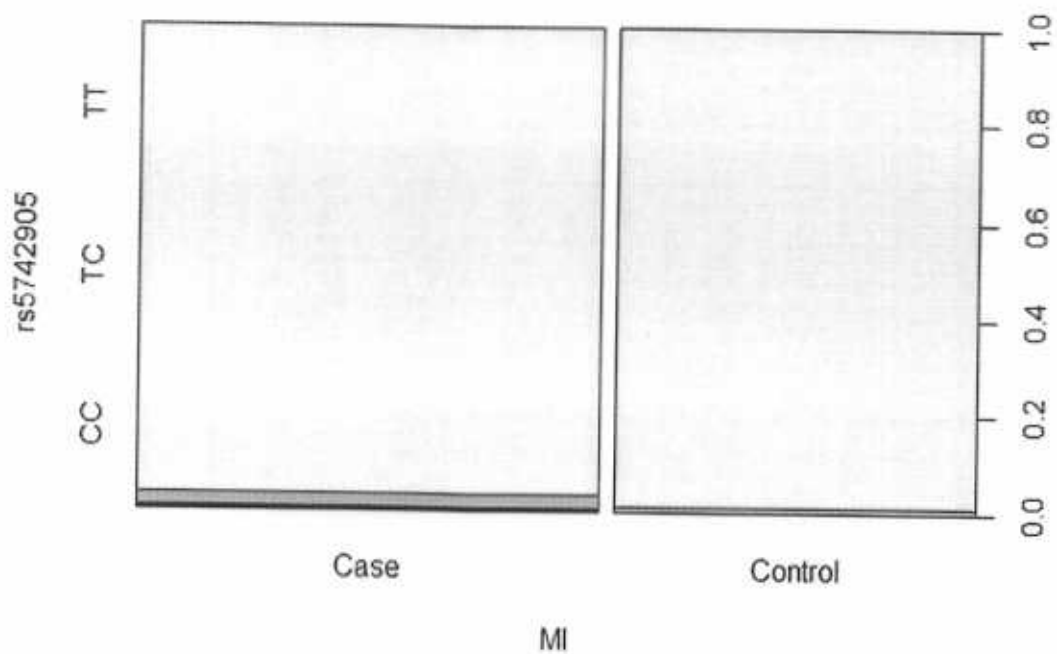

Fig. 3.17. Genotype frequency of SNP rs5742905 with disease status.

Table 3.5. Allele frequencies for SNP variants.

Alleles	Allele Frequencies		Alleles	Allele Frequencies	
	Controls	Cases		Controls	Cases
rs1801133–C	0.85	0.77	Rs1801133–T	0.15	0.23
rs1801131–A	0.68	0.66	Rs1801131–C	0.32	0.34
rs5742905–T	0.995	0.985	Rs5742905–C	0.005	0.015
rs662–A	0.69	0.69	Rs662–G	0.31	0.31
rs1805087–A	0.74	0.64	Rs1805087–G	0.26	0.36
ACE–In	0.56	0.47	ACE–Del	0.44	0.53

DISCUSSION

In the current study, use of tetra primer ARMS–PCR for identification of important SNPs in the homocysteine pathway related genes associated with CAD is reported for the first time. Additional analysis included logistic regression analysis, and investigation of gene–gene interactions for targeting the variants that could be used for CAD stratification. Tetra primer ARMS–PCR technique is relatively easy, cost effective and reliable genotyping technique and has been used to resolve the genotypes for various diseases in a number of studies (Zhang et al., 2006; Yang et al., 2007).

Various detection techniques for select Homocysteine pathway genes SNPs

Earlier studies mention a variety of genotyping methods for the variants studied in the present study. The RFLP analysis has been used for identification of the: *MTHFR* alleles rs1801133, and rs1801131 (Hanson et al., 2001; Almawi et al., 2004; Kerkeni et al., 2006; Vijaya Lakshmi et al., 2011), *CBS* allele rs5742905 (Wu et al., 2004; Dutta et al., 2005; Dutta et al., 2009), *PON1* variant rs662 (Dasgupta et al., 2011), and *MTR* variant rs1805087 (Vinukonda et al., 2009; Vijaya Lakshmi et al., 2011).

The RT-PCR based detections have been performed for: rs1801133 and rs1801131 (Callejon et al., 2007; Moon et al., 2007; Hambaba et al., 2008; Kristensen and Dobrovic, 2008; Patino-Garcia et al., 2009; Tao et al., 2009; Palomino-Morales et al., 2010); rs662 (Dasgupta et al., 2011); and rs1805087 (Kristensen and Dobrovic, 2008; Patino-Garcia et al., 2009; Tao et al., 2009) respectively.

The DHPLC assay analysis has also been reported for homocysteine gene polymorphisms but only for the *MTHFR* variants rs1801133 and rs1801131 (Fang et al., 2004; Sadewa et al., 2004). The SSCP analysis has also been used for SNPs used in present study: for *MTHFR* rs1801133 and rs1801131 (Ren et al., 1997; Porto et al., 2005; Sang et al., 2006; Real et al., 2009; Cheng et al., 2010); for *CBS* rs5742905 and for *MTR* rs1805087 (Tsai et al., 1996; Ren et al., 1998; Porto et al., 2005). As regards the genome wide association studies (GWAS), this approach has also revealed the significance of rs1801133 with disease phenotypes. The rs1801133 has been identified in several GWAS (Kiel et al., 2007; Collin et al., 2009; Hazra et al., 2009;

Tanaka et al., 2009). A thorough literature search revealed no study mentions tetra primer ARMS–PCR for allelic discrimination of SNPs targeted in the present study.

Genetic variations and hyperhomocysteinemia

The investigated SNPs included two in the *MTHFR* gene, rs1801133 C→T, and rs1801131 A→C, rs5742905 T→C in *CBS* gene; rs1805087 A→G in *MTR* gene; and the SNP in *PON1* gene was rs662 A→G. These 5 SNPs were from homocysteine pathway genes. The *ACE* gene I/D (rs4646994) polymorphism, however, was part of the angiotensin aldosterone system. Three out of the five genes that include the *MTHFR*, *MTR*, and *ACE* showed strong association with CAD cases in the studied Pakistani population.

Hyperhomocysteinemia is considered as an independent risk factor for the atherosclerotic disease and for the ischemic heart disease (Christen et al., 2000; Wald et al., 2002). The B vitamins, specifically B6, B12, and folate are vital for metabolism of homocysteine, and their deficiency can result in hyperhomocysteinemia. The elevated homocysteine levels can also result from genetic differences (SNPs in homocysteine pathway genes), or due to disease conditions such as chronic renal failure (CRF). The pathologic manifestations of hyperhomocysteinemia are dependent on production of ROS, reduction in NO, endothelial dysfunction, and enhanced production of proinflammatory genes (Antoniades et al., 2009).

The rs1801133 and rs1805087 with CAD

Presently the rs1801133 and *MTR* rs1805087 were found consistently associated with CAD. The *MTHFR* rs1801133 (C677T) polymorphism was first demonstrated as a SNP and was found related to vascular disease in 1995 (Frosst et al., 1995). Subsequent data determined inconclusively the association of this SNP with CAD. The C allele codes for alanine whereas T allele codes for valine; the latter allele encodes for a thermolabile enzyme that is highly dependent on circulating folate levels. An earlier study (with participants from a different geographical distribution area of Pakistan) failed to determine association of C677T polymorphism with CAD, but reported strong association of TT genotype with plasma homocysteine levels (Iqbal et al., 2005). Later the authors concluded that folate and vitamin deficiency, mild hyperhomocysteinemia and *MTHFR* C677T variant are risk factors for CAD in Pakistani population (Iqbal, 2006). A more recent study for glaucoma that recruited the participants from the same geographical distribution area as was considered

presently (Punjab) revealed statistically significant C677T allelic differences between cases and controls (Micheal et al., 2009). Contrasting results between the current study and a previous CAD study of Iqbal et al. (2005) may be attributed to differences in participant inclusion criteria, different distribution areas and differences in participant characteristics.

Several studies have highlighted that rs1801133 SNP is associated with high homocysteine levels, low folate levels and a consequent risk for hypertension and CAD (Klerk et al., 2002; Faria-Neto et al., 2006; Ilhan et al., 2008). High intake of folate and vitamins decreases homocysteinemia levels as well as the risk of CVD (Selhub et al., 2000), and in support of this observation, the T allele variant exhibits a blunted response in those countries where folic acid diet fortification is enforced (Klerk et al., 2002; Lewis et al., 2005; Antoniades et al., 2009). Likewise, the *MTR* rs1805087 (A2756G) variant results in hyperhomocysteinemia and is associated with CVD (Laraqui et al., 2006; Laraqui et al., 2007; Vinukonda et al., 2009; Vijaya Lakshmi et al., 2011). Vitamin B12 is a necessary cofactor for *MTR*, therefore, vitamin B12 is an additional modulator of hyperhomocysteinemia and vascular disease (in those countries where folate diet fortification is enforced) (Liaugaudas et al., 2001; Robertson et al., 2005). There is no folic acid diet fortification in Pakistan, and the use of multivitamin/folate pills is also limited, the two SNP variants 'rs1801133 and rs1805087', therefore, pose a higher risk for cardiovascular diseases in our indigenous population (as observed in the current study).

The rs1801131 and rs5742905 with CAD

The previous studies mention *MTHFR* rs1801131 and *CBS* rs5742905 associations with hyperhomocysteinemia and early onset CAD (Tsai et al., 1999; Szczeklik et al., 2001; Laraqui et al., 2007), yet in the present study these SNPs had modest relationship with CAD. The rs5742905 *CBS* SNP is rare, and the minor allele frequency for C allele shows individual variations according to global geographical distribution. Study by Franco et al (1998b) reported complete absence of mutant (C) allele in Asians, whereas current CAD cases revealed minor allele frequency (MAF) = 0.01, concordant with a CAD study by Dutta et al (2005). The rs1801131 SNP is associated with CAD in Pakistani population and reveals core genetic interactions in disease causation whereas the rs5742905 is not associated with CAD in the studied participants.

The PON1 rs662 and CAD

Although there were found no genotype and allelic differences for *PON1* rs662 SNP between cases and controls, yet this SNP, through strong genetic interactions, appeared to be strongly associated with CAD (Table 3.4). The *PON1* 'A' allele codes for glutamine (Q) whereas the 'G' allele encodes arginine (R). Conflicting reports exist as regards the association of this allelic variant with the CAD. A few studies have demonstrated that the arginine residue codes for the atherosclerotic potential and enhanced coronary risk (Mohamed et al., 2010), while others indicate glutamine residue to contribute to the atherosclerotic potential (Koubaa et al., 2009). In a previous meta-analysis, rs662 was found strongly associated with CAD (Wheeler et al., 2004). Studies in Pakistan have also found that this SNP has significant genetic and epistatic association with CAD/MI (Saeed et al., 2007). Interestingly the *PON1* rs662 SNP was involved in both gene interaction networks in the current study, substantiating the results of earlier studies.

The ACE I/D allele and CAD

Various studies have revealed combined associations of *ACE* I/D and homocysteine pathway genes in relation to disease susceptibility (Mendonca et al., 2008; Mendonca et al., 2009; Pizza et al., 2010). In this study, the genetic association of this variant with CAD was observed in three of the four genetic models; the dominant, additive and recessive. Previous meta–analyses and studies have also stressed the association and higher risk of coronary vascular disease with this polymorphism (Agerholm-Larsen et al., 2000; Zintzaras et al., 2008). The study by Zintzaras et al (2008) verified that this variant was highly heterogeneous, had strong association with CAD through dominant and recessive genetic models, but the strongest genetic association with CAD was through the additive model. The present study also draws similar conclusion and also stresses that *ACE* variant has strong gene–gene interactions with homocysteine/folate pathway genes.

Significance of current study in gene–gene interactions

This study demonstrates that gene–gene interactions modulate CAD in selected Pakistani population. The gene clusters that enhance disease susceptibility include: (i) *MTHFR* rs1801133, *PON1* rs662, and *MTR* rs1805087 (additive model), and (ii) *MTHFR* rs1801131, *PON1* rs662, ACE I/D (genotype model), respectively. The genes in the first cluster (*MTHFR*, *PON1*, *MTR*) encode important enzymes of

the homocysteine remethylation pathway. These three genes catalyze adjacent reactions and previous studies mention that epistatic interactions of these genes modulate the disease phenotypes (Bathum et al., 2007; Laraqui et al., 2007; Giusti et al., 2008; Vinukonda et al., 2009). In the second interacting gene cluster (for the genotype model), *ACE* gene replaced *MTR* gene, while the other two genes were the same as in the additive model. The *ACE* I/D variant along with the homocysteine pathway *MTHFR* gene are strong risk factors for vascular disorders (Tietjen et al., 2009; Bentley et al., 2010). The genetic interactions of *ACE* I/D and homocysteine genes confer enhanced CAD risk (Mendonca et al., 2008; Mendonca et al., 2009; Pizza et al., 2010), and same holds true for the currently studied population.

In conclusion, this study reports that tetra primer ARMS–PCR is simple, economic, fast, and reliable technique for genetic diagnosis of CAD. The genes identified presently include *MTHFR*, *MTR*, and *ACE*; for these genes, there were significant allelic differences between CAD patients and controls. The *PON1* gene was important in epistasis, alongwith the above three mentioned gene variants. Further studies are required to get an insight into the disease associations, to delineate contributory influences of genetic differences, and downstream pathways that control homocysteine concentration and mediate Hcy based vascular diseases.

CHAPTER 4: GENERAL DISCUSSION

GENERAL DISCUSSION

Inflammation, endothelial dysfunction, and plaque instability (in the atherosclerotic vessels), results in cardiovascular disease and manifests as coronary artery disease or peripheral artery disease (Drexler, 1997; Widlansky et al., 2003; Hansson, 2005). The vascular disorders are 'complex multifactorial' disorders, with environmental and polygenic inheritance (Givelber et al., 1998; Kumar, 2008). Previous investigations revealed that patients requiring coronary revascularization also had PAD, similarly many patients who required surgical management for obstructed peripheral vessels were affected with CAD (Widlansky et al., 2003; Allie et al., 2005). Despite overlap of a few genetic variants, differences exist and guide progression of atherosclerosis in one vascular bed relative to the other. Through the current study, a range of molecular biology techniques has been used to investigate the genes and enzymes underlying the specific cardiovascular disorders, PAD, and CAD.

Through transcriptomic analyses, the entire transcriptome of peripheral blood mononuclear cells (PBMC) was analyzed to highlight differences between PAD cases and normal subjects, through whole genome expression (3' IVT, Affymetrix HG-U133 Plus 2.0) microarray technology. The use of PBMC for differential gene expression analysis in CAD, stroke, and hypertension is already reported (Waehre et al., 2004; Timofeeva et al., 2006; Wingrove et al., 2008; Stamova et al., 2010). Earlier whole genome expression studies for PAD, however, used the atherosclerotic plaques and carotid, and lower limb vessels which are difficult to obtain in routine clinical practice (Dahl et al., 2007; Evans et al., 2008; Fu et al., 2008). The current study pioneers the use of peripheral blood mononuclear cells for genomic signatures of peripheral artery occlusive disease. The analyses, by dividing the participants into two groups, allowed the recognition and confirmation of thirty genes and transcripts as strongly associated with the peripheral artery disease. Seven of the twenty two upregulated genes and one of the seven downregulated genes, in the present study, have known association with PAD (Dahl et al., 2007; Evans et al., 2008; Fu et al., 2008). The upregulated genes in both the current study and in previous PAD studies include: *ATF3*, *CDKN1A*, *CFH*, *DUSP1*, *NAMPT*, *NR4A2*, *SAMSN1*, *SLC2A3*, while the downregulated gene is *OGT* (Dahl et al., 2007; Evans et al., 2008; Fu et al., 2008).

The current study, therefore, validates the previous PAD expression studies, and at the same time, adds a novel group of fourteen upregulated and six downregulated genes to polygenic inheritance of PAD. This study also highlights the reliable use of PBMC for expression analyses instead of obtaining atherosclerotic plaques and diseases vessels.

The gene interactions were analyzed by IPA and involvement of three gene interaction networks was observed as underlying mechanism of PAD. The networks included: (i) cell death, genetic disorder, (ii) gene expression, cellular and hematological development and function, and (iii) behavior, metabolism, and inflammatory disease. Interestingly, majority of the gene variants that overlap, in the present and previous CAD studies, were highly enriched in the first network. Thirteen upregulated genes formed the first IPA network, including *ATF3*, *CDKN1A*, *CFH*, *DUSP1*, *G0S2*, *ID1*, *IL8*, *KLF6*, *NAMPT*, *NR4A2*, *OSM*, *PTP4A1*, and *SLC2A3*. The inflammatory and immune basis of PAD was strengthened by observation that genes in the present study interacted with NF-κB which was present at the heart of gene interaction network. *ATF3* has direct interaction with NF-κB which in turn enhances E selectin expression (important mediator of diapedesis) (Kaszubska et al., 1993). Additionally, NF-κB induces TLR production and induces downstream effectors and inflammatory genes of TLR activated cells (Edfeldt et al., 2002; Janeway and Medzhitov, 2002). *ID1* and *KLF6* interact with and increase expression of *CDKN1A* (Kimmelman et al., 2004; Nickoloff et al., 2000). *ID1* through its interaction with NF-κB, enhances inflammation, endothelial cell migration, angiogenesis, and inhibits apoptosis (Klein et al., 2002; Nishiyama et al., 2005; Chen et al., 2010). The identified genes, through NF-κB, modulate the progression of peripheral artery disease. A potent proinflammatory cytokine, *IL8*, was upregulated in the current study. In the atherosclerotic plaques, *IL8* is highly expressed by activated macrophages and in turn drives activation and differentiation of more immune cells (Frostegard et al., 1999). *NAMPT*, *NR4A2*, and *OSM* share interacting networks with *IL8*, and enhance its release. These four genes form an important interacting network guiding progression of atherosclerotic disease, immune signaling, and PAD (Hurst et al., 2002; Davies et al., 2005; Dahl et al., 2007). These interactions form part of explanation for genetic predisposition to peripheral artery disease.

The current study exclusively provides a new mechanism of genetic interactions that forms the basis of PAD. The fourteen upregulated genes and six

downregulated genes had no known previous association with PAD (in expression studies or GWAS). The upregulated genes included *C5orf41*, *CDV3*, *DNAJB6*, *FCAR*, *FFAR2*, *G0S2*, *HIST1H2BC*, *ID1*, *IL8*, *KLF6*, *OSM*, *PTP4A1*, *STX11*, and *TPR*, whereas, regressed expression was observed for *ARHGEF7*, *C5orf28*, *CFLAR*, *MLL3*, *PDS5B*, and *TRAF3IP3*. The second IPA network was titled 'gene expression, cellular, hematological development and function'. It included *C5orf41*, *DNAJB6*, *FCAR*, *FFAR2*, *SAMSN1*, *STX11*, *TPR* (upregulated genes), and *PDS5B* (downregulated gene). In the second network again, the inflammatory mechanism in PAD was highlighted, as proinflammatory cytokines *TNFα* and *IL6*, and transcription activators *STAT3* and *FOS*, formed the core component in the network. *DNAJB6*, *FCAR*, and *FFAR2* (*GPR43*), the upregulated genes, all have direct interactions with *TNFα*, *IL6*, *STAT3*, and *FOS*, these cytokines and transcription activators have pivotal role in immune modulation (Beyer et al., 2001; Senga et al., 2003; Dai et al., 2005). The genes in the present study modulate inflammation, endothelial dysfunction and oxidative stress. It was noted that *ATF3*, *DUSP1*, *IL8*, *NR4A2*, and *SLC2A3* (genes upregulated in current study), as well as *IL6* and *FOS* were upregulated in patients with coronary vessel bypass grafts and cardiac arrest (Voisine et al., 2004). This microarray gene expression study for PAD, therefore, reports on the prospect that two separate pathways (cell death- genetic disorders, and gene expression-cellular and hematological development and function) mutually interact and result into peripheral artery disease.

The second part of current study focused on a new approach for allele discrimination in genes of homocysteine pathway and aimed for an insight into gene variants that modulate CAD in Pakistan. The polymorphisms studied include rs1801133 and rs1801131 SNPs (*MTHFR*), rs5742905 SNP (*CBS*), rs1805087 SNP (*MTR*), rs662 SNP (*PON1*), and *ACE* gene I/D (rs4646994) polymorphisms. A study carried out at Agha Khan University Karachi could not link *MTHFR* gene C677T allele with the coronary artery disease (Iqbal et al., 2005). Later on, however, the author included C677T in the risk factors for hyperhomocysteinemia and CAD in Pakistani population (Iqbal, 2006). In a study by Min Shi et al. (2003), constituting more than one thousand DNA samples of different human populations from around the continent were analyzed for genotypes, allele frequencies and linkage in the homocysteine pathway genes. The study reported that the highest linkage disequilibrium for the '*MTHFR*' C677T (rs1801133) and A1298C (rs1801131) SNPs

was observed for the Pakistani population, and suggested that these variants may be important candidates for genetic studies in this region (Shi et al., 2003).

MTR rs1805087 variant has not been previously studied in relation to CAD in the local population, however, in the present study it revealed strong association with CAD. Saeed et al. (2007) reported that PON1 gene cluster and rs662 *PON1* polymorphism were strongly associated with risk of MI, the present study further adds to this observation. Finally, in the present study, *ACE* gene I/D polymorphism (rs4646994) was found to modulate CAD in the Pakistani population. An earlier study documented that this variant was associated with early onset hypertension (Ismail et al., 2004), but to date no local study has mentioned this variant in relation to MI risk.

The analysis of the SNPs through tetra primer ARMS–PCR allowed the visualization and analysis of the many studied polymorphisms in the studied participants and this method allows rapid allele detection in limited time with limited resources. The use of simple thermal cyclers allows the molecular analysis in relatively less equipped laboratories. The problems with PCR amplifications due to intrinsic (deliberate) primer mismatches require troubleshooting (Ye et al., 2001; Okayama et al., 2004), thereafter, this method can detect alleles rapidly and reliably for a large number of samples.

The next aim of the current study was to determine if the gene-gene interactions were involved in CAD risk (in the studied participants). There exists no study that mentions the genetic interactions (epistasis) of homocysteine pathway genes for the local population. Two interactions involving three genes each were observed in association with CAD. The genetic network that comprised *MTHFR*, *PON1*, and *MTR* genes was not independently associated, whereas network that comprised *MTHFR*, *PON1*, and *ACE* genes, was independently associated with CAD in local studied population. This is another significant finding and can be focused for CAD studies in future.

In conclusion, the findings observed and presented in this thesis substantiate the involvement of immune and inflammatory reactions, apoptosis, endothelial dysfunction, oxidative damage, and plaque destabilization in the genesis and progression of peripheral artery disease. The involved genes have, known, immune mediated effects and genetic interactions, modulating vascular disease and PAD. Strict study designs and stringent statistical scrutiny followed by validation experiments, greatly enhance specificity of microarray studies. Likewise, for CAD

associations, the detection and validation of genes and allelic variants, allows determination of allele frequencies and their comparison among different world populations. The analyses of genes in homocysteine pathway and the gene variants, in local population revealed significant association with CAD risk.

Microarray technology allows detection of thousands of genes simultaneously, illuminating the mechanisms and pathways leading to disease states. The use of modified PCR technique, tetra primer ARMS–PCR, allows rapid and reliable discrimination of allele variants at genomic loci. The genes involved in PAD and allelic variants in CAD are strong nominees for PAD and CAD risk stratification, respectively, and may serve as targets for earlier therapeutic intervention, and in addition, as strong candidates for gene therapy.

For more definitive conclusion of PAD microarray study, the experiments can be repeated in different geographical regions, such as for the European, African, and Asian population. The highly differentiated genes can also be used for real time analyses of PAD association studies. An alternative approach may be the use of the PAD cases and control samples for genome wide association studies (GWAS) for a more comprehensive list of SNP loci (as markers for elucidation of quantitative trait loci), that are associated with PAD. The overlapping genes between the current study and future PAD microarray studies in Europe, Africa, and in Asia, can provide a list of global genetic modulators of PAD.

The tetra primer ARMS–PCR can be similarly used for allelic variants in homocysteine pathway, in different human populations, for a more comprehensive use for detection of alleles and analyses of results. Additional experiments may require investigation on larger sample sets, with stricter strategies to decrease non genetic effects, for a more definitive elucidation of genetic effects in the etiology of CAD in local and world populations.

MANUSCRIPTS AND PRESENTATIONS FROM PRESENT STUDY

Manuscripts

1. **Masud R, Qureshi IZ.** (2011) Tetra primer ARMS-PCR relates folate/homocysteine pathway genes and *ACE* gene polymorphism with coronary artery disease. *Mol Cell Biochem.* **355**: 289-297.

2. Circulating transcriptome whole genome microarray analysis for identifying genes associated with peripheral arterial disease. Finalized for submission.

Presentations

1 'Homocysteine pathway genomic signatures in coronary artery disease', presentation in National Symposium on Current Trends in Cellular, Medical and Environmental Physiology, 17th – 19th May, 2010.

2 'Genetic analysis of folate pathway genes in myocardial disease using allele specific PCR', abstract accepted and the study presented at 12th Biennial PPS conference held on April 11-12, 2011 at King Edward Medical University and CMH Lahore Medical College, Lahore.

REFERENCES

REFERENCES

Aboyans, V., R.L. McClelland, M.A. Allison, M.M. McDermott, R.S. Blumenthal, K. Macura, and M.H. Criqui. (2011). Lower extremity peripheral artery disease in the absence of traditional risk factors. The Multi-Ethnic Study of Atherosclerosis. *Atherosclerosis*. **214**: 169-173.

Acampa, W., M.D. Di Taranto, A. Morgante, B. Salvatore, L. Evangelista, F. Ricci, P. Costanzo, E. De Sisto, P.P. Filardi, M. Petretta, G. Fortunato, and A. Cuocolo. (2011). C-reactive protein levels are associated with paraoxonase polymorphism L55M in patients undergoing cardiac SPECT imaging. *Scand J Clin Lab Invest*. doi: 10.3109/00365513.2010.548529

Agerholm-Larsen, B., B.G. Nordestgaard, and A. Tybjaerg-Hansen. (2000). ACE gene polymorphism in cardiovascular disease: meta-analyses of small and large studies in whites. *Arterioscler Thromb Vasc Biol*. **20**: 484-492.

Akinkugbe, O.O. (1990). Epidemiology of cardiovascular disease in developing countries. *J Hypertens Suppl*. **8**: S233-S238.

Al-Allawi, N.A., A.S. Avo, and J.M. Jubrael. (2009). Methylenetetrahydrofolate reductase C677T polymorphism in Iraqi patients with ischemic stroke. *Neurol India*. **57**: 631-635.

Alderton, W.K., C.E. Cooper, and R.G. Knowles. (2001). Nitric oxide synthases: structure, function and inhibition. *Biochem J*. **357**: 593-615.

Ali, F.M., A. May, G.D. McLaren, and A. Jacobs. (1982). A two-step procedure for obtaining normal peripheral blood T-lymphocytes using continuous equilibrium density gradient centrifugation on percoll. *J Immunol Methods*. **49**: 185-191.

Allie, D.E., C.J. Hebert, M.D. Lirtzman, C.H. Wyatt, V.A. Keller, M.H. Khan, M.A. Khan, P.S. Fail, K. Vivekananthan, S.E. Allie, E.V. Mitran, G. Chaisson, S.J. Stagg, 3rd, A.A. Allie, M.W. McElderry, E.A. Barker, and C.M. Walker. (2005). A safety and feasibility report of combined direct thrombin and GP IIb/IIIa inhibition with bivalirudin and tirofiban in peripheral vascular disease intervention: treating critical limb ischemia like acute coronary syndrome. *J Invasive Cardiol*. **17**: 427-432.

Almawi, W.Y., G. Ameen, H. Tamim, R.R. Finan, and N. Irani-Hakime. (2004). Factor V G1691A, prothrombin G20210A, and methylenetetrahydrofolate reductase [MTHFR] C677T gene polymorphism in angiographically documented coronary artery disease. *J Thromb Thrombolysis*. **17**: 199-205.

Amento, E.P., N. Ehsani, H. Palmer, and P. Libby. (1991). Cytokines and growth factors positively and negatively regulate interstitial collagen gene expression in human vascular smooth muscle cells. *Arterioscler Thromb.* **11**: 1223-1230.

Anitschkow, N., Chalatow, S., (translated by Pelias, M. Z.) (1983). Classics in arteriosclerosis research: On experimental cholesterin steatosis and its significance in the origin of some pathological processes by N. Anitschkow and S. Chalatow, translated by Mary Z. Pelias, 1913. *Arteriosclerosis.* **3**: 178-182.

Andersson, J., P. Libby, and G.K. Hansson. (2010). Adaptive immunity and atherosclerosis. *Clin Immunol.* **134**: 33-46.

Andreoli, V., R.C. Gehrau, and J.L. Bocco. (2010). Biology of Kruppel-like factor 6 transcriptional regulator in cell life and death. *IUBMB Life.* **62**: 896-905.

Ansari, K.I., and S.S. Mandal. (2010). Mixed lineage leukemia: roles in gene expression, hormone signaling and mRNA processing. *FEBS J.* **277**: 1790-1804.

Antoniades, C., A.S. Antonopoulos, D. Tousoulis, K. Marinou, and C. Stefanadis. (2009). Homocysteine and coronary atherosclerosis: from folate fortification to the recent clinical trials. *Eur Heart J.* **30**: 6-15.

Archacki, S., and Q. Wang. (2004). Expression profiling of cardiovascular disease. *Hum Genomics.* **1**: 355-370.

Ardigo, D., C.A. Gaillard, and B. Braam. (2007). Application of leukocyte transcriptomes to assess systemic consequences of risk factors for cardiovascular disease. *Clin Chem Lab Med.* **45**: 1109-1120.

Arking, D.E., K. Reinier, W. Post, J. Jui, G. Hilton, A. O'Connor, R.J. Prineas, E. Boerwinkle, B.M. Psaty, G.F. Tomaselli, T. Rea, N. Sotoodehnia, D.S. Siscovick, G.L. Burke, E. Marban, P.M. Spooner, A. Chakravarti, and S.S. Chugh. (2010). Genome-wide association study identifies GPC5 as a novel genetic locus protective against sudden cardiac arrest. *PLoS One.* **5**: e9879.

Arya, M., I.S. Shergill, M. Williamson, L. Gommersall, N. Arya, and H.R. Patel. (2005). Basic principles of real-time quantitative PCR. *Expert Rev Mol Diagn.* **5**: 209-219.

Atkins, G.B., and M.K. Jain. (2007). Role of Kruppel-like transcription factors in endothelial biology. *Circ Res.* **100**: 1686-1695.

Audas, T.E., Y. Li, G. Liang, and R. Lu. (2008). A novel protein, Luman/CREB3 recruitment factor, inhibits Luman activation of the unfolded protein response. *Mol Cell Biol.* **28**: 3952-3966.

Austrup, F., D. Vestweber, E. Borges, M. Lohning, R. Brauer, U. Herz, H. Renz, R. Hallmann, A. Scheffold, A. Radbruch, and A. Hamann. (1997). P- and E-selectin mediate recruitment of T-helper-1 but not T-helper-2 cells into inflammed tissues. *Nature*. **385**: 81-83.

Aziz, H., A. Zaas, and G.S. Ginsburg. (2007). Peripheral blood gene expression profiling for cardiovascular disease assessment. *Genomic Med.* **1**: 105-112.

Baris, I., O. Etlik, V. Koksal, and S.T. Arican-Baris. (2010). Rapid diagnosis of spinal muscular atrophy using tetra-primer ARMS–PCR assay: simultaneous detection of SMN1 and SMN2 deletion. *Mol Cell Probes*. **24**: 138-141.

Barnes, M., J. Freudenberg, S. Thompson, B. Aronow, and P. Pavlidis. (2005). Experimental comparison and cross-validation of the Affymetrix and Illumina gene expression analysis platforms. *Nucleic Acids Res*. **33**: 5914-5923.

Bathum, L., I. Petersen, L. Christiansen, A. Konieczna, T.I. Sorensen, and K.O. Kyvik. (2007). Genetic and environmental influences on plasma homocysteine: results from a Danish twin study. *Clin Chem*. **53**: 971-979.

Beaudet, A.L., and J.W. Belmont. (2008). Array-based DNA diagnostics: let the revolution begin. *Annu Rev Med*. **59**: 113-129.

Belch, J.J., E.J. Topol, G. Agnelli, M. Bertrand, R.M. Califf, D.L. Clement, M.A. Creager, J.D. Easton, J.R. Gavin, 3rd, P. Greenland, G. Hankey, P. Hanrath, A.T. Hirsch, J. Meyer, S.C. Smith, F. Sullivan, and M.A. Weber. (2003). Critical issues in peripheral arterial disease detection and management: a call to action. *Arch Intern Med*. **163**: 884-892.

Beltowski, J. (2005). Protein homocysteinylation: a new mechanism of atherogenesis? *Postepy Hig Med Dosw (Online)*. **59**: 392-404.

Bemmo, A., D. Benovoy, T. Kwan, D.J. Gaffney, R.V. Jensen, and J. Majewski. (2008). Gene expression and isoform variation analysis using Affymetrix Exon Arrays. *BMC Genomics*. **9**: 529.

Bemmo, A., C. Dias, A.A. Rose, C. Russo, P. Siegel, and J. Majewski. (2010). Exon-level transcriptome profiling in murine breast cancer reveals splicing changes specific to tumors with different metastatic abilities. *PLoS One*. **5**: e11981.

Benagiano, M., M.M. D'Elios, A. Amedei, A. Azzurri, R. van der Zee, A. Ciervo, G. Rombola, S. Romagnani, A. Cassone, and G. Del Prete. (2005). Human 60-kDa heat shock protein is a target autoantigen of T cells derived from atherosclerotic plaques. *J Immunol*. **174**: 6509-6517.

Benjamini, Y., and Y. Hochberg. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. Roy. Stat. Soc. B 57*. **1**: 289-300.

Bentley, P., G. Peck, L. Smeeth, J. Whittaker, and P. Sharma. (2010). Causal relationship of susceptibility genes to ischemic stroke: comparison to ischemic heart disease and biochemical determinants. *PLoS One*. **5**: e9136.

Beyer, T., M. Herrmann, C. Reiser, W. Bertling, and J. Hess. (2001). Bacterial carriers and virus-like-particles as antigen delivery devices: role of dendritic cells in antigen presentation. *Curr Drug Targets Infect Disord*. **1**: 287-302.

Bielecki, M., K. Kowal, A. Lapinska, J. Chwiecko, J. Skowronski, S. Sierakowski, L. Chyczewski, and O. Kowal-Bielecka. (2009). Diminished production of TWEAK by the peripheral blood mononuclear cells is associated with vascular involvement in patients with systemic sclerosis. *Folia Histochem Cytobiol*. **47**: 465-469.

Binder, C.J., M.K. Chang, P.X. Shaw, Y.I. Miller, K. Hartvigsen, A. Dewan, and J.L. Witztum. (2002). Innate and acquired immunity in atherogenesis. *Nat Med*. **8**: 1218-1226.

Binder, C.J., K. Hartvigsen, M.K. Chang, M. Miller, D. Broide, W. Palinski, L.K. Curtiss, M. Corr, and J.L. Witztum. (2004). IL-5 links adaptive and natural immunity specific for epitopes of oxidized LDL and protects from atherosclerosis. *J Clin Invest*. **114**: 427-437.

Bjork, K.E., and K. Kafadar. (2007). Systematic order-dependent effect in expression values, variance, detection calls and differential expression in Affymetrix GeneChips. *Bioinformatics*. **23**: 2873-2880.

Blankenberg, S., S. Barbaux, and L. Tiret. (2003). Adhesion molecules and atherosclerosis. *Atherosclerosis*. **170**: 191-203.

Blaschke, F., D. Bruemmer, F. Yin, Y. Takata, W. Wang, M.C. Fishbein, T. Okura, J. Higaki, K. Graf, E. Fleck, W.A. Hsueh, and R.E. Law. (2004). C-reactive protein induces apoptosis in human coronary vascular smooth muscle cells. *Circulation*. **110**: 579-587.

Blazej, R.G., B.M. Paegel, and R.A. Mathies. (2003). Polymorphism ratio sequencing: a new approach for single nucleotide polymorphism discovery and genotyping. *Genome Res*. **13**: 287-293.

Blum, A., and N. Blum. (2009). Coronary artery disease: Are men and women created equal? *Gend Med*. **6**: 410-418.

Bonta, P.I., T.W. Pols, C.M. van Tiel, M. Vos, E.K. Arkenbout, J. Rohlena, K.T. Koch, M.P. de Maat, M.W. Tanck, R.J. de Winter, H. Pannekoek, E.A. Biessen, I. Bot, and C.J. de Vries. (2010). Nuclear receptor Nurr1 is expressed in and is associated with human restenosis and inhibits vascular lesion formation in mice involving inhibition of smooth muscle cell proliferation and inflammation. *Circulation*. **121**: 2023-2032.

Boontha, B., J. Nakkuntod, N. Hirankarn, P. Chaumpluk, and T. Vilaivan. (2008). Multiplex mass spectrometric genotyping of single nucleotide polymorphisms employing pyrrolidinyl peptide nucleic acid in combination with ion-exchange capture. *Anal Chem.* **80**: 8178-8186.

Borradaile, N.M., and J.G. Pickering. (2010). Polyploidy impairs human aortic endothelial cell function and is prevented by nicotinamide phosphoribosyltransferase. *Am J Physiol Cell Physiol.* **298**: C66-C74.

Botstein, D., R.L. White, M. Skolnick, and R.W. Davis. (1980). Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am J Hum Genet.* **32**: 314-331.

Boushey, C.J., S.A. Beresford, G.S. Omenn, and A.G. Motulsky. (1995). A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes. *JAMA.* **274**: 1049-1057.

Bowe, D.B., A. Sadlonova, C.A. Toleman, Z. Novak, Y. Hu, P. Huang, S. Mukherjee, T. Whitsett, A.R. Frost, A.J. Paterson, and J.E. Kudlow. (2006). O-GlcNAc integrates the proteasome and transcriptome to regulate nuclear hormone receptors. *Mol Cell Biol.* **26**: 8539-8550.

Brandt, S., K. Ellwanger, C. Beuter-Gunia, M. Schuster, A. Hausser, I. Schmitz, and S. Beer-Hammer. (2010). SLy2 targets the nuclear SAP30/HDAC1 complex. *Int J Biochem Cell Biol.* **42**: 1472-1481.

Braunwald, E. (1997). Shattuck lecture--cardiovascular medicine at the turn of the millennium: triumphs, concerns, and opportunities. *N Engl J Med.* **337**: 1360-1369.

Bull, T.M., C.D. Coldren, M. Moore, S.M. Sotto-Santiago, D.V. Pham, S.P. Nana-Sinkam, N.F. Voelkel, and M.W. Geraci. (2004). Gene microarray analysis of peripheral blood cells in pulmonary arterial hypertension. *Am J Respir Crit Care Med.* **170**: 911-919.

Buono, C., C.J. Binder, G. Stavrakis, J.L. Witztum, L.H. Glimcher, and A.H. Lichtman. (2005). T-bet deficiency reduces atherosclerosis and alters plaque antigen-specific immune responses. *Proc Natl Acad Sci USA.* **102**: 1596-1601.

Buono, C., C.E. Come, G. Stavrakis, G.F. Maguire, P.W. Connelly, and A.H. Lichtman. (2003). Influence of interferon-gamma on the extent and phenotype of diet-induced atherosclerosis in the LDLR-deficient mouse. *Arterioscler Thromb Vasc Biol.* **23**: 454-460.

Butz, L.W., and V. de Vigneaud. (1932). The formation of a homologue of cystine by the decomposition of methionine with sulfuric acid. *J Biol Chem.* **99**: 135–142.

Cai, J., X.L. Zhao, A.W. Liu, H. Nian, and S.H. Zhang. (2010). Apigenin inhibits hepatoma cell growth through alteration of gene expression patterns. *Phytomedicine*. doi: 10.1016/j.phymed.2010.08.006

Caligiuri, G., A. Nicoletti, B. Poirier, and G.K. Hansson. (2002). Protective immunity against atherosclerosis carried by B cells of hypercholesterolemic mice. *J Clin Invest*. **109**: 745-753.

Callejon, G., A. Mayor-Olea, A.J. Jimenez, M.J. Gaitan, A.R. Palomares, F. Martinez, M. Ruiz, and A. Reyes-Engel. (2007). Genotypes of the C677T and A1298C polymorphisms of the MTHFR gene as a cause of human spontaneous embryo loss. *Hum Reprod*. **22**: 3249-3254.

Campbell, L.A., and C.C. Kuo. (2004). Chlamydia pneumoniae--an infectious risk factor for atherosclerosis? *Nat Rev Microbiol*. **2**: 23-32.

Cardinal, H., M.A. Raymond, M.J. Hebert, and F. Madore. (2007). Uraemic plasma decreases the expression of ABCA1, ABCG1 and cell-cycle genes in human coronary arterial endothelial cells. *Nephrol Dial Transplant*. **22**: 409-416.

Carman, C.V., and T.A. Springer. (2004). A transmigratory cup in leukocyte diapedesis both through individual vascular endothelial cells and between them. *J Cell Biol*. **167**: 377-388.

Casey, D.P., W.W. Nichols, C.R. Conti, and R.W. Braith. (2010). Relationship between endogenous concentrations of vasoactive substances and measures of peripheral vasodilator function in patients with coronary artery disease. *Clin Exp Pharmacol Physiol*. **37**: 24-28.

Castro-Sanchez, A.M., C. Moreno-Lorenzo, G.A. Mataran-Penarrocha, B. Feriche-Fernandez-Castanys, G. Granados-Gamez, and J.M. Quesada-Rubio. (2009). Connective Tissue Reflex Massage for Type 2 Diabetic Patients with Peripheral Arterial Disease: Randomized Controlled Trial. *Evid Based Complement Alternat Med*. doi:10.1093/ecam/ nen040.

Chan, D., M.E. Anderson, and B.L. Dolmatch. (2010). Imaging evaluation of lower extremity infrainguinal disease: role of the noninvasive vascular laboratory, computed tomography angiography, and magnetic resonance angiography. *Tech Vasc Interv Radiol*. **13**: 11-22.

Chan, D., and L.L. Ng. (2010). Biomarkers in acute myocardial infarction. *BMC Med*. **8**: 34.

Chandrasekharan, U.M., M. Waitkus, C.M. Kinney, A. Walters-Stewart, and P.E. DiCorleto. (2010). Synergistic induction of mitogen-activated protein kinase phosphatase-1 by thrombin and epidermal growth factor requires vascular endothelial growth factor receptor-2. *Arterioscler Thromb Vasc Biol*. **30**: 1983-1989.

Chen, F., J. Guo, Y. Zhang, Y. Zhao, N. Zhou, S. Liu, Y. Liu, and D. Zheng. (2009a). Knockdown of c-FLIP(L) enhanced AD5-10 anti-death receptor 5 monoclonal antibody-induced apoptosis in human lung cancer cells. *Cancer Sci.* **100**: 940-947.

Chen, K.J., W.H. Pan, F.L. Yang, I.L. Wei, N.S. Shaw, and B.F. Lin. (2005). Association of B vitamins status and homocysteine levels in elderly Taiwanese. *Asia Pac J Clin Nutr.* **14**: 250-255.

Chen, S.C., Y.C. Liu, K.G. Shyu, and D.L. Wang. (2008). Acute hypoxia to endothelial cells induces activating transcription factor 3 (ATF3) expression that is mediated via nitric oxide. *Atherosclerosis.* **201**: 281-288.

Chen, Y., A.B. Rabson, and D.H. Gorski. (2010). MEOX2 regulates nuclear factor-kappaB activity in vascular endothelial cells through interactions with p65 and IkappaBbeta. *Cardiovasc Res.* **87**: 723-731.

Chen, Y.L., Y.S. Chang, J.G. Chang, and S.M. Wu. (2009b). Genotyping of single nucleotide polymorphism in MDM2 genes by universal fluorescence primer PCR and capillary electrophoresis. *Anal Bioanal Chem.* **394**: 1291-1297.

Cheng, H.L., S.S. Chiou, Y.M. Liao, Y.L. Chen, and S.M. Wu. (2010). Genotyping of two single nucleotide polymorphisms in 5,10-methylenetetrahydrofolate reductase by multiplex polymerase chain reaction and capillary electrophoresis. *J Chromatogr A.* doi: 10.1016/j.chroma.2010.08.080

Christen, W.G., U.A. Ajani, R.J. Glynn, and C.H. Hennekens. (2000). Blood levels of homocysteine and increased risks of cardiovascular disease: causal or casual? *Arch Intern Med.* **160**: 422-434.

Christensen, B., A. Mosdol, L. Retterstol, S. Landaas, and D.S. Thelle. (2001). Abstention from filtered coffee reduces the concentrations of plasma homocysteine and serum cholesterol--a randomized controlled trial. *Am J Clin Nutr.* **74**: 302-307.

Cimmino, G., P. Golino, and J.J. Badimon. (2011). Pathophysiological role of blood-borne tissue factor: should the old paradigm be revisited? *Intern Emerg Med.* **6**: 29-34.

Claudio, J.O., Y.X. Zhu, S.J. Benn, A.H. Shukla, C.J. McGlade, N. Falcioni, and A.K. Stewart. (2001). HACS1 encodes a novel SH3-SAM adaptor protein differentially expressed in normal and malignant hematopoietic cells. *Oncogene.* **20**: 5373-5377.

Collin, S.M., C. Metcalfe, L. Zuccolo, S.J. Lewis, L. Chen, A. Cox, M. Davis, J.A. Lane, J. Donovan, G.D. Smith, D.E. Neal, F.C. Hamdy, J. Gudmundsson, P. Sulem, T. Rafnar, K.R. Benediktsdottir, R.A. Eeles, M. Guy, Z. Kote-Jarai, J. Morrison, A.A. Al Olama, K. Stefansson, D.F. Easton, and R.M. Martin. (2009). Association of folate-pathway gene polymorphisms with the risk of prostate cancer: a population-based nested case-control study,

systematic review, and meta-analysis. *Cancer Epidemiol Biomarkers Prev.* **18**: 2528-2539.

Comerota, A.J., R.C. Throm, P. Kelly, and M. Jaff. (2003). Tissue (muscle) oxygen saturation (StO2): a new measure of symptomatic lower-extremity arterial disease. *J Vasc Surg.* **38**: 724-729.

Costandi, J., M. Melone, A. Zhao, and S. Rashid. (2011). Human Resistin Stimulates Hepatic Overproduction of Atherogenic ApoB-Containing Lipoprotein Particles by Enhancing ApoB Stability and Impairing Intracellular Insulin Signaling. *Circ Res.* doi: 10.1161/CIRCRESAHA.110.238949.

Creager, M.A., S.J. Gallagher, X.J. Girerd, S.M. Coleman, V.J. Dzau, and J.P. Cooke. (1992). L-arginine improves endothelium-dependent vasodilation in hypercholesterolemic humans. *J Clin Invest.* **90**: 1248-1253.

Cunningham, M.A., V. Swanson, R.E. O'Carroll, and R.J. Holdsworth. (2010). Increasing walking in patients with intermittent claudication: protocol for a randomised controlled trial. *BMC Cardiovasc Disord.* **10**: 49.

Dafni, C., N. Drakoulis, O. Landt, D. Panidis, M. Reczko, and D.V. Cokkinos. (2010). Association of the eNOS E298D polymorphism and the risk of myocardial infarction in the Greek population. *BMC Med Genet.* **11**: 133.

Dahl, T.B., A. Yndestad, M. Skjelland, E. Oie, A. Dahl, A. Michelsen, J.K. Damas, S.H. Tunheim, T. Ueland, C. Smith, B. Bendz, S. Tonstad, L. Gullestad, S.S. Froland, K. Krohg-Sorensen, D. Russell, P. Aukrust, and B. Halvorsen. (2007). Increased expression of visfatin in macrophages of human unstable carotid and coronary atherosclerosis: possible role in inflammation and plaque destabilization. *Circulation.* **115**: 972-980.

Dai, Y.S., J. Xu, and J.D. Molkentin. (2005). The DnaJ-related factor Mrj interacts with nuclear factor of activated T cells c3 and mediates transcriptional repression through class II histone deacetylase recruitment. *Mol Cell Biol.* **25**: 9936-9948.

Das, A., M.E. Fernandez-Zapico, S. Cao, J. Yao, S. Fiorucci, R.P. Hebbel, R. Urrutia, and V.H. Shah. (2006). Disruption of an SP2/KLF6 repression complex by SHP is required for farnesoid X receptor-induced endothelial cell migration. *J Biol Chem.* **281**: 39105-39113.

Dasgupta, S., F.Y. Demirci, A.S. Dressen, A.H. Kao, E.Y. Rhew, R. Ramsey-Goldman, S. Manzi, C.M. Kammerer, and M.I. Kamboh. (2011). Association analysis of PON2 genetic variants with serum paraoxonase activity and systemic lupus erythematosus. *BMC Med Genet.* **12**: 7.

Daugherty, A., E. Pure, D. Delfel-Butteiger, S. Chen, J. Leferovich, S.E. Roselaar, and D.J. Rader. (1997). The effects of total lymphocyte deficiency on the extent of atherosclerosis in apolipoprotein E-/- mice. *J Clin Invest.* **100**: 1575-1580.

Daugherty, A., and D.L. Rateri. (2002). T lymphocytes in atherosclerosis: the yin-yang of Th1 and Th2 influence on lesion formation. *Circ Res.* **90**: 1039-1040.

Davenport, P., and P.G. Tipping. (2003). The role of interleukin-4 and interleukin-12 in the progression of atherosclerosis in apolipoprotein E-deficient mice. *Am J Pathol.* **163**: 1117-1125.

Davies, M.J. (1996). Stability and instability: two faces of coronary atherosclerosis. The Paul Dudley White Lecture 1995. *Circulation.* **94**: 2013-2020.

Davies, M.R., C.J. Harding, S. Raines, K. Tolley, A.E. Parker, M. Downey-Jones, and M.R. Needham. (2005). Nurr1 dependent regulation of pro-inflammatory mediators in immortalised synovial fibroblasts. *J Inflamm (Lond).* **2**: 15.

Davis, A.H., W. Jianhua, T.C. Tsang, and D.T. Harris. (2007). Direct sequencing is more accurate and feasible in detecting single nucleotide polymorphisms than RFLP: using human vascular endothelial growth factor gene as a model. *Biol Res Nurs.* **9**: 170-178.

De Bock, C.E., Z. Lin, A.H. Mekkawy, J.A. Byrne, and Y. Wang. (2010). Interaction between urokinase receptor and heat shock protein MRJ enhances cell adhesion. *Int J Oncol.* **36**: 1155-1163.

de Boer, O.J., A.C. van der Wal, M.A. Houtkamp, J.M. Ossewaarde, P. Teeling, and A.E. Becker. (2000). Unstable atherosclerotic plaques contain T-cells that respond to Chlamydia pneumoniae. *Cardiovasc Res.* **48**: 402-408.

de Bree, A., W.M. Verschuren, H.J. Blom, and D. Kromhout. (2001). Association between B vitamin intake and plasma homocysteine concentration in the general Dutch population aged 20-65 y. *Am J Clin Nutr.* **73**: 1027-1033.

Delves, P.J., and I.M. Roitt. (2000). The immune system. First of two parts. *N Engl J Med.* **343**: 37-49.

Demyanets, S., C. Kaun, K. Rychli, S. Pfaffenberger, S.P. Kastl, P.J. Hohensinner, G. Rega, K.M. Katsaros, T. Afonyushkin, V.N. Bochkov, M. Paireder, I. Huk, G. Maurer, K. Huber, and J. Wojta. (2011). Oncostatin M-enhanced vascular endothelial growth factor expression in human vascular smooth muscle cells involves PI3K-, p38 MAPK-, Erk1/2- and STAT1/STAT3-dependent pathways and is attenuated by interferon-gamma. *Basic Res Cardiol.* **106**: 217-231.

Demyanets, S., C. Kaun, K. Rychli, G. Rega, S. Pfaffenberger, T. Afonyushkin, V.N. Bochkov, G. Maurer, K. Huber, and J. Wojta. (2007). The inflammatory cytokine oncostatin M induces PAI-1 in human vascular smooth muscle cells in vitro via PI 3-kinase and ERK1/2-dependent pathways. *Am J Physiol Heart Circ Physiol.* **293**: H1962-H1968.

Denes, V., M. Pilichowska, A. Makarovskiy, G. Carpinito, and P. Geck. (2010). Loss of a cohesin-linked suppressor APRIN (Pds5b) disrupts stem cell programs in embryonal carcinoma: an emerging cohesin role in tumor suppression. *Oncogene*. **29**: 3446-3452.

Dennis, G., Jr., B.T. Sherman, D.A. Hosack, J. Yang, W. Gao, H.C. Lane, and R.A. Lempicki. (2003). DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol*. **4**: P3.

Deutsch, S., R. Lyle, E.T. Dermitzakis, H. Attar, L. Subrahmanyan, C. Gehrig, L. Parand, M. Gagnebin, J. Rougemont, C.V. Jongeneel, and S.E. Antonarakis. (2005). Gene expression variation and expression quantitative trait mapping of human chromosome 21 genes. *Hum Mol Genet*. **14**: 3741-3749.

DiFeo, A., G. Narla, and J.A. Martignetti. (2009). Emerging roles of Kruppel-like factor 6 and Kruppel-like factor 6 splice variant 1 in ovarian cancer progression and treatment. *Mt Sinai J Med*. **76**: 557-566.

Djousse, L., D. Levy, L.A. Cupples, J.C. Evans, R.B. D'Agostino, and R.C. Ellison. (2001). Total serum bilirubin and risk of cardiovascular disease in the Framingham offspring study. *Am J Cardiol*. **87**: 1196-1200; A4, 7.

Dollery, C.M., and P. Libby. (2006). Atherosclerosis and proteinase activation. *Cardiovasc Res*. **69**: 625-635.

Dormandy, J.A. (1995). [Epidemiology and natural history of arterial diseases of the lower limbs]. *Rev Prat*. **45**: 32-36.

Drexler, H. (1997). Endothelial dysfunction: clinical implications. *Prog Cardiovasc Dis*. **39**: 287-324.

Du, R., K. Tantisira, V. Carey, S. Bhattacharya, S. Metje, A.T. Kho, B.J. Klanderman, R. Gaedigk, R. Lazarus, T.J. Mariani, J.S. Leeder, and S.T. Weiss. (2009). Platform dependence of inference on gene-wise and gene-set involvement in human lung development. *BMC Bioinformatics*. **10**: 189.

Dutta, S., A. Chatterjee, S. Sinha, A. Chattopadhyay, and K. Mukhopadhyay. (2009). Correlation between cystathionine beta synthase gene polymorphisms, plasma homocysteine and idiopathic mental retardation in Indian individuals from Kolkata. *Neurosci Lett*. **453**: 214-218.

Dutta, S., S. Sinha, A. Chattopadhyay, P.K. Gangopadhyay, J. Mukhopadhyay, M. Singh, and K. Mukhopadhyay. (2005). Cystathionine beta-synthase T833C/844INS68 polymorphism: a family-based study on mentally retarded children. *Behav Brain Funct*. **1**: 25.

Eady, J.J., G.M. Wortley, Y.M. Wormstone, J.C. Hughes, S.B. Astley, R.J. Foxall, J.F. Doleman, and R.M. Elliott. (2005). Variation in gene expression

profiles of peripheral blood mononuclear cells from healthy volunteers. *Physiol Genomics*. **22**: 402-411.

Edfeldt, K., J. Swedenborg, G.K. Hansson, and Z.Q. Yan. (2002). Expression of toll-like receptors in human atherosclerotic lesions: a possible pathway for plaque activation. *Circulation*. **105**: 1158-1161.

Eiberg, J.P., J.B. Gronvall Rasmussen, M.A. Hansen, and T.V. Schroeder. (2010). Duplex ultrasound scanning of peripheral arterial disease of the lower limb. *Eur J Vasc Endovasc Surg*. **40**: 507-512.

Eisen, M.B., P.T. Spellman, P.O. Brown, and D. Botstein. (1998). Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci USA*. **95**: 14863-14868.

Empana, J.P., F. Canoui-Poitrine, G. Luc, I. Juhan-Vague, P. Morange, D. Arveiler, J. Ferrieres, P. Amouyel, A. Bingham, M. Montaye, J.B. Ruidavets, B. Haas, A. Evans, and P. Ducimetiere. (2008). Contribution of novel biomarkers to incident stable angina and acute coronary syndrome: the PRIME Study. *Eur Heart J*. **29**: 1966-1974.

Evans, D.C., B. Sileshi, A.M. Zakaria, D. Giangiacomo, R.J. Manson, and J.H. Lawson. (2008). Genomic modeling of atherosclerosis in peripheral arterial disease and its variant phenotype in patients with diabetes. *Vascular*. **16**: 225-235.

Falchi, A., L. Giovannoni, I.S. Piras, C.M. Calo, P. Moral, G. Vona, and L. Varesi. (2005). Prevalence of genetic risk factors for coronary artery disease in Corsica island (France). *Exp Mol Pathol*. **79**: 210-213.

Falk, E., P.K. Shah, and V. Fuster. (1995). Coronary plaque disruption. *Circulation*. **92**: 657-671.

Fang, N., L. Lin, J. Ren, and D. Wu. (2004). Detection of C677T mutation in methylenetetrahydrofolate reductase gene by denaturing high performance liquid chromatography. *Biomed Chromatogr*. **18**: 625-629.

Faria-Neto, J.R., A.C. Chagas, S.P. Bydlowski, P.A. Lemos Neto, D.A. Chamone, J.A. Ramirez, and P.L. da Luz. (2006). Hyperhomocystinemia in patients with coronary artery disease. *Braz J Med Biol Res*. **39**: 455-463.

Fernandez-Suarez, X.M., and M.K. Schuster. (2010). Using the ensembl genome server to browse genomic sequence data. *Curr Protoc Bioinformatics*. Chapter 1:Unit1 15.

Ferreira, V.P., M.K. Pangburn, and C. Cortes. (2010). Complement control protein factor H: the good, the bad, and the inadequate. *Mol Immunol*. **47**: 2187-2197.

Ferretti, G., T. Bacchetti, C. Moroni, A. Vignini, L. Nanetti, and G. Curatola. (2004). Effect of homocysteinylation of low density lipoproteins on lipid peroxidation of human endothelial cells. *J Cell Biochem.* **92**: 351-360.

Finkelstein, J.D. (1998). The metabolism of homocysteine: pathways and regulation. *Eur J Pediatr.* **157 Suppl 2**: S40-S44.

Foulds, C.E., A. Tsimelzon, W. Long, A. Le, S.Y. Tsai, M.J. Tsai, and B.W. O'Malley. (2010). Research resource: expression profiling reveals unexpected targets and functions of the human steroid receptor RNA activator (SRA) gene. *Mol Endocrinol.* **24**: 1090-1105.

Fowkes, F.G., E. Housley, C.C. Macintyre, R.J. Prescott, and C.V. Ruckley. (1988). Variability of ankle and brachial systolic pressures in the measurement of atherosclerotic peripheral arterial disease. *J Epidemiol Community Health.* **42**: 128-133.

Franco, R., F. Maffei, D. Lourenco, C. Piccinato, V. Morelli, I. Thomazini, and M. Zago. (1998a). The frequency of 844ins68 mutation in the cystathionine beta-synthase gene is not increased in patients with venous thrombosis. *Haematologica.* **83**: 1006-1008.

Franco, R.F., J. Elion, J. Lavinha, R. Krishnamoorthy, M.H. Tavella, and M.A. Zago. (1998b). Heterogeneous ethnic distribution of the 844ins68 in the cystathionine beta-synthase gene. *Hum Hered.* **48**: 338-342.

Friesel, R., A. Komoriya, and T. Maciag. (1987). Inhibition of endothelial cell proliferation by gamma-interferon. *J Cell Biol.* **104**: 689-696.

Frosst, P., H.J. Blom, R. Milos, P. Goyette, C.A. Sheppard, R.G. Matthews, G.J. Boers, M. den Heijer, L.A. Kluijtmans, L.P. van den Heuvel, and Rozen, R. (1995). A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet.* **10**: 111-113.

Frostegard, J., A.K. Ulfgren, P. Nyberg, U. Hedin, J. Swedenborg, U. Andersson, and G.K. Hansson. (1999). Cytokine expression in advanced human atherosclerotic plaques: dominance of pro-inflammatory (Th1) and macrophage-stimulating cytokines. *Atherosclerosis.* **145**: 33-43.

Fryer, R.H., B.D. Wilson, D.B. Gubler, L.A. Fitzgerald, and G.M. Rodgers. (1993). Homocysteine, a risk factor for premature vascular disease and thrombosis, induces tissue factor activity in endothelial cells. *Arterioscler Thromb.* **13**: 1327-1333.

Fu, S., H. Zhao, J. Shi, A. Abzhanov, K. Crawford, L. Ohno-Machado, J. Zhou, Y. Du, W.P. Kuo, J. Zhang, M. Jiang, and J.G. Jin. (2008). Peripheral arterial occlusive disease: global gene expression analyses suggest a major role for immune and inflammatory responses. *BMC Genomics.* **9**: 369.

Furst, R., C. Brueckl, W.M. Kuebler, S. Zahler, F. Krotz, A. Gorlach, A.M. Vollmar, and A.K. Kiemer. (2005). Atrial natriuretic peptide induces mitogen-activated protein kinase phosphatase-1 in human endothelial cells via Rac1 and NAD(P)H oxidase/Nox2-activation. *Circ Res.* **96**: 43-53.

Furuno, K., H. Takada, K. Yamamoto, K. Ikeda, T. Ohno, V. Khajoee, Y. Mizuno, and T. Hara. (2007). Tissue inhibitor of metalloproteinase 2 and coronary artery lesions in Kawasaki disease. *J Pediatr.* **151**: 155-160, 160 e1.

Galis, Z.S., R. Kranzhofer, J.W. Fenton, 2nd, and P. Libby. (1997). Thrombin promotes activation of matrix metalloproteinase-2 produced by cultured vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol.* **17**: 483-489.

Galis, Z.S., G.K. Sukhova, M.W. Lark, and P. Libby. (1994). Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. *J Clin Invest.* **94**: 2493-2503.

Galmozzi, E., B.D. Menico, R. Rametta, P. Dongiovanni, A.L. Fracanzani, L. Benedan, V. Borroni, P. Maggioni, S. Fargion, and L. Valenti. (2010). A tetra-primer amplification refractory mutation system polymerase chain reaction for the evaluation of rs12979860 IL28B genotype. *J Viral Hepat.* doi: 10.1111/j.1365-2893.2010.01349.x.

Gardina, P.J., T.A. Clark, B. Shimada, M.K. Staples, Q. Yang, J. Veitch, A. Schweitzer, T. Awad, C. Sugnet, S. Dee, C. Davies, A. Williams, and Y. Turpaz. (2006). Alternative splicing and differential gene expression in colon cancer detected by a whole genome exon array. *BMC Genomics.* **7**: 325.

Gargalovic, P.S., M. Imura, B. Zhang, N.M. Gharavi, M.J. Clark, J. Pagnon, W.P. Yang, A. He, A. Truong, S. Patel, S.F. Nelson, S. Horvath, J.A. Berliner, T.G. Kirchgessner, and A.J. Lusis. (2006). Identification of inflammatory gene modules based on variations of human endothelial cell responses to oxidized lipids. *Proc Natl Acad Sci USA.* **103**: 12741-12746.

Gentleman, R.C., V.J. Carey, D.M. Bates, B. Bolstad, M. Dettling, S. Dudoit, B. Ellis, L. Gautier, Y. Ge, J. Gentry, K. Hornik, T. Hothorn, W. Huber, S. Iacus, R. Irizarry, F. Leisch, C. Li, M. Maechler, A.J. Rossini, G. Sawitzki, C. Smith, G. Smyth, L. Tierney, J.Y. Yang, and J. Zhang. (2004). Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.* **5**: R80.

Ghandri, N., S. Gabbouj, K. Farhat, N. Bouaouina, H. Abdelaziz, A. Nouri, L. Chouchane, and E. Hassen. (2011). Association of HLA-G polymorphisms with nasopharyngeal carcinoma risk and clinical outcome. *Hum Immunol.* **72**: 150-158.

Giusti, B., C. Saracini, P. Bolli, A. Magi, I. Sestini, E. Sticchi, G. Pratesi, R. Pulli, C. Pratesi, and R. Abbate. (2008). Genetic analysis of 56 polymorphisms in 17 genes involved in methionine metabolism in patients with abdominal aortic aneurysm. *J Med Genet.* **45**: 721-730.

Givelber, R.J., N.N. Couropmitree, D.J. Gottlieb, J.C. Evans, D. Levy, R.H. Myers, and G.T. O'Connor. (1998). Segregation analysis of pulmonary function among families in the Framingham Study. *Am J Respir Crit Care Med.* **157**: 1445-1451.

Goligorsky, M.S. (2000). Endothelial cell dysfunction and nitric oxide synthase. *Kidney Int.* **58**: 1360-1376.

Golimbet, V., G. Korovaitseva, L. Abramova, and V. Kaleda. (2009). The 844ins68 polymorphism of the cystathionine beta-synthase gene is associated with schizophrenia. *Psychiatry Res.* **170**: 168-171.

Graham, I.M., L.E. Daly, H.M. Refsum, K. Robinson, L.E. Brattstrom, P.M. Ueland, R.J. Palma-Reis, G.H. Boers, R.G. Sheahan, B. Israelsson, C.S. Uiterwaal, R. Meleady, D. McMaster, P. Verhoef, J. Witteman, P. Rubba, H. Bellet, J.C. Wautrecht, H.W. de Valk, A.C. Sales Luis, F.M. Parrot-Rouland, K.S. Tan, I. Higgins, D. Garcon, G. Andria, and et al. (1997). Plasma homocysteine as a risk factor for vascular disease. The European Concerted Action Project. *JAMA.* **277**: 1775-1781.

Grainger, D.J., C.M. Witchell, and J.C. Metcalfe. (1995). Tamoxifen elevates transforming growth factor-beta and suppresses diet-induced formation of lipid lesions in mouse aorta. *Nat Med.* **1**: 1067-1073.

Gresele, P., E. Falcinelli, F. Loffredo, G. Cimmino, T. Corazzi, L. Forte, G. Guglielmini, S. Momi, and P. Golino. (2011). Platelets release matrix metalloproteinase-2 in the coronary circulation of patients with acute coronary syndromes: possible role in sustained platelet activation. *Eur Heart J.* **32**: 316-325.

Griffin, T.J., J.G. Hall, J.R. Prudent, and L.M. Smith. (1999). Direct genetic analysis by matrix-assisted laser desorption/ionization mass spectrometry. *Proc Natl Acad Sci USA.* **96**: 6301-6306.

Griffin, T.J., and L.M. Smith. (2000a). Genetic identification by mass spectrometric analysis of single-nucleotide polymorphisms: ternary encoding of genotypes. *Anal Chem.* **72**: 3298-3302.

Griffin, T.J., and L.M. Smith. (2000b). Single-nucleotide polymorphism analysis by MALDI-TOF mass spectrometry. *Trends Biotechnol.* **18**: 77-84.

Grodzicker, T., C. Anderson, P.A. Sharp, and J. Sambrook. (1974). Conditional lethal mutants of adenovirus 2-simian virus 40 hybrids. I. Host range mutants of Ad2+ND1. *J Virol.* **13**: 1237-1244.

Grubben, M.J., G.H. Boers, H.J. Blom, R. Broekhuizen, R. de Jong, L. van Rijt, E. de Ruijter, D.W. Swinkels, F.M. Nagengast, and M.B. Katan. (2000). Unfiltered coffee increases plasma homocysteine concentrations in healthy volunteers: a randomized trial. *Am J Clin Nutr.* **71**: 480-484.

Gruber, J.V., and R. Holtz. (2010). Examining the genomic influence of skin antioxidants in vitro. *Mediators Inflamm.* doi:10.1155/2010/230450.

Guerzoni, A.R., P.M. Biselli, M.F. Godoy, D.R. Souza, R. Haddad, M.N. Eberlin, E.C. Pavarino-Bertelli, and E.M. Goloni-Bertollo. (2009). Homocysteine and MTHFR and VEGF gene polymorphisms: impact on coronary artery disease. *Arq Bras Cardiol.* **92**: 263-268.

Gupta, S., A.M. Pablo, X. Jiang, N. Wang, A.R. Tall, and C. Schindler. (1997). IFN-gamma potentiates atherosclerosis in ApoE knock-out mice. *J Clin Invest.* **99**: 2752-2761.

Guttman, M., P. Fules, and A. Guttman. (2003). Analysis of site-directed mutagenesis constructs by capillary electrophoresis using linear polymer sieving matrices. *J Chromatogr A.* **1014**: 21-27.

Ha, K., J. Coulombe-Huntington, and J. Majewski. (2009). Comparison of Affymetrix Gene Array with the Exon Array shows potential application for detection of transcript isoform variation. *BMC Genomics.* **10**: 519.

Hambaba, L., S. Abdessemed, M. Yahia, S. Laroui, and F. Rouabah. (2008). [Relationship between hyperhomocysteinemia and C677T polymorphism of methylene tetrahydrofolate reductase gene in a healthy Algerian population]. *Ann Biol Clin (Paris).* **66**: 637-641.

Hanson, N.Q., O. Aras, F. Yang, and M.Y. Tsai. (2001). C677T and A1298C polymorphisms of the methylenetetrahydrofolate reductase gene: incidence and effect of combined genotypes on plasma fasting and post-methionine load homocysteine in vascular disease. *Clin Chem.* **47**: 661-666.

Hansson, G.K. (2005). Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med.* **352**: 1685-1695.

Hansson, G.K., M. Hellstrand, L. Rymo, L. Rubbia, and G. Gabbiani. (1989). Interferon gamma inhibits both proliferation and expression of differentiation-specific alpha-smooth muscle actin in arterial smooth muscle cells. *J Exp Med.* **170**: 1595-1608.

Harpel, P.C., V.T. Chang, and W. Borth. (1992). Homocysteine and other sulfhydryl compounds enhance the binding of lipoprotein(a) to fibrin: a potential biochemical link between thrombosis, atherogenesis, and sulfhydryl compound metabolism. *Proc Natl Acad Sci USA.* **89**: 10193-10197.

Hata, N., K. Yoshimoto, N. Yokoyama, M. Mizoguchi, T. Shono, Y. Guan, T. Tahira, Y. Kukita, K. Higasa, S. Nagata, T. Iwaki, T. Sasaki, and K. Hayashi. (2006). Allelic losses of chromosome 10 in glioma tissues detected by quantitative single-strand conformation polymorphism analysis. *Clin Chem.* **52**: 370-378.

Hatanaka, H., M. Tsukui, S. Takada, K. Kurashina, Y.L. Choi, M. Soda, Y. Yamashita, H. Haruta, T. Hamada, T. Ueno, K. Tamada, Y. Hosoya, N. Sata, Y. Yasuda, H. Nagai, K. Sugano, and H. Mano. (2010). Identification of transforming activity of free fatty acid receptor 2 by retroviral expression screening. *Cancer Sci.* **101**: 54-59.

Hayashi, T., G. Honda, and K. Suzuki. (1992). An atherogenic stimulus homocysteine inhibits cofactor activity of thrombomodulin and enhances thrombomodulin expression in human umbilical vein endothelial cells. *Blood.* **79**: 2930-2936.

Hazra, A., P. Kraft, R. Lazarus, C. Chen, S.J. Chanock, P. Jacques, J. Selhub, and D.J. Hunter. (2009). Genome-wide significant predictors of metabolites in the one-carbon metabolism pathway. *Hum Mol Genet.* **18**: 4677-4687.

Heid, C.A., J. Stevens, K.J. Livak, and P.M. Williams. (1996). Real time quantitative PCR. *Genome Res.* **6**: 986-994.

Helfand, M., D.I. Buckley, M. Freeman, R. Fu, K. Rogers, C. Fleming, and L.L. Humphrey. (2009). Emerging risk factors for coronary heart disease: a summary of systematic reviews conducted for the U.S. Preventive Services Task Force. *Ann Intern Med.* **151**: 496-507.

Helgadottir, A., G. Thorleifsson, K.P. Magnusson, S. Gretarsdottir, V. Steinthorsdottir, A. Manolescu, G.T. Jones, G.J. Rinkel, J.D. Blankensteijn, A. Ronkainen, J.E. Jaaskelainen, Y. Kyo, G.M. Lenk, N. Sakalihasan, K. Kostulas, A. Gottsater, A. Flex, H. Stefansson, T. Hansen, G. Andersen, S. Weinsheimer, K. Borch-Johnsen, T. Jorgensen, S.H. Shah, A.A. Quyyumi, C.B. Granger, M.P. Reilly, H. Austin, A.I. Levey, V. Vaccarino, E. Palsdottir, G.B. Walters, T. Jonsdottir, S. Snorradottir, D. Magnusdottir, G. Gudmundsson, R.E. Ferrell, S. Sveinbjornsdottir, J. Hernesniemi, M. Niemela, R. Limet, K. Andersen, G. Sigurdsson, R. Benediktsson, E.L. Verhoeven, J.A. Teijink, D.E. Grobbee, D.J. Rader, D.A. Collier, O. Pedersen, R. Pola, J. Hillert, B. Lindblad, E.M. Valdimarsson, H.B. Magnadottir, C. Wijmenga, G. Tromp, A.F. Baas, Y.M. Ruigrok, A.M. van Rij, H. Kuivaniemi, J.T. Powell, S.E. Matthiasson, J.R. Gulcher, G. Thorgeirsson, A. Kong, U. Thorsteinsdottir, and K. Stefansson. (2008). The same sequence variant on 9p21 associates with myocardial infarction, abdominal aortic aneurysm and intracranial aneurysm. *Nat Genet.* **40**: 217-224.

Helgadottir, A., G. Thorleifsson, A. Manolescu, S. Gretarsdottir, T. Blondal, A. Jonasdottir, A. Jonasdottir, A. Sigurdsson, A. Baker, A. Palsson, G. Masson, D.F. Gudbjartsson, K.P. Magnusson, K. Andersen, A.I. Levey, V.M. Backman, S. Matthiasdottir, T. Jonsdottir, S. Palsson, H. Einarsdottir, S. Gunnarsdottir, A. Gylfason, V. Vaccarino, W.C. Hooper, M.P. Reilly, C.B. Granger, H. Austin, D.J. Rader, S.H. Shah, A.A. Quyyumi, J.R. Gulcher, G. Thorgeirsson, U. Thorsteinsdottir, A. Kong, and K. Stefansson. (2007). A common variant on chromosome 9p21 affects the risk of myocardial infarction. *Science.* **316**: 1491-1493.

Henikoff, S., J.G. Henikoff, A. Sakai, G.B. Loeb, and K. Ahmad. (2009). Genome-wide profiling of salt fractions maps physical properties of chromatin. *Genome Res.* **19**: 460-469.

Hennrikus, D., A.M. Joseph, H.A. Lando, S. Duval, L. Ukestad, M. Kodl, and A.T. Hirsch. (2010). Effectiveness of a smoking cessation program for peripheral artery disease patients: a randomized controlled trial. *J Am Coll Cardiol.* **56**: 2105-2112.

Higo, M., K. Uzawa, T. Kawata, Y. Kato, Y. Kouzu, N. Yamamoto, T. Shibahara, J.E. Mizoe, H. Ito, H. Tsujii, and H. Tanzawa. (2006). Enhancement of SPHK1 in vitro by carbon ion irradiation in oral squamous cell carcinoma. *Int J Radiat Oncol Biol Phys.* **65**: 867-875.

Hindle, A.K., C. Edwards, T. McCaffrey, S. Fu, and F. Brody. (2010). Identification of cardiovascular genes in omentum from morbidly obese patients with type 2 diabetes. *Int J Obes (Lond).* **34**: 1020-1027.

Hirsch, A.T., M.H. Criqui, D. Treat-Jacobson, J.G. Regensteiner, M.A. Creager, J.W. Olin, S.H. Krook, D.B. Hunninghake, A.J. Comerota, M.E. Walsh, M.M. McDermott, and W.R. Hiatt. (2001). Peripheral arterial disease detection, awareness, and treatment in primary care. *JAMA.* **286**: 1317-1324.

Hirsch, A.T., Z.J. Haskal, N.R. Hertzer, C.W. Bakal, M.A. Creager, J.L. Halperin, L.F. Hiratzka, W.R. Murphy, J.W. Olin, J.B. Puschett, K.A. Rosenfield, D. Sacks, J.C. Stanley, L.M. Taylor, Jr., C.J. White, J. White, R.A. White, E.M. Antman, S.C. Smith, Jr., C.D. Adams, J.L. Anderson, D.P. Faxon, V. Fuster, R.J. Gibbons, S.A. Hunt, A.K. Jacobs, R. Nishimura, J.P. Ornato, R.L. Page, and B. Riegel. (2006). ACC/AHA 2005 Practice Guidelines for the management of patients with peripheral arterial disease (lower extremity, renal, mesenteric, and abdominal aortic): a collaborative report from the American Association for Vascular Surgery/Society for Vascular Surgery, Society for Cardiovascular Angiography and Interventions, Society for Vascular Medicine and Biology, Society of Interventional Radiology, and the ACC/AHA Task Force on Practice Guidelines (Writing Committee to Develop Guidelines for the Management of Patients With Peripheral Arterial Disease): endorsed by the American Association of Cardiovascular and Pulmonary Rehabilitation; National Heart, Lung, and Blood Institute; Society for Vascular Nursing; TransAtlantic Inter-Society Consensus; and Vascular Disease Foundation. *Circulation.* **113**: e463-e654.

Hobbs, A.J., J.M. Fukuto, and L.J. Ignarro. (1994). Formation of free nitric oxide from l-arginine by nitric oxide synthase: direct enhancement of generation by superoxide dismutase. *Proc Natl Acad Sci USA.* **91**: 10992-10996.

Holland, P.M., R.D. Abramson, R. Watson, and D.H. Gelfand. (1991). Detection of specific polymerase chain reaction product by utilizing the 5'----3'

exonuclease activity of Thermus aquaticus DNA polymerase. *Proc Natl Acad Sci USA*. **88**: 7276-7280.

Homer, N., W.D. Tembe, S. Szelinger, M. Redman, D.A. Stephan, J.V. Pearson, S.F. Nelson, and D. Craig. (2008). Multimarker analysis and imputation of multiple platform pooling-based genome-wide association studies. *Bioinformatics*. **24**: 1896-1902.

Hong, Y.H., Y. Nishimura, D. Hishikawa, H. Tsuzuki, H. Miyahara, C. Gotoh, K.C. Choi, D.D. Feng, C. Chen, H.G. Lee, K. Katoh, S.G. Roh, and S. Sasaki. (2005). Acetate and propionate short chain fatty acids stimulate adipogenesis via GPCR43. *Endocrinology*. **146**: 5092-5099.

Hu, G., H.Y. Wang, D.M. Greenawalt, M.A. Azaro, M. Luo, I.V. Tereshchenko, X. Cui, Q. Yang, R. Gao, L. Shen, and H. Li. (2006). AccuTyping: new algorithms for automated analysis of data from high-throughput genotyping with oligonucleotide microarrays. *Nucleic Acids Res*. **34**: e116.

Huang da, W., B.T. Sherman, and R.A. Lempicki. (2009). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc*. **4**: 44-57.

Huang, Y., Y. Lei, H. Zhang, M. Zhang, and A. Dayton. (2010). Role of interleukin-18 in human natural killer cell is associated with interleukin-2. *Mol Immunol*. **47**: 2604-2610.

Huber, S.A., P. Sakkinen, C. David, M.K. Newell, and R.P. Tracy. (2001). T helper-cell phenotype regulates atherosclerosis in mice under conditions of mild hypercholesterolemia. *Circulation*. **103**: 2610-2616.

Hung, K., X. Sun, H. Ding, M. Kalafatis, P. Simioni, and B. Guo. (2002). A matrix-assisted laser desorption/ionization time-of-flight based method for screening the 1691G --> A mutation in the factor V gene. *Blood Coagul Fibrinolysis*. **13**: 117-122.

Huo, Y., and L. Xia. (2009). P-selectin glycoprotein ligand-1 plays a crucial role in the selective recruitment of leukocytes into the atherosclerotic arterial wall. *Trends Cardiovasc Med*. **19**: 140-145.

Hurst, S.M., R.M. McLoughlin, J. Monslow, S. Owens, L. Morgan, G.M. Fuller, N. Topley, and S.A. Jones. (2002). Secretion of oncostatin M by infiltrating neutrophils: regulation of IL-6 and chemokine expression in human mesothelial cells. *J Immunol*. **169**: 5244-5251.

Hurt, E., G. Bondjers, and G. Camejo. (1990). Interaction of LDL with human arterial proteoglycans stimulates its uptake by human monocyte-derived macrophages. *J Lipid Res*. **31**: 443-454.

Iakoubova, O.A., C.H. Tong, A.P. Chokkalingam, C.M. Rowland, T.G. Kirchgessner, J.Z. Louie, L.M. Ploughman, M.S. Sabatine, H. Campos,

J.J. Catanese, D.U. Leong, B.A. Young, D. Lew, Z. Tsuchihashi, M.M. Luke, C.J. Packard, K.E. Zerba, P.M. Shaw, J. Shepherd, J.J. Devlin, and F.M. Sacks. (2006). Asp92Asn polymorphism in the myeloid IgA Fc receptor is associated with myocardial infarction in two disparate populations: CARE and WOSCOPS. *Arterioscler Thromb Vasc Biol.* **26**: 2763-2768.

International Human Genome Sequencing Consortium. (2004). Finishing the euchromatic sequence of the human genome. *Nature*. **431**: 931-945.

International HapMap Consortium. (2005). A haplotype map of the human genome. *Nature*. **437**: 1299-1320.

Ilhan, N., M. Kucuksu, D. Kaman, N. Ilhan, and Y. Ozbay. (2008). The 677 C/T MTHFR polymorphism is associated with essential hypertension, coronary artery disease, and higher homocysteine levels. *Arch Med Res*. **39**: 125-130.

Iqbal, M.P. (2006). Hyperhomocysteinemia and coronary artery disease in Pakistan. *J Pak Med Assoc*. **56**: 282-285.

Iqbal, M.P., T. Fatima, S. Parveen, F.A. Yousuf, M. Shafiq, N. Mehboobali, A.H. Khan, I. Azam, and P.M. Frossard. (2005). Lack of association of methylenetetrahydrofolate reductase 677C>T mutation with coronary artery disease in a Pakistani population. *J Mol Genet Med*. **1**: 26-32.

Irizarry, R.A., B. Hobbs, F. Collin, Y.D. Beazer-Barclay, K.J. Antonellis, U. Scherf, and T.P. Speed. (2003). Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics*. **4**: 249-264.

Ishibashi, S., J.L. Goldstein, M.S. Brown, J. Herz, and D.K. Burns. (1994). Massive xanthomatosis and atherosclerosis in cholesterol-fed low density lipoprotein receptor-negative mice. *J Clin Invest*. **93**: 1885-1893.

Ismail, M., N. Akhtar, M. Nasir, S. Firasat, Q. Ayub, and S. Khaliq. (2004). Association between the angiotensin-converting enzyme gene insertion/deletion polymorphism and essential hypertension in young Pakistani patients. *J Biochem Mol Biol*. **37**: 552-555.

Jafar, T.H., Z. Qadri, and N. Chaturvedi. (2008). Coronary artery disease epidemic in Pakistan: more electrocardiographic evidence of ischaemia in women than in men. *Heart*. **94**: 408-413.

Jakubowski, H. (2006). Pathophysiological consequences of homocysteine excess. *J Nutr*. **136**: 1741S-1749S.

Jakubowski, H. (2008). The pathophysiological hypothesis of homocysteine thiolactone-mediated vascular disease. *J Physiol Pharmacol*. **59 Suppl 9**: 155-167.

Janeway, C.A., Jr., and R. Medzhitov. (2002). Innate immune recognition. *Annu Rev Immunol.* **20**: 197-216.

Jensen, L.J., M. Kuhn, M. Stark, S. Chaffron, C. Creevey, J. Muller, T. Doerks, P. Julien, A. Roth, M. Simonovic, P. Bork, and C. von Mering. (2009). STRING 8--a global view on proteins and their functional interactions in 630 organisms. *Nucleic Acids Res.* **37**: D412-D416.

Jiang, Z., J.M. Akey, J. Shi, M. Xiong, Y. Wang, Y. Shen, X. Xu, H. Chen, H. Wu, J. Xiao, D. Lu, W. Huang, and L. Jin. (2001). A polymorphism in the promoter region of catalase is associated with blood pressure levels. *Hum Genet.* **109**: 95-98.

Johnson, A.D., M. Kavousi, A.V. Smith, M.H. Chen, A. Dehghan, T. Aspelund, J.P. Lin, C.M. van Duijn, T.B. Harris, L.A. Cupples, A.G. Uitterlinden, L. Launer, A. Hofman, F. Rivadeneira, B. Stricker, Q. Yang, C.J. O'Donnell, V. Gudnason, and J.C. Witteman. (2009). Genome-wide association meta-analysis for total serum bilirubin levels. *Hum Mol Genet.* **18**: 2700-2710.

Jonasson, L., J. Holm, O. Skalli, G. Bondjers, and G.K. Hansson. (1986). Regional accumulations of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque. *Arteriosclerosis.* **6**: 131-138.

Jones, R., M.B. Baker, M. Weber, D.G. Harrison, G. Bao, and C.D. Searles. (2009). Molecular beacons can assess changes in expression and 3'-polyadenylation of human eNOS mRNA. *Am J Physiol Cell Physiol.* **296**: C498-C504.

Joshi, P., S. Islam, P. Pais, S. Reddy, P. Dorairaj, K. Kazmi, M.R. Pandey, S. Haque, S. Mendis, S. Rangarajan, and S. Yusuf. (2007). Risk factors for early myocardial infarction in South Asians compared with individuals in other countries. *JAMA.* **297**: 286-294.

Jylhava, J., C. Eklund, T. Pessi, O.T. Raitakari, M. Juonala, M. Kahonen, J.S. Viikari, T. Lehtimaki, and M. Hurme. (2009). Genetics of C-reactive protein and complement factor H have an epistatic effect on carotid artery compliance: the Cardiovascular Risk in Young Finns Study. *Clin Exp Immunol.* **155**: 53-58.

Kabakchiev, B., D. Turner, J. Hyams, D. Mack, N. Leleiko, W. Crandall, J. Markowitz, A.R. Otley, W. Xu, P. Hu, A.M. Griffiths, and M.S. Silverberg. (2010). Gene expression changes associated with resistance to intravenous corticosteroid therapy in children with severe ulcerative colitis. *PLoS One.* **5**: e13085.

Kadoglou, N.P., N. Sailer, A. Moumtzouoglou, A. Kapelouzou, H. Tsanikidis, I. Vitta, C. Karkos, P.E. Karayannacos, T. Gerasimidis, and C.D. Liapis. (2010). Visfatin (nampt) and ghrelin as novel markers of carotid atherosclerosis in patients with type 2 diabetes. *Exp Clin Endocrinol Diabetes.* **118**: 75-80.

Kanehisa, M., S. Goto, M. Furumichi, M. Tanabe, and M. Hirakawa. (2010). KEGG for representation and analysis of molecular networks involving diseases and drugs. *Nucleic Acids Res.* **38**: D355-D360.

Kannel, W.B. (1990). Influence of multiple risk factors on the hazard of hypertension. *J Cardiovasc Pharmacol.* **16 Suppl 5**: S53-S57.

Kanwar, Y.S., J.R. Manaligod, and P.W. Wong. (1976). Morphologic studies in a patient with homocystinuria due to 5, 10-methylenetetrahydrofolate reductase deficiency. *Pediatr Res.* **10**: 598-609.

Karas, M., and F. Hillenkamp. (1988). Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. *Anal Chem.* **60**: 2299-2301.

Kaszubska, W., R. Hooft van Huijsduijnen, P. Ghersa, A.M. DeRaemy-Schenk, B.P. Chen, T. Hai, J.F. DeLamarter, and J. Whelan. (1993). Cyclic AMP-independent ATF family members interact with NF-kappa B and function in the activation of the E-selectin promoter in response to cytokines. *Mol Cell Biol.* **13**: 7180-7190.

Kerkeni, M., F. Addad, M. Chauffert, A. Myara, M. Gerhardt, D. Chevenne, F. Trivin, M.B. Farhat, A. Miled, and K. Maaroufi. (2006). Hyperhomocysteinaemia, methylenetetrahydrofolate reductase polymorphism and risk of coronary artery disease. *Ann Clin Biochem.* **43**: 200-206.

Khandanpour, N., B. Jennings, M.P. Armon, A. Wright, G. Willis, A. Clark, and F.J. Meyer. (2011). Do novel risk biomarkers reflect the severity of peripheral arterial disease? *Angiology.* **62**: 126-133.

Khoo, S.K., K. Dykema, N.M. Vadlapatla, D. LaHaie, S. Valle, D. Satterthwaite, S.A. Ramirez, J.A. Carruthers, P.T. Haak, and J.H. Resau. (2011). Acquiring genome-wide gene expression profiles in Guthrie card blood spots using microarrays. *Pathol Int.* **61**: 1-6.

Kiel, D.P., S. Demissie, J. Dupuis, K.L. Lunetta, J.M. Murabito, and D. Karasik. (2007). Genome-wide association with bone mass and geometry in the Framingham Heart Study. *BMC Med Genet.* **8 Suppl 1**: S14.

Kim, J.J., Y.M. Hong, S. Sohn, G.Y. Jang, K.S. Ha, S.W. Yun, M.K. Han, K.Y. Lee, M.S. Song, H.D. Lee, D.S. Kim, J.E. Lee, E.S. Shin, J.H. Jang, Y.S. Lee, S.Y. Kim, J.Y. Lee, B.G. Han, J.Y. Wu, K.J. Kim, Y.M. Park, E.J. Seo, I.S. Park, and J.K. Lee. (2011). A genome-wide association analysis reveals 1p31 and 2p13.3 as susceptibility loci for Kawasaki disease. *Hum Genet.* doi: 10.1007/s00439-010-0937-x.

Kim, S.W., J.H. Heo, C.H. Kim, D.C. Yoo, D.H. Won, S.G. Lee, K.J. Cho, J.H. Song, S.J. Park, Y.G. Yang, and D.W. Choi. (2010). Rapid and direct detection of apolipoprotein E genotypes using whole blood from humans. *J Toxicol Environ Health A.* **73**: 1502-1510.

Kimmelman, A.C., R.F. Qiao, G. Narla, A. Banno, N. Lau, P.D. Bos, N. Nunez Rodriguez, B.C. Liang, A. Guha, J.A. Martignetti, S.L. Friedman, and A.M. Chan. (2004). Suppression of glioblastoma tumorigenicity by the Kruppel-like transcription factor KLF6. *Oncogene*. **23**: 5077-5083.

Kinney, C.M., U.M. Chandrasekharan, L. Mavrakis, and P.E. DiCorleto. (2008). VEGF and thrombin induce MKP-1 through distinct signaling pathways: role for MKP-1 in endothelial cell migration. *Am J Physiol Cell Physiol*. **294**: C241-C250.

Klein, S., A.R. de Fougerolles, P. Blaikie, L. Khan, A. Pepe, C.D. Green, V. Koteliansky, and F.G. Giancotti. (2002). Alpha 5 beta 1 integrin activates an NF-kappa B-dependent program of gene expression important for angiogenesis and inflammation. *Mol Cell Biol*. **22**: 5912-5922.

Klerk, M., P. Verhoef, R. Clarke, H.J. Blom, F.J. Kok, and E.G. Schouten. (2002). MTHFR 677C-->T polymorphism and risk of coronary heart disease: a meta-analysis. *JAMA*. **288**: 2023-2031.

Kluijtmans, L.A., and A.S. Whitehead. (2001). Methylenetetrahydrofolate reductase genotypes and predisposition to atherothrombotic disease; evidence that all three MTHFR C677T genotypes confer different levels of risk. *Eur Heart J*. **22**: 294-299.

Ko, N.U., P. Rajendran, H. Kim, M. Rutkowski, L. Pawlikowska, P.Y. Kwok, R.T. Higashida, M.T. Lawton, W.S. Smith, J.G. Zaroff, and W.L. Young. (2008). Endothelial nitric oxide synthase polymorphism (-786T->C) and increased risk of angiographic vasospasm after aneurysmal subarachnoid hemorrhage. *Stroke*. **39**: 1103-1108.

Koenig, W., D. Rothenbacher, A. Hoffmeister, M. Griesshammer, and H. Brenner. (2001). Plasma fibrin D-dimer levels and risk of stable coronary artery disease: results of a large case-control study. *Arterioscler Thromb Vasc Biol*. **21**: 1701-1705.

Kohli, U., B.L. Grayson, T.M. Aune, L.V. Ghimire, D. Kurnik, and C.M. Stein. (2009). Change in mRNA Expression after Atenolol, a Beta-adrenergic Receptor Antagonist and Association with Pharmacological Response. *Arch Drug Inf*. **2**: 41-50.

Kooperberg, C., M. LeBlanc, and V. Obenchain. (2010). Risk prediction using genome-wide association studies. *Genet Epidemiol*. **34**: 643-652.

Koriyama, H., H. Nakagami, T. Katsuya, K. Sugimoto, H. Yamashita, Y. Takami, S. Maeda, M. Kubo, A. Takahashi, Y. Nakamura, T. Ogihara, H. Rakugi, Y. Kaneda, and R. Morishita. (2010). Identification of evidence suggestive of an association with peripheral arterial disease at the OSBPL10 locus by genome-wide investigation in the Japanese population. *J Atheroscler Thromb*. **17**: 1054-1062.

Koubaa, N., A. Nakbi, S. Hammami, N. Attia, S. Mehri, K. Ben Hamda, M. Ben Farhat, A. Miled, and M. Hammami. (2009). Association of homocysteine thiolactonase activity and PON1 polymorphisms with the severity of acute coronary syndrome. *Clin Biochem.* **42**: 771-776.

Kozich, V., J. Sokolova, V. Klatovska, J. Krijt, M. Janosik, K. Jelinek, and J.P. Kraus. (2010). Cystathionine beta-synthase mutations: effect of mutation topology on folding and activity. *Hum Mutat.* **31**: 809-819.

Kozlowski, P., and W.J. Krzyzosiak. (2001). Combined SSCP/duplex analysis by capillary electrophoresis for more efficient mutation detection. *Nucleic Acids Res.* **29**: E71.

Krishnaswamy, G. (2010). The inflammation paradigm and coronary artery disease: What Celsus, Virchow and gene knock outs have taught us. *Cardiovasc Hematol Disord Drug Targets.* **10**: 246-256.

Kristensen, L.S., and A. Dobrovic. (2008). Direct genotyping of single nucleotide polymorphisms in methyl metabolism genes using probe-free high-resolution melting analysis. *Cancer Epidemiol Biomarkers Prev.* **17**: 1240-1247.

Krull, S., J. Dorries, B. Boysen, S. Reidenbach, L. Magnius, H. Norder, J. Thyberg, and V.C. Cordes. (2010). Protein Tpr is required for establishing nuclear pore-associated zones of heterochromatin exclusion. *EMBO J.* **29**: 1659-1673.

Kullo, I.J., K.R. Bailey, S.L. Kardia, T.H. Mosley, Jr., E. Boerwinkle, and S.T. Turner. (2003). Ethnic differences in peripheral arterial disease in the NHLBI Genetic Epidemiology Network of Arteriopathy (GENOA) study. *Vasc Med.* **8**: 237-242.

Kullo, I.J., K. Ding, H. Jouni, C.Y. Smith, and C.G. Chute. (2010). A genome-wide association study of red blood cell traits using the electronic medical record. *PLoS One.* **5**.

Kumar, D. (2008). Disorders of the genome architecture: a review. *Genomic Med.* **2**: 69-76.

LaBonte, M.J., P.M. Wilson, W. Fazzone, S. Groshen, H.J. Lenz, and R.D. Ladner. (2009). DNA microarray profiling of genes differentially regulated by the histone deacetylase inhibitors vorinostat and LBH589 in colon cancer cell lines. *BMC Med Genomics.* **2**: 67.

Lai, E. (2001). Application of SNP technologies in medicine: lessons learned and future challenges. *Genome Res.* **11**: 927-929.

Lakshmy, R., D. Ahmad, R.A. Abraham, M. Sharma, K. Vemparala, S. Das, K.S. Reddy, and D. Prabhakaran. (2010). Paraoxonase gene Q192R & L55M

polymorphisms in Indians with acute myocardial infarction & association with oxidized low density lipoprotein. *Indian J Med Res.* **131**: 522-529.

Lander, E.S., L.M. Linton, B. Birren, C. Nusbaum, M.C. Zody, J. Baldwin, K. Devon, K. Dewar, M. Doyle, W. FitzHugh, R. Funke, D. Gage, K. Harris, A. Heaford, J. Howland, L. Kann, J. Lehoczky, R. LeVine, P. McEwan, K. McKernan, J. Meldrim, J.P. Mesirov, C. Miranda, W. Morris, J. Naylor, C. Raymond, M. Rosetti, R. Santos, A. Sheridan, C. Sougnez, N. Stange-Thomann, N. Stojanovic, A. Subramanian, D. Wyman, J. Rogers, J. Sulston, R. Ainscough, S. Beck, D. Bentley, J. Burton, C. Clee, N. Carter, A. Coulson, R. Deadman, P. Deloukas, A. Dunham, I. Dunham, R. Durbin, L. French, D. Grafham, S. Gregory, T. Hubbard, S. Humphray, A. Hunt, M. Jones, C. Lloyd, A. McMurray, L. Matthews, S. Mercer, S. Milne, J.C. Mullikin, A. Mungall, R. Plumb, M. Ross, R. Shownkeen, S. Sims, R.H. Waterston, R.K. Wilson, L.W. Hillier, J.D. McPherson, M.A. Marra, E.R. Mardis, L.A. Fulton, A.T. Chinwalla, K.H. Pepin, W.R. Gish, S.L. Chissoe, M.C. Wendl, K.D. Delehaunty, T.L. Miner, A. Delehaunty, J.B. Kramer, L.L. Cook, R.S. Fulton, D.L. Johnson, P.J. Minx, S.W. Clifton, T. Hawkins, E. Branscomb, P. Predki, P. Richardson, S. Wenning, T. Slezak, N. Doggett, J.F. Cheng, A. Olsen, S. Lucas, C. Elkin, E. Uberbacher, M. Frazier, et al. (2001). Initial sequencing and analysis of the human genome. *Nature*. **409**: 860-921.

Laraqui, A., A. Allami, A. Carrie, A.S. Coiffard, F. Benkouka, A. Benjouad, A. Bendriss, N. Kadiri, N. Bennouar, A. Benomar, A. Guedira, A. Raisonnier, S. Fellati, J.E. Srairi, and M. Benomar. (2006). Influence of methionine synthase (A2756G) and methionine synthase reductase (A66G) polymorphisms on plasma homocysteine levels and relation to risk of coronary artery disease. *Acta Cardiol.* **61**: 51-61.

Laraqui, A., A. Allami, A. Carrie, A. Raisonnier, A.S. Coiffard, F. Benkouka, A. Bendriss, A. Benjouad, N. Bennouar, N. El Kadiri, A. Benomar, S. Fellat, and M. Benomar. (2007). Relation between plasma homocysteine, gene polymorphisms of homocysteine metabolism-related enzymes, and angiographically proven coronary artery disease. *Eur J Intern Med.* **18**: 474-483.

Laudes, M., F. Oberhauser, D.M. Schulte, S. Freude, R. Bilkovski, J. Mauer, G. Rappl, H. Abken, M. Hahn, O. Schulz, and W. Krone. (2010). Visfatin/PBEF/Nampt and resistin expressions in circulating blood monocytes are differentially related to obesity and type 2 diabetes in humans. *Horm Metab Res.* **42**: 268-273.

Lawall, H., P. Bramlage, and B. Amann. (2011). Treatment of peripheral arterial disease using stem and progenitor cell therapy. *J Vasc Surg.* **53**: 445-453.

Lee, E., D.E. Vaughan, S.H. Parikh, A.J. Grodzinsky, P. Libby, M.W. Lark, and R.T. Lee. (1996). Regulation of matrix metalloproteinases and plasminogen activator inhibitor-1 synthesis by plasminogen in cultured human vascular smooth muscle cells. *Circ Res.* **78**: 44-49.

Lee, L.G., C.R. Connell, S.L. Woo, R.D. Cheng, B.F. McArdle, C.W. Fuller, N.D. Halloran, and R.K. Wilson. (1992). DNA sequencing with dye-labeled terminators and T7 DNA polymerase: effect of dyes and dNTPs on incorporation of dye-terminators and probability analysis of termination fragments. *Nucleic Acids Res.* **20**: 2471-2483.

Lee, M.J., D.W. Gong, B.F. Burkey, and S.K. Fried. (2011). Pathways regulated by glucocorticoids in omental and subcutaneous human adipose tissues: a microarray study. *Am J Physiol Endocrinol Metab.* **300**: E571-E580.

Lee, S., D.H. Kim, Y.H. Goo, Y.C. Lee, S.K. Lee, and J.W. Lee. (2009). Crucial roles for interactions between MLL3/4 and INI1 in nuclear receptor transactivation. *Mol Endocrinol.* **23**: 610-619.

Lee, S.H., H. Sterling, A. Burlingame, and F. McCormick. (2008a). Tpr directly binds to Mad1 and Mad2 and is important for the Mad1-Mad2-mediated mitotic spindle checkpoint. *Genes Dev.* **22**: 2926-2931.

Lee, T., R. Schwandner, G. Swaminath, J. Weiszmann, M. Cardozo, J. Greenberg, P. Jaeckel, H. Ge, Y. Wang, X. Jiao, J. Liu, F. Kayser, H. Tian, and Y. Li. (2008b). Identification and functional characterization of allosteric agonists for the G protein-coupled receptor FFA2. *Mol Pharmacol.* **74**: 1599-1609.

Lee, T.S., H.C. Yen, C.C. Pan, and L.Y. Chau. (1999). The role of interleukin 12 in the development of atherosclerosis in ApoE-deficient mice. *Arterioscler Thromb Vasc Biol.* **19**: 734-742.

Leeuwenburgh, C., M.M. Hardy, S.L. Hazen, P. Wagner, S. Oh-ishi, U.P. Steinbrecher, and J.W. Heinecke. (1997). Reactive nitrogen intermediates promote low density lipoprotein oxidation in human atherosclerotic intima. *J Biol Chem.* **272**: 1433-1436.

Leiper, J., and P. Vallance. (1999). Biological significance of endogenous methylarginines that inhibit nitric oxide synthases. *Cardiovascular Research.* **43**: 542–548.

Lekakis, J.P., S. Papathanassiou, T.G. Papaioannou, C.M. Papamichael, N. Zakopoulos, V. Kotsis, A.G. Dagre, K. Stamatelopoulos, A. Protogerou, and S.F. Stamatelopoulos. (2002). Oral L-arginine improves endothelial dysfunction in patients with essential hypertension. *Int J Cardiol.* **86**: 317-323.

Lentz, S.R., and J.E. Sadler. (1991). Inhibition of thrombomodulin surface expression and protein C activation by the thrombogenic agent homocysteine. *J Clin Invest.* **88**: 1906-1914.

Lewis, S.J., S. Ebrahim, and G. Davey Smith. (2005). Meta-analysis of MTHFR 677C->T polymorphism and coronary heart disease: does totality of evidence

support causal role for homocysteine and preventive potential of folate? *BMJ.* **331**: 1053.

Li, A., S. Dubey, M.L. Varney, B.J. Dave, and R.K. Singh. (2003). IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis. *J Immunol.* **170**: 3369-3376.

Li, B., D. Ge, Y. Wang, W. Zhao, X. Zhou, D. Gu, and R. Chen. (2004). Lipoprotein lipase gene polymorphisms and blood pressure levels in the Northern Chinese Han population. *Hypertens Res.* **27**: 373-378.

Li, C., X. Li, Y. Miao, Q. Wang, W. Jiang, C. Xu, J. Li, J. Han, F. Zhang, B. Gong, and L. Xu. (2009). SubpathwayMiner: a software package for flexible identification of pathways. *Nucleic Acids Res.* **37**: e131.

Liaugaudas, G., P.F. Jacques, J. Selhub, I.H. Rosenberg, and A.G. Bostom. (2001). Renal insufficiency, vitamin B(12) status, and population attributable risk for mild hyperhomocysteinemia among coronary artery disease patients in the era of folic acid-fortified cereal grain flour. *Arterioscler Thromb Vasc Biol.* **21**: 849-851.

Libby, P., and M. Aikawa. (2002). Stabilization of atherosclerotic plaques: new mechanisms and clinical targets. *Nat Med.* **8**: 1257-1262.

Lima, L.M., M.G. Carvalho, A.P. Fernandes, P. Sabino Ade, A.A. Loures-Vale, C.P. da Fonseca Neto, J.C. Garcia, J.A. Saad, and M.O. Sousa. (2007). Homocysteine and methylenetetrahydrofolate reductase in subjects undergoing coronary angiography. *Arq Bras Cardiol.* **88**: 167-172.

Lima, V.V., F.R. Giachini, F.S. Carneiro, Z.N. Carneiro, M.A. Saleh, D.M. Pollock, Z.B. Fortes, M.H. Carvalho, A. Ergul, R.C. Webb, and R.C. Tostes. (2010). O-GlcNAcylation contributes to augmented vascular reactivity induced by endothelin 1. *Hypertension.* **55**: 180-188.

Lind, P.A., S. Macgregor, J.M. Vink, M.L. Pergadia, N.K. Hansell, M.H. de Moor, A.B. Smit, J.J. Hottenga, M.M. Richter, A.C. Heath, N.G. Martin, G. Willemsen, E.J. de Geus, N. Vogelzangs, B.W. Penninx, J.B. Whitfield, G.W. Montgomery, D.I. Boomsma, and P.A. Madden. (2010). A genomewide association study of nicotine and alcohol dependence in Australian and Dutch populations. *Twin Res Hum Genet.* **13**: 10-29.

Linton, K., Y. Hey, S. Dibben, C. Miller, A. Freemont, J. Radford, and S. Pepper. (2009). Methods comparison for high-resolution transcriptional analysis of archival material on Affymetrix Plus 2.0 and Exon 1.0 microarrays. *Biotechniques.* **47**: 587-596.

Littlejohn, M.D., B.R. Palmer, A.M. Richards, C.M. Frampton, A.P. Pilbrow, R.W. Troughton, V.A. Cameron, and M.A. Kennedy. (2008). Ile164

variant of beta2-adrenoceptor does not influence outcome in heart failure but may interact with beta blocker treatment. *Eur J Heart Fail.* **10**: 55-59.

Liu, D.S., X.O. Li, B.W. Ying, L. Chen, T. Wang, D. Xu, and F.Q. Wen. (2010). Effects of single nucleotide polymorphisms 869 T/C and 915 G/C in the exon 1 locus of transforming growth factor-beta1 gene on chronic obstructive pulmonary disease susceptibility in Chinese. *Chin Med J (Engl).* **123**: 390-394.

Liu, W., D.I. Smith, K.J. Rechtzigel, S.N. Thibodeau, and C.D. James. (1998). Denaturing high performance liquid chromatography (DHPLC) used in the detection of germline and somatic mutations. *Nucleic Acids Res.* **26**: 1396-1400.

Locasale, J.W., and A. Wolf-Yadlin. (2009). Maximum entropy reconstructions of dynamic signaling networks from quantitative proteomics data. *PLoS One.* **4**: e6522.

Loscalzo, J. (2003). Adverse effects of supplemental L-arginine in atherosclerosis: consequences of methylation stress in a complex catabolism? *Arterioscler Thromb Vasc Biol.* **23**: 3-5.

Lu, X., J. Shao, H. Li, and Y. Yu. (2010). Temporal gene expression changes induced by a low concentration of benzo[a]pyrene diol epoxide in a normal human cell line. *Mutat Res.* **684**: 74-80.

Lusis, A.J. (2000). Atherosclerosis. *Nature.* **407**: 233-241.

Ma, X., X. Wang, X. Gao, L. Wang, Y. Lu, P. Gao, W. Deng, P. Yu, J. Ma, J. Guo, H. Cheng, C. Zhang, T. Shi, and D. Ma. (2007). Identification of five human novel genes associated with cell proliferation by cell-based screening from an expressed cDNA ORF library. *Life Sci.* **81**: 1141-1151.

Mach, F., U. Schonbeck, J.Y. Bonnefoy, J.S. Pober, and P. Libby. (1997). Activation of monocyte/macrophage functions related to acute atheroma complication by ligation of CD40: induction of collagenase, stromelysin, and tissue factor. *Circulation.* **96**: 396-399.

Mach, F., U. Schonbeck, G.K. Sukhova, E. Atkinson, and P. Libby. (1998). Reduction of atherosclerosis in mice by inhibition of CD40 signalling. *Nature.* **394**: 200-203.

MacMahon, M., C. Kirkpatrick, C.E. Cummings, A. Clayton, P.J. Robinson, R.H. Tomiak, M. Liu, D. Kush, and J. Tobert. (2000). A pilot study with simvastatin and folic acid/vitamin B12 in preparation for the Study of the Effectiveness of Additional Reductions in Cholesterol and Homocysteine (SEARCH). *Nutr Metab Cardiovasc Dis.* **10**: 195-203.

Majek, P., Z. Reicheltova, J. Stikarova, J. Suttnar, A. Sobotkova, and J.E. Dyr. (2010). Proteome changes in platelets activated by arachidonic acid, collagen, and thrombin. *Proteome Sci.* **8**: 56.

Major, A.S., S. Fazio, and M.F. Linton. (2002). B-lymphocyte deficiency increases atherosclerosis in LDL receptor-null mice. *Arterioscler Thromb Vasc Biol.* **22**: 1892-1898.

Maldonado, C., C.V. Soni, N.D. Todnem, S. Pushpakumar, D. Rosenberger, S. Givvimani, J. Villafane, and S.C. Tyagi. (2010). Hyperhomocysteinemia and sudden cardiac death: potential arrhythmogenic mechanisms. *Curr Vasc Pharmacol.* **8**: 64-74.

Mallat, Z., H. Ait-Oufella, and A. Tedgui. (2007). Regulatory T-cell immunity in atherosclerosis. *Trends Cardiovasc Med.* **17**: 113-118.

Mandal, K., M. Jahangiri, and Q. Xu. (2004). Autoimmunity to heat shock proteins in atherosclerosis. *Autoimmun Rev.* **3**: 31-37.

Marcucci, R., D. Prisco, T. Brunelli, G. Pepe, A.M. Gori, S. Fedi, M. Capanni, I. Simonetti, G. Federici, A. Pastore, R. Abbate, and G.F. Gensini. (2000). Tissue factor and homocysteine levels in ischemic heart disease are associated with angiographically documented clinical recurrences after coronary angioplasty. *Thromb Haemost.* **83**: 826-832.

Markoff, A., A. Savov, V. Vladimirov, N. Bogdanova, I. Kremensky, and V. Ganev. (1997). Optimization of single-strand conformation polymorphism analysis in the presence of polyethylene glycol. *Clin Chem.* **43**: 30-33.

Martin-Fuentes, P., F. Civeira, D. Recalde, A.L. Garcia-Otin, E. Jarauta, I. Marzo, and A. Cenarro. (2007). Individual variation of scavenger receptor expression in human macrophages with oxidized low-density lipoprotein is associated with a differential inflammatory response. *J Immunol.* **179**: 3242-3248.

Marx, N., B. Kehrle, K. Kohlhammer, M. Grub, W. Koenig, V. Hombach, P. Libby, and J. Plutzky. (2002). PPAR activators as antiinflammatory mediators in human T lymphocytes: implications for atherosclerosis and transplantation-associated arteriosclerosis. *Circ Res.* **90**: 703-710.

Masuda, Y., A. Kubo, A. Kokaze, M. Yoshida, N. Fukuhara, and Y. Takashima. (2008). Factors associated with serum total homocysteine level in type 2 diabetes. *Environ Health Prev Med.* **13**: 148-155.

Mathers, C.D., and D. Loncar. (2006). Projections of global mortality and burden of disease from 2002 to 2030. *PLoS Med.* **3**: e442.

Matsuda, S., A. Yamashita, Y. Sato, S. Kitajima, T. Koike, C. Sugita, S. Moriguchi-Goto, K. Hatakeyama, M. Takahashi, C. Koshimoto, Y. Matsuura, T. Iwakiri, Y.E. Chen, J. Fan, and Y. Asada. (2011). Human C-

reactive protein enhances thrombus formation after neointimal balloon injury in transgenic rabbits. *J Thromb Haemost.* **9**: 201-208.

Maugeri, N., A.A. Manfredi, and A. Maseri. (2010). Clinical and experimental evidences on the prothrombotic properties of neutrophils. *Srp Arh Celok Lek.* **138 Suppl 1**: 50-52.

McClain, D.A., W.A. Lubas, R.C. Cooksey, M. Hazel, G.J. Parker, D.C. Love, and J.A. Hanover. (2002). Altered glycan-dependent signaling induces insulin resistance and hyperleptinemia. *Proc Natl Acad Sci USA.* **99**: 10695-10699.

McCully, K.S. (1969). Vascular pathology of homocysteinemia: implications for the pathogenesis of arteriosclerosis. *Am J Pathol.* **56**: 111-128.

McCully, K.S. (2009). Chemical pathology of homocysteine. IV. Excitotoxicity, oxidative stress, endothelial dysfunction, and inflammation. *Ann Clin Lab Sci.* **39**: 219-232.

McCully, K.S., and R.B. Wilson. (1975). Homocysteine theory of arteriosclerosis. *Atherosclerosis.* **22**: 215-227.

McDermott, M.M., L. Ferrucci, K. Liu, J.M. Guralnik, L. Tian, M. Kibbe, Y. Liao, H. Tao, and M.H. Criqui. (2011). Women with peripheral arterial disease experience faster functional decline than men with peripheral arterial disease. *J Am Coll Cardiol.* **57**: 707-714.

McEver, R.P., and R.D. Cummings. (1997). Perspectives series: cell adhesion in vascular biology. Role of PSGL-1 binding to selectins in leukocyte recruitment. *J Clin Invest.* **100**: 485-491.

McKeigue, P.M. (1992). Coronary heart disease in Indians, Pakistanis, and Bangladeshis: aetiology and possibilities for prevention. *Br Heart J.* **67**: 341-342.

McPherson, R., A. Pertsemlidis, N. Kavaslar, A. Stewart, R. Roberts, D.R. Cox, D.A. Hinds, L.A. Pennacchio, A. Tybjaerg-Hansen, A.R. Folsom, E. Boerwinkle, H.H. Hobbs, and J.C. Cohen. (2007). A common allele on chromosome 9 associated with coronary heart disease. *Science.* **316**: 1488-1491.

Medzhitov, R. (2001). Toll-like receptors and innate immunity. *Nat Rev Immunol.* **1**: 135-145.

Meier, P., J. Antonov, R. Zbinden, A. Kuhn, S. Zbinden, S. Gloekler, M. Delorenzi, R. Jaggi, and C. Seiler. (2009). Non-invasive gene-expression-based detection of well-developed collateral function in individuals with and without coronary artery disease. *Heart.* **95**: 900-908.

Melquist, S., D.W. Craig, M.J. Huentelman, R. Crook, J.V. Pearson, M. Baker, V.L. Zismann, J. Gass, J. Adamson, S. Szelinger, J. Corneveaux, A. Cannon, K.D. Coon, S. Lincoln, C. Adler, P. Tuite, D.B. Calne, E.H. Bigio, R.J. Uitti, Z.K. Wszolek, L.I. Golbe, R.J. Caselli, N. Graff-Radford, I. Litvan, M.J. Farrer, D.W. Dickson, M. Hutton, and D.A. Stephan. (2007). Identification of a novel risk locus for progressive supranuclear palsy by a pooled genomewide scan of 500,288 single-nucleotide polymorphisms. *Am J Hum Genet.* **80**: 769-778.

Mendonca, M.I., R.P. Dos Reis, A.I. Freitas, A.C. Sousa, A. Pereira, P. Faria, S. Gomes, B. Silva, N. Santos, M. Serrao, I. Ornelas, S. Freitas, J.J. Araujo, A. Brehm, and A.A. Cardoso. (2008). Human paraoxonase gene polymorphisms and coronary artery disease risk. *Rev Port Cardiol.* **27**: 1539-1555.

Mendonca, M.I., R.P. Dos Reis, A.I. Freitas, A.C. Sousa, A. Pereira, P. Faria, S. Gomes, B. Silva, N. Santos, M. Serrao, I. Ornelas, S. Freitas, C. Freitas, J.J. Araujo, A. Brehm, and A.A. Cardoso. (2009). Gene-gene interaction affects coronary artery disease risk. *Rev Port Cardiol.* **28**: 397-415.

Mengel-Jorgensen, J., J.J. Sanchez, C. Borsting, F. Kirpekar, and N. Morling. (2004). MALDI-TOF mass spectrometric detection of multiplex single base extended primers. A study of 17 y-chromosome single-nucleotide polymorphisms. *Anal Chem.* **76**: 6039-6045.

Meyer, B.C., T. Werncke, E. Foert, M. Kruschewski, W. Hopfenmuller, C. Ribbe, K.J. Wolf, and T. Albrecht. (2010). Do the cardiovascular risk profile and the degree of arterial wall calcification influence the performance of MDCT angiography of lower extremity arteries? *Eur Radiol.* **20**: 497-505.

Mhawech-Fauceglia, P., D. Wang, J. Kesterson, K. Clark, L. Monhollen, K. Odunsi, S. Lele, and S. Liu. (2010). Microarray analysis reveals distinct gene expression profiles among different tumor histology, stage and disease outcomes in endometrial adenocarcinoma. *PLoS One.* **5**: e15415.

Micheal, S., R. Qamar, F. Akhtar, M.I. Khan, W.A. Khan, and A. Ahmed. (2009). MTHFR gene C677T and A1298C polymorphisms and homocysteine levels in primary open angle and primary closed angle glaucoma. *Mol Vis.* **15**: 2268-2278.

Miller, Y.I., M.K. Chang, C.J. Binder, P.X. Shaw, and J.L. Witztum. (2003). Oxidized low density lipoprotein and innate immune receptors. *Curr Opin Lipidol.* **14**: 437-445.

Millis, M.P. (2011). Medium-throughput SNP genotyping using mass spectrometry: multiplex SNP genotyping using the iPLEX(R) Gold assay. *Methods Mol Biol.* **700**: 61-76.

Min, S.H., D.M. Kim, Y.S. Heo, Y.I. Kim, H.M. Kim, J. Kim, Y.M. Han, I.C. Kim, and O.J. Yoo. (2009). New p53 target, phosphatase of regenerating liver 1 (PRL-1) downregulates p53. *Oncogene*. **28**: 545-554.

Mirshahi, F., M. Vasse, A. Tedgui, H. Li, R. Merval, E. Legrand, J.P. Vannier, J. Soria, and C. Soria. (2002). Oncostatin M induces procoagulant activity in human vascular smooth muscle cells by modulating the balance between tissue factor and tissue factor pathway inhibitor. *Blood Coagul Fibrinolysis*. **13**: 449-455.

Mitui, M., C. Campbell, G. Coutinho, X. Sun, C.H. Lai, Y. Thorstenson, S. Castellvi-Bel, L. Fernandez, E. Monros, B.T. Carvalho, O. Porras, G. Fontan, and R.A. Gatti. (2003). Independent mutational events are rare in the ATM gene: haplotype prescreening enhances mutation detection rate. *Hum Mutat*. **22**: 43-50.

Miyauchi, S., A. Hirasawa, A. Ichimura, T. Hara, and G. Tsujimoto. (2010). New frontiers in gut nutrient sensor research: free fatty acid sensing in the gastrointestinal tract. *J Pharmacol Sci*. **112**: 19-24.

Mohamed, R.H., R.H. Mohamed, R.A. Karam, and T.A. Abd El-Aziz. (2010). The relationship between paraoxonase1-192 polymorphism and activity with coronary artery disease. *Clin Biochem*. **43**: 553-558.

Moon, H.W., T.Y. Kim, B.R. Oh, H.C. Min, H.I. Cho, S.M. Bang, J.H. Lee, S.S. Yoon, and D.S. Lee. (2007). MTHFR 677CC/1298CC genotypes are highly associated with chronic myelogenous leukemia: a case-control study in Korea. *Leuk Res*. **31**: 1213-1217.

Morray, B., I. Goldenberg, A.J. Moss, W. Zareba, D. Ryan, S. McNitt, S.W. Eberly, G. Glazko, and J. Mathew. (2007). Polymorphisms in the paraoxonase and endothelial nitric oxide synthase genes and the risk of early-onset myocardial infarction. *Am J Cardiol*. **99**: 1100-1105.

Morris, M.S., M.F. Picciano, P.F. Jacques, and J. Selhub. (2008). Plasma pyridoxal 5'-phosphate in the US population: the National Health and Nutrition Examination Survey, 2003-2004. *Am J Clin Nutr*. **87**: 1446-1454.

Morrison, T.B., J.J. Weis, and C.T. Wittwer. (1998). Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. *Biotechniques*. **24**: 954-958, 960, 962.

Mudd, S.H., J.D. Finkelstein, F. Irreverre, and L. Laster. (1964). Homocystinuria: an Enzymatic Defect. *Science*. **143**: 1443-1445.

Mudd, S.H., F. Skovby, H.L. Levy, K.D. Pettigrew, B. Wilcken, R.E. Pyeritz, G. Andria, G.H. Boers, I.L. Bromberg, R. Cerone, B. Fowler, H. Grobe, H. Schmidt, and L. Schweitzer. (1985). The natural history of homocystinuria due to cystathionine beta-synthase deficiency. *Am J Hum Genet*. **37**: 1-31.

Muggerud, A.A., M. Hallett, H. Johnsen, K. Kleivi, W. Zhou, S. Tahmasebpoor, R.M. Amini, J. Botling, A.L. Borresen-Dale, T. Sorlie, and F. Warnberg. (2010). Molecular diversity in ductal carcinoma in situ (DCIS) and early invasive breast cancer. *Mol Oncol.* **4**: 357-368.

Muglia, P., F. Tozzi, N.W. Galwey, C. Francks, R. Upmanyu, X.Q. Kong, A. Antoniades, E. Domenici, J. Perry, S. Rothen, C.L. Vandeleur, V. Mooser, G. Waeber, P. Vollenweider, M. Preisig, S. Lucae, B. Muller-Myhsok, F. Holsboer, L.T. Middleton, and A.D. Roses. (2010). Genome-wide association study of recurrent major depressive disorder in two European case-control cohorts. *Mol Psychiatry.* **15**: 589-601.

Mukherjee, T.K., H. Dinh, G. Chaudhuri, and L. Nathan. (2002). Testosterone attenuates expression of vascular cell adhesion molecule-1 by conversion to estradiol by aromatase in endothelial cells: implications in atherosclerosis. *Proc Natl Acad Sci USA.* **99**: 4055-4060.

Murphy, M.E., and H. Sies. (1991). Reversible conversion of nitroxyl anion to nitric oxide by superoxide dismutase. *Proc Natl Acad Sci USA.* **88**: 10860-10864.

Nakashima, M., and J.S. Lazo. (2010). Phosphatase of regenerating liver-1 promotes cell migration and invasion and regulates filamentous actin dynamics. *J Pharmacol Exp Ther.* **334**: 627-633.

Nasir, K., Guallar, E., Navas-Acien, A., Criqui, M. H. and Lima, J. A. (2005). Relationship of monocyte count and peripheral arterial disease: results from the National Health and Nutrition Examination Survey 1999-2002. *Arterioscler Thromb Vasc Biol.* **25**: 1966-1971.

Nathan, L., and G. Chaudhuri. (1997). Estrogens and atherosclerosis. *Annu Rev Pharmacol Toxicol.* **37**: 477-515.

Nathan, L., S. Pervin, R. Singh, M. Rosenfeld, and G. Chaudhuri. (1999). Estradiol inhibits leukocyte adhesion and transendothelial migration in rabbits in vivo : possible mechanisms for gender differences in atherosclerosis. *Circ Res.* **85**: 377-385.

Nawa, T., M.T. Nawa, M.T. Adachi, I. Uchimura, R. Shimokawa, K. Fujisawa, A. Tanaka, F. Numano, and S. Kitajima. (2002). Expression of transcriptional repressor ATF3/LRF1 in human atherosclerosis: colocalization and possible involvement in cell death of vascular endothelial cells. *Atherosclerosis.* **161**: 281-291.

Newton, C.R., A. Graham, L.E. Heptinstall, S.J. Powell, C. Summers, N. Kalsheker, J.C. Smith, and A.F. Markham. (1989a). Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). *Nucleic Acids Res.* **17**: 2503-2516.

Newton, C.R., L.E. Heptinstall, C. Summers, M. Super, M. Schwarz, R. Anwar, A. Graham, J.C. Smith, and A.F. Markham. (1989b). Amplification

refractory mutation system for prenatal diagnosis and carrier assessment in cystic fibrosis. *Lancet*. **2**: 1481-1483.

Nibbe, R.K., M. Koyuturk, and M.R. Chance. (2010). An integrative -omics approach to identify functional sub-networks in human colorectal cancer. *PLoS Comput Biol*. **6**: e1000639.

Nickoloff, B.J., V. Chaturvedi, P. Bacon, J.Z. Qin, M.F. Denning, and M.O. Diaz. (2000). Id-1 delays senescence but does not immortalize keratinocytes. *J Biol Chem*. **275**: 27501-27504.

Nicolas, L., G. Milon, and E. Prina. (2002). Rapid differentiation of Old World Leishmania species by LightCycler polymerase chain reaction and melting curve analysis. *J Microbiol Methods*. **51**: 295-299.

Nicoletti, A., G. Caligiuri, I. Tornberg, T. Kodama, S. Stemme, and G.K. Hansson. (1999). The macrophage scavenger receptor type A directs modified proteins to antigen presentation. *Eur J Immunol*. **29**: 512-521.

Niederstatter, H., M.D. Coble, P. Grubwieser, T.J. Parsons, and W. Parson. (2006). Characterization of mtDNA SNP typing and mixture ratio assessment with simultaneous real-time PCR quantification of both allelic states. *Int J Legal Med*. **120**: 18-23.

Niedoszytko, M., J.N. Oude Elberink, M. Bruinenberg, B. Nedoszytko, J.G. de Monchy, G.J. Te Meerman, R.K. Weersma, A.B. Mulder, E. Jassem, and J.J. van Doormaal. (2011). Gene expression profile, pathways, and transcriptional system regulation in indolent systemic mastocytosis. *Allergy*. **66**: 229-237.

Nielsen, Y.J. (2010). Whole-body MR angiography in patients with peripheral arterial disease. *Dan Med Bull*. **57**: B4231.

Nielsen, Y.W., J.P. Eiberg, V.B. Logager, T.V. Schroeder, S. Just, and H.S. Thomsen. (2009). Whole-body magnetic resonance angiography at 3 tesla using a hybrid protocol in patients with peripheral arterial disease. *Cardiovasc Intervent Radiol*. **32**: 877-886.

Niki, E. (2004). Antioxidants and atherosclerosis. *Biochem Soc Trans*. **32**: 156-159.

Nishiyama, K., K. Takaji, K. Kataoka, Y. Kurihara, M. Yoshimura, A. Kato, H. Ogawa, and H. Kurihara. (2005). Id1 gene transfer confers angiogenic property on fully differentiated endothelial cells and contributes to therapeutic angiogenesis. *Circulation*. **112**: 2840-2850.

Norgren, L., W.R. Hiatt, J.A. Dormandy, M.R. Nehler, K.A. Harris, F.G. Fowkes, K. Bell, J. Caporusso, I. Durand-Zaleski, K. Komori, J. Lammer, C. Liapis, S. Novo, M. Razavi, J. Robbs, N. Schaper, H. Shigematsu, M. Sapoval, C. White, J. White, D. Clement, M. Creager, M. Jaff, E. Mohler, 3rd, R.B. Rutherford, P. Sheehan, H. Sillesen, and K. Rosenfield. (2007).

Inter-Society Consensus for the Management of Peripheral Arterial Disease (TASC II). *Eur J Vasc Endovasc Surg.* **33 Suppl 1**: S1-S75.

Nurk, E., G.S. Tell, O. Nygard, H. Refsum, P.M. Ueland, and S.E. Vollset. (2001). Plasma total homocysteine is influenced by prandial status in humans: the Hordaland Hhomocysteine Sstudy. *J Nutr.* **131**: 1214-1216.

Nurk, E., G.S. Tell, S.E. Vollset, O. Nygard, H. Refsum, R.M. Nilsen, and P.M. Ueland. (2004). Changes in lifestyle and plasma total homocysteine: the Hordaland Homocysteine Study. *Am J Clin Nutr.* **79**: 812-819.

O'Donnell, N., N.E. Zachara, G.W. Hart, and J.D. Marth. (2004). Ogt-dependent X-chromosome-linked protein glycosylation is a requisite modification in somatic cell function and embryo viability. *Mol Cell Biol.* **24**: 1680-1690.

Oefner, P.J., and P.A. Underhill. (1995). Comparative DNA sequencing by denaturing high-performance liquid chromatography (DHPLC). *Am J Hum Genet.* **57 (Suppl.)**: A266.

Ohashi, K., V. Burkart, S. Flohe, and H. Kolb. (2000). Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. *J Immunol.* **164**: 558-561.

Okayama, H., D.T. Curiel, M.L. Brantly, M.D. Holmes, and R.G. Crystal. (1989). Rapid, nonradioactive detection of mutations in the human genome by allele-specific amplification. *J Lab Clin Med.* **114**: 105-113.

Okayama, N., K. Fujimura, J. Nakamura, Y. Suehiro, Y. Hamanaka, and Y. Hinoda. (2004). Evaluation of a new efficient procedure for single-nucleotide polymorphism genotyping: tetra-primer amplification refractory mutation system-polymerase chain reaction. *Clin Chem Lab Med.* **42**: 13-16.

Okoniewski, M.J., Y. Hey, S.D. Pepper, and C.J. Miller. (2007). High correspondence between Affymetrix exon and standard expression arrays. *Biotechniques.* **42**: 181-185.

Orita, M., H. Iwahana, H. Kanazawa, K. Hayashi, and T. Sekiya. (1989). Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proc Natl Acad Sci USA.* **86**: 2766-2770.

Pacanowski, M.A., Y. Gong, R.M. Cooper-Dehoff, N.J. Schork, M.D. Shriver, T.Y. Langaee, C.J. Pepine, and J.A. Johnson. (2008). beta-adrenergic receptor gene polymorphisms and beta-blocker treatment outcomes in hypertension. *Clin Pharmacol Ther.* **84**: 715-721.

Palareti, G., S. Salardi, S. Piazzi, C. Legnani, M. Poggi, F. Grauso, A. Caniato, S. Coccheri, and E. Cacciari. (1986). Blood coagulation changes in homocystinuria: effects of pyridoxine and other specific therapy. *J Pediatr.* **109**: 1001-1006.

Palomino-Morales, R., C. Gonzalez-Juanatey, T.R. Vazquez-Rodriguez, L. Rodriguez, J.A. Miranda-Filloy, B. Fernandez-Gutierrez, J. Llorca, J. Martin, and M.A. Gonzalez-Gay. (2010). A1298C polymorphism in the MTHFR gene predisposes to cardiovascular risk in rheumatoid arthritis. *Arthritis Res Ther.* **12**: R71.

Papazafiropoulou, A., Kardara, M., Sotiropoulos, A., Bousboulas, S., Stamataki, P. and Pappas, S. (2010). Plasma glucose levels and white blood cell count are related with ankle brachial index in type 2 diabetic subjects. *Hellenic J Cardiol.* **51**: 402-406.

Pasalic, D., N. Marinkovic, B. Grskovic, G. Ferencak, R. Bernat, and A. Stavljenic-Rukavina. (2009). C-reactive protein gene polymorphisms affect plasma CRP and homocysteine concentrations in subjects with and without angiographically confirmed coronary artery disease. *Mol Biol Rep.* **36**: 775-780.

Patino-Garcia, A., M. Zalacain, L. Marrodan, M. San-Julian, and L. Sierrasesumaga. (2009). Methotrexate in pediatric osteosarcoma: response and toxicity in relation to genetic polymorphisms and dihydrofolate reductase and reduced folate carrier 1 expression. *J Pediatr.* **154**: 688-693.

Patino, W.D., O.Y. Mian, J.G. Kang, S. Matoba, L.D. Bartlett, B. Holbrook, H.H. Trout, 3rd, L. Kozloff, and P.M. Hwang. (2005). Circulating transcriptome reveals markers of atherosclerosis. *Proc Natl Acad Sci USA.* **102**: 3423-3428.

Pearson, A.M. (1996). Scavenger receptors in innate immunity. *Curr Opin Immunol.* **8**: 20-28.

Peiser, L., S. Mukhopadhyay, and S. Gordon. (2002). Scavenger receptors in innate immunity. *Curr Opin Immunol.* **14**: 123-128.

Peng, L., A.J. Barczak, R.A. Barbeau, Y. Xiao, T.J. LaTempa, C.A. Grimes, and T.A. Desai. (2010). Whole genome expression analysis reveals differential effects of TiO2 nanotubes on vascular cells. *Nano Lett.* **10**: 143-148.

Perla-Kajan, J., and H. Jakubowski. (2010). Paraoxonase 1 protects against protein N-homocysteinylation in humans. *FASEB J.* **24**: 931-936.

Perna, A.F., D. Ingrosso, C. Lombardi, F. Acanfora, E. Satta, C.M. Cesare, E. Violetti, M.M. Romano, and N.G. De Santo. (2003). Possible mechanisms of homocysteine toxicity. *Kidney Int Suppl.* **63**: S137-S140.

Peruzzi, B., M. Serra, C. Pescucci, M. Sica, S. Lastraioli, T. Rondelli, S. Pedemonte, A. Maria Risitano, M. De Angioletti, P. Piccioli, and R. Notaro. (2010). Easy genotyping of complement C3 'slow' and 'fast' allotypes by tetra-primer amplification refractory mutation system PCR. *Mol Cell Probes.* **24**: 401-402.

Petidis, K., S. Douma, M. Doumas, I. Basagiannis, K. Vogiatzis, and C. Zamboulis. (2008). The interaction of vasoactive substances during exercise modulates platelet aggregation in hypertension and coronary artery disease. *BMC Cardiovasc Disord.* **8**: 11.

Pfeufer, A., C. van Noord, K.D. Marciante, D.E. Arking, M.G. Larson, A.V. Smith, K.V. Tarasov, M. Muller, N. Sotoodehnia, M.F. Sinner, G.C. Verwoert, M. Li, W.H. Kao, A. Kottgen, J. Coresh, J.C. Bis, B.M. Psaty, K. Rice, J.I. Rotter, F. Rivadeneira, A. Hofman, J.A. Kors, B.H. Stricker, A.G. Uitterlinden, C.M. van Duijn, B.M. Beckmann, W. Sauter, C. Gieger, S.A. Lubitz, C. Newton-Cheh, T.J. Wang, J.W. Magnani, R.B. Schnabel, M.K. Chung, J. Barnard, J.D. Smith, D.R. Van Wagoner, R.S. Vasan, T. Aspelund, G. Eiriksdottir, T.B. Harris, L.J. Launer, S.S. Najjar, E. Lakatta, D. Schlessinger, M. Uda, G.R. Abecasis, B. Muller-Myhsok, G.B. Ehret, E. Boerwinkle, A. Chakravarti, E.Z. Soliman, K.L. Lunetta, S. Perz, H.E. Wichmann, T. Meitinger, D. Levy, V. Gudnason, P.T. Ellinor, S. Sanna, S. Kaab, J.C. Witteman, A. Alonso, E.J. Benjamin, and S.R. Heckbert. (2010). Genome-wide association study of PR interval. *Nat Genet.* **42**: 153-159.

Piatkiewicz, P., A. Czech, J. Taton, and A. Gorski. (2010). Investigations of cellular glucose transport and its regulation under the influence of insulin in human peripheral blood lymphocytes. *Endokrynol Pol.* **61**: 182-187.

Piccioli, P., M. Serra, V. Gismondi, S. Pedemonte, F. Loiacono, S. Lastraioli, L. Bertario, M. De Angioletti, L. Varesco, and R. Notaro. (2006). Multiplex tetra-primer amplification refractory mutation system PCR to detect 6 common germline mutations of the MUTYH gene associated with polyposis and colorectal cancer. *Clin Chem.* **52**: 739-743.

Pinderski, L.J., M.P. Fischbein, G. Subbanagounder, M.C. Fishbein, N. Kubo, H. Cheroutre, L.K. Curtiss, J.A. Berliner, and W.A. Boisvert. (2002). Overexpression of interleukin-10 by activated T lymphocytes inhibits atherosclerosis in LDL receptor-deficient Mice by altering lymphocyte and macrophage phenotypes. *Circ Res.* **90**: 1064-1071.

Pinto, L.A., M. Depner, N. Klopp, T. Illig, C. Vogelberg, E. von Mutius, and M. Kabesch. (2010). MMP-9 gene variants increase the risk for non-atopic asthma in children. *Respir Res.* **11**: 23.

Piscione, F., G. Iaccarino, G. Galasso, E. Cipolletta, M.A. Rao, G. Brevetti, R. Piccolo, B. Trimarco, and M. Chiariello. (2008). Effects of Ile164 polymorphism of beta2-adrenergic receptor gene on coronary artery disease. *J Am Coll Cardiol.* **52**: 1381-1388.

Pizza, V., A. Bisogno, E. Lamaida, A. Agresta, G. Bandieramonte, A. Volpe, R. Galasso, L. Galasso, M. Caputo, M.F. Tecce, and A. Capasso. (2010). Migraine and coronary artery disease: an open study on the genetic polymorphism of the 5, 10 methylenetetrahydrofolate (MTHFR) and

angiotensin I-converting enzyme (ACE) genes. *Cent Nerv Syst Agents Med Chem.* **10**: 91-96.

Platt, N., and S. Gordon. (2001). Is the class A macrophage scavenger receptor (SR-A) multifunctional? - The mouse's tale. *J Clin Invest.* **108**: 649-654.

Pluddemann, A., C. Neyen, and S. Gordon. (2007). Macrophage scavenger receptors and host-derived ligands. *Methods.* **43**: 207-217.

Plump, A.S., J.D. Smith, T. Hayek, K. Aalto-Setala, A. Walsh, J.G. Verstuyft, E.M. Rubin, and J.L. Breslow. (1992). Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell.* **71**: 343-353.

Pols, T.W., P.I. Bonta, and C.J. de Vries. (2007). NR4A nuclear orphan receptors: protective in vascular disease? *Curr Opin Lipidol.* **18**: 515-520.

Porto, M.P., L.C. Galdieri, V.G. Pereira, N. Vergani, J.C. da Rocha, C. Micheletti, A.M. Martins, A.B. Perez, and V.D. Almeida. (2005). Molecular analysis of homocystinuria in Brazilian patients. *Clin Chim Acta.* **362**: 71-78.

Potier, L., C. Abi Khalil, K. Mohammedi, and R. Roussel. (2011). Use and utility of ankle brachial index in patients with diabetes. *Eur J Vasc Endovasc Surg.* **41**: 110-116.

Pradervand, S., A. Paillusson, J. Thomas, J. Weber, P. Wirapati, O. Hagenbuchle, and K. Harshman. (2008). Affymetrix Whole-Transcript Human Gene 1.0 ST array is highly concordant with standard 3' expression arrays. *Biotechniques.* **44**: 759-762.

Prati, F., M.T. Mallus, L. Broglia, and M. Albertucci. (2010). Integrated non-invasive imaging techniques. *EuroIntervention.* **6 Suppl G**: G161-G168.

Prekeris, R., J. Klumperman, and R.H. Scheller. (2000). Syntaxin 11 is an atypical SNARE abundant in the immune system. *Eur J Cell Biol.* **79**: 771-780.

Pritchard, K.A., L. Groszek, D.M. Smalley, W.C. Sessa, M. Wu, P. Villalon, M.S. Wolin, and M.B. Stemerman. (1995). Native Low-Density Lipoprotein Increases Endothelial Cell Nitric Oxide Synthase Generation of Superoxide Anion. *Circulation Research.* 77: 510-518.

Puskas, L.G., L.Z. Feher, C. Vizler, F. Ayaydin, E. Raso, E. Molnar, I. Magyary, I. Kanizsai, M. Gyuris, R. Madacsi, G. Fabian, K. Farkas, P. Hegyi, F. Baska, B. Ozsvari, and K. Kitajka. (2010). Polyunsaturated fatty acids synergize with lipid droplet binding thalidomide analogs to induce oxidative stress in cancer cells. *Lipids Health Dis.* **9**: 56.

Rajappa, M., S.K. Sen, and A. Sharma. (2009). Role of pro-/anti-inflammatory cytokines and their correlation with established risk factors in South Indians with coronary artery disease. *Angiology*. **60**: 419-426.

Rajavashisth, T.B., A. Andalibi, M.C. Territo, J.A. Berliner, M. Navab, A.M. Fogelman, and A.J. Lusis. (1990). Induction of endothelial cell expression of granulocyte and macrophage colony-stimulating factors by modified low-density lipoproteins. *Nature*. **344**: 254-257.

Real, J.T., S. Martinez-Hervas, A.B. Garcia-Garcia, F.J. Chaves, M. Civera, J.F. Ascaso, and R. Carmena. (2009). Association of C677T polymorphism in MTHFR gene, high homocysteine and low HDL cholesterol plasma values in heterozygous familial hypercholesterolemia. *J Atheroscler Thromb*. **16**: 815-820.

Refsum, H., P.M. Ueland, O. Nygard, and S.E. Vollset. (1998). Homocysteine and cardiovascular disease. *Annu Rev Med*. **49**: 31-62.

Ren, J., A. Ulvik, H. Refsum, and P.M. Ueland. (1998). Chemical mismatch cleavage combined with capillary electrophoresis: detection of mutations exon 8 of the cystathionine beta-synthase gene. *Clin Chem*. **44**: 2108-2114.

Ren, J., A. Ulvik, P.M. Ueland, and H. Refsum. (1997). Analysis of single-strand conformation polymorphism by capillary electrophoresis with laser-induced fluorescence detection using short-chain polyacrylamide as sieving medium. *Anal Biochem*. **245**: 79-84.

Rhead, B., D. Karolchik, R.M. Kuhn, A.S. Hinrichs, A.S. Zweig, P.A. Fujita, M. Diekhans, K.E. Smith, K.R. Rosenbloom, B.J. Raney, A. Pohl, M. Pheasant, L.R. Meyer, K. Learned, F. Hsu, J. Hillman-Jackson, R.A. Harte, B. Giardine, T.R. Dreszer, H. Clawson, G.P. Barber, D. Haussler, and W.J. Kent. (2010). The UCSC Genome Browser database: update 2010. *Nucleic Acids Res*. **38**: D613-D619.

Riches, L.C., A.M. Lynch, and N.J. Gooderham. (2010). A molecular beacon approach to detecting RAD52 expression in response to DNA damage in human cells. *Toxicol In Vitro*. **24**: 652-660.

Ridker, P.M., N.J. Brown, D.E. Vaughan, D.G. Harrison, and J.L. Mehta. (2004). Established and emerging plasma biomarkers in the prediction of first atherothrombotic events. *Circulation*. **109**: IV6-IV19.

Rieder, M.J., S.L. Taylor, V.O. Tobe, and D.A. Nickerson. (1998). Automating the identification of DNA variations using quality-based fluorescence re-sequencing: analysis of the human mitochondrial genome. *Nucleic Acids Res*. **26**: 967-973.

Rigat, B., C. Hubert, P. Corvol, and F. Soubrier. (1992). PCR detection of the insertion/deletion polymorphism of the human angiotensin converting enzyme gene (DCP1) (dipeptidyl carboxypeptidase 1). *Nucleic Acids Res*. **20**: 1433.

Riis, M.L., T. Luders, A.J. Nesbakken, H.S. Vollan, V. Kristensen, and I.R. Bukholm. (2010). Expression of BMI-1 and Mel-18 in breast tissue - a diagnostic marker in patients with breast cancer. *BMC Cancer*. **10**: 686.

Ririe, K.M., R.P. Rasmussen, and C.T. Wittwer. (1997). Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Anal Biochem*. **245**: 154-160.

Risbano, M.G., C.A. Meadows, C.D. Coldren, T.J. Jenkins, M.G. Edwards, D. Collier, W. Huber, D.G. Mack, A.P. Fontenot, M.W. Geraci, and T.M. Bull. (2010). Altered immune phenotype in peripheral blood cells of patients with scleroderma-associated pulmonary hypertension. *Clin Transl Sci*. **3**: 210-218.

Risueno, A., C. Fontanillo, M.E. Dinger, and J. De Las Rivas. (2010). GATExplorer: genomic and transcriptomic explorer; mapping expression probes to gene loci, transcripts, exons and ncRNAs. *BMC Bioinformatics*. **11**: 221.

Robertson, J., F. Iemolo, S.P. Stabler, R.H. Allen, and J.D. Spence. (2005). Vitamin B12, homocysteine and carotid plaque in the era of folic acid fortification of enriched cereal grain products. *CMAJ*. **172**: 1569-1573.

Robinette, C.D., and J.F. Fraumeni, Jr. (1977). Splenectomy and subsequent mortality in veterans of the 1939-45 war. *Lancet*. **2**: 127-129.

Rodgers, G.M., and M.T. Conn. (1990). Homocysteine, an atherogenic stimulus, reduces protein C activation by arterial and venous endothelial cells. *Blood*. **75**: 895-901.

Rodgers, G.M., and W.H. Kane. (1986). Activation of endogenous factor V by a homocysteine-induced vascular endothelial cell activator. *J Clin Invest*. **77**: 1909-1916.

Rodriguez, I., E. Coto, J.R. Reguero, P. Gonzalez, V. Andres, I. Lozano, M. Martin, V. Alvarez, and C. Moris. (2007). Role of the CDKN1A/p21, CDKN1C/p57, and CDKN2A/p16 genes in the risk of atherosclerosis and myocardial infarction. *Cell Cycle*. **6**: 620-625.

Romacho, T., V. Azcutia, M. Vazquez-Bella, N. Matesanz, E. Cercas, J. Nevado, R. Carraro, L. Rodriguez-Manas, C.F. Sanchez-Ferrer, and C. Peiro. (2009). Extracellular PBEF/NAMPT/visfatin activates pro-inflammatory signalling in human vascular smooth muscle cells through nicotinamide phosphoribosyltransferase activity. *Diabetologia*. **52**: 2455-2463.

Roodink, I., J. Raats, B. van der Zwaag, K. Verrijp, B. Kusters, H. van Bokhoven, M. Linkels, R.M. de Waal, and W.P. Leenders. (2005). Plexin D1 expression is induced on tumor vasculature and tumor cells: a novel target for diagnosis and therapy? *Cancer Res*. **65**: 8317-8323.

Rosamond, W., K. Flegal, G. Friday, K. Furie, A. Go, K. Greenlund, N. Haase, M. Ho, V. Howard, B. Kissela, S. Kittner, D. Lloyd-Jones, M. McDermott, J. Meigs, C. Moy, G. Nichol, C.J. O'Donnell, V. Roger, J. Rumsfeld, P. Sorlie, J. Steinberger, T. Thom, S. Wasserthiel-Smoller, and Y. Hong. (2007). Heart disease and stroke statistics--2007 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation.* **115**: e69-e171.

Rosenberg, S., M.R. Elashoff, P. Beineke, S.E. Daniels, J.A. Wingrove, W.G. Tingley, P.T. Sager, A.J. Sehnert, M. Yau, W.E. Kraus, L.K. Newby, R.S. Schwartz, S. Voros, S.G. Ellis, N. Tahirkheli, R. Waksman, J. McPherson, A. Lansky, M.E. Winn, N.J. Schork, and E.J. Topol. (2010). Multicenter validation of the diagnostic accuracy of a blood-based gene expression test for assessing obstructive coronary artery disease in nondiabetic patients. *Ann Intern Med.* **153**: 425-434.

Rosenkranz-Weiss, P., W.C. Sessa, S. Milstien, S. Kaufman, C.A. Watson, and J.S. Pober. (1994). Regulation of nitric oxide synthesis by proinflammatory cytokines in human umbilical vein endothelial cells: elevations in tetrahydrobiopterin levels enhance endothelial nitric oxide synthase specific activity. *J. Clin. Invest.* **93**: 2236-2243.

Rossi, L., I. Lapini, A. Magi, G. Pratesi, M. Lavitrano, G.M. Biasi, R. Pulli, C. Pratesi, R. Abbate, and B. Giusti. (2010). Carotid artery disease: novel pathophysiological mechanisms identified by gene-expression profiling of peripheral blood. *Eur J Vasc Endovasc Surg.* **40**: 549-558.

Rozanov, D.V., A.Y. Savinov, R. Williams, K. Liu, V.S. Golubkov, S. Krajewski, and A.Y. Strongin. (2008). Molecular signature of MT1-MMP: transactivation of the downstream universal gene network in cancer. *Cancer Res.* **68**: 4086-4096.

Rudkowska, I., C. Raymond, A. Ponton, H. Jacques, C. Lavigne, B.J. Holub, A. Marette, and M.C. Vohl. (2011). Validation of the use of peripheral blood mononuclear cells as surrogate model for skeletal muscle tissue in nutrigenomic studies. *Omics.* **15**: 1-7.

Sadewa, A.H., R. Sutomo, M. Istiadjid, K. Nishiyama, T. Shirakawa, M. Matsuo, and H. Nishio. (2004). C677T mutation in the MTHFR gene was not found in patients with frontoethmoidal encephalocele in East Java, Indonesia. *Pediatr Int.* **46**: 409-414.

Saeed, M., M. Perwaiz Iqbal, F.A. Yousuf, S. Perveen, M. Shafiq, J. Sajid, and P.M. Frossard. (2007). Interactions and associations of paraoxonase gene cluster polymorphisms with myocardial infarction in a Pakistani population. *Clin Genet.* **71**: 238-244.

Saghir, F.S., I.M. Rose, A.Z. Dali, Z. Shamsuddin, A.R. Jamal, and N.M. Mokhtar. (2010). Gene expression profiling and cancer-related pathways in type I endometrial carcinoma. *Int J Gynecol Cancer*. **20**: 724-731.

Sallinen, R., M.A. Kaunisto, C. Forsblom, M. Thomas, J. Fagerudd, K. Pettersson-Fernholm, P.H. Groop, and M. Wessman. (2010). Association of the SLC22A1, SLC22A2, and SLC22A3 genes encoding organic cation transporters with diabetic nephropathy and hypertension. *Ann Med*. **42**: 296-304.

Samani, N.J., J. Erdmann, A.S. Hall, C. Hengstenberg, M. Mangino, B. Mayer, R.J. Dixon, T. Meitinger, P. Braund, H.E. Wichmann, J.H. Barrett, I.R. Konig, S.E. Stevens, S. Szymczak, D.A. Tregouet, M.M. Iles, F. Pahlke, H. Pollard, W. Lieb, F. Cambien, M. Fischer, W. Ouwehand, S. Blankenberg, A.J. Balmforth, A. Baessler, S.G. Ball, T.M. Strom, I. Braenne, C. Gieger, P. Deloukas, M.D. Tobin, A. Ziegler, J.R. Thompson, and H. Schunkert. (2007). Genomewide association analysis of coronary artery disease. *N Engl J Med*. **357**: 443-453.

Sang, F., H. Ren, and J. Ren. (2006). Genetic mutation analysis by CE with LIF detection using inverse-flow derivatization of DNA fragments. *Electrophoresis*. **27**: 3846-3855.

Santos, M.G., M. Pegoraro, F. Sandrini, and E.C. Macuco. (2008). Risk factors for the development of atherosclerosis in childhood and adolescence. *Arq Bras Cardiol*. **90**: 276-283.

Saren, P., H.G. Welgus, and P.T. Kovanen. (1996). TNF-alpha and IL-1beta selectively induce expression of 92-kDa gelatinase by human macrophages. *J Immunol*. **157**: 4159-4165.

Scharrer, U., M. Skrzypczak-Zielinska, W. Wituszynska, M. Mierzejewski, K. Krause, C. Cybulski, and U.G. Froster. (2010). A simple method of investigating mutations in CHEK2 by DHPLC: a study of the German populations of Saxony, Saxony-Anhalt, and Thuringia. *Cancer Genet Cytogenet*. **199**: 48-52.

Schmidt, H.H., H. Hofmann, U. Schindler, Z.S. Shutenko, D.D. Cunningham, and M. Feelisch. (1996). No .NO from NO synthase. *Proc Natl Acad Sci USA*. **93**: 14492-14497.

Schnare, M., G.M. Barton, A.C. Holt, K. Takeda, S. Akira, and R. Medzhitov. (2001). Toll-like receptors control activation of adaptive immune responses. *Nat Immunol*. **2**: 947-950.

Schosser, A., K. Pirlo, D. Gaysina, S. Cohen-Woods, L.C. Schalkwyk, A. Elkin, A. Korszun, C. Gunasinghe, J. Gray, L. Jones, E. Meaburn, A.E. Farmer, I.W. Craig, and P. McGuffin. (2010). Utility of the pooling approach as applied to whole genome association scans with high-density Affymetrix microarrays. *BMC Res Notes*. **3**: 274.

Schwientek, P., P. Ellinghaus, S. Steppan, D. D'Urso, M. Seewald, A. Kassner, R. Cebulla, S. Schulte-Eistrup, M. Morshuis, D. Rofe, A. El Banayosy, R. Korfer, and H. Milting. (2010). Global gene expression analysis in nonfailing and failing myocardium pre- and postpulsatile and nonpulsatile ventricular assist device support. *Physiol Genomics*. **42**: 397-405.

Sebregts, E.H., P.R. Falger, and F.W. Bar. (2000). Risk factor modification through nonpharmacological interventions in patients with coronary heart disease. *J Psychosom Res*. **48**: 425-441.

Segel, G.B., M.W. Halterman, and M.A. Lichtman. (2011). The paradox of the neutrophil's role in tissue injury: a review. *J Leukoc Biol*. **89**: 359-372.

Seimon, T.A., M.J. Kim, A. Blumenthal, J. Koo, S. Ehrt, H. Wainwright, L.G. Bekker, G. Kaplan, C. Nathan, I. Tabas, and D.G. Russell. (2010). Induction of ER stress in macrophages of tuberculosis granulomas. *PLoS One*. **5**: e12772.

Selhub, J., P.F. Jacques, A.G. Bostom, P.W. Wilson, and I.H. Rosenberg. (2000). Relationship between plasma homocysteine and vitamin status in the Framingham study population. Impact of folic acid fortification. *Public Health Rev*. **28**: 117-145.

Senga, T., S. Iwamoto, T. Yoshida, T. Yokota, K. Adachi, E. Azuma, M. Hamaguchi, and T. Iwamoto. (2003). LSSIG is a novel murine leukocyte-specific GPCR that is induced by the activation of STAT3. *Blood*. **101**: 1185-1187.

Sensier, Y., T. Hartshorne, A. Thrush, S. Nydahl, A. Bolia, and N.J. London. (1996). A prospective comparison of lower limb colour-coded Duplex scanning with arteriography. *Eur J Vasc Endovasc Surg*. **11**: 170-175.

Shack, S. (2011). Gene expression profiling of tissues and cell lines: a dual-color microarray method. *Methods Mol Biol*. **700**: 125-143.

Shahar, E., L.E. Chambless, W.D. Rosamond, L.L. Boland, C.M. Ballantyne, P.G. McGovern, and A.R. Sharrett. (2003). Plasma lipid profile and incident ischemic stroke: the Atherosclerosis Risk in Communities (ARIC) study. *Stroke*. **34**: 623-631.

Shannon, P., A. Markiel, O. Ozier, N.S. Baliga, J.T. Wang, D. Ramage, N. Amin, B. Schwikowski, and T. Ideker. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res*. **13**: 2498-2504.

Shi, M., D. Caprau, P. Romitti, K. Christensen, and J.C. Murray. (2003). Genotype frequencies and linkage disequilibrium in the CEPH human diversity panel for variants in folate pathway genes MTHFR, MTHFD, MTRR, RFC1, and GCP2. *Birth Defects Res A Clin Mol Teratol*. **67**: 545-549.

Shi, M.M. (2001). Enabling large-scale pharmacogenetic studies by high-throughput mutation detection and genotyping technologies. *Clin Chem*. **47**: 164-172.

Shi, M.M. (2002). Technologies for individual genotyping: detection of genetic polymorphisms in drug targets and disease genes. *Am J Pharmacogenomics*. **2**: 197-205.

Shintomi, K., and T. Hirano. (2009). Releasing cohesin from chromosome arms in early mitosis: opposing actions of Wapl-Pds5 and Sgo1. *Genes Dev*. **23**: 2224-2236.

Siasos, G., D. Tousoulis, C. Antoniades, E. Stefanadi, and C. Stefanadis. (2007). L-Arginine, the substrate for NO synthesis: an alternative treatment for premature atherosclerosis? *Int J Cardiol*. **116**: 300-308.

Skaggs, H.S., H. Xing, D.C. Wilkerson, L.A. Murphy, Y. Hong, C.N. Mayhew, and K.D. Sarge. (2007). HSF1-TPR interaction facilitates export of stress-induced HSP70 mRNA. *J Biol Chem*. **282**: 33902-33907.

Skalen, K., M. Gustafsson, E.K. Rydberg, L.M. Hulten, O. Wiklund, T.L. Innerarity, and J. Boren. (2002). Subendothelial retention of atherogenic lipoproteins in early atherosclerosis. *Nature*. **417**: 750-754.

Skinner, A.L., A.A. Vartia, T.D. Williams, and J.S. Laurence. (2009). Enzyme activity of phosphatase of regenerating liver is controlled by the redox environment and its C-terminal residues. *Biochemistry*. **48**: 4262-4272.

Smedley, D., S. Haider, B. Ballester, R. Holland, D. London, G. Thorisson, and A. Kasprzyk. (2009). BioMart--biological queries made easy. *BMC Genomics*. **10**: 22.

Smith, J.D., E. Trogan, M. Ginsberg, C. Grigaux, J. Tian, and M. Miyata. (1995). Decreased atherosclerosis in mice deficient in both macrophage colony-stimulating factor (op) and apolipoprotein E. *Proc Natl Acad Sci USA*. **92**: 8264-8268.

Sniderman, A., S. Shapiro, D. Marpole, B. Skinner, B. Teng, and P.O. Kwiterovich, Jr. (1980). Association of coronary atherosclerosis with hyperapobetalipoproteinemia [increased protein but normal cholesterol levels in human plasma low density (beta) lipoproteins]. *Proc Natl Acad Sci USA*. 77: 604-608.

Song, L., C. Leung, and C. Schindler. (2001). Lymphocytes are important in early atherosclerosis. *J Clin Invest*. **108**: 251-259.

Stamova, B., H. Xu, G. Jickling, C. Bushnell, Y. Tian, B.P. Ander, X. Zhan, D. Liu, R. Turner, P. Adamczyk, J.C. Khoury, A. Pancioli, E. Jauch, J.P. Broderick, and F.R. Sharp. (2010). Gene expression profiling of blood for the prediction of ischemic stroke. *Stroke*. **41**: 2171-2177.

Steed, M.M., and S.C. Tyagi. (2010). Mechanisms of cardiovascular remodeling in hyperhomocysteinemia. *Antioxid Redox Signal*. doi:10.1089/ars.2010.3721.

Stefan, P., E. Moritz, W. Alexander, M. Weber, D. Norbert, W. Annette, F. Thomas, and P. Andreas. (2009). Development of a high-throughput method for screening the dopamine D2 (DRD2) receptor gene polymorphisms based on the LightCycler system. *Clin Lab*. **55**: 353-358.

Stefanius, K., T. Kantola, A. Tuomisto, P. Vahteristo, T.J. Karttunen, L.A. Aaltonen, M.J. Makinen, and A. Karhu. (2011). Downregulation of the hedgehog receptor PTCH1 in colorectal serrated adenocarcinomas is not caused by PTCH1 mutations. *Virchows Arch*. **458**: 213-219.

Steinberg, D. (1997). Low density lipoprotein oxidation and its pathobiological significance. *J Biol Chem*. **272**: 20963-20966.

Steinberg, D. (2002). Atherogenesis in perspective: hypercholesterolemia and inflammation as partners in crime. *Nat Med*. **8**: 1211-1217.

Stemme, S., B. Faber, J. Holm, O. Wiklund, J.L. Witztum, and G.K. Hansson. (1995). T lymphocytes from human atherosclerotic plaques recognize oxidized low density lipoprotein. *Proc Natl Acad Sci USA*. **92**: 3893-3897.

Stuhlinger, M.C., P.C. Tsao, J.H. Her, M. Kimoto, R.F. Balint, and J.P. Cooke. (2001). Homocysteine Impairs the Nitric Oxide Synthase Pathway: Role of Asymmetric Dimethylarginine. *Circulation*. **104**: 2569-2575.

Su, Z.G., S.Z. Zhang, Y.P. Hou, L. Zhang, D.J. Huang, L.C. Liao, and C.Y. Xiao. (2002). Relationship between a novel polymorphism of hepatic lipase gene and coronary artery disease. *Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao (Shanghai)*. **34**: 780-785.

Su, Z.G., S.Z. Zhang, L. Zhang, Y. Tong, C.Y. Xiao, Y.P. Hou, and L.C. Liao. (2003). A novel polymorphism A(+884)-->G in the hepatic lipase gene and its association with coronary artery disease. *Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao (Shanghai)*. **35**: 606-610.

Sun, X., H. Ding, K. Hung, and B. Guo. (2000). A new MALDI-TOF based mini-sequencing assay for genotyping of SNPS. *Nucleic Acids Res*. **28**: E68.

Swaminath, G. (2008). Fatty acid binding receptors and their physiological role in type 2 diabetes. *Arch Pharm (Weinheim)*. **341**: 753-761.

Syvanen, A.C. (2005). Toward genome-wide SNP genotyping. *Nat Genet*. **37 Suppl**: S5-S10.

Szabo, S.J., B.M. Sullivan, S.L. Peng, and L.H. Glimcher. (2003). Molecular mechanisms regulating Th1 immune responses. *Annu Rev Immunol*. **21**: 713-758.

Szczeklik, A., M. Sanak, M. Jankowski, J. Dropinski, R. Czachor, J. Musial, I. Axenti, M. Twardowska, T. Brzostek, and M. Tendera. (2001). Mutation A1298C of methylenetetrahydrofolate reductase: risk for early coronary disease not associated with hyperhomocysteinemia. *Am J Med Genet*. **101**: 36-39.

Szelinger, S., J.V. Pearson, and D.W. Craig. (2011). Microarray-based genome-wide association studies using pooled DNA. *Methods Mol Biol*. **700**: 49-60.

Szperl, M., Z. Dzielinska, M. Roszczynko, L.A. Malek, M. Makowiecka-Ciesla, M. Demkow, J. Kadziela, A. Prejbisz, E. Florczak, T. Zielinski, A. Januszewicz, and W. Ruzyllo. (2008). Genetic variants in hypertensive patients with coronary artery disease and coexisting atheromatous renal artery stenosis. *Med Sci Monit*. **14**: CR611-CR616.

Tanaka, T., P. Scheet, B. Giusti, S. Bandinelli, M.G. Piras, G. Usala, S. Lai, A. Mulas, A.M. Corsi, A. Vestrini, F. Sofi, A.M. Gori, R. Abbate, J. Guralnik, A. Singleton, G.R. Abecasis, D. Schlessinger, M. Uda, and L. Ferrucci. (2009). Genome-wide association study of vitamin B6, vitamin B12, folate, and homocysteine blood concentrations. *Am J Hum Genet*. **84**: 477-482.

Tao, M.H., P.G. Shields, J. Nie, C. Marian, C.B. Ambrosone, S.E. McCann, M. Platek, S.S. Krishnan, B. Xie, S.B. Edge, J. Winston, D. Vito, M. Trevisan, and J.L. Freudenheim. (2009). DNA promoter methylation in breast tumors: no association with genetic polymorphisms in MTHFR and MTR. *Cancer Epidemiol Biomarkers Prev*. **18**: 998-1002.

Tayo, B.O., R.A. DiCioccio, Y. Liang, M. Trevisan, R.S. Cooper, S. Lele, L. Sucheston, S.M. Piver, and K. Odunsi. (2009). Complex segregation analysis of pedigrees from the Gilda Radner Familial Ovarian Cancer Registry reveals evidence for mendelian dominant inheritance. *PLoS One*. **4**: e5939.

The International HapMap Project. (2003). The International HapMap Project. *Nature*. **426**: 789-796.

Thorgeirsson, T.E., F. Geller, P. Sulem, T. Rafnar, A. Wiste, K.P. Magnusson, A. Manolescu, G. Thorleifsson, H. Stefansson, A. Ingason, S.N. Stacey, J.T. Bergthorsson, S. Thorlacius, J. Gudmundsson, T. Jonsson, M. Jakobsdottir, J. Saemundsdottir, O. Olafsdottir, L.J. Gudmundsson, G. Bjornsdottir, K. Kristjansson, H. Skuladottir, H.J. Isaksson, T. Gudbjartsson, G.T. Jones, T. Mueller, A. Gottsater, A. Flex, K.K. Aben, F. de Vegt, P.F. Mulders, D. Isla, M.J. Vidal, L. Asin, B. Saez, L. Murillo, T. Blondal, H. Kolbeinsson, J.G. Stefansson, I. Hansdottir, V. Runarsdottir, R. Pola, B. Lindblad, A.M. van Rij, B. Dieplinger, M. Haltmayer, J.I. Mayordomo, L.A. Kiemeney, S.E. Matthiasson, H. Oskarsson, T. Tyrfingsson, D.F. Gudbjartsson, J.R. Gulcher, S. Jonsson, U. Thorsteinsdottir, A. Kong, and K. Stefansson. (2008). A variant

associated with nicotine dependence, lung cancer and peripheral arterial disease. *Nature*. **452**: 638-642.

Thorneycroft, I.H. (1990). Oral contraceptives and myocardial infarction. *Am J Obstet Gynecol*. **163**: 1393-1397.

Tietjen, G.E., N.A. Herial, C. Utley, L. White, S. Yerga-Woolwine, and B. Joe. (2009). Association of von Willebrand factor activity with ACE I/D and MTHFR C677T polymorphisms in migraine. *Cephalalgia*. **29**: 960-968.

Timofeeva, A.V., L.E. Goryunova, G.L. Khaspekov, D.A. Kovalevskii, A.V. Scamrov, O.S. Bulkina, Y.A. Karpov, K.A. Talitskii, V.V. Buza, V.V. Britareva, and R. Beabealashvilli. (2006). Altered gene expression pattern in peripheral blood leukocytes from patients with arterial hypertension. *Ann N Y Acad Sci*. **1091**: 319-335.

Touyz, R.M. (2004). Reactive oxygen species and angiotensin II signaling in vascular cells -- implications in cardiovascular disease. *Braz J Med Biol Res*. **37**: 1263-1273.

Touyz, R.M., and A.M. Briones. (2011). Reactive oxygen species and vascular biology: implications in human hypertension. *Hypertens Res*. **34**: 5-14.

Trabetti, E. (2008). Homocysteine, MTHFR gene polymorphisms, and cardio-cerebrovascular risk. *J Appl Genet*. **49**: 267-282.

Tregouet, D.A., I.R. Konig, J. Erdmann, A. Munteanu, P.S. Braund, A.S. Hall, A. Grosshennig, P. Linsel-Nitschke, C. Perret, M. DeSuremain, T. Meitinger, B.J. Wright, M. Preuss, A.J. Balmforth, S.G. Ball, C. Meisinger, C. Germain, A. Evans, D. Arveiler, G. Luc, J.B. Ruidavets, C. Morrison, P. van der Harst, S. Schreiber, K. Neureuther, A. Schafer, P. Bugert, N.E. El Mokhtari, J. Schrezenmeir, K. Stark, D. Rubin, H.E. Wichmann, C. Hengstenberg, W. Ouwehand, A. Ziegler, L. Tiret, J.R. Thompson, F. Cambien, H. Schunkert, and N.J. Samani. (2009). Genome-wide haplotype association study identifies the SLC22A3-LPAL2-LPA gene cluster as a risk locus for coronary artery disease. *Nat Genet*. **41**: 283-285.

Tsai, M.Y., N.Q. Hanson, M.K. Bignell, and K.A. Schwichtenberg. (1996). Simultaneous detection and screening of T833C and G919A mutations of the cystathionine beta-synthase gene by single-strand conformational polymorphism. *Clin Biochem*. **29**: 473-477.

Tsai, M.Y., B.G. Welge, N.Q. Hanson, M.K. Bignell, J. Vessey, K. Schwichtenberg, F. Yang, F.E. Bullemer, R. Rasmussen, and K.J. Graham. (1999). Genetic causes of mild hyperhomocysteinemia in patients with premature occlusive coronary artery diseases. *Atherosclerosis*. **143**: 163-170.

Tsang, M.W., C.L. Chu, Y.W. Kam, K.H. Kwong, C.K. Lam, S.Y. Ngan, and Y.K. Yu. (2005). Characterising atherothrombosis in Hong Kong: results of

the Hong Kong data from a global atherothrombosis epidemiological survey. *Hong Kong Med J.* **11**: 36-41.

Tyagi, S., and F.R. Kramer. (1996). Molecular beacons: probes that fluoresce upon hybridization. *Nat Biotechnol.* **14**: 303-308.

Undas, A., J. Brozek, M. Jankowski, Z. Siudak, A. Szczeklik, and H. Jakubowski. (2006). Plasma homocysteine affects fibrin clot permeability and resistance to lysis in human subjects. *Arterioscler Thromb Vasc Biol.* **26**: 1397-1404.

Underhill, P.A., L. Jin, A.A. Lin, S.Q. Mehdi, T. Jenkins, D. Vollrath, R.W. Davis, L.L. Cavalli-Sforza, and P.J. Oefner. (1997). Detection of numerous Y chromosome biallelic polymorphisms by denaturing high-performance liquid chromatography. *Genome Res.* 7: 996-1005.

Underhill, P.A., P. Shen, A.A. Lin, L. Jin, G. Passarino, W.H. Yang, E. Kauffman, B. Bonne-Tamir, J. Bertranpetit, P. Francalacci, M. Ibrahim, T. Jenkins, J.R. Kidd, S.Q. Mehdi, M.T. Seielstad, R.S. Wells, A. Piazza, R.W. Davis, M.W. Feldman, L.L. Cavalli-Sforza, and P.J. Oefner. (2000). Y chromosome sequence variation and the history of human populations. *Nat Genet.* **26**: 358-361.

Urgert, R., T. van Vliet, P.L. Zock, and M.B. Katan. (2000). Heavy coffee consumption and plasma homocysteine: a randomized controlled trial in healthy volunteers. *Am J Clin Nutr.* **72**: 1107-1110.

Uyemura, K., L.L. Demer, S.C. Castle, D. Jullien, J.A. Berliner, M.K. Gately, R.R. Warrier, N. Pham, A.M. Fogelman, and R.L. Modlin. (1996). Cross-regulatory roles of interleukin (IL)-12 and IL-10 in atherosclerosis. *J Clin Invest.* **97**: 2130-2138.

Valdez, A.C., J.P. Cabaniols, M.J. Brown, and P.A. Roche. (1999). Syntaxin 11 is associated with SNAP-23 on late endosomes and the trans-Golgi network. *J Cell Sci.* **112** (Pt 6): 845-854.

van der Heul-Nieuwenhuijsen, L., R.C. Padmos, R.C. Drexhage, H. de Wit, A. Berghout, and H.A. Drexhage. (2010). An inflammatory gene-expression fingerprint in monocytes of autoimmune thyroid disease patients. *J Clin Endocrinol Metab.* **95**: 1962-1971.

van Hinsbergh, V.W., E.A. van den Berg, W. Fiers, and G. Dooijewaard. (1990). Tumor necrosis factor induces the production of urokinase-type plasminogen activator by human endothelial cells. *Blood.* **75**: 1991-1998.

Venter, J.C., M.D. Adams, E.W. Myers, P.W. Li, R.J. Mural, G.G. Sutton, H.O. Smith, M. Yandell, C.A. Evans, R.A. Holt, J.D. Gocayne, P. Amanatides, R.M. Ballew, D.H. Huson, J.R. Wortman, Q. Zhang, C.D. Kodira, X.H. Zheng, L. Chen, M. Skupski, G. Subramanian, P.D. Thomas, J. Zhang, G.L. Gabor Miklos, C. Nelson, S. Broder, A.G. Clark, J. Nadeau, V.A.

McKusick, N. Zinder, A.J. Levine, R.J. Roberts, M. Simon, C. Slayman, M. Hunkapiller, R. Bolanos, A. Delcher, I. Dew, D. Fasulo, M. Flanigan, L. Florea, A. Halpern, S. Hannenhalli, S. Kravitz, S. Levy, C. Mobarry, K. Reinert, K. Remington, J. Abu-Threideh, E. Beasley, K. Biddick, V. Bonazzi, R. Brandon, M. Cargill, I. Chandramouliswaran, R. Charlab, K. Chaturvedi, Z. Deng, V. Di Francesco, P. Dunn, K. Eilbeck, C. Evangelista, A.E. Gabrielian, W. Gan, W. Ge, F. Gong, Z. Gu, P. Guan, T.J. Heiman, M.E. Higgins, R.R. Ji, Z. Ke, K.A. Ketchum, Z. Lai, Y. Lei, Z. Li, J. Li, Y. Liang, X. Lin, F. Lu, G.V. Merkulov, N. Milshina, H.M. Moore, A.K. Naik, V.A. Narayan, B. Neelam, D. Nusskern, D.B. Rusch, S. Salzberg, W. Shao, B. Shue, J. Sun, Z. Wang, A. Wang, X. Wang, J. Wang, M. Wei, R. Wides, C. Xiao, C. Yan, et al. (2001). The sequence of the human genome. *Science*. **291**: 1304-1351.

Verma, S., and T.J. Anderson. (2002). Fundamentals of endothelial function for the clinical cardiologist. *Circulation*. **105**: 546-549.

Verstraelen, S., I. Nelissen, J. Hooyberghs, H. Witters, G. Schoeters, P. Van Cauwenberge, and R. Van Den Heuvel. (2009). Gene profiles of a human alveolar epithelial cell line after in vitro exposure to respiratory (non-) sensitizing chemicals: identification of discriminating genetic markers and pathway analysis. *Toxicol Lett*. **185**: 16-22.

Vet, J.A., B.J. Van der Rijt, and H.J. Blom. (2002). Molecular beacons: colorful analysis of nucleic acids. *Expert Rev Mol Diagn*. **2**: 77-86.

Vijaya Lakshmi, S.V., S.M. Naushad, Y. Rupasree, D. Seshagiri Rao, and V.K. Kutala. (2011). Interactions of 5'-UTR Thymidylate Synthase Polymorphism with 677C--> T Methylene Tetrahydrofolate Reductase and 66A--> G Methyltetrahydrofolate Homocysteine Methyl-transferase Reductase Polymorphisms Determine Susceptibility to Coronary Artery Disease. *J Atheroscler Thromb*. **18**: 56-64.

Villanueva, A., D.Y. Chiang, P. Newell, J. Peix, S. Thung, C. Alsinet, V. Tovar, S. Roayaie, B. Minguez, M. Sole, C. Battiston, S. Van Laarhoven, M.I. Fiel, A. Di Feo, Y. Hoshida, S. Yea, S. Toffanin, A. Ramos, J.A. Martignetti, V. Mazzaferro, J. Bruix, S. Waxman, M. Schwartz, M. Meyerson, S.L. Friedman, and J.M. Llovet. (2008). Pivotal role of mTOR signaling in hepatocellular carcinoma. *Gastroenterology*. **135**: 1972-1983, 1983 e1-e11.

Vinukonda, G., N. Shaik Mohammad, J. Md Nurul Jain, K. Prasad Chintakindi, and R. Rama Devi Akella. (2009). Genetic and environmental influences on total plasma homocysteine and coronary artery disease (CAD) risk among South Indians. *Clin Chim Acta*. **405**: 127-131.

Violi, F., Criqui, M., Longoni, A. and Castiglioni, C. (1996). Relation between risk factors and cardiovascular complications in patients with peripheral vascular disease. Results from the A.D.E.P. study. *Atherosclerosis*. **120**: 25-35.

Vogiatzi, K., S. Apostolakis, V. Voudris, S. Thomopoulou, G.E. Kochiadakis, and D.A. Spandidos. (2010). Interleukin 8 gene polymorphisms and susceptibility to restenosis after percutaneous coronary intervention. *J Thromb Thrombolysis*. **29**: 134-140.

Vogler, C., L. Gschwind, B. Rothlisberger, A. Huber, I. Filges, P. Miny, B. Auschra, A. Stetak, P. Demougin, V. Vukojevic, I.T. Kolassa, T. Elbert, D.J. de Quervain, and A. Papassotiropoulos. (2010). Microarray-based maps of copy-number variant regions in European and sub-Saharan populations. *PLoS One*. **5**: e15246.

Voisine, P., M. Ruel, T.A. Khan, C. Bianchi, S.H. Xu, I. Kohane, T.A. Libermann, H. Otu, A.R. Saltiel, and F.W. Sellke. (2004). Differences in gene expression profiles of diabetic and nondiabetic patients undergoing cardiopulmonary bypass and cardioplegic arrest. *Circulation*. **110**: II280-II286.

Waehre, T., A. Yndestad, C. Smith, T. Haug, S.H. Tunheim, L. Gullestad, S.S. Froland, A.G. Semb, P. Aukrust, and J.K. Damas. (2004). Increased expression of interleukin-1 in coronary artery disease with downregulatory effects of HMG-CoA reductase inhibitors. *Circulation*. **109**: 1966-1972.

Wagner, A.M., J.C. Wiebe, M. Boronat, P. Saavedra, D. Marrero, F. Varillas, and F.J. Novoa. (2010). Insulin Resistance Explains the Relationship between Novel Cardiovascular Risk Factors and Hypertension. the Telde Study. *J Endocrinol Invest*. doi: 10.3275/7325.

Wald, D.S., M. Law, and J.K. Morris. (2002). Homocysteine and cardiovascular disease: evidence on causality from a meta-analysis. *BMJ*. **325**: 1202.

Walsh, T.J., A. Francesconi, M. Kasai, and S.J. Chanock. (1995). PCR and single-strand conformational polymorphism for recognition of medically important opportunistic fungi. *J Clin Microbiol*. **33**: 3216-3220.

Wang, C.C., H.L. Liang, C.C. Hsiao, M.C. Chen, T.H. Wu, C.J. Wu, J.S. Huang, Y.H. Lin, and H.B. Pan. (2010). Single-dose time-resolved contrast enhanced hybrid MR angiography in diagnosis of peripheral arterial disease: compared with digital subtraction angiography. *J Magn Reson Imaging*. **32**: 935-942.

Wang, P., P.M. Vanhoutte, and C.Y. Miao. (2011). Visfatin and Cardio-Cerebro-Vascular Disease. *J Cardiovasc Pharmacol* .doi: 10.1097/FJC.0b013e31820eb8f6.

Wang, Y., and N. Sheibani. (2002). Expression pattern of alternatively spliced PECAM-1 isoforms in hematopoietic cells and platelets. *J Cell Biochem*. **87**: 424-438.

Ward, K.J., S. Ellard, C.S. Yajnik, T.M. Frayling, A.T. Hattersley, P.N. Venigalla, and G.R. Chandak. (2006). Allelic drop-out may occur with a primer binding site polymorphism for the commonly used RFLP assay for the

-1131T>C polymorphism of the Apolipoprotein AV gene. *Lipids Health Dis.* **5**: 11.

Warde-Farley, D., S.L. Donaldson, O. Comes, K. Zuberi, R. Badrawi, P. Chao, M. Franz, C. Grouios, F. Kazi, C.T. Lopes, A. Maitland, S. Mostafavi, J. Montojo, Q. Shao, G. Wright, G.D. Bader, and Q. Morris. (2010). The GeneMANIA prediction server: biological network integration for gene prioritization and predicting gene function. *Nucleic Acids Res.* **38 Suppl**: W214-W220.

Weiss, N. (2005). Mechanisms of increased vascular oxidant stress in hyperhomocysteinemia and its impact on endothelial function. *Curr Drug Metab.* **6**: 27-36.

Weiss, N., C. Keller, U. Hoffmann, and J. Loscalzo. (2002). Endothelial dysfunction and atherothrombosis in mild hyperhomocysteinemia. *Vasc Med.* **7**: 227-239.

Weiss, N., Y.Y. Zhang, S.R. Heydrick, C. Bierl, and J. Loscalzo. (2001). Overexpression of cellular glutathione peroxidase rescues homocyst(e)ine-induced endothelial dysfunction. *Proc Natl Acad Sci USA.* **98**: 12503–12508.

Welch, C., M.K. Santra, W. El-Assaad, X. Zhu, W.E. Huber, R.A. Keys, J.G. Teodoro, and M.R. Green. (2009). Identification of a protein, G0S2, that lacks Bcl-2 homology domains and interacts with and antagonizes Bcl-2. *Cancer Res.* **69**: 6782-6789.

Welch, G.N., and J. Loscalzo. (1998). Homocysteine and atherothrombosis. *N Engl J Med.* **338**: 1042-1050.

Welch, G.N., G.R. Upchurch, Jr., R.S. Farivar, A. Pigazzi, K. Vu, P. Brecher, J.F. Keaney, Jr., and J. Loscalzo. (1998). Homocysteine-induced nitric oxide production in vascular smooth-muscle cells by NF-kappa B-dependent transcriptional activation of Nos2. *Proc Assoc Am Physicians.* **110**: 22-31.

Welcome Trust Case Control Consortium. (2007). Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature.* **447**: 661-678.

Wheeler, J.G., B.D. Keavney, H. Watkins, R. Collins, and J. Danesh. (2004). Four paraoxonase gene polymorphisms in 11212 cases of coronary heart disease and 12786 controls: meta-analysis of 43 studies. *Lancet.* **363**: 689-695.

Whitney, A.R., M. Diehn, S.J. Popper, A.A. Alizadeh, J.C. Boldrick, D.A. Relman, and P.O. Brown. (2003). Individuality and variation in gene expression patterns in human blood. *Proc Natl Acad Sci USA.* **100**: 1896-1901.

Widlansky, M.E., N. Gokce, J.F. Keaney, Jr., and J.A. Vita. (2003). The clinical implications of endothelial dysfunction. *J Am Coll Cardiol.* **42**: 1149-1160.

Wiederholt, T., R. Heise, C. Skazik, Y. Marquardt, S. Joussen, K. Erdmann, H. Schroder, H.F. Merk, and J.M. Baron. (2009). Calcium pantothenate modulates gene expression in proliferating human dermal fibroblasts. *Exp Dermatol.* **18**: 969-978.

Wilcken, D.E., and B. Wilcken. (1976). The pathogenesis of coronary artery disease. A possible role for methionine metabolism. *J Clin Invest.* **57**: 1079-1082.

Williams, K.J., and I. Tabas. (1995). The response-to-retention hypothesis of early atherogenesis. *Arterioscler Thromb Vasc Biol.* **15**: 551-561.

Wingrove, J.A., S.E. Daniels, A.J. Sehnert, W. Tingley, M.R. Elashoff, S. Rosenbery, L. Buellesfeld, E. Grube, L.K. Newby, G.S. Ginsburg, and M.E. Kraus. (2008). Correlation of peripheral blood gene expression with the extent of coronary artery stenosis. *Circ Cardiovasc Genet.* **1**: 31-38.

Winter-Warnars, H.A., Y. van der Graaf, and W.P. Mali. (1996). Interobserver variation in duplex sonographic scanning in the femoropopliteal tract. *J Ultrasound Med.* **15**: 421-428; discussion 329-330.

Wittchen, E.S. (2009). Endothelial signaling in paracellular and transcellular leukocyte transmigration. *Front Biosci.* **14**: 2522-2545.

Wu, C., C. Orozco, J. Boyer, M. Leglise, J. Goodale, S. Batalov, C.L. Hodge, J. Haase, J. Janes, J.W. Huss, 3rd, and A.I. Su. (2009). BioGPS: an extensible and customizable portal for querying and organizing gene annotation resources. *Genome Biol.* **10**: R130.

Wu, J.M., T.G. Wang, Y.Q. Li, X.W. Song, Y.Y. Liu, H.R. Yun, Z.Y. Zhong, and T.H. Zhou. (2004). [Genetic mutations of homocysteine metabolism related enzymes in patients with ischemic stroke]. *Yi Chuan.* **26**: 298-302.

Wyler von Ballmoos, M., D. Dubler, M. Mirlacher, G. Cathomas, J. Muser, and B.C. Biedermann. (2006). Increased apolipoprotein deposits in early atherosclerotic lesions distinguish symptomatic from asymptomatic patients. *Arterioscler Thromb Vasc Biol.* **26**: 359-364.

Xiao, Y., M.R. Segal, Y.H. Yang, and R.F. Yeh. (2007). A multi-array multi-SNP genotyping algorithm for Affymetrix SNP microarrays. *Bioinformatics.* **23**: 1459-1467.

Xie, P., Y.S. Cao, P. Su, Y.H. Li, Z.L. Gao, and M.M. Borst. (2010). Expression of toll-like receptor 4, tumor necrosis factor- alpha, matrix metalloproteinase-9 and effects of benazepril in patients with acute coronary syndromes. *Clin Med Insights Cardiol.* **4**: 89-93.

Xiong, Z., Y. Yan, J. Song, P. Fang, Y. Yin, Y. Yang, A. Cowan, H. Wang, and X.F. Yang. (2009). Expression of TCTP antisense in CD25(high) regulatory T cells aggravates cuff-injured vascular inflammation. *Atherosclerosis.* **203**: 401-408.

Xu, Q. (2002). Role of heat shock proteins in atherosclerosis. *Arterioscler Thromb Vasc Biol.* **22**: 1547-1559.

Yang, B., Q. Xu, F. Wu, F. Liu, X. Ye, G. Liu, Z. Shao, X. Meng, B. Mougin, and J. Wu. (2010a). Using peripheral blood mRNA signature to distinguish between breast cancer and benign breast disease in non-conclusive mammography patients. *Cancer Biol Ther.* **10**: 1235 - 1239.

Yang, J., X. Li, R.S. Al-Lamki, M. Southwood, J. Zhao, A.M. Lever, F. Grimminger, R.T. Schermuly, and N.W. Morrell. (2010b). Smad-dependent and smad-independent induction of id1 by prostacyclin analogues inhibits proliferation of pulmonary artery smooth muscle cells in vitro and in vivo. *Circ Res.* **107**: 252-262.

Yang, X., X. Lu, M. Lombes, G.B. Rha, Y.I. Chi, T.M. Guerin, E.J. Smart, and J. Liu. (2010c). The G(0)/G(1) switch gene 2 regulates adipose lipolysis through association with adipose triglyceride lipase. *Cell Metab.* **11**: 194-205.

Yang, X.F., Y. Yin, and H. Wang. (2008). Vascular inflammation and atherogenesis are activated via receptors for PAMPS and suppressed by regulatory T cells. *Drug Discov Today Ther Strateg.* **5**: 125-142.

Yang, Y.G., J.Y. Kim, S.J. Park, S.W. Kim, O.H. Jeon, and D.S. Kim. (2007). Apolipoprotein E genotyping by multiplex tetra-primer amplification refractory mutation system PCR in single reaction tube. *J Biotechnol.* **131**: 106-110.

Ye, S., S. Dhillon, X. Ke, A.R. Collins, and I.N. Day. (2001). An efficient procedure for genotyping single nucleotide polymorphisms. *Nucleic Acids Res.* **29**: E88-E88.

Ye, Z., Smith, C. and Kullo, I. J. (2011). Usefulness of red cell distribution width to predict mortality in patients with peripheral artery disease. *Am J Cardiol.* **107**: 1241-1245.

Yesilkaya, H., F. Meacci, S. Niemann, D. Hillemann, S. Rusch-Gerdes, M.R. Barer, P.W. Andrew, and M.R. Oggioni. (2006). Evaluation of molecular-Beacon, TaqMan, and fluorescence resonance energy transfer probes for detection of antibiotic resistance-conferring single nucleotide polymorphisms in mixed Mycobacterium tuberculosis DNA extracts. *J Clin Microbiol.* **44**: 3826-3829.

Yu, B., N.A. Sawyer, C. Chiu, P.J. Oefner, and P.A. Underhill. (2006). DNA mutation detection using denaturing high-performance liquid chromatography (DHPLC). *Curr Protoc Hum Genet.* Chapter 7:Unit7 10.

Yu, J.T., C.X. Mao, H.W. Zhang, Q. Zhang, Z.C. Wu, N.N. Yu, N. Zhang, Y. Li, and L. Tan. (2011). Genetic association of rs11610206 SNP on chromosome

12q13 with late-onset Alzheimer's disease in a Han Chinese population. *Clin Chim Acta.* **412**: 148-151.

Yusuf, S., S. Ounpuu, and S. Anand. (2002). The global epidemic of atherosclerotic cardiovascular disease. *Med Princ Pract.* **11 Suppl 2**: 3-8.

Yusuf, S., S. Reddy, S. Ounpuu, and S. Anand. (2001a). Global burden of cardiovascular diseases: part I: general considerations, the epidemiologic transition, risk factors, and impact of urbanization. *Circulation.* **104**: 2746-2753.

Yusuf, S., S. Reddy, S. Ounpuu, and S. Anand. (2001b). Global burden of cardiovascular diseases: Part II: variations in cardiovascular disease by specific ethnic groups and geographic regions and prevention strategies. *Circulation.* **104**: 2855-2864.

Zakkar, M., H. Chaudhury, G. Sandvik, K. Enesa, A. Luong le, S. Cuhlmann, J.C. Mason, R. Krams, A.R. Clark, D.O. Haskard, and P.C. Evans. (2008). Increased endothelial mitogen-activated protein kinase phosphatase-1 expression suppresses proinflammatory activation at sites that are resistant to atherosclerosis. *Circ Res.* **103**: 726-732.

Zeng, Q., X.N. Sun, L.H. Ma, L. Chen, Y. Wang, C. Chen, Z.Y. Ye, Z.H. Wu, Y.Z. Li, and Y. Xu. (2010). Biomarkers for the prediction of major adverse cardiovascular events in patients with acute coronary syndrome. *Anat Rec (Hoboken).* **293**: 1512-1518.

Zhan, L., A. Rosenberg, K.C. Bergami, M. Yu, Z. Xuan, A.B. Jaffe, C. Allred, and S.K. Muthuswamy. (2008). Deregulation of scribble promotes mammary tumorigenesis and reveals a role for cell polarity in carcinoma. *Cell.* **135**: 865-878.

Zhang, B., J. Chang, M. Fu, J. Huang, R. Kashyap, E. Salavaggione, S. Jain, S. Kulkarni, M.A. Deardorff, M.L. Uzielli, D. Dorsett, D.C. Beebe, P.Y. Jay, R.O. Heuckeroth, I. Krantz, and J. Milbrandt. (2009a). Dosage effects of cohesin regulatory factor PDS5 on mammalian development: implications for cohesinopathies. *PLoS One.* **4**: e5232.

Zhang, C., J. Kawauchi, M.T. Adachi, Y. Hashimoto, S. Oshiro, T. Aso, and S. Kitajima. (2001). Activation of JNK and transcriptional repressor ATF3/LRF1 through the IRE1/TRAF2 pathway is implicated in human vascular endothelial cell death by homocysteine. *Biochem Biophys Res Commun.* **289**: 718-724.

Zhang, D.Y., G. Sabla, P. Shivakumar, G. Tiao, R.J. Sokol, C. Mack, B.L. Shneider, B. Aronow, and J.A. Bezerra. (2004). Coordinate expression of regulatory genes differentiates embryonic and perinatal forms of biliary atresia. *Hepatology.* **39**: 954-962.

Zhang, H., W.P. Wang, T. Guo, J.C. Yang, P. Chen, K.T. Ma, Y.F. Guan, and C.Y. Zhou. (2009b). The LIM-homeodomain protein ISL1 activates insulin gene promoter directly through synergy with BETA2. *J Mol Biol.* **392**: 566-577.

Zhang, L., R.X. Yin, W.Y. Liu, L. Miao, D.F. Wu, L.H. Aung, X.J. Hu, X.L. Cao, J.Z. Wu, and S.L. Pan. (2010a). Association of methylenetetrahydrofolate reductase C677T polymorphism and serum lipid levels in the Guangxi Bai Ku Yao and Han populations. *Lipids Health Dis.* **9**: 123.

Zhang, Q., W.D. Huang, X.Y. Lv, and Y.M. Yang. (2010b). The association of ghrelin polymorphisms with coronary artery disease and ischemic chronic heart failure in an elderly Chinese population. *Clin Biochem.* doi:10.1016/j.clinbiochem.2010.12.013.

Zhang, S., D. Ma, X. Wang, T. Celkan, M. Nordenskjold, J.I. Henter, B. Fadeel, and C. Zheng. (2008a). Syntaxin-11 is expressed in primary human monocytes/macrophages and acts as a negative regulator of macrophage engulfment of apoptotic cells and IgG-opsonized target cells. *Br J Haematol.* **142**: 469-479.

Zhang, X., X. Miao, Y. Guo, W. Tan, Y. Zhou, T. Sun, Y. Wang, and D. Lin. (2006). Genetic polymorphisms in cell cycle regulatory genes MDM2 and TP53 are associated with susceptibility to lung cancer. *Hum Mutat.* **27**: 110-117.

Zhang, Y., Z. Yang, Y. Cao, S. Zhang, H. Li, Y. Huang, Y.Q. Ding, and X. Liu. (2008b). The Hsp40 family chaperone protein DnaJB6 enhances Schlafen1 nuclear localization which is critical for promotion of cell-cycle arrest in T-cells. *Biochem J.* **413**: 239-250.

Zhang, Y., K.X. Zhang, X. He, W.T. Yuan, G.L. Wang, S.Y. Mao, P.J. Gao, W. Huang, and D.L. Zhu. (2005). [A polymorphism of kynureninase gene in a hypertensive candidate chromosomal region is associated with essential hypertension]. *Zhonghua Xin Xue Guan Bing Za Zhi.* **33**: 588-591.

Zhao, Y., and D. Bruemmer. (2010). NR4A orphan nuclear receptors: transcriptional regulators of gene expression in metabolism and vascular biology. *Arterioscler Thromb Vasc Biol.* **30**: 1535-1541.

Zhou, G.H., M. Gotou, T. Kajiyama, and H. Kambara. (2005). Multiplex SNP typing by bioluminometric assay coupled with terminator incorporation (BATI). *Nucleic Acids Res.* **33**: e133.

Zhou, Y., P. Abidi, A. Kim, W. Chen, T.T. Huang, F.B. Kraemer, and J. Liu. (2007). Transcriptional activation of hepatic ACSL3 and ACSL5 by oncostatin m reduces hypertriglyceridemia through enhanced beta-oxidation. *Arterioscler Thromb Vasc Biol.* **27**: 2198-2205.

Zhu, Y.X., S. Benn, Z.H. Li, E. Wei, E. Masih-Khan, Y. Trieu, M. Bali, C.J. McGlade, J.O. Claudio, and A.K. Stewart. (2004). The SH3-SAM adaptor HACS1 is up-regulated in B cell activation signaling cascades. *J Exp Med.* **200**: 737-747.

Zintzaras, E., G. Raman, G. Kitsios, and J. Lau. (2008). Angiotensin-converting enzyme insertion/deletion gene polymorphic variant as a marker of coronary artery disease: a meta-analysis. *Arch Intern Med.* **168**: 1077-1089.

zur Stadt, U., S. Schmidt, B. Kasper, K. Beutel, A.S. Diler, J.I. Henter, H. Kabisch, R. Schneppenheim, P. Nurnberg, G. Janka, and H.C. Hennies. (2005). Linkage of familial hemophagocytic lymphohistiocytosis (FHL) type-4 to chromosome 6q24 and identification of mutations in syntaxin 11. *Hum Mol Genet.* **14**: 827-834.