Vitamin D influences calcium dependent cardiovascular functions with reference to NOS3 and VEGF

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My Hero, Inspiration,Beloved father

Late.Sri.M.K.Mullur

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LIST OF ABBREVIATIONS

Abbreviations:

%	- Percent
. OH	- hydroxyl radical
ALP	- Alkaline Phosphatase Level
ALT	- Alanine Transaminase
ANOVA	- Analysis of Variance
ATP	- Adenosine Tri Phosphate
B.wt	- Body weight
BMI	- body mass index
BP	- blood pressure
bpm	- beat per minute
BSA	- body surface area
CAD	- Coronary artery disease
CCF	- Congestive cardiac failure
CHF	- Congestive heart failure
Cm	- Centimeter
Conc	- Concentration
CVD	- Cardiovascular disease
CV%	- Co-efficient of variability
DBP	- Diastolic systolic pressure
DNA	- Deoxy ribonucleic acid
ECAM	- Endothelial leucocyte adhesion molecule
ECG	- Electrocardiogram
Echo	- Echocardiograph
EDRF	- Endothelium derived relaxing factor
eNOS	- Endothelial nitric oxide synthase
FFA	- Free Fatty Acids
g/dl	- Grams/Desi litre
g/L	- Grams/Litre
gm	- Gram
gms	- Grams

HHD	- Hypertensive heart diease
H0	- Null hypothesis
H1	- Alternate hypothesis
H2O	- Water
H_2O_2	- Hydrogen peroxide
Hb%	- Haemoglobin Percentage
HDLC	- High Density Lipoprotein fraction of cholesterol
HIF-1α	- Hypoxia inducible factor-1α
IEC	- Institutional Ethical Committee
IHD	- Ischemic Heart Disease
Kg	- kilograms
LDLC	- Low Density Lipoprotein fraction of cholestrol
m 2	- square meter
MCH	- Mean Corpuscular Hemoglobin
MCHC	- Mean Corpuscular Hemoglobin Concentration
MCV	- Mean Corpuscular volume
MDA	- Malondialdehyde
mg/dl	- Milligram per decilitre
mg/kg	- Milligram per Kilogram
MI	- Myocardial infarction
ml	- Millilitrer
mmHg	- Millimetre of mercury
mmol/L	- Milimole per Liter
NCDs	- Non-Communicable Diseases
NO	- Nitric Oxide
NOx	- Total Nitric Oxide Concentration
O 2	- Oxygen
$O2^{-}$	- Superoxide radicals
ONOO -	- Peroxynitrite
PCV	- Packed Cell Volume

pg/mL	- Pictogram per millilitre
PP	- Pulse pressure
PR	- Pulse rate
RBC	- Red Blood Corpuscles
RHD	- Rheumatic heart disease
RNA	- Ribonucleic acid
ROS	- Reactive Oxygen Spaces
Rpm	- Rotation Per Minute
SBP	- Systolic blood pressure
SMCs	- Smooth Muscle Cells
SOD	- superoxide dismutase
SV	- Stroke volume
TC	- Total Cholesterol
TGs	- Triglycerides
VCAM1	- Vascular cell adhesion molecule-1
VEGF	- Vascular endothelial growth factor
VLDL C	- Very Low Density Lipoprotein fraction of cholesterol
VSMCs	- Vascular smooth muscle cells
WBC	- White Blood Corpuscles
WHO	- World health organization
WR	- Working reagent
µl	- Micro liter
µm	- Micrometer
µmol/L	- Micromole/Liter

ABSTRACT

ABSTRACT

Objective: To assess the link between vitamin D and cardiovascular diseases (CVD) in the percpective of angiogenic factors among the various types of CVD patients admitted in ICCU.

Methods: Cross sectional study was conducted on CVD patients age ranging from 40 to 80 years who were diagnosed for first time and admitted in ICCU of BLDE(DU) Shri.B.M.Patil Medical College , Hospital and Research Centre Vijayapur ,Karnataka(India).

The following parameters were tested: Anthropometric parameters: height (cms), weight (kgs), BMI (kg/m^2) and BSA (m^2); Physiological parameters: pulse rate in (beats/min), systolic blood pressure (mmHg), diastolic blood pressure (mmHg), pulse pressure (mmHg) and mean arterial pressure (mmHg); Hematological parameters: RBC, WBC, HB%, PCV, Platelet count and blood indices like MCV, MCH, MCHC; echocardiography; electrocardiography; biochemical parameters: triglyceride, cholesterol, HDL, LDL, VLDL, serum creatinine, CPK-MB, serum sodium, potassium, calcium and vitamin D; Oxidative and nitrosative stress measure: serum malondialdehyde (MDA), nitric oxide (NOx) concentration; antioxidant capacity: serum superoxide dismutase (SOD) activity, and vascular endothelial growth factor (VEGF) and nitric oxide synthase (NOS3).

Results: The increase in white blood cells (WBCs) level in present study indicate the inflammatory process in the physiological system. Further increase in the ESR is indicative of myocardial damage with leakage of proteins. As dyslipidemia is associated with various types of stress factors and MI, HHD and IHD are definitely generate more stress among all the types of CVD.Our results show significantly lower levels of vitamin D in all groups of CVD patients. Results also found higher levels of MDA , significantly lower levels SOD and lower

levels NO. We found decreased VEGF levels in present study in groups 3(IHD),4(Angina),6(RHD) and 7(Cardiomyopathy) and decreased levels of NOS3 in all groups of CVD patients

Conclusion: Results clearly shows that vitamin D is an important factor for the regulation of cardiovascular health. Deficiency of vitamin D leads to most types of CVD in present study. Possibly vitamin D influences angiogenic factor like VEGF and alter cardiovascular remodeling. Further it may be concluded that the role of vitamin D induced cardiovascular remodeling depends on nature and types of CVD.

CHAPTER 1

INTRODUCTION

INTRODUCTION

CARDIOVASCULAR DISEASES (CVD):

The cardiovascular diseases (CVD) like heart failure (HF), myocardial infarction (MI), acute coronary syndromes (ACS), are on the frontline, when it comes to morbidity and mortality, worldwide.(Munzel *et al*, 2015)

According to Global Burden of Disease study, during the last two decades, the incidence of non-communicable diseases snatched the limelight due to its high morbidity and mortality. It replaced the more common and deadlier , infectious diseases, due to an overall improvement in the standard of living conditions, with respect to water quality, sanitation, nutrition, socio-economic conditions, and lastly the advancement in the scientific field, which contributed to broadening the pathway to cure of many severe infectious diseases, paving a way to reduce its respective burden across the whole world. (Lim *et al* 2010; Murray *et al*, 2012)

However, with this improvement of standards of living, a curse followed in the form of lifestyle-associated disease patterns categorised under various disorders like obesity, hypercholesterolemia, hypertension, smoking, diabetes mellitus etc. This kind of lifestyle slowly encroached into the people's lives like a silent killer and soon spread throughout the world like a pandemic, harbouring the various risk factors within, like a time bomb to be exploded at any time in near future. It finally culminated in various cardiovascular diseases like HF, MI, ACS and its sequelae and complications associated with huge morbidity and premature mortality, in the form of greater than 50% of deaths worldwide and also loss of 20% life years. (Lim *et al* 2010; Murray *et al*, 2012)

VITAMIN D:

During the last decade, it became clear that deficient serum concentrations of vitamin D metabolites are prevalent not only in specific patients groups but also in the general population in western countries and throughout the world. The by far most important reason for this phenomenon is an inadequate skin exposure to solar ultraviolet B radiation, as ultraviolet B-induced skin synthesis is the major source of vitamin D for humans. Ecological studies have reported higher rates of CHD with increasing distance from the equator, a phenomenon that can be attributed to the higher prevalence of vitamin D deficiency in regions with less exposure to sunlight(Palacios and Gonzalez,2014)

Due to the above mentioned enormous disease pattern, showing an increasing trend in its incidence pattern, a necessity was created to solve this enigma of an up rise in the respective diseased population, which led them to explore at the molecular level for bringing about an improvement in the trajectory of the natural history of the disease, and change it for

the better, by suitable interventions during its progression. In this constant and untiring search, vitamin D emerged as the winner, as it was found to be the chief mediator of vascular health, through its potential beneficial effects on the same. (Palacio and Gonzalez, 2014)

Before embarking on its various beneficial effects, one has to acknowledge its physiological actions in the body. Secondly, it is found to be important for regulation of cardiovascular function. In the blood, 85% to 90% of 25-hydroxy vitamin D is bound to vitamin D binding protein (VDBP), and 10%-15% is bound to albumin and the remaining portion circulates freely. However, the metabolically active form of vitamin D is 1,25 dihydroxy vitamin D formed from 25-hydroxy vitamin D by the action of the enzyme, 1-hydroxylase. (Bikle *et al*, 2017). It is this form of vitamin D which is the chief mediator of various physiological effects in the body, which gets disrupted due to stressful situations, occurring as the final common pathway of abnormal lifestyle patterns.

According to various recent studies, reduced vitamin D levels in the body was the core operator of cancers, autoimmune diseases, inflammatory diseases, depression, cardiovascular diseases (Hypertension, coronary artery disease, left ventricular hypertrophy, increased arterial stiffness, endothelial dysfunction) (Holick, 2007). However, its molecular mechanism remains elusive and mysterious enough to catapult the emergence of various other studies focussed on it. One of the research made with respect to the effect of vitamin D on vascular health was that, it promoted downregulation of proinflammatory cytokines like IL-1, IL-6 and TNFα. So therefore, we can say that adequate vitamin D is very much essential for maintenance of vascular homeostasis. (Talmor *et al*,2008). This particular study contradicted the previous notion that vascular calcification was a passive process, that occurred as a non-specific response to any kind of vascular damage. However, recent studies found that vascular calcification is an active process, which occurs due to the loss of specific neuroprotective effect of vitamin D. Furthermore studies stated its positive effects on growth,

proliferation, and morphology of murine cardiomyocytes. (Talmor *et al*,2008) As mentioned earlier, it has a beneficial effect on the cardiovascular health, mediated by its action on the endothelium. With respect to this mode of action, one has to acknowledge the power of the endothelium to act as both endocrine and paracrine organ, directly bringing about vascular homeostasis.

Researchers found that it was the endothelial dysfunction which culminated into impaired cardiovascular function. It is also the target structure of various risk factors which mediate the pathogenesis of the various cardiovascular diseases. Therefore, as we now know that vitamin D can maintain the endothelial health, we can alter the trajectory of entire CVDs for the better, by external supplementation of vitamin D, the evidence of which has to be accumulated through various clinical trials.

Epidemiological studies identified the deficiency or low level of vitamin D in most part of world population irrespective of age, ethnicity, and geographical location. (Palacio and Gonzalez, 2014)

CALCIUM:

In search of various mechanisms through which vitamin D mediates the maintenance of vascular health, the latest achievement by the researchers has been role of calcium in it. According to studies, calcium homeostasis operates at the heart of cardiovascular health as it alters or dictates the physiological microenvironment of the cardiac musculature. If we briefly refer to the details of its role in our body, one must not forget that calcium dietary intake is the only way of maintaining the calcium homeostasis, with its RDA being 1000-1500 mg/day, with no endogenous synthesis of it within our body. Three main organs play a chief role in its functioning in the body, bone, kidney and intestine. They do so by

coordination in between each other, by the virtue of hormonal interaction in between parathormone and vitamin D. (Berridge *et al*, 2000)

The physiological effects of calcium ions in the body is reflected in the form of its diverse control of various functions like muscle contraction, hormonal secretion, neuronal circuits, immune responses and gene expression. Therefore, now we can say that vitamin D is one such extrinsic factor which is amenable to modification, thereby, having the potential of affecting the prognosis of various disorders, just by repletion of its stores in the body. (Munro, 2010)

NITRIC OXIDE SYNTHASE (NOS3/eNOS) & NITRIC OXIDE (NO):

One of the most potent effector on endothelium is the NOS3 or eNOS expression within it. It is imperative to understand the mode of action of NOS3 on the endothelium to enlighten the roadway of management of various CVDs, which is invariably associated with endothelial dysfunction, irrespective of the type of CVD. The most important proven effect of NO on endothelium is the vasodilatation, decreased in the various CVDs, due to decreased expression of NOS3 in them. (Förstermann et al,1994)

There are three isoenzyme forms of NOS, namely eNOS (endothelial NOS), iNOS (inducible NOS), & nNOS (neuronal NOS). Out of these, it's the iNOS which when released excessively can regulate blood pressure (BP) and heart rate (HR). Therefore, despite the physiological vasodilatation produced by eNOS, the presence of iNOS totally dampens the beneficial effect of eNOS.(Das et al,2017)

This increased expression of iNOS occurs due to accumulation of various reactive Oxygen species(O_2^-,H_2O_2), leading to its expression through transcriptional and post-transcriptional mechanisms. On contrary, eNOS exerts its cardioprotective effect through increase of cGMP in the smooth muscle cells culminating in physiological vasodilatation.

eNOS is also that type of isoenzyme which when released by the vascular endothelium brings about inhibition of platelet adhesion and aggregation. It also inhibits the leucocyte adhesion to the vessel wall (Kuhlencordt *et al*, 2001)

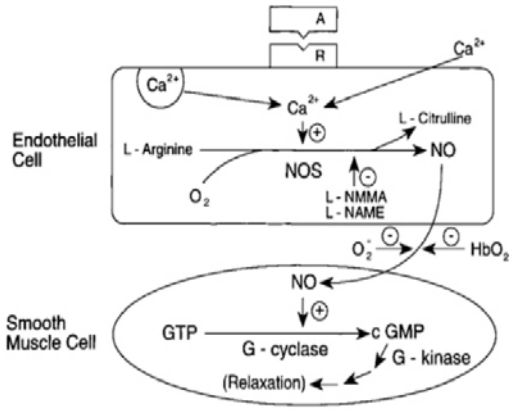


Fig.1.1 Mechanism of NO synthesis and its action on smooth muscle cell.

Source: Taylor CT. Mitochondria and cellular oxygen sensing in the HIF pathway. Biochem J. 2008;409:19-26

Vascular endothelial growth factors (VEGF):

Vascular endothelial growth factors (VEGF) are primary angiogenic molecules controlling vascular homeostasis, vascular growth and function. It plays very important role in the development of collateral vessels and angiogenesis.

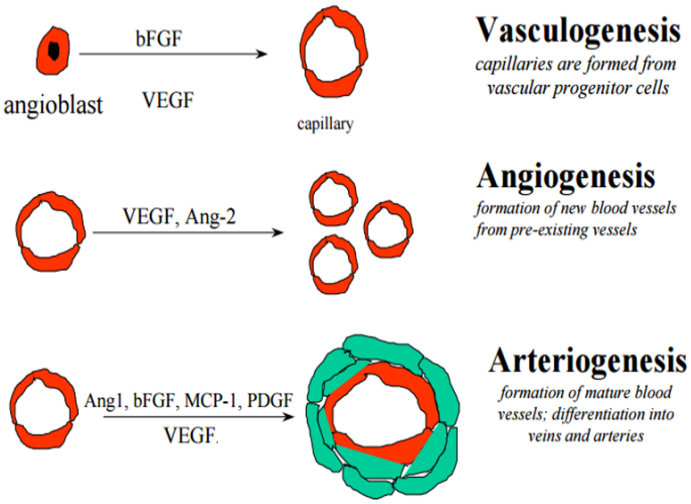


Fig.1.2 VEGF and vasculogenesis, angiogenesis and arteriogenesis.

Source: Taylor CT. Mitochondria and cellular oxygen sensing in the HIF pathway. Biochemical Journal. 2008;409:19-26

Very less information is available from randomized controlled trials concerning the effects of VEGF on CVD in the general population.

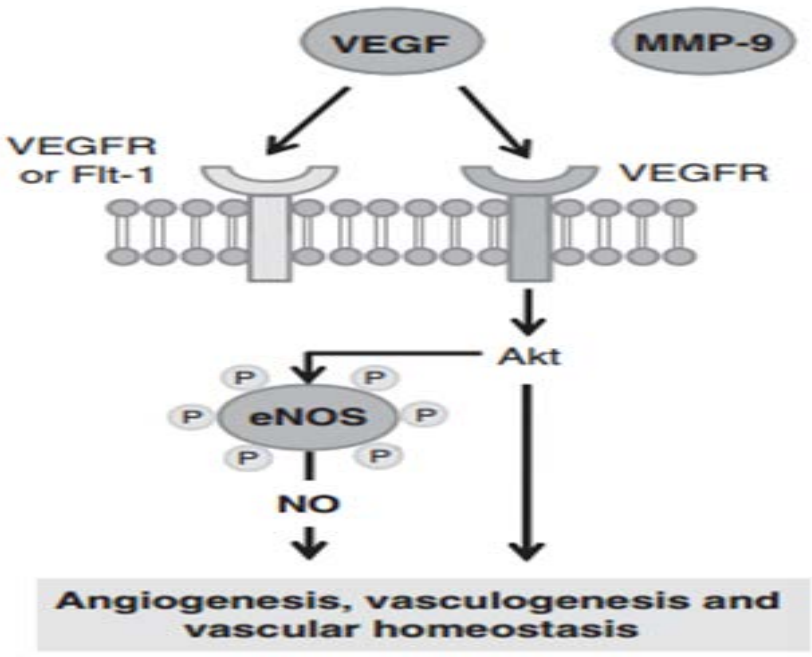


Fig.1.3. Role of VEGF on stimulation NO production subsequently angiogenesis, vasculogenesis and vascular homeostasis.

Source: Das KK, Das S, Ambekar JG. Hypoxia and oxidative stress: Cell signaling mechanism and protective role of vitamin C and cilnidipine. In: Catala A, editor. Lipid Peroxidation:Inhibition, Effects and Mechanisms. Chapter 11, ISBN 978-1-53610-506-3. NY: Nova Science Publishers; 2017. pp. 249-262

Vitamin D and cardiovascular health:

The endothelium is a complex endocrine and paracrine organ that plays a crucial role in the maintenance of vascular homeostasis. Impaired endothelial function has been postulated to provide a final common pathway by which multiple risk factors exert their deleterious effects on cardiovascular health (Cohn *et al*,2004)

Vitamin D deficiency (VDD) has been associated with endothelial dysfunction and CVD. It has attracted recent attention for its potential cardio-protective properties especially its actions on the endothelium.

The calcium homeostasis is directly linked with physiological microenvironment of cardiac myocytes. This link is expected to have a relation with endothelial functions of vascular system. The calcium link is also suspected with functional aspect of NOS3 protein synthesis.

Further, a link between vitamin D and vascular health is also a current hot topic for cardiovascular research where the relationship between vitamin D and VEGF in the perceptive CVD yet to be ascertained.

Researchers began noticing that men (and women) with vitamin D deficiencies had a host of symptoms that appeared to be related to decreased nitric oxide output: "hypertension, left ventricular hypertrophy, increased arterial stiffness, and endothelial dysfunction in normal subjects and in patients with chronic kidney disease and type 2 diabetes." (Marianne *et al,* 2011) This was verified by a fairly recent animal study showing that vitamin D

deficient rats in the womb and early life were much more likely to develop hypertension and had a decreased ability to create nitric oxide. (Olena *et al*, 2014)

NOS3 protein deficiency was found to be associated with various pathological consequences like hypertension, increased vascular smooth muscle cell proliferation in response to vessel injury, increased leucocyte-endothelial interactions, hypercoagulability and finally increased diet-induced atherosclerosis. (Kuhlencordt *et al*,2001). However, researchers finally found a link between vitamin D and NO through the studies showing the vitamin D deficiency associated with host of disorders occurring as the consequence of decreased NO output like hypertension, left ventricular hypertrophy (LVH), increased arterial stiffness, endothelial dysfunction etc. This revelation was a part of study which showed that vitamin D deficient rats in the womb and early life were much more likely to develop hypertension and a decreased ability to produce NO.

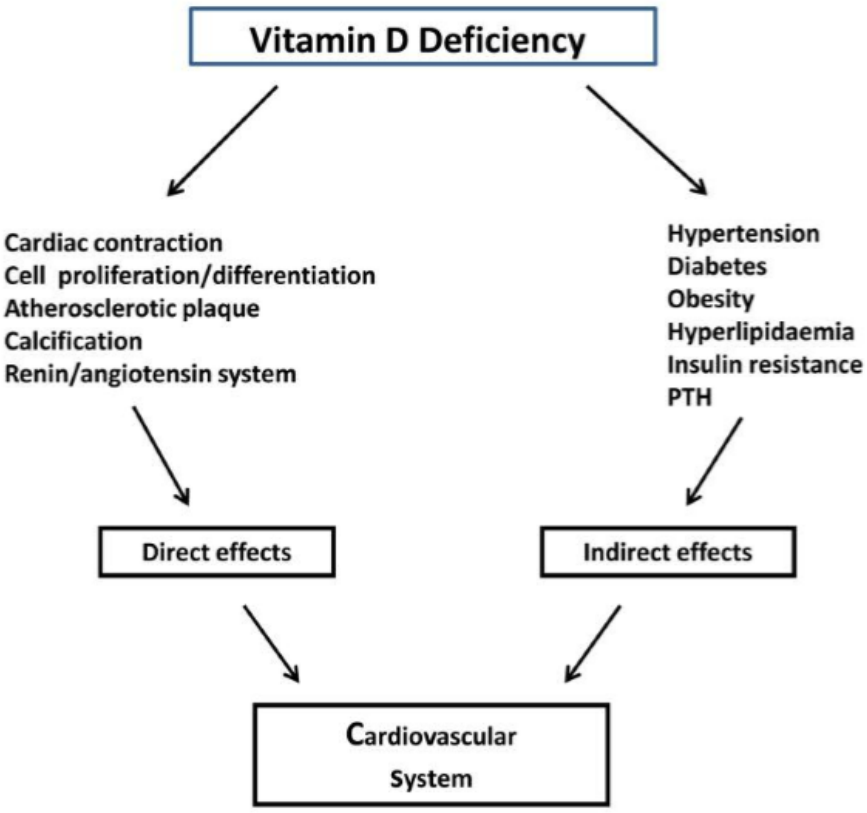


Figure1.4.Associations between vitamin D deficiency and cardiovascular disorders.

Source: Nitsa et al: Vitamin D and Cardiovascular Disease (Review). 32: 977-981 (2018)

Vitamin D deficiency affects the cardiovascular system both directly and indirectly through the multiple roles that it plays in various conditions and pathologies associated with cardiovascular system (see text for details). PTH: Parathyroid hormone.

CHAPTER 2

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Vitamin D,Calcium and CVD:

There is continuous interest to assess the role of vitamin D in development and management of cardiovascular disease (CVD). There exist a relationship between low vitamin D levels and obesity, diabetes mellitus, dyslipidaemia, endothelial dysfunction and hypertension. Though vitamin D has protective mechanism and therapeutic use in CVD but exact mechanism is not much known.

Smokers who show dyslipidemia are at high risk for cardiovascular disease and these are associated with vitamin D deficiency and myocardial infarction.

However, in an analysis of a cohort study, 454 men who reported nonfatal acute MI or fatal CHD had significantly lower levels of 25(OH)D when compared with 900 matched controls without cardiovascular disease; this risk remained significant after adjustment for

other risk factors including family history, diabetes, hypertension, race/ethnicity, body mass index, and others.[4]

Boonstra (2001) and Rahman (2007) in their experimental study observed that there was evidence that vitamin D deficiency results in maladaptive cardiac remodelling attributable to progressive myocyte hypertrophy and interstitial fibrosis. Also VDR knock out mice exhibited ventricular hypertrophy and increased matrix turnover (Boonstrar *et al*, 2001 and Rahman *et al*, 2007).

Scragg and Sowers (2007) in their large population study 'The Third National Health and Nutrition Examination Survey' (NHANES III), showed that systolic BP and pulse pressure correlate inversely with levels of vitamin D. (Scragg and Bell, 2007)

Griffith and van Mierlo (2006) in their epidemiological data has shown that supplementation of calcium is associated with small reductions in systolic and diastolic blood pressure.(Griffith *et al*,1999 and van Mierlo *et al*, 2006)

In a cohort study, 3299 Caucasian patients who were candidates of coronary angiography were followed up for a median of 7.7 years; those patients with 25(OH)D levels below 10 ng/mL had a significantly increased risk for death due to heart failure and sudden cardiac death when compared with patients with optimal levels of vitamin D, after adjustment for multiple known CVS risk factors.(Pila *et al*, 2008)

Wang et al (2010), in their data from 10 prospective studies and 8 RCTs, reported CVD outcomes of supplemental calcium alone, calcium plus vitamin D, and vitamin D alone. The collected data did not demonstrate any apparent effect, either beneficial or harmful, of calcium supplementation with or without vitamin D. (Wang *et al*, 2010)

Lipid Profile and CVD:

Rajmohan *et al* (2000) in their study found that the prevalence of coronary artery disease was significantly high among patients with isolated hypercholesterolemia , isolated high LDL and isolated low HDL when compared to individuals with normal lipid profile.

Castelli and Anderson (1986) found that the level of total cholesterol proved to be an excellent predictor of coronary heart disease in those aged less than 50 years. However, in those aged over 50 years, more accurate predictors of coronary heart disease risk were serum lipoprotein measurements.(Castelli and Anderson,1986)

In India only limited studies exist on epidemiology of cholesterol and other lipoprotein lipids on large samples in the last 20 years.

Blood Pressure:

The Asia Pacific Cohort Studies Collaboration have demonstrated the log-linear relationships of BP with ischaemic and haemorrhagic stroke, IHD and total cardiovascular death.(Lawes *et al*, 2003)

In the ICMR study on 5537 individuals (3050 urban residents and 2487 rural residents) demonstrated 25% and 29% prevalence of hypertension (Criteria: >=140/90 mm of Hg) among males and females respectively in urban Delhi and 13% and 10% in rural Haryana.

Altered endothelial function leads to thickening of the intimal layer, especially in the peripheral muscular arteries and can contribute to raised peripheral vascular resistance, a characteristic of hypertension in the elderly population (Taddei S *et al*., 2001; Torregrossa AC *et al*., 2011).

Pulse pressure is an easy, independent and finest instrument for measuring arterial stiffness and a good indicator for cardiovascular hazards. Studies have shown a strong association between PP and arterial stiffness (Safar ,2000; Safar *et al*., 2003; Cecelja *et al* 2009).

Vascular Endothelium:

Dynamic nature of endothelium cell layer has multiple physiological functions, like blood perfusion regulation, fluid exchange, coagulation mechanism, inflammatory responses, vasculogenesis and angiogenesis (Aird, 2004; Pries & Kuebler, 2006). Endothelium by secreting various mediators is involved in both synthetic and metabolic functions .Endothelial system is the prime short-term regulator of BP like baroreceptor reflex (Stauss & Persson, 2000). Basal vascular tone and BP are regulated by normal levels of NO produced by endothelial cells (Jin& Loscalzo , 2010).

In adults, approximately ten trillion (10^{13}) single layered endothelial cells form an 'organ' with a large surface of approximately about 350m 2 area and about 110g weight (Pries & Kuebler,2006). Structural and functional integrity of endothelial cell are required for various vital CV functions and integrity (Galley & Webster,2004). The vasodilator function of endothelium was demonstrated by removing endothelial cells and observing acetylcholine induced dilator response from among isolated arteries (Furchgott & Zawadzki , 1980). The key factor responsible for arterial relaxation was nitric oxide (NO) which was first discovered as endothelium derived relaxing factor (EDRF) (Vanhoutte *et al*., 2009). NO, being an autocoid derived from endothelium is a primary factor of vascular homoeostasis and is a simple molecule that regulates vascular tone, vascular permeability and antithrombotic properties (Jin & Loscalzo, 2010). A review by El Assar *et al*. (2012) stated the different processes which alter endothelial function.

Oxidative Stress:

Concept of effect of oxygen free radicals and nitric oxide (NOS) in the pathogenesis of CVD is well known. However, the cardiovascular system equipped with defence mechanism which include antioxidant enzymes like super-oxide dismutase (SOD), etc. and other free radical scavengers to protect the cell against cytotoxic ROS, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\cdot}$). (Das, 2000)

An increase in production of ROS such as Superoxide radicals ($O2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$) and singlet oxygen causes oxidative stress. ROS also participate in physiological functions like endothelium-dependent functions, smooth muscle and endothelial cell growth and survival, and regulation of remodeling of the vascular wall (Fortuno A et al., 2005).

Importantly, oxidative stress can be altered by the inequality between antioxidant defenses and reactive oxygen species (ROS) that are produced in vessel walls and regulate cell functions and cellular senescence (Erusalimsky JD., 2009). Imbalance in the regulation of oxidative stress contributes to vascular pathology characterized by loss of mitochondrial function and increased ROS production, and, eventually, leads to the development of cardiovascular pathological alterations, such as increased BP and heart attacks. Nitric oxide (NO) is as an endothelium-derived relaxing factor was invented by Furchgott and Zawadzki. NO documented as an important molecule that regulates of vascular internal environment including vascular permeability, vascular compliance and antithrombotic properties (Palmer *et al.,* 1987).

Superoxide molecules are produced by transferring an electron to oxygen; superoxide concentrations can be reduced to the picomolar levels by superoxide dismutase (SOD). However, superoxide molecules react with nitric oxide (NO) at least

ten times faster than SOD can scavenge NO (Beckman , 2001). This reaction may have some biological significance when the concentration of superoxide molecules rises in blood vessels with advancing age (Faraci and Didion, 2004). The higher value of superoxide inhibits the generation of NO by vascular cells, resulting in the impairment of endothelium-dependent relaxation. On the other hand,both eNOS activation and NO bioavailability are decreased with age (Guzik *et al*., 2002; Soucy *et al*., 2006); lower NO levels further increase ROS production.

Oxidative stress contributes to inactivation of NO resulting in its reduction in bioavailability and endothelial dysfunction. Loss of normal endothelial function associated with decreased NO production results in altered vasorelaxation and hypertension (Schulz *et al*., 2011; Silva *et al*., 2012).

Various interventions, including administration of antioxidant vitamins and antihypertensive agents and exercise training, will enhance the protein levels and enzymatic activities of SOD, such as Cu/Zn SOD and Mn SOD, in the vascular endothelium and smooth muscle cells of the aorta in experimental animal models. (Yamashita et al, 1999)

Vascular endothelial Growth Factor (VEGF):

Blood vessel development consists of two distinct phases – vasculogenesis and angiogenesis. Vasculogenesis is the assembly of vessels de novo and angiogenesis arises through the proliferation, movement, and incorporation of endothelial cells into existing vessels (Geudens and Gerhardt,2011).

Vascular endothelial growth factor (VEGF), is a signal protein produced by cells that stimulates the formation of blood vessels (Senger *et al*., 1983). The growth of new blood vessels requires the activation of specific signal transduction pathways mediated in endothelial cells by the vascular endothelial growth factor (VEGF) and

angiopoietin families of growth factors (Ferrara *et al.,* 2003; Yancopoulos *et al*., 2004). VEGF is also implicated in the survival of newly formed blood vessels and blood vessels in tumors (Alon *et al*., 1995; Benjamin and Keshet .,1997; Benjamin *et al*., 1999).

NEED OF THE STUDY

The calcium homeostasis is directly linked with physiological microenvironment of cardiac myocytes. This link is expected to have a relation with endothelial functions of vascular system. The link is also suspected with functional aspect of NOS3 gene expression pathways. Further, a link between calcitriol and vascular health is also a current hot topic for cardiovascular research. In view of a complex scenario to understand cardiac disorders and health linking with calcium homeostasis the current study has been undertaken on the patients of heart disease in Vijayapur district of Karnataka (India).

HYPOTHESIS

Lower vitamin D concentration differentially induces cardiovascular dysfunction through molecular alterations of VEGF and NOS3 signalling pathways

CHAPTER 3

AIM AND OBJECTIVES OF STUDY

AIM

To assess the link between vitamin D and cardiovascular diseases (CVD) in the percpective of angiogenic factors among the various types of CVD patients admitted in ICCU

OBJECTIVES OF THE STUDY

1. To evaluate physiological parameters in all types CVD patients admitted in ICCU.
2. To evaluate electrocardiographic and echocardiographic changes in all types CVD patients admitted in ICCU.
3. To evaluate serum calcium and Vitamin D levels in all types CVD patients and compare with each other.
4. To find out oxidative and nitrosative stress parameter in all types CVD patients and its relation to serum calcium and vitamin D levels.
5. To find out NOS3 and VEGF protein synthesis in all types CVD patients in relation to serum calcium and vitamin D levels.

CHAPTER 4

MATERIALS AND METHODS

1. STUDY DESIGN:

A cross sectional observational study was conducted on male patients who were diagnosed for cardiovascular disease and admitted in ICCU of BLDE(Deemed to be University) Shri.B.M.Patil Medical College , Hospital and Research Center. Vijayapur ,Karnataka(India) aged between 40-80 years. Patients were screened and thorough clinical examination has been done. Baseline examination and recordings were done followed by grouping of patients according to type of CVD they are suffering with.

Table 4.1. Groups of CVD patients admitted in ICCU and control.

Group 1	Hypertensive heart disease (HHD)	n=39	
Group 2	Myocardial infarction (MI)	n=49	
Group 3	Ischemic heart disease (IHD) (other than MI)	n=44	**Control**
Group 4	Angina pectoris (Angina)	n=40	n=40

Group 5	Congestive cardiac failure (CCF)	n=35	For molecular marker parameters
Group 6	Rheumatic heart disease (RHD)	n=33	
Group 7	Cardiomyopathy	n=37	

2. STUDY POPULATION:

1.1. Participants

The study participants were acquired CVD male patients aged between 40 to 80 years from ICCU of BLDE (Deemed to be University) Shri.B.M.Patil Medical College , Hospital and Research Center Vijayapur city, Karnataka(India).

1.2.Sample size

A total sample size of 270 as per consultation with Statistician of BLDE (Deemed to be University). Due to limitation of getting female participants, only males were taken into consideration. Just to provide reference for some selected parameters, 40 controls also were included into the study.

With 95% confidence level, anticipated prevalence of cardiovascular diseases is 3% and desired precision as $\pm$ 5.5%.the minimum sample size is 35 per group [7 groups- HHD, MI, IHD, ANGINA, CCF, RHD, and Cardiomyopathy] with finite population correction(Maximum patients (N) =500).

After adjusting 10% lost to follow up

So, total sample size will be 245 +25 = 270.

$$n= \frac{Z^2P(1-P)}{d^2}$$

- where-

  n= sample size

  Z=Z statistics for level of significance

  P= Expected prevalence

  d=desired precision

3. INCLUSION AND EXCLUSION CRITERIA:

3.1.Inclusion criteria: Acquired Cardiovascular disease male patients age ranging from 40 to 80 years who were diagnosed for first time and admitted in ICCU of BLDE(DU) Shri.B.M.Patil Medical College , Hospital and Research Center. Vijayapur ,Karnataka(India).

3.2.Exclusion criteria: Patients with

- ✓ Congenital heart disease
- ✓ Thyroid disorders
- ✓ Diabetes mellitus
- ✓ Chronic kidney disease
- ✓ Metabolic and malignant bone diseases which affect calcium homeostasis,
- ✓ Supplementation with calcium, vitamin D, calcium containing antacids, antihypertensive drugs and female patients were excluded from the study.

4. CRITERIA FOR DISCONTINUATION:

- ✓ Immediate death of patients
- ✓ Shifting of patients from ICCU

5. ETHICS:

5.1. Informed consent:

Written informed consent was obtained from all patients/ guardians for participation in the study (Appendix I).

5.2. Institutional approval

Study was approved by the institutional ethical committee of BLDE(Deemed to be University) (IEC No-111/2015-16, dated 10/04/2015 and IEC No/2017-18, dated 27/03/2018). India, as per the guidelines (2006) of Indian Council of Medical Research (ICMR ethical guidelines for biomedical research on human participants, 2006).

5.3.Declaration of Helsinki & ICMR guidelines:

We followed the declaration of Helsinki during the entire study.

6. STUDY SUBJECTS SELECTION PROCEDURE:

The patients were selected who were diagnosed for acquired heart disease and admitted in ICCU between December 2016 to March 2018.

7. METHOD OF DATA COLLECTION:

A data collection sheet was designed to gather all the necessary information of the patients. The written official permission was also taken from the hospital administrator. Detail clinical history from all the patients was noted. All the recordings and blood sample collection were done immediately after admission into the ICCU. Reference values for each of parameters were noted. Age and gender matched control subjects were separately taken for evaluation of oxidative stress markers and molecular markers.

7.1 Measurement of anthropometric and physiological parameters:

7.1.1. Height (cms): This was measured with the subject in standing position without footwears, nearest to 0.1cms.

7.1.2. Weight (kgs): The subjects were weighed in a standard machine with minimum of clothing, nearest to 0.1 kgs.

7.1.3. Body Surface Area (BSA, m^2 **)**This was calculated in each subject by using Dubois Nomogram.

7.1.4. Body Mass Index (**BMI,** Kg/m^2): This was calculated for each subject from his height and weight by using formula BMI = weight in Kg divided by height in m^2

7.1.5. Respiratory rate(RR,cycles/min**)**: Without the knowledge of the subject, the upward and downward excursions of anterior chest wall and anterior abdomen wall were confirmed by palpation for one minute.

7.1.6. Heart rate(PR, Beats/min**):** calculation was done from R-R interval of ECG and also counted by manual method.

7.1.7. Systolic blood pressure(SBP,mmHg**) and Diastolic blood pressure(DBP,** mmHg**):** by using Diamond mercury sphygmomanometer. SBP and DBP are recorded in the lying down position by both Palpatory and Auscultatory methods.

7.1.8. Pulse pressure(PP, mmHg): It is the pulsatile component of the blood pressure. It was estimated as the difference between systolic and diastolic blood pressure and expressed in mmHg.

7.1.9. Mean Arterial Pressure (MAP, mmHg**):** It is an average arterial pressure in an individual during single cardiac cycle. It is calculated by formula : DBP + 1/3 Pulse Pressure (PP)

7.2. Haematological analysis:

7.2.1.Complete Blood Count (CBC): 1ml of blood was collected in commercial tubes containing about 40 µl potassium EDTA as anticoagulant and the blood cell count was analysed within 24 hours by automated haematology cell counter (CYSMAX K4500 of Transasia). The following parameters were analysed i.e. Red blood cell(RBC), white blood cells (WBC), haemoglobin(HB),packed cell volume (PCV), Platelet count and blood indices like mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration(MCHC) (Garcia-Manzano AU et al., 2001)

7.2.2.Erythrocyte sedimentation rate (ESR, mm at the end of 1 hour**)** :

Westergren method: The Westergren tube is open at both ends. It is 30 cm in length and 2.5 mm in diameter. The lower 20 cm are marked with 0 at the top and 200 at the bottom. It contains about 2 ml of blood.

PROCEDURE :

1. 3mlof blood collected in EDTA vaccutaner.
2. Mix the anticoagulated blood thoroughly.
3. The blood is drawn into the tube up to 0 mark with the help of rubber bulb.
4. Blood from bottom of the tube is wiped out with cotton.
5. Set the tube upright in stand. Make sure the pipette fits snugly to eliminate possible leakage and that the pipette is in vertical position.
6. Leave the tube undisturbed for 1 hour.
7. At the end of 1 hour, the result is read.

The reading obtain is magnified as the column is lengthier.(McPherson and Pinncus)

7.3. Electrocardiography:

Equipment: 12-lead electrocardiograph (Cardiart 6108T)

Procedure :

The device should be pre-programmed in accordance with American Heart Association (AHA) specifications.(Kossman *et al*,1967 and Pipberger *et al,* 1975)

Prior to undertake the procedure the following should be checked:

- ✓ That electrocardiograph is safe and ready to use.
- ✓ The patient area is clean and tidy.
- ✓ There is sufficient paper, electrodes, razors and skin preparation equipment.

In order to achieve clinically accurate recordings with minimal artefact it is essential for patients to be comfortable and relaxed. This may be achieved through optimising the environmental conditions and providing sufficient explanation to the patient. Skin preparation is often required to help produce an artefact-free and accurate ECG.

Electrode placement:

The following electrode sites should be correctly identified and the placement of the electrodes must conform to AHA recommendations.

Limb leads:

Right arm limb lead (RA, red) - right forearm, proximal to wrist

Left arm limb lead (LA, yellow) - left forearm, proximal to wrist

Left leg limb lead (LL, green) - left lower leg, proximal to ankle

Right leg limb lead (RL, black) - right lower leg, proximal to ankle

Chest leads :

Table.4.2. Positions of chest leads.

Electrode	Position
V1	Fourth intercostal space at the right sternal edge
V2	Fourth intercostal space at the left sternal edge
V3	Midway between V2 and V4
V4	Fifth intercostal space in the mid-clavicular line
V5	Left anterior axillary line at same horizontal level as V4
V6	Left mid-axillary line at same horizontal level as V4 & V5

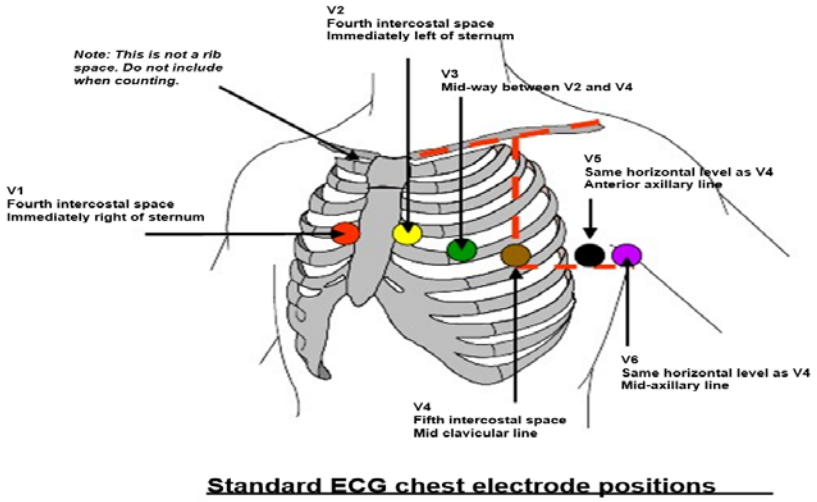


Fig.4.1 positions of chest leads.

Source: Recording a standard 12-lead electrocardiogram: An Approved Methodology, 2010; Clinical Guidelines by Consensus.p2-9

Recording:

In order to record a good quality ECG the patient must be relaxed and comfortable. To be recorded at 25mm/s with a gain setting of 10mm/mV. Press the appropriate button on the machine to initiate a recording. If the ECG is technically correct and of good quality, ensure that it is fully and correctly labelled (patient identification information, relevant clinical details) then remove all of the electrodes from the patient.

7.4. Echocardiography: by using echocardiograph(SIEMENS- MCMDD01AA)

Echocardiography (EchoCG) is a method of non-invasive cardiac imaging with the help of reflected ultrasound signals, which allows to evaluate the morpho-functional parameters of the heart structures. Doppler echocardiography allow evaluation of heart contractility, phase analysis, measurement of wall and cavity parameters, determine chamber pressure, etc.

The diagnostic significance of echocardiography is extremely high. Being a highly informative non-invasive technique, echocardiography serves to detect changes in the heart that do not manifest themselves clinically and are not detected in the ECG.

Echocardiography is performed on special echocardiographs, equipped with an ultrasound generator with a frequency of 1-10 MHz, a sensor that senses reflected ultrasound signals, a transducer of ultrasonic waves into electromagnetic and a recording device that displays the studied structures of the heart as an echocardiogram.

7.5. Biochemical parameters:

7.5.1.Estimation of lipid profile

a. Estimation of Serum triglyceride

Serum triglyceride was estimated by glycerol phosphatase-oxidase (GPO-PAP) method (Bucolo & David , 1973; Fossati & Prencipe, 1982; McGowan *et al*, 1983).

Principle

Triglycerides were enzymatically hydrolysed by lipase to glycerol and free fatty acids. The glycerol was subsequently measured by a coupled enzymatic reaction system. The glycerol released was phosphorylated to glycerol-3-phosphate by glycerol kinase. The glycerol-3-phosphate was oxidized by glycerol phosphate oxidase to produce dihydroxyacetone phosphate and hydrogen peroxide. Peroxidase catalysed the reaction of hydrogen peroxide with 4-aminoantipyrine and 3, 5-Dichloro-2-hydroxybenzene sulfonate. The absorbance of chromogen formed was measured at 505 nm. The intensity of the chromogen (Quinoneimine) formed was proportional to the triglycerides concentration in the sample.

Reagents

1.Triglyceride reagent: ATP (2.5 mmol/L), Mg^{2+} (2.5 mmol/L), 4-aminoantipyrine (0.8 mmol/L), 3, 5-Dichloro-2-hydroxybenzene sulfonate (1 mmol/L), Peroxidase (>2000U/L), Glycerol Kinase (>550 U/L), Glycerol phosphate oxidase (>8000U/L), Lipoprotein Lipase (>3500 U/L), Buffer (53mmol/L, pH 7.0 ± 0.1 at 20^0C).

2.Triglyceride standard (200mg/100ml).

Procedure

1.Three test tubes were taken and labelled as blank, standard and test. The procedure of the assay was as follows.

	Blank	Standard	Test
Sample	--	--	10 µl
Standard	--	10 µl	--
Distilled water	10 µl	--	--
Working Reagent	1000 µl	1000 µl	1000

2.Mixed well and incubated at 37^0C for 10 minutes.

3.Absorbance of test and standard was read against blank at 505nm.

Calculation:Triglycerides(mg/dl)= $\frac{\text{OD of test}}{\text{OD of standard}}$ x Concentration of standard (200mg/dl)

Precision of the assay

a. Inter-assay co-efficient of variability (CV): 4.15%

	Level 1	Level 2
No. of samples	10	10
Mean (mg/dl)	81	140
SD	3.2	6.1
CV %	3.95	4.35

b. Intra-assay co-efficient of variability (CV): 4.15 %

	Level 1	Level 2
No. of samples	10	10
Mean (mg/dl)	82.1	139.5
SD	3.4	5.8
CV %	4.14	4.16

b. Estimation of Serum cholesterol:

Cholesterol was estimated by cholesterol oxidase-peroxidase (CHOD-PAP) enzymatic method (Allian *et al*, 1974; Roeschlau *et al*, 1974)

Principle

Cholesterol esters were hydrolyzed by Cholesterol esterase to cholesterol and free fatty acids. Free cholesterol was oxidized by cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxide. This hydrogen peroxide combined with 4-aminoantipyrine to form a chromophore (quinoneimine dye) which was measured at 505 nm.

Reagents

1.Reagent

Good's buffer (50mmol/L), Phenol (5 mmol/L),4-aminoantipyrine (0.3 mmol/L),Cholesterol esterase (≥200 U/L), Cholesterol oxidase (≥ 50 U/L),Peroxidase (≥ 3 kU/L)

2.Standard: Cholesterol (200mg/100ml)

Procedure

1.Three test tubes were taken and labelled as blank, standard and test. The procedure of the assay was as follows.

	Blank	Standard	Test
Sample	--	--	10 µl
Standard	--	10 µl	--
Reagent	1.0 ml	1.0 ml	1.0 ml
Distilled water	10 µl	--	--

2.Mixed well and incubated at 37^0C for 10 minutes.

3.Absorbance of test and standard was read against blank at 505nm.

Calculation

$$\text{Cholesterol (mg/dl)} = \frac{\text{OD of test}}{\text{OD of standard}} \text{ x Concentration of standard (200mg/dl)}$$

Precision of the assay

a.Inter-assay co-efficient of variability (CV): 2.38%

	Level 1	Level 2
No. of samples	10	10
Mean (mg/dl)	122.2	216.02
SD	3.1	4.82
CV %	2.53	2.23

c. Intra-assay co-efficient of variability (CV): 2.44 %

	Level 1	Level 2
No. of samples	10	10
Mean (mg/dl)	116.25	196.83
SD	2.61	4.69
CV %	2.5	2.38

c. Estimation of HDL cholesterol

High density lipoprotein (HDL) cholesterol was estimated by phosphotungstic acid (PTA) method (Burstein *et al*., 1970).

Principle: Phosphotungstic acid precipitates low and very low density lipoproteins (LDL & VLDL) in the presence of divalent cations such as magnesium. The high density lipoprotein (HDL) cholesterol which remains unaffected in the supernatant was estimated using cholesterol reagent.

Reagents

1.Precipitating reagent: Phosphotungstic acid (0.77 mmol/l) & Magnesium chloride (17.46 mmol/l)

2.Cholesterol working reagent: Good's buffer (50mmol/L),Phenol (5 mmol/L), 4-aminoantipyrine (0.3 mmol/L),Cholesterol esterase (≥200 U/L),Cholesterol oxidase (≥ 50 U/L), Peroxidase (≥ 3 kU/L)

3.HDL cholesterol standard (50mg/dl)

Procedure

1.Precipitation: 500 µl of precipitating reagent was added to 250 µl serum and standard. Mixed well and kept for 10 minutes at room temperature to allow reaction, and centrifuged at 4000 rpm for 10 minutes. The clear supernatant was used for further reaction.

2.Three test tubes were taken and labeled as blank, standard and test. The procedure of the assay was as follows.

	Blank	Standard	Test
Supernatant	--	--	50 µl
Standard	--	50 µl	--
Distilled water	50 µl	--	--
Cholesterol working reagent	1.0 ml	1.0 ml	1.0 ml

3.Mixed well and incubated at 37^0C for 10 minutes.

4.Absorbance of test and standard was read against blank at 500nm.

Calculations

$$\text{HDL Cholesterol (mg/dl)} = \frac{\text{OD of test}}{\text{OD of standard}} \text{ x Concentration of standard (50mg/dl)}$$

Precision of the assay

a. Inter-assay co-efficient of variability (CV): 5.76%

	Level 1	Level 2
No. of samples	10	10
Mean (mg/dl)	36.8	62.08
SD	1.86	4.02
CV %	5.05	6.47

b. Intra-assay co-efficient of variability (CV): 5.3%

	Level 1	Level 2
No. of samples	10	10
Mean (mg/dl)	41.2	68.2
SD	2.01	3.9
CV %	4.88	5.71

- LDL and VLDL levels were estimated by calculation using Friedwald formula;

  LDL mg/dl= Total cholesterol – HDL cholesterol- TG/5

  VLDL= TG/5

7.5.2.Serum creatinine: Jaff kinetic method

Principal, Reagent, and Chemicals used in Jaffe's Method:

Creatinine. Standard solutions were prepared daily by dilution of a stock solution containing 400 mg. creatinine/100 ml. of 0 1N-HCl. The stock solution was stored at 0° and was never kept for longer than 4 weeks.

Picric acid. An aqueous solution, saturated at room temperature (18-20%), was prepared from picric acid (A.R.) which had been recrystallized twice from water and satisfied the criteria of purity proposed by Folin & Doisy (1917). The solution was kept in dark bottles and was made up at intervals of a few days.

Other reagents. Sodium hydroxide, (A.R.) 2-5N. Sodium tungstate, (A.R.) a 10% (w/v) solution of Na2WO4, 2H20. Sulfuric acid, (A.R.) 0-66N. Phosphate buffer, 1 m, pH 7 0, Oxalic acid, (A.R.) saturated aqueous solution. Lloyd's reagent hydrated aluminium silicate.

Procedure:

1.To 40 ml of the solution containing creatinine were added 2-0ml of alkaline picrate solution and the colour was allowed to develop for 20 minutes in a water bath at 20 + 0-20.

2.The alkaline picrate solution was made up immediately before use by adding 1 vol. of sodium hydroxide to 5 vol. of picric acid.

3.The optical density of the developed colour was measured at a wavelength of 520 ml using water as reference optical density.

Light absorption by alkaline creatinine picrate. The absorption spectrum of alkaline creatinine picrate shows maximum absorption in the region of 490 mu. But at wavelengths below 500 ml the optical density of alkaline picrate (i.e. the reagent blank) is also high. Consequently filters having maximum transmission at wavelengths above 500 mi. have generally been used. When measured with absorption meters employing diffraction gratings which provide light with a relatively broad waveband, the developed colour is reported not to obey Beer's Law, but with instruments providing light with a narrow waveband Beer's Law is reported to be obeyed over the required concentration range. It has also been reported, however, that even with monochromatic light Beer's Law is not obeyed at all wavelengths.

7.5.3.Serum electrolytes(Na^+ and K^+): VITROS slide method:

Serum Na^+:

Principles:

The VITRO Na^+ slide is a multi-layered, analytical element coated on a polyester support that uses direct potentiometry for measurement of sodium ion. The slide consists of two ion-selective electrode, each containing methyl monensin, a reference layer, a silver and silver chloride layer coated on polyester support.

Procedure:

A drop of patient sample and a drop of VITROS reference fluid on separate halves of the slide results in migration of both fluids toward the centre of the paper bridge. A stable liquid junction is formed connecting the reference electrode to the sample indicator electrode.

Each electrode produces an electrical potential in response to the activity of sodium applied to it. The potential difference poised between the two electrodes is proportional to the sodium concentration in the sample.

Serum K^+:

Principle:

The VITROS K^+ slide is multi-layered, analytical element coated on a polyester support that uses direct potentiometry for measurement of ionic potassium. The slide consists of two ion-selective electrode, each containing valinomycin, a reference layer, a silver and silver chloride layer coated on polyester support.

Procedure:

A drop of patient sample and a drop of VITROS reference fluid on separate halves of the slide results in migration of both fluids toward the centre of the paper bridge. A stable liquid junction is formed connecting the reference electrode to the sample indicator electrode.

Each electrode produces an electrical potential in response to the activity of potassium applied to it. The potential difference poised between the two electrodes is proportional to the potassium concentration in the sample.

7.5.4 Serum CPK-MB

Principle:

The VITROS CKMB slide method is performed using the VITROS CKMB slides and VITROS chemistry products calibrated kit 6on VITROS 250/350/950 and 5, 1 FS/4600 chemistry systems and the VITROS 5600 Integrated system.

Procedure:

A drop of patient sample is deposited on the slide and is evenly distributed by the spreading layer to the underlying layers. This layer contains surfactants; N-acetylecysteine (NAC), which activates CK without pretreatement of the sample; and goat antihuman CK-MM antibodies, which inhibit CK-MM(muscle) activity and nearly 50% of the CK-MB(heart) activity. The remaining CK activity represents 50% of the total CK-MB isoenzyme concentration. In the reagent layer, creatine kinase in the sample catalyzes the conversion of creatine phosphate and adenosine diphosphate (ADP) to creatine and adenosine triphosphate (ATP). I n the presence of glycerol kinase, glycerol is phosphorylated to L- α-glycerolphosphate which is then oxidized to dihydroxyacetone phosphate and H_2O_2 in the reaction catalysed by L- α-glycerolphosphate oxidase. Finally, leuco dye is oxidized by hydrogen peroxide in the presence of peroxidase to form dye.

The low wavelength cut off filter on the slide support minimizes the blank rate effects of incident light during dye development.

The rate of change in reflection density is converted to enzyme activity.

Test type	VITROS system	Approximate incubation period	Temperature	Wavelength	Reaction sample volume
Multiple-point rate	5600,4600, 5,1FS,	5 minutes	37^0 C	670nm	11μL

	950,250/350				

Calculation:

$$\% \text{ CPK-MB} = \frac{\text{CPK-MB}}{\text{CPK}} \times 100$$

7.5.5.Serum calcium:

Ortho- Cresol phthalein Complexone (OCPC) method

Principle:

OCPC reacts with calcium in alkaline solution to form a purple coloured complex. The intensity of purple colour formed is proportional to the calcium concentration and is measured photometrically between 540 nm and 600 nm with maximum absorbance at 575nm.

Reagents: Reagent 1: AMP reagent

2-Amino-2-methyl-1-propanalol	505 mmol/L
Surfactant	---

Reagent 2:OCPC reagent

OCPC	0.06mmol/L
8-Hydroxy Quinoline	6.9mmol/L
HCl	45mmol
Surfactant	---

Assay parameters:

- Mode: End point
- Wavelength(nm) : 578
- Sample volume (µl): 5/10
- Reagent volume(µl): 500/1000
- Incubation time(min): 1
- Incubation temp(^{0}C): 37
- Concentration of standard(mg/dl): 10
- Blank with: Reagent
- Units: mg/dl

Procedure:

Pipette the reagents in test tubes labelled as follows,

	Blank (µl)	**Standard (µl)**	**Sample (µl)**
Working reagent	1000	1000	1000
Standard	--	10	--
Sample	--	--	10

Mix well and read at 578nm against reagent blank.

Calculations:

$$\text{Concentration of serum calcium} = \frac{\text{Absorbance of Test}}{\text{Absorbance of standard}} \text{ x Concentration of standard (10)}$$

= ……..mg/dl

Reference range: Serum: 8.4-10.4 mg/dl

Linearity- the assay is linear up to 20mg/dl(5mmol/L).For higher values dilute the sample with normal saline and repeat the assay. Multiply the results with the dilution factor.

7.5.6. Serum vitamin D:

Chemiluminiscent Immunoassay (CLIA)

25-hydroxyvitamin D in serum was measured with automated chemiluminiscent immunoassay technology (VITROS eci, Johnson and Johnson Ortho Clinical Diagnostics). CV for inter-assay analyses is 5.8% at a 25- hydroxyvitamin D level of 39.5 nmol/L and 3.1% at 121.25 nmol/L.(Aslan and Geddes,2009)

Principle:

A competitive immunoassay of technique is used which involves the release 25-OH Vitamin D in the sample from the binding protein using a low Ph denaturant and the subsequent competition of the free 25-OH Vitamin D with horseradish peroxide (HRP) labelled 25-OH vitamin D reagent for monoclonal anti-vitamin D bound to the well. Unbound materials are removed by washing. The bound HRP conjugate is measured by a luminescent reaction. A reagent containing luminogenic substrate and electron transfer agent is added to the wells. The HPR in the bound conjugate catalyzes the oxidation of the luminol derivative, producing light. The electron transfer agent increases the level of light produced and prolongs its emission. The light signals are read by the system. The amount of HPR conjugate bound is indirectly proportional to the concentration of 25-OH vitamin D present.

Materials:

- VITROS Immunodiagnostic products 25-OH vitamin D total reagent pack.

- VITROS Immunodiagnostic products 25-OH vitamin D total calibrator.
- VITROS Immunodiagnostic products signal reagent.
- VITROS Immunodiagnostic products universal wash reagent.

Procedure:

- Collect specimens using standard procedures.
- Sample should be thoroughly separated from all cellular materials.
- Thoroughly mix samples by inversion and bring to 15-30^{o}C before use.

Measuring range:

System	Measurable (reportable) range
ECi/ECiQ, 3600, 5600	8.00-150ng/Ml (20.0-375nmol/L)

Results:

Reporting units and unit conversion:

Conversional	alternate
ng/mL(nmol/L×0.4)	nmol/L (ng/mL ×2.5)

Inferences:

Interferent	Interferent concentration		Units = ng/mL		Unit = nmol/L	
			Analyte concentration	Bias”	Analyte concentration	Bias”
Paricalcitol (Zemplar)	24ng/mL	57.6nmol/L	10.5	125	26.3	313

”estimate of the average difference observed.

Calculation:

$$\% \text{ cross reactivity} = \frac{\text{Mean value spiked(ng/mL)} - \text{mean value un-spiked(ng/mL)}}{} \times 100$$

Concentration of cross reactant(ng/mL)

7.6.Evaluation of oxidative and nitrosative stress and antioxidant status :

7.6.1.Estimation of Serum malondialdehyde (MDA)

Serum malondialdehyde (MDA), a marker of oxidative stress was estimated by Kei Satoh method (Satoh, 1978).

Principle

Auto-oxidation of unsaturated fatty acids involves the formation of semi-stable peroxides, which then undergo a series of reactions to form malondialdehyde. Malondialdehyde reacts with Thiobarbituric acid to form pink coloured chromogen. The resulting chromogen was extracted with 4.0 ml of n-butyl alcohol and the absorbance of which was measured at 530 nm.

Reagents

1.Trichloroacetic acid (TCA) reagent: 20g/dl TCA in 100 ml distilled water to prepare 20% TCA.

2.Sodium sulphate solution (2M): 28.4 gm of anhydrous sodium sulfate was mixed in 90 ml of distilled water by heating and stirring. Then distilled water was added to make final volume of 100 ml.

3.Thiobarbituric acid (TBA) reagent: 670 mg of TBA in 100ml of 2M sodium sulphate solution, 2.Sulphuric acid (0.05M), 3.N-butyl alcohol

No.	Working standard	Distilled water	MDA (nmol/ml)
1	3.0 ml	----	10
2	2.5 ml	0.5 ml	8.3
3	2.0 ml	1.0 ml	6.7
4	1.5 ml	1.5 ml	5

5	1.0 ml	2.0 ml	3.3
6	0.5 ml	2.5 ml	1.7
7	0 ml	3.0 ml	0

Standards

Following calibrators were prepared from the working standard (10nmol/ml).

Standard Graph

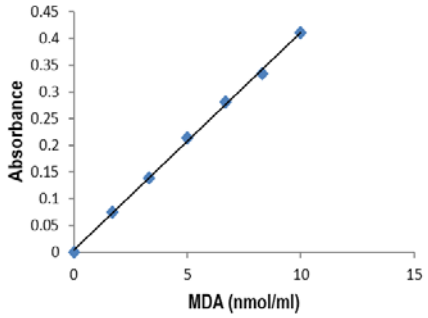


Figure.4.3.Standard curve of MDA. LVPM(Laboratory of Vascular Physiology and Medicine).

Procedure

1.300 µl of serum and 1.5 mL of TCA was taken in a test tube and kept for 10 min at room temperature.

2. Centrifugation at 3500 rpm for 10 min was done.

3.The supernatant was decanted and the precipitate obtained was washed with 0.05M Sulphuric acid.

4.1.5 mL of 0.05M Sulphuric acid and 3 mL of TBA reagent were added to the precipitate.

5.The test tube containing the mixture was kept in a boiling water bath for 30 min.

6.Then the tube was cooled in cold water followed by addition of 2.4 mL of n-butyl alcohol with vigorous shaking to extract the chromogen.

7.Separation of organic phase was facilitated by centrifugation at 3000 rpm for 10 min.

8.The absorbance (OD) was read at the 530 nm wavelength using spectrophotometer.

Calculation

Concentration of serum MDA (nmol/ml)

$$= \frac{\text{OD of Test}}{\text{Nano}-\text{molar Extinction Co}-\text{efficient}} \text{ X } \frac{\text{Total volume of solution in cuvette}}{\text{sample volume}}$$

$$= \frac{\text{OD of Test}}{1.56 \text{ x } 105} \text{ X } \frac{109}{1000} \text{ X} \frac{2.4}{0.3}$$

= OD of the Test x 51.28 nmol/ml.

7.6.2.Estimation of superoxide dismutase (SOD)

Superoxide dismutase (SOD) activity was measured by Marklund and Marklund method (Marklund S & Marklund, 1998).

Principle: Superoxide anion is involved in auto-oxidation of pyrogallol at alkalike pH (8.5). The superoxide dismutase inhibits auto-oxidation of pyrogallol which can be determined as an increase in absorbance at 420 nm.

Reagents

1.Tris buffer (0.05M): 50 mM of Tris buffer and 1 mM of EDTA was mixed with distilled water and HCL was added to adjust the pH at 8.5. A final volume of 100 ml solution at pH 8.5 was prepared.

2. Pyrogallol (20mM): 25 mg pyrogallol was dissolved in 10 mL distilled water.

Procedure

1.Control: 2.9 ml of Tris buffer was taken in a cuvette to which 0.1 ml of Pyrogallol was added. Then absorbance (OD) was read at 420 nm after 1min 30 sec and 3 min 30 sec.

2.Test: 2.8 ml of Tris buffer and 0.1 ml of serum was taken in a cuvette to which 0.1 ml of Pyrogallol was added. Then absorbance (OD) was read at 420 nm after 1min 30 sec and 3 min 30 sec.

3.Difference in absorbance (ΔA/min) was calculated as

$$\Delta A/\min = \frac{\text{OD at 3 min 30 sec} - \text{OD at 1 min 30 sec}}{2}$$

Calculation

$$\text{Serum SOD activity} = \frac{\Delta A/\text{ min of control} - \Delta A/\text{ min of Test}}{\Delta A/\text{ min of control x 50}} \text{ x 100 x } \frac{1}{\text{volume of sample}}$$

$$= \frac{C-T}{C \text{ x } 50} \text{ x 100 x } \frac{1}{0.1} \quad = \frac{C-T}{C \text{ x } 50} \text{ x 1000} \quad = \text{------- U/ml}$$

One unit of SOD is defined as the amount of enzyme required to cause 50% inhibition of pyrogallol auto-oxidation.

7.6.3.Estimation of serum nitric oxide concentration

Total serum nitric oxide concentration (NOx) was measured as an index of endothelial function. Serum NOx was estimated by improved Griess method using vanadium chloride as a reducing agent for reduction of nitrate to nitrite (QuantiChrom^{TM} Nitric Oxide Assay Kit: D2NO-100, BioAssay Systems, USA). The subjects were advised to abstain from foods such as cured meat, fish, cheese, herbal or black tea, beer, wine and malted beverages on the previous day to avoid dietary effect on NOx (Choi *et al*, 2001). To avoid change in the serum NO levels secondary to physical activity, subjects were given rest for at least 10 minutes before collection of blood sample.

Principle

Since NO is unstable and oxidized to nitrite and nitrate, it is common practice to quantitate total NO_2/NO_3 as a measure for NO level. Nitrate was reduced to nitrite by vanadium chloride (VCl_3) after deproteinization of serum sample by somogyi reagent (NaoH & $ZnSO_4$). The nitrite produced was determined by diazotization of sulfanilamide and coupling to napthylethyline diamine.

Reagents

$ZnSO_4$ Solution (75mMol/L),NaOH solution (55mMol/L),Vanadium chloride III, Griess reagent (Sulfanilamide and N-Naphthylethylene diamine), $NaNO_2$ standard (1.0 mM/L)

Procedure

1.Deproteination:150 µl of sample was mixed with 8 µl $ZnSO_4$ in 1.5 ml eppendorf tube. 8 µl of NaOH was added following vortex for one minute. The mixture was vortexed again and centrifuged for 10 min at 14,000 rpm. Clear supernatant obtained was transferred to a clean tube.

2.Standards: 0.1ml of working standard (100 µM/L) was prepared by mixing 0.1 mL of 1.0 mM/L $NaNO_2$ standard with 0.9 mL of distilled water.

Following calibrators were prepared from the working standard.

No.	Working standard	Distilled water	Nitrite (µmol/L)
1	500 µL	----	100
2	300 µL	200 µL	60
3	150 µL	350 µL	30
4	---	500 µL	0 (blank)

3.Reaction

i. Working reagent (WR) for all samples and standards was prepared by mixing per reaction tube

a. 400 µL - Sulfanilamide
b. 400 µL - N-Naphthylethylene diamine
c. 200 µL - Vanadium chloride III

ii.400 µL of deproteinated sample and calibrators were added in a separate labeled eppendorf tubes.

iii. Then 800 µL of working reagent was added to each tubes.

iv. Incubated for 10 min at 60^0C.

Measurement

Optical density (OD) was read at 540 nm (UV-1700, UV-visible spectrophotometer, Scimadzu).

Standard graph

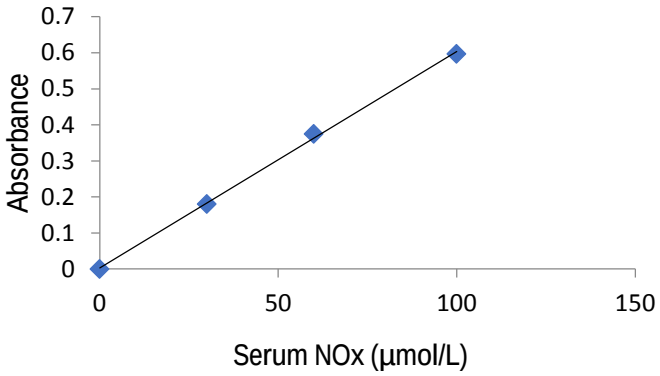


Figure.4.4.Standard curve of Nitric Oxide(NO). LVPM(Laboratory of Vascular Physiology and Medicine)

Calculation

i. Standard graph was plotted using OD against standard concentrations.

ii. Slope was determined using linear regression fitting.

iii. The NO concentration of sample was calculated as

$$Serum\ NO\ (\mu M) = \frac{ODsample - OD\ blank}{\text{Slope}}$$

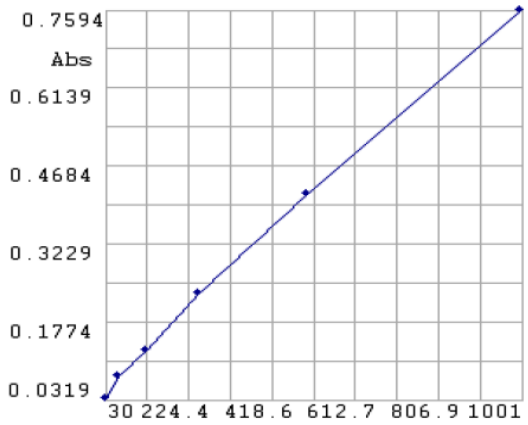
7.7 Molecular markers:

7.7.1.Serum Vascular endothelial growth factor (VEGF):

Was estimated based on the principle of a solid phase enzyme-linked immunosorbent assay (ELISA) by using a commercially available kit.

Blood was collected into Vacutainer CPTT 8 mL tubes containing 0.1 mL of molar sodium citrate (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) and centrifuged at room temperature for 20 minutes at 1500g. The top layer corresponding to plasma was decanted using sterile transfer pipettes. The plasma was immediately frozen and stored at –40 C in polypropylene cryo- preservation vials (Nalgene, Nalge Nunc, Rochester, NY).VEGF in plasma was measured using commercially available kits. VEGF concentrations was quantified using enzyme-linked immunosorbent assay (ELISA). The sensitivity of the assay is 7 pg/ml. Intra and inter-assay CV in serum samples were 4.5 and 7%, respectively (Hormbrey *et al*, 2002)

Standard Graph



VEGF in pg/ml

Figure.4.5. Standard curve of VEGF,LVPM (Laboratory of Vascular Physiology and Medicine).

7.7.2. Serum eNOS:

Evaluated by ELISA method by using standard kit.

Principle:

The purified anti-eNOS antibody was pre-coated onto96-well plates. the HRP conjugated anti-eNOS antibody was used as detection antibodies. The standards, test samples and HRP conjugated detection antibody were added to the wells subsequently, mixed and incubated, then unbound conjugates were washed away with wash buffer. TBM substrate (A&B) were used to visualize HRP enzymatic reaction. TMB was catalysed by HRP to produce a blue colour product that changes into yellow after adding acidic stop solution. The density of yellow is proportional to the eNOS amount captured in plate. Read the O.D. absorbance at 450nm in a microplate reader, and then the concentration of eNOS can be calculated.

Kit components:

One 96-well plate pre-coated with anti-human eNOS antibody, Standard: 0.5ml (270pg/ml)**,** Standard diluent buffer:1.5 ml, Wash buffer (30×): 20 ml. Dilution:1:30, Sample diluent buffer:6 ml**,** HRP conjugate anti-human eNOS antibody (RTU):6ml**,** Stop solution: 6ml**,**TMB substrate A: 6ml**,** TMB substrate B: 6ml**,**Plate sealer: 2**,** Hermetic bag:1

Other material required: 37˚C incubator, Microplate reader(wavelength:450nm), Precise pipette and disposable pipette tips, Automated plate washer, ELISA shaker, 1.5ml of Eppendorf tubes, Absorbent filter papers, Plastic or glass container with volume of above 1L.

Procedure:

- **Preparation of sample and reagents**

1.Sample

Isolate the test samples soon after collecting, then, analyse immediately(within 2 hours). Or aliquot and store at -20˚C for long term. Avoid multiple freeze-thaw cycles.

Serum: Coagulate at room temperature for 10-20 min, then, centrifuge at the speed of 2000-3000 r.p.m for 20 min to collect supernatant. If precipitation appeared, centrifuge again.

2.Wash Buffer

Dilute concentrated Wash Buffer(Kit Component 4) 30-fold (1:30) with

distilled water(i.e add 20ml of concentrated wash buffer into 580 ml distilled water).

3.Standard

Dilution of Standard: set 10 Standard wells on the pre-coated plates, add100µl of Standard to the 1^{st} and 2^{nd} well, then add 50µl of Standard Diluent buffer to the above two wells and mix thoroughly; transfer 100µl from the 1^{st} and 2^{nd} well to the 3^{rd} and 4^{th} well respectively, then add 50µl of Standard diluent buffer to the 3^{rd} and the 4^{th} well and mix thoroughly; take out 50µl from the 3^{rd} and the 4^{th} well respectively and discard, and transfer 50µl to the 5^{th} and the 6^{th} well to the 7^{th} and the 8^{th} well, then add 50µl of Standard Diluent buffer to the 7^{th} and 8^{th} well and mix thoroughly; transfer 50µl from the 7^{th} and the 8^{th} well to the 9^{th} and the 10^{th} well, add 50µl of Standard Diluent buffer to the 9^{th} and 10^{th} well and mix thoroughly, take out 50µl from the 9^{th} and the 10^{th} well and discard (After diluting, the loading volume for each well is 50µl, and the concentrations are 180 pg/ml, 120pg/ml, 60pg/ml,15pg/ml).

Assay procedure

1.Equilibrate kit components for 15-30 min at room temperature.

2.Set standard, test sample and control (zero) wells on the pre-coated plate respectively, and then, record their positions. Add 50µl of diluted standard (180 pg/ml, 120pg/ml, 60pg/ml, 30pg/ml, 15pg/ml) into the standard wells. Add 50µl of standard diluent buffer (Kit Component 3) into the control (zero) well. Do not add sample and HRP conjugated antibody into the control (zero) well.

3.For test sample wells, add 40µl of Sample diluent buffer (Kit Component 5) first, then, add 10µl of sample. Add the solution at the bottom of each well without touching the side wall. Shake the plate mildly to mix thoroughly.

4.Cover the plate with Plate sealer (Kit Component 10) and incubate at 37˚C for 30 mins.

5.Remove the sealer, and wash plate using the following method:

Automated Washing: Aspirate all wells, then wash plates 5 times using Wash Buffer (1×). After the final wash, invert plate, and clap the plate on absorbent filter papers until no moisture remained. It is recommended that the washer be set for a soaking time of 10 seconds or shaking 6.Add 50µl of HRP conjugated anti- e NOS antibody (Kit Component 6) into each well (except control well).

7.Cover the plate with Plate sealer (Kit Component 10) and incubate at 37˚C for 30 min.

8.Remove the sealer, and wash the plate.(See step 5).

9.Add 50µl of TMB substrate A (Kit Component 8) into each well, and then, add 50µl of TMB substrate B (Kit Component 9), vortex gently the plate on ELISA shaker for 30 seconds (Or shake gently by hand for 30 seconds), and Incubate in dark at 37˚C for 15 min. The shades of blue can be seen in the wells.

10.Add 50µl of Stop solution (Kit Component 7) into each well, and mix thoroughly. The colour changes into yellow immediately.

11.Read the O.D. absorbance at 450 nm in a microplate reader within 15 min after adding the stop solution.

Calculation:

The relative $O.D._{450}$ = (The $O.D._{450}$ of each well) – (The $O.D._{450}$ of zero well)

STATISTICAL ANALYSIS:

All characteristics were summarized descriptively. For continuous variables, the summary statistics of mean± standard deviation (SD) were used. For categorical data, the number and percentage were used in the data summaries and diagrammatic presentation.The difference of the means of analysis variables between two independent groups was tested

by unpaired t test. The difference of the means of analysis variables between more than two independent groups was tested by ANOVA and F test of testing of equality of Variance. Tukey's post-hoc test was used for multiple comparison. Pearson's correlation coefficient (r) was used to test the strength and direction of relationships between the interval levels of variables. Linear regression was used for predictive analysis. If the p-value was < 0.05, then the results were considered to be statistically significant otherwise it was considered as not statistically significant. Data were analyzed using SPSS software v.23.0. and Microsoft office 2007.

CHAPTER 5

RESULTS AND DISCUSSION

5.1. Anthropometric parameters

5.1.Results and Discussion

Table 5.1: Comparison of mean anthropometric parameters among study groups

Parameters	NORMAL RANGE	CARDIAC PATIENTS							F value	ANOVA p value
		HHD (n=39)	MI (n=49)	IHD (n=44)	ANGINA (n=40)	CCF (n=35)	RHD (n=33)	CARDIOMYOPATHY(n=37)		
AGE (years)	-	50.77±7.44[a]	55.16±7.18[f]	50.64±7.43[b]	51.38±6.89[c]	52.18±7.81[d]	48.27±8.5[e,f]	58.03±8.46[a,b,c,d,e]	6.827	<0.001*
HEIGHT (cms)	-	159.67±5.3	160.98±5.93	161.09±5.83	161.13±5.49	160.73±4.86	160±5.95	161.3±5.39	0.476	0.826
WEIGHT (kgs)	-	63.59±10.53	66.39±10.82	66.75±10.69	67.58±10.58	65.12±10.1	63.58±9.58	65.46±11.32	0.814	0.56
BMI (kg/m2)	18.5-24.9	24.87±3.36	25.53±3.22	25.62±3.04	25.94±3.1	25.16±3.28	24.79±3	25.05±3.33	0.685	0.662
BSA(m2)	1.7-1.9	1.92±0.14[a]	1.81±0.15[b]	1.71±0.15[c]	1.72±0.14[d]	1.63±0.15	1.57±0.15[a,b,c,d]	1.67±0.16	5.323	<0.001*

Hypertensive heart disease (HHD); myocardial infarction (MI); ischemic heart disease (IHD); Angina pectoris(Angina); congestive cardiac failure (CCF); rheumatic heart disease (RHD).

* significant at 5% level of significance ($p<0.05$), a,b,c,d,e,f,g represent significant difference in multiple comparison by tukey's test ($p<0.05$)

Table 5.1a. Anthropometric parameters of control group

Parameters	Control group (n=40)
AGE (years)	52.36±5.831
HEIGHT (cms)	158±5.95
WEIGHT (kgs)	60.58±9.58
BMI(kg/m2) 18.5-24.9	22.79±3
BSA(m2) 1.7-1.9	1.67±0.15

Table 5.1 shows mean age and anthropometric parameters of various cardiovascular disease patients.

BSA shown significant difference in different cardiac disease groups although the values remain within the normal range.

BMI of all groups of CVD patients are within normal range.

Table 5.1a. depicts anthropometric parameters of control group (for comparing oxidative and molecular marker parameters) which shows lower BMI as compared to study groups.

5.2 Physiological parameters

5.2 Results and Discussion

Table5.2.1: Comparison of mean physiological parameters among study groups

Parameters	NORMAL RANGE	CARDIAC PATIENTS							F value	ANOVA p value
		HHD (n=39)	MI (n=49)	IHD (n=44)	ANGINA (n=40)	CCF (n=35)	RHD (n=33)	CARDIOMYOPATHY (n=37)		
RR(cpm)	12-20	20.87±9.28	20.16±8.43	20.82±7.66	21.55±8.67	17.39±2.03	20.3±3.68	19.84±7.95	1.11	0.356
HR(bpm)	60-100	89.08±23.23	86.41±21.39	85.09±19.11	86.15±25.15	85.94±22.09	83.33±29.12	91.73±27.45	0.49	0.058
SBP (mmHg)	≤120	**142.87±25.21**[a,e]	**136.73±21.31**	**139.91±22.89**[c]	124.35±31.55[a,b,c]	**130.42±26.64**	**133.03±29.51**[d e],	**141.35±23.96**[b d],	5.858	<0.001*
DBP (mmHg)	≤ 80	**108.51±33.78**[a,b,c,d,e,f]	**87.18±22.03**[a]	84.27±23.71[b]	79.9±27.85[c]	82.67±20.79[d]	81.03±29.3[e]	88.38±24.86[f]	5.364	<0.001*
MAP (mmHg)	≤90	**115.82±16.68**[a,b,c,d,e,f]	**98.59±12.67**[a]	**100.22±17.97**[b]	**100.4±16.89**[c]	**98.48±17.81**[d]	**98.94±16.98**[e]	**104.04±18.64**[f]	5.409	<0.001*
PP(mmHg)	≤40	**58.87±13.48**[a,b,c]	**51.1±12.5**[d]	**47.41±15.78**[a]	37.2±15.41[b,d,e]	**46.3±14**[c]	**49.82±15.36**	**51.73±17.44**[e]	4.411	<0.001*

Hypertensive heart disease (HHD); myocardial infarction (MI); ischemic heart disease (IHD); Angina pectoris(Aangina); congestive cardiac failure (CCF); rheumatic heart disease (RHD). RR, respiratory rate; PR, pulse rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; PP, pulse pressure.

* significant at 5% level of significance ($p<0.05$), a,b,c,d,e,f,g represent significant difference in multiple comparison by tukey's test ($p<0.05$)

Results from physiological parameters like blood pressures depict a significant changes between the groups ($p<0.05$). (Table 5.2).

Results clearly shows a higher blood pressure values in all the components of blood pressure in case of group 1(HHD) and 2 (MI) patients as compared to normal blood pressure values. Groups 3 (IHD), 4 (angina),5 (CCF), 6 (RHD) and 7 (Cardiomyopathy) shows higher values of SBP, MAP and PP compared to normal blood pressure values.

Hypertension is one of the strongest risk factors for most of different CVD acquired during life.

A recent publication by Rapsomaniki *et al* (2014) from the United Kingdom based on an analysis of the electronic records of 1.25 million people from 1997 to 2010 reported that people aged 30 years with hypertension have a higher lifetime risk of angina, myocardial infarction, heart failure and cardiac arrest/sudden cardiac death than people with normal BP and also found that individuals with normal BP have a lower lifetime risk of CHD than individuals with hypertension.

Asia Pacific Cohort Studies Collaboration (APCSC) by Lawes *et al* (2003). have clearly demonstrated a linear relationships of blood pressure with ischaemic stroke, haemorrhagic stroke, ischaemic heart disease and total cardiovascular death.

As in our study systolic blood pressure is high in most of CVD than diastolic pressure which is in agreement with study by Kannel (1999) which shows that systolic pressure is more important than diastolic pressure as a determinant of cardiovascular sequelae. Mild or moderate elevations of systolic blood pressure, without increase in diastolic blood pressure, are associated with an increased risk of cardiovascular disease.

Hypertension doubles the risk of CVD.It plays a crucial role in the development and accelerates other processes of atherosclerosis. According to many epidemiologic data, CVD mortality increases with blood pressure. Czech, European, and American guidelines from the early 21st century recommend that blood pressure should be maintained below 130/80 mmHg in patients with ischemic heart disease (spinar,2004)

5.3 Haematological parameters

5.3.Results and Discussion

Table 5.3: Comparison of mean hematological parameters among study groups

Parameters	NORMAL RANGE	CARDIAC PATIENTS							F value	ANOVA p value
		HHD (n=39)	MI (n=49)	IHD (n=44)	ANGINA (n=40)	CCF (n=35)	RHD (n=33)	CARDIO MYOPATHY (n=37)		
RBC count (millions)	4.5-5.5	5.51±0.88	4.45±0.81	4.42±0.71	4.55±0.76	4.54±0.67	4.65±0.95	4.72±0.86	0.69	<0.001*
WBC count (Thousands)	4000-11000	**12982.64± 5302.64**	10478.39± 3840.66[a,b]	**14676.36± 7928.12[a]**	11510.03± 3800.1	**12690.06± 4352.37**	**14894.24± 7588.93[b]**	**12404.32± 6037.71**	3.225	0.004*
HB%	1.25-15.5	13.24±2.54	12.0±2.18	12.25±2.36	12.04±2.04	12.04±2.04	13.27±2.97	13.53±2.59	3.168	0.005*
PCV%	36-46	49.84±7.38	38.28±7.09	37.9±6.88	37.92±6.91	37.92±5.91	40.02±8.16	41.07±7.75	1.239	0.004*
Platelet count(lakhs)	1.5-4	4.84±0.83	2.58±0.81	2.61±0.79	2.68±0.88	2.55±0.93	2.77±0.8	3.01±0.96	1.416	0.021*
MCV(fl)	80-100	86.17±9.74	84.99±9.25	83.09±10.52	83.59±9.48	83.72±9.69	83.99±12.29	85.46±10.38	0.49	0.816
MCHC(%)	31.5-34.5	33.01±2.13	32.79±2.34	32.59±1.53	32.87±2.06	32.89±1.29	33.02±1.62	33.47±1.82	0.823	0.553
MCH(pg)	27-32	28.53±4.18	28.18±4.1	27.15±3.71	27.66±3.89	27.19±3.66	27.81±4.71	28.83±4.3	0.951	0.459
ESR (mm/1stHr)	5-20	**39.03±28.64**	**32.16±23.98**	**47.84±38.54**	**37.15±23.86**	**47.85±35.85**	**41.73±40.54**	**47.05±36.13**	1.487	0.0183*

Hypertensive heart disease (HHD); myocardial infarction (MI); ischemic heart disease (IHD); Angina pectoris(Angina); congestive cardiac failure (CCF); ,rheumatic heart disease (RHD), RBC; red blood corpuscles, WBC; white blood corpuscles, Hb; hemoglobin, PCV; packed cell volume, MCV; mean corpuscular volume, MCHC; mean cell hemoglobin concentration, MCH; mean corpuscular hemoglobin, ESR; erythrocyte sedimentation rate.

* significant at 5% level of significance ($p<0.05$), a,b,c,d,e,f,g represent significant difference in multiple comparison by tukey's test ($p<0.05$)

Among haematological parameters, total WBC count is significantly high except in group 2(MI) and group 4 (angina) as compared to normal range and ESR is also significantly high in all groups of CVD patients. (Table.5.3)

The increase in white blood cells (WBCs) level in present study indicate the inflammatory process in the physiological system. Low-grade inflammation is associated with most cardiovascular diseases and it contributes significantly to oxidative stress. Thereby, inflammation represents an independent cardiovascular risk factor. Further increase of ESR in present study in all the types of CVD patients indicate myocardial damage with leak of proteins. Previously researchers reported that the erythrocyte sedimentation rate may be a good indicator for coronary heart disease, mortality, and the risk of death from coronary heart disease.(Karbach *et al*,2014; Harrison *et al*, 2011; Kossmann *et al*, 2017)

5.4 Electrocardiographic findings

5.4.Results and Discussion

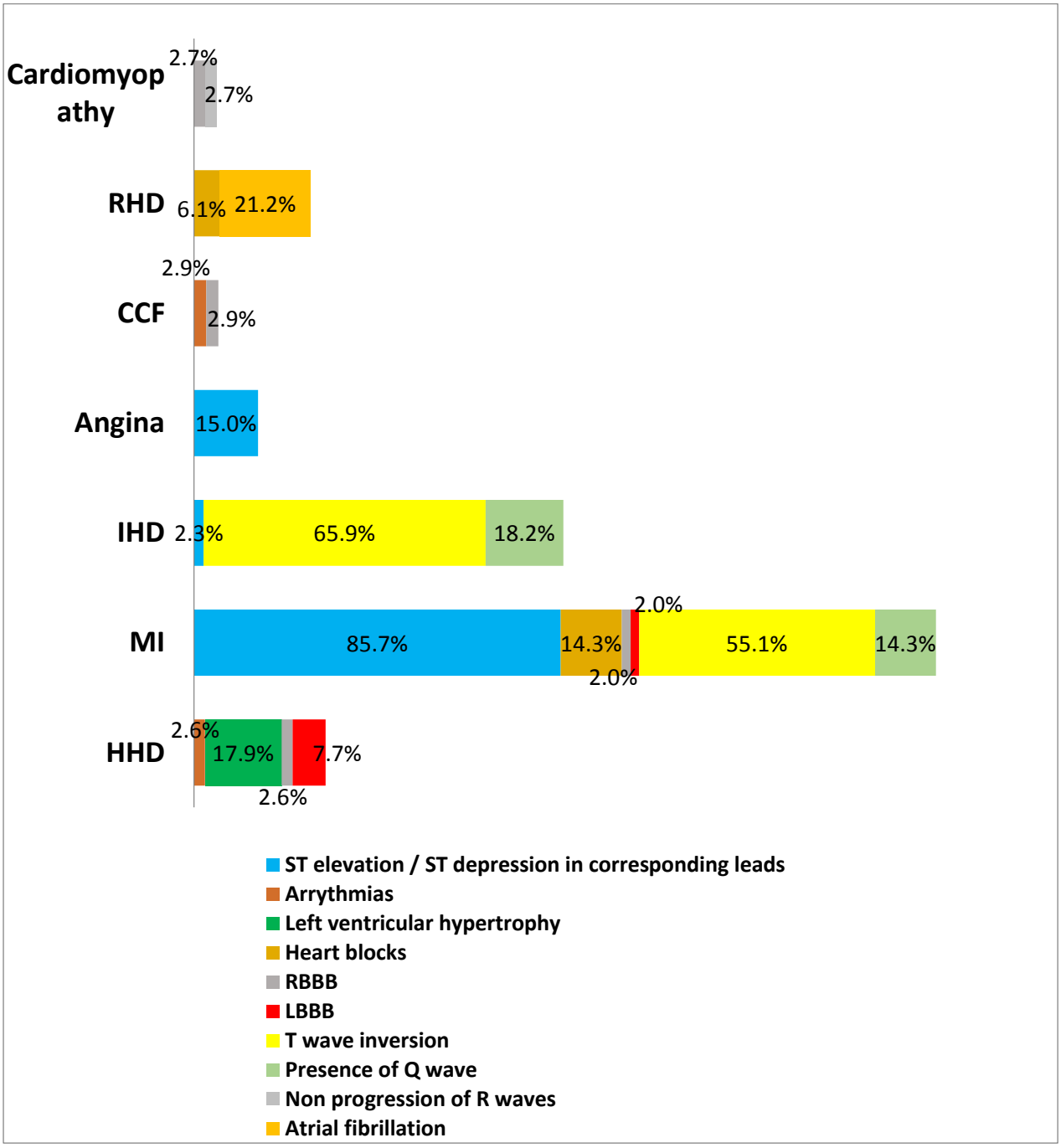


Fig.5.4.1:Electrocardiographic findings among study groups. Hypertensive heart disease (HHD); myocardial infarction (MI); ischemic heart disease (IHD); Angina pectoris(Angina); congestive cardiac failure (CCF),rheumatic heart disease (RHD), RBBB; right bundle branch block; LBBB; left bundle branch block.

Our results shows ST elevation/ST depression in 15% of angina, 2.3% of IHD and 87.7% of MI patients .Arrythmia was shown in 2.9 % of CCF and 2.6% of HHD patients. 17.9% of HHD patients shown left ventricular failure. Heart block was found in 14.3% of MI and 6.1% of RHD patients. RBBB was seen in 2.7% of cardiomyopathy, 2.9% of CCF, 2% OF MI and 2.6% of HHD patients. LBBB was seen in 2% of MI and 2.6% of HHD patients. T wave inversion was seen in 65.9% of IHD and 55.6% of MI patients.Q wave was noted in 18.2% of IHD and 55.1% of MI patients.

Apart from its use in the clinical context, as a part of routine examination procedure in heart patients,the resting ECG has proved its value as a diagnostic tool for detecting CVD. ECGs of apparently healthy individuals have been used for studying the prevalence, correlates, and the predictive value of asymptomatic heart diseases in the general population.(Higgins *et al*,1965)

Our findings are in agreement with study of Blackburn *et al*(1970) who reported that highly significant increased risks for CHD morbidity and mortality are associated with presence of large Q waves, inverted T waves, atrial fibrillation, premature beats, first degree AV blocks, and minor T waves in 12 770 middle aged working men of the seven countries study.

The majority of the ST elevation MI (STEMI) anterior patients present ST-segment elevations in derivations, reflecting the anterior (V1–V4) and, partially, the lateral wall (I, aVL), as well as reciprocal ST-changes in the inferior derivations. Our results are also in agreement with those of Kosuge *et al*.(2010).

5.5.Echocardiographic findings

5.5.Results and Discussion

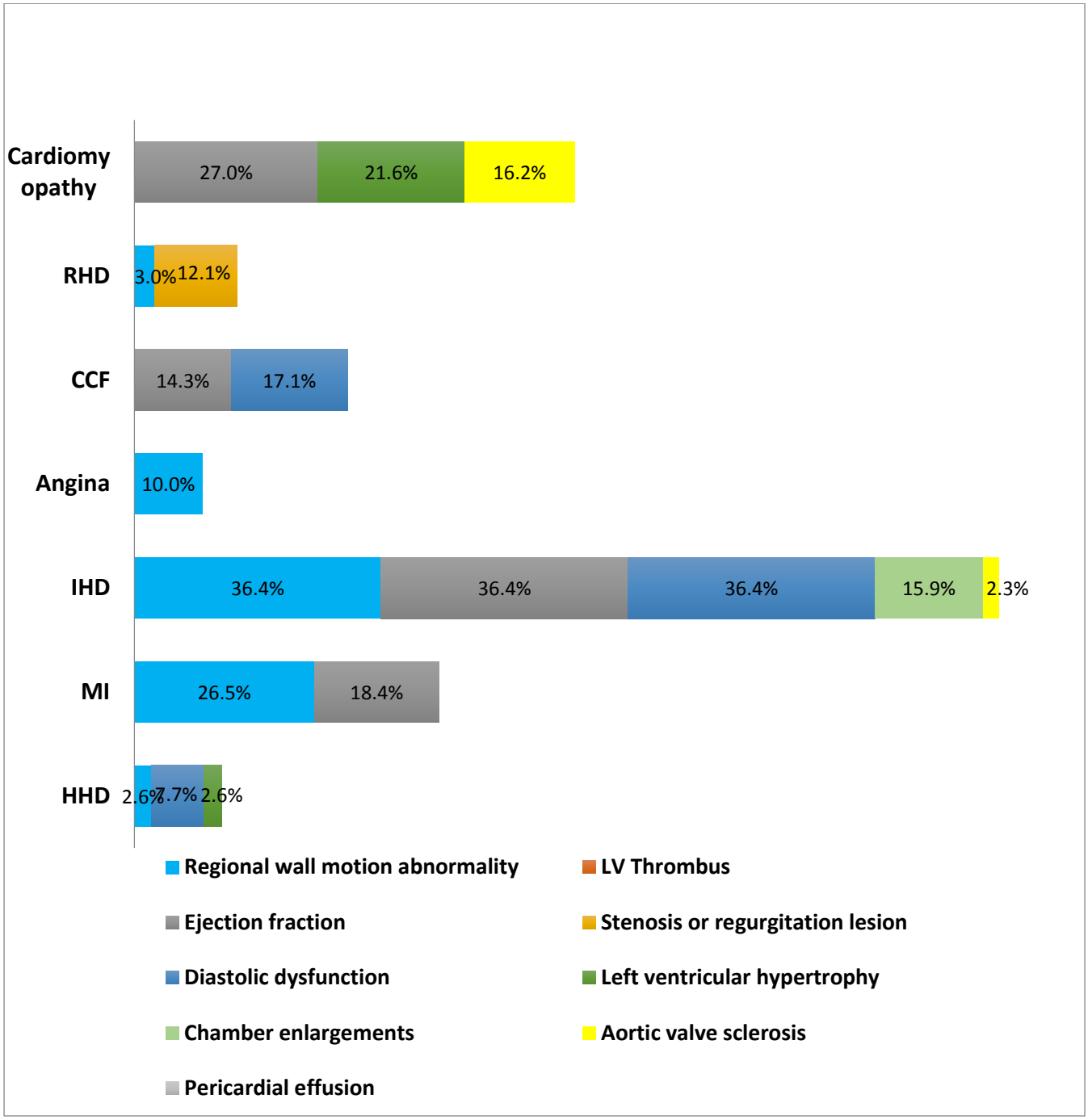


Fig.5.5.1: Echocardiographic findings among study groups. Hypertensive heart disease (HHD); myocardial infarction (MI); ischemic heart disease (IHD); Angina pectoris(Angina); congestive cardiac failure (CCF); ,rheumatic heart disease (RHD)

Results of our study show regional wall motion abnormality in 3% of RHD patients, 10% of angina patients, 36.4% of IHD, 26.5% of MI patients and 2.6% of HHD patients. 27% of cardiomyopathy patients, 14.3% CCF patients, 36.4% of IHD and 18.4% of MI patients shown decreased ejection fraction (< 60%) in present study.Diastolic dysfunction was found in 17.1% of CCF patients, 36.4% of IHD and 7.7% of HHD patients. Left ventricular hypertrophy was found in 21.6% of cardiomyopathy and 2.6% of HHD patients. 15.9% of IHD patients were also shown chamber enlargement. Aortic valve sclerosis were found in 16.2% and 2.3% of IHD patients. Results of our study are similar line of findings of Seher *et al* (2019).

5.6 Biochemical parameters

5.6.1.Results and Discussion

Table 5.6.1: Comparison of mean lipid profile parameters among study groups

Parameters	NORMAL RANGE	CARDIAC PATIENTS							F value	ANOVA p value
		HHD (n=39)	MI (n=49)	IHD (n=44)	ANGINA (n=40)	CCF (n=35)	RHD (n=33)	CARDIOMYOPATHY (n=37)		
CHOLESTEROL (mg/dl)	150-220	**228.49±40.21**[a,b,c,d,e]	**223.63±36.36**	221.82±38.34[a]	192.03±36[b]	187.27±26[c]	195.42±36.03[d]	202.7±42.44[e]	3.354	0.003*
TRIGLYCERIDES (mg/dl)	50-150	**170.77±44.73**[a,b,c,d,e]	**157.71±40.09**[a,f]	**168.61±41.41**[g,h]	137.35±51.17[b,h]	146.09±47.69[c]	140.61±48.75[d]	131.65±40.04[e,f,g]	1.313	0.025*
LDL (mg/dl)	90-129	**139.02±18.9**[a,b,c]	**136.68±18.19**	**134.49±18.87**	124.95±19.06[a]	129.05±13.41[b]	**134.84±19.01**	129.88±15.58[c]	3.023	0.007*
HDL (mg/dl)	37-71	81.62±14.03[a,c,d,e,f]	72.96±11.2[a]	73.95±14.48	66.8±14.77[c]	65.91±11.62[d]	67.48±8.97[e]	68.57±11.56[f]	1.525	0.017*
VLDL (mg/dl)	15-40	37.89±18.45[a,b,c]	34.27±15.82	39.14±14.49	28.14±15.54[a]	35.7±18.62	32.88±13.14[b]	32.48±15.05[c]	2.161	0.047*

hypertensive heart disease (HHD); myocardial infarction (MI); ischemic heart disease (IHD); Angina pectoris(Angina); congestive cardiac failure (CCF); rheumatic heart disease (RHD). LDL, low-density lipoprotein; HDL, high-density lipoprotein; VLDL, very low-density lipoprotein.

* significant at 5% level of significance ($p<0.05$), a,b,c,d,e,f,g represent significant difference in multiple comparison by tukey's test ($p<0.05$)

A higher cholesterol level from normal range has been observed in case of group 1(HHD)and 2(MI). Similarly in case of triglycerides, groups 1(HHD),2 (MI) and 3(IHD) also shows higher triglyceride limit from normal range. LDL levels in groups 1(HHD), 2 (MI), 3 (IHD) and 6 (RHD) showed statistically significant higher levels compared to other groups . Though HDL and VLDL levels showed statistically significant difference among cardiac patient groups but values are within the normal range.

Hence it may be stated that dyslipidemia among CVD patients are extremely specific and depending on their types and nature. The Framingham Off-spring study followed a cohort with finding that low levels of HDL-C, high levels LDL-C and TG in any combination, were associated with increased CVD risk. (Anderson *et al.*2014)

As dyslipidemia is associated with various types of stress factors and MI, HHD and IHD definitely generate more stress among all the types of CVD hence it may be the possible reason behind dyslipidemia in groups 1(HHD), 2 (MI) and 3(IHD) of CVD patients in the present study.

According to Adak *et al.* (2010) dyslipidemia is one of the primary causes for CAD. Increased TC, TG, LDL-C and decreased HDL-C are the important risk factors in myocardial infarction. (Adak *et al.* 2010)

Our findings are in agreement with numerous studies reported that hypercholesterolemia, hypertriglyceridemia or both carry an increased risk of developing premature CAD.(Adak *et al.* 2010)

5.6.2.Results and Discussion

Table 5.6.2: Comparison of mean biochemical parameters among study groups

Parameters	NORMAL RANGE	CARDIAC PATIENTS							F value	ANOVA p value
		HHD (n=39)	MI (n=49)	IHD (n=44)	ANGINA (n=40)	CCF (n=35)	RHD (n=33)	CARDIOMYOPATHY (n=37)		
S.creatinine (mg/dl)	0.9-1.4	1.45±0.83[a,b,c,d,e]	1.21±0.83[a]	1.24±0.79[b]	1.03±0.65[c]	1.35±0.76	1.02±0.53[d]	1.2±0.66[e]	1.604	0.146
S.sodium (mmol/l)	135-145	149.72±14.84[a,b,c]	142.43±9.55[a]	143.73±11.83	142.08±10.68[b]	143.12±11.47	142.88±11.81[c]	144.78±12.51	1.948	0.073
S.pottasium (mmol/l)	2.6-5.2	5.87±1.92	4.21±0.99	4.47±1.33	4.37±1.03	4.54±1.07	4.72±1.11	4.85±1.34	7.356	<0.001*
CPK-MB (U/L)	0-26	27.15±9.65[a,c]	27±9.87[b]	24.23±9.62	20.95±6.45[a,b]	24.97±7.32	22.7±9.69[c]	23.62±8.35	2.614	0.018*
S.calcium (mg/dl)	8.5-10.2	11.83±4.83[a,b]	9.37±3.23	9.51±2.86	8.55±2.33[a]	9.05±2.49	9.57±3.24	8.82±2.47[b]	4.505	<0.001*
Vitamin D (ng/ml)	20-50	**11.81±2.14**	**12.06±1.89**	**12.29±1.54**	**12.12±1.78**	**12.64±1.67**	**12.01±1.21**	**11.73±1.77**	1.085	0.372

hypertensive heart disease (HHD); myocardial infarction (MI); ischemic heart disease (IHD); Angina pectoris (Angina);congestive cardiac failure (CCF); rheumatic heart disease (RHD).

* significant at 5% level of significance ($p<0.05$), a,b,c,d,e,f,g represent significant difference in multiple comparison by tukey's test ($p<0.05$)

Chemical parameters shows statistically significant difference in the serum calcium level among all the groups of CVD patient although these are within normal range.

Major aspects of cardiac functions like, excitation, contraction, excitation-contraction coupling as well as relaxation requires maintenance of intracellular Ca^{2+} homeostasis in the heart.

It is well accepted that intracellular calcium release from the sarcoplasmic reticulum (SR) is required for cardiac muscle contraction. Impaired calcium release causes decreased muscle contraction (systolic dysfunction) and alteration of exocytosis of calcium removal affects relaxation (diastolic dysfunction) (Viola and Hool, 2014)

The key regulator of cardiac function is intracellular calcium (Ca^{2+}), as Ca^{2+} acts as a second messenger and plays an important role in regulating cardiac physiology and pathophysiology. Altered expression and activity of Ca^{2+}-related proteins are associated with cardiac dysfunction, which is observed in many patients with heart failure.(Kho *et al*, 2012)

Our results show statistically significantly lower levels of vitamin D in all groups of CVD patients in comparison to normal range.Vitamin D deficiency has been found to contribute to the development of various cardiometabolic conditions such as hypertension , and coronary artery disease. The connection between cardiovascular homeostasis and vitamin D status using a rat model of vitamin D deficiency was first accomplished as early as in 1987 (Weisher and Simpson, 1987) .

Prospective studies have also found a high prevalence of vitamin D deficiency in patients hospitalized with AMI. A multicenter study carried out with 239 patients with acute coronary syndrome (ACS) showed that 96% of the individuals had low vitamin D levels at hospital admission.(Lee *et al*, 2011)

Our results from present study clearly indicate sever deficiency of Vitamin D levels in all the groups of CVD patients admitted in ICCU. These results further indicate the importance of vitamin D on cardiovascular functions.

5.6.3.Results and Discussion

Table 5.6.3: Comparison of mean oxidative stress parameters among study groups

Parameters	Control values	CARDIAC PATIENTS							F value	ANOVA p value
		HHD (n=39)	MI (n=49)	IHD (n=44)	ANGINA (n=40)	CCF (n=35)	RHD (n=33)	CARDIOMYOPATHY(n=37)		
MDA (nmol/L)	1.1±0.5 §	**2.03±0.73**	**2.09±0.83**	**1.8±0.71**	**1.94±0.65**	**1.88±0.68**	**2±0.83**	**1.84±0.77**	0.854	0.53
SOD (U/mL)	1.8±0.6 §	**0.37±0.09**[a]	**0.73±0.66**[a,b,c,d,e,f]	**0.36±0.11**[b]	**0.4±0.13**[c]	**0.39±0.09**[d]	**0.41±0.1**[e]	**0.38±0.09**[f]	9.524	<0.001*
NO (µmol/L)	6.4±1.6 §	**4.12±1.72**	**4.4±3.58**	**3.63±1.88**	**3.88±2.17**	**5.27±4.12**	**4.62±2.04**	**3.67±1.59**	1.837	0.092

hypertensive heart disease (HHD); myocardial infarction (MI); ischemic heart disease (IHD); Angina pectoris (Angina); congestive cardiac failure (CCF); rheumatic heart disease (RHD); §All groups showing significant difference with control values.ANOVA done with seven CVD groups and control group.

* significant at 5% level of significance ($p<0.05$), a,b,c,d,e,f,g represent significant difference in multiple comparison by tukey's test ($p<0.05$)

Table 5.6.3 shows statistically significant ($p<0.05$) higher levels of MDA in all groups of CVD as compared to control group. MI group shows highest value (2.09±0.83) of MDA among all CVD groups. All groups of CVD also show statistically significant ($p<0.05$) lower levels of SOD and NO as compared to control group. SOD value (0.36±0.11) and NO value (3.63±1.88) were found least in IHD patients.

A lot of oxygenated compounds, particularly aldehydes such as Malondialdehyde (MDA) are produced during the attack of free radicals to membranes, lipoprotein and polyunsaturated fatty acids. Thus degree of lipid peroxidation in the blood provides useful information for the prognosis of CVD patients.This imbalance will cause damage to cellular components and tissues in the body leading to oxidative stress as well as decrease in total antioxidant capacity. (Mahbood *et al*,2005)

Several authors also have reported that increased levels of MDA in CVD patients. (Ramprasad, 2014)

Decreased SOD level indicate possibility of higher generation of superoxide. SOD is well known endogenous antioxidant present in cell membrane which protect cell membrane against oxidative stress of any kind. Low SOD level in all CVD groups also indicate a serious oxidative stress in the cell membrane of myocyte.

Decreased activity of the antioxidant system, including decreased antioxidant enzyme(SOD) activity may contribute to oxidative stress in patients with atherosclerosis. (Stralin *et al*. 1995)

Lower NO levels in all groups of CVD patients admitted in ICCU in present study as compared to control indicate a possible formation of peroxynitrate by binding available NO with generated superoxide or hydrogen peroxide which itself may be considered as cardiotoxic. Further lowering of NO definitely reduces circulation of blood to deep interior of myocardial tissue and lead to further aggravation of cardiac damage. The decrease in synthesis and bioavailability of NO is an important step in the development of atherosclerosis. (Furchgott and Zawadzki, 1980)

5.7 Molecular parameters

5.7.Results and Discussion

Table 5.7.: Comparison of mean molecular parameters among study groups

Hypertensive heart disease (HHD); myocardial infarction (MI); ischemic heart disease (IHD); Angina pectoris (Angina); congestive cardiac failure (CCF); rheumatic heart disease (RHD); §All groups showing

Parameters	Control values	CARDIAC PATIENTS							F value	ANOVA p value
		HHD (n=39)	MI (n=49)	IHD (n=44)	ANGINA (n=40)	CCF (n=35)	RHD (n=33)	CARDIOMYOPATHY (n=37)		
VEGF (pg/ml)	420.9±79.7§	424.99±104.23[a,b,c]	461.7±173.37[d,e,f]	**365.65±97.42[a,d]**	**395.05±89.71**	421.27±117.7[g]	**358.09±85.8[b,e]**	**355.13±68.98c,f,g**	5.358	<0.001*
NOS3 (pg/ml)	187.56±18.96	**106.46±13.43**	**107.06±23.36**	**100.58±14.62**	**106.69±19.6**	**106.52±17.9**	**96.72±14.3**	**95.69±17.56**	3.024	0.007*

Significant difference with control values.ANOVA done with seven CVD groups and control group.

* significant at 5% level of significance ($p<0.05$), a,b,c,d,e,f,g represent significant difference in multiple comparison by tukey's test ($p<0.05$)

Our results shows statistically significant ($p<0.05$) increase VEGF levels in groups 3 (IHD, 365.65±97.42), 4 (Angina, 395.05±89.71**)**, 6 (RHD, 358.09±85.8) and 7 (Cardiomyopathy, 355.13±68.98) as compared to control group (Table.5.7). NOS3 levels were also found to be significantly reduced in all CVD groups as compared to control groups. The present study clearly indicate an impaired protective mechanism on myocardial tissues against ischemic insult due to lower VEGF, lower NOS3 and NO (Table 5.6.3).

VEGF influences vasculogenesis, angiogenesis and cell signalling to protect intracellular homeostasis against low oxygen microenvironment. Most of cardiac disorders are linked to low oxygen microenvironment. Hence VEGF over expression facilitate an adoptive mechanism in vascular smooth muscle as well as myocardial tissues to promote angiogenesis and vasculogenesis. Further VEGF influences NOS3 mechanism in endothelial cell as well as vascular smooth muscle to generate more NO to increase circulation especially to the ischemic part or circulatory deficient part of MI.(ref)

In present study decrease of NOS3 activity support these views. The low NO as we have mentioned earlier possibly may not be only due to NOS3 effect but its interaction with superoxide reduce its bioavailability. Numerous therapies have been investigated to assess the possibility of reversing endothelial dysfunction by enhancing the release of nitric oxide from the endothelium, either through stimulation of nitric oxide synthesis or protection of nitric oxide from oxidative inactivation and conversion to toxic molecules such as peroxynitrite. Accordingly, causal relationships between improved endothelial function, reduction in myocardial ischemia and acute coronary events need further to be investigated. (Cannon, 1998)

5.8 Correlations

5.8.1&1a.Result and Discussion

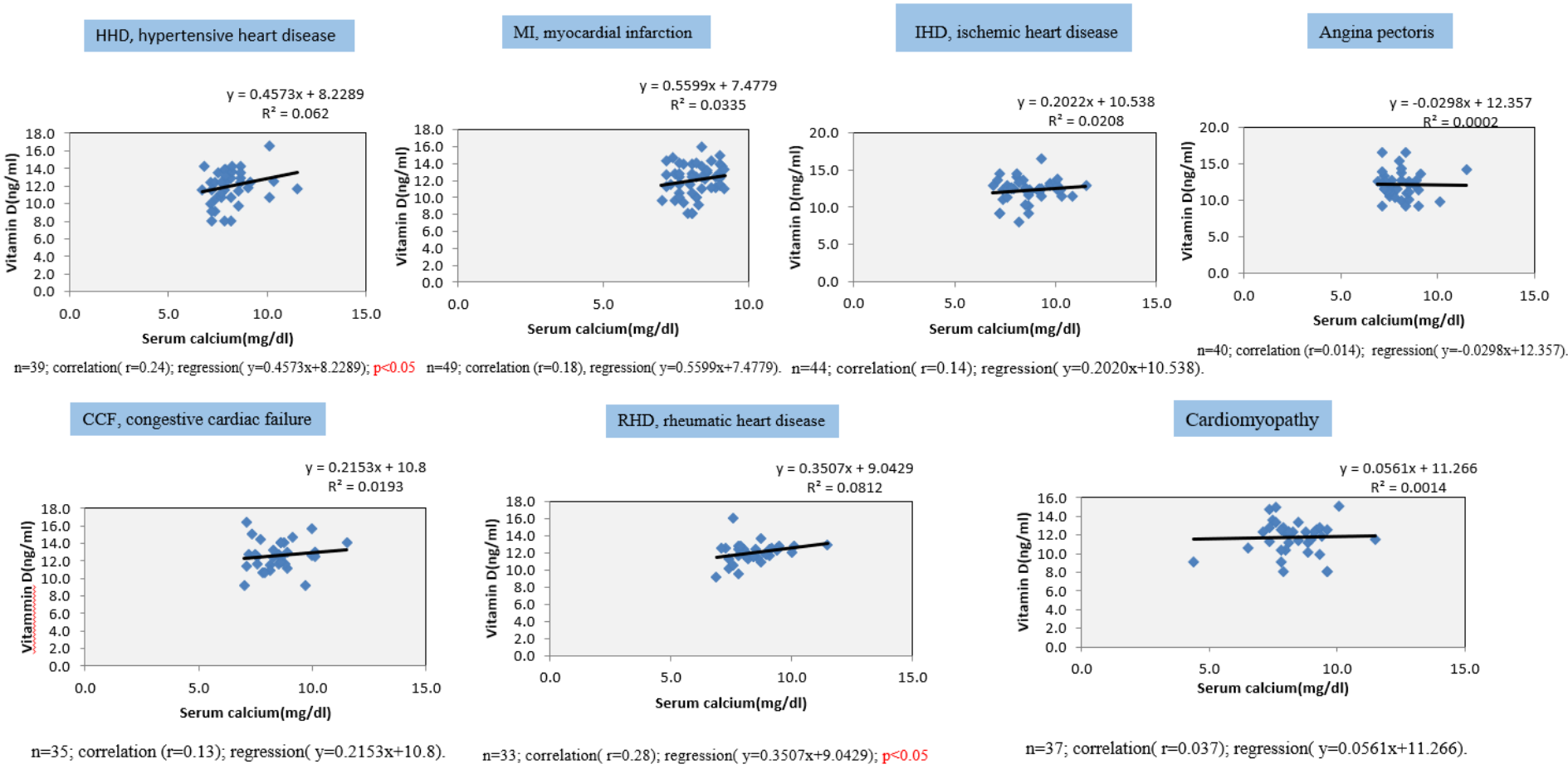


Fig 5.8.1: Correlation between vitamin D & serum calcium among cardiac patients

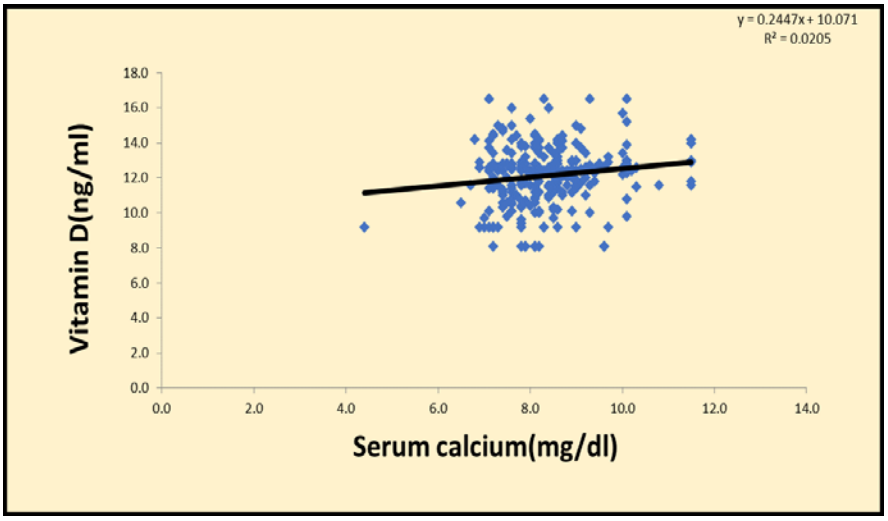


Fig.5.8.1a: Correlation between vitamin D & serum calcium of all cardiac patients.

n=278; correlation(r=0.14); regression(y= -0.2447x+10.071);NS

Our results showed significant correlation ($p<0.05$) between serum calcium and vitamin D in group 2 (MI) and 6 (cardiomyopathy) but remaining groups did not show any such correlation (Fig.5.8.1 and 5.8.1a). When correlation between serum calcium and vitamin D of all the CVD groups together were analysed it also did not show any such significance. It indicates serum calcium and vitamin D are two independent variables in case of cardiac diseases.

5.8.2.Results and Discussion

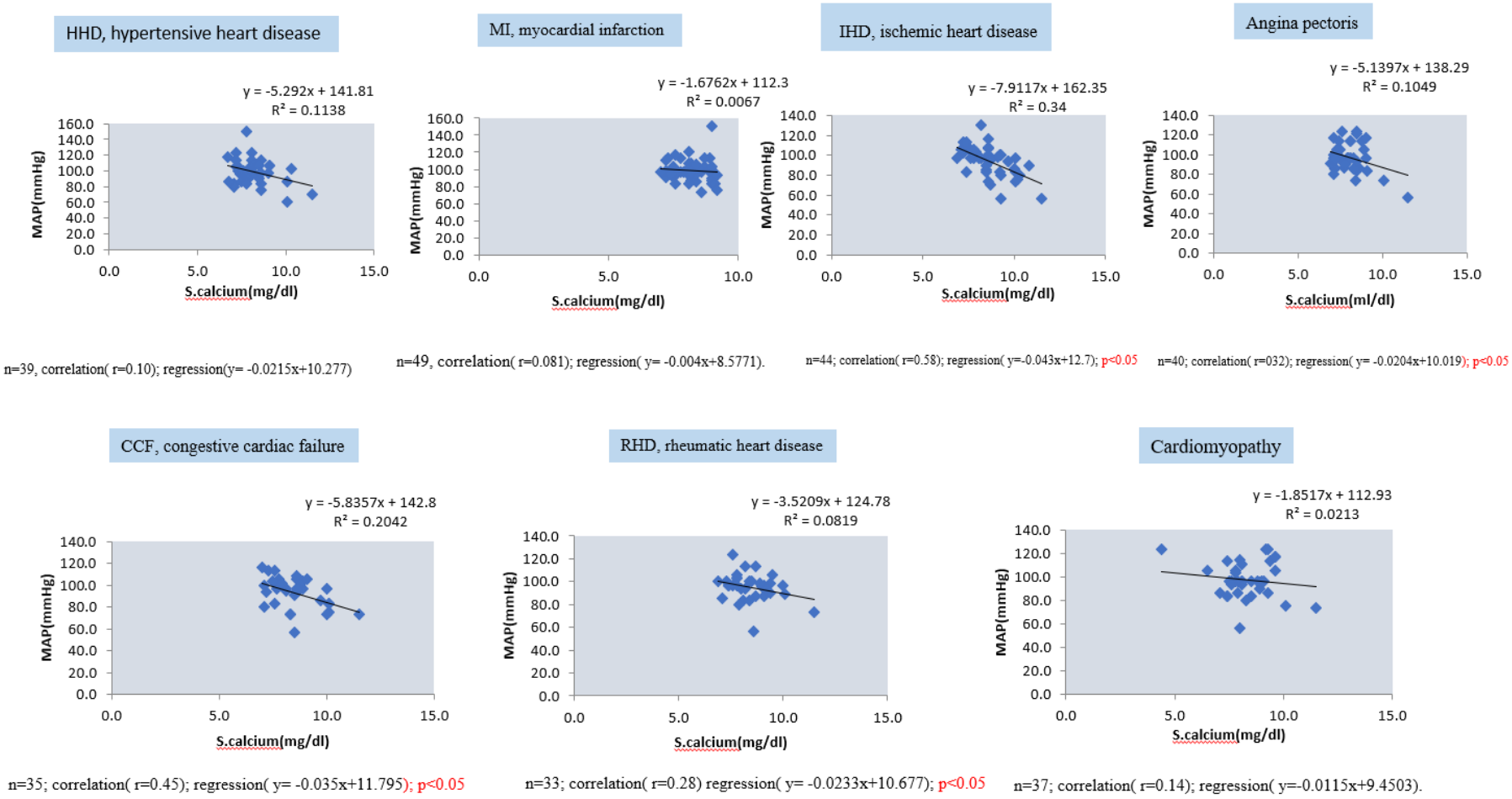


Fig.5.8.2.: Correlation between MAP & serum calcium of cardiac patients

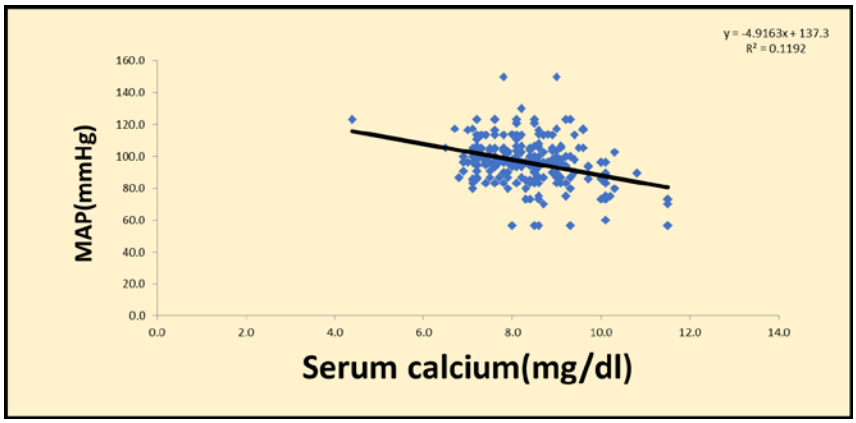


Fig.5.8.2a: Correlation between MAP & serum calcium of all cardiac patients.

n=278; correlation (r=0.36); regression(y= -0.0263x+10.857); $p<0.05$

MAP and serum calcium in general found to be correlated in all groups together ($p<0.05$) although individually groups 1(HHD), 2 (MI), 5 (CCF) and 7(cardiomyopathy) did not show any significant correlation (Fig.5.8.2). Hence values of BP in any form of CVD may not be completely ruled out while establishing a relationship between serum calcium and BP.

5.8.3.Results and Discussion

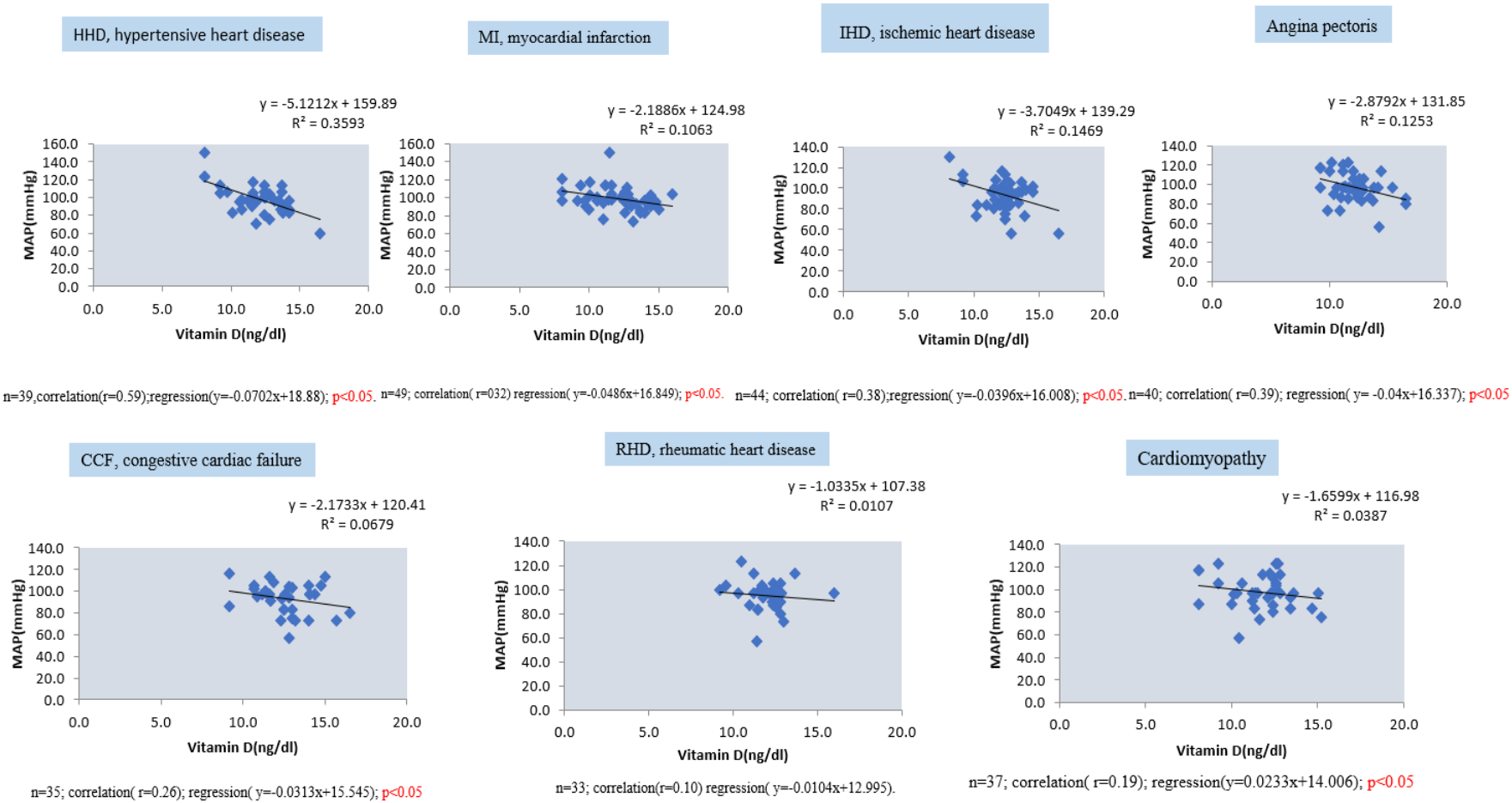


Fig.5.8.3: Correlation between MAP & vitamin D of cardiac patients

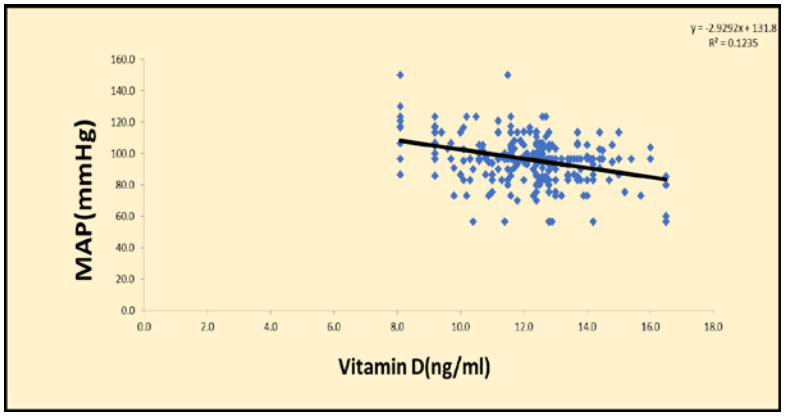


Fig.5.8.3a: Correlation between MAP & vitamin D of all cardiac patients.

n=278; correlation (r=0.35); regression(y= -0.0438x+16.328); $p<0.05$

Vitamin D is negatively correlated with MAP in all types of CVD patients except in group 6 (CCF)(Fig.5.8.3). While the analysis of correlashionship between vitamin D and MAP of all types of the CVD patient were done it also showed the similar negative correlation between the variables.(Fig.5.8.3a).

The results indicates that the lower the vitamin D level in serum are associated with higher the MAP values in all the types of CVD patients. The relationship between vitamin D and BP clearly indicate further the significance of vitamin D on regulation of cardiac hemodynamics. The Third National Health and Nutrition Examination Survey (NHANES III),performed a large on sample of population 12,644 North-Americans, showed an inverse relationshipbetween systolic BP and pulse pressure with levels of vitamin D.(Sragg *et al*, 2007)

van Ballegooijen *et al*. in their follow up study on 5,066 normotensive individuals had their serum vitamin D level measured which was low compared to normal values and were followed up for 6.4 years. At the end of follow-up, 1,036 (20.5%) developed hypertension, low levels of vitamin D were associated with a greater risk of development of the disease.

5.8.4.Results and Discussion

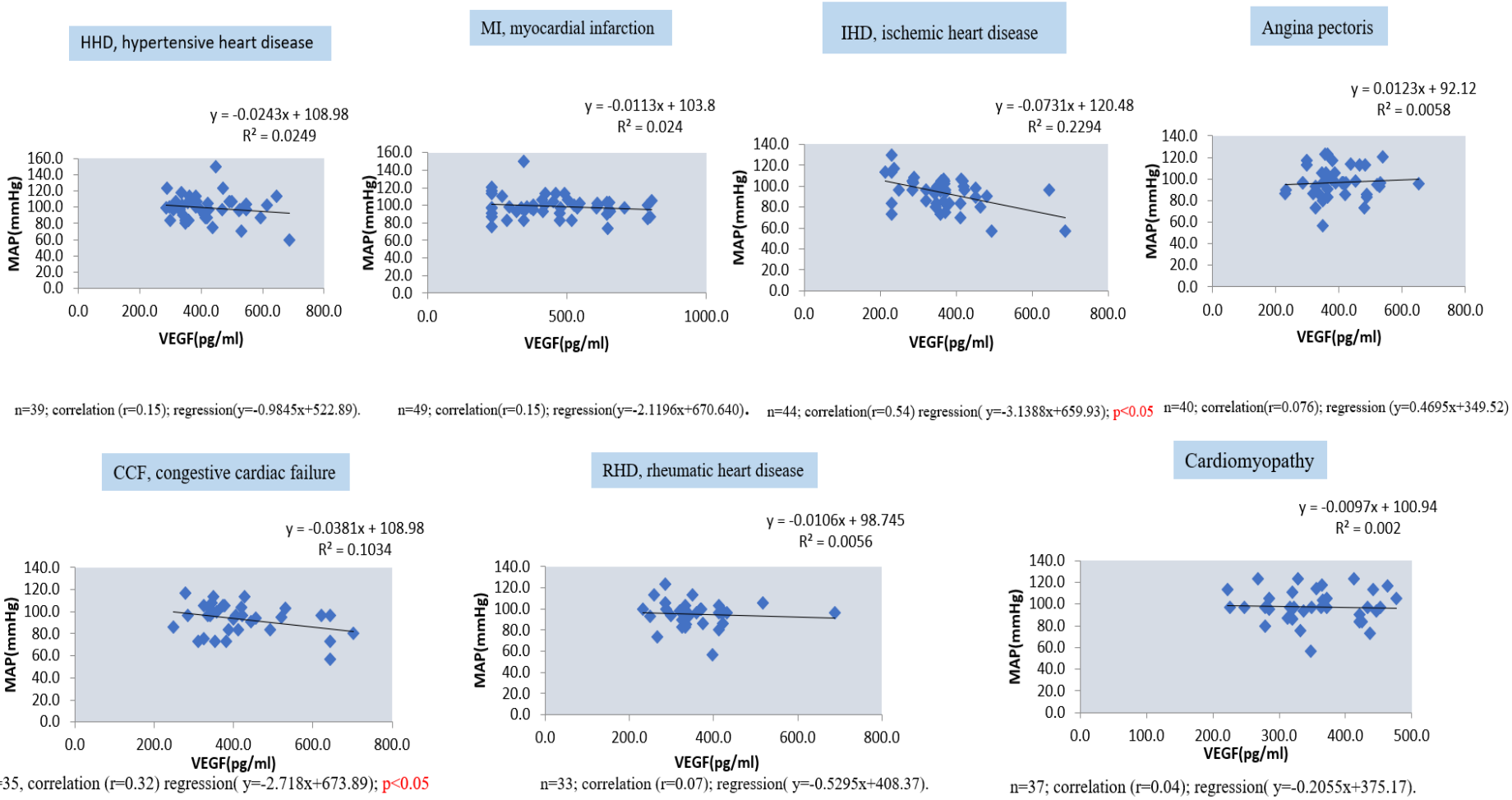


Fig.5.8.4: Correlation between MAP & VEGF of cardiac patients

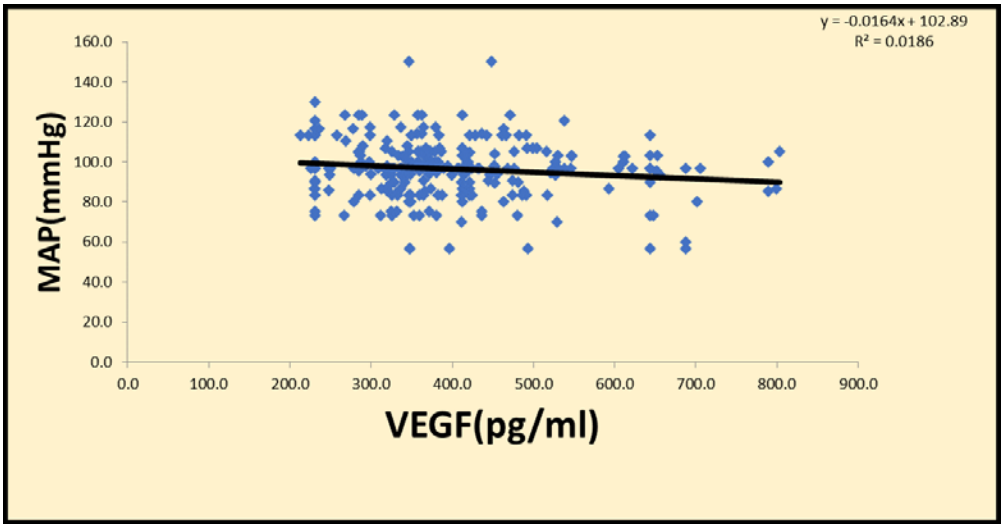


Fig.5.8.4a: Correlation between MAP & VEGF of all cardiac patients n=278; correlation (r=0.13); regression(y= -1.1451x+510.08) NS

The study clearly shows VEGF has no relationship with MAP in group 3(IHD) and 5(CCF). Further in case of all the group combination did not show any correlation between VEGF and MAP in the present study. Although VEGF influences MAP in normal condition but results from our study reflect in case of CVDs VEGF can not be considered as a predictor for MAP

5.8.5.Results and Discussion

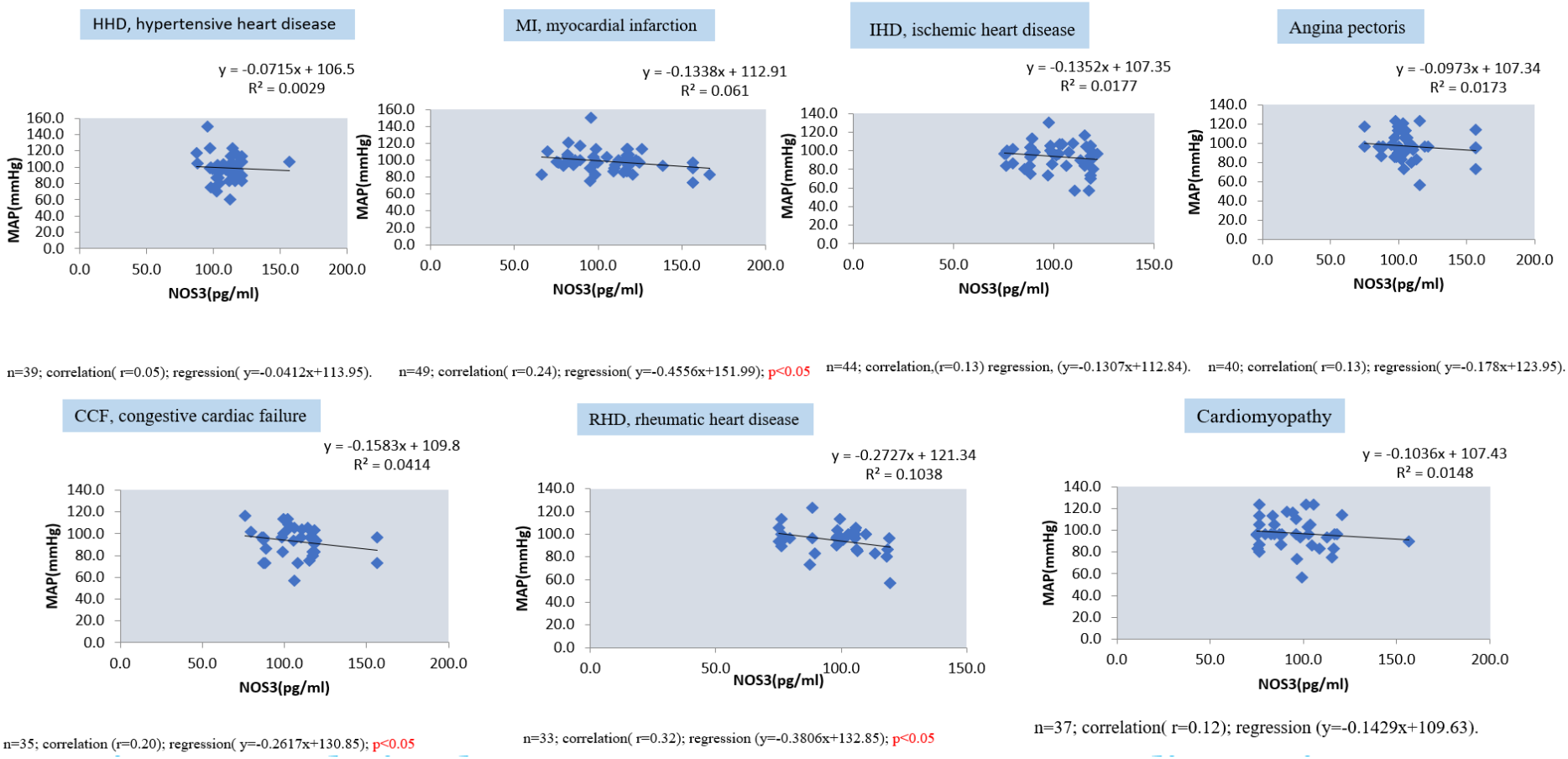


Fig.5.8.5: Correlation between MAP & NOS3 of cardiac patients

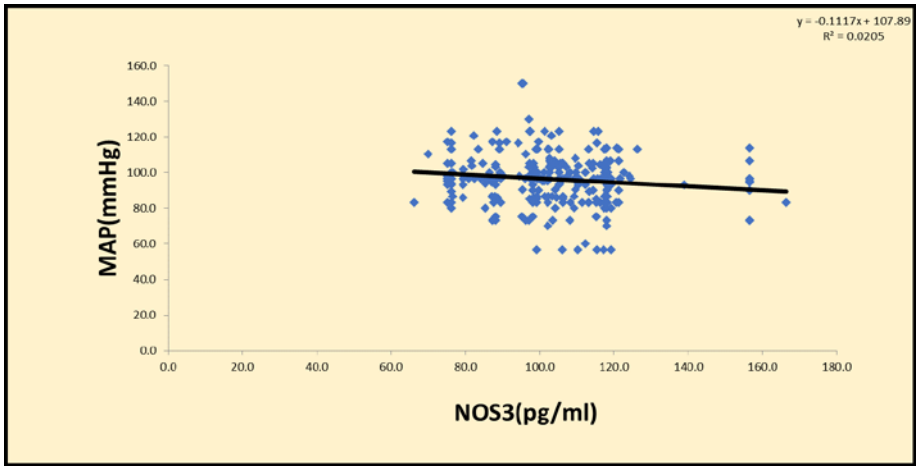


Fig.5.8.5a. Correlation between MAP & NOS3 of all cardiac patients. n=278; correlation(r=0.14); regression(y= -0.1117x+107.89) NS

The study clearly shows NOS3 has no relationship with MAP in group 2(MI), 5(CCF) and 6 (HHD). Furthure in case of all the group combination did not show any correlation between NOS3 and MAP in the present study. Although NOS3 regulates arterial dilatation by promoting secretion of NO but the action of NOS3 is secondary to VEGF. Perhaps non-influence of VEGF on MAP may be the secondary factor for non significant correlation between NOS3 and MAP.

5.8.6.Results and Discussion

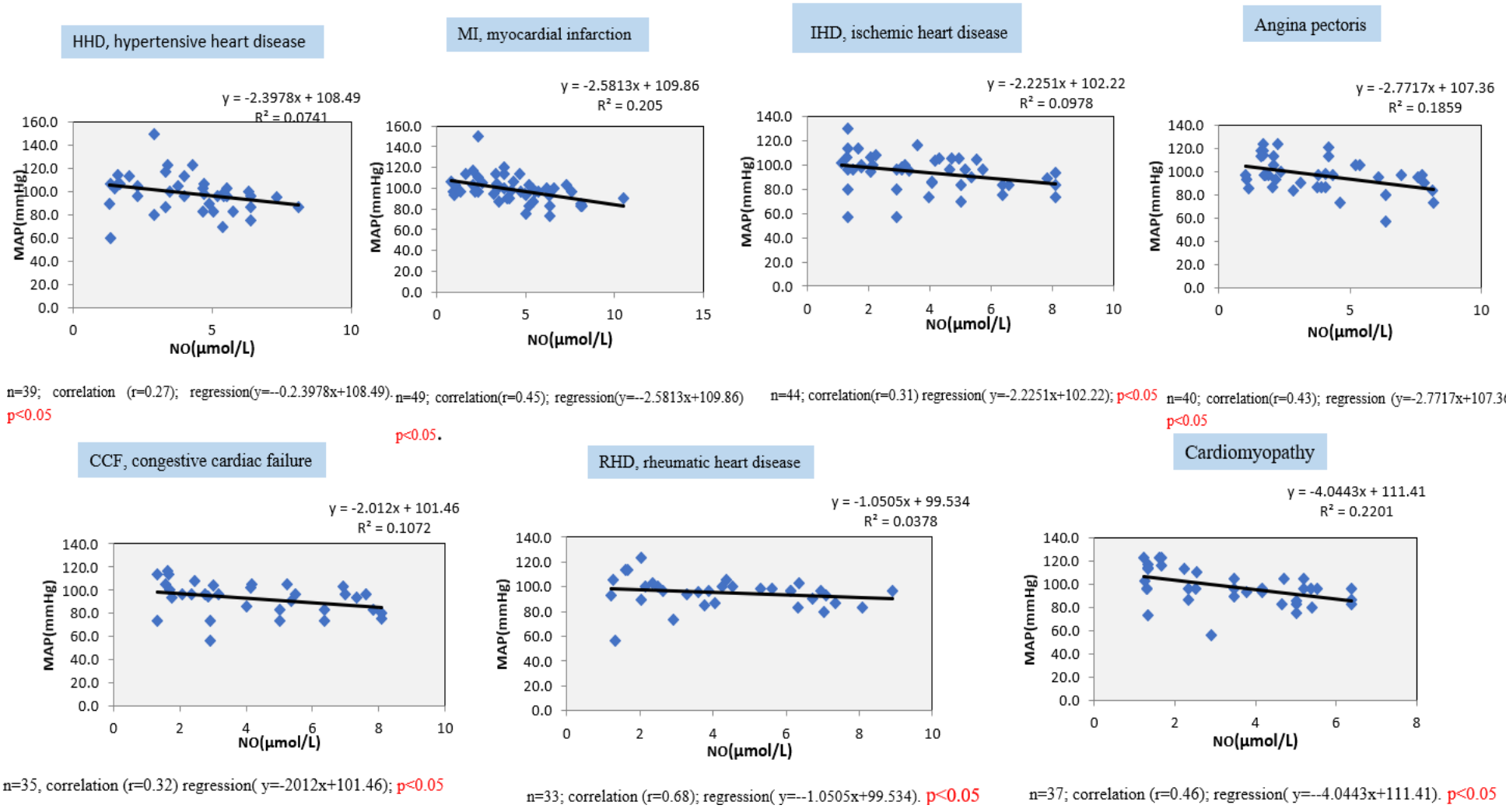


Fig.5.8.6: Correlation between MAP & NO of cardiac patients

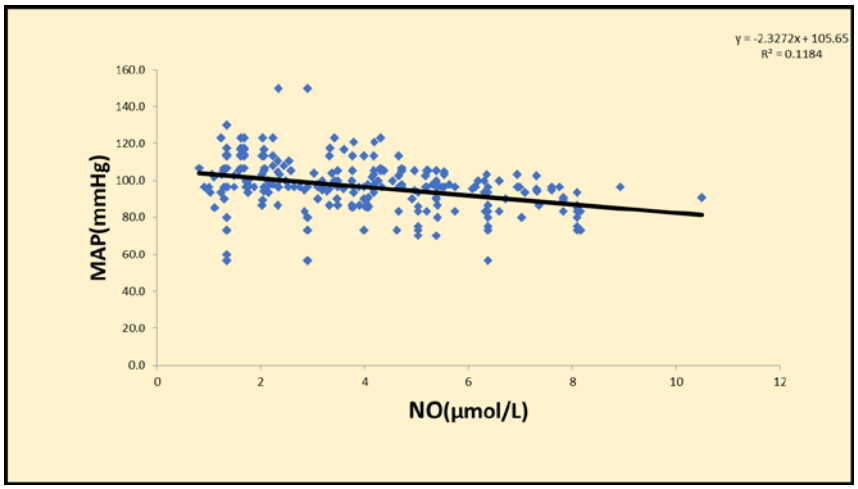


Fig.5.8.6a: Correlation between MAP & NO of all cardiac patients. n=278; correlation(r=0.34) regression(y= -0.2.3272x+105.65) $p<0.05$

Present study shows inverse relation between NO and MAP in CVD i.e lower the NO is having higher MAP in patients admitted in ICCU.

As NO is known vasodilator hence decrease in NO indicate greater vascular resistance and increase in BP. Reduced NO production causes systemic vasoconstriction and elevated blood pressure.

Most common conditions, which are associated as risk factors for atherosclerosis such as hypertension and hypercholesterolemia, are involved in diminished release of nitric oxide into the arterial wall either because of impaired synthesis or excessive oxidative degradation.(Cannon III, 1998)

5.8.7.Results and Discussion

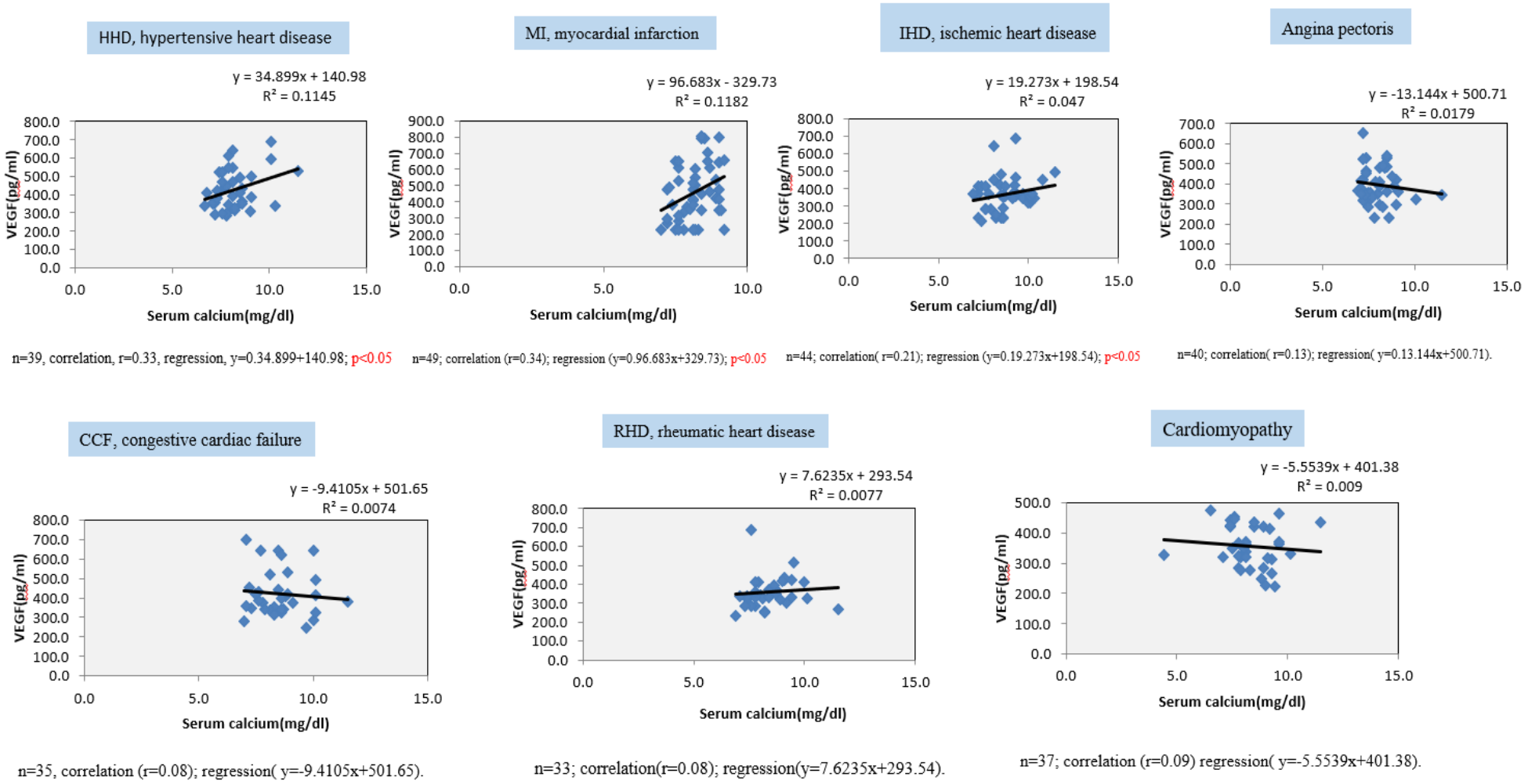


Fig.5.8.7a: Correlation between VEGF & serum calcium of all cardiac patients

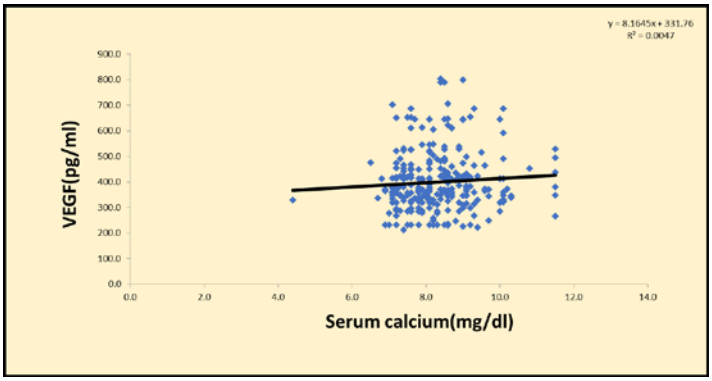


Fig.5.8.7a: Correlation between VEGF & serum calcium of all cardiac patients. n=278; correlation(r=0.06); regression(y= -8.1645x+331.76) NS

There is no correlation between VEGF and serum calcium in except in group 1(HHD), 2(MI) and 3(IHD) of CVD patients but in combination of all CVD patients also no correlation found. This clearly indicate both VEGF and serum calcium are independent variables for any types of CVD.

Normally VEGF and calcium have relationship through calmoduline protein II kinase and cAMP responsive element binding protein as well as extracellular signal regulated protein kinase through VEGFR2. However the present study did not show such relationship rather it indicate a possible disruption of calcium regulatory pathway.(Touyz *et al*, 2017)

5.8.8.Results and Discussion

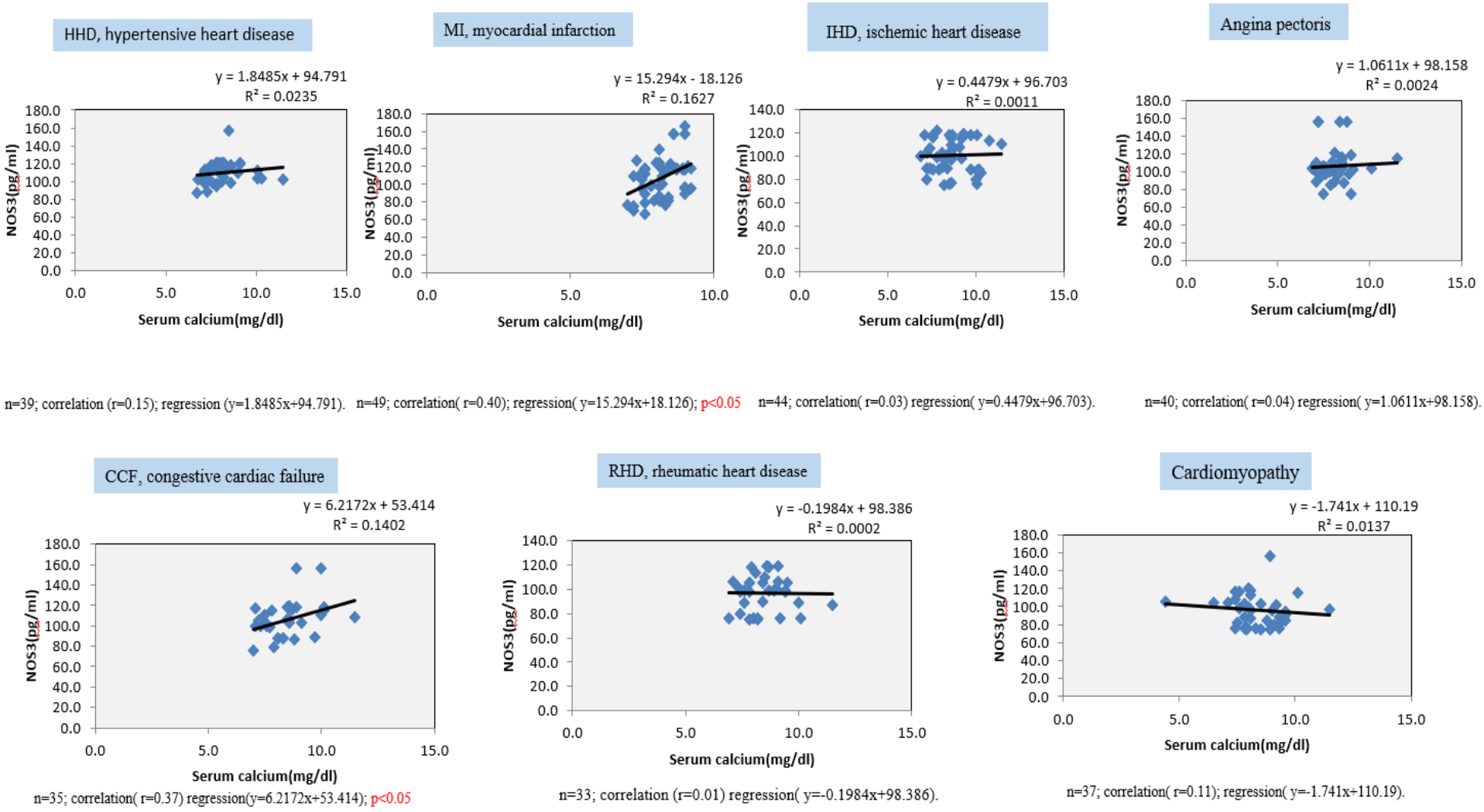


Fig.5.8.8a: Correlation between NOS3 & serum calcium of all cardiac patients

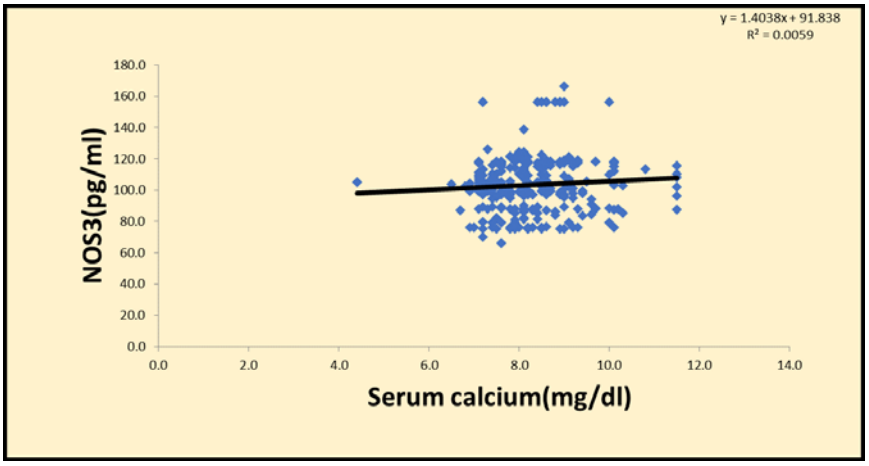


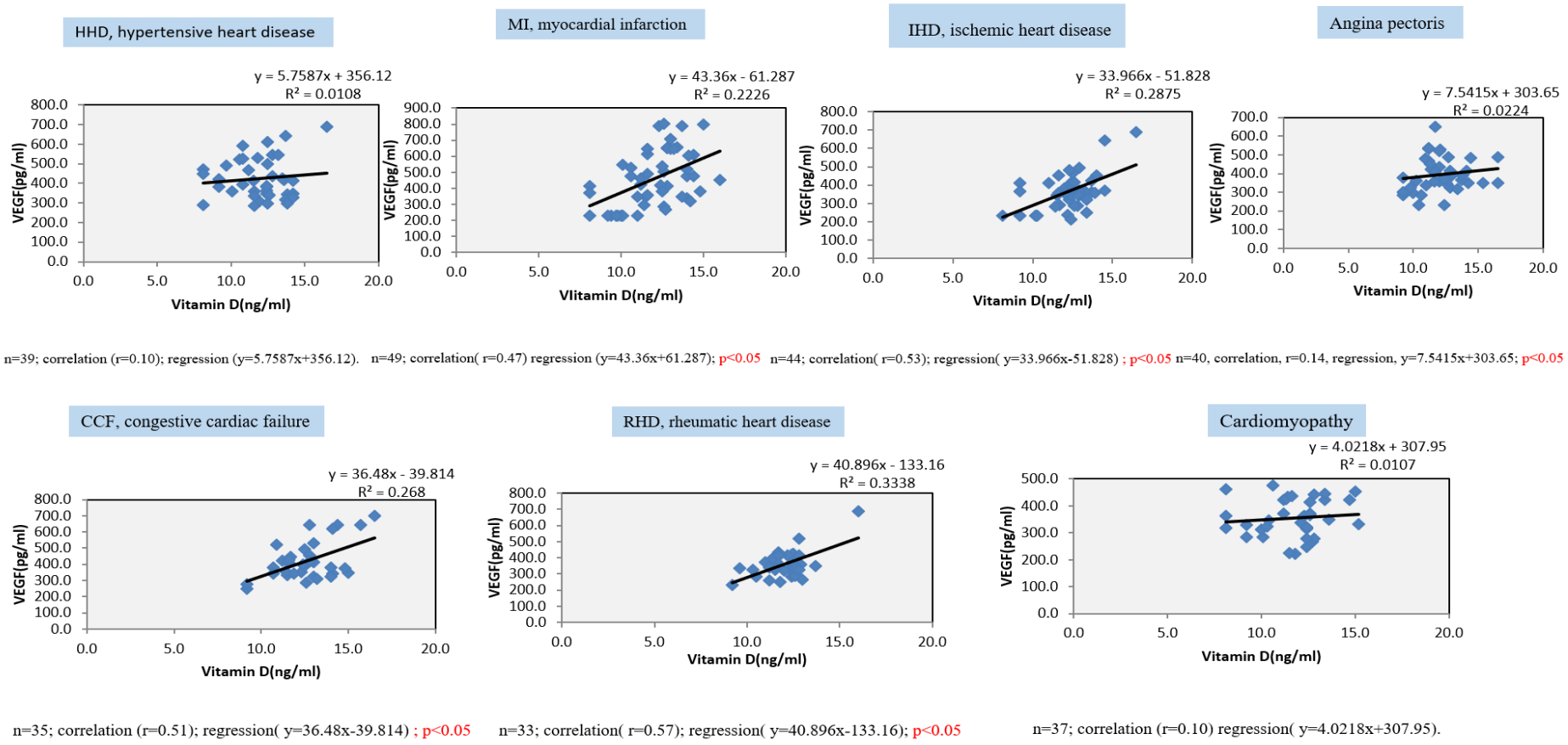
Fig.5.8.8a: Correlation between NOS3 & serum calcium of all cardiac patients. n=278; correlation(r=0.078); regression(y= 1.4038x+91.838) NS

There is no correlation between NOS3 and serum calcium except in group 5 (CCF) of CVD patients but in combination of all the CVD ptient also there is no correlation.

NOS3 induce myocardial calcium 2 cell signalling mechanism is not yet clear. It is observed that increase in cytoplasmic calcium activate calcium-calmoduline system and changes the alignment of oxygenase and reductase of NOS3.(William,2004)

No correlation between NOS3 and serum calcium probably indicate that these parameters are independent in calcium signalling mechanism in CVD patients.

5.8.9.Results and Discussion



n=39; correlation (r=0.10); regression (y=5.7587x+356.12). n=49; correlation(r=0.47) regression (y=43.36x+61.287); p<0.05 n=44; correlation(r=0.53); regression(y=33.966x-51.828) ; p<0.05 n=40, correlation, r=0.14, regression, y=7.5415x+303.65; p<0.05

n=35; correlation (r=0.51); regression(y=36.48x-39.814) ; p<0.05 n=33; correlation(r=0.57); regression(y=40.896x-133.16); p<0.05 n=37; correlation (r=0.10) regression(y=4.0218x+307.95).

Fig.5.8.9: Correlation between vitamin D & VEGF of cardiac patients

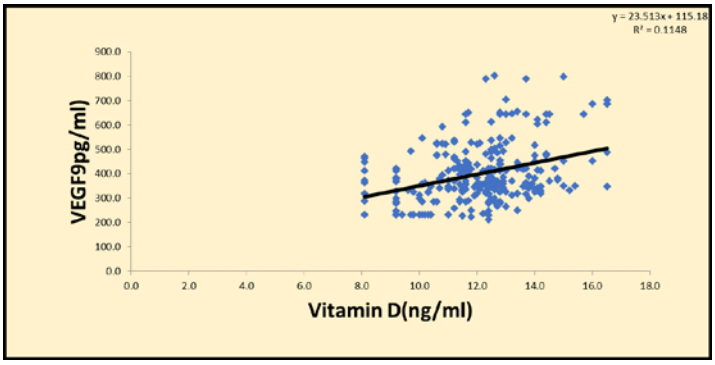


Fig.5.8.9a: Correlation between vitamin D & VEGF of all cardiac patients.

n=278; correlation(r=0.33); regression(y= 23.513x+115.18) $p<0.05$

Vitamin D and VEGF shows significant correlation in all CVD patients admitted in ICCU in present study. Results indicate that lower the vitamin D, coincided with lower VEGF in case of most of CVD patient groups. **This is one of the unique and rare observation in relation to vitamin D with VEGF among CVD patients.** Lower vitamin D with lower VEGF in present study among CVD patients clearly indicate a possible lack of angiogenesis and vasculogenesis in vascular smooth muscle as well as cardiac tissues. Hence cardiovascular protection against any type of cardiac injuries may remain unprotected due to lack of vitamin D. This correlation suggests vitamin D is essential for cardiovascular health. Endothelial cells express vitamin D receptor (VDR)and its activation affects the development of immature cells, partly by modulating response elements in the VEGF promoter.(Merke *et al*, 1989)

Vitamin D metabolites reduced endothelium-dependent vascular smooth muscle contractions and vascular tone in hypertensive models, an effect mediated by affecting calcium influx across endothelial cells.(Wong et al,2008)

5.8.10.Results and Discussion

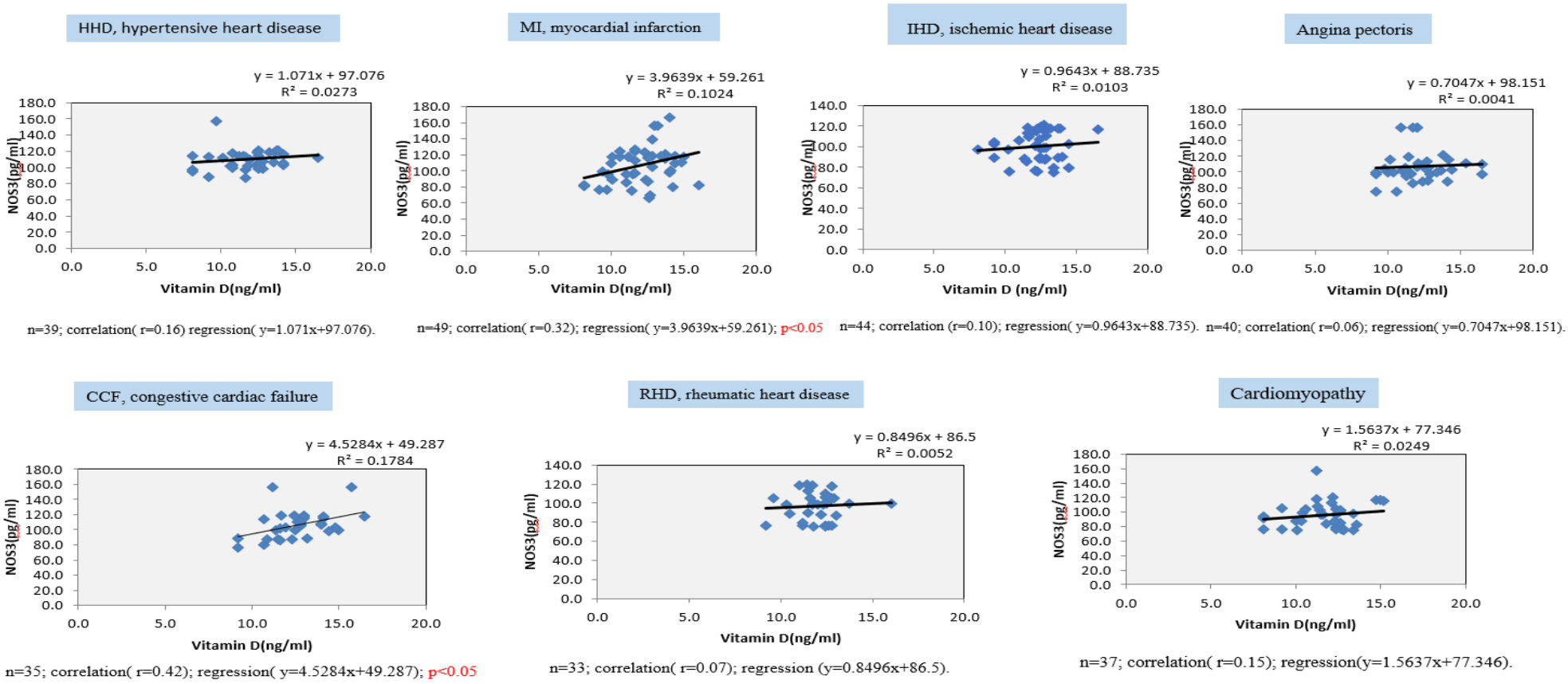


Fig.5.8.10: Correlation between vitamin D & NOS3 of cardiac patients

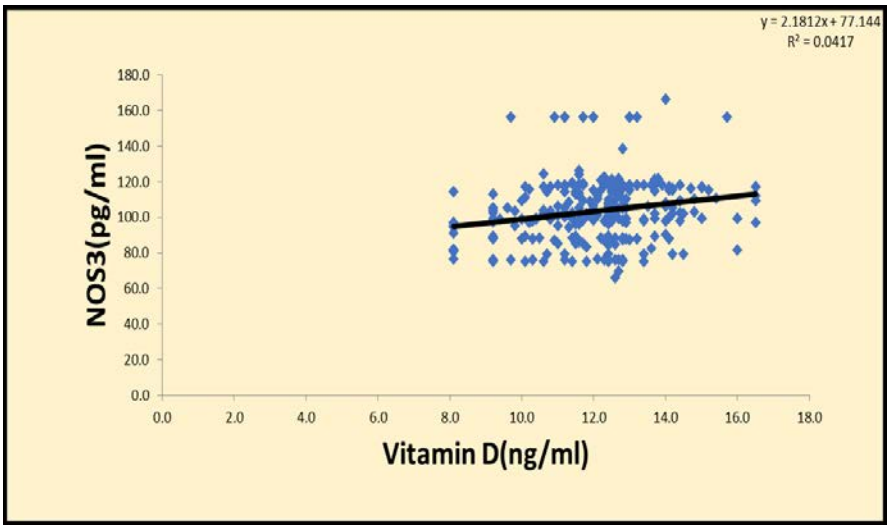


Fig.5.8.10a: Correlation between vitamin D & NOS3 of all cardiac patients. n=278; correlation (r=0.20); regression(y= 2.1812x+77.144) $p<0.05$

Although there were no significant correlation between Vitamin D and NOS3 in all the groups of CVD patients except group 2 and 5 but in Fig 5.8.10a, a significant relationship has been shown in combine smple of CVD patients. Results clearly indicate a relationship between vitamin D and NOS3. Lower the vitamin D correlates with lower NOS3 in present study among CVD patients. **This is once again an unique and rare observation between vitamin D and NOS3 among CVD patients.** This relationship may be due to the lower expression of VEGF protein induced lack of cell signalling pathways for NOS3 expression. Hence a link between vitamin D, VEGF, NOS3 and NO are very important for regulation of cardiovascular health. A disruption at any level of this chain may lead to CVD in any form. The present study support the existence of this possible link among these four components.

5.8.11.Results and Discussion

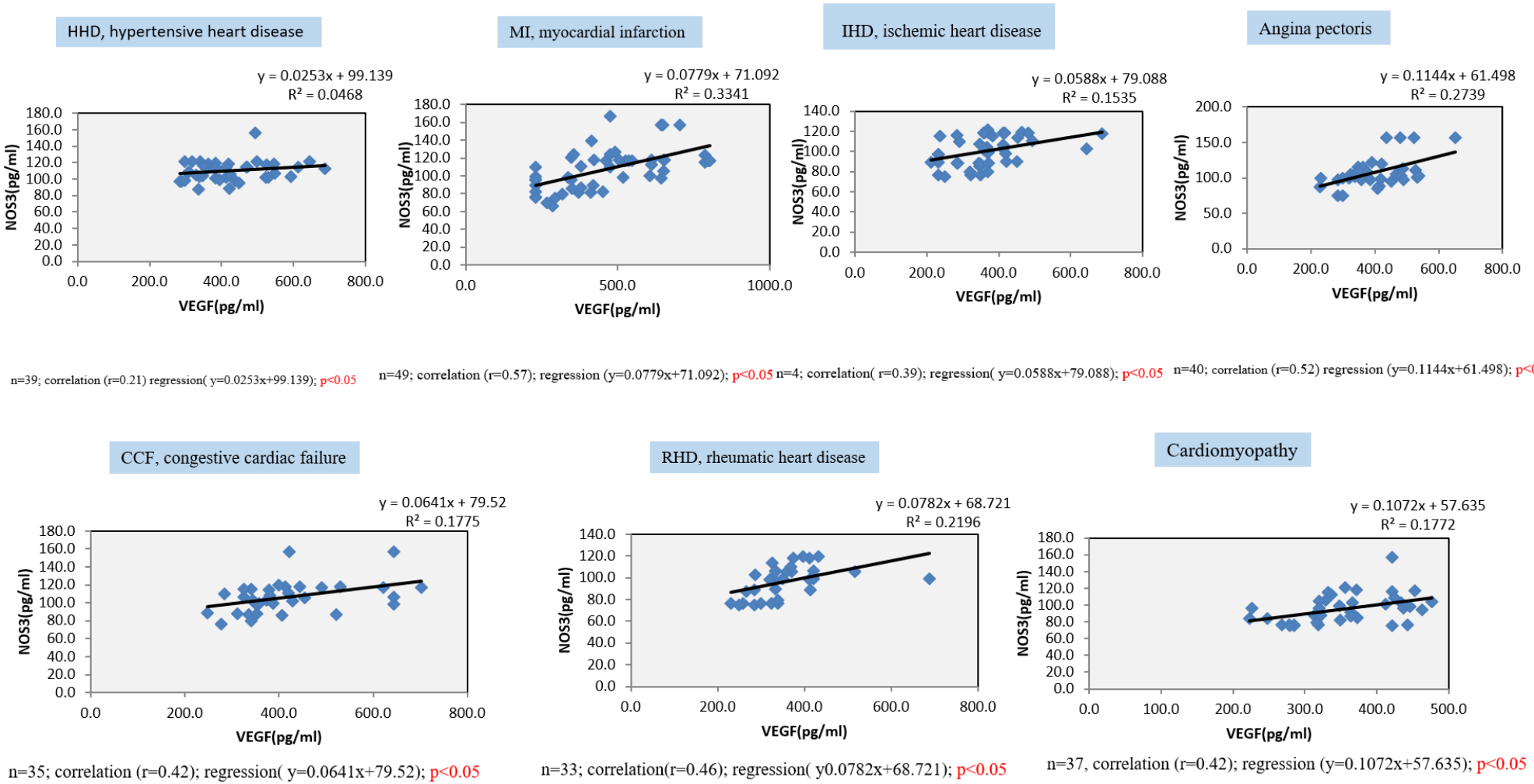


Fig.5.8.11a: Correlation between VEGF & NOS3 of all cardiac patients

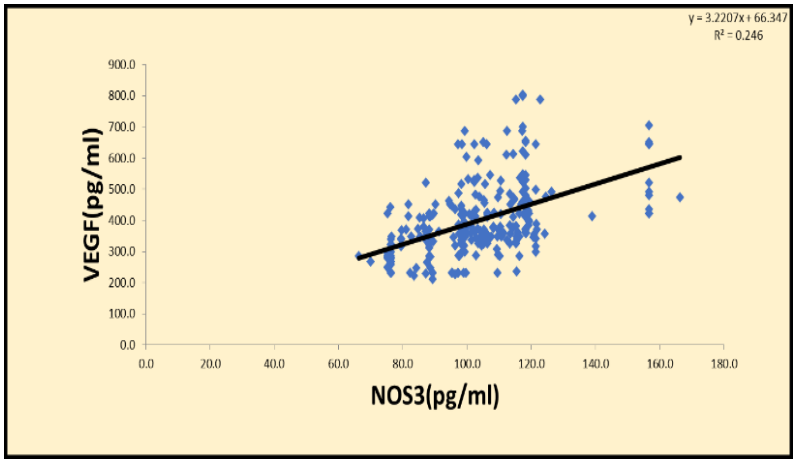


Fig.5.8.11a: Correlation between VEGF & NOS3 of all cardiac patients.n=278; correlation (r=0.49); regression(y= 3.2207x+66.347) $p<0.05$

There is a significant correlation between VEGF and NOS3 in all CVD patients in present study (Table 5.8.11).

Further our statement proves the link between VEGF and NOS3 is unique and significant.

VEGF, which is an angiogenic protein is related to regulation of NO. Together VEGF and NOS3 play an impartant role in pathogenesis of cardiovascular complications. Together VEGF and NOS3 causes the alteration of the signal transection and result in cardiovascular consequences and clinincal impact.(Kim *et al*, 2008)

Further research is also required to understand the molecular mechanism of VEGF induced signalling pathway for cardiovascular injuries.

Table. 5.8.12 .Overall correlation between various parameters among all cardiac patients.

	Serum calcium	**Vitamin D**	**MAP**	**NO**	**NOS3**	**VEGF**
Serum calcium		r=0.143 p=0.017*	r=-0.345 p=<0.001*	r=0.211 p=0.747	r=0.086 p=0.153	r=0.069 p=0.255

In Table. 5.8.12 the overall correlation between various parameters among all CVD patients is shown which has been discussed earlier.

Vitamin D			r=-0.351 p=<0.001*	r=0.019 p=0.754	r=0.179 p=0.003*	r=0.339 p=<0.001*
MAP				r=-0.253 p=0.723	r=-0.142 p=0.018*	r=-0.136 p=0.024*
NO					r=0.184 p=0.002*	r=0.043 p=0.473
NOS3						r=0.503 p=<0.001*
VEGF						

Chapter 6

SUMMARY AND CONCLUSION

SUMMARY:

Cardiovascular disease (CVD) is considered as the leading cause of disability and death worldwide. CVDs represent significant health risk factors and they are major contributors to global death and chronic illness/disability. Epidemiological data in humans have shown that vitamin D insufficiency is associated with hypertension, left ventricular hypertrophy, increased arterial stiffness, and endothelial dysfunction.Vitamin D in relation to the calcium homeostasis is directly linked with physiological microenvironment to regulate cardiovascular functions. This vitamin D regulated cardiovascular functions perhaps may be influenced by VEGF and NOS3 proteins. Very few information is available on vitamin D in relationship with VEGF in the perspective of CVD. In view of a complex scenario to understand cardiac disorders in relation to vitamin D through molecular regulatory system the

current study has been undertaken in the patients of cardiac diseases in Vijayapur district of Karnataka.

We hypothesized that lower vitamin D concentration differentially induces cardiovascular dysfunction through molecular alterations of VEGF and NOS3 signaling pathways in different types of CVD patients.

Cross sectional study was conducted on CVD patients age ranging from 40 to 80 years who were diagnosed for first time and admitted in ICCU of BLDE(DU) Shri.B.M.Patil Medical College , Hospital and Research Centre Vijayapur ,Karnataka(India).

The following parameters were tested: Anthropometric parameters: height (cms), weight (kgs), BMI (kg/m^2) and BSA (m^2); Physiological parameters: pulse rate in (beats/min), systolic blood pressure (mmHg), diastolic blood pressure (mmHg), pulse pressure (mmHg) and mean arterial pressure (mmHg); Hematological parameters: RBC, WBC, HB%, PCV, Platelet count and blood indices like MCV, MCH, MCHC; echocardiography; electrocardiography; biochemical parameters: triglyceride, cholesterol, HDL, LDL, VLDL, serum creatinine, CPK-MB, serum sodium, potassium, calcium and vitamin D; Oxidative and nitrosative stress measure: serum malondialdehyde (MDA), nitric oxide (NOx) concentration; antioxidant capacity: serum superoxide dismutase (SOD) activity, and vascular endothelial growth factor (VEGF) and nitric oxide synthase (NOS3).

The increase in white blood cells (WBCs) level in present study indicate the inflammatory process in the physiological system. Further increase in the ESR is indicative of myocardial damage with leakage of proteins. As dyslipidemia is associated with various types of stress factors and MI, HHD and IHD are definitely generate more stress among all the types of CVD

Our results show significantly lower levels of vitamin D in all groups of CVD patients. Results also found higher levels of MDA , significantly lower levels SOD and lower levels NO. We found decreased VEGF levels in present study in groups 3(IHD),4(Angina),6(RHD) and 7(Cardiomyopathy) and decreased levels of NOS3 in all groups of CVD patients.

The risk of cardiovascular complications increases continually along with the high blood pressure. As NO is known vasodilator hence decrease in NO indicate greater vascular resistance and increase in BP. Reduced NO production causes systemic vasoconstriction and elevated blood pressure. The inverse relationship between vitamin D and BP clearly enlighten further support that vitamin D is an important hormone or vitamin to regulate cardiac haemodynamic.

Higher MDA with lower SOD in all CVD patients in present study clearly indicate an oxidative stress. Low SOD level also indicates a serious oxidative stress in the cell membrane of myocyte. Nitric oxide (NO) is an important cellular signaling molecule. Lower NO levels in all groups of CVD patients indicate a possible formation of peroxynitrate by binding available NO with generated superoxide or hydrogen peroxide which itself may be considered as cardiotoxic. Decreased VEGF level in present study in IHD, angina, RHD and cardiomyopathy indicate a possible angiogenesis in myocardial or vascular tissues among CVD patients. Results further indicate that VEGF is an important molecular marker in case of CVD. Decreased plasma NOS3 level is an important indicator of endothelial dysfunction. Hence low serum VEGF and low serum NOS3 among different types of CVD patients clearly indicate an alteration of cardiovascular pathophysiology.

Correlation between serum vitamin D and VEGF is one of the unique and rare observation. Lower vitamin D with lower VEGF in present study among CVD patients clearly indicate a possible lack of angiogenesis and vasculogenesis in vascular smooth muscle as well as

cardiac tissues. Hence cardiovascular protection against any type of cardiac assaults remain unprotected due to lack of vitamin D. Positive relationship between vitamin D and NOS3 among CVD patients may be due to the lower expression of VEGF protein induced lack of cell signaling pathways for NOS3 expression.

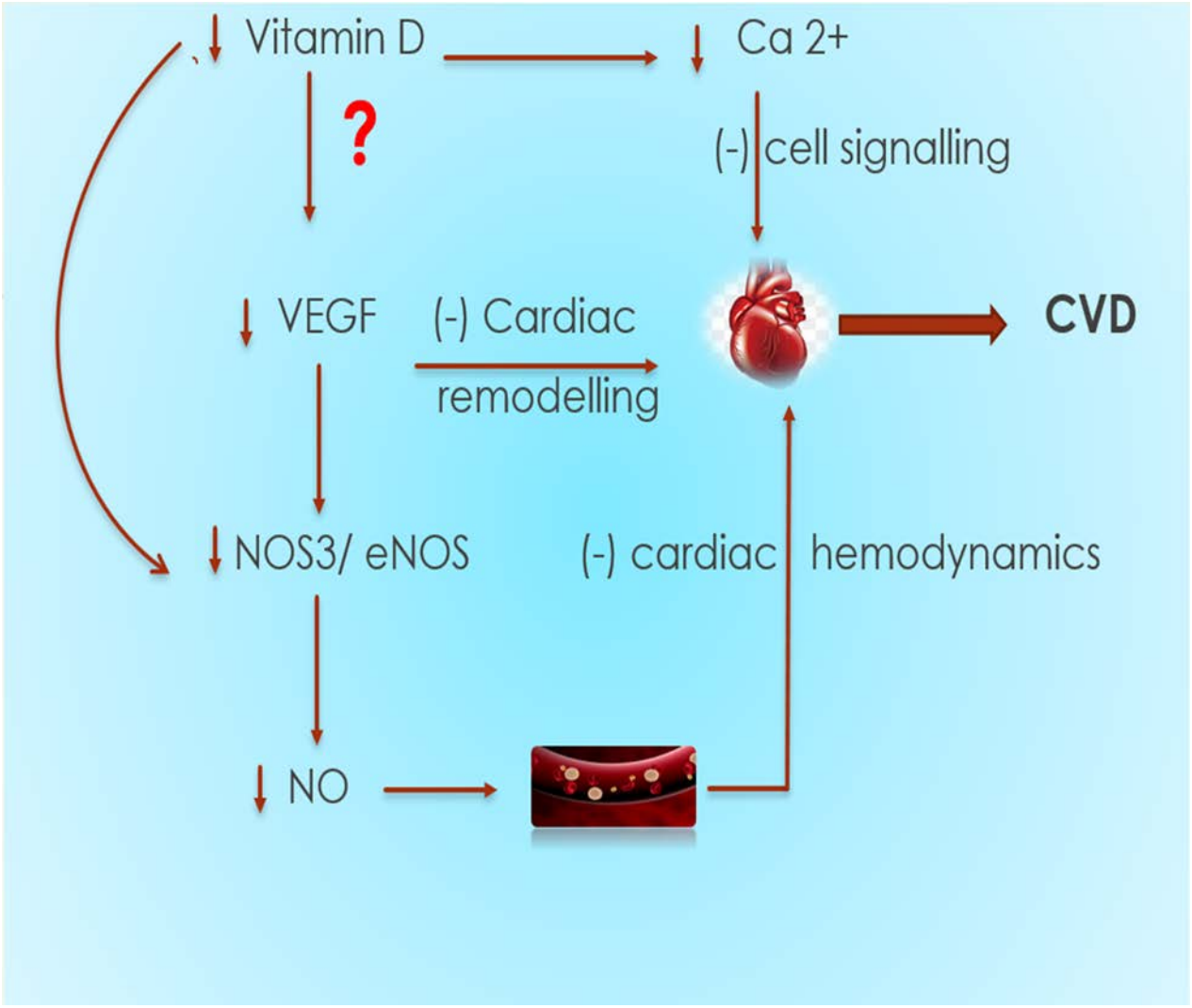
Hence a link between vitamin D, VEGF, NOS3 and NO are very important for regulation of cardiovascular health. A disruption at any level of this chain will lead to CVD in any form. The present study supports a unique link between these four components.

CONCLUSION

Results clearly shows that vitamin D is an important factor for the regulation of cardiovascular health. Deficiency of vitamin D leads to most types of CVD in present study. Possibly vitamin D influences angiogenic factor like VEGF and alter cardiovascular remodeling. Further it may be concluded that the role of vitamin D induced cardiovascular remodeling depends on nature and types of CVD.

Limitations and future perspectives of the study:

Graphical Abstract


Vitamin D
Ca 2+
?
(-) cell signalling
VEGF
(-) Cardiac remodelling
CVD
NOS3/ eNOS
(-) cardiac hemodynamics
NO

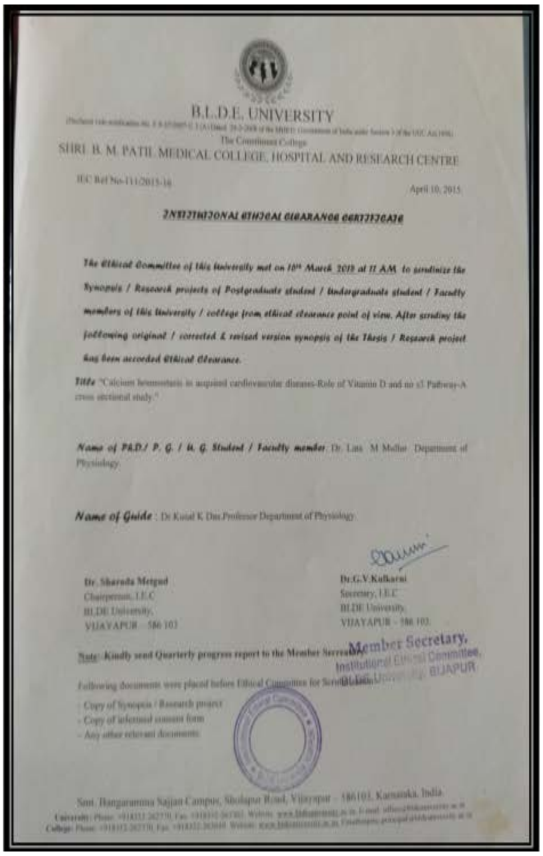
ANNEXURES

ANNEXURE –I

PLIGARISM CERTIFICATE

ANNEXURE – II

INSTITUTIONAL ETHICAL CLEARANCE CERTIFICATE



B.L.D.E. UNIVERSITY

The Constituent College

SHRI. B. M. PATIL MEDICAL COLLEGE, HOSPITAL AND RESEARCH CENTRE

IEC Ref No-111/2015-16

April 10, 2015

INSTITUTIONAL ETHICAL CLEARANCE CERTIFICATE

The Ethical Committee of this University met on 10th March 2015 at 11 AM to scrutinize the Synopsis / Research projects of Postgraduate student / Undergraduate student / Faculty members of this University / college from ethical clearance point of view. After scrutiny the following original / corrected & revised version synopsis of the Thesis / Research project has been accorded Ethical Clearance.

Title "Calcium homeostasis in acquired cardiovascular diseases-Role of Vitamin D and [illegible] Pathway-A cross sectional study."

Name of PhD/ P. G. / U. G. Student / Faculty member: Dr. Lata M Mullur Department of Physiology

Name of Guide : Dr Kusal K Das Professor Department of Physiology

Dr. Sharada Metgud
Chairperson, I.E.C
BLDE University,
VIJAYAPUR – 586 103

Dr.G.V.Kulkarni
Secretary, I.E.C
BLDE University,
VIJAYAPUR – 586 103.

Member Secretary,
Institutional Ethical Committee,
BLDE University, BIJAPUR

Note: Kindly send Quarterly progress report to the Member Secretary.

Following documents were placed before Ethical Committee for Scrutinization.

- Copy of Synopsis / Research project
- Copy of informed consent form
- Any other relevant documents

Smt. Bangaramma Sajjan Campus, Sholapur Road, Vijayapur – 586101, Karnataka, India.

BLDE

(DEEMED TO BE UNIVERSITY)

The Constituent College

SHRI. B. M. PATIL MEDICAL COLLEGE, HOSPITAL & RESEARCH CENTRE, VIJAYAPURA

IEC Ref No-/2017-18 / 251

27/03/2018

INSTITUTIONAL ETHICAL CLEARANCE CERTIFICATE

The Ethical Committee of this University met on 27th March 2018 at 11.AM to scrutinize the Synopsis / Research projects of Postgraduate student / Undergraduate student / Faculty members of this University / college from ethical clearance point of view. After scrutiny the following original / corrected & revised version synopsis of the Thesis / Research project has been accorded Ethical Clearance

Title Vitamin D influences calcium dependent cardiovascular functions with reference to NOS3 and VEGF

Name of (PhD./P.G./U.G.Student/Facultymember).Dr Lata.M.Mullur.Asso.Prof,Dept of Physiology

Name of Guide Dr Kusal K Das Professor Dept of Physiology

Dr. Sharada Metgud
Chairperson, I.E.C
BLDE University,
VIJAYAPUR – 586 103

Dr.G.V.Kulkarni
Secretary, I.E.C
BLDE University,
VIJAYAPUR – 586103.

MEMBER SECRETARY
Institutional Ethics Committee
BLDE (Deemed to be University)
Vijayapura-586103, Karnataka

Note:-Kindly send Quarterly progress report to the Member Secretary.

Following documents were placed before Ethical Committee for Scrutinization.

- Copy of Synopsis / Research project
- Copy of informed consent form
- Any other relevant documents.

Smt. Bangaramma Sajjan Campus, B. M. Patil Road (Sholapur Road), Vijayapura - 586103, Karnataka, India.

ANNEXURE –III

SAMPLE WRITTEN INFORMED CONSENT FORM

B. L. D. E.(Deemed to be University) SHRI B.M. PATIL MEDICAL COLLEGE, HOSPITAL AND RESEARCH CENTRE, VIJAYPURA.

RESEARCH INFORMED CONSENT FORM

TITLE OF THE PROJECT : Vitamin D influences calcium dependent cardiovascular functions with reference to NOS3 and VEGF.

PRINCIPAL INVESTIGATOR: Dr.Lata.M.Mullur
Ph D Scholar

GUIDE'S NAME : Dr Kusal K Das
Professor
Department of Physiology

CO-GUIDE: Dr.M.S.Biradar
Professor
Dept. of Medicine

1: PURPOSE OF RESEARCH: I have been informed that this study will assess Vitamin D influences calcium dependent cardiovascular functions with reference to NOS3 and VEGF. This study will be useful academically as well as clinically.

2: PROCEDURE: I understand that, the procedure of the study will involve recording of various physiological, physical, vascular, biochemical and molecular parameters. The procedure will not interfere with any of my physiological parameters.

3: RISK AND DISCOMFORTS: I understand determination of Vitamin D influences calcium dependent cardiovascular functions with reference to NOS3 and VEGF will not cause any discomfort to me and do not involve any risk to my health.

4: BENEFITS: I understand that my participation in the study may not have a direct benefit to me but this may have a potential beneficial effect in the future.

5: CONFIDENTIALITY: I understand that medical information produced by this study will become part of institutional records and will be subject to the confidentiality and privacy regulation of the said institute. Information of a sensitive personal nature will not be a part of medical record, but will be stored in investigators research file and identified only by a code number. The code key connecting name two numbers will be kept in a separate secured location.

If the data are used for publication in the medical literature and for teaching purposes no names will be used and other identities such as photographs, audio and video tapes will be

used only with my special written permission. I understand I may see the photographs and the video tapes and have the audio tapes before giving this permission.

6: REQUEST FOR MORE INFORMATION:

I understand that I may ask more questions about the study at any time. Concerned researcher is available to answer my questions or concerns. I understand that I will be informed of any significant new findings discovered during the course of this study which might influence my continued participation. If during the study or later, I wish to discuss my participation in all concerns regarding this study with a person not directly involved, I am aware that the social worker of the hospital is available to talk with me. A copy of this consent form will be given to me to keep for careful re-reading.

7: REFUSAL OR WITHDRAWAL OF PARTICIPATION: I understand that my participation is voluntary and may refuse to participate or may withdraw my consent and discontinue participation in the study at any time without prejudice to my present or future care at this hospital. I also understand that researcher may terminate my participation in this study at any time after she/he has explained the reasons for doing so and had helped arrange for my continued care by my physician or physical therapist if this is appropriate.

8: INJURY STATEMENT: I understand that in unlikely event of injury to me resulting directly from my participation in this study, if such injury were reported promptly, then medical treatment will be available to me, but no further compensation would be provided. I understand that by my agreement to participate in this study I am not waiving any of my legal rights.

I have explained to _____ (Patient/Relevant guardian)

the purpose of the research, procedures required and the possible risk and benefits to the best of my ability.

Investigator/ Guide Date:

I confirm that _____ (Name of the P.G. Guide /Chief researcher) has explained to me the purpose of research, the study procedure that I will undergo, and the possible risk and discomforts as well as benefits that I may experience. Alternative to my participation in the study have also been to give my consent from. Therefore I agree to give consent to participate as a subject and this research project.

Participant / Guardian Date:

Witness to signature Date:

Modified from Portney L.G, Watkins M.P., in Foundation of Clinical Research, Second Edition, New Jersey, Prentice Hall Health 2000. (APPENDIX – E)

ANNEXURE –IV

PRESENTATIONS AND AWARDS

LOCAL :

1. Alteration of vitamin d and serum calcium in cardiac patients with reference to ECG.

in SARS on 8th Dec 2017 in Shri. B.M.Patil Medical college,Hospital and Research centre,

Vijayapura.

2. "Alteration of serum vitamin D (1,25 DHCC) and calcium in patients of myocardial infarction and ischemic heart disease of tertiary hospital in vijayapura district(karnataka)" on 6/6/2018 at BLDE(Deemed to be University) Research Day, and won '**Research Day award'** (Poster)

3. Presented poster on 'Vitamin D on Possible Regulatory Action of VEGF in Cardiovascular Diseases' at SARS Gold medal competition on 28/3/19.

4. Presented poster on 'Impact of automobile emission exposure on respiratory functions of road side vendors and shopkeepers' at Dr. P.G.Halakatti college of Engineering and Technology, Vijayapura on 25/5/19.

NATIONAL CONFERENCE:

1. Serum lipid profile among different cardiac patients admitted in ICCU of the tertiary hospital at-"3rd Annual conference of association of Physiologists of India, ASSOPICON 2016, during 14th to 17th September, 2016 held at Department of Physiology, BLDE (Deemed to be University), Shri. B.M.Patil Medical college,Hospital and Research centre,

Vijayapura.

INTERNATIONAL CONFERENCE:

1. Alteration of vitamin d and serum calcium in cardiac patients with reference to ECG at-FIPSPHYSIOCON-2017, during 5th to 7th November, 2017 held at DRDO, New Delhi.

2. Presented e-poster on 'A comparative assessment of perception and learning by traditional, smart board and interactive teaching' at International conference APMEC-2019 at Singapore from 9th -13th Jan 2019 organised by National University of Singapore.

ANNEXURE – V

PUBLICATIONS

1. Mullur L.M .Dietary salt intake matters for development of hypertension. BJHS; 2017;Jan-June|2(1)

2.Das KK, Reddy RC, Bhagoji IB, Das S, Bagali, Mullur L, Khodnapur JP, and Biradr MS, primary concept of nickel toxicity-an overview. J Basic Clin Physiol Pharmacol. 2018. DOI: https://doi.org/10.1515/jbcpp-2017-0171

[Indexed in pubmed & scopus].

3.Kusal K Das, Rajesh Honnutagi,Lata Mullur,R.Chandramouli Reddy, Swastika Das, Dewan Syed Abdul Majid, M.S.Biradar. Heavy Metal and Low-Oxygen Microenvironment-Its impact on Liver Metabolism and Dietary Supplementation.2019, Dietary Interventions in Liver Disease.Chapter 26.

4.Mullur L.M, Das K K Biradar M.S.Alteration of serum vitamin d (1,25 DHCC) and calcium in patients of myocardial infarction and ischemic heart diseases.IJPHRD:2019,10(9) [SCOPUS]

5.Mullur L.M, KumavatV, Biradar M.S and Das K K. Serum lipid profile among the patients of various cardiac diseases admitted in intensive cardiac care unit (ICCU). IJPHRD:2019,10(9) (In press) [SCOPUS]

ANNEXURE VI

PERFORMA FOR COLLECTION OF SAMPLE

Name: Age: Sex:

Occupation: Religion/Caste: Address:

Past History:

Family History:

Personal History:

Appetite: Diet (Veg/Non Veg): Sleep:

Nourishment: Bowel habbits: Bladder Habits:

Sleep pattern:

Habits: Chewing pan/Gutkha/tobacco, H/O Alcohol intake, H/O Smoking

Social history:

Drug intake history:

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