CELLULAR AND MOLECULAR INFLUENCES ON PRO-THROMBOTIC AND PRO-INFLAMMATORY STATES IN YOUNG PATIENTS WITH CORONARY ARTERY DISEASE

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A THESIS PRESENTED BY

REEMA GEORGE

TO

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY, TRIVANDRUM

THIRUVANANTHAPURAM

IN PARTIAL FULFILMENT OF THE REQUIREMENT
FOR THE AWARD OF

DOCTOR OF PHILOSOPHY

2016

CERTIFICATE

I**, Reema George,** hereby certify that I had personally carried out the work depicted in the thesis entitled, **"Cellular and molecular influences on pro-thrombotic and pro-inflammatory states in young patients with coronary artery disease"** under the supervision of **Dr. S. Harikrishnan,** except where external help was sought and acknowledged. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

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This is to certify that Reema George, in the Department of Cardiology of this Institute, has fulfilled the requirements of the regulations relating to the nature and prescribed period of research for the PhD degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum. The study entitled **"Cellular and molecular influences on pro-thrombotic and pro-inflammatory states in young patients with coronary artery disease"** was carried out under my direct supervision. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date. Clearance was obtained from the Institutional Ethics Committee for carrying out the study.

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ACS	Acute coronary syndrome
AGE	Advanced glycation end product
AMI	Acute myocardial infarction
apo(a)	Apo lipoprotein(a)
Apo A1	Apo lipoprotein A1
Apo B	Apo lipoprotein B
AT-III	Antithrombin-III
Atg gene	AuTophaGy related gene
CABG	Coronary artery bypass graft surgery
CAD	Coronary artery disease
CAG	Coronary angiogram
CAM	Cell adhesion molecule
CCL2	CC motif chemokine ligand 2
CCL5	CC motif chemokine ligand 5
CCR	CC chemokine receptor
CCR2	C-C chemokine receptor type 2
CCR5	C-C chemokine receptor type 5
CD14	Cluster of differentiation 14
CD 14 FITC	CD14 antibody conjugated with fluorescent isothiocyanite
CD16	Cluster of differentiation 16
CD16 PE	CD16 antibody conjugated with phycoerythrin

CD45	Cluster of differentiation 45
CD62	Cluster of differentiation 62
CD62 p	Cluster of differentiation 62-platelets
CD 62p PE	CD62 p antibody conjugated with phycoerythrin
CD62 L	Cluster of differentiation 62-leukocytes
CETP	Cholesterol ester transport protein
CHD	Coronary Heart disease
CHOP	C/EBP homologous protein expression
CK	Cytokines
CK-MB	Creatine kinase-muscle brain
CM	Classical Monocytes
COX-1	Cycloxygenase-1
COX-2	Cycloxygenase-2
C-RP	Cross-reactive protein
CVD	Cardiovascular diseases
CX_3CR1	CX_3C chemokine receptor 1
DALY	Disability adjusted life years
DVD	Double vessel disease
EA	Effort angina
EBCT	Electron beam computerized tomography
ECG	Electro cardiogram
ECM	Extracellular matrix
ELISA	Enzyme linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase

ESM	Esterase-sensitive chemical motif
ESI	Electro spray ionization
FACS	Fluorescence activated cell sorting
FCT	Fibrous cap thickness
FcγIII IgG receptors	Functional class III receptor of immunoglobulin G
FGF-2	Fibroblast growth factor 2
FSC	Forward Scatter
FITC	Fluorescent isothiocyanite
GMCSF	Granulocyte Macrophage Colony Stimulating Factor
hCE-1	Human carboxyl esterase-1
Hcy	Homocysteine
HDAC	Histone deacetylase
HDL	High density lipoprotein
HDL-C	High density lipoprotein cholesterol
HMG-CoA reductase	3- hydroxyl-3-methyl glutaryl Co-enzyme A reductase
HLA-DR	Human Leukocyte Antigen - antigen D Related
HRP	Horse-radish peroxidase
hs-CRP	High-sensitivity cross reactive protein
HSP60	Heat shock protein 60
ICAM	Intracellular adhesion molecule
IFN-γ	Interferon- Gamma
IL-1	Interleukin-1
IL-2	Interleukin-2

IL-8	Interleukin-8
IL-10	Interleukin-10
IL-16	Interleukin-16
IL-1-β	Interleukin-1-beta
IL-6	Interleukin-6
IMPase	Inositol mono phosphatase
IP3	Inositol tri phosphate
iNOS	Induced nitric oxide synthase
JAM	Junctional adhesion molecule
LC-MS/MS	Liquid chromatography- tandem mass spectrometry
LAD	Left anterior descending artery
LFA-1	Lymphocyte function associated antigen-1
LCX	Left circumflex artery
LDL	Low density lipoprotein
LDL-C	Low density lipoprotein cholesterol
LMCA	Left main coronary artery
Lp(a)	Lipoprotein (a)
LPS	Lipopolysaccharide
Mac-1	Macrophage-1 antigen
MAP kinase	Mitogen activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
MI	Myocardial infarction
MMPs	Matrix metalloproteinases
MPO	Myeloperoxidase

mRNA	messenger RNA
mTOR	Mechanistic Target of Rapamycin inhibitor
MVD	Multi-vessel disease
МΦ	Macrophage
MCSF	Macrophage colony stimulating factor
MHC	Major histocompactabilty complex
NCD	Non-communicable disease
NCM	Non-Classical Monocytes
NFκB	Nuclear factor kappa B
NO	Nitric oxides
NSTEMI	Non ST segment elevated myocardial infarction
OH^-	hydroxyl radical
OCT	Optical coherence tomography
Ox. LDL	Oxidised LDL
PCSK 9	Proprotein convertase subtilisin kexin 9
PAI-1	Plasminogen activator inhibitor-1
PAR	Protease activating receptors
PBMNC	Peripheral Blood Mononuclear cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
PECAM-1	Platelet endothelial cell adhesion molecule1
PDGF	Platelet derived growth factor
PCI	Percutaneous coronary intervention

PF-4	Platelet factor 4
PPAR	Peroxisome proliferative active receptor
PSGL-1	P-selectin glycoprotein ligand 1
PTCA	Percutaneous trans coronary angioplasty
RANTES	Regulated on activation normal T-cell expressed and secreted
RCA	Right coronary artery
ROS	Reactive oxygen species
sCD40L	Soluble CD 40 ligand
SMC	Smooth muscle cell
SSC	Side scatter
STEMI	ST segment elevated myocardial infarction
SVD	Single vessel disease
TGFβ	Transforming growth factor beta
TF	Tissue factor
Th Cells	T helper cells
TLR	Toll like receptor
TMB	Tetramethyl benzidine
TGF-β	Tumor growth Factor-β
TNF-α	Tumour necrosis factor-alpha
TNF	Tumour necrosis factor
t-PA	Tissue-plasminogen activator
TRAIL	TNF-Related Apoptosis Inducing Ligand
TREM-1	Triggering receptor expressed on myeloid cells-1
TVD	Three or triple vessel disease

UA	Unstable angina
u-PA	Urokinase plasminogen activator
VCAM- 1	Vascular-cell adhesion molecule-1
VLA-4	Very late antigen-4
VLDL-C	Very low density lipoprotein cholesterol
v-WF	von-Willebrand factor
WBC	White blood cells/corpuscles
WHO	World Health Organization

SYNOPSIS

Coronary artery disease (CAD) is a worldwide health epidemic. Over 80% of cardiovascular disease (CVD) related deaths take place in developing countries, and occur almost equally in men and women. Indians have the highest risk rates for CAD among all ethnic groups at young ages. In the majority of the cases CAD occur associated with conventional risk factors (such as age, sex, family history, diabetes mellitus, hypertension and dyslipidemia), but in many young patients, CAD occur without any marked conventional risk factors. Thus, there is an increase in interest in the identification of new cardiovascular biomarkers, especially thrombotic and inflammatory markers that have the potential to improve risk assessment for CAD.

The major cause of CAD is atherosclerosis, which is a chronic inflammatory and fibro-proliferative disease of the vessel wall, where thrombotic factors play a major role in the progression of the disease. There is ample proof that monocytes, the key inflammatory cells, involved are heterogeneous population of cells which are segregating into different phenotypes and play different roles in the propagation of atherosclerosis leading to CAD. Thus whether they represent biomarkers or therapeutic targets is an important question.

No in-depth studies have been done on the role of thrombotic and inflammatory factors in the progression of CAD, especially in patients on treatment, as major coronary events recur in patients undergoing treatment. Thus it was hypothesized that *identification of thrombotic and inflammatory risk factors along with conventional risk factors, would improve risk assessment for CAD especially in young population*. So the

current study was mainly focused on to create a database of young CAD patients and to evaluate important thrombotic inflammatory risk factors in young CAD patients on treatment in our hospital in comparison to healthy controls.

The major objectives of the study were-

1. To develop a database of young CAD patients (≤55 years) with angiographically proven CAD based on conventional risk factor profile, mode of presentation and angiographic profile.

2. To assay thrombotic, inflammatory risk factors and platelet activation along with conventional risk factors.

3. To identify monocyte phenotypes in peripheral blood by flow cytometry and their relation to CAD.

4. To assess whether there is a relation between inflammatory markers and monocyte phenotypes

To develop database of 5467 CAD patients (≤ 55 years of age), who were treated at SCTIMST from 2000-2010, details regarding mode of presentation of CAD, risk factor profile, angiographic profile and follow up record were collected. Further, detailed thrombotic and inflammatory risk factors were analysed in a sub- group of 209 CAD patients who were admitted consecutively during the period 2011-2015. Controls consisted of 160 healthy volunteers, who were asymptomatic for CAD and recruited during the same time as patients. Blood levels of Fibrinogen, platelet activation [based

on p-selectin exposure], lipoprotein(a) [lp(a)], antithrombin-III [AT-III], plasminogen activator inhibitor-1 [PAI-1], tissue-plasminogen activator [t-PA], von-Willebrand factor [v-WF], Protein C and homocysteine were quantitated as major thrombotic factors. In addition, the two important monocyte phenotypes in blood- classical monocytes ($CD14^{++}CD16^{-}$ or $CD14/CD16^{-}$ monocytes) and non-classical monocytes ($CD14^{+}CD16^{+}$ or CD16 monocytes) were characterized by flow cytometry and specific gating strategy. Inflammatory markers including tumor necrosis factor-alpha (TNF-α), Interleukin-1-beta (IL-1β), and myeloperoxidase (MPO) activity were assessed to define whether there is a relation between monocyte phenotypes and inflammatory markers.

The Data-base generated from 5467 young CAD patients [male 87.7%; female 12.3%] showed that females had more risk factors for CAD, particularly hypertension, dyslipidemia and diabetes mellitus; compared to male patients. Mode of presentations of CAD, angiographic findings, mode of treatment, follow up and final outcome were different for both sexes. Among males, dyslipidemia as low HDL-C and smoking emerged as major risk factors and they had more severe form of disease [multiple vessel disease] compared to females. Majority of females had single vessel disease. These findings indicate that there is a need for intensive primary prevention to prevent early occurrence of risk factors like smoking and dyslipidemia. There should be efforts for early detection along with meticulous control of risk factors.

Thrombotic and inflammatory risk factors in CAD revealed that patients who were on treatment with anti-atherosclerotic drugs, (also on fat restricted diet) showed

improved lipid profile (normal levels of total cholesterol, LDL-C and triglycerides) compared to controls. However, they had low level of HDL-C as the only form of dyslipidemia. Thrombotic factors including fibrinogen, platelet activation and Lp(a) were significantly elevated in patients, while antithrombotic factor, AT-III and anti-fibrinolytic factor PAI-1 were significantly lower in patients compared to controls. No significant difference was observed in the levels of other thrombotic factors. An important observation was a significant negative correlation between platelet activation and AT-III, indicating that these factors act antagonistically in thrombosis and inflammation as reported by earlier studies. Patients admitted with acute coronary syndrome (ACS) were found to have more thrombotic risk factors (low ATIII, high platelet activation, lipoprotein(a) and fibrinogen) than patients with effort angina (EA). Similarly, based on coronary angiogram findings, a trend towards increased prevalence of thrombotic risk factors were seen in patients having more severe CAD (including double vessel disease and triple/multi vessel disease) compared to mild CAD (normal CAD and single vessel disease). Event rate of CAD (consisting of mortality, admission with ACS, need for repeated revascularization and other cardiovascular conditions such as stroke) in patients were also lower (6%), with two deaths, one ACS, four requiring CABG and three patients requiring repeated angiography, on a median follow up of 26 months. These results suggest that treatment for CAD might have lowered major risk factors and probably occurrence of adverse events in patients as reported by earlier studies. But the current treatment seems to be not effective in reducing the level of Lp(a), platelet activation, fibrinogen and/or elevating AT-III and raising HDL-C.

Characterization of blood Monocyte phenotypes demonstrated a trend towards an increased percentage of non-classical monocytes in CAD patients compared to controls. Moreover when these patients were classified based on the presentation of recent coronary artery events, as ACS and EA, a remarkable increase in non-classical monocytes was observed in ACS patients than that of CAD with EA. In terms of disease severity an inverse trend was observed as patients with less severe disease were presented with more recent ACS. Generally Classical monocytes are phagocytic monocytes which are recruited initially during the process of atherosclerosis, while non-classical monocytes are more mature monocytes involved in the production of more pro-inflammatory cytokines and also showed homing behavior and enhanced affinity to endothelium. In view of these, the observed increase in the non-classical monocytes was indicative of the inflammatory state and its association with ACS.

Analysis of inflammatory markers clearly showed a significant increase in IL-1β and myeloperoxidase in patients, with no difference in the level of TNF-α in comparison to controls. None of these inflammatory markers showed any correlation with monocyte phenotypes. Different inflammatory factors may be activated at different phases of the disease. This could be the reason for non-significant levels of TNF-α compared to other inflammatory factors. However, fibrinogen levels in CAD patients showed positive correlation ($r=0.5$, $p=0.0006$) with total $CD14^+$ monocyte count.

The study provide evidence that there is cross-talk between conventional (low HDL-C, diabetes mellitus, smoking and hypertension) and thrombotic risk factors [low AT-III, Lp(a), platelet activation and fibrinogen]. These factors could be a trigger for

monocyte recruitment (from bone marrow) and activation to a mature phenotype (non classical monocytes) in the peripheral blood itself. As the non-classical monocyte phenotypes are known to be activated phenotypes, its association with ACS might be indicating their association with myocardial recovery, which is always a part of ACS. So future strategies, aiming at lowering the levels of thrombotic and inflammatory risk factors (in addition to control of conventional risk factors) may improve the outcome of the disease.

The study is significant as it has made an effort to create one of the largest data-base of young CAD patients in India. To the best of our knowledge, the combined analyses of thrombotic, inflammatory risk factors along with monocyte phenotypes in young patients, was done for the first time in the country. This also seems to be one of the first reports from the region, on monocyte phenotype characterization in CAD patients based on mode of presentation of CAD symptoms.

The future implications of the study points to the initiation of more research on the combined role of thrombotic and inflammatory risk factors in CAD. More exploration is needed with regard to the role of thrombotic factors such as Lp(a), fibrinogen and AT-III in monocyte recruitment and activation. There is also a need to correlate monocyte phenotype and plaque vulnerability (using methods like optical coherence tomography) in view of association of non-classical monocytes with CAD, based on presentation of symptoms. Moreover platelet monocyte interactions and platelet-monocyte microparticles in circulation may be studied as potential biomarkers for CAD.

I. INTRODUCTION

Coronary artery disease [CAD] is now described as a worldwide health epidemic. About 7.3 million people die due to CAD every year. Over 80% of these deaths take place in developing countries and occur almost equally in men and women. This is creating a huge economic burden to these countries as the disability adjusted life years [DALYs] lost affects the productivity as the affected population is much younger.

The costly treatments push most of the poor patients into poverty as they had to resort to distress financing and catastrophic health spending. The number of people who die from cardiovascular diseases/CVD is projected to reach 23.3 million by 2030. When deaths due to CVD [one of the major component being CAD] have shown a declining trend in the Western Countries, its prevalence in developing countries is increasing. The phenomenon is not different in India. Out of the 53% death attributable to non-communicable disease/NCD [excluding death due to injury], 24% of the deaths were related to CVD (WHO, 2015).

Indians have the highest risk rates for CAD among all ethnic groups (Joshi *et al.*, 2007) They also have very high prevalence of risk factors for CAD at young ages (Panwar *et al.*, 2011). CAD occurs in Indians 10 years earlier than their western counterparts with incidence of acute coronary syndrome/ ACS being higher and more in young patients.

Factors responsible for premature CAD in Indian subjects could be multiple, ranging from economic, social, psychological, lifestyle [smoking, sedentary habits, improper diet] and biochemical factors [abnormal lipids, hypertension, diabetes, obesity] (Gupta *et al.*, 2008), along with recently reported thrombotic (Panwar *et al.*, 2011) and inflammatory risk factors.

Within India the southern states are having a higher prevalence of CAD and conventional CAD risk factors such as tobacco use, hypertension, diabetes mellitus, dyslipidaemia and family history of premature CAD (Thankappan *et al.*, 2010).

Prevalence rate of CAD in the south Indian state of Kerala is reported to be 11% and 7% in urban and rural population, respectively (Zachariah *et al.*, 2013). However, a recent report (Krishnan *et al.*, 2016) has pointed out that there is no difference in prevalence rate of definite CAD in the urban and rural population of Kerala. Thus there is a need for population level and individual level approach to reduce CAD risk factors and prevalence. The CAD prevalence in Kerala is comparable to industrialized countries and age adjusted CAD prevalence was 0.9% for definite CAD in patients below 45 years (Krishnan *et al.*, 2016). The total cholesterol is also very high compared to national average (Kerala Health Statistics, 2010)

In view of the above facts, it is evident that India, especially southern states like Kerala is facing an epidemic of premature CAD. So more data regarding causative factors, clinical profile and treatment patterns are highly relevant in the population. Currently the data is very limited, especially from the younger population.

Thus, this study made an effort to formulate a data base of young patients with angiographically proven CAD; documenting their risk factor profile, mode of presentation, angiographical profile, and follow up data. A sub-group of this cohort underwent a detailed analysis of non-conventional risk factors [thrombotic and inflammatory risk factors along with monocyte phenotype characterization] and the data was compared with healthy matched controls.

1.1 CAD and Atherosclerosis

Atherosclerosis is the major cause of CAD. Word atherosclerosis is derived from Greek 'athero' meaning gruel and 'sclerosis' meaning thickening. Atherosclerotic plaques can progressively narrow coronary artery lumen and may damage ante grade blood flow. The patient may be symptomatic or asymptomatic depending on the size and nature of the plaque. The plaque may also become unstable by plaque rupture or erosion developing ACS.

Earlier atheroma [thickening of artery wall] formation was attributed to lipid deposition, but now there are evidence of involvement of thrombotic, inflammatory and cellular components in the progression of atherosclerosis (Libby, 2002).

In majority of the cases, CAD can be attributed to conventional risk factors (Yusuf *et al.*, 2004). But in significant number of cases, especially in the younger age group, CAD occurs without any conventional risk factors (Kassam & Stewart, 2001) .

Atherosclerosis is now described as a multi-factorial progressive disease that is influenced by inflammation at every step from beginning to progression, and that each of the risk factors contribute to pathogenesis by aggravating the underlying process of inflammation (Mallika et.al., 2007). So the progression of CAD needs to be understood on the basis of inflammatory pathways.

The atherosclerotic and the intravascular thrombotic processes appear somewhat interdependent and may therefore be able to be integrated under the term 'athero-thrombosis'. It has been established that there is bi-directional relation between inflammation and thrombotic factors in the pathogenesis of atherosclerotic CAD (Corti *et al.*, 2004) (Levi, 2004).

Thus in this study we are investigating the relationship between inflammation and thrombosis by assessing various thrombotic and inflammatory factors in young patients with CAD.

1.2 Risk factors of CAD

The major risk factors for CAD are mainly based on the pathogenesis of CAD as was observed in earlier studies. Presence of diabetes, smoking, dyslipidaemia, male gender and family history of premature CAD were found to be significantly associated with CAD and are referred to as conventional risk factors (O'Donnell & Elosua, 2008).

With the new evidences about involvement of inflammation and thrombosis in atherosclerosis, "inflammation theory of atherosclerosis"(Libby, 2002) (Libby *et al.*,

2010) and the concept of Virchow's triad involving thrombosis (Bagot & Arya, 2008) is given importance as non-conventional factors responsible for the pathogenesis of CAD.

In atherosclerotic CAD, inflammation and thrombosis are intertwined (Krychtiuk *et al.*, 2013). Chronic inflammation may lead to immune response and production of inflammatory cytokines. Pre-existing prothrombotic factors can also enhance inflammatory cytokine production. All these can promote endothelial dysfunction and eventually platelet activation. These processes can ultimately trigger monocyte recruitment and activation, which could also enhance the production of prothrombotic factors.

Inflammatory mechanisms involving fatty streak and foam cell formation could activate intrinsic and extrinsic coagulation pathway with thrombin activation and excess fibrinogen production. These effects result in activation of other coagulation factors making endothelial surface thrombogenic and inflammatory, along with inhibition of antithrombotic factors. There is also a release of protease activating receptors [PARs]. This could contribute to mobilization of P-selectin, excessive chemokine production, expression of endothelial adhesion molecules, expression of leukotrienes, production of prostaglandins, platelet activating factors, nitric oxide/ NO, induction of cycloxygenase-2/ COX-2 and endothelial shape change (Levi, 2004) (Davì & Patrono, 2007). The flow chart below represents a summary of important mechanism acting in the progression of atherosclerotic CAD [Figure 1]

Figure 1: Flow chart showing important events in the progression of atherosclerotic CAD involving thrombosis and inflammation [AGE: advanced glycation end product; ROS: Reactive oxygen species; SMC: Smooth muscle cells; CAMs: Cell adhesion molecules; CKs: cytokines, MΦ: Macrophages; MMPs: Matrix metalloproteinases; MPO: Myeloperoxidase; TF: Tissue factor; ECM: Extracellular matrix]

1.2.1 Thrombotic and inflammatory risk factors in CAD

Inflammation can trigger and perpetuate thrombosis which can lead to the presentation of the patients as ACS. Many of the thrombotic risk factors are also important inflammatory activators. There are many thrombotic markers implicated in CAD. The important thrombotic-inflammatory risk factors included in the present study are fibrinogen, lipoprotein (a) [Lp(a)], homocysteine [Hcy], antithrombin-III [AT-III],

protein C, von-Willebrand factor [v-WF], tissue-plasminogen activator [t-PA], plasminogen activator inhibitor-1 [PAI-1] and high-sensitivity cross reactive protein [hs-CRP].

1.2.2 Platelet activation and CAD

Platelets play an important role in the pathogenesis of CAD. For the platelet to be functional it has to be activated. During platelet activation, it undergoes shape change, become irregular, sticky and exposes granules to the surface releasing growth factors and releasing surface markers like P-selectin. This activation process is triggered by inflammatory and thrombotic processes which also play a role in atherosclerosis. In general, a bidirectional inflammatory interaction exists between platelets, leukocytes and endothelial cells, promoted by thrombotic factors in the progression of CAD (Aukrust *et al.*, 2010). Variety of platelet activation markers is present. In the present study P-selectin expressed by platelets are used as a marker for CAD. The most potential function of platelet is monocyte recruitment. The figure 2 below represents the involvement of platelet in atherothrombotic process. Activated platelets release inflammatory, mitogenic substances into the microenvironment. There is time-dependent synthesis of protein mediators [tissue factor/TF and interleukin-1-β/IL-1-β] primarily altering the adhesive, chemotactic and proteolytic properties of the endothelium. P-selectin released by platelets bind to the monocyte receptor, P-selectin glycoprotein ligand 1 [PSGL-1], enhancing the monocyte adhesion to endothelial adhesion molecules like vascular-cell adhesion molecule [VCAM]- 1 expressed on activated endothelial cells. These processes can induce the production of TF by monocytes (Davì & Patrono 2007).

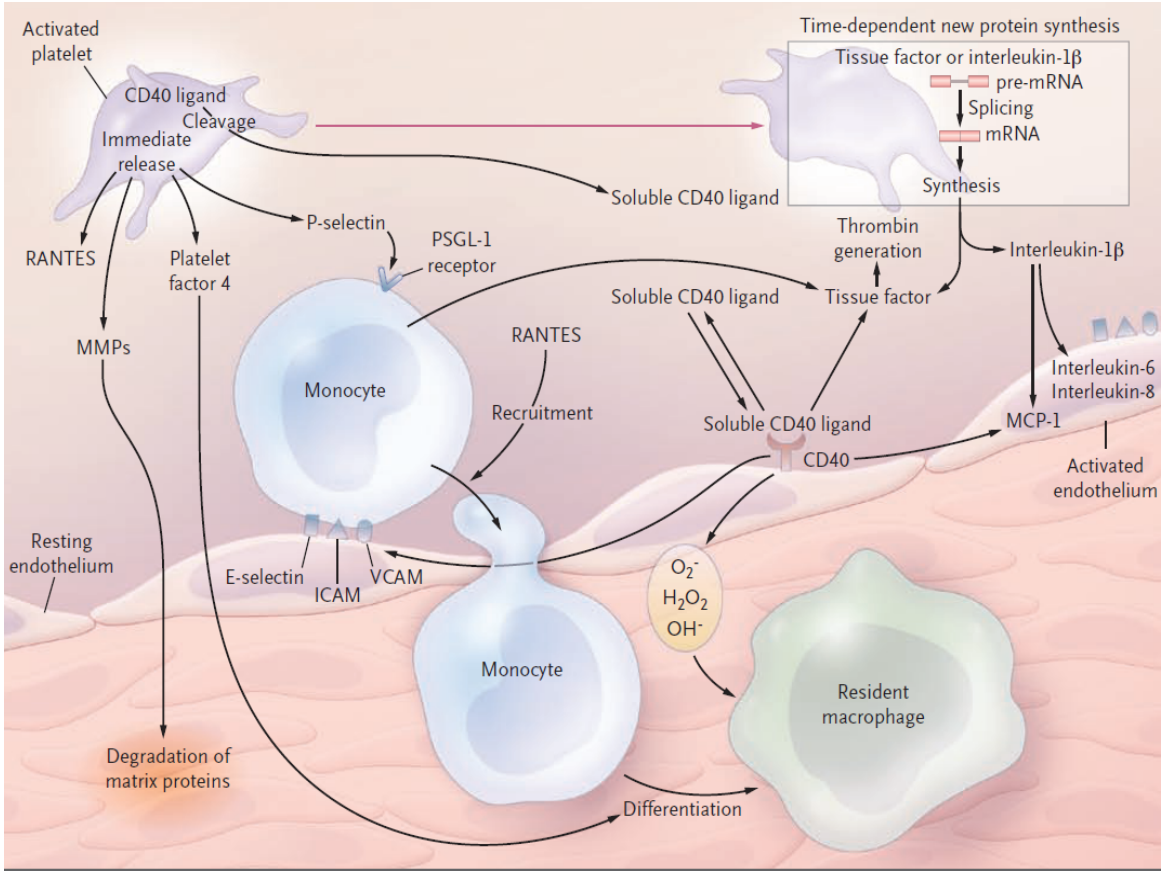


Figure 2: Platelet involvement in thrombotic and inflammatory processes in the progression of CAD [P-selectin glycoprotein ligand 1 (PSGL-1); vascular-cell adhesion molecule (VCAM), regulated on activation normal T-cell expressed and secreted (RANTES)., platelet factor 4 (PF-4), matrix metalloproteinase (MMP 2 or 9). intracellular adhesion molecule (ICAM), messenger RNA (mRNA), monocyte chemoattractant protein 1 (MCP-1), and hydroxyl radical (OH^-)].“Reproduced with permission from (Davì and Patrono, 2007), Copyright Massachusetts Medical Society.

1.2.3 Monocyte phenotypes and their influence in disease state

Leukocytes plays important role in atherosclerotic progression and leukocytosis is a common reaction during CAD. Monocytes are principle leukocytes involved in atherosclerosis. They are the inflammatory cells recruited at the endothelium by the chemokines during the initial phases of lipid deposition and atherogenesis and gets activated in the sub endothelium. Subsequently they transform into macrophages [MΦs]

and engulf oxidised lipids, forming foam cells (Woollard & Geissmann, 2010). Activated monocytes are also potent initiator of thrombosis as described earlier (Davì & Patrono, 2007). Monocytes are also involved in the production of matrix metalloproteinases [MMPs] involved in plaque destabilization (Newby, 2008) and also produce myeloperoxidase/MPO (Tavora *et al.*, 2009).

The earlier concept regarding monocyte is that they are homogeneous cells (Stansfield & Ingram, 2015), but research in the last 20-30 years have proved that they are heterogeneous in nature with different subsets based on the expression of surface markers as well as their function as evident in variety of inflammatory processes (Geissmann *et al.*, 2003).

Monocytes consist of at least two subsets / phenotypes (Passlick *et al.*, 1989) whose proportion in blood fluctuates in response to diseases like CAD (Tsujioka *et al.*, 2009) and other inflammatory diseases (Stansfield and Ingram, 2015).

Specific shift in monocyte subsets can influence certain disease states. Thus selective & rapid enumeration of monocytes & their subsets/ phenotypes has the potential to develop as new biomarkers for the identification of different disease conditions (Wildgruber *et al.*, 2009).

Monocytes are also involved in initiation of atherosclerosis, plaque destabilization and may play important roles in heart remodelling: Figure 3 (Ghattas *et al.*, 2013).

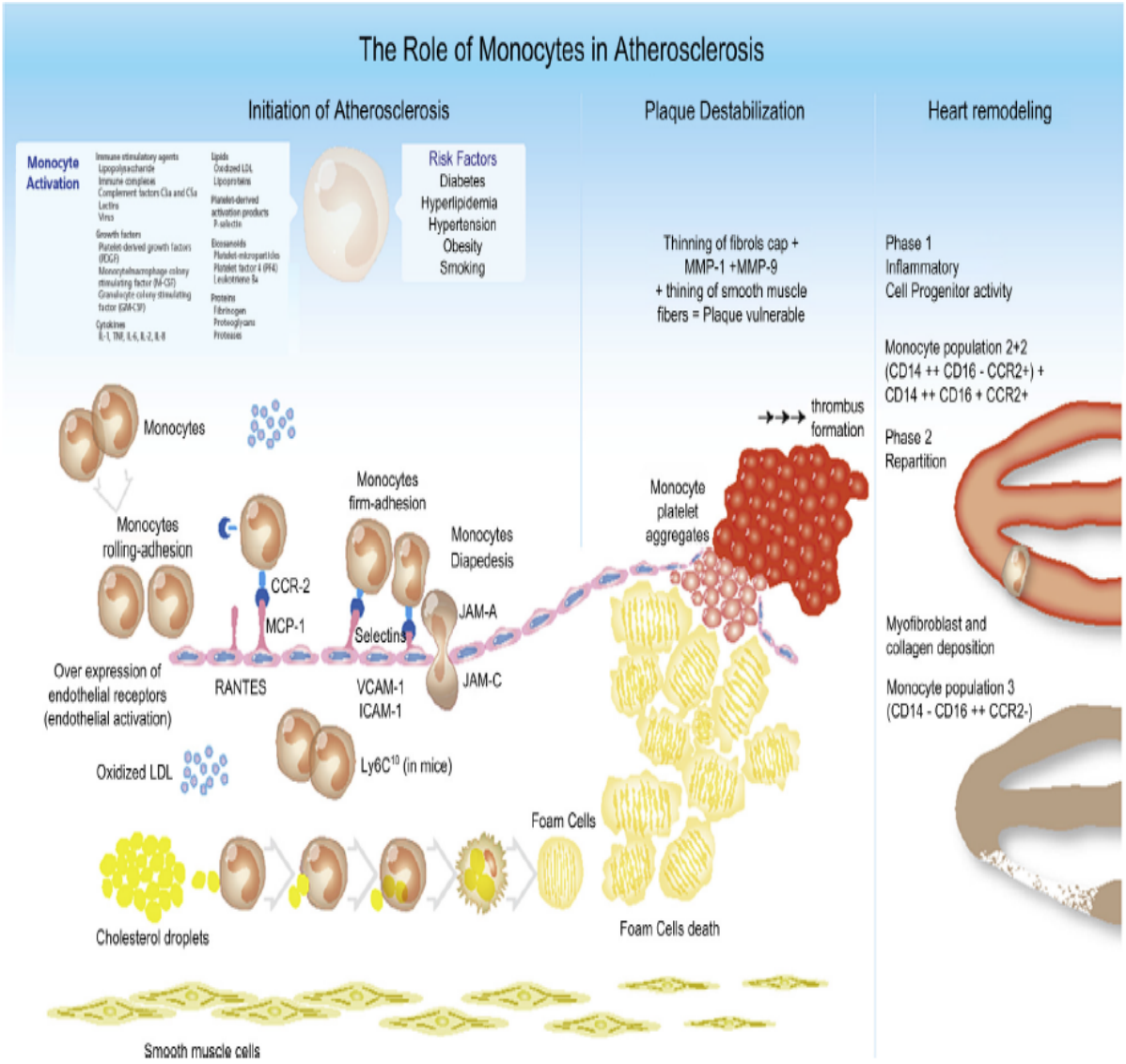


Figure 3: Monocyte function in atherosclerosis. Monocytes patrolling in the blood are activated by different factors. They traffic to the damaged/activated endothelium. The dysfunctional endothelium over-express MCP-1, VCAM-1and ICAM-1 on its surface. After rolling and attachment to the endothelium, the monocytes cross the endothelial surface by diapedesis. Monocytes are involved in intiation, plaque destabilization and heart remodeling during atherosclerosis.CCR: CC chemokine receptor; JAM: junctional adhesion molecule; **Adapted from** (Ghattas *et al.*, 2013)**[** ' *Journal of the American College of Cardiology*, 62: 1541–51. doi: 10.1016/j.jacc.2013.07.043.]

In the study monocyte subsets were characterized based on the expression of CD14, a lipopolysaccharide /LPS receptor and CD16, a functional class III receptor of

immunoglobulin G/ FcγIII receptors of Ig G as classical monocytes [CM] with higher expression of CD14 and absence of expression of CD16 [$CD14^{++}CD16^{-}$] and non-classical monocytes [NCM] with expression of both CD14 and CD16 [$CD14^{+}CD16^{+}$] based on the earlier classification (Passlick *et al.,* 1989).

Different subsets of monocytes, based on their phenotypic characteristics, secrete pro and anti-inflammatory factors which may also depend on the physiological conditions. In the study, monocyte phenotypes were correlated with the expression of inflammatory factors such as tumour necrosis factor-alpha [TNF-α], IL-1-β and MPO activity in the peripheral blood along with WBC count and thrombotic factor fibrinogen.

The thesis made an effort to develop a data on young angiographically proven CAD patients, one of the largest data bases from the region [Part –I of the study]. So far, to the best of our knowledge, combined thrombotic inflammatory risk factor analysis in relation to conventional risk factor with angiographically proven CAD patients is one of the first endeavours from the region, which correlated conventional and non-conventional risk factors with mode of presentation and severity of the disease [Part II-case-control study 1].

Phenotypic characterisation of monocytes and correlation with inflammatory markers was a novel attempt made in identifying the monocyte functionality in the population as there is no clear consensus regarding exact role of these monocyte phenotypes in disease as well as at basal level [Part II –case –control study 2].

There could be extensive cross-talk between conventional and non-conventional risk factors in the progression of CAD as evidenced by many studies. On the basis of this we have designed our study hypothesis and objectives as described below. The evidence obtained from the study shall be contributing to the improved risk assessment of young CAD patients and may open new avenues to improve the efficacy of current treatment methods.

The details of disease and current knowledge in the field are described in Review of Literature [Chapter 2]. Design and methods adopted for the study is explained in Materials and Methods section [Chapter 3]. The study results [Chapter 4] are critically examined and discussed [Chapter 5: Discussion] and clear cut summary and conclusion are provided [Chapter 6].

Hypothesis

“Identification of thrombotic and inflammatory risk factors along with conventional risk factors improve risk assessment of CAD in young population”

Objectives of the study

- To develop a database of young patients (≤55 years) with angiographically proven CAD based on conventional risk factor profile, mode of presentation and angiographic profile.
- To assay thrombotic, inflammatory risk factors and platelet activation in selected young patients with angiographically proven CAD compared to healthy controls and to correlate with conventional risk factor levels.
- To identify monocyte phenotypes in peripheral blood by flow cytometry in patients with CAD and to compare with controls.
- To assess whether there is a relation between inflammatory markers and monocyte phenotypes in peripheral blood of patients by comparing with controls.

II. REVIEW OF LITERATURE

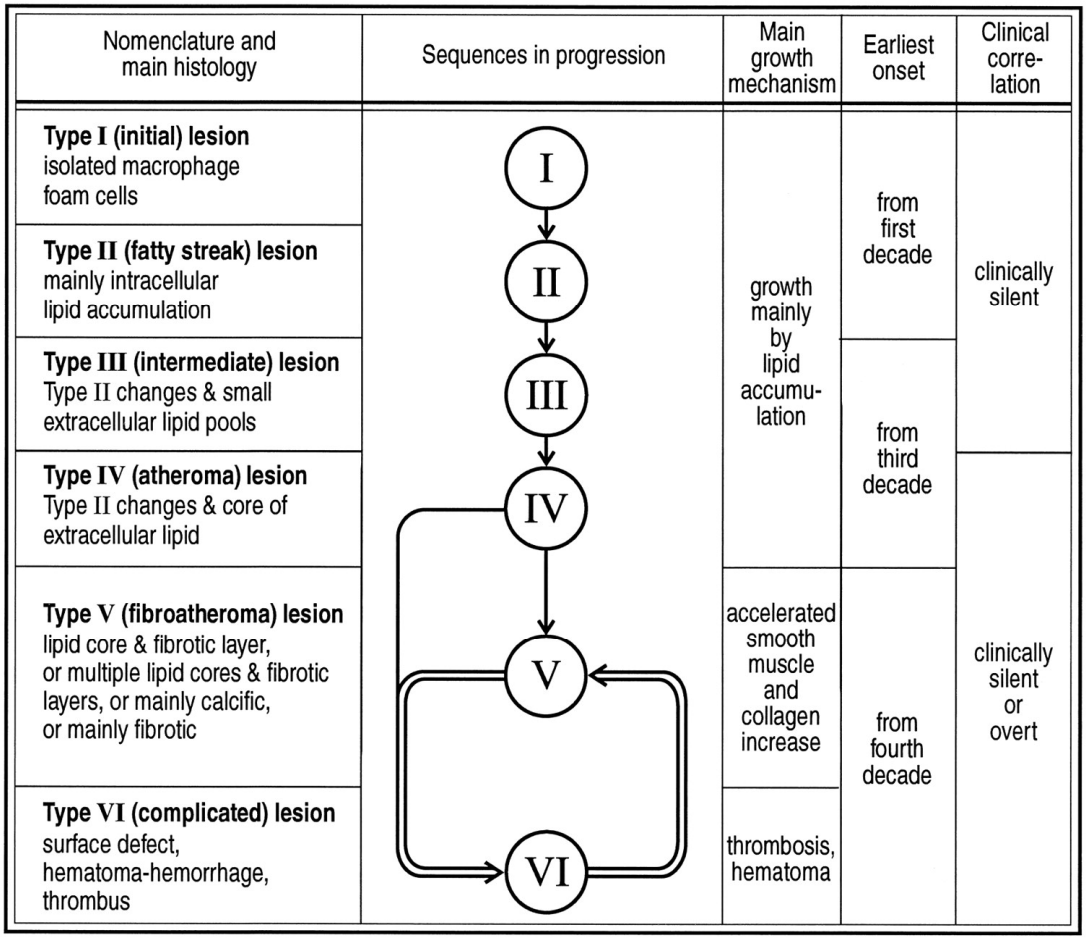
Atherosclerotic CAD is presently considered as a multi factorial disease manifested by the focal development of lesions within the arterial wall known as atherosclerotic plaques. This is mainly in response to various deleterious effects on the arterial wall. It could be due to dyslipidaemia, vasoconstrictor hormones, hypertension, hyperglycaemia (and associated manifestations) or pro-inflammatory cytokines. Inflammation and thrombosis play a major role in the progression of atherosclerosis finally leading to plaque rupture (Libby *et al.* , 2009).

2.1 Different stages of atherosclerotic lesions

Atherosclerosis starts as initial fatty streak formation which progresses to fibro-lipid plaque and ultimately can end-up in plaque rupture and athero-thrombosis. The initial fatty streak formation starts to appear even during infancy, although major manifestation like atheroma formation starts to be visible only during third or fourth decade of life.

Atherosclerotic lesions are classified into various stages based on the appearance of arterial wall and cellular changes. They are classified as six types based on the clinical guidelines put forward by American Heart Association as Type I to Type VI. Table 1 provide summary of classification of different types of atherosclerotic lesion

Table 1: Classification of atherosclerotic lesion: Main histology and sequence of events. Adapted from (Stary *et al.*, 1995) **[***Circulation* 92:1355–174. doi: 10.1161/01.CIR.92.5.1355].



[Flow diagram in centre column indicates pathways in evolution and progression of human atherosclerotic lesions. The direction of arrows indicates sequence in which characteristic morphologies may change. From type I to type IV, changes in lesion morphology occur primarily because of increasing accumulation of lipid. The loop between types V and VI illustrates how lesions increase in thickness when thrombotic deposits form on their surfaces. Thrombotic deposits may form repeatedly over varied time spans in the same location and may be the principal mechanism for gradual occlusion of medium-sized arteries].

During atherosclerotic lesion formation, gross changes occur in the morphology of a normal coronary artery [Figure 4]. Coronary artery consists of three layers: Tunica intima, Tunica media and Tunica adventitia. The intima consists of a monolayer of epithelial like

cells known as endothelial cells, which is in constant contact with blood and its components.

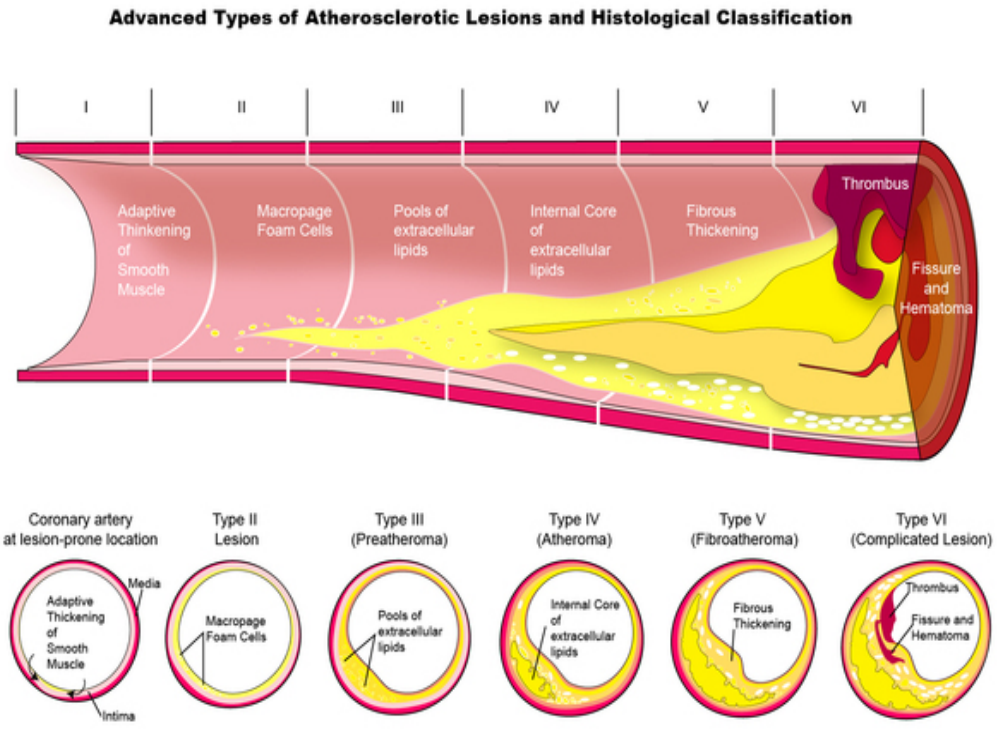


Figure 4: Advanced types of atherosclerotic lesion and histological classification. Adapted from Riley (2011)[*https://www.behance.net/gallery/181439/ Advanced-Atherosclerotic –Lesions- and –Classifications]*

Endothelial cells rest on the basal lamina, which they themselves produce. In humans, the basal lamina consists of scattered resident smooth muscle cells also. The internal elastic lamina separates media from the intima. Media consists mainly of numerous layers of smooth muscle cells [SMCs]. External elastic lamina separates media and the third layer adventitia. Adventitia is made of fibroblasts, mast cells, microvessels, lymphatic vessels and nerves. All these are housed within the extra cellular matrix. Endothelial cells and SMC are resident cells of the arterial wall. They, along with cells emigrated from the

blood like T-lymphocytes, monocytes, dendritic cells and mast cells secrete many products like chemokines, cytokines, and enzymes which plays a major role in atherosclerosis. These substances lead to many processes which can contribute to initiation, evolution and decide on the fate of atherosclerotic plaque.

2.1.1 Anatomy of Coronary Arteries

Coronary arteries supply blood to heart muscle. Then run along the outside of the heart and have small branches. The two main coronary arteries are the **left main coronary artery (LMCA)** and **right coronary artery (RCA).** They come off the ascending aorta at the aortic sinuses. The left main coronary artery is divided into a) the **left anterior descending artery (LAD)**-descends along the anterior interventricular sulcus. b) **The circumflex artery (LCX)** curving to left in coronary sulcus branches off from the left coronary artery and encircles the heart in the atrio-ventricular groove. The artery supplies blood to the lateral side and back of the heart.

RCA supplies blood to right ventricle, right atrium and the posterior interventriclar septum. The RCA divides into smaller branches including the **right posterior descending artery** and the **acute marginal artery** (Coronary Heart Disease, 2016).

CAD can be assessed by coronary angiograms based on the extend of thrombosis/stenosis or spasm. CAD is reported when the stenosis exceeds 50% and when it reaches >70% or when there is >50% stenosis of LMCA, it is considered as significant CAD.

A study show that young (age <40 years) patients who had angiographic evaluation had less extensive CAD, with a high incidence of angiographically normal vessels and relative paucity of LMCA involvement compared to older patients who are reported to have greater increase in multi-vessel disease [MVD] (Khan *et al.*, 2014). Similar trend was again seen when women from South India was compared based on age. Young women had a higher incidence of normal coronaries with non significant lesions (Ezhumalai & Jayaraman, 2014).

2.2 Cardiovascular disease/CAD-Prevalence and risk factors

According to the World Health Organization [WHO], globally more people die annually from CVD than any other disease.CAD is one of the major forms of CVD. It is estimated that in 2012, 17.5 million people died from CVD, about 31% of the total death in the world. Of these 7.4 million deaths were due to coronary heart disease/ CHD and 6.7 million deaths were due to stroke.

Over three fourth of these deaths, take place in low and middle income countries. 37% of non communicable disease mortality is due to CVD in these countries. Economic transition, urbanization, industrialization and globalization promote life style changes in these countries leading to the increased prevalence of CVD.

Over the years life expectancy has increased, which naturally can increase the prevalence of CVD. The impact of CAD/CVD on socio-economic prospects of individual as well as society is huge leading to catastrophic health spending and physical burden of

disease during the mid-life of the individual, leading to loss of productivity. WHO recommends facilities for prevention and control of risk factors like tobacco use, high blood pressure, diabetes, increase lipid levels even at the primary care facilities by providing advice to have a heart healthy life style, by introducing daily regular physical activity, eating adequate amounts of fruits and vegetables, by avoiding excess salt intake and also by providing pharmacotherapy (WHO, 2015).

INTERHEART study was a case control study involving 52 countries across the globe. It studied the effect of potentially modifiable risk factors associated with myocardial infarction or MI. 15152 cases and 14820 controls were enrolled for the study. According to the findings smoking, Apo B [Apo lipoprotein B]/ Apo A1 [Apo lipoprotein A1] ratio, history of hypertension, diabetes, abdominal obesity, psychosocial factors, lack of daily consumption of fruits and vegetables, regular alcohol consumption and lack of physical activity were all related to acute MI/ AMI. These risk factors were analysed for men, women, old and young. Overall population attributable risk (PAR) for 9 risk factors in men was at 90% and for women it was 94%. The findings show that worldwide strategies should be employed to prevent risk factors and thus prevent premature cases of MI (Yusuf *et al.*, 2004)

2.2.1 CAD prevalence in India

Conventional CAD/CVD risk factors are highly prevalent among Indians. India is considered as diabetic capital of the world. Along with that there is higher prevalence of hypertension and it is predicted that these risk factors will be on the rise in the coming

years (Kearney *et al.*, 2005). The higher prevalence of risk factors makes Indians more prone to CVDs as indicated in the INTERHEART study.

Study on epidemiology and causation of CHD and stroke in India revealed that CHD and stroke have increased significantly in both urban and rural areas over the last 50 years [Figure 5]

There is a strong correlation between urbanization and increase in CVD across the globe. Major risk factors of CAD were found high in urban population, while smoking was more common in rural subjects. There is also evidence of higher prevalence of hypertension in rural population in different parts of the country. In addition to this high prevalence of coagulation and platelet function is also being reported (Gupta *et al.*, 2008).

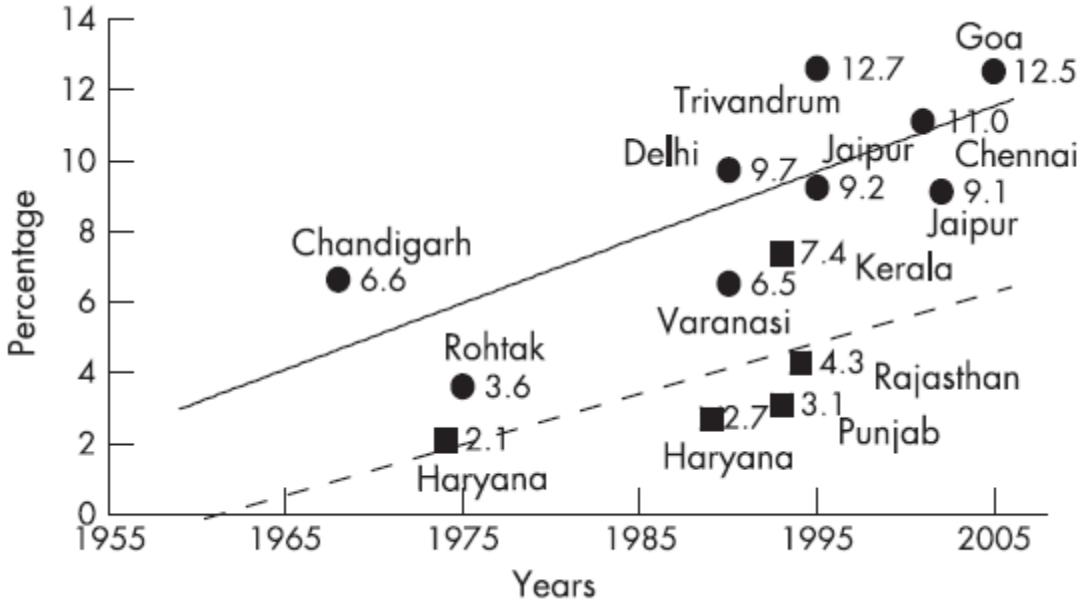


Figure 5: Coronary heart disease (CHD) prevalence (%) in Indian urban and rural subjects aged >30 years as reported in epidemiological studies. Figure represents increasing trend of CHD in both

urban and rural population Adapted from (Gupta *et al.*, 2008)[*Heart* 94: 16–26. doi: 10.1136/hrt.2007.132951.]

India is going through an epidemiological transition where by burden of communicable diseases are declining slowly while non communicable disease is rising rapidly. There has been fourfold rises in CHD during the past 40 years. Prevalence of CHD is between 7% and 13% in urban regions while, it is between 2% and 7% in rural areas. The DALY lost during 1990 was 5.6 million in men and 4.5 million in women. It is expected that by 2020 it may reach 14.4 million and 7.7 million respectively in men and women (Krishnan, 2012).

To control further increase in risk factors, it is important to introduce preventive measures. There should be national interventions involving increase in tobacco taxes, labelling of unhealthy foods, reduction of salt in processed food and better urban design to promote physical activity.

2.2.2 CAD prevalence in young Indians

Studies on CAD have shown that South Asians, especially Indians have high rates of acute MI [AMI] at younger ages compared with individuals from other countries. The earlier age of AMI in South Asians is due to higher risk factor levels at younger ages. The mean age for AMI in South Asians is 53.0±11.4 when in other countries is 58.2±12.2 years. The mortality rate is also high in Indians at young ages (Joshi *et al.*, 2007) (Reddy, 2007).

2.2.3 CAD prevalence in South India

South Indians have higher incidence of CAD among Indians. A verbal autopsy study among 45 villages of a South Indian State in 2004 showed that 32% of all deaths were due to CVD out-ranking the infectious diseases which were responsible for only 13% mortality(Joshi *et al.*, 2006)

Kerala state is a harbinger to rest of India on the course of chronic non communicable diseases (NCD)-heart diseases, stroke and cancer. The high burden of conventional risk factors was comparable to that of United States (Thankappan *et al.*, 2010). Even age standardized mortality due to CVD was comparable to that of industrialized countries (Soman *et al.*, 2011).

A recent community based cross sectional study from Kerala on prevalence of CAD and its risk factors have shown that there is no urban rural divide in the prevalence of CAD. Women showed a high prevalence of 14.3% compared to men (9.8%). Conventional risk factors were in high prevalence. The prevalence of definite CAD in Kerala increased nearly three times since 1993 (Krishnan *et al.*, 2016).

A prospective study on presentation, management and outcomes of 25748 acute coronary syndrome admissions in 125 hospitals in Kerala (Kerala ACS registry, largest ACS registry from India) from 2007 to 2009 revealed that the mean age of patients with ACS was 60± 12 years and STEMI predominated in the cohort. In-hospital mortality, major adverse cardiovascular events and interventions were high in this group (Mohanan *et al.*, 2013).

2.2.4 Gender based difference in CAD

There is a gender difference in occurrence of CAD, its symptoms and treatment in men and women. Women present more with angina symptoms, while men present with ACS (Solimene, 2010). Women are less prescribed anti-platelets, may not undergo timely catheterization and fail to reach target dose of drugs like beta blockers. There is a greater need for research in the treatment and diagnosis adopted for women with better understanding of patho-physiology of CAD (Solimene, 2010)(Sharma & Gulati, 2013).

The data from West clearly indicates that there is higher prevalence of heart disease in women reaching up to 40% of the total mortality (Solimene, 2010). On the whole, death due to CAD is decreasing in men, but it is increasing in young women [<55 years] (Sharma & Gulati, 2013). The higher mortality in women with CAD is due to multiple factors, heavier risk factor burden, more involvement of inflammatory factors, smaller vessel size of coronary arteries and less aggressive treatment of diabetes in women (Maas & Appelman, 2010)

2.2.5 CAD prevalence in young women

Women are protected from CAD because of the effect of oestrogen in their reproductive years. This protection is lost when women develop diabetes. When CAD develops in women, the prognosis is worse in women and the mortality in young women (< 50 years) is twofold compared to men of the same age group(Solimene, 2010)(Sharma & Gulati, 2013).

In young women from the west, (average age 43 years) smoking and hypertension were found to be important risk factors (Adão *et al.*, 2004).

Women may have more atypical presentations of CAD. Moreover the interpretation of non-invasive diagnostic testing is not as much as reliable in young women compared to men(Maas & Appelman, 2010).

A recent study on young women (< 55 years) substantiates that if modifiable risk factors such as smoking, obesity, diabetes and hypertension could be controlled, we can prevent the development of CAD. (Davis *et al.*, 2015)

2.3 Management of CAD

Management of CAD involves screening and diagnosis of the disease along with administration of prescribed modality of treatment. It also involves control of conventional and non-conventional risk factors, mainly by life-style changes and by medications. Flow chart below explains the important management strategies used for the control of CAD [Figure 6]

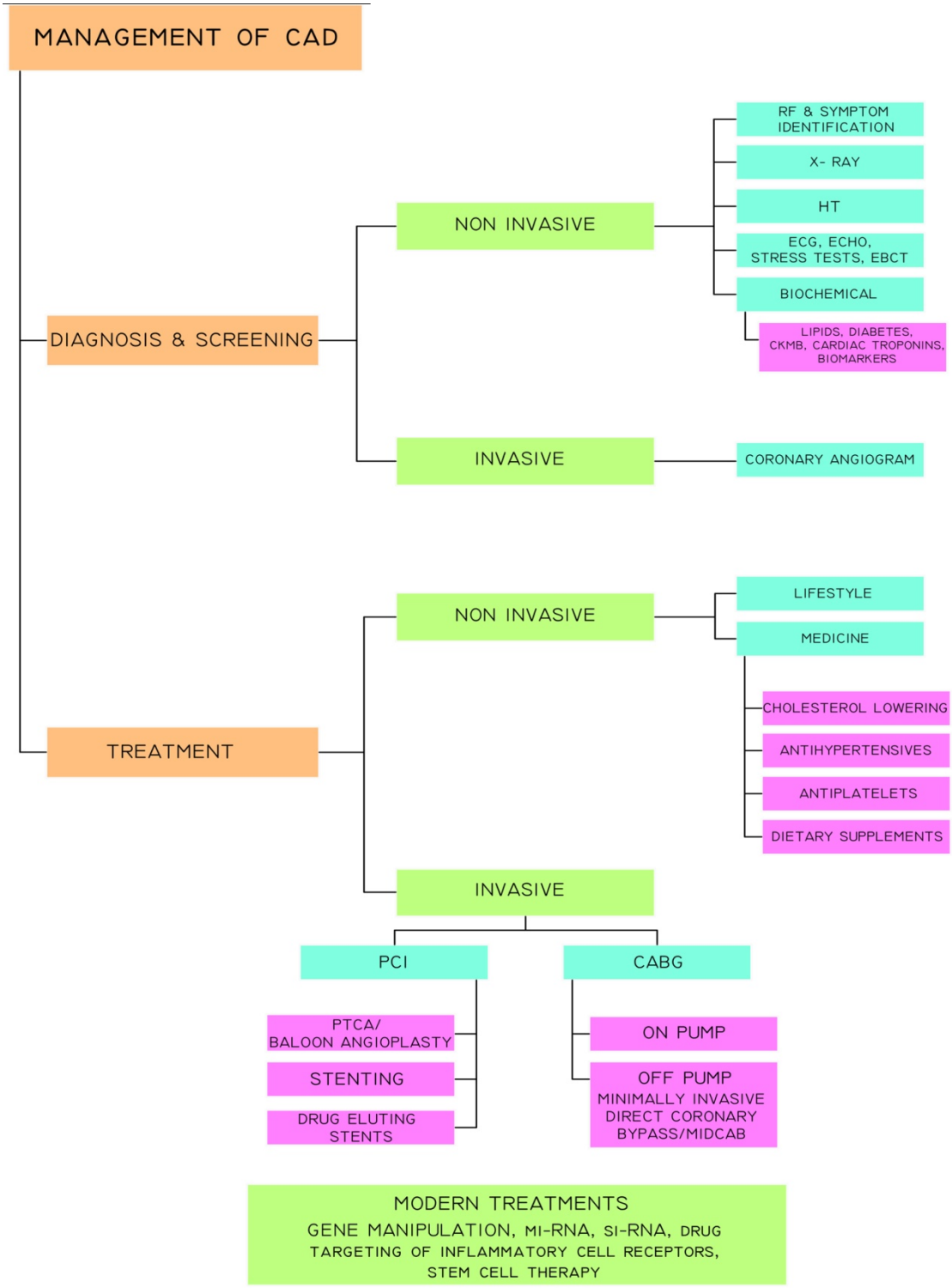


Figure 6: Management of CAD- diagnosis and treatment. [HT: hypertension, ECG: electrocardiogram, Echo: Echo cardiogram, EBCT: electron beam computer tomography, PCI: percutaneous coronary intervenrion, CABG: Coronary arteries bypass graft, PTCA: percutaneous trans coronary angioplasty

2.3.1 Diagnosis

Diagnosis of CAD is done when the medical practitioner suspect the presence of CAD based on the life style, biochemical changes (especially hyperglycemia and dyslipidemia), hypertension, physical conditions like obesity, sedentary life style, increased BMI, smoking habits, family history of CVD in nearest relative and also when patient is brought to emergency medical care due to angina, shortness of breath, suspected heart attack symptoms such as conditions of unstable angina/UA, ACS, MI, heart failure and arrhythmia [Figure 6].

CAD has got varied presentations. Most commonly the patient present with either effort angina [EA] or angina like symptoms or with acute chest pain, in the case of ACS. The other mode of presentation is sudden cardiac death (SCD). ACS can be presented as STEMI or NSTEMI (Non-STEMI) – when there is cardiac enzyme elevation or presented as UA when there is no such symptom. Patients may appear asymptomatic, when the disease is detected during evaluation for some other disease.

The non-invasive investigations for the evaluation of CAD include ECG, echocardiography and stress tests including Treadmill exercise test. The invasive investigations include coronary angiography and ventriculography.

One of the non-invasive techniques used for detection of CAD is electron beam computerized tomography or EBCT. In CAD patients, plaques which block coronary arteries contain significant amounts of calcium. Based on calcium scoring physicians can

plan the treatment strategies. Another method used for screening CAD is CT angiography or ultrafast CT; this is a non-invasive form of coronary angiography. (Kulick, 2015)

Clinical significance of Cardiac troponins

Cardiac troponins are measured to assess whether myocardial necrosis has happened or not. These tests are done when patient arrive at emergency room with suspected signs of heart attack. Troponins are regulatory proteins and are important for muscle contraction. The amino acid sequences of cardiac and skeletal muscle troponins differ and hence variety of monoclonal antibodies is developed to facilitate rapid assay of troponins. Tropnin I and Troponin T are determined for screening. It is now considered as gold standard biomarker replacing the earlier test using enzyme creatine Kinase MB [CKMB] to detect myocardial damage and estimate prognosis of patients with CAD. The detection and quantification allows risk stratification of patients with chest pain and ACS (Al-Otaiby, *et al.,* 2011).

Biomarkers elevated in different forms of CAD are described in the table 2 below

Table 2: Elevation of specific markers at different stages of CAD (Vasan, 2006)

Different stages	**Markers**
Plaque	LDL, ox.LDL, CRP, IL-6, IL-10, IL-16, Fibrinogen, TNF-α
Unstable plaque	MMP-9, MPO, ICAM, VCAM
Plaque rupture	Soluble CD40 ligand (sCD40L), Placental growth factor, Pregnancy associated plasma protein A (PAPP-A), VCAM
Thrombosis	PAI-1, sCD40L, v-WF, D-dimer
Ischemia	Ischemia modified albumin, Free fatty acid, Choline, Brain natriuretic peptide (BNP), IL-6/ TF
Necrosis	Cardiac troponinT (cTNT), cTNI, CK-MB, Myoglobin
LV remodeling	BNP, NT-ProBNP, MMP

2.3.2 Treatment

Treatment of CAD starts with life style modifications. Non-invasive treatment involves use of medications to prevent excessive plaque progression, plaque stabilisation, lysis of the thrombus inside the coronary arteries and also to treat risk factors of CAD, especially diabetes and hypertension. Invasive modalities of treatment mainly involve coronary angioplasty and coronary bypass surgery or graft/ CABG [Figure 6].

2.3.2.1 Lipid lowering drugs

Statin is the most important group of drugs widely used for lipid reduction. It interferes with cholesterol biosynthetic pathway by inhibiting 3- hydroxyl-3-methyl glutaryl CoA (HMG-CoA) reductase and thus prevents the production of mevalonate an important precursor for cholesterol. The first statin drug was mevastatin. Now around seven pharmaceutical forms of statins are available: lovastatin, simvastatin, pravastatin, fluvastatin, atorvastatin, rosuvastatin and pitavastatin. Statins are structural analogues of HMG CoA and hence competitively inhibits the enzyme 1000-10000 times than that of the original substrate. It can also lower serum cholesterol by up regulation of LDL receptor. An important role of statins is inhibition of small G protein activation. These proteins thus modulate the functions of multiple downstream signalling molecules. Statins help in the suppression of pro-inflammatory cytokines especially nuclear factor kappa B/ NFκB, TNF-α, IL-1-β and IL-6 along with inhibition of expression of induced Nitric oxide synthase/ iNOS after LPS stimulation.

The statins can effectively stimulate endothelial nitric oxide synthase/eNOS and enhance the production of endothelial NO. It also inhibits migration and proliferation of

muscle cells; by their inhibitory action on MAP kinase [Mitogen activated protein kinase] pathway as they inhibit activation of Rho proteins. They also inhibit excessive ROS production. Statins are also found to switch T helper [Th] cell balance between Th1/Th2 to Th2 polarization and there by prevent Th1 type autoimmune disease.

Thus statins are proven to act at various levels in patients with atherosclerosis. As explained earlier suppression of pro-inflammatory molecules, enhancement of NO production, and prevention of SMC migration and proliferation and reduction of ROS have profound effect on retarding atherosclerosis progression. Statins have proven to reduce vascular inflammation and adverse event in atherosclerotic CAD. Statins also may have anti-thrombotic and anti-oxidative effects (Pahan, 2006).

Important side effects of statins are liver failure and rhabdomyolosis. It is also found to raise Hb A1c and fasting blood glucose. Other possible side effects of statins are memory loss, amnesia, confusion and memory impairment.(Vos, 2005)

Fibrates are the next most commonly used anti-lipid drug after statins. In contrast to statins, fibrates are not involved in the inhibition of cholesterol biosynthesis. It stimulates β-oxidation of fatty acids mainly in peroxisomes and partly in mitochondria. Main action of fibrate drugs is in the activation of peroxisome proliferator activated receptor [PPAR – alpha]. They are involved in lowering fatty acids and tri acyl glycerols. The first fibrate used was clofibrate. Other fibrate drugs available are bezafibrate, fenofibrate and gemfibrozil. They are mostly used to bring down triglyceride levels.

The main action of fibrate is in increased clearance of VLDL-C, an increase in HDL-C and decrease in triglycerides. LDL-C reduction by these drugs is only marginal.

However fenofibrate can lower small dense LDL more efficiently than statins. Because of these actions, they are used in diabetic or atherogenic dyslipidemia.

Fibrates can also inhibit IL-6 which is actively involved monocyte migration and foam cell formation an important step in the pathogenesis of atherosclerotic CAD. Fibrates can be used in combination with statins. These combinations improve endothelial function (Pahan, 2006).

An important drug used in lipid lowering is nicotinic acid. It is added to statin therapy when HDL-C is low or when triglyceride levels are above normal. National Cholesterol Education Program's Adult Panel III guidelines recommend adding the drug along with statins when triglycerides are above 200 mg/dl and HDL is below 40 mg/dl (Pflieger *et al.*, 2011).

Other commonly used drugs used in lipid lowering are ezetimibe which selectively inhibits intestinal absorption of cholesterol, torcetrapib which inhibits cholesterol ester transfer protein [CETP] and Proprotein Convertase Subtilisin Kexin 9 [PCSK-9] inhibitors (Pahan, 2006).

2.3.2.2 Platelet lowering drugs

Platelets play an important role in the thrombotic processes which occur in relation to CAD. Platelet lowering drugs are essential for primary and secondary prevention of CAD. Table 3 gives description of important anti-platelet drugs used in CAD.

Table 3: Important anti-platelet drugs (Patrono and Rocca, 2010)

Drug	**Chemical name/function**	**Advantage/ use**	**Disadvantage**
Aspirin	Acetyl salicilicylic acid. Inhibits COX-1. Prevents thrombaxane production and prevents activation of its receptor on platelets and hence platelet activation	Reduce mortality due to CAD in combination with statins. Given to patients undergoing CABG and PTCA to prevent adverse events, reduce angina symptoms.	Stomach ulcers and bleeding
Ticlopidine, **clopidogrel**, prasugrel,ticagrelor	Thienopyridine derivative. Prodrug, targets ADP receptor $P2Y_{12}$ and reduce platelet activation	Used during invasive procedure, ACS and in those showing aspirin insensitivity or allergy	Pro-drug, need cytochrome P450 isozymes of liver for activation. Genetic variation of enzymes affects function, less predictive than aspirin. More risk of bleeding
Cangrelor	Active drug, reversible inhibitor of ADP receptor $P2Y_{12}$ and reduce platelet activation. Modified ATP derivative	Complete inhibition of ADP induced platelet aggregate	Risk of bleeding
Abciximab, Tirofiban and Epitifibatide	$\alpha_{IIb}\beta_3$ – Integrin inhibitors. Reduce platelet aggregation by inhibiting the binding of activated platelets to fibrinogen and other ligands	intravenous administration, short term treatment of patients with ACS and in those undergoing PCI	Increased mortality

Table 4 shows different drugs used in the treatment of CAD

Table 4: Common medications for treatment of CAD. Adapted from (Pflieger *et al.*, 2011)[Medical management of stable coronary artery disease.' *American Family Physician* 83: 819–26]

Medication/Class	***Use***	***Comments***
Antihypertensives		
Angiotensin Converting Enzyme [ACE inhibitors]	All patients with hypertension, diabetes mellitus, chronic kidney disease, or left ventricular dysfunction	Decrease mortality
Angiotensin receptor blockers	All patients with hypertension, diabetes mellitus, chronic kidney disease, or left ventricular dysfunction and in whom ACE inhibitors are not tolerated	No additional benefit compared to ACE inhibitors. Used in those who cannot tolerate ACEI.
Beta Blockers	All patients with history of MI, ACS or left ventricular dysfunction	Decrease mortality
Calcium channel blockers	Patients who do not tolerate beta blockers	Indicated in hypertension and also to control angina.
Nitrates	Patients with angina	No evidence of mortality benefit, symptomatic benefit.
Antiplatelet Agents: See table 3		
Lipid lowering agents		
Ezetimibe (Zeita)	Patients who have not achieved LDL goal after statin therapy or who are intolerant to statins	No evidence of mortality benefits
Fibrates	Patients with triglycerides between 200-400 mg/dl and non HDL >130 mg/dl, Triglycerides ≥500mg/dl	Reduction of non-HDL <100mg/dl
Statins	Patients with baseline LDL ≥ 100 mg/dl	Drug initiated with lifestyle measures.

2.3.2.3 Coronary angioplasty and Coronary Artery Bypass Graft surgery

Patients who are still symptomatic and on optimal medical management or showing inducible ischemia, needs revascularisation procedures like coronary angioplasty or coronary bypass graft surgery/ CABG. This can restore coronary circulation and control ischemia. Coronary angioplasty and stenting has become the procedure of choice in single and two vessel CAD where the anatomy is suitable. CABG surgery is the choice when there is three vessel disease, especially in patients with diabetes [Figure 6].

2.3.2.4 New insights into the management of premature CAD

In the case of premature CAD, it is important to study the family history and thus screen patients with genetic pre-disposition. Candidate gene studies for CAD include genes that control pathways of lipid metabolism, coagulation cascade, endothelial function, smooth muscle cells, vascular growth, inflammation, oxidation and anti-oxidation balance in the arterial wall, glucose metabolism, control of insulin resistance and homocysteine production and metabolism (Chaer, *et al.,* 2004). Some of the candidate genes studied are endothelin-1 [EDN1] (Pare' *et al.,* 2007), APOB, IL6R, LDLR (Baixeras *et al.,* 2014)

Therapeutic angiogenesis can be considered as a biological coronary artery bypass mechanism in the treatment of CAD. These may theoretically be effective in patients with diffuse CAD, as in these patients traditional revascularization procedures may not be feasible. Early clinical trials in patients with these conditions have been done using angiogenic therapeutic products. The results have shown to reduce angina symptoms, increase in exercise time and improve left ventricular function (Syed, *et.al,* 2004).

Stem cell therapy is another modality which is shown in small clinical trials to improve CAD treatment outcomes. But this mode of therapy still remains experimental. Research during the past 10 to 20 years is providing early evidence that adult and embryonic stem cells may be able to replace damaged heart muscle cells and establish new blood vessels to supply them (Can stem cells Repair a Damaged Heart, 2015). An experimental application of hematopoietic stem cells for the regeneration of the tissues in

the heart has been done by inducing heart attack in mice by tying off a major blood vessel, the LMCA. Through the identification of unique cellular surface markers, the investigators isolated a selected group of adult primitive bone marrow cells with a high capacity to develop into cells of multiple types. When injected into the damaged wall of the ventricle, these cells led to the formation of new cardiomyocytes, vascular endothelium, and SMCs, thus generating de novo myocardium, including coronary arteries, arterioles, and capillaries (Orlic *et al.,* 2001). In another model, investigators purified a "side population" of hematopoietic stem cells from a genetically altered mouse strain and then transplanted into the marrow of lethally irradiated mice approximately 10 weeks before the recipient mice were subjected to heart attack via tying off LAD. The survival rate was 26% (Jackson *et al.,* 2001). A study also showed that human adult stem cells taken from the bone marrow are capable of giving rise to vascular endothelial cells when transplanted into rats (Kochler *et al.,* 2001)

2.4 Pathogenesis of CAD-Bidirectional relation between thrombosis and inflammation

Atherosclerotic CAD was earlier considered as a consequence of lipid deposition in the arterial wall, but now it is also considered as an inflammatory disorder. The earliest lesion of atherosclerosis, the fatty streak is a pure inflammatory lesion consisting of monocyte derived MΦs and T lymphocytes.

The initiation of atherosclerotic processes starts with endothelial dysfunction. The major causes for endothelial dysfunction include elevated and modified LDL; free

radicals caused by cigarette smoking, hypertension and diabetes mellitus; possibly elevated plasma Hcy concentrations; infectious microorganisms such as herpes virus or *Chlamydia pneumonia* or combination of these factors.

The different forms of endothelial injury increase the adhesiveness of endothelium with respect to platelets and leukocytes resulting in increased permeability. The changes in endothelial permeability to lipoproteins and other plasma constituents are mediated by NO, prostacyclin, platelet derived growth factor [PDGF], angiotensin II and endothelin. During the process there is up regulation of leukocyte adhesion molecules [L-selectin, integrins and platelet endothelial cell adhesion molecule1 (PECAM-1)] and the up regulation of endothelial adhesion molecules [E-selectin, P-selectin, ICAM-1, VCAM-1]. Migration of leukocytes into the arterial wall is mediated by ox.LDL, MCP-1, IL-8, PDGF, Macrophage colony stimulating factor/MCSF and osteopontin.

Foam cell formation from fatty streak is mediated by ox.LDL, MCSF, TNF-α and IL-1. There is also active involvement of T lymphocytes and SMCs. SMC migration is stimulated by PDGF, fibroblast growth factor 2 [FGF-2] and transforming growth factor β [TGFβ]. TNF-α, IL-2 and Granulocyte-MΦ colony stimulating factor/ GMCSF may also mediate T-cell activation.

Platelet adhesion and aggregation are stimulated by integrins, P-selectin, fibrin, thrombaxane A_2, TF and other inflammatory mediators. The inflammatory responses have a profound effect on lipoprotein movement within the artery. TNF-α, IL-1 and MCSF increase binding of LDL to endothelium and smooth muscle and increase transcription of

LDL-receptor gene. LDL in turn can initiate induction of urokinase and inflammatory cytokines such as IL-1. Thus atherosclerosis progression is a vicious cycle involving interaction between lipids and inflammatory mediators, also involving thrombosis/ coagulation along with ROS (Ross, 1999).

The disrupted atherosclerotic plaque is known as a "solid state" stimulus to thrombosis while prothrombotic and anti-fibrinolytic factors in the blood is referred to as "fluid phase" stimulus for the same. These insights has changed the concept of atherosclerotic lesions to be diffuse in nature, rather than localized. Thus current treatment should focus on addressing culprit lesion as well as stabilization of other plaques which can result in recurrent events.

Two morphological extremes of coronary atherosclerotic plaques exist. First type is stenotic lesions, which have smaller lipid cores, more fibrosis tissue and calcification, thick fibrous caps and less compensatory enlargement. Clinical manifestation is ischemia characterized by angina and positive exercise test. The ischemia produced by the lesion is managed by medical therapy and revascularization.

The second type of lesions is non-stenotic and outnumbers the stenotic lesions and has large lipid cores with thin fibrous caps which are susceptible to plaque rupture and thrombosis. They undergo compensatory enlargement (positive remodelling) to a greater extent leading to under recognition of luminal stenosis by angiogram. These types of lesions mostly lead onto UA and MI.

The major outcome of plaque disruption is thrombosis. It is mainly caused by superficial erosion, plaque rupture or erosion of calcified nodule. Thrombosis which results in thrombin generation, also amplifies platelet activation. Subsequently there is conversion of fibrinogen to fibrin and also release of v-WF from activated platelets. All these result in cross linking of platelets with fibrin mesh to form a three dimensional white arterial thrombus.

PAI-1 disrupts natural fibrinolytic mechanism by inhibiting t-PA and urokinase plasminogen activator/ u-PA. PAI-1 levels are increased in diabetes, obesity and hypertension [Figure 7]. Disrupted plaques also produce TF increasing thrombogenicity of the blood, leading to activation of coagulation mechanisms (Libby, 2005)

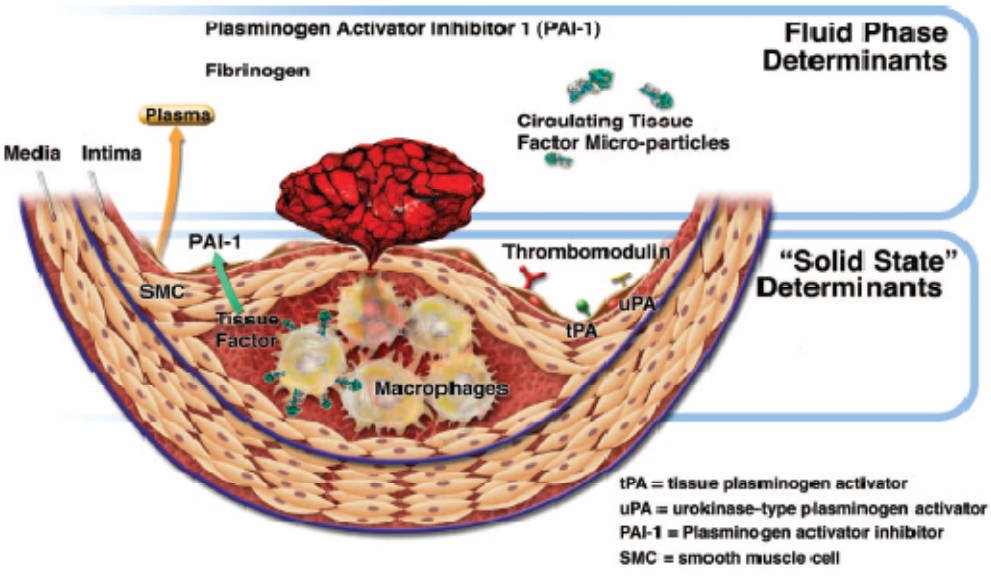


Figure 7: Determinants of thrombosis in coronary atherosclerotic plaques ;adapted from (Libby, 2005)[*Circulation* 111: 3481–88. doi: 10.1161/CIRCULATIONAHA.105.537878.]

Along with the conventional cardiovascular risk factors, inflammatory markers such as C-reactive protein (C-RP) and cytokines can accelerate CAD. LDL particle size, number and composition also have a decisive role in atherosclerosis.

There are certain protective factors against atherosclerosis, which include regular physical activity, HDL and apo A1. ApoA1 in HDL prevents LDL modification and promotes reverse cholesterol transport which can effectively reduce plaque progression and may even induce regression of plaques.

Although the cells associated with atherosclerosis, -MΦs, SMCs and endothelial cells are derived from the intimal regions of the artery, there is a wide held notion that reparative cells of atherosclerosis are derived from bone marrow especially reparative MΦs. Mobilization of atheroprotectve cells from the bone marrow and promoting their homing to thrombosis-prone plaques may be a new way to stabilize atherosclerosis against thrombosis and its devastating consequences (Hansson, 2005) (Falk, 2006).

During ACS, inflammatory and coagulation pathways work hand in hand or more specifically bidirectional. Chronic inflammation in the vascular endothelium is affected by external and internal factors resulting in immune response. Several studies substantiate bi-directional relation between thrombosis and inflammation(Corti *et al.*, 2004)(Levi, 2004)(Esmon, 2005).

For the initiation of coagulation cascade the major stimulus is injury to vessel, resulting in platelet activation. Platelet activation is also crucial for atherosclerotic lesion formation also. Thrombin action on platelets results in platelet shape change and exposure

of P-selectin from alpha granules into platelet surface through the open canalicular system of platelets. P-selectin is a receptor for ligands in leukocytes and endothelial cells and it more specifically bind to PSGL-1 of monocytes.

Thus monocyte platelet aggregates are frequently formed and it stimulates excessive TF expression by monocytes and in turn activates inflammatory cytokine NFκB also. Activated platelets also produce CD40 l, IL-1-β (a pro-inflammatory cytokine), RANTES and PF-4 which activates monocytes, allowing its recruitment to endothelium and transform into MΦs (Davì & Patrono, 2007).

In short in atherosclerotic CAD, inflammation and thrombosis are intertwined promoting each other. Coagulation process and inflammation are active during all stages of atherosclerosis and is seen aggravated or enhanced during atherothrombosis during plaque rupture leading to ACS.

Immune system also plays an active role in inflammation. Potential antigens involved in activating toll like receptors (TLRs) within the plaque include endogenous stress molecule heat shock protein 60 (HSP 60) and Ox. LDL. The complement system is also activated during atherosclerosis. Patients with atherosclerotic lesions have elevated levels of C3a and C4a. There is also involvement of dendritic cells, T cells, B cells and immunogobulins in the control as well as triggering of atherosclerotic CAD (Krychtiuk *et al.*, 2014).

In addition to its activating role in atherosclerosis, immune system also plays important role in protecting arteries against atherosclerotic CAD. Two anti-inflammatory

cytokines acting as atheroprotective signals are Tumor growth Factor-β [TGF-β] and IL-10. TGF-β promotes collagen production leading to plaque stability. It can also modulate T-cell activation.

Vaccination using antibodies against modified LDL, heat shock proteins have been proven effective in preventing CAD experimentally and might be a breakthrough in atherosclerosis prevention, although research is still undergoing.

When coming to secondary prevention several immunosuppressive agents have been known to be used in drug eluting stents, such as sirolimus (Rapamycin), which has shown effectiveness in reducing in-stent re-stenosis. Another immune suppressant cyclosporine reduces intimal cell proliferation in response to arterial injury. Statins also have many immuno-suppressive actions in addition to cholesterol reduction. Similarly PPAR family also has immuno-modulatory action and can reduce T-cell activation and SMC activation. (Hansson & Libby, 2006)

2.5 Thrombotic –inflammatory risk factors in CAD

Several thrombotic and inflammatory risk factors have been identified in CAD and most are emerging as new biomarkers for early CAD detection. Therapeutic reduction of these risk factors may also pave way for prevention and control of premature CAD.

The important thrombotic factors which we analysed in the study are fibrinogen, Lp(a), Platelet activation by P-selectin expression, Hcy, t-PA, PAI-1, v-WF and the

antithrombotic factors AT-III and Protein C. Additionally inflammatory marker hs-CRP was also analysed. In a separate cohort of patients and controls, monocyte phenotype characterization was done based on the phenotypic expression of CD14 and CD16 receptors. In addition, total and differential WBC count and inflammatory markers TNF-α, IL-1-β and MPO activity were analysed in these subjects along with thrombotic factor fibrinogen. All these factors are having implications in the pathogenesis of atherosclerosis.

Analysis of both thrombotic and inflammatory risk factors are reported in many studies from India, (Deepa *et al.*, 2002), (Khare *et al.*, 2004), (Shalia *et al.*, 2010)(Panwar *et al.*, 2011), (Aggarwal *et al.*, 2012)(Mishra *et al.*, 2013) and abroad (Thompson *et al.*, 1995), (Pineda *et al.*, 2009)

All these reports revealed that thrombotic-inflammatory risk factors were high in CAD patients from the Indian sub-continent. .

Most of the studies from India are based on North Indian population and there are only few studies from South India. Thus we planned case–control study to evaluate the role of thrombotic and atherogenic factors in young patients with angiographically proven CAD who are on treatment with statins and antiplatelet drugs. The treatment using statins and anti-platelet drugs might contribute to the control of some of the thrombotic risk factors. Thus the study was intended to assess the residual risk in those patients who are on statins and anti-platelets (George *et al.*, 2015).

In addition to the thrombotic –inflammatory risk factors we are evaluating as part of this study, there are many non-conventional risk factors which are being found to be

important in the progression of atherosclerotic CAD. These factors include Factor V Leiden, Prothrombin, G20210A mutation (Feinbloom, 2005); smaller LDL particle size (Renjith & Jayakumari, 2011) ; air pollution, and functionally defective HDL (Sini., 2013).

2.5.1 Fibrinogen as an important thrombotic risk factor for CAD

Several studies have established fibrinogen as an important thrombotic risk factor in CAD. Fibrinogen is an acute phase protein synthesized by the liver. It is an important glycoprotein triggering thrombus formation. It binds with platelets by means of glycoprotein IIb/IIIa, forming platelet aggregates. It is the Factor I of coagulation pathway. Coagulation pathway is involved in conversion of fibrinogen to fibrin (activated Factor I) by thrombin. In a thrombogenic environment, excessive fibrinogen can easily trigger thrombosis. Fibrinogen also have a role in immune stimulation [innate immunity] and stimulate monocyte to secrete cytokines and also interact with integrins of immune cells (Delvaeye & Conway, 2009). It is involved in the formation of atherosclerotic plaque during early stages of CAD by triggering leukocyte migration and production of adhesion molecules (Papageorgiou *et al.*, 2010).

There is no established treatment to bring down high fibrinogen levels, but cholesterol lowering drugs such as atorvastatin (Leibovitz *et al.*, 2004) and Bezafibrate (Madrid-Miller *et al.*, 2010) have their influence on controlling fibrinogen and the reduction was associated with a lower incidence of major cardiovascular events .

2.5.2 Lipoprotein (a) as a thrombotic inflammatory marker

Lp(a) consists of an LDL-like particle and the specific apolipoprotein(a) [apo(a)], which is covalently bound to the apo B of the LDL like particle. It is synthesised in the liver. Lp(a)- apo(a) is involved in defective fibrinolysis and increased thrombosis (Levi, 2004). Lp(a) also contribute to mononuclear recruitment by means of 'CC chemokine': I-309 and enhance intracellular cholesterol accumulation and foam cell formation (Riches and Porter, 2012). Lp(a) has also been linked to oxidized phospholipid burden and thus have a pro-inflammatory role, along with its atherogenic and thrombogenic role (Dubé *et al.*, 2012).

Elevated Lp(a) is often associated with premature CVD/CHD, which was independent of cholesterol levels. Several studies points to the role of Lp(a) in CAD. Lp(a) is also elevated in Indians with angiographically defined CAD and is highly predictive of the presence of disease and severity of CAD(Rajasekhar *et al.*, 2004)(Ashfaq *et al.*, 2012)

Niacin treatment is generally advised to lower Lp(a)(Nordestgaard *et al.*, 2010). Aspirin is able to reduce Lp(a) production by cultured hepatocytes via the reduction of apo lipoprotein(a) [apo(a)] gene transcription(Akaike *et al.*, 2002).

2.5.3 Homocysteine in CAD

Hcy is an intermediary amino acid formed by the conversion of methionine to cysteine. Homocystinuria or severe hyper homocysteinemia is a rare autosomal recessive disorder characterized by severe elevations in plasma and urine Hcy concentrations.

Clinical manifestation of homocystinuria includes severe premature atherosclerosis. Moderate hyper homocysteinemia is an independent risk factor for atherosclerotic vascular disease and for recurrent venous thromboembolism. Homocysteine is metabolized by one of two divergent pathways: trans-sulphuration [catalyzed by cystathionine-β-synthase requiring vitamin B6 as a cofactor] and remethylation producing methionine [catalyzed either by methionine synthase or by betaine-homocysteine methyltransferase with Vitamin B 12 as co-factor].

Elevations in the plasma Hcy concentration can occur due to genetic defects in the enzymes involved in homocysteine metabolism, to nutritional deficiencies (vitamin co-factors), or to other situations like fibrate and nicotinic acid treatment, smoking and chronic kidney failure (Rosenson &Kang, 2016).

Hcy and its metabolic intermediates can produce excessive methylation to cellular compounds including nucleic acids, proteins and lipids. These have adverse effect on their function (Tehlivets, 2011).

However the role of Hcy in promoting CAD still remains controversial as there are conflicting reports about its association with CAD. (Panwar *et al.*, 2011) (Shenoy *et al.*, 2014) (Deepa *et al.*, 2001) (Mehta & Shah 2012).

Recently our group, have standardized a direct, rapid and sensitive method for homocysteine assay in urine sample [liquid chromatography–tandem mass spectrometry coupled with electro spray ionization/LC-MS/MS coupled with ESI].Using this

technique, urine samples from CAD patients showed elevated Hcy levels compared to that of controls (Gopu *et al.*, 2013).

2.5.4 Fibrinolytic factors (t-PA and PAI-1) in CAD

Fibrinolytic factors play important role in the progression of CAD. t-PA is a fibrinolytic agent while PAI-1 is a potential inhibitor of fibrinolysis. In patients with CAD, both PAI-1 and t-PA are elevated (Deepa *et al.*, 2002). PAI-1 in platelets are also elevated during MI (Soeki *et al.*, 2000). t-PA is elevated during acute inflammation (Chia *et al.*, 2003), while PAI-1 is elevated in patients with metabolic syndrome (Palomo *et al.*, 2009).

Treatment of SMCs and endothelial cells with simvastatin have shown that there was marked reduction of PAI-1 with increase in t-PA (Bourcier & Libby, 2000).

2.5.5 v-WF as a thrombotic risk factor CAD

v-WF is an important thrombotic risk factor. It is secreted by endothelium as well as platelets. On endothelial damage collagen is exposed and v-WF is released, which bind to Glycoprotein Ib receptor of platelets. Its activation is associated with acute ischemic syndromes. It is needed for platelet adhesion to endothelium. Its blood levels are elevated in association with higher levels of cardiovascular risk factors, atrial fibrillation, and during PCI and stent implantation.

Various approaches have been taken to block the interaction of v-WF and the platelet GPIb receptor and thereby to inhibit v-WF function in thrombogenesis. Heparins have been shown to significantly impair v-WF dependent platelet haemostatic

mechanisms by binding to a site on the v-WF molecule that overlaps its A1 domain responsible for GPIb binding. GPIIb/IIIa inhibitors, the monoclonal antibody c7E3 Fab (abciximab), eptifibatide or tirofiban can suppress the v-WF-mediated platelet activation (Spiel, *et al.,* 2008).

Statins are also found to effectively reduce, v-WF in peripheral atherosclerosis (Furukawa, 2006) and in patients with hypercholesterolemia (Joukhadar *et al.,* 2001)

Plasma v-WF levels are determined by genetic factors including ABO blood groups and v-WF mutations, and by non-genetic factors including aging, impaired NO production, inflammation, free radical production and diabetes (Vischer, 2006). Individuals with non-type O blood group have found to have an increased thrombotic risk probably because v-WF-FVIII levels were found to be higher in them (Franchini *et al.*, 2007).

Patients with lower v-WF are reported to have lower prevalence of arterial thrombosis compared to patients with higher v-WF (Sanders *et al.*, 2013) and again there was no ethnic difference between South Asians and Europeans in the case of v-WF (Jaumdally *et al.*, 2007).

2.5.6 Antithrombin –III/ AT-III as an antithrombotic factor in CAD

AT-III is a serpin and is the natural inhibitor of thrombin activity. It has many anti-inflammatory functions in models of sepsis, septic shock and disseminated intravascular coagulation. Low level of AT-III is a risk factor for CAD.

AT-III potentially blocks the activation of NFκB in human monocytes and endothelial cells (Oelschläger *et al.*, 2002).

It is a plasmatic α-glycoprotein formed by a single peptide chain. It inhibits thrombin (first target) and free Xa, IXa, VIIa plasmatic factors. In plasma it is found fewer than two forms: α-antithrombin and β-antithrombin. Deficiency of AT III represents a risk factor for thrombo-embolic disease. There are two types of AT-III defeciencies - quantitative and qualitative. Incidence of AT- III inherited deficiency is relative rare (1:10.000). Acquired deficiency of AT- III is more frequent. The transmission of AT- III deficiency is autosomal dominant with variable shield factor. Treatment of AT- III deficiency is administration of AT- III concentrates (when plasmatic level of AT-III is below 80% from normal value) and heparin therapy. The treatment with AT- III concentrates is for patients who have to undergo major surgical interventions and pregnant women with AT-III deficiency (Găman & Găman, 2014).

AT-III deficiency has been reported in individuals with CAD as reported in many studies (Celik *et al.*, 2008) (Fennich *et al.*, 2013). But an earlier study has shown it to have an important role in the prognosis of patients with angina pectoris undergoing clinical assessment(Thompson *et al.*, 1996).

An antithrombin mutation A3488 has been found to be relatively high in British population. This is a prevalent genetic risk factor for venous thrombosis and is the most frequent cause of antithrombin deficiency (Corral *et al.*, 2007). In a study from India,

several variants of antithrombin were identified in an Indian population of 1950 patients with deep vein thrombosis (Bhakuni *et al.*, 2015).

2.5.7 Protein C as an antithrombotic factor in CAD

Protein C is an important vitamin K dependent antithrombotic factor. During atherosclerotic processes, antithrombotic factors such as Protein C may be impaired or blocked due to excessive activation of inflammation and thrombosis. In a study from India, Protein C promoter region CG polymorphisms showed statistically significant association with reduced Protein C levels. Thus protein C deficiency was largely associated with Protein C gene promoter CG polymorphisms (Pai *et al.*, 2012).

However an earlier case control study from India gave evidence that human body synthesises increased amounts of Protein C in ischemic heart disease to compensate for the hypercoagulable state that exists in this disorder, thus playing a protective role (Ganapathyraman *et al.*, 1996).

2.5.8 P-selectin, a marker for platelet activation in promoting thrombosis and inflammation in CAD

During thrombosis and inflammation platelets are activated. Platelet activation is a critical component in the pathogenesis of atherosclerotic CAD. Variety of markers for platelet activation have been established and CD 62p (P selectin) is one of them (Ferroni *et al.*, 2012).

P-selectin is the largest of the selectins (140 KD), which are generally stored in the alpha granules of the platelets. Subsequent to platelet activation, exposed P-selectin can

bind with PSGL-1 of monocytes. These aggregates can further enhance activation of monocyte, facilitating inflammation and thrombosis (Davì and Patrono, 2007).

Monocyte-platelet aggregate formation, as occurs in the blood under pro-inflammatory conditions, expands the pool of circulating $CD14^{++}CD16^{+}$ pro-inflamatory monocytes in a COX-2 dependent manner, and these monocytes exhibit increased adhesion to endothelium, indicating the pro-inflammatory role of platelet activation (Passacquale *et al.*, 2011).

The role of platelet P-selectin is not solely adhesive; its binding to PSGL-1 induces platelet activation that enhances platelet aggregation and thrombus formation. Therefore, targeting platelet P-selectin or its ligand PSGL-1 could provide a potential therapeutic approach in the management of thrombotic disorders (Théorêt *et al.*, 2011).

Platelet bound P-selectin is reported to be high in conditions like ACS compared to stable angina. P-selectin is independent of age, gender, anti-platelet medication and is associated with molecular markers of MI and infarct size (Stellos *et al.*, 2010). Patients with ACS and multivessel disease had increased P-selectin expression(George *et al.*, 2016). P-selectin is also elevated in hypercholesterolemia (Chan *et al.*, 2015).

Indices of platelet activation: P-selectin, platelet monocyte aggregate, platelet neutrophils aggregate especially the PFA-100 CT, may reflect plaque instability, an ongoing thrombotic state and/or reduced responsiveness to aspirin (Linden *et al.*, 2007).

Statins can blunt the exercise-induced increase in P-selectin following a marathon (Zaleski *et al.*, 2013). There are also conflicting reports on the effect of anti-platelets on P-selectin, as some reports supports role of aspirin (Kaufmann *et al.*, 2013), while others support clopidogrel (Klinkhardt *et al.*, 2003). Overall there is no conclusive evidence on the role of aspirin (Pernerstorfer *et al.*, 2001), (Linden *et al.*, 2007) or clopidogrel in lowering P-selectin (George *et al.*, 2016) (Stellos *et al.*, 2010).

Platelet reactivity/platelet leukocyte aggregates, platelet activation (Patel *et al.*, 2007), soluble P-selectin (Gokulakrishnan *et al.*, 2006) and Platelet bound P-selectin (Banerjee *et al.*, 2012) (George *et al.*, 2016) has been found to impact the progression of CAD in Indians. These factors are found to be high in our population who have high levels of CAD. So elevation of platelet activation could be considered when planning therapeutic strategies in future.

It has been widely established by many studies that platelet-monocytes aggregates are emerging as a better marker for platelet activation than P-selectin expression(Michelson *et al.*, 2001), especially in acute MI (Furman *et al.*, 2001) (Sarma, 2002). They are highly influenced by endothelial activation (He, 2006) and can facilitate monocyte tethering to endothelium (da Costa Martins, 2004). It can link haemostasis or thrombosis to inflammation and is induced by PAR agonist by regulation of NO and MMPs (Chung *et al.*, 2004) They can be easily estimated using flow cytometry techniques (Harding *et al.*, 2007).

Interaction between platelets and monocytes can also trigger platelet monocyte micro-particle formation, which are highly potent in enhancing thrombotic and inflammatory reactions in the vascular wall (McGregor *et al.*, 2006).

Interactions with platelets stimulate monocyte to express Mac-1 [Macrophage-1 antigen], activate NFκB, and increase the production of IL-1-β, IL-6, IL-8, and TF. MCP-1 changes were suggested to be an important factor of microvascular reflow abnormalities after reperfusion. Enhanced generation of monocyte platelet aggregate is associated with high risk of future cardiovascular events (Shantsila & Lip, 2009)

2.6 Role of Leukocytes in Atherosclerotic CAD

The coronary heart disease risk ratios associated with a high white blood cell count are comparable to those of other inflammatory markers, including C-RP. In addition, other components of the complete blood count, such as hematocrit and the erythrocyte sedimentation rate also are associated with CHD, and the combination of the complete blood count with the WBC can improve our ability to predict CHD risk. These tests are inexpensive, widely available, and easy to order and interpret. They merit further research (Madjid & Fatemi, 2013).

Many studies showed that WBC and differential count was associated with CAD (Rasouli *et al.*, 2007) and they also were found to be correlating with mortality of patients (Núñez *et al.*, 2005). Many studies claim higher risk for CHD and CVD associated with increased total leucocyte count seems to be accounted for by the increased granulocyte count (Rana *et al.*, 2007) (Hong *et al.*, 2014)

Endothelial cell activation by a variety of stimuli including proinflammatory cytokines (eg, IL-1-β, TNF-α, IFN-γ), certain bacterial endotoxins, hemodynamic factors, viruses, and thrombin can predispose to local thrombosis, loss of vessel barrier function, and rapid and robust leukocyte recruitment. Chemokines in leukocyte recruitment are depicted in the table [Table 5] below

Table 5: Chemokines implicated in leukocyte recruitment; adapted from (Rao *et al.*, 2007) [*Circulation Research* 101: 234–47. doi: 10.1161/CIRCRESAHA.107.151860b**.]**

Receptor	Chemokine Ligands	Leukocyte targets
CCR2	CCL2 (MCP-1), CCL8(MCP-2), CCL7 (MCP-3)	Monocytes, dendritic cells, memory T cells
CCR5	CCL3 (MIP-1α), CCL5 (RANTES), CCL4 (MIP-1β)	T cells, monocytes
CX_3CR1	CX_3CL1 (fractalkine)	Monocytes

A study by Tani *et al.* (2009) has demonstrated that monocyte count was the only leukocyte type significantly and independently associated with coronary atherosclerotic regression, even after adjustment for lipid levels. Thus the decrease in monocyte count is a non lipid-lowering effect of statins and it may be used as a novel marker of coronary atherosclerotic regression.

2.7 Monocyte phenotypes in disease, inflammation and CAD

There is ample evidence, of involvement of leukocytes in almost all stages of atherosclerosis. Monocytes being the principle leukocytes recruited during atherogenesis,

there is enough and more evidence over the involvement of monocytes in atherosclerosis. They have multiple roles in pathogenesis and disease progression.

Recently the focus has shifted onto the heterogeneity of these monocytes. Monocytes form about 3-5% of the total leukocytes in the blood. The recent evidence has shown that monocyte heterogeneity may predict disease progression and might be used as a biomarker in CAD. These monocytes are precursors for MΦs, which play a very significant role in atherosclerotic CAD. The monocytes/MΦs can be pro or anti-inflammatory (Imhof & Lions, 2004)

The exact functions of each monocyte subsets are not clearly defined even today. So it is important to determine the subsets as well as its functionality to clearly target these subsets for the prevention and control of CAD.

The identification of non-classical monocyte with the help of CD14 and CD16 conjugated monoclonal antibodies in peripheral human blood was done by Passlick *et al* (1989) with aid of two colour immuno fluorescence and flow cytometry. This was one of the first studies which classified monocytes into two functionally distinct phenotypes.

CD14 (Cluster of Differentiation 14) is a lipopolysaccharide co-receptor along with TLR 4. It is mainly expressed by all monocytes. CD16 (Cluster of differentiation 16) is a FcγRIII receptor for Immunoglobulin G. It is expressed by monocytes, MΦs, natural killer cells and neutrophils. Several classifications based on other markers are also available but differentiation based on CD14 and CD16 is the mostly accepted.

According to this classification, two monocyte subsets are present: one with higher CD14 expression and no CD16 expression designated as $CD14^{++}CD16^{-}$ [classical monocytes/CM] and the other with CD14 expression and CD16 expression designated as $CD14^{+}CD16^{+}$ [non classical monocytes/NCM] (Passlick *et al* 1989). In our discussion, for convenience, CM are designated as $CD16^{-}$ monocytes and other [NCM] as $CD16^{+}$ monocytes.

The nomenclature of monocytes and dendritic cells [DCs] in blood has become quite confusing. To resolve this, a group of experts drafted a nomenclature proposal under the auspices of the International Union of Immunological Societies [IUIS] and the WHO.

In humans, monocytes were initially defined on the basis of morphology and cytochemistry [monocyte-specific esterase] and later by flow cytometry that was based on light scatter properties and on cell-surface markers such as CD14. This technology enabled the identification of a $CD16^{+}$ subpopulation, which is characterized by higher expression of major histocompatibility complex [MHC] class II and after stimulation by TLR ligands by higher TNF production. In addition, these cells were shown to expand in inflammatory diseases.

The classical $CD16^{-}$ monocytes and the $CD16^{+}$ cells were shown to share morphology, cytochemistry, and many cell-surface markers. The more recent approach of expression profiling and hierarchical clustering has substantiated the close relationship of the 2 types of cells. In addition, monocytes with an intermediate phenotype between classical and $CD14^{low}CD16^{+}$ monocyte subsets have been described. These are found in low frequency, but they have unique features and expand with cytokine treatment and in

inflammation. The use of popular terms such as “inflammatory monocytes,” or “proinflammatory monocytes” is not recommended by the new recommendation, because this leads to confusion as the label inflammatory has been used for different subpopulations in humans and mice.

So, human blood monocytes are subdivided into 3 subsets, that is, classical [$CD14^{++}CD16^{-}$], intermediate [$CD14^{++}CD16^{+}$], and nonclassical [$CD14^{+}CD16^{++}$], as discussed above. CMs are the cells known to haematologists for a century as monocytes on the basis of structure, whereas the somewhat smaller, NCMs, which account for only 10% of all monocytes, were described only 20 years ago.

There appears to be a developmental relationship between these cells - from classical to intermediate to non classical sub types. During the course of an infection or with M-CSF treatment, there is an increase of the intermediate cells initially followed by a rise of the non classical $CD14^{+}CD16^{++}$ monocytes. Here, $^{+}$ denotes an expression level that is ~ 10-fold above the isotype control and $^{++}$ is ~ 100-fold above the isotype control. With a gradual development from classical to non classical monocytes, it may be difficult at times to determine the boundaries between the subpopulations. The CD14 and CD16 markers have proven useful in many studies in the literature, and their use is recommended for determination of subpopulations. For human monocytes CD14 and CD16 antibodies targeting different epitopes are available (Ziegler-Heitbrock *et al.*, 2010).

There are many assays which are developed for the sub-typing of monocytes. In a novel single-platform assay for determination of the absolute number of human blood monocyte subpopulations, i.e., the $CD14^{++}CD16^{-}$ /classical and the $CD14^{+}CD16^{++}$/ non-classical monocytes, a four-color combination of antibodies to CD14, CD16, CD45, and HLA-DR [Human Leukocyte Antigen - antigen D Related] was used to reduce the spill-over of natural killer cells and of granulocytes into the $CD14^{+}CD16^{++}$ monocyte gate. There was no age dependence, but monocytes showed an effect of gender, in that, females had lower NCMs compared to males. The study confirmed that exercise will lead to more than three-fold increase of the NCMs and also showed that therapy with low doses of glucocorticoids will deplete these cells. This robust single-platform assay may be a useful tool for monitoring the absolute number of monocyte subpopulations in health and disease (Heimbeck *et al.*, 2010).

When monocytes are defined by CD14 and CD16 expression, there should be careful gating strategies and use of other surface markers such as HLA-DR, CCR2/ C-C chemokine receptor type 2 and CX_3CR1/ CX_3C chemokine receptor 1 to define monocytes in the blood or peripheral blood mononuclear cells. Freezing and thawing may lead to CD16 and CX_3CR1 expression. There is also differences in monocyte phenotypes based on ethnicity and exposure of parasites such as *Plasmodium falciparum* and *Schistosoma haematobium* (Appleby *et al.*, 2013).

The mobilization of CD16 positive monocytes during exercise is stimulated by catecholamine production, which may enhance this phenotype during hypertension and

this is mainly due to desensitisation of beta adrenergic receptors by monocytes (Dimitrov *et al.*, 2013).

$CD16^+$/ non classical and intermediate cells are better at phagocytosis and at producing microbicidal reactive nitrogen intermediates than are $CD16^-$/ CMs. The $CD16^-$ subset, which is the predominant monocyte population in the circulation in a healthy person in the absence of infection, has more effective antimicrobial capacity and is more efficient at producing microbicidal ROS. The ratio of $CD16^+$ to $CD16^-$ monocytes changes significantly in disease states. Results accumulated over the past decade suggest that the $CD16^+$ subset is expanded in a vast number of inflammatory diseases, irrespective of their etiology. The increase in numbers of $CD16^+$ monocytes are such a general feature of inflammatory states, although it can indicate severity and outcome, it cannot suggest a specific diagnosis.

There are much contradictory findings regarding the role of monocyte phenotypes in different stages of CAD. Various explanations have been proposed for the increase in circulating $CD16^+$ cells in inflammatory disease: the maturation of $CD16^-$ cells into $CD16^+$ cells; the increased movement of $CD16^-$ monocytes out of the blood vessels into tissues; and even the stimulation of a putative $CD16^+$ monocyte developmental pathway. There exist functional differences of the two monocyte subsets are summarized in the figure below [Figure 8].

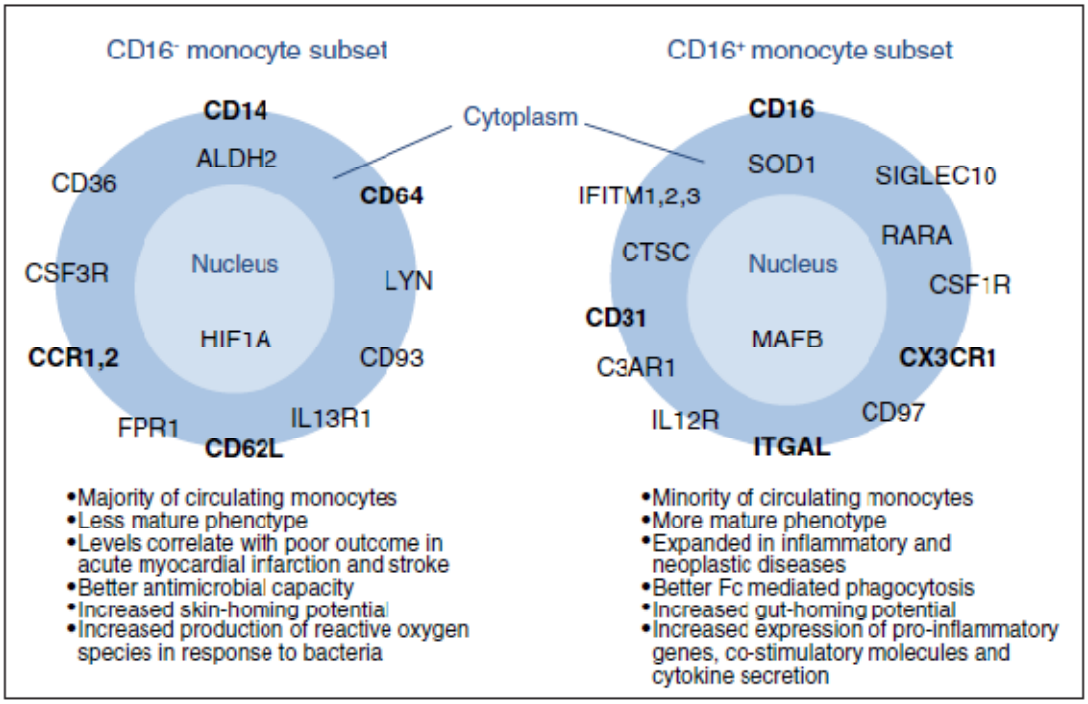


Figure 8: Functional differences between CD14^{+}CD16^{-} (CD16^{-}) monocyte subset and CD14^{+}CD16^{+} (CD16^{+}) monocyte subset based on the gene expression of different markers. [Abbreviations: ALDH2-aldehyde dehydrogenase 2 family; C3AR1-complement component 3a receptor 1; CSF1R-Colony stimulating factor1 receptor; CSF3R- Colony stimulating factor 3 Receptor, CTSC-Cathespin C; FPR1-formyl peptide receptor-1, HIF1A-hypoxiainducible factor 1 A, IFITM1-3- interferon induced transmembrane protein1,2 and 3; IL12R- interleukin 12 receptor or Interleukin 13 Receptor; LYN,v-yes-1-Yamaguchi Sarcoma viral related oncogene homolog; MAFB, v.maf musculoponeurotic fibrosarcoma oncogene homolog B (avian), RARA-retinoic acid receptor alpha subunit;SIGLEC10- Sialic acid binding Immunoglobulin like lectin 10, SOD 1-Soluble super oxide dismutase 1] Adapted from(Martinez, 2009)[*Journal of Biology* 8: 99. doi: 10.1186/jbiol206.]

Understanding the role and full potential of the monocyte subsets in the inflammatory response will be essential for creating novel and directed therapeutic approaches(Martinez, 2009).

There is overlap as well as differences in the function of monocyte phenotypes in both humans and mice, table below show fundamental difference between two established monocyte subsets in mice and humans.

Table 6: Monocyte subsets/phenotypes in Humans and mice-a comparison

HUMANS	**MICE**
CD14 &CD16 PHENOTYPES	Ly-6 C,Gr1 PHENOTYPES
$CD14^{hi}CD16^{-}CCR2^{+}CX_3CR1^{lo}$ [CCR2 is MCP-1 receptor] **Anti-inflammatory function** CD62L CD64, CD11b CCR1 expression. May also produce IL-1, IL-6, ROS, Prostaglandin E2, Plasminogen activator	$Ly6C^{hi}Gr1^{+}CCR2^{+}CX_3CR1^{lo}$ **Proinflammatory function** CCR5/Macrophage inflammatory protein-1 (MIP-1)α, CCR1 expression
$CD14^{+}CD16^{+}CCR2^{-}CX_3CR1^{hi}$ **Proinflammatory function produce TNFα** lack CCR2, Higher levels of MHCII and CD32. Considerable heterogeneity, Antigen presenting capacity **.Resident subset** Produce IFN-α, TLR-4	$Ly\text{-}6C^{lo}Gr1^{lo}$, $CCR2^{-}CX_3CR1^{hi}$ **Anti-inflammatory function produce IL10, resident subset**

(Woollard & Geissmann, 2010) (Shantsila &Lip, 2009)

A study on three classes of circulating human monocytes using microarray, flow cytometry, and cytokine production analysis revealed that intermediate monocytes expressed a large majority (87%) of genes and surface proteins at levels between classical and nonclassical monocytes, establishing their intermediary nature at the molecular level. This unveils the close relationship between the intermediate and NCMs, along with features that separate them. Thus genetic studies could clearly define monocyte subsets on the basis of its function and biology(Wong *et al.*, 2011).

2.7.1 Monocyte / Macrophage system in atherosclerosis

Heterogeneity within the monocyte/MΦ population, have important implications for plaque development and regression. A better insight into how specific phenotypes may influence plaque progression should facilitate the development of novel methods of treatments (Saha *et al.*, 2009).

Patients with CAD have higher numbers of $CD14^{+}CD16^{+}$/NCMs than healthy cohorts. Furthermore, $CD14^{+}CD16^{+}$ counts correlate negatively with the concentration of HDL and positively with levels of atherogenic lipids. Peak levels of $CD14^{hi}CD16^{lo}$/ intermediate monocytes after acute MI were found to correlate negatively with the recovery of left ventricular function (Woollard & Geissmann, 2010). Similarly Krychtiuk *et al* (2014) showed that small HDL which was dysfunctional showed positive correlation in CAD with NCMs and inverse correlation with CMs.

When non classical and intermediate monocytes were analysed in obese and lean subjects, NCMs were associated with glycemic levels, independent of fat mass. Drastic weight loss led to a sharp decrease of this subset, the variations of which were strongly related to fat mass changes. A reduction of at least 5% of fat mass was sufficient to produce a significant decrease in NCMs. A reduction in the population of the intermediate subset was also observed during weight loss and was associated with a decrease in intima-media thickness (Poitou *et al.*, 2011).

These observations in human cohorts provide evidence for a direct link between adiposity, inflammation, and monocyte maturation. There are also reports of mobilisation

of intermediate monocytes during exercise with regular physical activity reducing its levels dependent on TNF-α, along with this intermediate monocytes are positively correlating with LDL and negatively correlating with HDL. Intermediate monocytes also correlate with other risk factors such as diabetes mellitus and hypertension. It represent an inflammatory monocyte population and implicated in the progression of CAD (Shantsila & Lip, 2009) and predictive of adverse events in cardiovascular diseases(Rogacev *et al.*, 2012)

Therefore targeting intermediate monocytes may emerge as a treatment avenue in CAD. Selective depletion of intermediate monocytes requires a highly specific surface marker related regimen. Therefore, uniform adoption of the recommended gating strategies for proper identification of each monocyte subpopulation is necessary to provide a clear picture of their role in human disease (Stansfield & Ingram, 2015)

Peripheral non-classical monocytes as assessed by flow cytometry were associated with severity of CAD based on Gensini score in patients with stable angina pectoris in a Japanese cohort (Ozaki *et al.*, 2012). Similarly a study on the relation between monocyte subsets and the presence, extent, and vulnerability characteristics of non-calcified coronary plaques [NCPs] as assessed by multi-detector computed tomography [MDCT] suggest that an increased subset of NCMs is related to coronary plaque vulnerability in patients with stable angina pectoris (Kashiwagi *et al.*, 2010).

A study showed that an up-shifting subset of $CD14^{+}CD16^{+}$ [including non classical and intermediate] monocytes might be induced by percutaneous coronary intervention

[PCI], which subsequently leads to peri-procedural myocardial injury. Moreover, statin loading before PCI could exert anti-inflammatory effects partly by modulating monocyte phenotype and thus prevent peri-procedural myocardial injury(Peng, 2013).

Another study examined whether distinct monocyte subsets relate in specific ways to coronary fibrous cap thickness [FCT] in patients with unstable angina pectoris [UAP]. The changes in the non-culprit FCT were assessed by optical coherence tomography [OCT] at baseline and after 9 months. The percentage change in FCT showed significant negative correlation with the percentage changes in NCMs [$CD14^{+}CD16^{+}CX3CR1^{+}$], but not in CMs [$CD14^{+}CD16^{-}CCR2^{+}$]. In addition, the percent change in NCMs was significantly decreased in the group of patients who received statin treatment compared with the group of patients who did not. Thus NCMs may have a role in coronary plaque vulnerability(Imanishi *et al.*, 2010).

NCMs are considered senescent cells with shortened telomeres versus CMs and increased expression of β-galactosidase. Senescent $CD14^{+}CD16^{+}$ monocytes are activated cells, with increased inflammatory activity and ability to interact with endothelial cells. Therefore, accumulation of senescent monocytes may explain, in part, the development of chronic inflammation and atherosclerosis in elderly subjects and in patients with chronic inflammatory diseases(Merino *et al.*, 2011).

A study by Tsujioka *et al.* (2009) pointed out that the peak levels of CMs affect both the extent of myocardial salvage and the recovery of left ventricular function after AMI, indicating that the manipulation of monocyte heterogeneity could be a novel therapeutic target for salvaging ischemic damage.

Another study showed that among the chemokine receptors CCR2, CX_3CR1, and CCR5 expression on NCMs was negatively associated with carotid intima-media thickness. This study by Berg *et al* (2012) also showed that CMs can predict future cardiovascular risk independently of other risk factors in a randomly selected population. This is one of the studies which contradict the predictive role of non-classical and intermediate monocyte in CAD, but in this study frozen and thawed samples were used for the analysis.

Monocytes are also involved in cardiac repair after plaque formation. Classical $CD14^{+}CD16^{-}$ monocytes are attracted initially to the repair site, while $CD16^{+}$ positive monocytes are recruited lately. It mainly involves $CD14^{+}CD16^{++}CCR2^{-}$ [NCMs]. There may be involvement of intermediate monocyte $CD14^{++}CD16^{+}$ $CCR2^{+}$ monocytes also. These may alter the extracellular matrix remodelling by myofibroblast deposition and angiogenesis, leading to thinning of the infracted region (Ghattas *et al.*, 2013).

2.7.2 Monocyte/Macrophage system and inflammatory markers

Monocyte/ MΦ system are highly influenced by the cytokines in the vascular environment and they themselves are characterised by the secretion of variety of cytokines and chemokine receptors. Cytokines may be pro-inflammatory or anti-inflammatory and may be used to define the functionality of monocyte and macrophage subsets or phenotypes.

TNF/ TNFα, cachexin, or cachectin is a cytokine involved in systemic inflammation and is one of the cytokines that make up the acute phase reaction. It is produced chiefly

by activated monocytes/MΦ, although it can be produced by many other cell types such as CD4+ lymphocytes, natural killer cells, neutrophils, mast cells, eosinophils, and neurons. The primary role of TNF is in the regulation of immune cells. TNF, being an endogenous pyrogen, is able to induce fever, apoptotic cell death, cachexia, inflammation and to inhibit tumorigenesis and viral replication and respond to sepsis via IL1 and IL6 producing cells.

TNF-α is a proinflammatory cytokine, which is implicated in some metabolic disorders and may play a role in the development of CVD. The plasma TNF-α concentration is associated with degrees of early atherosclerosis and correlates with metabolic and cellular perturbations that are considered important for the vascular process (Skoog, 2002). The levels are high in acute ischemia, post MI patients and stable patients at risk of coronary events (Ridker *et al.*, 2000). There are many reports of TNF-α production by non classical monocytes [which also includes intermediate phenotypes] in vivo (Schlitt *et al.*, 2004) or based on LPS stimulation (Belge *et al.*, 2002) and also during exercise and hypertension (Dimitrov *et al.*, 2013).

In a study by Skrzeczyńska-Moncznik *et al* (2008) intermediate monocytes exhibited an increased phagocytic activity and a decreased antigen presentation in comparison with NCMs. LPS-stimulated NCMs produced TNF-α but little IL-10. By contrast, LPS-stimulated intermediate subpopulation produced significantly more IL-10 than non classical and classical monocytes.

IL-1-β is the most studied member of the IL-1 family because of its role in mediating autoinflammatory diseases. The IL-1 family also includes members that

suppress inflammation, and also those involved in the innate immune response (Dinarello, 2009).

Circulating levels of IL-1 are associated with the presence of traditional cardiac risk factors, such as diabetes mellitus, hypertension, smoking, and dyslipidemia. Elevated levels of IL-1 result in secretion of chemokines and other cytokines [eg, IL-6], increased expression of adhesion molecules, activation of endothelial and SMC proliferation, MΦ activation, and increased vascular permeability. This cascade promotes atherosclerosis and plaque destabilization.

IL-1 and other proinflammatory cytokines have also been implicated in the progression of heart failure, as a result of their negative inotropic effects and deleterious effects on left ventricular remodelling. Another important mechanism by which IL-1 may enhance atherogenesis and exacerbate left ventricular dysfunction is by contributing to endothelial dysfunction. IL-1 stimulates release of endothelin-1, a potent vasoconstrictor, and IL-1 stimulates i.NOS, which increases the formation of ROS and reactive nitrogen species [eg, nitrotyrosine], which leads to oxidative and so-called nitrosative stress and endothelial dysfunction.

Balloon angioplasty results in increased levels of IL-1-β at the injured segment of the porcine coronary artery but not at uninvolved sites. In clinical studies, IL-1-β has been found in greater concentration in atherosclerotic human coronary arteries. An association between certain IL-1ra gene polymorphisms and the presence and extent of coronary disease, as well as the occurrence of restenosis after coronary stenting, has also been

identified. One of the body's responses to acute inflammatory processes, such as acute coronary syndromes, is to upregulate IL-1ra (Fearon & Fearon, 2008).

LPS, concanavalin A (Con A), and phorbol myristate acetate [PMA] stimuli induces production and release of IL-1-β from human monocytes in vitro. Of the three, LPS demonstrated the greatest potency for IL-1-β production (Jessop & Hoffman, 1993).

Neutrophils respond to IL-1- β signalling more compared to monocyte/ MΦ system. This is by activation of NLRC4 inflammasome within the neutrophils, without undergoing cell death during bacterial infection and sustains pro-inflammatory response by producing IL-1- β in itself. Thus neutrophils are also active source of IL-1-β (Chen *et al.*, 2014).

There are reports that statins and fibrates effectively reduced the release of TNF-α, IL-1-β and lower hs-CRP levels in dyslipidemia patients. The statin- and fibrate-induced suppression of pro-inflammatory cytokine release from monocytes seems to play a role in their beneficial effect on the incidence of cardiovascular events (Okopień *et al.*, 2005). Statin therapy attenuated the production of monocyte pro-inflammatory cytokines especially TNF-α in chronic heart failure [CHF] patients with dyslipidemia (Nakagomi *et al.*, 2012).

MPO is an important pro- inflammatory enzyme analysed in the study. It is a member of the heme peroxidase super family. It is stored within the azurophilic granules of leukocytes that generate reactive intermediates, leading to oxidative damage of host lipids and proteins. It has been shown that MPO is present within atherosclerotic plaque in

human arteries and contributes to atherogenesis by catalyzing oxidative reactions in the vascular wall (Karakas & Koenig, 2012).

MPO -containing macrophages and neutrophils have been described at sites of plaque rupture. MPO-positive cells in thrombi adjacent to disrupted plaques are numerous in diabetic patients (Tavora *et al.*, 2009).

Numerous lines of evidence implicate a role for MPO in the pathogenesis of atherosclerosis. Enriched within vulnerable plaque, MPO serves as an enzymatic source of eicosanoids and bioactive lipids and generates atherogenic forms of both low- and high-density lipoproteins.

Increased systemic levels of MPO and its oxidation products may predict increased cardiovascular risk. During leukocyte activation, MPO amplifies the oxidative potential of the respiratory burst by using hydrogen peroxide as a co-substrate to form more ROS. This can result in the generation of a number of potent oxidant compounds capable of promoting oxidative modification of host tissues. Production of reactive chlorinating species, such as hypochlorous acid, is an activity specific to the MPO pathway.

The antimicrobial activities of these products provide the rationale for the role of MPO in the innate immune response to foreign invasion. Generation of oxidized bioactive lipids provides additional mechanisms linking MPO and inflammatory pathways. The enzyme plays an important role in the formation of arachidonic acid oxidation products involved in the promotion of inflammatory cascades. While this provides evidence that

MPO and its products are important homeostatic factors, evidence suggests that excessive activity of MPO can play a role in inflammatory tissue injury.

Given that MPO adversely influences LDL atherogenicity and HDL functionality, it is possible that inhibiting MPO activity may provide a therapeutic approach to management of both LDL and HDL. The finding that statins partially reduce MPO expression and reduce systemic levels of protein modification by MPO-catalyzed pathways suggests that perhaps some of the so-called pleiotropic benefit of statins may be due in part to influence on MPO levels and activity (Nicholls & Hazen, 2008).

Statins strongly inhibit MPO mRNA expression in human and murine monocyte-MΦs. Reduction of MPO mRNA levels was observed in vivo in leukocytes from statin-fed mice, correlating with reductions in MPO protein and enzyme activity (Kumar & Reynolds, 2005).

2.7.3 Monocyte- Macrophage system as therapeutic target

Monocyte-MΦ system is also emerging as a target for therapy due to their extensive role as mediators of the disease. The role of monocyte phenotypes in various stages of atherosclerosis [in progression/regression] also point to its potential importance in therapeutics.

So the important question whether the cytokines and micro-environment within the vascular wall or bone marrow could be modified to synthesize phenotypes which allow regression of atherosclerosis.

Micro-RNA [mi-R] treatment to target vulnerable atherosclerotic lesions and plaque rupture intend to explore mi-Rs that are able to control the pro-inflammatory function of monocytes and MΦs, targeted through either silencing of pro-atherogenic or augmentation of anti-atherogenic pathways (Martin *et al.*, 2011).

Similarly Si-RNA technology is also emerging as an important tool in therapeutics. They explore cellular signalling mechanisms controlling migration, activation, proliferation, and death of MΦs during development of atherosclerosis (Leuschner *et al.*, 2011).

Selective delivery of drugs to cells of the monocyte-MΦ lineage, using the intracellular carboxyl esterase, human carboxyl esterase-1 [hCE-1], which is expressed predominantly in these cells, is an emerging therapeutic strategy. Selective delivery of many types of intracellular targeted small molecules to monocytes and MΦs by attaching a small esterase-sensitive chemical motif (ESM) that is selectively hydrolyzed within these cells to a charged, pharmacologically active drug is experimented.

ESM versions of histone deacetylase (HDAC) inhibitors, for example, are extremely potent anti-cytokine and anti-arthritic agents with a wider therapeutic window than conventional HDAC inhibitors. In human blood, effects on monocytes [hCE-1-positive] are seen at concentrations 1000-fold lower than those that affect other cell types [hCE-1-negative]. Chemical conjugates of this type, by limiting effects on other cells, could find widespread applicability in the treatment of human diseases where monocyte- MΦs play a key role in disease pathology(Needham *et al.*, 2011).

Mechanisms to deplete macrophages in atherosclerotic lesions involve several mechanisms [Table 7] along with selective targeting of macrophages to cell death [Figure 8]

Table 7: Mechanism to deplete Macrophages in the plaque

Important strategies	Mechanism
I. LIPID LOWERING MECHANISM	
a. Statins & Ezetimibe	Direct lowering of LDL ; pleiotropic effect- reduce the inflammatory reactions involving monocyte-MΦ system
b. Proprotein convertase subtilisin kexin 9 (PCSK 9)	Regulates circulating LDL concentration by inhibiting hepatic LDL receptor mediated LDL uptake
II. INCREASE CHOLESTEROL EFFLUX FROM MACROPHAGE	
a. Reverse cholesterol transport	HDL and apo lipoprotein A-1.
b. Cholesterol ester transport protein (CETP) inhibition	Increasing HDL concentrations, inhibiting CETP, promoting expression of cholesterol transporters
III. DECREASE MONOCYTE RECRUITMENT	
a. Level of adhesion molecules	VCAM-1, ICAM, lymphocyte function associated antigen-1/LFA-1, very late antigen-4/VLA-4 blocking by use of antibodies
b. Level of junctional proteins	Genetic knock out of junctional adhesion molecules and connexins
c. Level of chemokines and their receptors	CCL2 (MCP-1)/CCR2, CCL5/CCR5 and CX_3CL1 CX_3CR5 along with reduction of RANTES and MMPs
d. Level of macrophage migration inhibitory factor (MIF).	Involved in the up regulation of adhesion molecules, chemokine receptors and toll like receptors (TLRs)
IV. INHIBITION OF MONOCYTE ACTIVATION: TARGETING PLATELETS	Affecting RANTES, PF-4, P-selectin, CD40L and triggering receptor expressed on myeloid cells-1/ TREM-1 ligand and monocyte platelet aggregates) by using antiplatelet drugs especially clopidogrel.

V.	INHIBITION OF MACROPHAGE ACTIVATION	By inhibiting IFN-γ and CD40L

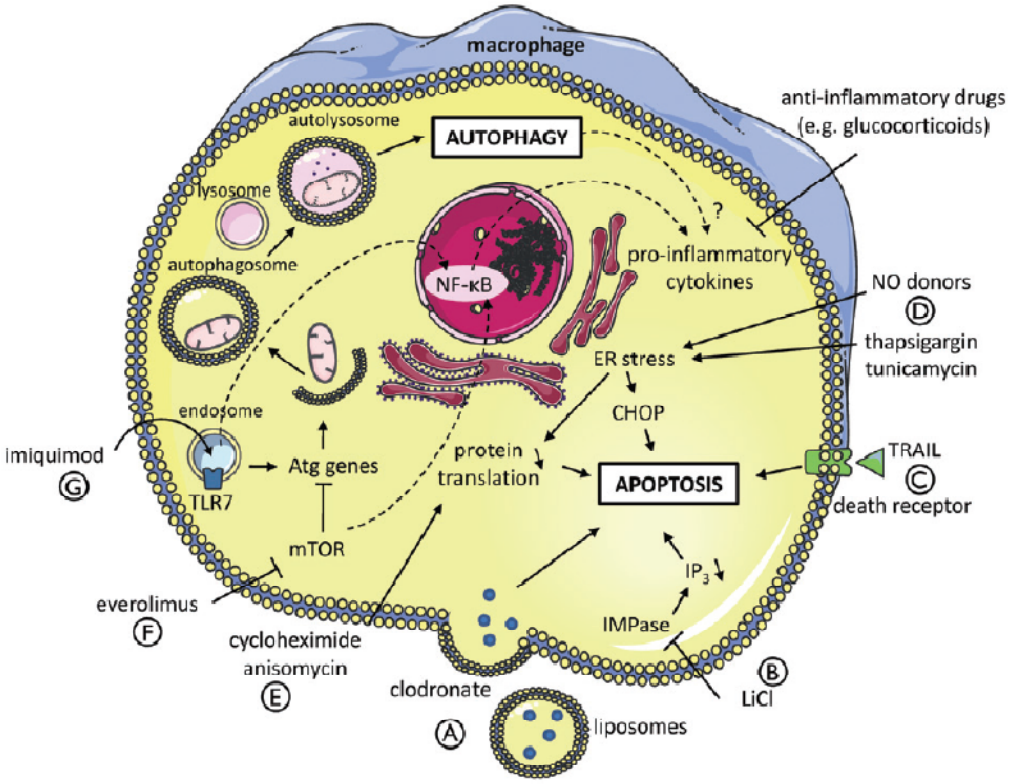


Figure 9: Targets to induce macrophage death

Clodronate-containing liposomes are ingested by phagocytes and the released clodronate induces apoptosis [A]. Interfering with phosphatidyl inositol cell-signalling through inhibition of IMPase/inositol mono phosphatase by lithium chloride [B] leads to decreased free inositol. The subsequent decrease in IP3/ inositol tri phosphate concentrations triggers apoptotic cell death. Systemic administration of recombinant TRAIL/ TNF-Related Apoptosis Inducing Ligand [C] induces apoptosis via its death receptor in infiltrating monocytes in atherosclerotic plaques but not in circulating

monocytes. Treatment of atherosclerotic plaques with an NO donor [D] clears MΦs via apoptosis. Induction of MΦ apoptosis is based on inhibition of *de novo* protein synthesis and induction of Endoplasmic reticulum stress/ER stress [CHOP or C/EBP homologous protein expression].

ER stress inducers, thapsigargin and tunicamycin [D], also initiate selective macrophage apoptosis. Protein translation inhibitors, cycloheximide and anisomycin selectively clear MΦs from atherosclerotic plaques by apoptosis [E]. Everolimus, the mTOR/ Mechanistic Target of Rapamycin inhibitor [F], induce autophagic cell death in MΦs via activation of Atg/ AuTophaGy related genes that attribute to autophagosome and ultimately auto lysosome formation. Stimulation of the endosomal receptor TLR7 with imiquimod [G] initiates autophagy exclusively in MΦs. TLR7 stimulation by imiquimod, as well as mTOR inhibition by everolimus, leads to the release of pro-inflammatory cytokines that can be abolished by adding anti-inflammatory drugs, such as glucocorticoids.

Even though results with these new drugs are promising in animal studies, clinical trials are so far disappointing due to side effects or the lack of additional benefits. Targeting plaque MΦs has been the focus of recent research, not only for cell-specific delivery of drugs, but also for the detection and imaging of vulnerable plaques. Recent progress in MΦ-targeted nanostructures and bio-resorbable drug-eluting scaffolds may facilitate therapeutic atherosclerosis treatment with minimal adverse effects (De Meyer *et al.*, 2012).

III. MATERIALS AND METHODS

This section describes the details regarding data collection, analytical procedures and the equipment used for the study

3.1 Study Design

We obtained data on young CAD patients based on their demography (gender and age) and conventional risk factor profile (family history, hypertension, diabetes, smoking habits, dyslipidaemia), angiographic profile, mode of presentation of CAD, treatment patterns and follow up for the entire study cohort.

The second part of the study consisted of two case-control studies. In the first case-control study thrombotic risk factors [fibrinogen, Lp(a), v-WF, t-PA, PAI and HCy] and antithrombotic factors [AT-III and Protein C] were determined along with platelet activation by P-selectin expression on platelets and inflammatory marker - hs-CRP level in a subset of CAD patients and controls.

The second case- control study consisted of monocyte phenotype characterization based on the expression of CD14 and CD16 markers and analysis of its relation with important inflammatory factors such as TNF-α, IL-1-β and MPO along with determination of white blood cell (WBC) count and thrombotic risk factor fibrinogen in a different cohort of CAD patients and controls.

In both the case control studies, conventional risk factors, mode of presentation, and angiographic profile were compared with non-conventional risk factors.

Subjects: Those patients, aged ≤55 years who were admitted to the department of Cardiology for coronary angiography during the year 2000-2012 were enrolled into the database [N= 5467]. A small subset of 209 [case- control study 1: n=152; case- control study 2: n=57] patients from this group who were admitted during 2011-2015 was selected for case-control studies. Controls were free living subjects who volunteered to participate and who belonged to the same age category (≤ 55 years), were enrolled during the same time period [N=169; case- control study 1: n=102; case- control study 2: n=67].

The entire study was conducted at the Departments of Cardiology, Biochemistry and Thrombosis Research Unit/ TRU [Biomedical Technology Wing] of Sree Chitra Tirunal Institute for Medical Sciences and Technology [SCTIMST], a tertiary care hospital in the southern state of Kerala, India

All the subjects who participated in the risk factor analysis had given written informed consent, before they provided samples for the study. The study was approved by the Institutional Ethics Committee **(IEC/014; November 28, 2009).**

3.2 Data collection from CAD patients

Clinical data of 5467 consecutive patients, with CAD were collected as they were admitted in SCTIMST for evaluation of CAD and had a coronary angiographic evaluation. Data regarding conventional risk factors such as positive family history [defined as those patients whose parents and/ or siblings had CAD before the age of 60], tobacco smoking [current and ex-smokers], diabetes mellitus [those with fasting blood

glucose level above 127 mg/dl or those taking anti-diabetic medications], dyslipidaemia [those with high total cholesterol, >200 mg/dl; LDL-C, > 100 mg/dl; Triglycerides >130 mg/dl and or those with HDL-C <40 mg/dl (males)/ <50mg/dl (females)] and hypertension [those whose systolic/ diastolic pressure above 140/90 mm of Hg and or taking hypertensive drugs] were collected (Yusuf *et al.*, 2004).

Data of all patients [including cas-control studies], regarding conventional risk factors were obtained from hospital records, while data regarding controls were obtained using a structured questionnaire. Data regarding mode of presentation of CAD, angiographic findings, treatment, follow up data including follow-up events [recurrence of MI/ ACS, repeated hospitalization due to angina, and re-admission for catheterization] and mortality were also obtained from all patients. All the patients were on guideline based therapy which included anti-platelets, statins and other anti-atherosclerotic drugs for the control of disease.

3.3 Inclusion and exclusion criteria

Criteria for patients to be included in the database in addition to the age criteria, were angiographically documented coronary artery disease (defined as >50% stenosis), or documented to have ACS or history of angina pectoris with positive treadmill stress test and angiographically normal coronaries. Patients with valvular diseases and those patients who underwent angiograms for other reasons other than for ruling out CAD were excluded from the study. Patients with infective endocarditis were also excluded. For the case control studies only patients documented to have obstructive CAD were included.

Controls were healthy volunteers, free living subjects, without any proven CAD and unrelated to patients. Control subjects, were recruited from the general population as they visited hospital as by-standers or as blood donors or they were hospital staff.

3.4 Sample collection

Blood sample collection was done, when the patients were admitted in the hospital for elective coronary angiography or angioplasty at least after three months of presentation of symptoms and after at least three months of treatment with statins and anti-platelet drugs. Controls were also recruited almost at the same time of patient enrolment.

Blood samples for thrombotic risk factor analysis [fibrinogen, Lp (a), homocysteine, t-PA, PAI-1, v-WF, AT-III and protein C], platelet activation and hs-CRP were obtained from 152 patients and 102 controls during the period from October 2011 to December 2013. Urine samples for Hcy analysis were collected from selected subjects [Patients n=9 & controls n=12] from the same cohort asthe blood samples were collected.

Blood samples for monocyte characterisation and inflammatory markers [TNF-α, IL-1-β and MPO, blood cell count] were collected during the period of July 2014 to April 2015, from a separate set of patients [n=57] and controls [n=67].

About 10 ml of blood samples were collected from both patients and controls after overnight fasting of 8-12 hours, aliquoted to appropriate vacutainer tubes [citrated, EDTA and plain tubes] and also to heparin sodium rinsed polypropylene tubes for obtaining citrated plasma, EDTA blood, serum and heparin blood respectively. Blood samples were subjected to centrifugation at 2500 rpm (for 10 minutes) - 4000 rpm (for 15 minutes)

speed for separating serum and plasma for different analyses, ensuring that samples are not haemolysed. Samples were processed immediately for the assay of platelet activation [EDTA blood], fibrinogen [citrated plasma], lipid profile, glucose [serum], blood cell count [EDTA blood] and monocyte characterization [Heparin blood]. For rest of the analyses, samples were aliquoted and stored at -20 ^{o} C or -80 ^{o} C and analyzed within one to two months of sample collection.

3.5 Materials

Vacutainer tubes- Plain tubes, K_2EDTA tubes (ethylene diamine tetra acetic acid), and Sodium citrate tubes were obtained from Becton Dickinson Franklin Lakes NJ USA. Heparin Sodium I.P, polypropelene centrifuge tubes, O-dianisidine dihydrochloride [3, 3'- Dimethoxybenzidine dihydrochloride], and HiSep^{TM} LSM with density 1.077±0.001 (density gradient solution for separation of PBMNC from peripheral blood) were purchased from Himedia Laboratories, Mumbai. Sodium chloride, Formaldehyde, Paraformaldehyde, Disodium hydrogen phosphate, Potassium dihydrogen phosphate, Di-potassium hydrogen phosphate, Hydrogen peroxide, Sulphuric acid and Hydrochloric acid, HPLC grade Methanol and Acetonitrile were purchased from Merck [India].

Reagent kits for Antithrombin-III [STACHROM® AT III 3 & AT III 6, Fibrinogen [FIBRI-PREST® 2], Protein C [STACLOT® PROTEIN C], tissue Plasminogen activator (t-PA) [Asserachrom® tPA ELISA], Plasminogen activator inhibitor-1 (PAI-1) [Asserachrom® PAI-1 ELISA and von-Willebrand factor (v-WF) [Asserachrom® VWF:Ag ELISA] were procured from Diagnostica Stago [France]. Reagent kit for Homocysteine [Auto Pure Homocysteine Enzyme Cycling Kit] was purchased from

Accurex Biomedicals Pvt Ltd Mumbai and reagent kit for lipoprotein (a) [Lp(a)-turbilatex] was obtained from SPINREACT [Spain].

P-selectin (CD62p) -Phycoerythrin/ PE conjugated antibody and CD16-Phycoerythrin/ PE conjugated antibody were procured from Beckman Coulter inc. [USA]. CD14-Fluorescent isothiocyanite/FITC conjugated antibody was purchased from Becton Dickinson/BD Pharmigen™ [USA]. Reagent kit for high-sensitivity cross reactive protein (hs-CRP) [ZYMUTEST CRP] was obtained from HYPHEN BioMed [France], ELISA kits for tumour necrosis factor-alpha (TNF-α) [ELH-TNFa] and Interleukin-1- beta (IL-1-β) [ELH-IL1b] were procured from RayBio® tech inc. [USA].

Reagents for glucose [GLU Flex® reagent Cartridge], total cholesterol [CHOL Flex® Reagent Cartridge], Triglycerides [TGL Flex® Reagent Cartridge] and HDL-C [A-HDL] for use in dimension clinical chemistry auto analyzer was purchased from Siemens [USA].

Reagents for measurement of blood cell count [WBC, RBC, Platelet count] and Haemoglobin for use in Beckman Coulter- A^C. T5 differential Haematology analyser [USA] were obtained from Horiba ABx [France]. DL-Homocysteine, Cystamine dihydrochloride and DL-Dithiothreitol were purchased from Sigma [USA]. Formic acid was purchased from Rankem [India] and Sodium hydroxide was obtained from S.D. Fine Chemicals [India]. Eppendorf tubes were procured from Tarson Pvt LTD [India].

3.6 Analysis of conventional risk factors

3.6.1 Assay of Fasting blood glucose

Blood glucose was quantitated by the hexokinase/glucose-6- phosphate dehydrogenase method using GLU FLEX® reagent cartridge [Siemens, USA] in Dimension® Clinical Chemistry auto analyser according to manufacturer's protocol. Reference range standardised for fasting blood glucose is 70-110 mg/dl.

3.6.2 Assay of Total Cholesterol

Total cholesterol was estimated in serum by the cholesterol esterase- cholesterol oxidase-peroxidase method using CHOL FLEX® reagent cartridge [Siemens, USA] in Dimension® Clinical Chemistry auto analyser according to manufacturer's protocol.

Normal range of total serum cholesterol should be less than 200 mg/dl of blood.

3.6.3 Assay of Triglycerides

Triglyceride in serum was quantitated by the enzymatic method using TGL FLEX® reagent cartridge [Siemens, USA] in Dimension® Clinical Chemistry auto analyser according to manufacturer's protocol.

Normal range of serum Triglycerides: 30-150 mg/dl.

3.6.4 Assay of High Density Lipoprotein-Cholesterol (HDL-C)

HDL-C in serum was determined by the enzymatic method using AHDL FLEX® reagent cartridge in Dimension® Clinical Chemistry auto analyser according to manufacturer's protocol.

Normal range of HDL-C is >40 mg/dl in males; >50 mg/dl in females.

3.6.5 Determination of Low Density Lipoprotein-Cholesterol (LDL-C)

LDL-C in serum was derived indirectly using Friedewald equation (Friedewald *et.al.*, 1972) LDL-C= Total Cholesterol- (VLDL-C+HDL-C, (where VLDL-C = Triglycerides/5)

Normal range of LDL-C was considered less than 100 mg/dl.

3.7 Analysis of thrombotic, antithrombotic factors, platelet activation by P-selectin and analysis of hs-CRP

3.7.1 Assay of Fibrinogen

Plasma fibrinogen levels were determined by the clotting method using FIBRI-PREST® reagent kit in *Starline* Analyzer [Diagnostica Stago France] according to manufacturer's protocol.

Principle: This test method involves measuring the rate of conversion of fibrinogen to fibrin in diluted sample under the influence of excess thrombin. The clotting time can be used as a measure of the concentration of the fibrinogen. The observed clotting time was

usually between 8 and 25 seconds, when plasma fibrinogen levels (fibrinogenaemia) were between 1.5 and 4 gram/litre (g/l).

The normal plasma range of fibrinogen is usually between 2 and 4 g/l.

3.7.2 Assay of Lipoprotein(a) [Lp(a)]

Lipoprotein (a) [Lp(a)] in serum was quantitated by turbidimetry method using Lp(a)-turbilatex kit [SPINREACT, Spain] in UV-1601PC-spectrometer [Shimadzu, Japan] , according to manufacturer's protocol.

Principle: Latex particles coated with antibodies to Lp(a)-anti-Lp(a) are agglutinated when mixed with samples containing Lp(a). The agglutination causes an absorbance change dependent upon the Lp(a) contents of the sample that can be quantified by comparison from a calibrator of known Lp(a) concentration.

Procedure: A fixed volume of diluted calibrators/ serum aliquoted and stored [frozen samples stored at -20 ^{o} C up to 1 month] was mixed with appropriate volume of reagents as directed by manufacturer's instruction. Absorbance of calibrator/sample was immediately read at first minute (A_1) and also after 4 minutes (A_2) at a wavelength of 570 nm. Calibration curve was prepared by calculating the absorbance differences (A_2-A_1) of each calibrator and plotting the values against Lp(a) concentration in a calibration curve. Lp(a) concentration in the sample was calculated by interpolation of its (A_2-A_1) in the calibration curve.

Normal value of Lp(a) should be less than 30 mg/dl of serum.

3.7.3 Assay of von-Willebrand factor (v-WF)

An enzyme-linked immunosorbent assay was employed for the measurement of the v-WF in plasma, using ASSERACHROM® VWF: Ag reagent kit from Diagnostica Stago France.

Principle: The v-WF to be measured is captured by rabbit anti-human v-WF antibodies coated on the internal walls of ELISA plate. Bound v-WF was detected by a second peroxidase- conjugated antibody, using a substrate that reacts with peroxidase complex to produce a measurable signal proportional to v-WF.

Procedure: Samples and standards were added to the wells of ELISA plate coated with rabbit anti-human v-WF antibodies, then treated with a second antibody, rabbit anti-v-WF antibody-coupled with peroxidase, which bind to the remaining free antigenic determinants of the bound v-WF. The bound enzyme peroxidase was revealed by its action on the substrate, TMB [tetramethyl benzidine]. The reaction was stopped with a strong acid. The intensity of the colour was read at 450 nm, after stopping the reaction, using an ELISA plate reader [iMark microplate absorbance reader, Bio-Rad Laboratories, Inc. USA]. The intensity of colour will be proportional to the concentration of v-WF present in the original specimen.

v-WF level in plasma is usually in the range of 50-160%.

3.7.4 Assay of tissue –Plasminogen activator (t-PA)

Tissue-plasminogen activator (t-PA) in plasma was measured by the enzyme-linked immunosorbent assay (ELISA) using Asserachrom® tPA reagent kit [Diagnostica Stago France] according to manufacturer's protocol.

Principle: t-PA to be measured is captured by t-PA specific antibody pre-coated onto ELISA plates. Subsequently it is treated with another t-PA specific antibody -conjugated with peroxidase and the peroxidase reaction is visualized using a substrate.

Procedure: Samples and standards were added to the wells of ELISA plate pre-coated with mouse monoclonal anti-human t-PA antibodies, then treated with a second mouse monoclonal anti-human t-PA antibody coupled with peroxidase, which bind to another antigenic determinant of the bound t-PA. The bound enzyme peroxidase was revealed by its action on the substrate, TMB. After stopping the reaction with a strong acid, the intensity of the colour was measured at 450 nm, using ELISA plate reader [iMark microplate absorbance reader, Bio-Rad Laboratories, Inc. USA] which is directly proportional to the concentration of t-PA initially present in the sample.

The t-PA plasma level is usually in the range of 2-12 ng/ml.

3.7.5 Assay of Plasminogen activator inhibitor-1(PAI-1)

PAI-1 is the principal inhibitor of tissue plasminogen activator (t-PA) and urokinase, the activators of plasminogen and hence it is an inhibitor of fibrinolysis.

Enzyme immunoassay was used for the determination of PAI-1 using Asserachrom® PAI-1 regent kit [Diagnostica Stago France], according to manufacturer's protocol.

Principle: This assay employs a quantitative enzyme immunoassay technique that measures the specified antigen, PAI-1, in plasma. PAI-1 to be measured is captured by a PAI-1 specific antibody pre-coated onto ELISA plates. Subsequently it is treated with another PAI-1 - specific antibody conjugated with peroxidase and the peroxidation reaction is visualized using a substrate.

Procedure: Samples and standards were added to the wells of ELISA plate coated with mouse monoclonal anti-human PAI-1 antibody, then treated with a second mouse monoclonal anti-human PAI-1 antibody coupled with peroxidase that bind to another antigenic determinant distant from the first one forming the "sandwich". The bound enzyme peroxidase was then revealed by its activity on the substrate, ortho phenylene diamine, in the presence of hydrogen peroxide. After stopping the reaction with a strong acid, the intensity of the colour was measured at 492 nm using ELISA plate reader [iMark microplate absorbance reader, Bio-Rad Laboratories, Inc. USA]

The PAI-1 level of platelet-free plasma is in the range of 4-43 ng/ml.

3.7.6 Assay of Homocysteine (Hcy) in serum

Total L-homocysteine in human serum was quantitated using Auto Pure Homocysteine Enzyme Cycling Kit [Accurex Biomedicals] in Autochem Nexgen semi-automated clinical chemistry analyzer [Span Diagnostics, India] according to manufacturer's instruction.

Principle: The assay is based on the measurement of co-substrate conversion product. Oxidized Hcy from the sample is reduced to free Hcy which then reacts with S-adenosyl methionine to form methionine and S-adenosyl homocysteine. S-adenosyl homocysteine is assessed by coupled enzyme reactions wherein adenosine is formed. The adenosine formed is hydrolysed into inosine and ammonia which reacts with glutamate dehydrogenase with concomitant conversions of NADH to NAD^+. The concentration of Hcy is proportional to the amount of NADH converted to NAD^+ and is measured as change in absorbance at 340 nm.

Serum Hcy values should be less than 15µmol/l.

3.7.7 Assay of Homocysteine in urine *(New method developed as part of the study)*

Hcy is a sulphur containing amino acid and has been shown to inhibit the activity of endothelial dimethyl amino hydrolase [DDAH], causing the accumulation of asymmetric dimethyl arginine [ADMA] and inhibition of nitric oxide synthesis. The concentrations of Hcy in biological fluids are used in the clinical diagnosis of cardiovascular diseases and this necessitates the development of a rapid and sensitive method for simultaneous determination of Hcy. A rapid, simple and sensitive method for simultaneous determination of Hcy by liquid chromatography–tandem mass spectrometry (LC–MS/MS) coupled with electro spray ionization (ESI) in human urine was developed for the study (Gopu *et al.*, 2013). Using this method Hcy was assayed in urine samples of patients and controls.

Principle: LC-MS/MS combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry (MS).

Sample preparation: An aliquot of diluted urine samples (1:9 with deionized water)/calibrator samples/QC samples were mixed with 10 µl of cystamine dihydrochloride [Internal Standard/IS], 10 µl of 0.1 N NaOH was added to adjust the urine pH > 7. The disulfide bonds of Hcy in the urine was reduced by the addition of freshly prepared 500 mmol L^{-1} DL- Dithiothreitol [DTT] solution, vortexed for 5 seconds and left at room temperature for 15 minutes. Then 0.1% formic acid in methanol was added to precipitate the proteins. The mixture was vortexed for 5 seconds then centrifuged for 10 minutes at 14,000 rpm. The supernatant was taken in an LC–MS certified sample vial [Waters, Milford, MA, USA] and injected for analysis.

LC–ESI–MS/MS analysis: High-performance liquid chromatography separations were performed on a Waters Alliance [Waters Co., Milford, MA, USA] system, using Atlantis HILIC silica column (100 mm × 2.1 mm) with a particle size of 5µ m. The mobile phase consisted of water/methanol/acetonitrile (35/35/30, v/v) containing 0.2% formic acid with a flow-rate of 0.2 ml min^{-1} and a total run time of 4.5 minutes. Positive multiple reactions monitoring [MRM] mode was chosen for quantification of each analyte and cystamine dihydrochloride [CYA] was used as the internal standard for the assay. Mass spectrometric analyses were performed with a Micromass Quattro micro triple quadrupole mass spectrometer [Micromass UK Limited, Manchester, England] equipped

with an electro spray ion source [ESI] operating in positive mode. Data were acquired with Mass Lynx 4.1 software.

Results are obtained in chromatogram based on mass to charge ratio as peaks in the chromatogram and results are expressed in micromoles /litre.

3.7.8 Assay of Antithrombin-III [AT-III]

Plasma AT-III activity was measured using an amidolytic method [Chromogenic substrate assay using STACHROM® AT III reagent kit] in auto analyser STA® line [Diagnostica Stago, France] according to manufacturer's instruction.

Principle: The test principle is based on the powerful and immediate inhibitory action of plasma AT- III on thrombin in the presence of heparin The plasma to be tested is incubated in a known excess of thrombin in the presence of heparin. Antithrombin in plasma exerts a powerful inhibitory action on thrombin. The residual thrombin is quantitated by its amidolytic action on the synthetic chromogenic substrate ethyl-malonyl-S-Pro-Arg-pNA.AcOH–CBS 61.50, resulting in the release of the product para nitro aniline (pNA), measured at 405 nm. The quantity of thrombin neutralized by the initial reaction step is proportional to antithrombin level in the plasma being tested. Then the residual thrombin in the second reaction step as measured by pNA release is inversely proportional to the antithrombin present in the plasma tested. Thus plasma AT-III is automatically calculated by the instrument.

The normal plasma antithrombin level is in the range of 80% to 120%.

3.7.9 Assay of Protein C in plasma

Protein C activity in plasma was measured by the clotting method using STACLOT® PROTEIN C reagent kit in auto- analyzer of STA® line [Diagnostica Stago France], based on manufacturer's instruction.

Principle: Protein C is activated in the presence of the specific activator extracted from *Agkistrodon c. contortrix* venom. The resulting activated protein C inhibits the clotting factors, factor -V and factor- VIII and thus prolongs the activated partial thromboplastin time (APTT) of a system in which all the factors are present[constant and in excess], except the Protein C which is derived from the sample being tested.

Procedure: A fixed volume of citrated plasma was taken from aliquot of citrated serum [frozen plasma samples stored at -80^{o}C up to 3 months] was mixed with the appropriate reagent and assayed using Analyzer of STA® line. The Protein C level is expressed as percentage (%).

The normal plasma range of Protein C is usually between 70% and 130%.

3.7.10 Assay of Platelet activation in whole blood

Platelet activation in blood was assayed by flow cytometry (Michelson, 1996) using antibody to P-selectin (CD62p) [Phycoerythrin/ PE conjugated antibody from Beckman Coulter (Ref: IM1759U)].

Principle: Platelets are generally circular or discoid in shape, but when activated their shape become irregular. Platelets are composed of variety of granules like alpha granules

and P-selectin is stored within these granules. During platelet activation, alpha granules coalesce with platelet membrane exposing P-selectin. This P-selectin can be detected using flow cytometry using flurochrome labelled P-selectin antibody. Platelets can be identified by appropriate gating procedure in flow cytometry and from these gated cells, percentage activation of platelets can be measured by determination of fluorescence. It is a cellular marker for thrombosis as well as inflammation.

Procedure: Appropriate dilutions were made on fresh EDTA blood using phosphate buffered saline [PBS- pH 7.4] to adjust the platelet count to 2-2.5 x 10^5 cells. To the tubes containing cells, 5 µl of CD62p PE antibody was added and incubated in dark for one hour. The sample was then diluted in 750 µl of PBS and analyzed directly or fixed in paraformalehyde [3.7%] for 20 minutes for storage at 4 ^{o} C and analyzed within seven days of storage. The sample was analysed using flow cytometry [Beckman-Coulter flow cytometre (COULTER Epics XL)] using 488 nm laser. When platelet was activated, P-selectin was exposed on to the platelet surface to which PE conjugated antibody bind. The intensity of fluorescence was expressed as percentage that indicates the level of platelet activation. Unstained cells were used to gate the negative population of cells to eliminate auto-fluorescence of the cells.

There is no definite reference range for P-selectin exposure as an indication of platelet activation. P-selectin exposure of 0.5% is considered basal, above this level there is increased platelet activation.

3.7.11 Assay of hs-CRP

Cross - reactive protein (CRP), also known as Pentraxin 1, is a secreted pentameric protein that functions as a sensor and activator for the innate immune response. In humans, it is a major acute-phase protein; its circulating concentration is dramatically elevated at the onset of inflammation. CRP level in serum was measured using the Zymutest CRP ELISA reagent kit, a two-site high sensitivity immuno assay for measuring CRP in serum as described by manufacturer‘s instruction (Hyphen Biomed, France).

Principle: Zymutest CRP is a ‘one step sandwich ELISA technique specific for CRP. Serum is allowed to react simultaneously with the two antibodies, specific to CRP, resulting in the CRP molecule being sandwiched between the solid phase antibody and enzyme-linked (peroxidase-conjugated) antibodies. The peroxidase is then visualized using a substrate.

Procedure: First, the immuno-conjugate, a goat polyclonal antibody, specific for human CRP and coupled to horse-radish peroxidase (HRP) was introduced into the microwell, precoated with a polyclonal antibody [F (ab‘) 2 fragments] specific for CRP. Then, the serum/standard was introduced, and the immunological reaction was initiated. CRP present in the sample bound to the coated microwell and reacted with the immuno-conjugate simultaneously. Following a washing step, the peroxidase substrate, TMB in the presence of hydrogen peroxide was added. The reaction was stopped with 0.45 M sulphuric acid and the absorbance was measured spectrophotometrically at 450 nm in

ELISA microplate reader [BioTEK ELISA reader: *ELx 800*(USA)]. The amount of colour developed is directly proportional to the concentration of CRP.

3.8 Monocyte Phenotype Characterization and assay of inflammatory markers

3.8.1 Determination of Blood cell count [Red Blood Cell (RBC) count, white blood cell count (WBC)-total and differential count, platelet count in blood] and Haemoglobin

Blood cell count: RBC count, WBC-total and differential count, platelet count and haemoglobin concentration were determined in fresh EDTA blood using automated A^C•T™ 5diff Cap Pierce Haematology Analyzer [Beckman Coulter, USA] using coulter principle, which is capable of measuring complete blood counts-WBC, WBC differentials (5 part differentials), RBC, platelets and level of haemoglobin**.**

Reference range:

RBC in males: 4.5-6 million cells/ mm^3 and in females are 4.1- 5.4 million cells/ mm^3 of blood.

Platelet count: 1.5 -4.5 lakh cells/ mm^3 of blood.

WBC count: 5-10 X 10^3 cells/µl of blood. It is generally expressed as 5000-10,000 cells/ mm^3 of blood.

Differential WBC count

Neutrophils: 2-6 X 10^3 cells/µl of blood or 40-60% of Total WBC count.

Lymphocytes: 1-4 X 10^3 cells/µl of blood or 20-40% of Total WBC count.

Eosnophils: 0.1- 0.6 X 10^3 cells/µl of blood or 2-6% of Total WBC count.

Monocytes: 0.1-0.8 X 10^3 cells/µl of blood or 2-8% of Total WBC count.

Basophils: 0-0.05 X 10^3 cells µl of blood or 0-1% of Total WBC count.

Reference range for Haemoglobin is 14-18 g/dl of blood in males and 12-16 g/dl of blood in females.

3.8.2 Monocyte Phenotype characterization in peripheral blood

Circulating monocytes can be subdivided into functionally distinct subpopulations based on differential expression of surface molecules. Blood monocyte phenotypes were characterized by flow cytometry technique (Passlick, *et.al.*, 1989) using fluorochrome labelled antibodies to CD14 [CD14 FITC] and CD16 [CD16 PE]; the surface markers expressed on monocytes (Strauss-Ayali *et.al.*, 2007). Flow cytometric analysis was performed by FACS *Aria* flow cytometre using FACS DEVA software [Becton Dickinson, Oxford, UK].

CD14 is generally expressed by all monocytes, while CD16 is expressed by monocytes, macrophages, natural killer cells and lymphocytes. There are mainly two subsets of monocyte phenotypes named as classical monocytes/CM [$CD14^{++}CD16^{-}$] and non classical monocytes [$CD14^{+}CD16^{+}$].

Principle: Phenotypic characterization of monocyte is mainly based on the expression intensity of surface markers (receptors) – namely CD14 [cluster of differentiation marker 14, a lipopolysacharide receptor] and CD16 [cluster of differentiation marker 16, Function class III receptor, binding to functional class (Fc) portion of Immunoglobulin G and hence the name FcγRIII].Using flow cytometry, human blood monocytes are characterized by forward and side scatter as well as the expression of the receptors- CD14 and CD16.

Procedure:

(a) Isolation of Peripheral Blood Mononuclear Cells [PBMNC]

Peripheral blood mononuclear cells [PBMNCs] were isolated from heparinised venous blood, collected from patients and controls, by density gradient centrifugation using polysucrose containing reagent HiSep^{TM} LSM with density 1.0770±0.0010 g/ml., [Himedia, India] according to the manufacturer's protocol. Briefly, the density solution was transferred to a clean polypropylene tube and to this an equal volume of blood was layered. The tubes were centrifuged at 400 x g at room temperature for 30 minutes. The band of mononuclear cells formed between density solution and plasma layer [buffy coat] was aspirated and transferred to another polypropylene centrifuge tube. An equal volume of isotonic PBS was added, mixed gently by aspiration, and centrifuged at 160-260 x g for 10 minutes at room temperature, to remove density solution and platelet contamination. The cells were suspended in 2 ml of PBS and the cell count of isolated

mononuclear cells was determined with haematology analyzer [A^C. T5 differential Haematology analyser, USA].

(b)Flow Cytometry for phenotypic analysis of monocyte population in blood

Based on cell count 100-300 µl of PBMNC suspension was taken in four different amber coloured eppendorf tubes of 1 ml capacity. To these tubes appropriate antibodies [2.5 µl of CD14 FITC. or CD16 PE, or CD14 FITC +CD16PE] or PBS were added. To all the tubes equal proportion of 3.4% formaldehyde (fixative) and PBS were added. For each samples four eppendorf tubes were prepared as described above and the tubes were incubated in dark for 15 to 20 minutes for fluorescent staining. After staining, samples were analysed by flow cytometry using FACS *Aria* flow cytometre with FACS- DEVA software.

Appropriate gating strategy was used to separate out monocytes ($CD14^+$ cells) from mononuclear cell population. From the gated $CD14^+$ cells, classical and non classical monocyte subsets were identified based on forward scatter-side scatter plot. When 10,000 cells were recorded, a plot of forward scatter (X-axis) and side scatter (Y-axis) were obtained. Forward scatter (FSC) was representative of cell size and side scatter (SSC) was representative cell shape and granularity. Cells represented as dot plots [quarter plots] appeared at four different quarters (gates) depending on the fluorochromes for which they were positive, thus giving the distribution and percentage of monocyte phenotypes. Table 6 and Figure 11 provide details of gating arrangement.

Table 8: Gating arrangement of PBMNC in flow cytometry

GATING	PBMNC phenotype
Q1 (Extreme Y axis PE fluorescence)	CD 16 positive cells
Q2 (Middle, fluorescence for both PE & FITC)	Cells double positive for CD 14 and CD 16
Q3 (Near to origin, cells with no fluorescence)	Cells which are unstained or negative for both CD14 and CD16
Q4 (Extreme X axis, FITC fluorescence)	CD14 positive cells

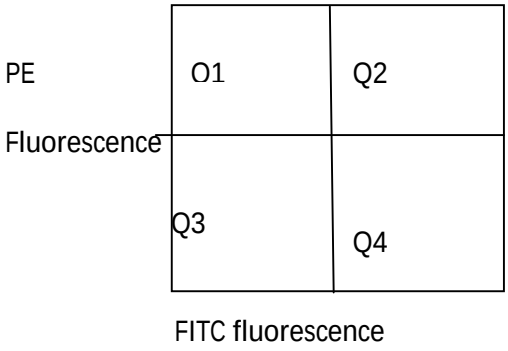


Figure 10: Gating of PBMNC into four quarters based on FITC/PE Fluorescence

PBMNC separated consist of mainly mononuclear cells- lymphocytes and monocytes. All monocytes are CD14 positive hence those cells which fall in CD14 positive gates, could be $CD14^{+}$ alone or $CD14^{+}$ $CD16^{+}$ (double positives). However, Lymphocytes are exclusively $CD16^{+}$ cells. So in a double stained sample CD14 positive cells are obtained on Q4 gate and CD16 positive cells at Q1 and Q2 gate represents cells double stained for CD14 and CD16 [Table 8].

The CD14 positive cells could be gated out from the PBMNC double stained with CD14 FITC and CD16PE antibody, using back gating (Shantsila and Lip 2009). The separated monocytes are again gated as quarter plots [PE fluorescence (Y axis) Vs FITC (X-axis)]. From the quarter plot CM [$CD14^{++}$ $CD16^{-}$ (Q4 Gate)] and NCM

[$CD14^{+}CD16^{+}$ (Q2 Gate)] could be easily identified. The percentage of fluorescence is representative of percentage of cells [Details are presented in the results section].

The expected range of CMs [$CD14^{++}CD16^{-}$] is 70-90% and NCMs [CD14+CD16+] is 10-30% of the total monocytes.

3.8.3 Analysis of Inflammatory markers

In this study, the levels of TNF-α, IL-1-β and MPO were analysed as important inflammatory markers, in order to identify, whether there is a relationship between monocyte subsets and the expression of inflammatory markers in patients and controls. Thrombotic factor-fibrinogen was also measured to examine its relation with monocyte phenotypes.

3.8.3.1 Assay of Tumour Necrosis Factor-alpha (TNF-α)

Human TNF-α is a non-glycosylated protein of 17.5 kDa with 157 amino acids. TNF-α in serum was quantitated using ELISA reagent kit [RayBio®, USA] according to the manufacturer's protocol.

Procedure: This assay employed an antibody specific for human TNF-α, which was coated on a 96-well plate. Aliquots of standards and samples were pipetted into the wells. TNF-α present in the sample was bound to the wells by the immobilized antibody. The wells were washed and biotinylated anti-human TNF-α antibody was added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin was pipetted to the wells. The wells were again washed, a TMB substrate was added and the colour

developed was in proportion to the amount of TNF-α, which was measured at 450 nm using ELISA plate reader [BioTEK ELISA reader: *ELx 800*(USA)].

There is no definite reference ranges as the levels of this inflammatory marker vary based on physiological and anthropological conditions.

3.8.3.2 Assay of Interleukin-1-beta (IL-1-β)

IL-1-β in serum was quantitated using the ELISA reagent kit [RayBio® USA] according to manufacturer's protocol.

Procedure: This assay employed an antibody specific for human IL-1-β, which was coated on a 96-well plate. Aliquots of standards and samples were pipetted into the wells. IL-1-β present in the sample was bound to the wells by the immobilized antibody. The wells were washed and biotinylated anti-human IL-1-β antibody was added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin was pipetted to the wells. The wells were again washed, a TMB substrate was added to the wells and the colour developed was in proportion to the amount of IL-1-β, which was measured at 450 nm in ELISA plate reader [BioTEK ELISA reader: *ELx 800*(USA)].

There is no definite reference ranges as the levels of this inflammatory marker vary based on physiological and anthropological conditions.

3.8.3.3 Assay of Myeloperoxidase (MPO)

MPO activity in human serum was measured spectrophotometrically, using O-dianisidine dihydrochloride and hydrogen peroxide (Bradley *et.al.*, 1982).

Principle: Myeloperoxidase (MPO,EC 1.11.1.7) is a green coloured haemoprotein, released by activated neutrophils, monocytes and tissue associated macrophages.

In the presence of H_2O_2 as oxidizing agent, MPO catalyses the oxidation of *o*-dianisidine yielding a brown coloured product, oxidized *o*-dianisidine, with a maximum absorbance at 470 nm, according to the following overall reaction:

$$2H_2O_2 + o\text{-dianisidine} \rightarrow \text{oxidized dianisidine} + 4H_2O.$$

One unit (U) of MPO activity is defined as that degrading 1 μmol of hydrogen peroxide per minute at 25° C.

Reagents: (a) 50 mM phosphate buffer, pH 6, (b) 0.0005% H_2O_2 in 50 mM phosphate buffer containing 16.7 mg/ 100 ml of O-dianisidine dihydrochloride.

Procedure: An aliquot (0.1 ml) of sample was added to 2.9 ml of reagent [H_2O_2- O-dianisidine dihydrochloride] and the absorbance was measured immediately at 470 nm in a spectrophotometer [UV-Shimadzu Japan] and a second reading was taken after 10 minutes. MPO activity was calculated using the following equation using molar extinction co-efficient (1.3×10^4 $LM^{-1}cm^{-1}$)

Myeloperoxidase activity (U/L) =

$$\frac{\frac{\Delta A}{min} \times total\ volume\ in\ \mu l \times 10^6 \mu\ mol/mol}{Molar\ extinction\ co-efficient\ of\ MPO \times path\ length \times sample\ volume\ in\ \mu l}$$

Where $\frac{\Delta A}{min}$ represents change in absorbance (difference between absorbance at 0^{th} minute and at 10^{th} minute) expressed per minute. , total volume is equivalent to 3000 μl

(2.9 ml reagent & 0.1 ml of sample, giving rise to 3 ml which is 3000 µl), path length in spectrophotometer expressed as 1.0 centimetre, and sample volume is 100 µl (0.1 ml).

3.9 Statistical analysis

Statistical analysis was carried out using Microsoft-office Excel, Graph-Pad prism demo 5 and STATA/IC 11.2 version software. Mean along with standard deviation (SD)/ Standard error of mean (SEM) and or median along with the range (lowest value to highest value) were used for the expression of continuous variables using Microsoft-office Excel. Unpaired Student's t-test and one way Annova was used to compare the difference between mean values. T-test, Annova and Chi-square analysis/ Fisher's exact test (Binary logistic regression analysis) were done using Graph-Pad prism demo 5; Multiple logistic regression analysis, using STATA/IC 11.2 version were used for group comparisons. $p<0.05$ was defined significant. Pearson's correlation analysis [using Graph-Pad prism demo 5] was used for the correlation between different parameters.

IV. RESULTS

(PART-I)

4.1 Database of young CAD patients

The clinical data of 5467 consecutive CAD patients, aged, ≤55 years were collected. These young patients [≤ 55 years] constituted 26.6% of the total CAD patients admitted in the hospital.

4.1.1 Gender and age distribution

Females constituted 12.3% (n=673) of the total population [N=5467]. The mean age of females was 48.98±5.3, while the mean age of males was 47.64±5.9 years (Mean age of the whole group was 47.8±6). Females, who were very young [≤ 45 years] constituted 25% of the total female population, while males of the same age group was 31.6% of the male population [Figure 11]. Thus in the study the females with CAD were slightly older than males. In the whole population of CAD patients [≤55 years], those ≤ 45 years constituted 31% of the total population.

Detailed age and gender based distribution was also calculated, there was an increase in the number of CAD patients as age increases. More patients were at the age group of 45-55 years. [Figure 12]

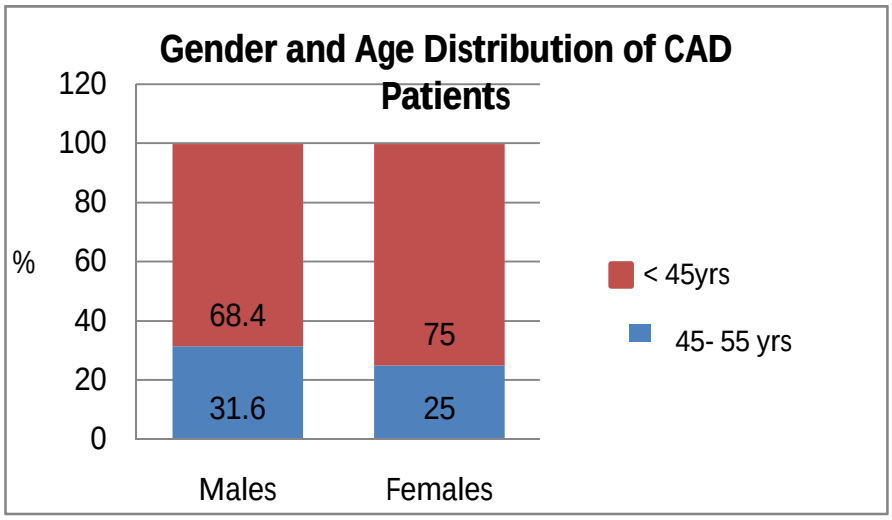


Figure 11: Gender and age distribution of CAD patients: very young patients, <45 years and patients aged 45-55 years

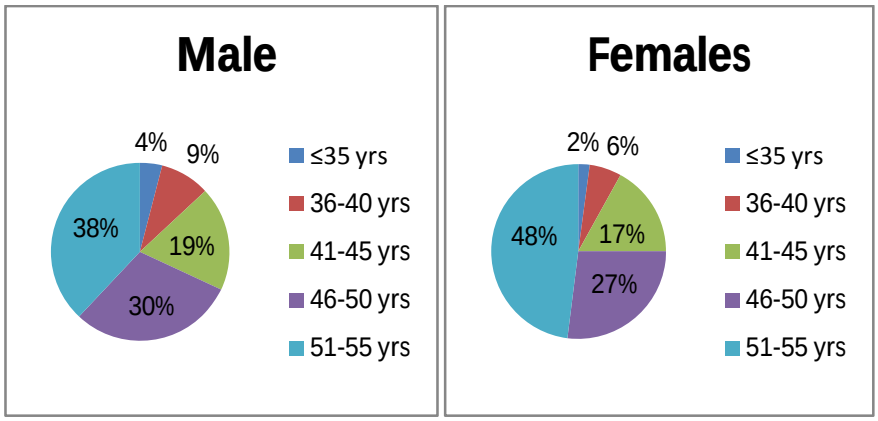


Figure 12 A Figure 12 B

Figure 12: Distribution of male and female patients in different age groups-males (Figure 12 A) and females (Figure 12 B)

4.1.2 Conventional risk factor profile

The conventional risk factor profile of patients was determined [Figure 13]. The data indicates that dyslipidaemia (defined in the methods) is an important risk factor among CAD patients. Among male patients, 65% were smokers, while in females less than 1% was using some form of tobacco. Smoking was the most important risk factor, in the population, after dyslipidaemia followed by hypertension, diabetes and positive family history of CAD.

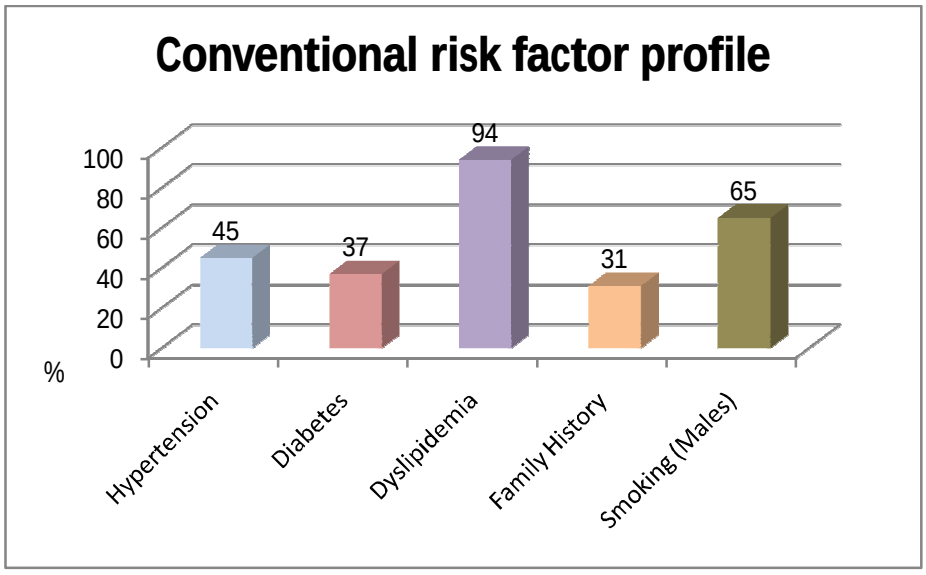


Figure 13: Conventional risk factor profile of whole patient population

When risk factor profiles were compared between male and female patients [Figure 14], dyslipidaemia was identified as a major risk factor for both groups. Apart from smoking and dyslipidaemia all other risk factors were significantly elevated in female patients [$p < 0.0001$].

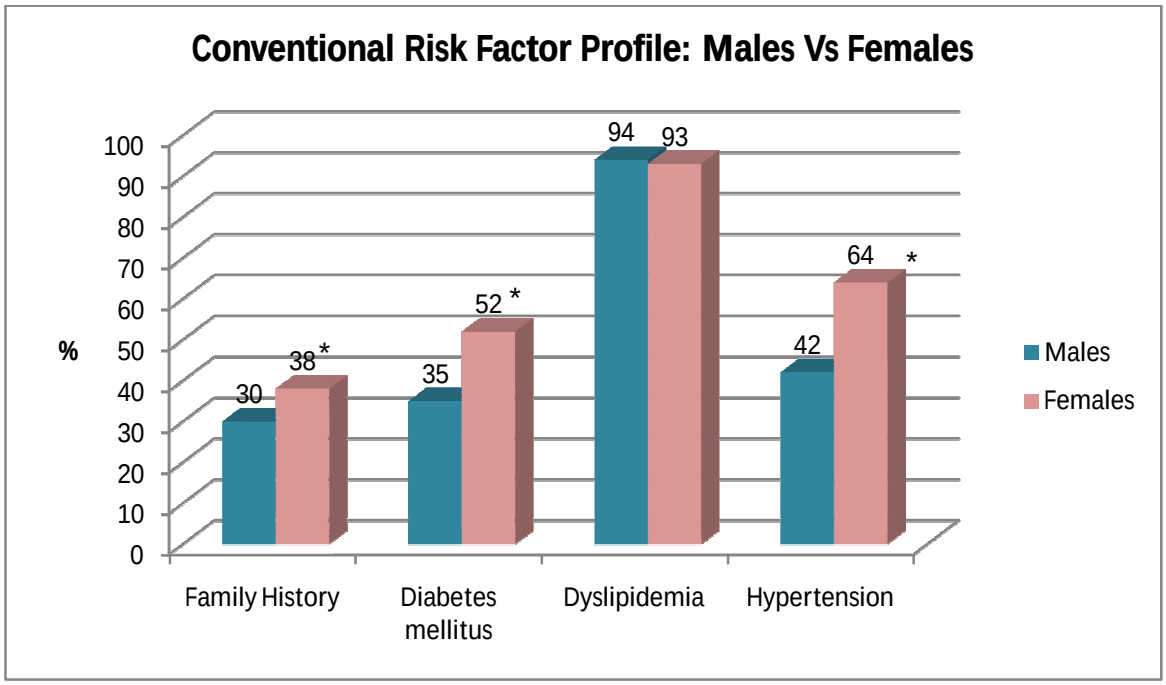


Figure 14: Comparison of conventional risk factors between males & females. [* indicates $p<0.0001$ as indicated by Chi square (χ^2) analysis]

Usually females develop CAD 10 years later than males, unless they are diabetic. Our data analysis revealed that, apart from dyslipidaemia, the high prevalence of risk factors like diabetes mellitus and hypertension predispose females to the development of early CAD.

4.1.3 Mode of presentation of CAD

Based on the mode of presentation with which patients were admitted to the hospital, the patients were categorised into those having ACS or those with EA. The term *acute coronary syndrome* [ACS] refers to any group of clinical symptoms compatible with AMI and it covers the spectrum of clinical conditions ranging from UA to NSTEMI to STEMI.

UA and NSTEMI are closely related conditions: their pathophysiologic origins and clinical presentations are similar, but they differ in severity. A diagnosis of NSTEMI can be made when the ischemia is sufficiently severe to cause myocardial damage that result in the release of a biomarker of myocardial necrosis into the circulation (cardiac-specific troponins T / I/ muscle and brain fraction of creatine kinase [CK-MB]).

In contrast, the patient is considered to have experienced UA if no such biomarker can be detected in the blood stream hours after the initial onset of symptoms of ischemia. UA exhibits 1 or more of 3 principal presentations: (1) rest angina (usually lasting >20 minutes), (2) new-onset (<2 months previously) severe angina, and (3) a crescendo pattern of occurrence (increasing in intensity, duration, frequency, or any combination of these factors).

EA is characterized by chest pain occurring during periods of exercise and relieved by rest. A variant of effort angina is dyspnoea on exertion, which can be another manifestation of myocardial ischemia.

We found that ACS [75%] was the most common mode of presentation of CAD in our group of patients. It was mainly characterized by STEMI and NSTEMI based on electrocardiogram changes. Next common mode of presentation was EA which included chest discomfort typical of EA, this was followed by dyspnoea on exertion (21%). Less than one percent (0.9%) of the patients presented atypical symptoms like back pain and many of them were evaluated due to ECG changes [Figure 15].

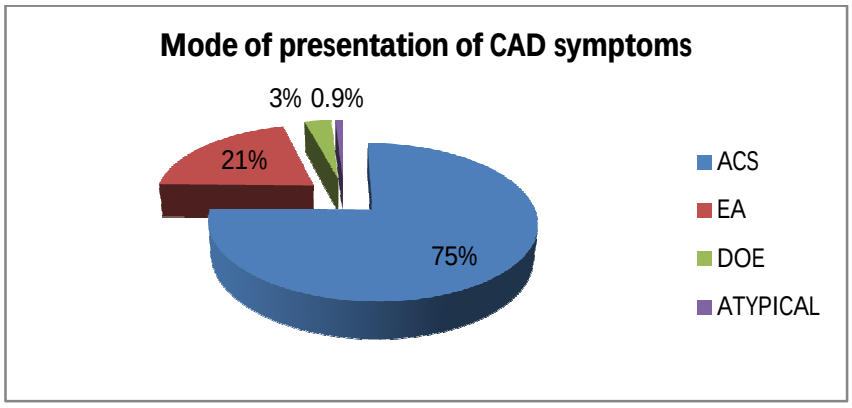


Figure 15: Mode of presentation of CAD in patients, majority presented with ACS.

The mode of presentation of CAD symptoms was found to be different between males and females [Figure 16]. ACS was the major mode of presentation among males [78%] as well as females [54%]. However, effort angina which was the second common mode of presentation, in the female group [35.3%] compared to males [18.6%]. Atypical symptoms were more common among females. Apart from ACS all other modes of presentation was significantly elevated in the female patients.

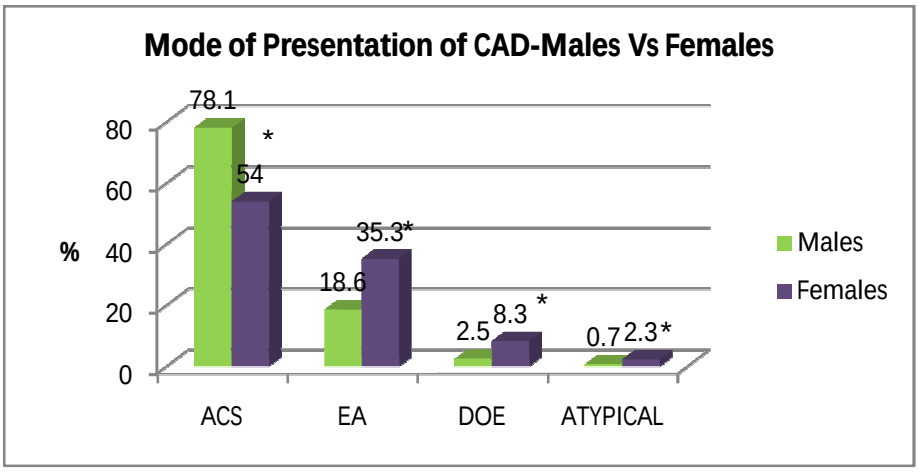


Figure 16: Mode of presentation of CAD in males and females *Significance /p<0.001 as indicated by Chi square (χ^2) analysis.

4.1.4 Angiographic findings

Coronary angiogram was performed using standard techniques by the radial or femoral route in the cardiac catheterization lab. Angiographic findings were classified as normal when there is no evidence of atherosclerotic disease or as minor CAD when the angiographic stenosis was less than 50%. Majority of the patients were diagnosed with single vessel disease (SVD). This was followed by double vessel disease/ two vessel disease (DVD). 19% of the patients had three vessel disease [TVD] [Figure 17]. When angiographic severity among males and females were compared, males had severe form of the disease with higher prevalence of DVD and TVD, while females had significantly increased incidence of mild CAD or normal coronaries compared to males [Figure 18].

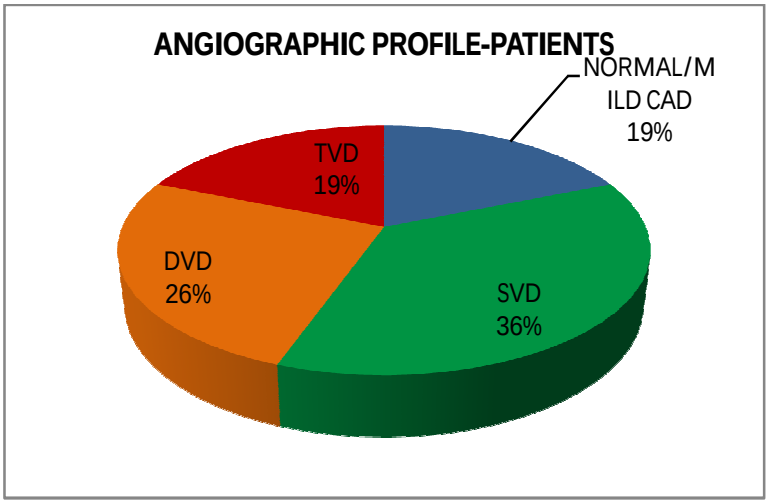


Figure 17: CAG findings in whole population

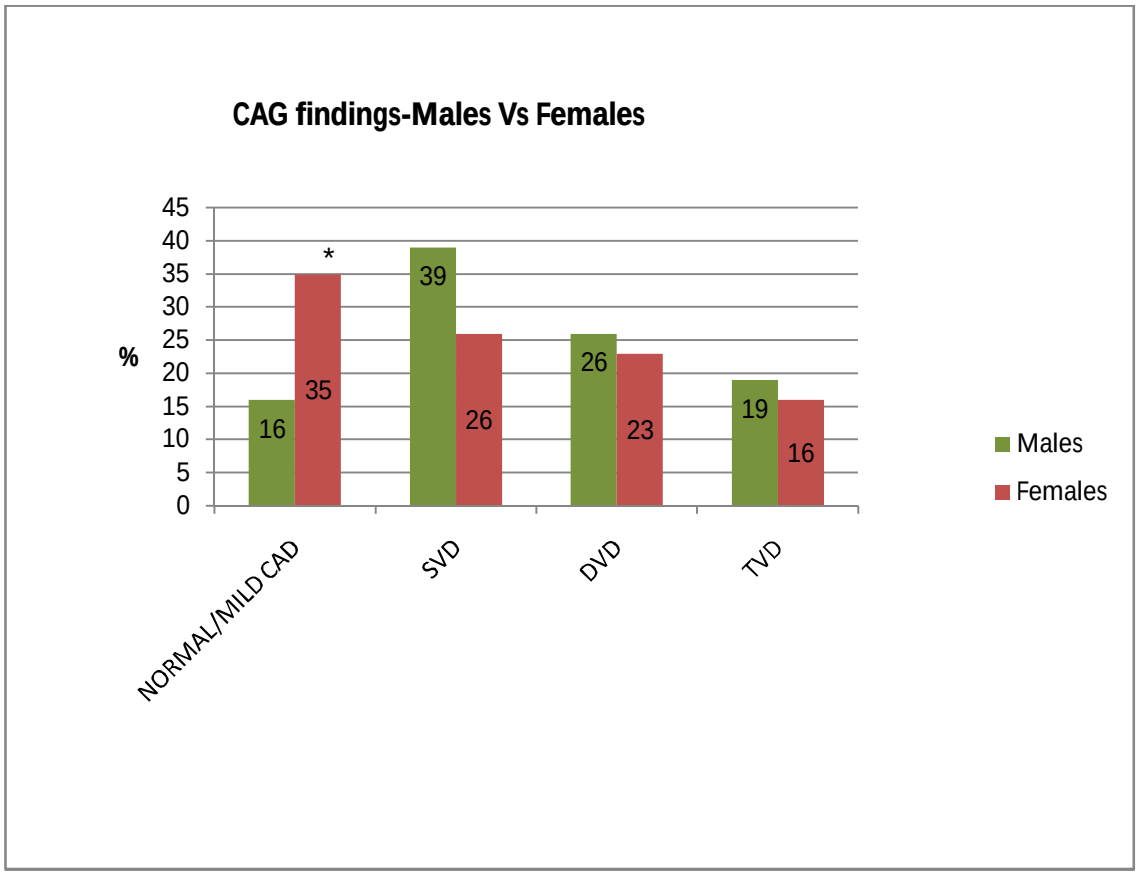


Figure 18: Comparison of coronary angiogram findings between males & females *- significance/ $p<0.001$ by Chi square (χ^2) analysis

4.1.5 Treatment and follow-up of CAD patients

The treatment plan advised by the Cardiologists for these patients following the coronary angiogram is described below. The treatment decisions were based on the currently accepted guidelines of ACC-AHA (Guidelines and Statements ACC/AHA Joint Guidelines 2016). If the patients had TVD or LMCA disease, they were referred for surgery. If the patients had DVD or SVD, they were referred for coronary angioplasty [Percutaneous transluminal coronary angioplasty/PTCA], if feasible. Patients with minor

lesions or diffusely diseased vessels which were poor targets for intervention were kept on medical treatment and reviewed regularly.

When treatment plans of male and female patients were compared, higher proportion of females was continued on medical treatment without any interventions [male-39% vs. females-53%], whereas higher proportion of males were advised for PTCA [male-39% vs females 22%]. Equal proportion [20%] of males and females were referred for CABG.
The average follow up duration of the whole patient group was 51.85±39.6 months with a median of 48 months. The mean follow up duration of males were 52.53 months and females were 46.70 months.

During follow up, it was found that majority [43.1%] of the patients underwent PTCA and a quarter [24.6%] had to undergo CABG. One third of the patients continued on medical management.

Sex-wise comparison based on treatment received during follow up, showed that more males underwent PTCA [male-46% vs. females-24%] and CABG [male-26% vs. female 15%] compared to females. On the other hand, higher proportion of females continued to be on medical management compared to males[male-30% vs. females-62%].

Mortality reported during follow up among the study population was 4% [Figure 19]. When mortality rate was compared between males and females, females had a slightly increased rate (3.6% vs. 5.3%).

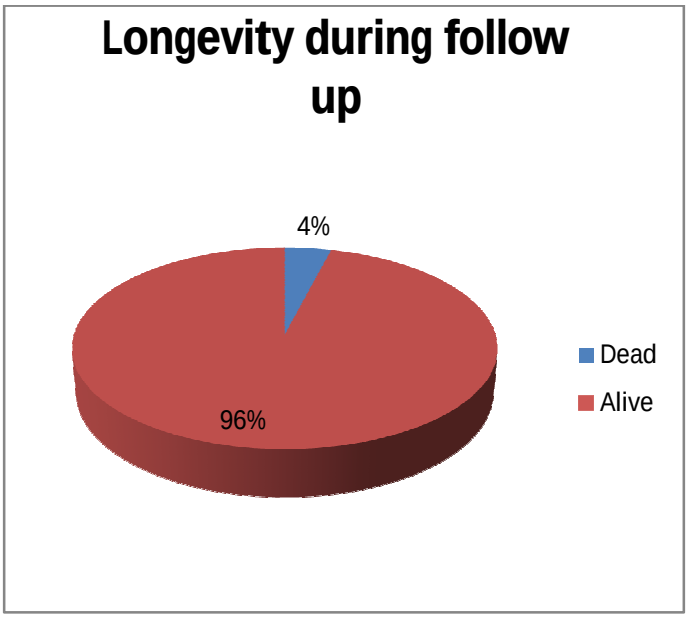


Figure 19: Mortality during follow up .

PART-II: Analysis of non-conventional risk factors for CAD

The analysis of non-conventional risk factors was done as two separate analysis, one involving analysis of important thrombotic, antithrombotic risk factors along with platelet activation by P-selectin expression and hs-CRP [152 patients and 102 controls] and the second one constituting monocyte phenotypic characterization and analysis of important inflammatory markers [57 patients and 67 controls]. These findings were correlated with their conventional risk factor profile, angiographic findings and mode of presentation.

4.2 Analysis of thrombotic-inflammatory risk factors *(Case-Control Study-1)*

The basic characteristics of subjects are described below.

4.2.1 Demography, conventional risk factors and biochemical analysis

Majority of the patients in this subgroup [84%] were males. Dyslipidaemia [as low HDL- C], history of tobacco smoking, diabetes mellitus, hypertension and family history of premature CAD were significantly higher in patients compared to controls [Table 9]. Among conventional risk factors, smoking emerged as an important risk factor among male patients [68% of the male patients gave a history of either current or past smoking]. Controls comparatively had a low risk factor profile [Table 9].

Table 9: Basic characteristics of Patients and controls

	Patients N=152	**Controls N=102**	**P-value**
Age: Mean± SD	45.8±6.6	43.4±9.0	0.027
	Frequency (%)	**Frequency (%)**	
Sex			
Male, N (%)	127 (84%)	77 (76%)	0.113
Female, N (%)	25(16%)	25 (24%)	
Conventional risk factors, N (%):			
Positive Family history to CAD	54(36%)	17 (17%)	<0.05
Smoking	85 (56%)	13 (13%)	<0.001
Diabetes mellitus	89(59%)	17(17%)	<0.001
Hypertension	79(52%)	5(5%)	<0.001
Low HDL	142(93%)	52 (52%)	<0.001
≥1 conventional risk factor	151	71	<0.001
≥2 conventional risk factor	133	22	<0.001

N: number of subjects, %: percentage of subjects from the set of patients or controls taken for thrombotic risk factor analysis, SD: standard deviation. P value indicates significance- Chi-square analysis

When lipid profile of patients and controls were analysed the results showed that, patients had higher rate of dyslipidaemia in the form of low HDL-C only, while levels of total cholesterol, LDL-C and triglycerides were significantly low among patients, compared to controls. This may be due to treatment effect of statins and dietary restrictions, and life style modifications in patients.

Mean while, controls were free living subjects with no dietary resctriction and/or any drugs. But mean fasting blood sugar was significantly higher in patients compared to controls along with HDL-C. Thus patients were more diabetic compared to controls [Table 10].

Table 10: Fasting blood sugar and lipid profile of patients and controls [Case-control study-I]

Parameters (Units)	Patients: Mean± SD	Controls: Mean± SD	'p'value
Fasting blood sugar (mg/dl)	159±85	100±33	<0.001
Total Cholesterol (mg/dl)	145±52	219±45	<0.001
Triglycerides (mg/dl)	127±54	147±69	<0.05
HDL-C (mg/dl)	34±8	41±7	<0.001
LDL-C (mg/dl)	83±33	151±54	<0.001

4.2.2 Analysis of Thrombotic, antithrombotic risk factors, P-selectin and hs-CRP

Thrombotic risk factor analysis showed that mean levels of fibrinogen, Lp(a) and platelet activation based on P-selectin expression were significantly higher in patients compared to controls. Another major finding was that mean value of antithrombotic factor, A-III was significantly lower in patients. PAI-1, (an inhibitor of fibrinolytic enzymes- t-PA and u-PA/urokinase- plasminogen activator), was found to be significantly lowered in patients. There were no significant differences between the levels of t-PA, v-WF, homocysteine and Protein C between patients and controls. Inflammatory factor hs-CRP also showed no significant difference between patients and controls. Summary of thrombotic, inflammatory risk factor analysis) are represented in the Table 11 below.

Table 11: Thrombotic risk factor profile – Patient Vs Control

Parameters[Normal range]	**Patients N=152**		**Controls N=102**		**'p' value**
	Frequency (n)	Mean± SD	Frequency (n)	Mean± SD	
Fibrinogen [2-4 g/l]	149	3.94±1.71	90	3.14±0.88	<0.001
Lp (a) [<30 mg/dl]	149	38.59±33.52	99	21.43±16.78	<0.001
Antithrombin-III [80-120%]	103	60.51±38.24	78	93.22±31.58	<0.001
V-WF [55-200%]	106	146.02±108.66	53	130.05±87.87	0.36
PAI-1 [4-43 ng/ ml]	107	27.42±15.27	53	38.97±22.19	<0.001
t-PA [2-12 ng/ml]	45	14.27±8.67	30	15.71±9.71	0.51

Homocysteine [<30 µM/ml]	45	25.06±13.35	33	22.94±13.86	0.50
Platelet Activation [<0.5%]	142	9.24±11.81	92	1.48±2.85	<0.001
Protein-C [70-130%]	8	130.4±19.99	8	145.5±36.53	0.32
Hs-CRP [0.2-10µg/ml]	33	40.18±5.4	23	30.56±5.6	0.23

SD: standard deviation, n: total number of subjects in which test were done within the total population of patients and controls[N]. Unpaired t test ofthrombotic risk factors with mean and Standard deviation represented as mean ± SD. Normal range is detailed in square brackets.

Initial logistic regression analysis with (un-adjusted odds ratio) for thrombotic risk factors showed similar trends as that of t-test [Table 12]. 84% of the patients had increased platelet activation, 61% of the patients had low antithrombin-III, while 44% of the patients had high Lp(a) and 34% of the patients had high fibrinogen. PAI-1 was significantly low in patients [90%] compared to controls [64%]. When 91% of the patients investigated had high Hcy, 76% of the controls also showed high homocysteine levels. [p=0.073, Odds ratio (OR):3.28 at 95% confidence interval (C.I): 0.90-12.03, (results not shown in the table)]. But the analysis of Hcy levels was limited by the small sample size.

When we analysed the urine samples of a small subset of patients, [n=9] and controls [n=12] using LC-MS/MS method, high levels of Hcy were found among patients compared to controls [Results provided in the next section].

Logistic regression analysis with adjusted odds ratio was done for important thrombotic risk factors [Fibrinogen, platelet activation, antithrombin-III, Lp(a), PAI-1 and v-WF]. The logistic regression analysis of adjusted odds ratio revealed that high Lp(a) and low antithrombin-III were significant independent risk factors among thrombotic risk factors analysed. This was followed by platelet activation and high fibrinogen levels among patients.

PAI-1 levels were significantly lower in patients even after multiple regression analysis, while v-WF levels showed no significant difference between patients and controls. For other thrombotic risk factors sample size was not uniform/ low to be included into the logistic analyses with adjusted odds ratio. The results of logistic regression analysis are given in Table 12 below.

Table 12: Logistic regression analysis of thrombotic risk factors

Parameters		**Patients n (%)**	**Controls n (%)**	**Binary logistic regression analysis**			
				Un adjusted Odds ratio	95% CI	*Adjusted odds ratio	95% CI
Fibrinogen levels:	Fibrinogen <4g/l	99 (67%)	77 (86%)	1		7.92	0.93-67.19
	Fibrinogen >4g/dl	50 (34%)	13 (14%)	2.99	1.52-5.90		
AT-III levels:	AT-III >80%	40 (39%)	61 (78%)	1			
	AT-III <80%	63 (61%)	17 (22%)	5.65	2.90-11.02	6.16	1.03-36.73
Lp(a)	Lp(a)	83	77 (78%)	1			

levels:	<30mg/dl	(56%)					
	Lp(a)>30mg/dl	66(44%)	22 (22%)	2.78	1.57-4.94	8.49	1.058-68.14
PAI-1 levels	PAI-1 <43ng/ml	96 (90%)	34 (64%)	1			
	PAI-1 >43 ng/ml	11 (10%)	19 (36%)	0.21	0.09-0.48	0.09	0.01-0.74
Platelet activation	P-selectin expression <0.5%	23 (16%)	55 (60%)	1			
	P-selectin expression >0.5%	119 (84%)	37 (40%)	8.04	4.34-14.90	11.50	0.93-142.27
v-WF levels	v-WF <200%	80 (75%)	42(79%)	1			
	v-WF > 200%	26(25%)	11(21%)	1.32	0.67-2.58	1.81	0.69-4.76

Logistic regression analysis of important thrombotic risk factors with odds ratio (OR) and confidence interval (CI) (n number of subjects in which thrombotic risk factors analysis was done representing number of subjects with low and high values. *All parameters were adjusted for each other in the multiple logistic regression.

4.2.2.1 Homocysteine analysis in urine: LC-MS/MS (electro spray ionization)

Hcy levels were assessed in urine samples of selected patients and controls by Liquid chromatography tandem mass spectrometry or LC-MS/MS by electro spray ionization. Electron spray ionization was obtained as chromatograms represented as peak values. From these plots, retention time and peak values were calculated. Retention time was similar while peak values were higher in patient samples. Thus Hcy values were comparatively higher in patients than controls. This result was different from that

obtained by enzymatic method in serum sample. Representative chromatograms of patients are presented in Figure 20; results of analysis are represented in Table 13.

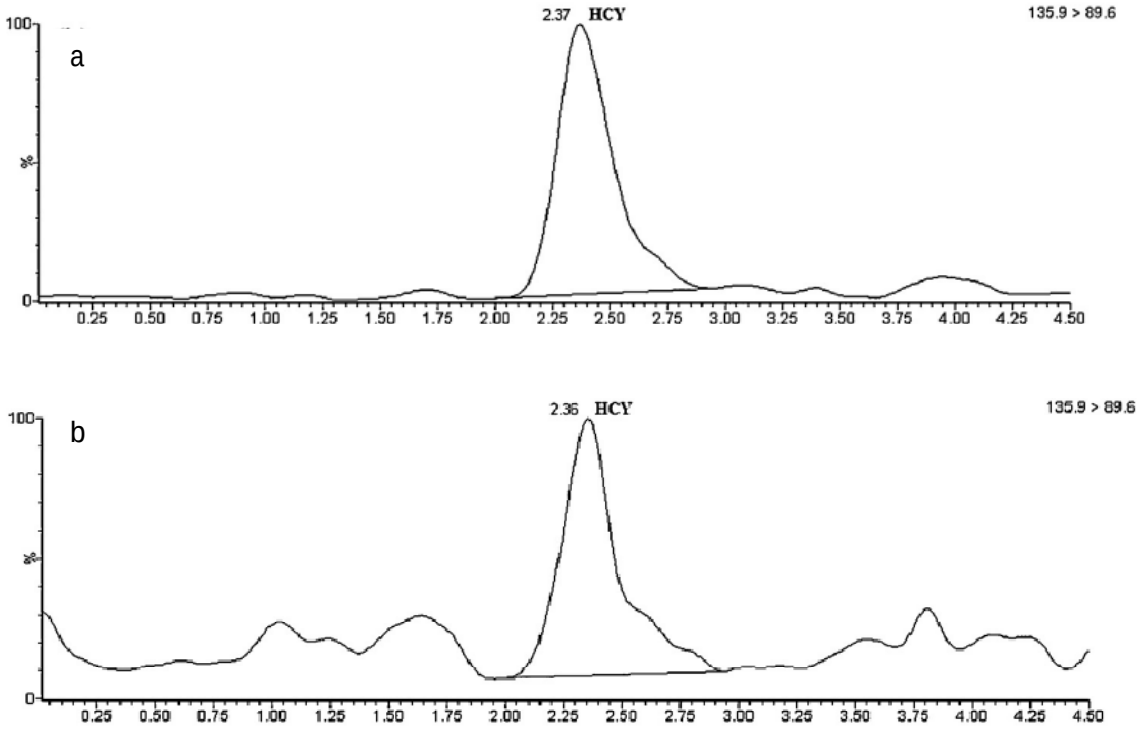


Figure 20: Representative LC–ESI–MS/MS chromatograms of Hcy in patient urine sample (a) and healthy volunteer sample (b)

Table 13: Mean chromatogram parameters of Homocysteine by LC-MS/MS (electron spray ionization)

Homocysteine		
	Patients(N=9)	**Controls (N=12)**
Retention time(Min)	**2.37**	**2.36**
Peak value	766	**213**
Concentration (µM/l)	20.1±5.7	8.9±2.2

4.2.2.2 Platelet activation by P-selectin expression using flow cytometry

Platelet activation was measured based on P-selectin expression on platelets using flow cytometry as described above in materials and methods section. [Figure 21 (A) show lower rate of expression and Figure 21 (B) show higher rate of expression].

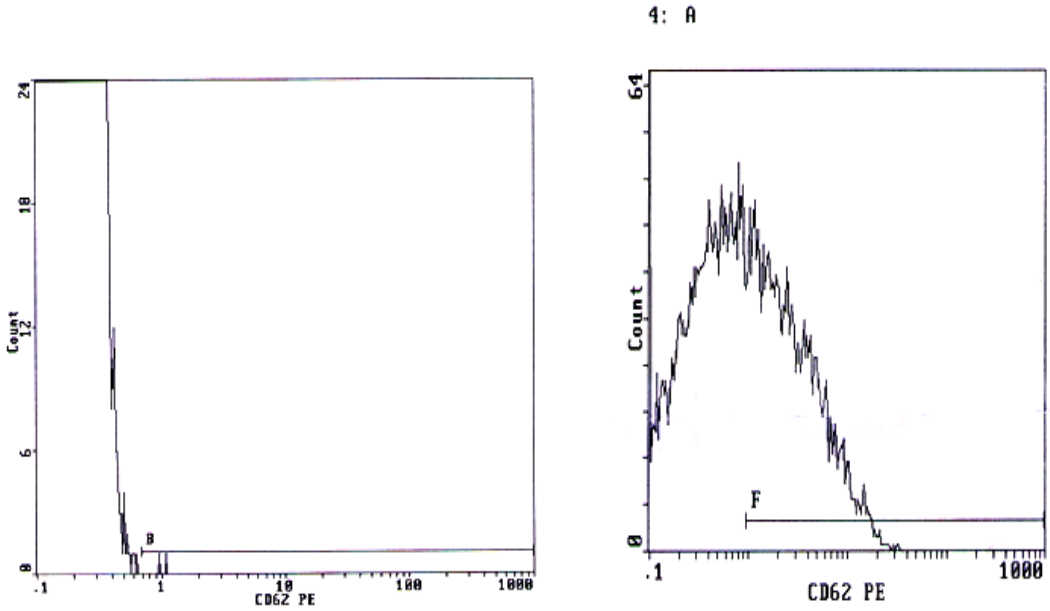


Figure 21: Representative flow cytometry histogram for CD62p PE expression. (A) Low P-selectin expression (0.01% platelet activation); (B)High P-selectin expression (35.7% platelet activation)

4.2.2.3 Correlation analysis

A significant negative correlation was observed between antithrombotic factor AT-III and platelet activation among patients[r=-0.4 and p<0.001] as well as controls also [r=-0.5 & p<0.001].

Fibrinogen and v-WF showed a significant positive correlation [r=0.3 & p<0.05] among patients. Similarly Lp(a) and hs-CRP [r=0.3 & p=0.0024] were positively correlated in patients.

55% of the patients had three of the five thrombotic risk factors (platelet activation, fibrinogen, Lp(a), homocysteine, v-WF & low antithrombin-III). Meanwhile 91% of the patients had at least two thrombotic risk factors, indicating higher prevalence of thrombotic risk factors in patients.

4.2.2.4 Association between conventional and thrombotic risk factors

Thrombotic risk factors were high in patients with conventional risk factors. More than 80% of the patients with conventional risk factors had their platelets activated. Similarly, nearly 60% of patients with conventional risk factors also had low AT-III. 56% of our patients were diabetic, with 56% of male patients being smokers. Figure 22 below represents thrombotic risk factors in patients with conventional risk-factors.

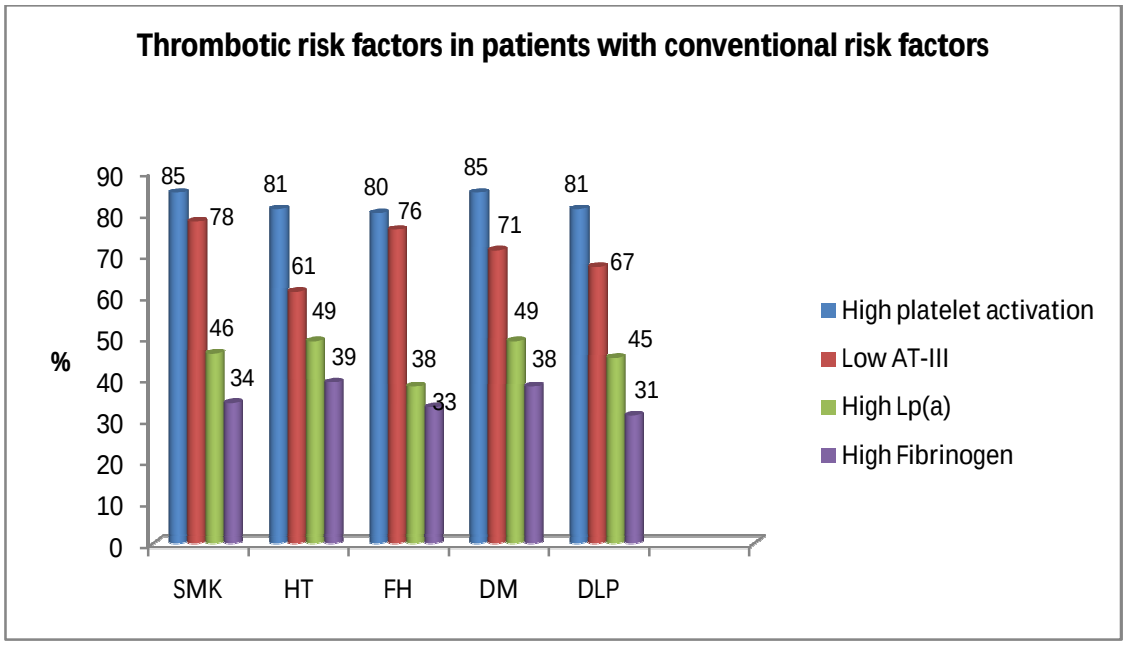


Figure 22: Thrombotic risk factors in patients with conventional risk factors: SMK-smokers, HT-patients with hypertension, FH - patients with positive family history, DM-diabetes mellitus patients DLP-Dyslipidaemia patients

A similar comparison done in the case of control subjects is shown in Figure 23. Platelet activation was the most significant risk factor in controls with conventional risk factors. [Figure 23].

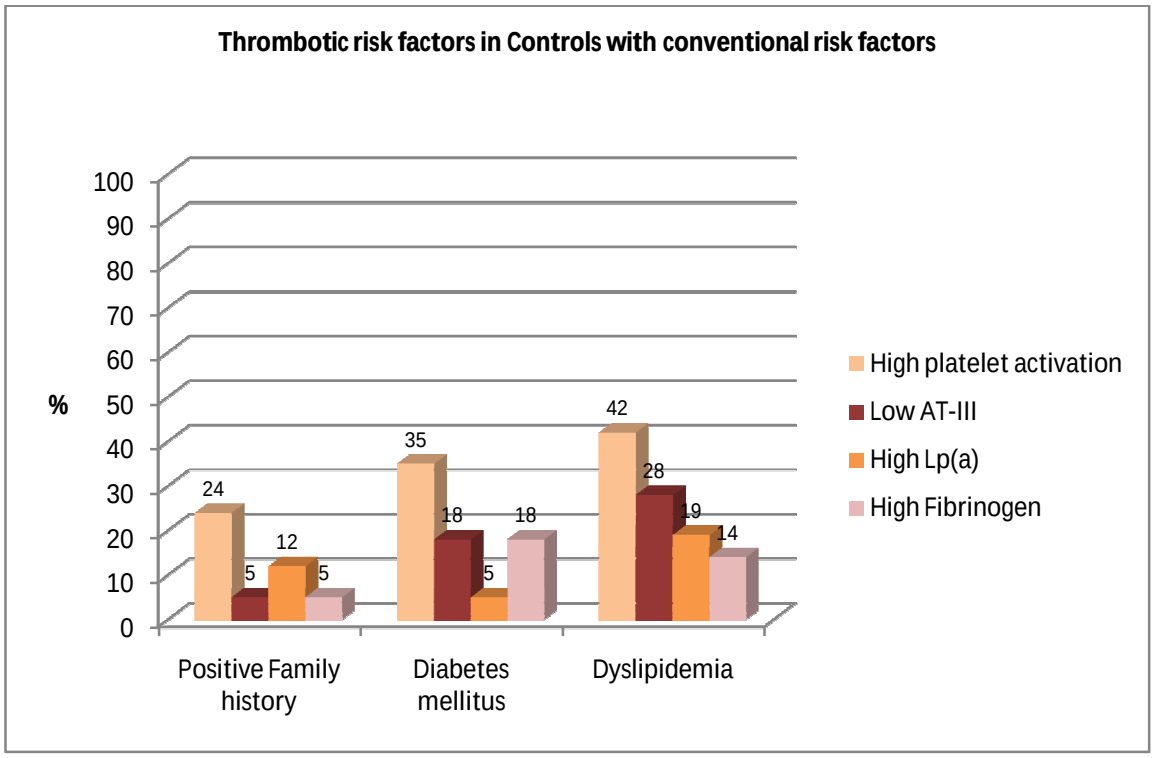


Figure 23: Thrombotic risk factors and conventional risk factors in controls

Proportion of controls who were smokers was comparatively low (13%), but within this group there was higher prevalence of thrombotic risk factors such as platelet activation (60%), low antithrombin-III(40%) and high fibrinogen(38%). This highlights the fact that smoking predisposes to thrombotic milieu even in healthy young adults. Fibrinogen was slightly higher in controls who were smokers than patients who were smokers [Figure 24].

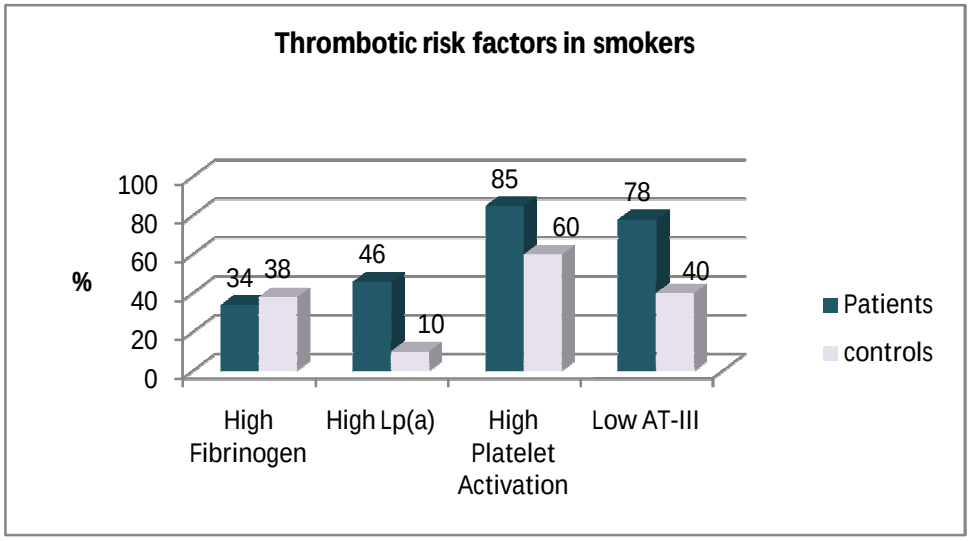


Figure 24: Smoking and thrombotic risk factors

Patients with low HDL-C also had high prevalence of platelet activation (85%) and low AT-III (78%) as represented in Figure 25.

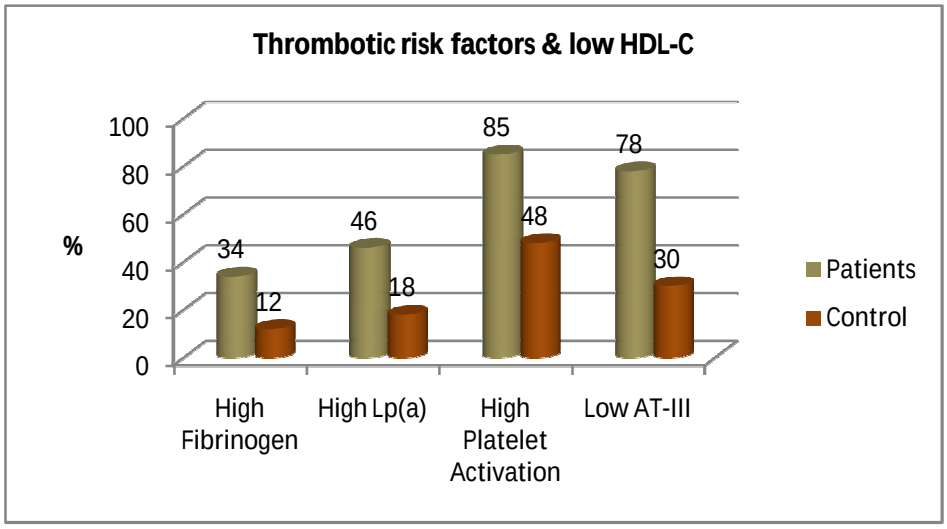
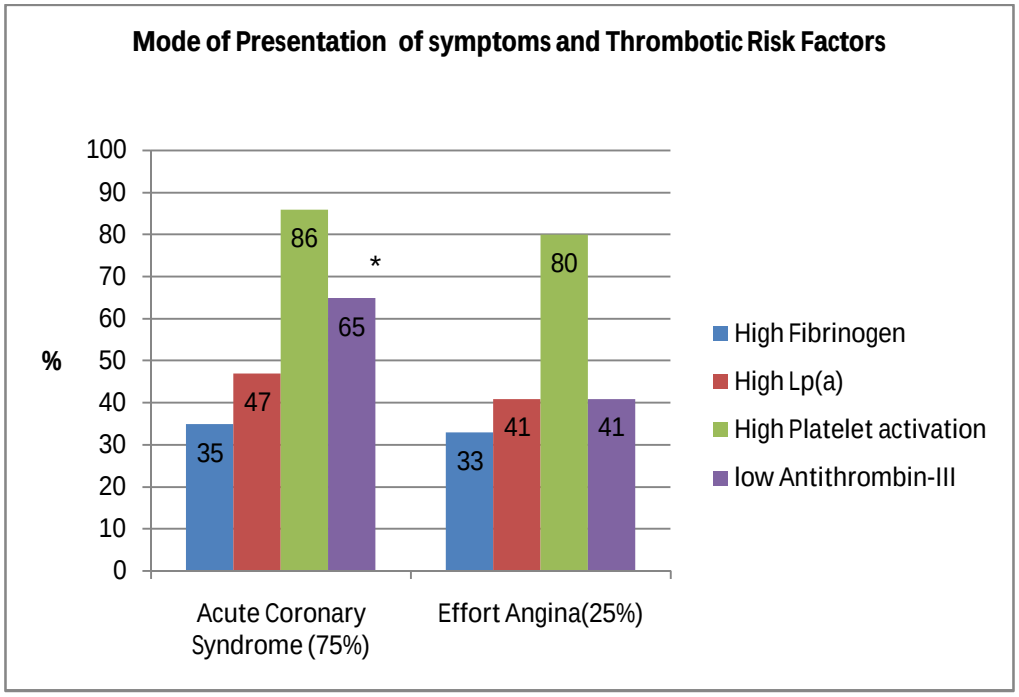


Figure 25: Thrombotic risk factors in subjects with low-HDL-C

4.2.2.5 Thrombotic risk factors were high in patients presenting with ACS

Majority of the patients (75%) whose thrombotic risk factors were analyzed presented with ACS. The percentage of thrombotic risk factor levels [high platelet activation, fibrinogen, Lp(a) and low AT-III] were higher in ACS group compared to EA, of this percentage of patients with low AT-III was significantly elevated among ACS group compared to EA. [Figure 26].



*represents p<0.05/significance in chi square analysis

Figure 26: Thrombotic risk factors in ACS and EA patients

Nine ACS patients and one EA patient had four thrombotic risk factors [high platelet activation, fibrinogen, Lp(a)and low AT-III] elevated and 84% of the ACS and 70% of the EA patients had at least two thrombotic risk factors at higher levels. This shows the clustering of thrombotic risk factors in patients who present with ACS than EA.Table 14

represents mean values of important thrombotic risk factors in ACSand EA. There is no significant difference in the case of mean values of thrombotic risk factors in ACS vs. EA, but a trend of increase in mean values are seen in ACS.

Table 14: Mean±SEM-Thrombotic risk factors ACS Vs EA

	Mode of presentation of Patients		
Risk Factors	**Effort Angina**	**ACS**	**P-value**
	Risk Factors± SEM	Risk Factors± SEM	
Platelet Activation	7.936 ± 1.744	9.737 ± 1.203	0.25
Antithrombin-III	72.53 ± 6.507	58.29 ± 4.535	0.082
Lipoprotein(a)	32.95± 4.911	40.73 ± 3.288	0.21
Fibrinogen	3.994 ± 0.3675	3.922 ± 0.1400	0.84

4.2.2.6 Coronary Angiogram Findings of Patients &relation to thrombotic factors

The association of the severity of CAD and thrombotic factor levels were analyzed. Based on the coronary angiography (CAG) findings, the patients were grouped into two- a) **Mild CAD:** minor CAD and or SVD (40%) and b) **Severe CAD**, with multi-vessel: double or three vessel disease (60%). Though there was a trend, associating the thrombotic risk factor levels and severity of CAD, it did not reach statistical significance [Figure 27].

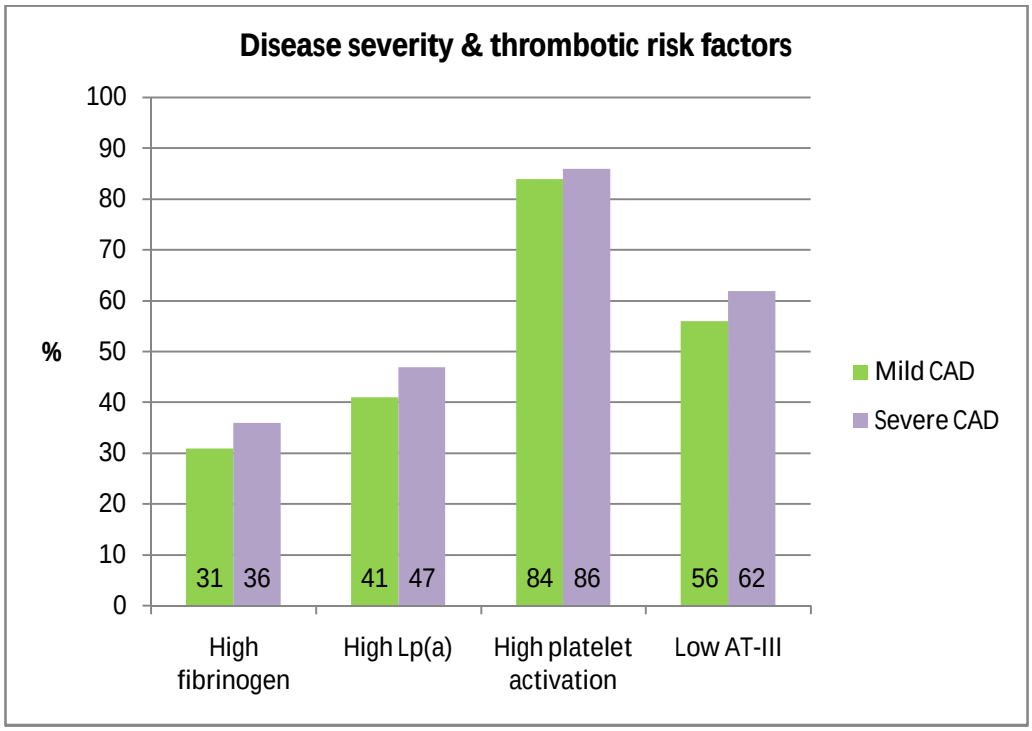


Figure 27: Thrombotic risk factors and vessel disease

4.2.2.7 Follow up and event rate

The total event rate of was calculated during their follow-up visits to the hospital or over telephone. The event rate in patients was 6% with two deaths, one admission with ACS, four patients to be subjected to CABG surgery and three patients requiring repeat angiography. Correlation between event rate and thrombotic risk factors were not practicable as the event rate was only 6% at a mean follow-up period of 26.23±5.4 months with a median of 26 months.

4.3 Characterization of monocyte phenotypes and inflammatory markers in young CAD patients and controls *(Case –Control study 2)*

Atherosclerosis has long been identified to have an inflammatory component contributing to its pathogenesis. Among the cells that gain access to the arterial wall, monocytes are the first to be recruited to arterial intima, where they differentiate into MΦ.

It has recently been reported that circulating monocytes constitute a heterogeneous population composed of mainly two types, the $CD14^{++}CD16^{-}$/ CM and $CD14^{+}CD16^{+}$ /NCM cells, which have different functions and migratory capacity. Different populations of monocytes may be associated with the presence of risk factors for development of coronary artery disease (CAD) or even predict cardiovascular events in individuals with CAD.

Given the important role of monocytes in all stages of development of the atheroma, the objective of this study was to characterise peripheral blood monocytes based on the phenotypic expression of CD14 and CD16 in patients with CAD compared with controls and to correlate it with inflammatory markers in the blood.

4.3.1 Basic characteristics, biochemistry and blood count analysis

Basic characteristics of patients including conventional risk factors lipid profile and blood count parameters are detailed in Table 13.

Table 15: Basic characteristic, lipid profile and blood count in patients and controls

A. BASIC CHARACTERISTICS			
	Patients N=57	**Controls N=67**	**'p' value**
Age[#]: Mean± SD	47.4±5.99	44.6±8.01	0.0513
	Frequency (%)	**Frequency (%)**	
Sex*			
Male, N (%)	49 (86%)	50(75%)	0.177
Female, N (%)	8(24%)	17 (25%)	
Conventional risk* factors, N (%):			
Positive Family history to CAD	22(39%)	19 (28%)	0.2544
Smoking	28 (49%)	6 (9%)	<0.001
Diabetes mellitus	33(58%)	7(11%)	<0.001
Hypertension	29(51%)	6 (9%)	<0.001
Low HDL	41 (71%)	21(31%)	<0.001
B. FBS, LIPID PROFILE AND BLOOD COUNT ANALYSIS[#]			
Parameters (units)	Patients: Mean±SD	Controls: Mean±SD	'p' value
Fasting blood glucose (mg/dl)	146.5±62.31	91.8±35.88	<0.001
Total Cholesterol (mg/dl)	140.8±45.56	200.7±36.33	<0.001
Triglycerides (mg/dl)	134.2±60.96	118.1±56.98	0.1632
HDL-C (mg/dl)	36.4±9.27	47.7±8.88	<0.001
Total WBC count (Cells/mm^3 of blood)	7813±1540	6580±1360	<0.001
Neutrophil (%)	61±10	49±9	<0.001
Lymphocyte (%)	33±9	41±9	<0.001
Eosnophil (%)	4±3	6±5	0.136
Monocyte (%)	2±0.8	3±1	0.1060
Platelet count (X 10^6 cells/µl of blood)	222.38±62.25	219.38±59.42	0.7939
RBC count (x10^3 cells/µl of blood)	4.65±0.65	4.85±0.53	0.1694
Haemoglobin concentration (g/dl)	13.15±1.4	13.88±2.64	0.069

N: number of subjects, %: percentage of subjects from the set of patients or controls taken for monocyte phenotype characterisation, SD: standard deviation. *-indicates analysis using $\chi 2$ analysis, #- indicates analysis using Student's t test.

Males predominated in the study. Majority of conventional risk factors were significantly elevated in patients, except positive family history. Fasting blood glucose was significantly high and HDL-C was significantly low in patients, while total cholesterol and LDL-C was significantly low in patients showing similar trends to case – control study-I. No significant difference was observed in the case of triglycerides. In patients significant increase in total WBC count and neutrophil count along with a significant decrease in lymphocyte count was observed, but no difference was observed in monocyte and eosinophil counts.

4.3.2 Flow cytometry of Peripheral Blood Mononuclear cells [PBMNC] for monocyte phenotypic characterization

PBMNC consists of two types of mononuclear cells- lymphocytes and monocytes. These cell suspensions were subjected to fluorescence activated cell sorting (FACS) analysis.

Representative dot plot obtained from flow cytometry analysis of unstained PBMNC by SSC (Y-axis) –FSC (X-axis) [Figure 28 (A)] and quarter plots of fluorescence of PE (Y-axis) Vs FITC (X-axis) in an unstained PBMNC is described below [Figure 28 (B)] More than 99% of PBMNC were in the Q3 [or unstained gate] and Q1=0.0%, Q2=0.3%, Q4=0.1% [Figure 28(B)]. Similarly, plots for PBMNC stained with CD14 FITC antibody alone and CD16 PE antibody alone were also obtained. Plots thus obtained from unstained, cells single stained with CD14 FITC and CD16 plots are generally used for

making arrangements for voltage settings, fluorescent compensations to allow appropriate gating.

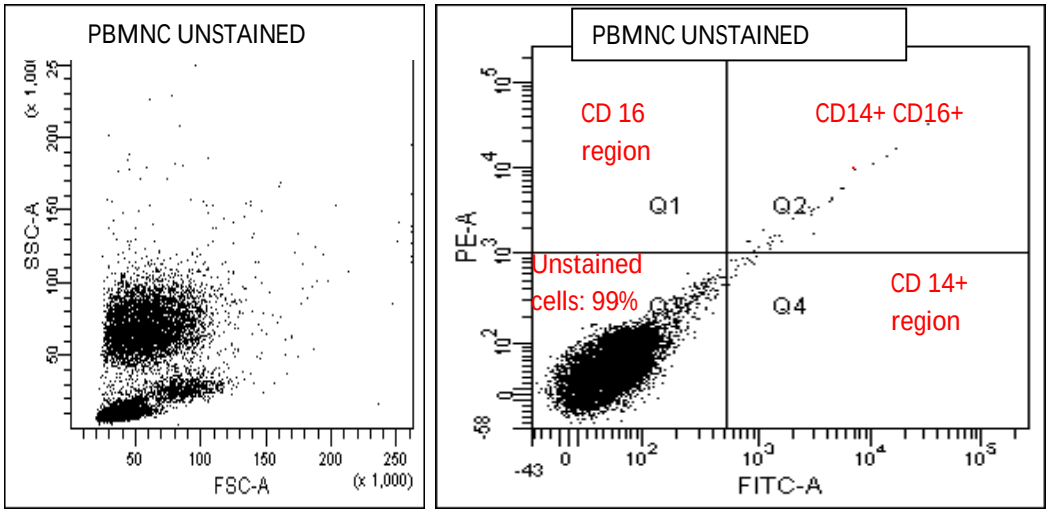


Figure 28(A)

Figure 28(B)

Figure28: Representative flow cytometry of PBMNC of unstained cell (without any antibody labelling) (A)- Side scatter (SSc) -Forward scatter(FSc) plot of unstained PBMNC. (B) quarter plots of PBMNC demonstrating the division of cells based on presence/ absence of CD14/CD16 cells.

CD14 FITC and CD16 PE double antibody stained cells were used for characterization of different phenotypes and their quarter plots were used to identify percentage of subsets in mononuclear cells. Figure 29 shows representative dot plots of patients double stained with CD14 FITC antibody and CD16 PE antibody. Plots of control samples were also obtained.

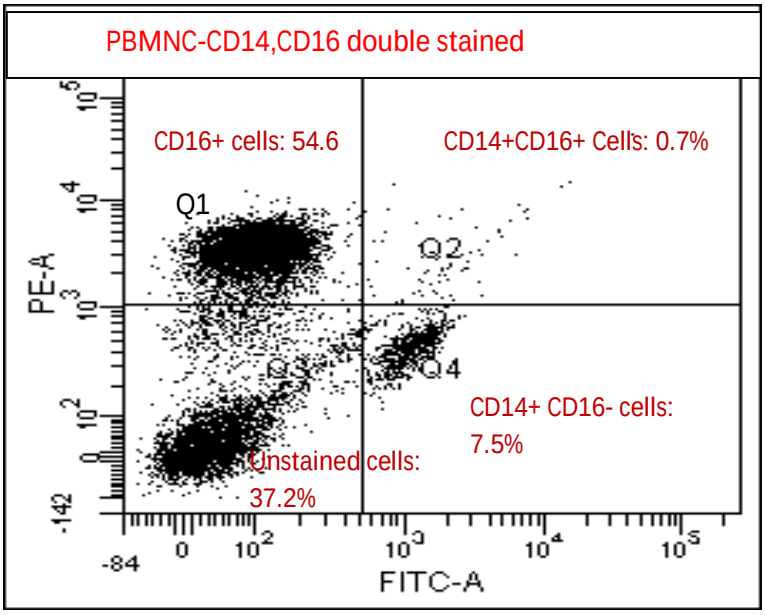


Figure 29: Representative quarter plot of PE Vs FITC for CD 14 FITC and CD16 PE double antibody labelled PBMNC – showing different phenotypes and their percentage when 10,000 cells were counted.

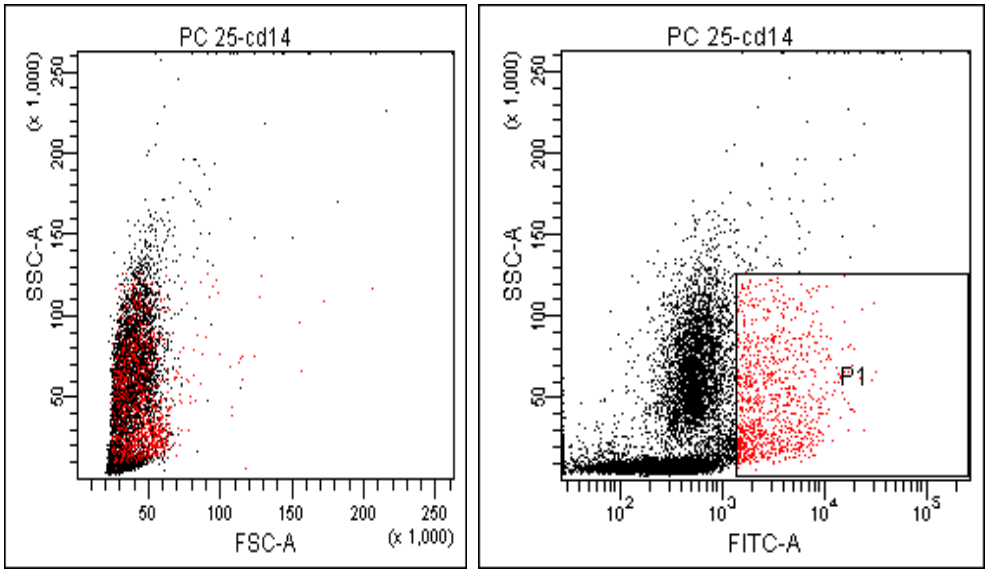
There was no significant difference between $CD14^{+}CD16^{-}$ phenotype and $CD14^{+}CD16^{+}$ phenotypes in patients and controls. The overall results of characterization of different cells of PBMNC in patients and controls are represented in the Table 16 below.

Table 16: Phenotypes of PBMNC based on CD14 FITC antibody and CD16 PE antibody labelling

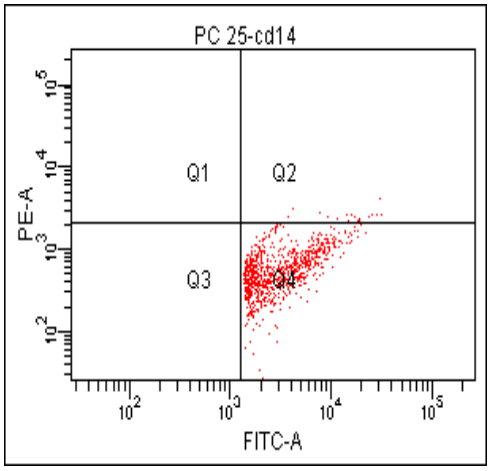
Phenotypes of cells in PBMNC	Patients: Mean±SD N=57	Controls: Mean±SD N=67	'p' value
$CD14^{+}CD16^{-}$	7.26±5.24	8.54±11.93	0.52
$CD16^{+}$	17.96±11.76	14.38±8.62	0.075
$CD14^{+}CD16^{-}$	4.089±4.022	5.22±8.46	0.2929

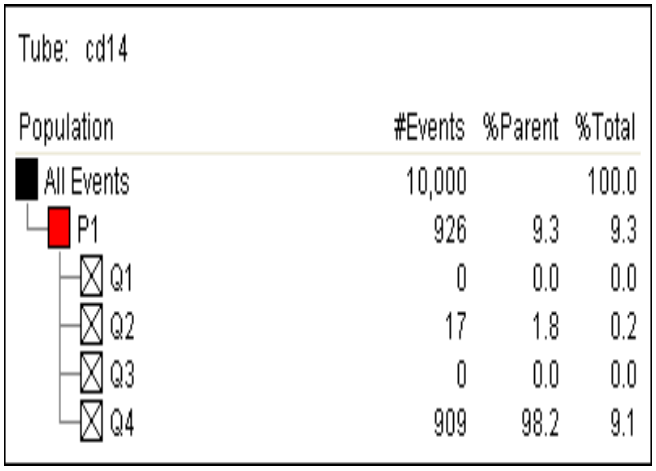
4.3.2.1 Gating strategy for monocyte phenotyping

Using FACS analysis the monocyte population was gated out and their characterization based on CD14 and CD16 expression was performed, from the data obtained from PBMNC. Appropriate gating strategy [FACS DIVA software] was used to separate out monocytes ($CD14^+$ cells) from mononuclear cell population [PBMNC] by plotting, CD14 FITC (X- axis) against SSC (Y-axis) and back gating it to FSC (X-axis) –SSC(Y-axis) plot, to completely gate out CD14 positive cells/monocytes from lymphocyte population.[Figure 30(A & B)] The gated $CD14^+$ cells, were further analysed to get different monocyte subsets in quarter plots as $CD14^{++}CD16^-$ and $CD14^+$ $CD16^-$[Figure 30 (C)].



(A) (B)





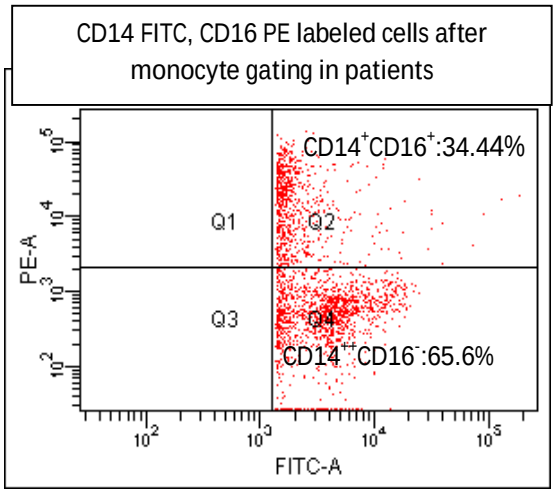
Tube: cd14

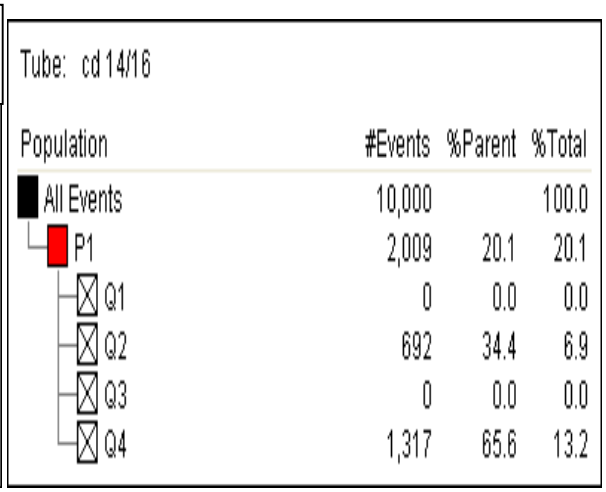
Population	#Events	%Parent	%Total
All Events	10,000		100.0
P1	926	9.3	9.3
Q1	0	0.0	0.0
Q2	17	1.8	0.2
Q3	0	0.0	0.0
Q4	909	98.2	9.1

(C) (D)

Figure 30: Representative plots showing gating strategy for the separation of monocyte population from PBMNC in CD14 FITC labeled cells. (A)&(B) –back gating strategy to gate out monocyte [P1 region (red dots)]. (C) Quarter plot of CD14+ cells showing ~100% CD14 positive cells as shown in (D)

Same gating strategy was used for double stained cells. CD14-FITC (X axis) fluorescence vs CD16-PE (Y axis) fluorescence plot in double stained cells of PBMNC gave the distribution and percentage of monocyte phenotypes in patients [Figure 31(A) & (B)] and controls [Figure 31 (C) & (D)] when appropriate gating strategy was applied.





Tube: cd14/16

Population	#Events	%Parent	%Total
All Events	10,000		100.0
P1	2,009	20.1	20.1
Q1	0	0.0	0.0
Q2	692	34.4	6.9
Q3	0	0.0	0.0
Q4	1,317	65.6	13.2

(A) (B)

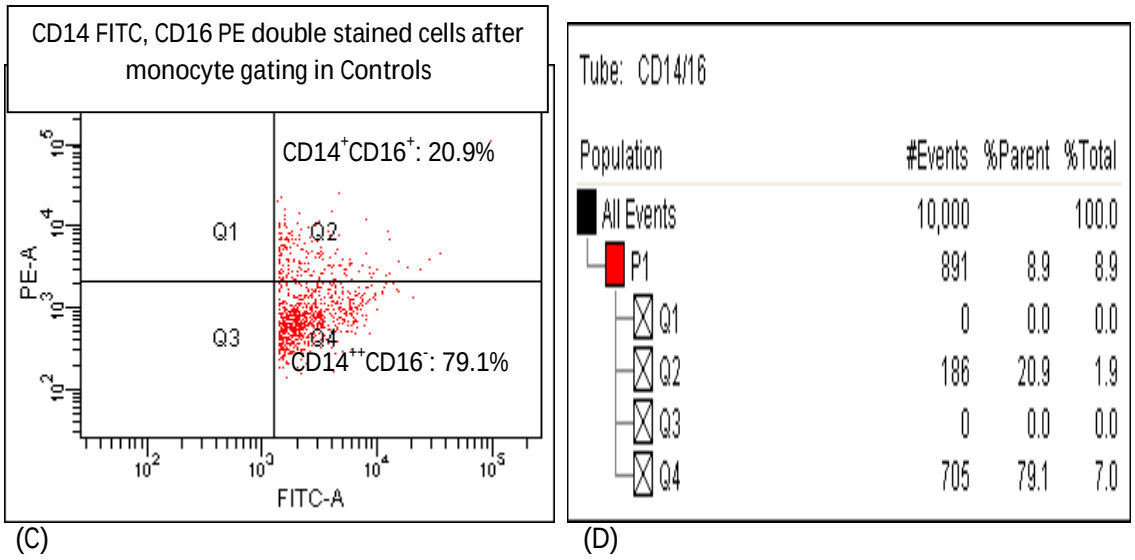


Figure 31: Representative quarter plots of double stained (CD14 FITC and CD16 PE antibody labelled) cells after monocytes were gated from PBMNC. (A) and (B) represents result of patient showing 65.6% $CD14^{++}CD16^{-/}$Classical monocytes at Q4 gate and 34.4% $CD14^{+}CD16^{+}$/non classical monocytes at Q2 gate. (C) and (D) represents result of a control showing 79.1% $CD14^{++}CD16^{-/}$Classical monocytes at Q4 gate and 20.1% $CD14^{+}CD16^{+}$/non classical monocytes at Q2 gate.

The results of monocyte characterization in patients and controls are represented in the table [Table 17].

Table 17: Monocyte phenotypes Classical Vs Non-classical in Patients and controls

MONOCYTE PHENOTYPES	**Patients (Mean±SD)**	**Controls (Mean±SD)**	**P-value**
Classical (%) $CD14^{++}CD16^{-}$	72±20	77±12	0.2131
Non Classical (%) $CD14^{+}CD16^{-}$	28±21	23±12	0.1678

The result shows that, there was no significant difference in classical and non classical monocyte phenotypes among patients and controls. But a trend towards increase in

classical monocytes was observed among controls. Similarly there was an opposite trend of decrease in classical and increase in non-classical monocytes among patients.

4.3.2.2 Monocyte phenotypes based on mode of presentation of CAD

Patients were categorized based on presentation of symptoms at the time of admission as having acute coronary syndrome and having effort angina. Patients who presented with ACS had more number of NCMs, while patients with EA had higher presence of CMs [Table 18]. There was significant difference in monocyte subsets among patients who presented with ACS vs. those who presented with EA.

Table 18: Monocyte phenotypes and CAD symptoms

Monocyte phenotype	CAD/ACS (N=27)	CAD/EA (N=29)	Controls (N=67)	'P'-value			
					't'-test		
				'Anova'	**	*	#
CM (%)	67±20	83±14	77±12	0.0043	0.007	0.015	0.09
NCM (%)	33±20	17±15	23±12	0.003	0.015	0.01	0.1

Values represented as mean ± standard deviation 'Anova'- (between three groups): shows significant difference; **-indicates p-value between ACS and EA,*indicates p-value between ACS and Controls; # - indicates p value between EA and controls

The difference in monocyte phenotypes between ACS patients and controls were also significant. There was no significant difference in monocyte subsets between controls and patients who presented with effort angina. Thus overall ACS patients had significantly increased NCMs and decreased CMs compared to those with EA and control subjects.

4.3.2.3 Monocyte phenotypes based on the CAG findings

When severity of CAD based on CAG findings was compared with distribution of monocyte phenotypes, the number of non-classical monocytes tend to decrease as the severity of disease increases (from single vessel disease to three vessel disease). . Table 19 provide details of monocyte phenotypes and its correlation with CAD severity.

Table 19: Monocyte phenotypes and severity of CAD

Vessel Disease	Class. Monocyte % [Mean ± SD]	Non Class.Mon % [Mean ± SD]	'p' value ('Anova')
SVD or minor CAD (N=26)	68±17	32±17	0.058
DVD (N=9)	79±11	21±11	
TVD (N=22)	82±17	18±17	

P value was calculated using anova

4.3.3 Relation between monocyte phenotypes and inflammatory markers

Monocytes are principle inflammatory cells in circulation. They transform into macrophages on inflammatory stimulus and can interact with other inflammatory cells especially platelets and endothelium or can itself secrete variety of inflammatory cytokines and chemokines. As monocytes are a heterogeneous population of cells, the cytokines and chemokines synthesized by monocytes can give insight into their functionality and may help to have clear cut idea on their role in variety of atherosclerotic process in CAD. In view of this in the present study, we investigated the level of important inflammatory markers: TNF-α, IL-1-β and MPO. We also tried to correlate

total the WBC count and the fibrinogen levels with these inflammatory markers related to monocyte-macrophage system.

4.3.3.1 Inflammatory markers-*TNF-ά, IL-1- β, Myeloperoxidase activity, WBC count & fibrinogen* in CAD patients and controls

The analysis of inflammatory markers related to monocyte-macrophages and fibrinogen showed significant differences in the levels of IL-1-β, myeloperoxidase, fibrinogen and WBC. No significant difference was observed in the case of TNF-α [Table 20].

Table 20: Inflammatory markers in patients and controls

INFLAMMATORY MARKERS (units)	Patients: Mean± SD [n=57]	Controls: Mean± SD [n=67]	'p'value
TNF-α (pg/ml)	39±13	35±15	0.25
IL-1-β (pg/ml)	0.7±0.5	0.5±0.2	0.0363
MPO activity (u/l)	37±25	27±16	0.0325
WBC count (cells/mm^3)	7813±1540	6580±1360	<0.0001
Fibrinogen (g/l)	5±3	3±1	0.0005

There was no significant difference between any of the inflammatory/thrombotic markers, among patients when they were categorized based on mode of presentation of CAD as ACS [n=27] and EA [n=29]. Similar trend was observed in the case of disease severity based on CAG findings.

4.3.3.2 Correlation analysis of monocyte phenotypes and inflammatory markers in CAD

Correlation analysis between monocytes and inflammatory markers as well as between different inflammatory markers are represented in Table 21 below.

Table 21 : Correlation Analysis of Monocytes and inflammatory markers

1. Correlation: Monocytes and fibrinogen	
a. $CD14^+$ Cells [Total monocytes] &Fibrinogen	
CAD: Positive correlation; r=0.5, p=0.0006	Controls: No correlation
b. $CD14^+CD16^-$ monocytes [CM] & Fibrinogen	
CAD: No correlation	Controls: - ve correlation r=-0.4, p=0.03
c. $CD14^+CD16^+$monocytes [NCM] & Fibrinogen	
CAD: No correlation	Controls: +ve correlation r=0.4, p=0.03
2. WBC , inflammatory markers and fibrinogen	
a**. WBC & IL-1β**	
CAD:+ve correlation, r=0.6, p=0.02	Controls: No correlation
b. Neutrophil count &IL-1β	
CAD: +ve correlation, r=0.5, p=0.03	Controls: No correlation

C. Neutrophil count & MPO	
CAD:+ve correlation, r=0.4, p=0.03	Controls: No correlation
d. Neutrophil count and fibrinogen	
CAD:+ve correlation, r=0.3, p=0.02	Controls: No correlation

Correlation analysis between monocyte phenotype and inflammatory markers revealed that there was no significant relation between monocyte phenotypes and inflammatory markers. However, fibrinogen levels showed positive correlation with total $CD14^{+}$ cells [which includes a combination of both classical and non-classical monocytes], in patients (r=0.5; p=0.0006). Classical monocytes showed negative correlation with fibrinogen among controls, while non-classical monocytes showed a positive correlation between fibrinogen levels among controls. Among patients, total WBC count and neutrophils showed a positive correlation with IL-1-β. Neutrophil count also showed positive correlation with myeloperoxidase activity (r=0.4; p=0.03) and with fibrinogen (r=0.3; p=0.02) in patients. No other significant correlation was observed with any of the inflammatory markers.

V. DISCUSSION

5.1 Database of young CAD patients

Coronary artery disease is considered as a worldwide epidemic, more importantly CAD strikes Indians at a young age (Reddy, 2007) (Krishnan, 2012) compared to developed world. CAD risk factors are prevalent at very young age in Indians (Joshi *et al.*, 2007).There are very limited studies on the prevalence of CAD and risk factor profile of CAD patients from India. The prevalence (Thankappan *et al.*, 2010) (Zachariah *et al.*, 2013) and mortality of CAD(Soman *et al.*, 2011) had been reported to be higher in this region.

Our study showed that 31% of the young CAD patients (defined as < 55 years) were very young (ie <45 years).The major risk factor in the population was dyslipidaemia in the form of low HDL-C. While total cholesterol, LDL-C and triglycerides remained comparatively normal. The lipid profile of the patients could have been altered by statins which almost all of the patients were on. Treatment using statins have reduced the total cholesterol levels and triglycerides, but did not have much effect on HDL. (Jeger & Dieterle, 2012), (McTaggart & Jones, 2008), this pattern –of near normal total cholesterol and triglycerides, near-target LDL cholesterol and low HDL is characteristic in patients on statins.

Second most important risk factor was smoking, but it was almost exclusive to male population, who constituted about 88 % of our study population. In many previous studies, smoking was reported as an important risk factor in young adults with CAD

(Imamura *et al.*, 2004) (Tamrakar *et al.*, 2013), (Davis *et al.*, 2015). But in our study, dyslipidaemia emerged as the most important risk factor. Culturally women restrain from smoking in Kerala and hence smoking was nearly absent in women, but on the contrary more than 50% of males were smokers.

Apart from smoking and dyslipidaemia, other conventional risk factors, namely diabetes, hypertension, family history of premature CAD were high in female patients (Hypertension> diabetes> family history), compared to males. In a previous report on very young (<50 years) female subjects from Portugal with acute coronary syndromes, hypertension (48.5%) was an important risk factor (Adão *et al.*, 2004).

In a recent study by Davis *et al* (2015) on young women under 55 years, it was reported that young women had more prevalence of diabetes and hypertension similar to our findings. Development of early risk factors among females was the reason for development of CAD in them. This leads to lose of protection offered by the oestrogens in females in their reproductive age. Females usually develop the disease 10 years later than men unless they are diabetic (Klein and Nathan, 2003).

There are many studies (Yusuf *et al.*, 2004) (Gupta *et al.*, 2008) which indicate that conventional risk factors are contributing to CAD in the Indian population and our observations were not different.

When mode of presentation was compared in patients, acute coronary syndrome was the most important mode of presentation. Myocardial infarction (Christus *et al.*, 2011) and chest pain (Baligar, 2015) was the most common mode of presentation in young patients in other studies also. Mode of presentation was different for males and females,

with males having significantly high ACS and females presenting more with effort angina and atypical symptoms. Previous reports also showed a different mode of presentation between males and females. (Sharma & Gulati, 2013). A Meta analysis had shown that women usually had more commonly atypical angina and silent MI (Hemingway *et al.*, 2008).

Coronary angiographic findings have shown that SVD predominated in the study group, which was in accordance with earlier observations in young CAD patients (Chua *et al.,* 2010) (Sricharan *et al.,* 2012) (Tamrakar *et al.*, 2013). Females had significantly higher incidence of single vessel disease, and minor CAD as well as normal coronaries. This further substantiated earlier reports on female CAD (Hemingway *et al.*, 2008) (Sharma & Gulati, 2013) (Davis *et al.*, 2015).

The treatment offered to these patients based on CAG findings relied on the currently accepted guidelines (Guidelines and Statements ACC/AHA Joint Guidelines 2016). Many of the female patients were kept on medical therapy as they had either more normal coronaries or minor CAD. Mean while equal number of males and females were referred for CABG. This could be due to the fact that since females were diabetic, the possibility of three vessel disease in them is higher.

Mortality rate was slightly high among females. There are many proposed reasons for the high mortality among females. Substantial delays in health care seeking behaviour and less use of treatment resources are seen in young women compared to men (Kumar *et al.*, 2012). A survey by European Society of Cardiology has pointed out women are less likely to undergo diagnostic and treatment procedures for CAD (Daly, 2005). The clinical

presentation and subsequent diagnostic procedures are less reliable in females at the age group of less than 55 years (Maas & Appelman, 2010). Another reason could be the socio-economic biases in the female health care spending in the society, where females do not receive the treatment they are advised.

5.2 Thrombotic and inflammatory risk factor analysis

The patho-biology of CAD is linked with inflammatory and thrombotic pathways which may contribute to its premature occurrence in young patients. Thrombotic risk factors (Feinbloom, 2005), inflammatory risk factors (Zakynthinos and Pappa, 2009) and monocyte phenotype characterization (Shantsila *et al.*, 2014) is being widely studied in the pathogenesis of CAD in the western world. There are not many studies which evaluated thrombotic risk factors associated with CAD in young Indians (Khare *et al.*, 2004) (Shalia *et al.*, 2010) . There was only one study from South India apart from the present study, which evaluated more than two thrombotic risk factors (Deepa *et al.*, 2002). There are studies which evaluated inflammatory markers associated with CAD in our country (Jha *et al.*, 2008) (Kumpatla *et al.*, 2014), while there are no reports on monocyte phenotype characterization in CAD from India and its association with inflammatory markers.

Present study is unique in that, it evaluated thrombotic risk factors [fibrinogen, Lp(a), t-PA, PAI-1, v-WF, homocysteine, AT-III Protein C], platelet activation by P-selectin exposure (cellular prothrombotic-infammatory marker) and the inflammatory marker hs-CRP. The study also characterized monocyte phenotypes (classical vs. non-classical monocytes) and assessed levels of inflammatory markers (WBC count, TNF-α, IL-1-β,

MPO) and thrombotic risk factor fibrinogen. Thrombotic and inflammatory marker levels were compared with presence of conventional risk factors and their association with mode of presentation of CAD and disease severity.

5.2.1 Preliminary analysis and lipid profile

Majority of the patients in this sub-study were also males. Conventional risk factors evaluate (dyslipidaemia, diabetes mellitus, hypertension, smoking and family history of CAD) were significantly elevated among patients compared to controls. Dyslipidaemia was observed as low HDL-C only, as found with the main study group. LDL and TG levels were low among patients for the reasons which we already discussed (Vide supra).

5.2.2 Thrombotic risk factor analysis

Thrombotic risk factor analysis revealed, that AT-III was significantly low in our patients compared to controls. Antithrombin is a serine protease inhibitor which inhibits thrombin and activated factor X. Inflammation lead to decrease in antithrombin levels due to its impaired synthesis and degradation by elastase produced by activated neutrophils. Low levels of AT-III indicate the bidirectional relation of inflammation and thrombosis in the progression of CAD (Levi, 2004).

In multivariate analysis, low AT-III emerged as an important thrombotic risk factor along with high Lp(a) levels and also high fibrinogen. The finding of low AT-III levels in patients is an important observation from the present study. So far there are only sporadic reports of low AT-III levels in cardiovascular disease (Celik *et al.*, 2008) (Mishra *et al.*, 2013). In an earlier study, the risk of cardio-vascular events was negatively related to AT-

III levels and positively related to fibrinogen levels in patients who had angina pectoris (Thompson *et al.*, 1996).

Fibrinogen has a predictive role in future coronary events as reported by Shi *et al*. (2010). In the present study 33 % of the patients had high fibrinogen levels and all were on treatment with statins. There is a report of low fibrinogen levels in hypercholesterolemia subjects on treatment with statins (Leibovitz *et al.*, 2004).In a study from North India (Singh & Singh, 2008),fibrinogen was elevated in 58 % of CAD patients who were not on statin treatment. Thus indicating that, statins might be reducing fibrinogen in some of the patients, but not in all.

Thrombotic risk factor Lp(a) was significantly elevated in our patients .Lp(a) , an apo B containing lipoprotein, has an additional apo(a) apolipoprotein. It is a pro-thrombotic agent and is a potent inhibitor of fibrinolysis, due to its structural homology to plasminogen (Berglund, 2004). Prospective studies indicate an association between elevated Lp(a) and angiographically proven CAD (Rajasekhar *et al.*, 2004) (Ashfaq *et al.*, 2012).In the present study, mean Lp(a) was significantly elevated, 44 % of the CAD patients showed high Lp(a) levels that is 30 mg/dl or more. Logistic regression revealed a higher odds ratio (Table 12) for Lp(a) indicating it as a significant risk factor in the population. There are reports that aspirin administration can reduce Lp(a) (Akaike *et al.*, 2002), as our patients were on aspirin, this might be the probable reason for normal Lp(a) levels in about 60 % of the patients.

PAI-1 levels were significantly lower in patients compared to controls, while no significant difference was seen in the case of t-PA. t-PA and PAI-1 levels are involved in fibrinolytic pathway and are generally elevated in CAD. A study from Chennai, south India (Deepa *et al.*, 2002) has shown, that elevation of t-PA and PAI-1 were associated with CAD along with fibrinogen. But a study from Western India [Mumbai] (Shalia *et al.*, 2010) showed that there was no significant elevation of t-PA levels in older [>40 years] patients with acute myocardial infarction (AMI) compared to control subjects. The study also showed significant lowering of PAI-1 in patients under treatment for CAD and also with stable angina. An observation found to be similar to our findings. Statin treatment in our patients could be contributing to the significant lowering of PAI-1 in patients and comparable levels of t-PA in patients and controls. Cell culture studies in human vascular smooth muscle and endothelial cells (Bourcier & Libby, 2000), show that statins could reduce PAI-1, and increase t-PA that inhibits release of PAI-1.

In the present study there was no significant difference in v-WF as well as homocysteine levels in the peripheral blood. Statin therapy in patients could have resulted in comparable v-WF levels in patients and controls. Studies on effect of statins on peripheral atherosclerosis (Furukawa, 2006) and hypercholesterolemic subjects (Joukhadar *et al.*, 2001) have shown that that statin induces reduction in v-WF levels.

Hcy is a thiol containing amino acid synthesized as a by-product of methionine metabolism. Hyperhomocystinaemia can damage the endothelium, resulting in a pro-thrombotic state. There are several contradictory findings about the association of Hcy and CAD (Deepa *et al.*, 2001) (Mehta & Shah, 2012) (Shenoy *et al.*, 2014).For Hcy

assay, we used indirect enzymatic assay for the estimation of homocysteine, in which coupled enzyme reaction using NAD/NADH was employed. Recently our group (Gopu *et al.*, 2013),have standardized a direct, rapid and sensitive assay for Hcy estimation in urine sample. This method used liquid chromatography– tandem mass spectrometry coupled with electro spray ionization or LC-MS/MS ESI. Using this technique, urine samples from CAD patients had elevated Hcy levels compared to that of controls.

An antithrombotic factor analysed in the present study was Protein C. Activated Protein C inhibit coagulation pathway at the level of factor V and VIII and have anti-inflammatory functions (Levi, 2004). There was no significant difference between Protein C levels in patients and control subjects in the present study, but other studies from India (Pai *et al*., 2012) (Mishra *et al.*, 2013) reported correlation between CAD and Protein C. The sample size for the analysis of Protein C in patients and controls were very low compared to other parameters studied and hence conclusive explanation for lack of significance or comparable levels in patients and controls is not feasible. Probably an increased sample size in future studies can provide reliable results on Protein C in our population.

hs-CRP, is an acute phase protein is an important inflammatory marker in CAD. The mean hs-CRP levels were similar and higher in patient and controls in our study. hs-CRP levels were higher in AMI patients in a study from Chennai, India and showed correlation with leptin (Rajendran *et al.*, 2012). In our study subjects including patients and controls we could not establish any positive correlation between lipid profile and hs-CRP, but lipid profile was elevated in controls. Elevated hs-CRP indicates the need for the

control of factors contributing to systemic inflammation, especially dislipidaemia in the general population for the prevention of CAD in the young. Increased sample size can also provide clear definition on hs-CRP levels in the population.

5.2.2.1 Platelet Activation by P-selectin exposure an important risk factor

Platelet activation is a critical component in the pathogenesis of atherosclerotic CAD (Davì and Patrono, 2007). Various components released by activated platelets may contribute myocardial dysfunction and vasoconstriction by the release of reactive oxygen species. Culmination of the cascade starting with platelet activation eventually causes plaque rupture and atherothrombosis, by means of cytokines, chemokines, matrix degrading enzymes and thrombotic factors released by platelets. Variety of markers for platelet activation have been established using different methods (Ferroni *et al.*, 2012). In our study, percentage expression of CD 62p (P-selectin) in fresh peripheral blood without any stimulation with platelet activating factors (ADP, collagen and thrombin) were estimated by means of flow cytometry, where we observed an inherent increase in platelet activation in patients compared to controls. Moreover there was wide range of variability in percentage P-selectin exposure values in both patients and controls.

P-selectin is the largest of the selectins (140 KD), which are generally stored in the alpha granules of the platelets. Subsequent to platelet activation, exposed P-selectin can bind with PSGL-1 of monocytes. These aggregates can further enhance activation of monocyte, facilitating activation of inflammation and thrombosis (Davì and Patrono, 2007). Soluble P-selectin (Gokulakrishnan *et al.*, 2006) and exposure of P-selectin upon

induction by platelet activating agents and platelet reactivity (Patel *et al.*, 2007) have been reported to be enhanced in Indians. Platelet activation (exposure of P-selectin) was significantly elevated in our patients, even though they were on anti-platelet drugs, aspirin and clopidogrel. Another study on platelet function of aspirin-treated patients undergoing cardiac catheterization (Linden *et al.*, 2007) showed similar trends to that of our study. Many of our patients had percentage platelet activation above 10 %. Basal levels of platelet activation in controls were much lower even in those patients with conventional risk factors, indicating the severity of inflammatory and thrombotic process, which might be prevalent in the patients.

There are conflicting reports regarding the role of aspirin (Kaufmann *et al.*, 2013) and clopidgrel (Klinkhardt *et al.*, 2003) in P-selectin inhibition. Aspirin is involved in the inhibition of cycloxygenase-1 (COX-1) pathway while clopidogrel is a thienopyridine derivative inhibiting ADP-dependent platelet function (Patrono and Rocca, 2010). A study on platelet function profiles on diabetic and non-diabetic patients with CAD on combination therapy with aspirin and clopidogrel treatment undergoing elective PCI revealed that diabetic patients had increased platelet reactivity and they had increased unresponsiveness to clopidogrel (Angiolillo *et al.*, 2005). Majority of our patients were also diabetic and hence increase in P-selectin expression might be suspected to be due to unresponsiveness to clopidogrel. There is also a report of statin influence on P-selectin expression, as statins can attenuate increase in P-selectin expression induced by exercise (Zaleski *et al.*, 2013). Statins are also being proved to effect endothelial NO synthesis, along with influence on pathways of inflammatory cells, mainly by inhibition of

isoprenylation of GTPase, thereby controlling downstream pathways of gene expression (Bu *et al.*, 2011) as also in platelet activation, not related to P-selectin exposure. It is known that P-selectin exposure is directly related to activation of thrombin and inflammatory factors (Levi, 2004),with activation of protease activation receptor-1 (PAR-1) (Malerba *et al.*, 2013). Thrombin activation is mainly a consequence of thrombotic inflammatory pathways during atherothrombosis as in acute coronary syndromes. It has been proven to have its effect on platelets and leukocytes (Popović *et al.*, 2012).

Another study by Stellos *et al*. (2010) has also found that molecular markers of myocardial necrosis and infarct size correlated with P-selectin expression(Stellos *et al.*, 2010). This explains the clinical relevance of P-selectin expression in patients. The low event rate of 6% in our patients may be due to meticulous follow up they were undergoing as they were part of a study and were in regular contact with physicians. All these ensured regular monitoring and control of risk factors and also ensuring drug compliance.

Many of the thrombotic factors were elevated in CAD patients, along with subsequent lowering of antithrombin-III as indicated in our study. Thus our study substantiate the fact that platelet mediated thrombotic-inflammatory processes are operational in CAD, even when they are on treatment with anti-platelet drugs and there is a need for new therapeutic strategies that specifically target P-selectin exposure which forms a bridge between platelet and inflammatory cells, as indicated by other studies also(Aukrust *et al.*, 2010).

5.2.2.2 Interrelation between thrombotic risk factors

Platelet activation as evidenced by P-selectin exposure was negatively correlated with the levels of AT-III among patients as well as controls. This provides evidence to the fact that low AT-III and elevated P-selectin act synergistically in the progression of thrombotic-inflammatory process as indicated by other studies (Levi, 2004) (Oelschläger *et al.*, 2002). The expression of P-selectin on platelet membrane mediates the adherence of platelets to leukocytes and endothelial cells and enhances the expression of TF on monocytes. This molecular mechanism relies on NFκB activation. The reaction induces binding of activated platelets to mononuclear cells and neutrophils.

P-selectin is easily shed from platelet surface and soluble P-selectin levels are increased in ACS and systemic inflammation. Interaction of P-selectin with monocytes is an important trigger for monocyte recruitment into atherosclerotic plaques. AT-III forms a part of three major mechanisms involved in anticoagulation. Inflammation impairs all anticoagulation mechanisms. AT can directly bind to neutrophils and other leukocytes and attenuate cytokine and chemokine receptor expression essential for inflammation (Levi, 2004).

Oelschläger *et al.* (2002) have reported that AT-III is having anti-inflammatory properties, which potentially blocks activation of NFκB. Their study found that AT-III inhibited agonist-induced DNA binding of NFκB in cultured human monocytes and endothelial cells in a dose dependent manner by preventing phosphorylation and proteolytic degradation of inhibitor protein IκBα. This factor was subsequently inhibiting interleukin-6, TNF-α and TF.

Similarly, a study on inhibitory effects of AT-III on blood cells and endothelial cells in the retinal ischemia - reperfusion injury model in rats proved that intravenous injection of AT-III (250 U/Kg) inhibited leukocyte rolling and suppressed effect of P- selectin as indicated by immuno-histochemistry (Nishijima *et al.*, 2003).

A positive, but weak correlation was observed between fibrinogen levels and v-WF. Fibrinogen and v-WF bind to platelets at different stages of coagulation (Davì and Patrono, 2007). Fibrinogen is bound to platelets by means of glycoprotein IIb/IIIa receptor and this binding is essential for platelet activation as well as triggering of coagulation process(Levi, 2004). During vascular injury, platelets are tethered to the particular site and this tethering is mediated by glycoprotein Ib/V/I. v-WF would bind to glycoprotein Ib. So fibrinogen plays a crucial role in maintaining the stability of thrombus, while v-WF is essential for inter-platelet bridging at low shear stress (Davì and Patrono, 2007). Thus the interaction of fibrinogen and v-WF is at different stages of platelet recruitment and activation and these interactions may lead to the weak correlation between them.

A similar correlation was also observed between Lp(a) and hs-CRP. Elevated hs-CRP and Lp(a) were associated with angiographically proven CAD patients from Malaysia (Shahid *et al.*, 2011). In our patients hs-CRP was not significantly elevated, probably due to the small sample size, but we observed a significant elevation in Lp (a) levels in patients.

A recent review has focused on the role of CRP in different pathways of atherogenesis. CRP have active functions in the processes such as in compliment activation, increased uptake and oxidation of LDL by monocytes, up regulation of adhesive molecules, induction of TF production, inhibition of NO production, inhibition of fibrinolysis by increasing the expression of PAI-1, and promotion of monocyte infiltration into the vessel wall (Shrivastava *et al.*, 2015).

Similarly Lp(a) is also involved in fibrinolytic pathway, as it is a competitive inhibitor of t-PA due to structural homology of apo(a) to plasminogen (Levi, 2004). Lp(a) is also claimed to be interlinked with monocyte activation as an adhesive substrate. Lp(a) interaction with monocyte is via apo(a) and Mac-1 [Macrophage-1 antigen / integrin $\alpha_M\beta_2$ / macrophage integrin]. The general consequence of the Mac-1/ Lp(a) interaction is increased recruitment of monocytes to atherosclerotic vessel [pro-inflammatory action] and increased TF [pro-thrombotic action] on monocytes (Sotiriou, 2006). Thus there is an interrelation between thrombotic risk factors along with inflammatory factors in the progression of atherosclerotic CAD.

There were differences in the level of thrombotic risk factors in patients, but overall thrombotic risk factor levels were elevated in patients. Also 50-90% of the patients had at least 2 to 5 thrombotic risk factors. This further proves the fact, that thrombotic risk factors are elevated in young Indians.

5.2.2.3 Thrombotic risk factors are prevalent in patients with conventional risk factors

Thrombotic risk factors, especially platelet activation and low AT-III were elevated in patients with major conventional risk factors such as hypertension, smoking, diabetes mellitus, positive family history of CAD and dyslipidaemia.

Platelet activation was also elevated to 42% in controls with conventional risk factors. Thus the result from the present study indicate that platelet activation was highly influenced by major conventional risk factors. In a study from Chennai, South India) on patients with CAD and diabetes, collagen induced GP IIb/IIIa binding (another marker for platelet activation) was significantly higher (Deepa *et al.*, 2006) similarly diabetes subjects from our study also had elevated P-selectin exposure.

Patients with low HDL-C also had elevated thrombotic risk factors. A recent review has focused on the role of HDL in cardio-protection along with its role in reducing platelet activation (Kontush, 2014). In another study on patients having CAD, urinary markers of platelet activation indicated that low HDL-C is associated with lipid peroxidation and platelet activation (Vazzana *et al.*, 2013). All these were in line with our observations.

Majority of smokers had their platelets activated irrespective of whether they were patients or controls, indicating tobacco smoking as a major contributor to platelet activation. An earlier study on healthy subjects on low dose aspirin also showed smokers had elevated P-selectin (Pernerstorfer *et al.*, 2001).

Positive family history of premature CAD was also found to correlate with higher levels of thrombotic markers. A study on human umbilical vein endothelial cells of new-born with a strong positive family history to MI expressed significantly high P-selectin compared to controls(Paez *et al.*, 2005). Other association which is reported is that between hypertension and platelet activation (Lip, 2003).

Low AT-III was also found to be an important risk factor, as it was found elevated in smokers. As discussed earlier it is a strong anti-thrombotic and anti-inflammatory agent(Levi, 2004), a probable reason for its reduction in smokers. Fibrinogen was also elevated among smokers indicating that smoking is an important contributing factor to elevation of fibrinogen levels. Smoking alters haemostatic process by multiple mechanisms, which involves alteration of fibrinogen and creating an imbalance in thrombotic, antithrombotic and anti-fibrinolytic pathways (Barua & Ambrose, 2013). All these indicate conventional risk factors could be contributing to thrombotic risk factors.

5.2.2.4 Association of thrombotic factors with ACS and severity of coronary artery disease

Thrombosis plays a critical role in the pathogenesis of ACS (Abbate *et al.*, 2012). Patients presenting with ACS had significantly low levels of AT-III, compared to patients presented with EA. This is a significant finding from our study. As discussed earlier one or two case reports are available in the case of AT-III (Fennich *et al.*, 2013). Other thrombotic risk factors: Platelet activation, Lp(a) and fibrinogen showed only an

increasing trend without any significant difference in the case of presentation as ACS compared to EA.

When disease severity was compared, only a rising trend was seen in the case of all important thrombotic risk factors, but it did not reach statistical significance. A previous study on relation of platelet activation to coronary angiographic severity could not establish a significant difference between platelet activation and coronary angiographic severity (Tan *et al.*, 2005). But a recent study on adverse cardiovascular events in patients with ACS treated with aspirin and clopidogrel found that $P2Y_{12}$ P-selectin test could predict adverse events(Thomas *et al.*, 2014) But in our study we have not determined platelet reactivity and function.

The association between AT-III, fibrinogen (Thompson *et al.*, 1996) (Shi *et al.*, 2010) and Lp(a) (Ashfaq *et al.*, 2012) on disease severity and predictability of the disease is already established, but event rate was comparatively low in our patients. The event rate of only 6% might probably be due to the guideline based treatment regime or may be the fact that the cohort is under close follow-up which will ensure patient compliance.

5.2.3 Leukocyte markers and inflammatory factors as emerging CAD risk factors

Atherosclerotic CAD is also considered as an inflammatory disease, where thrombotic as well as immune mechanisms work hand in hand in the disease pathogenesis, progression and regression. In these processes immune cells dominate in early

atherosclerotic lesions, with their effector molecules accelerating the progression of lesions along with activation of inflammation leading to ACS.

Thus in CAD immune mechanisms interact with metabolic risk factors to initiate, propagate and activate lesions in the arterial tree (Hansson, 2005). Leukocytes play an important role in atherosclerotic CAD (Madjid and Fatemi, 2013) as they are principal cells with immune function in the human body. These cells function in response to variety of adhesion molecules, inflammatory markers secreted during vascular injury produced by endothelium, platelets and resident cells. Whenever there is vascular injury one of the primary responses is leukocytosis. During an acute inflammation primary cells recruited are the neutrophils. Neutrophil engagement is transient and once the inflammation persists, there is increased recruitment of mononuclear cells, especially monocytes (Swirski & Robbins, 2013).

Differential and total leukocyte counts are studied as markers for inflammatory conditions and also CAD. In the present study total WBC count was significantly elevated in patients compared to controls. This was mainly contributed by the significant increase in neutrophil population. There were no significant differences between monocyte population and eosnophil population in both patients and controls. Elevated WBC count have also found to have future predictive value in cardiovascular disease (Madjid and Fatemi, 2013). A study on relation of leukocytes and its subsets with the severity of stable coronary artery disease in patients with diabetes mellitus have shown that leukocyte count

was an independent predictor for high Gensini Scores in patients after adjusting for conventional risk factors of CAD (Hong *et al.*, 2014).

However, monocytes [monocyte- macrophage system] are the principle cells recruited at the endothelium during atherogenesis and they are involved in the uptake of oxidized LDL, foam cell formation (Ley *et.al.*, 2011) and contribute to the production of MMPs ,Tissue Factors(TF), endothelial activation along with platelet activation (Davì & Patrono, 2007). Thus monocytes are actively involved in thrombosis, inflammation (Woollard & Geissmann, 2010) and ROS production (Pamukcu *et al.*, 2010) .

These are important processes that aggravate atherosclerosis, the principal pathological manifestation of CAD. Monocytes are also involved in various processes of atherosclerosis regression as well as angiogenesis (Pamukcu *et al.*, 2010), (Ghattas *et al.*, 2013). The reduction in monocyte population could be considered as a response to anti-atherogenic treatment in patients (Tani *et al.*, 2009).

But the main focus has shifted to the heterogeneity of these monocytes. Research of 20-30 years have proved that monocytes are heterogenous in nature with different subsets based on the expression of surface markers as well as their function in variety of inflammatory processes (Woollard & Geissmann, 2010) (Zawada *et al.*, 2012) (Stansfield & Ingram, 2015).

Monocytes consist of at least two subsets / phenotypes whose proportion in blood fluctuates in response to inflammatory states. Specific shift in subsets may occur in response to different disease processes. Thus selective & rapid enumeration of monocytes

& their subsets/ phenotypes can emerge as potential biomarkers for the identification of different disease conditions (Wildgruber *et al.*, 2009). In view of this, we made an attempt to characterize monocyte phenotypes in patients and controls and correlated with levels of inflammatory markers.

5.2.3.1 Monocyte phenotypic characterization

Monocytes express variety of surface receptors (Martinez, 2009) and also heterogenous in function. The most accepted description of monocytes is based on the surface expression of CD14 and CD16.

The percentage of CM [$CD14^{++}CD16^{-}$] and NCM [$CD14^{+}CD16^{++}$] (Passlick *et al.*, 1989) differ based on different physiological conditions.. Their percentages in blood varies between 70-90% and 10-30% respectively (Ozaki *et al.*, 2012). Later on a third or intermediate subset of monocyte was also included as $CD16^{+}$ along with NCM which exhibited differential expression of CD14 and CD16 phenotypes.

Thus based on the nomenclature committee of the international Union of immunological societies two population of $CD16^{+}$ cells were identified [intermediate/ $CD14^{++}CD16^{+}$ and non-classical monocytes $CD14^{+}CD16^{++}$], along with classical $CD14^{++}$CD16- monocytes (Ziegler-Heitbrock *et al.*, 2010).

But in the present study, we followed the earlier classification (Passlick *et al.*, 1989), as there is confusion regarding the role of CD16 positive monocytes, and there was need to purchase additional antibodies (due to financial constrains) for the clear identification of three phenotypes.

In a similar study from Japan on the role of monocyte subsets on the severity of CAD in patients with stable angina, monocytes were defined as classical and non-classical monocytes alone (Ozaki *et al.*, 2012).

In most of the studies involving monocyte subset characterization, monocyte marker specific antibodies were added to 100 µl of anticoagulated blood and appropriate RBC lysis solution was added for RBC lysis followed by FACS analysis (Ozaki *et al.*, 2012) (Heimbeck *et al.*, 2010) (Rogacev *et al.*, 2012). But in the present study we first separated out mononuclear cells (monocytes and lymphocytes) from the peripheral blood using commercially available ficoll density solution, specific for mononuclear cells. From this $CD14^{+}$ cells were gated out. The method adopted was from a study on monocyte diversity on myocardial infarction (Shantsila and Lip, 2009).

CMs ($CD14^{++}CD16^{-}$) and NCMs ($CD14^{+}CD16^{+}$) were separated from the $CD14^{+}$ cells. The NCMs obtained by this method were a mixture of intermediate and NCMs, based on the current classification of monocytes.

Zawada *et al.*, (2012), used antibody to CD86 marker for the separation of non classical and intermediate phenotypes, which would completely stain monocyte population from the whole blood and gate cells based on the expression of CD14 and CD16. Other studies also used HLA-DR staining (Heimbeck *et al.*, 2010) (Dimitrov *et al.*, 2013). These methods extract all monocytes without contamination from neutrophils and natural killer cells – (a type of lymphocytes, which are also CD16 positive).

Since we separated PBMNC using density centrifugation, contamination from neutrophils were avoided and we could avoid the step of RBC lysis. PBMNC, we separated mainly consisted of lymphocytes along with CD 14 positive monocytes. CD14 positive cells consisting monocytes were completely gated out (as described in Materials and Methods & Results section) from the PBMNC. In many previous studies cryo-preserved samples were used for monocyte phenotype characterization (Berg *et al.*, 2012)(Appleby *et al.*, 2013). Thus several methods are available for monocyte characterization.

Monocyte phenotypes in CAD

There was a trend towards increase in the number of non classical monocytes in patients, while classical monocytes were found lower compared to controls. But the difference was not significant. The absence of significant difference between subsets could probably be due to statin treatment in patients as there are reports of statins affecting monocyte phenotype and production of cytokines.

Peng, (2013) has observed that statin therapy could modulate a shift of NCM, induced during percutaneous coronary intervention to CM. Another study has found that rosuvastatin treatment reduced monocyte activation in HIV patients (Funderburg *et al.*, 2014). A study by Imanishi *et al*. (2010) on association of monocyte phenotype and coronary fibrous cap thickness in patients with unstable angina pectoris showed that NCMs were associated with the particular condition. The same study also showed that percent change in NCM phenotype [$CD14^{+}CD16^{+}CX_3CR1^{+}$] was significantly decreased

in the group of patients who received statin treatment compared with the group of patients who did not received treatment.

Non-classical monocyte pro-inflammatory and Classical monocyte anti-inflammatory?

There is still no consensus over which monocyte phenotype is pro-inflammatory and which one is anti-inflammatory. Non classical monocytes produce inflammatory markers like TNF-α and hence they are considered pro-inflammatory (Belge *et al.*, 2002) (Schlitt *et al.*, 2004) (Skrzeczyńska-Moncznik *et al.*, 2008) . A study has established NCMs as senescent monocytes with shortened telomeres,increased expression of beta-galactosidase and are activated cells which can interact more with endothelium and involved in inflammation in atherosclerosis (Merino *et al.*, 2011).

It has been found that non classical and intermediate monocytes (based on new classification) have more endothelial affinity and they exhibit a homing behaviour. Non classical monocytes which expresses fractalkine receptor or CX_3CR1 is established to have patrolling nature, and while intermediate monocyte is found to have CCR5 receptor expression along with HLA-DR (Zawada *et al.*, 2012). Recent reports have also pointed out monocytes may also involved in the repair of atherosclerotic tissue (Ghattas *et al.*, 2013).

CD16 positive monocytes as predictor of coronary artery disease events

In the present study non classical monocytes were significantly elevated in patients who presented with ACS compared to those with EA and also compared to control subjects. To the best of our knowledge this is the first report which link occurrence of acute events with CAD.

The NCMs, which we describe in our study, are a mixture of non-classical and intermediate monocytes based on the current classification. Rogacev *et al.* (2012) has established intermediate monocytes as predictor of cardiovascular events, almost in line with our observation. But another study published in the same year provided evidence that the classical monocytes ($CD14^{++}CD16^{-}$) can also be predictors of cardiovascular events (Berg *et al.*, 2012) contrary to our findings.

There was no correlation between monocyte subsets and disease severity as established by CAG findings. This is mainly because of the fact that patients with SVD were more likely to present with ACS compared to those with MVD.

In a study on patients with stable angina pectoris, $CD14^{+}CD16^{+}$/ NCMs were associated with disease severity, correlated positively with Gensini score and multivariate logistic regression analysis revealed that the proportion of $CD14^{+}CD16^{+}$ / NCM was an independent contributor to MVD (Ozaki *et al.*, 2012).

Increased CD16 positive cells, mainly consisting of NCMs were also implicated in myocardial salvage and angiogenesis in a study on the impact of human peripheral blood

monocyte subsets in patients with AMI. The presence of excess CMs were implicated in reduction in myocardial salvage or recovery (Tsujioka *et al.*, 2009). Evidence is also emerging on the role of CD16 positive monocytes in heart remodelling, angiogenesis and thinning of infracted myocardium (Ghattas *et al.*, 2013).

5.2.3.2 Monocyte phenotypes and its association with inflammatory markers

We measured TNF-α, IL-1β and MPO activity, inflammatory markers released by leukocytes in the serum and plasma of patients and controls. There was no significant difference between mean TNF-α level between patients and controls. We could not establish a correlation between TNF-α and monocyte subsets. NCMs are considered to be an important source of TNF-α (Schlitt *et al.,* 2004). However in the same study they reported that there was no significant difference in TNF-α production between patients and controls.

Significantly increased levels of TNF- α were seen in subjects within higher quartiles of $CD14^{+}CD16^{+}$/ non-classical monocyte subsets. Plasma levels of TNF-α had been shown to correlate with burden of atherosclerosis as assessed by carotid ultrasound (Skoog, 2002). Another study has associated TNF-α with increased risk of recurrent coronary events in patients in the stable phase after MI (Ridker *et al.*, 2000). These were in contrary to our findings as we could not establish a relation between TNF- α and disease severity as well as coronary events. But in a study on differential TNF production by monocytes under physical stress it was established that Lipopolysaccharide /LPS stimulation was essential for TNF- α production (Dimitrov *et al.*, 2013).

In a study on effects of statin therapy on the production of monocyte pro-inflammatory cytokines, cardiac function and long term prognosis in chronic heart failure in patients with dyslipidaemia, statin therapy attenuated the production of TNF- α and other pro-inflammatory cytokines in monocytes. This was in line with our observation, statin therapy could be the reason for reduced TNF- α production as monocyte being an important source of these cytokines and therapy could have significantly reduced its levels in the systemic circulation (Okopien *et al*.,2005).

A significant increase in serum IL-1-β was observed in our patients compared to controls. IL-1-β also known as catabolin is produced by activated macrophages. It is an important mediator of inflammatory response. Caspase 1 is needed for its proteolytic activation. It is also involved in cell proliferation, differentiation, apoptosis, pain due to inflammation and hypersensitivity. It functions by the production of inflammasome NALP3 (Dinarello, 2009).Traditional CV risk factors increase IL-1-β levels. It can also stimulate iNOS and increase ROS production (Fearon & Fearon, 2008). . IL-1-β can also trigger platelet activation and activated platelets are also major source of IL-1-β a (Davì & Patrono, 2007).

There was no correlation between monocyte subsets and IL-1-β in our study. There are earlier reports which postulate IL-1-β is produced in response to LPS like TNF-α (Jessop & Hoffman, 1993). There is a report of statin and fibrates reducing the production of TNF-α and IL-1-β by monocytes in dyslipidaemic patients on treatment (Okopień *et al.*, 2005).

A significant correlation was observed between IL-1-β and total WBC count and neutrophils count in our CAD patients. Neutrophils are a major source of IL-1-β along with monocytes and respond well to produce the cytokine than monocytes (Chen *et al.*, 2014); probably neutrophils are activated to produce the cytokines in CAD as in other inflammatory conditions (Wright *et al.*, 2010).

Myeloperoxidase (MPO) is a haeme peroxidase. It is an enzyme produced mainly by neutrophils and also circulating monocytes. MPO produce hypocholorous acid and increase oxidative stress. MPO level is reported to correlate with adverse risk in ACS. Monocytes expressing MPO occur in fibrous caps and thrombi in unstable plaque. MPO deficiency provides protection from CAD (Tavora *et al.*, 2009).

MPO levels were significantly elevated in our patients compared to controls, but no difference could be observed between MPO activity based on the disease severity and presentation of symptoms. MPO activity is implicated in variety of atherosclerotic processes such as production of ROS, LDL oxidation, HDL dysfunctionality, inhibition of NO, including formation of foam cells (Karakas & Koenig, 2012). MPO is hence involved in the formation of atherogenic lipids (Nicholls & Hazen, 2008).

MPO is also affected by statin administration. Reduction of MPO mRNA levels was observed in vivo in leukocytes, especially macrophages from statin-fed mice, correlating with reductions in MPO protein and enzyme activity (Kumar & Reynolds, 2005). Similarly, we also could not establish a correlation between monocyte subsets and MPO production. But a positive correlation was observed between MPO activity and neutrophil

count. Neutrophils are major producers of MPO as explained earlier and since we measured serum MPO, the activity was also contributed by the neutrophils too.

None of the inflammatory markers showed any correlation with monocyte phenotypes. Different inflammatory factors may be activated at different phases of the disease. This could be the reason for non-significant levels of TNF-α compared to other inflammatory factors.

Fibrinogen is the only thrombotic risk factor measured and correlated with monocyte subsets in this group of patients and controls. Fibrinogen did not show any correlation with different monocyte subsets, but its levels correlated positively with whole of monocytes (CD14 positive cells of PBMNC) in CAD patients. Fibrinogen is having chemotactic function, which can attract monocytes to atherosclerotic lesion (Papageorgiou *et al.*,2010), a probable reason for increase in monocytes being associated with higher fibrinogen levels.

Thus there is an extensive cross-talk between conventional, thrombotic and inflammatory risk factors in the progression of CAD as explained extensively in our present study.

VI. SUMMARY AND CONCLUSION

There is rising incidence and prevalence of atherosclerotic vascular disease in India and other developing world. Risk factors, both conventional and non-conventional (especially thrombotic and inflammatory) may contribute to the pathogenesis of atherosclerotic CAD. Our hypothesis was "Identification of thrombotic and inflammatory risk factors in addition to estimation of conventional risk factors improves risk assessment for CAD especially in young population". Our major objectives were 1) to develop a data base of young angiographically proven CAD patients less than 55 years, 2) to analyse thrombotic factors in patients and 3) to characterise monocyte phenotypes in young patients along with inflammatory markers and to find whether is any relation between them .

The data of 5467 patients were collected. The study provided evidence that dyslipidaemia especially in the form of low HDL-C was important risk factor in the whole population. Smoking (65%) was the most important risk factor in males. Overall, the risk factor levels were high among female patients, with higher incidence of hypertension, diabetes mellitus and positive family history. The early occurrence of these risk factors is predisposing females to early CAD.

Majority (75%) of the patients were presented with acute coronary syndrome. Based on coronary angiogram findings, single vessel disease was the most common form of angiographic pattern. Severity of disease was more in the case of males, while females generally had single vessel disease and milder forms of the disease.

So the higher prevalence of risk factors contributes to the early development of CAD. Thus there is a need for primordial and primary prevention initiatives to prevent the earlier occurrence of CAD risk factors. Early detection and meticulous control of risk factors is the need of the hour.

Thrombotic risk factor analysis and inflammatory risk factor studies were case control studies. Thrombotic risk factors, fibrinogen, Lp(a) and platelet activation - P-selectin expression were significantly elevated in patients, while antithrombotic risk factor, AT-III was significantly low in patients. PAI-1 an anti-fibrinolytic factor was significantly low in patients. 55% of the patients had three out of five thrombotic risk factors, which included platelet activation, fibrinogen, Lp(a), homocysteine and low AT-III. 91% of the patients had at least two thrombotic risk factors. Thus there was clustering of thrombotic risk factors in the population.

A negative correlation was observed between p-selectin expression, a marker of platelet activation and AT-III as expected in CAD. In general, thrombotic risk factors were high in patients with conventional risk factors. Smokers: whether patients or controls had higher incidence of platelet activation, low AT-III and fibrinogen.

The patients included in the study were on treatment with anti-platelets and statins and on dietary restriction, at least for a period of three months, however this could not lower major thrombotic risk factors such as fibrinogen, platelet activation and Lp(a) and also could not elevate low antithrombin-III and low HDL-C. But treatment had modifying effect over lipid profile other than HDL-C and some of thrombotic risk factors which

were comparable between patients and controls. Another important phenomenon was significant lowering of PAI-1 in patients, which is generally found, elevated in CAD.

The present study clearly shows a cross talk between conventional risk factors and thrombotic risk factors. Meanwhile, a trend of increase in thrombotic risk factors was observed in patients with acute coronary syndrome and severity of disease as evidenced by CAG findings. Adverse events reported after a mean period of 26±3 months were only 6%. This could be due to short follow up period or might be due to effect of treatment.

So the major findings of thrombotic risk factor analysis proposes that there is a need for further studies to understand the fact that whether the strategies aiming at lowering the levels of thrombotic risk factors (in addition to control of conventional risk factors) could improve the outcomes of CAD prevention.

Monocyte sub-typing and inflammatory marker estimation was done in 57 patients and 67 controls. Peripheral blood mononuclear cells were separated from heparinised whole blood, for the FACS analysis of monocytes and two monocyte phenotypes (classical: $CD14^{+}CD16^{-}$ and non classical: $CD14^{+}CD16^{+}$) were characterized. EDTA blood was used for complete blood count analysis and serum/plasma was used for the analysis of inflammatory markers (TNF-α, IL-1β and myeloperoxidase) along with fibrinogen. In CAD, significant increase in total WBC & neutrophils were observed. An important observation was a trend towards increase in non-classical monocytes in patients compared to controls. The difference in monocyte phenotypes in patients and controls were not significant and this could be due to effect of treatment using statins and reduced sample size. A significant difference in monocyte phenotypes were observed within the

patient group, when they were analysed as patients with ACS and effort angina. Patients with ACS had significantly elevated non classical monocytes compared to effort angina patients. Thus monocyte phenotype characterization showed a trend towards an association of non-classical monocytes and CAD. A significant association between non classical monocytes and mode of presentation of CAD was observed and this was associated with ACS. Non –classical monocytes are proven to be activated monocytes, and they have more endothelial affinity, resulting in its correlation with ACS rather than effort angina. These non-classical monocytes also have role in angiogenesis and myocardial recovery another probable reason for its association with ACS as there are evidence of recruitment of these phenotype during myocardial salvage. As we conclude there is a clear need to specify the role of monocyte phenotypes in plaque vulnerability and whether they can be used for biomarker for CAD, which requires long term studies.

Inflammatory risk factors, IL-1-β and myeloperoxidase along with fibrinogen were significantly elevated in our patients, while no significant difference was observed in the case of TNF-α. Different inflammatory factors may be activated at different phases of the disease. This could be the reason for non-significant levels of TNF-α compared to other inflammatory factors. The effect of treatment was significant in the study as none of these inflammatory markers showed correlation with monocyte subset, as there is evidence that statins can attenuate inflammatory cytokine production in monocytes. But a positive correlation was observed in the case of CD14 positive cells and fibrinogen. Significant positive correlation was also observed between IL-1-β and neutrophils as well as between

myeloperoxidase and neutrophils, indicating the involvement of neutrophils in the production of inflammatory markers in CAD.

The entire study provides evidence of involvement of conventional, thrombotic and inflammatory markers in CAD. A cross-talk between conventional risk factors and thrombotic risk factors was observed in the study. The elevated risk factors could be recruiting monocytes in the peripheral blood. These monocytes are activated to mature inflammatory phenotype in acute coronary syndrome and might be triggering adverse events or driving myocardium to salvage, which need more evidence. A significant observation from the study was elevation of non classical monocytes in patients with ACS; hence it is highly essential to elaborate the role of monocyte phenotypes as a marker for CAD and plaque vulnerability. Treatment in patients is contributing to reduction of many of the risk factors in the population. So there is a need for long term studies considering combined evaluation of conventional and non-conventional risk factors to get clear cut idea about the role of these factors in the progression of the disease along with existing treatment modalities. The study also approves the fact that current treatment regimes should be continued, but there should be effort to improve the efficacy of the treatment as many of the thrombotic and inflammatory risk factors were elevated in patients on treatment.

Significance of the study

The first objective of the study to develop a database of young CAD patients was fulfilled. The database demonstrated high prevalence of conventional risk factors in the young population which have significance in primordial and primary prevention.

The study provides evidence for the presence of multiple thrombotic risk factors in the young cohort of CAD patients, a first study from the region. No other study from the region have analysed the association of thrombotic risk factors and various inflammatory markers as analysed from the region.

The study showed there was heightened platelet activation as evidenced by increase in P-selectin expression. This study also demonstrated antithrombin-III deficiency in patients with CAD from the region. Characterisation of monocyte phenotypes in young patients with CAD and controls is one of the first attempts from our country, to the best of our knowledge. It provided evidence that increase in non-classical monocytes is associated with ACS, probably one of the first reports of a direct association of monocyte phenotypes and ACS. A correlation was also observed between fibrinogen levels, leukocyte counts and monocyte subsets.

Limitations of the study

All patients had angiographically proven CAD and were undergoing treatment, for at least, a minimum period of three months with anti-platelets and statins. We could not study patient population who are not on treatment as it was unethical to withdraw medications in patients, as it may lead to adverse events. The sample size was not uniform for all of the risk factors analysed. This was mainly due to financial constraints, as most of the reagent kits were very expensive. Major part of the study was in case-control mode and hence the study was not free from biases in sample selection. Gender bias was evident in our sample selection as majority of the patients were males which prevented gender specific studies.

6.1 Future Directions

- Multiple thrombotic risk factors were elevated in CAD patients. So there is need to initiate long term studies on the prevalence and control of important thrombotic risk factors, based on current treatment regimes or find whether there is a need to improve the efficacy of current treatment modalities or to introduce new strategies of treatment and finally, to see whether how far reduction of thrombotic risk factors can help improving the outcomes.
- Monocyte subsets can have different functions in the progression and regression of atherosclerotic CAD. In the present study a trend towards increase in non-classical monocytes was observed. In vitro cell culture techniques can be employed for the culture of specific monocyte subsets under experimental conditions. These studies may certainly shed light into the different functional aspects of monocyte subsets and may provide evidence on their role in inflammation and finally atherogenesis.
- Platelet activation as evidenced by P-selectin exposure was significantly elevated in patients; this P-selectin is a receptor for the monocyte ligand. Thus in atherosclerotic CAD there is a clear evidence of formation of monocyte –platelet aggregates. These aggregates are more potent in the progression of thrombosis and inflammation in CAD than individual cell aggregates. Thus role of monocyte –

platelet aggregates, their interaction and microparticle production should be elaborated further for better understanding of pathogenesis of CAD.

- Monocyte phenotypes are related to plaque vulnerability, as there is evidence of involvement of particular phenotypes *in situ* at atherosclerotic plaque site. New imaging techniques such as optical coherence tomography should be employed to identify plaque morphology like plaque rupture or plaque erosion and correlate it with monocyte phenotyping in the peripheral blood, so that it could be used in future to identify the morphology of the lesions. There are few reports, which state that statins can attenuate monocyte cytokine production and statins could change monocyte phenotype from pro-inflammatory to anti-inflammatory. These reports points to the fact that monocyte phenotypes could be used as efficient treatment targets. For this understanding monocyte subset functionality and biology is very essential. Elaborate transcriptome and gene expression studies may provide light into the variety of markers expressed by monocytes and many of the targets have been identified. Thus there is a need to develop new target specific drugs, which would efficiently cleave out harmful phenotype *in situ* and drive vascular endothelium to recovery without advancing to plaque vulnerability.

VII. BIBLIOGRAPHY

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VIII. LIST OF PUBLICATION AND CONFERENCES/ SEMINARS

8.1 Journal Publications

1. **"Simultaneous determination of homocysteine and asymmetric dimethylarginine in human urine by liquid chromatography–tandem mass spectrometry"–** Authors: C.L. Gopu, P.R. Hari, **Reema George**, S. Harikrishnan, K. Sreenivasan. *Journal of Chromatography B,* 939 (2013) 32– 37 **http://dx.doi.org/10.1016/j.jchromb.2013.09.010**
2. "**Circulating Thrombotic Risk Factors in Young Patients with Coronary Artery Disease Who Are on Statins and Anti-platelet Drugs"-** Authors: **Reema George**, Harikrishnan S., N. Jayakumari, Anugya Bhatt, Jissa V.T., Jaganmohan A. Tharakan. *Indian Journal of Clinical Biochemistry* (July-Sept 2016) 31(3):302–309. **DOI 10.1007/s12291-015-0540-y**
3. **"Enhanced P-selectin expression on platelet-a marker of platelet activation, in young patients with angiographically proven coronary artery disease"-** Authors: **Reema George,** Anugya Bhatt, N. Jayakumari, Jissa V.T., Harikrishnan S. *Journal of Molecular and Cellular Biochemistry* (2016) 419:125–133. **DOI 10.1007/s11010-016-2756-4.**

8.2 Conferences and Seminars

International

- Abstract titled **"Coronary Artery Disease In The Young In India – A Gender Based Comparison**" was presented as poster in the World Congress of Cardiology 2014 held at Melbourne, World Heart Federation Australia on May 04 - 05. Authors: Harikrishnan S, Jaganmohan Tharakan, Jayakumari N, **Reema George**, Jeemon George, Manas Chacko, Krishna Sankar, Vivek Narayanan, Suresh Babu, Vineeth CP.

- Abstract titled **"Profile of symptomatic CAD in patients younger than 30 years of age"** was presented as Oral Abstract in Asia Pacific Society of Cardiology Congress held at AbuDhabi on May 2nd 2015. Authors: Arun GK, Harikrishnan.S, Sanjay G, S Sivasankaran, Ajithkumar VK, **Reema George**. Presented as oral abstract.

- Abstract titled "**Thrombotic risk factors were high in young CAD patients who are on statins and anti-platelet drugs"** was presented as Poster in 6th International Conference on Recent Advances on Cardiovascular Sciences (January 31st-February 1st 2014), Organized by International Academy of Cardiovascular Sciences India, at Delhi Institute of Pharmaceutical Sciences and Research, New Delhi. Authors: **Reema George**, Anughya Bhatt, N. Jayakumari, S. Harikrishnan.

- Presented a paper titled **"Platelet Activation, a thrombotic- inflammatory factor playing a prominent role for Coronary artery disease"** (as first author) at International Seminar on recent biochemical approaches in therapeutics, organized by Department of Biochemistry, University of Kerala at Trivandrum, January 2013. Authors: **Reema George,** Anughya Bhatt, N. Jayakumari, S. Harikrishnan.

National

- Abstract titled **"Thrombotic risk factors [Antithrombin-III, Platelet Activation, Lipoprotein(a) and Fibrinogen] are playing prominent role for CAD in the young**" was presented in the Cardiological Society of India, Kerala chapter meeting held at Thodupuzha, on November 02, 2013. Authors: **Reema George**, Anughya Bhatt, N. Jayakumari, S. Harikrishnan, Jagan Mohan Tharakan. Presented by Arun Gopalakrishnan.
- **Abstract titled "P-selectin exposure on platelets and its relation with Antithrombin-III in CAD patients on statin antiplatelet therapy"** was presented at Institute day of SCTIMST (April 2015).Authors: **Reema George**, Anughya Bhatt, N. Jayakumari, S. Harikrishnan, Jagan Mohan Tharakan.