RELATIONSHIP BETWEEN OXYGEN TENSION, OXIDATIVE STRESS AND VASCULAR AGEING AMONG THE GENERAL POPULATION OF VIJAYAPUR URBAN AREA: A CROSS SECTIONAL APPROACH.

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BLDE (DEEMED TO BE) University, Vijayapur, Karnataka, India

Under the Faculty of Medicine

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DOCTOR OF PHILOSOPHY (Medical)

IN

Physiology

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DECLARATION BY THE CANDIDATE

I hereby declare that this thesis entitled "***Relationship between oxygen tension, oxidative stress and vascular ageing among the general population of Vijayapur urban area: A cross sectional approach.***" is a bonafide and genuine research work carried out by me under the guidance of Professor Kusal K. Das and Dr Manjunatha R. Aithala, Department of Physiology, BLDE (Deemed to be University), Shri B.M.Patil Medical College, Hospital and Research Centre, Vijayapur, Karnataka, India. No part of this thesis has been formed the bases for the award of any degree or fellowship previously.

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Endorsement by the Principal/Head of Institution

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Abbreviations:

%	- Percent
·OH	- hydroxyl radical
aASI	- Arterial Stiffness Index at tibial artery
ACEIs	- Angiotensin converting enzyme inhibitors
Ach	- acetylcholine
AGE	- advanced glycation end products
Aix	- augmentation index
AIx@75	- Augmentation Index normalized for a heart rate of 75 beat per minute
ALP	- Alkaline Phosphatase Level
ALT	- Alanine Transaminase
ANOVA	- Analysis of Variance
ASI	- Arterial Stiffness Index
ATP	- Adenosine Tri Phosphate
B.wt	- Body weight
b-a PWV	- Brachial-Ankle pulse wave velocity
bASI	- Arterial Stiffness Index at Brachial artery
BMI	- body mass index
BP	- blood pressure
bpm	- beat per minute
BSA	- body surface area
CAD	- Coronary artery disease
c-f PWV	- carotid-femoral pulse wave velocity
cGMP	- Cyclic guanosine monophosphate
CHF	- Congestive heart failure
Cm	- centimeter
Conc	- Concentration
cSBP	- central systolic BP
CT	- Computed Tomography

CV	- Cardiovascular
CV%	- Co-efficient of variability
DBP	- Diastolic systolic pressure
DNA	- Deoxy ribonucleic acid
ECAM	- Endothelial leucocyte adhesion molecule
ECG	- Electrocardiogram
ED	- Endothelin
EDRF	- Endothelium derived relaxing factor
eNOS	- Endothelial nitric oxide synthase
EPC	- Endothelial progenitor cell
Epo	- Erythropoietin
ESRD	- End stage renal disease
FBS	- Fasting blood sugar
FFA	- Free Fatty Acids
FMD	- Flow mediated dilatation
g/dl	- Grams/Desi litre
g/L	- Grams/Litre
gm	- Gram
gms	- Grams
GSH	- Reduced glutathione
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
HPA axis	- Hypothalamo-pituitary-adrenal axis
HR	- Hour
HRV	- Heart rate variability
ICAM	- Intercellular adhesion moledule
ICAM1	- Intercellular Adhesion Molecule 1
ICMR	- Indian Council of Medical Research

IDLC	- Intermediate Density Lipoprotein fraction of cholesterol
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
ml	- Millilitrer
mmHg	- Millimetre of mercury
mmol/L	- Milimole per Liter
MMPs	- Matrix metalloproteinases
MRI	- Magnetic resonance imaging
mU/mL	- Milliunits per millilitre
NAD	- Nicotinamide Adenine Dinucleotide.
NCDs	- Non-Communicable Diseases
NO	- Nitric Oxide
NOx	- Total Nitric Oxide Concentration
O_2	- Oxygen
$O2^{-}$	- Superoxide radicals
OD	- Optical density
$ONOO^{-}$	- Peroxynitrite
PCV	- Packed Cell Volume
pg/mL	- Pictogram per millilitre
PP	- Pulse pressure
PR	- Pulse rate
PTT	- Pulse transit time
PWV	- Pulse wave velocity

RBC	- Red Blood Corpuscles
RIx	- Reflection index
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: We aimed to assess the influence of oxygen tension or oxygen microenvironment *in vivo* including oxidative stress on age and gender associated changes in vascular health among general population of Vijayapur urban area.

Methods: The present cross-sectional study conducted in Sri B.M. Patil Medical College (October 2016 to April 2017) on 204 healthy subjects male (n= 102) and female (n=102) subjects (20-95 years) were randomly selected among general population of Vijayapur city, Karnataka, India. Subjects were divided into six group: Group I (20-29 years), II (30-39 years), III (40-49 years), IV (50-59 years), V (60-69 years) and VI (>70 years). The following parameters were tested: Anthropometric parameters: height (cms), weight (kg), 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; biochemical parameters: fasting blood glucose (FBS), serum triglyceride, serum cholesterol, serum HDL, serum LDL, serum VLDL; Arterial stiffness parameters: Brachial-ankle pulse wave velocity (baPWV), carotid-femoral pulse wave velocity (c-f PWV), augmentation index (AIx@75), arterial stiffness index at brachial (bASI) and tibial arteries (aASI); Oxidative and nitrosative stress measure: serum malondialdehyde (MDA), serum nitric oxide (sNOx) concentration; and Antioxidant capacity: serum superoxide dismutase (SOD) activity, erythrocyte reduced glutathione (GSH), serum ascorbic acid or vitamin C; Oxygen sensing molecular markers: serum erythropoietin (Epo) and vascular endothelial growth factor (VEGF). Statistical analysis was done by using one-way ANOVA followed by post-hoc t-test and unpaired t-test by using SPSS software.

Results: Group I to group VI showed significant ($p<0.001$) steady increase of PWV, ASI, AIx and MAP in both male and females with concomitant significant decrease of serum NOx in both male and female subjects. Further a significant ($p=0.000$) negative correlation between b-aPWV and c-f PWV with NOx in both male and female subjects were also observed. Observed significant ($p<0.001$) increased MDA and decreased SOD, GSH and Vit C levels in both males and females in association with age. Also observed significant ($p<0.001$) increased serum VEGF and decreased Epo level with ageing among both male and female participants.

Conclusion: PWV may be considered as more reliable marker than MAP to evaluate age associated arterial stiffness. Epo might be playing a crucial homeostatic role in ageing. Females have an augmented protection against age related alteration of vascular pathophysiology due to greater VEGF concentration as compared to their male counterparts. Endothelial function is not the final protection for arterial function as role of VEGF and its expression in arterial smooth muscles is very important for vascular stability. Understanding of these mechanisms may support greater pharmaco-physiological understanding of arterial stiffness which may possibly improve cardiovascular health of an individual irrespective to their sex.

CHAPTER I

INTRODUCTION

Introduction:

Ageing is a privilege and a societal achievement. It is also a challenge which will impact on all aspects of 21st century society (Park K., 2005). Globally, 10 % (600 million) of the world's population is elderly and it is expected to increase to 21 % (1.97 billion) in 2051 (Department of economic and social affairs, New York, United Nations. World Population Ageing; 1950-2050). Demographically, Asia is the most important continent in the world, where the population is growing both larger and older. India's older population is estimated to grow from close to 8% (76 million) to about 9% (113 million) in 2016, and almost 20% in 2050 (Kowal P et al., 2012).

Old age should be regarded as a normal inevitable biological phenomenon (Park K., 2005). Ageing can be defined as the "time related deterioration of the physiological functions necessary for survival and fertility" (Khurana Indu., 2006).

Arterial ageing is a normal physiological process that develops gradually with age. The researchers worldwide are just beginning to understand the complexity of this phenomenon (Mirea O et al., 2012).

There are medical conditions due to age-related physiological changes that occur exclusively among the elderly which affect the quality of life. The diseases associated with older age groups are often non-communicable diseases (NCDs) that include CV diseases (hypertension, heart attacks and stroke), cancers, chronic respiratory diseases (such as chronic obstructed pulmonary disease and asthma) and diabetes (Boutayeb A & Boutayeb S.,2005; Hunter DJ & Reddy KS., 2013).

Arterial wall stiffens with age (Izzo JL Jr et al., 2001). So, age is one of the most powerful determinants of cardiovascular risk and is associated with a number of deleterious changes in the cardiovascular system (Cassel CK et al., 1997). Cardiovascular diseases are the leading causes of morbidity, mortality, and disability

in most of the countries (Mirea O et al., 2012). Since ageing has a profound influence on the risk of cardiovascular diseases even death hence it is important to understand the oxygen sensing mechanism in the process of ageing (Mirea O et al., 2012).

The age-associated changes related to the endothelium are also a subject of great interest. It has been demonstrated that endothelial function and its regenerative capacity decline with age (Mirea O et al., 2012).

Studies are needed to find out the various factors and mechanisms which regulate progression of ageing as it may be genetically predispose or express in their own way by changing internal physiological environment including vascular system, genetics and epigenetics.

This study further explores the relationship between age & oxygen sensing mechanism with special reference to vascular integrity.

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CHAPTER II

REVIEW OF LITERATURE

1. AGEING

Ageing is an inevitable physiological process (Kirkwood TB and Holliday R., 1979). According to Seneca "old age is an incurable disease". Whereas Sir James Starling Ross stated "you do not heal old age, you protect it: you promote it: you extend it" (Park K., 2005).

According to literature there were 0.6 billion people aged 60 years in 2000, and above and it will be 1.2 & 2 billion by 2025 & 2050 respectively (Cohen JE., 2003).

Even though ageing is defined as functional declination but chronologic & biologic ageing begins at conception and after sexual maturation (Cassel CK., 1997).

Once the ageing begins altered homeostatic balance and increased incidence of pathology which will lead to decreased ability to respond to stress and death remains the ultimate consequence. Achievement of desirable phenotypes of human longevity and healthy ageing remain among the principal challenges of biology and medicine (Mirea O et al., 2012).

1.1. THEORIES OF AGEING:

Ageing can be explained by different theories.

1.1.1. Evolutionary theories

The evolutionary theory was first formulated in the year 1940. According to this theory "accumulation of late acting mutations in the population might lead to pathology and senescence". Life span is species specific and it functions as survivability and reproductive strategy in a competitive environment (Medvedev ZA., 1961; Johnson TE and Hutchison EW., 1990).

1.1.2. Molecular theories

According to this theory senescence results from changes in gene expression (Harrison BJ and Holliday R., 1967). Research studies on worms, flies, and mice showed that life span is regulated by an insulin-like signalling pathway (Levy MZ et al., 1992). A study proved a locus on chromosome 4 that may contain gene(s) that promote exceptional longevity (Friedman DB and Johnson TE., 1988).

1.1.3. Cellular theories

Cell senescence/telomere theory: The age process occurs after a characteristic number of cell divisions which leads to terminally arrested cells with altered physiology (Bick MD and Strehler BL., 1972).

1.1.4. Free radical theory

This theory states that ageing is mainly due to accumulation of free radicals in cells over time (Hekimi S., 2011). Each living species existing in this environment which contains reactive oxygen species (ROS) which are the leaking intermediates during mitochondrial respiration (Wheeler KT and Lett JT., 1974).

1.1.5. System-based theories of ageing:

1.1.5.1. ***Neuroendocrine theory.*** According to this theory hypothalamo-pituitary-adrenal (HPA) axis is the master regulator, acts as the "pacemaker" which signals the of each stage of life (Reitz MS and Sanadi DR., 1972; Efstratiadis A et al., 1977). A long term exposure to physical, biological, or emotional stress may weaken the ability to repair and lead to death (Brown WT., 1985; Holliday R., 1986).

1.1.5.2. ***Immune theory:*** According to this theory ageing is characterized by a decreased sensitivity against infection, protection against cancer, and an enhanced failure to recognize autocells (Hnawalt PC., 1987; Cutler RG., 1973).

1.2. CHANGES IN VARIOUS SYSTEMS WITH AGEING (Bijlani RL., 2004)

1.2.1. Blood

The physiological reserve for erythropoietic & leucopoietic capacity is reduced in elderly as active haemopoieticbone marrow slowly replaced with fat.

1.2.2. Immune mechanisms

There is a definite decline in immune competence associated with ageing. Both cell mediated & humoral immunity are affected. The elderly are more susceptible to infections, to autoimmune diseases & there is higher incidence of malignancies in old age.

1.2.3. Respiratory system

Literature reported that four percent reduction in the alveolar surface area for every 10 years after the age of 30**.** The compliance of total respiratory system is reduced by age of 60 years due to rigidity of the chest wall. There is a reduction in pulmonary diffusing capacity and the respiratory response to hypoxia & hypercapnia is sluggish in the elderly.

1.2.4. Cardiovascular system

The elasticity of the aorta & other large arteries decreases with increasing age. The systolic & pulse pressure are increased, where as slight alteration in diastolic pressure. There is myocardial atrophy accompanied by deposition of a brown pigment, lipofuscin. There is prolongation of the myocardial contraction & relaxation time. There is decrease in ventricular compliance, structural alterations in the valves. Pacemaker cells in sinoatrial node are reduced, which is related to reduced heart rate response to both sympathetic & parasympathetic stimuli.

1.2.5. The alimentary canal

With advancing age there is decline in the digestive & absorptive function of the GIT due to diminution of masticatory efficiency, dysphagia, and reduced absorptive surface and reduced enzyme secretion.

1.2.6. Excretory system

Microscopically, there is a reduction in the number & size of nephrons. There is a 10% reduction in renal plasma flow per decade after the age of 30. There is a progressive age related decrease in glomerular filtration rate, the secretory & reabsorptive functions of renal tubules.

1.2.7. Endocrines

Ageing of the endocrines has often been considered to be underlying mechanism of ageing. There may be a decrease in the blood concentration of the hormone itself or the binding protein involved in the transport of the hormone, decreased responsiveness of the target cells, and alteration in the number or sensitivity of hormone receptors or diminished response to physiological stimuli for secretion of the hormone. The response of the hypothalamo hypophyseal axis to stress is diminished with age.

In females, there is a decrease in serum estrogen & progesterone levels after menopause & an increase in FSH & LH levels. In males, there is a decrease in testosterone output & a decrease in gonadotropin levels.

1.2.8. Nervous system

During aging process there will be accumulation of lipofuscin in the cells & death of synapses & dendrites which leads to brain atrophy. The function of various neurotransmitter systems is impaired.

1.2.9. Special senses

Presbyopia is a constant feature after the age of 40. The intraocular pressure also rises with age.

The ear shows diminished sensitivity after the age of 30. The sensation of smell & taste also decline with age.

1.2.10. Effect of ageing on autonomic nervous system(Allen S C., 1998)

There is reduced response to sympathetic nerve stimulation or infusion of catecholamine and there is an age related decrease in the β1 & β2 adrenoreceptor sensitivity. The observation that many old people have higher base line levels of plasma noradrenaline than the young people may be in part a compensatory mechanism. Morphological changes include reduction in the autonomic neuron cell number, higher density of lipofuscin in neuronal cytoplasm & degenerative changes in Vasa Nervorum. In both the sympathetic & parasympathetic systems there is evidence of reduced nerve conduction velocity. In the parasympathetic nervous system, cardiac muscarinic receptor responses decline, probably due to reduction in the receptor sensitivity to acetylcholine (ACh). More recently evidence has come to light which indicates that flux of Ca2+ ions across neuronal membranes is progressively impaired with age. The net effect of these changes is reduced efficiency in the sympathetic and the parasympathetic systems which reduce the ability to put into effect the responses required to maintain physiological homeostasis.

2. VASCULAR AGEING

There is a progressive declination of all bodily functions including vascular function with ageing. Arterial ageing is a normal physiological process that develops gradually with age. Some studies have shown that endothelial function and its regenerative capacity decline with age (Mirea O et al., 2012).

2.1.Age-associated structural change in arterial system:

According to Sir William Osler (1898), "Longevity is a vascular question, which has been well expressed in the axiom that man is only as old as his arteries. To a majority of men death comes primarily or secondarily through this portal. The onset of what may be called physiological arteriosclerosis depends, in the first place, upon the quality of arterial tissue which the individual has inherited, and secondly upon the amount of wear and tear to which he has subjected it."

Cardiovascular ageing process is related to various factors that act on muscle cells and intimal layer of the artery leads to increased ventricular and vascular stiffness (Cefalu CA., 2011) (Figure 1).

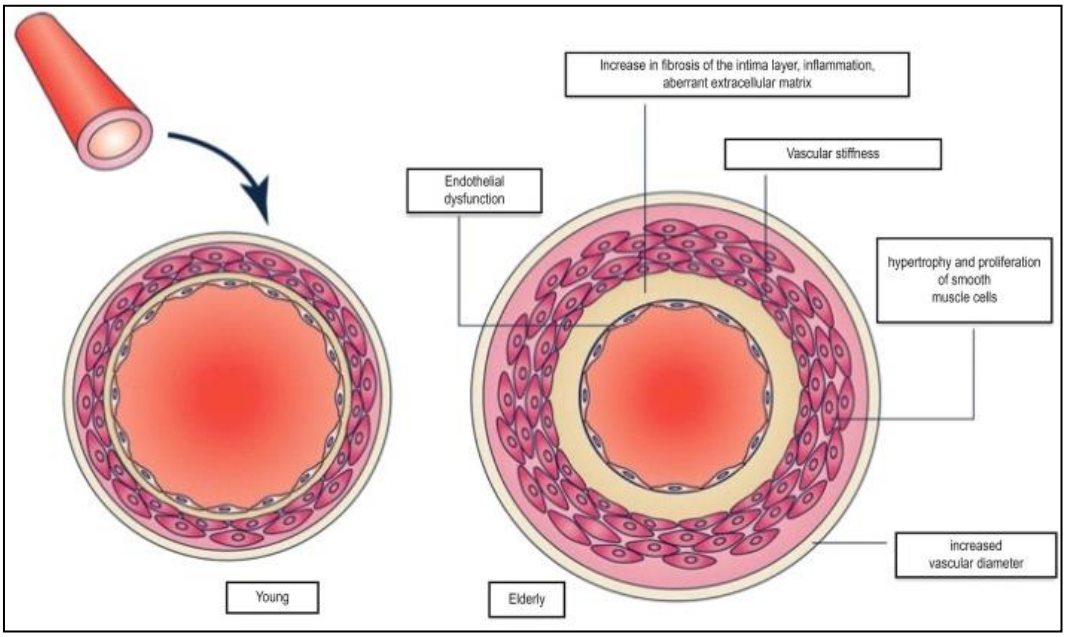

Figure 1: Pathophysiology of vascular ageing (Reference: Costantino S et al., 2016)

Major alteration in the basic structure and function of arterial bed due to calcification, elasticity loss and wall diameter increase in the media layer. Even though it is more evident in large vessels, it might see in peripheral vascular bed (Stratton JR et al., 2003; Costantino S et al., 2016). These alterations with age lead to decreased arterial compliance and its ability to withstand stress (Benetos A., 2011).

Blood vessel walls, especially large elastic arteries stiffen with age. Ten percent increase in the diameter of the aorta and proximal elastic arteries with each beat, while three percent increase in the diameter of the muscular arteries in response to every beat (O'Rourke MF & Hashimoto J., 2007). The heterogeneity in stiffness process with age among vessels can be explained on the basis of severity of exersion exerted by the different degree of stretch (O'Rourke MF & Hashimoto J., 2007; Lionakis N et al., 2012).

Age associated changes on vascular functioning leads to cardiovascular disease. Studies are needed to know how ageing has an impact on cardiovascular system. A study on Chinese and European subjects showed relationship between age-related stiffness, raised SBP, and cardiovascular health hazards (Wojciechowska et al., 2012)).

Decreased arterial compliance with age related stiffness is the prime contributor for raised SBP. Both structural and functional alterations in the arterial system are correlated to ageing. These alterations result in reduced expansion of aorta in return to ventricular contraction which shows raised SBP and failure to recoil results in reduction in DBP thus causing widening of PP (Lee HY & Oh BH., 2010). Hence, an increase in PP, a pulsatile component creates increased pulsatile tension on the arterial system even in the normotensive individuals (Millar JA et al.,2000).

The causes of arterial stiffness are summarized in the figure 2. The principal structural change with age occurs in the intima (hyperplasia) and the media (degeneration). The structural changes in the media of elastic arteries (medial degeneration) includes increase in collagen component and cross linking, increase in fragmented elastin and reduced in elastin component (Lim MA & Townsend RR et al. 2009). The age-related structural changes in the elastin (thinning and fragmentation) and collagen are not seen in the muscular arteries. These changes in media are associated with increased expression of matrix metalloproteinases (MMPs). Matrix metalloproteinases regulate collagen and elastin molecules of the vessel wall. The factors those determine the stiffness of arteries and its ability to expand and recoil are structural proteins and pressure exerted by blood on their wall (Cecelja M & Chowienczyk P., et al 2012). Chronic pulsatile tension on the structural proteins, collagen component and elastin content in the arterial wall results in disruption of muscular attachments and fracture of elastin fibers (Lee HY & Oh BH., 2010).

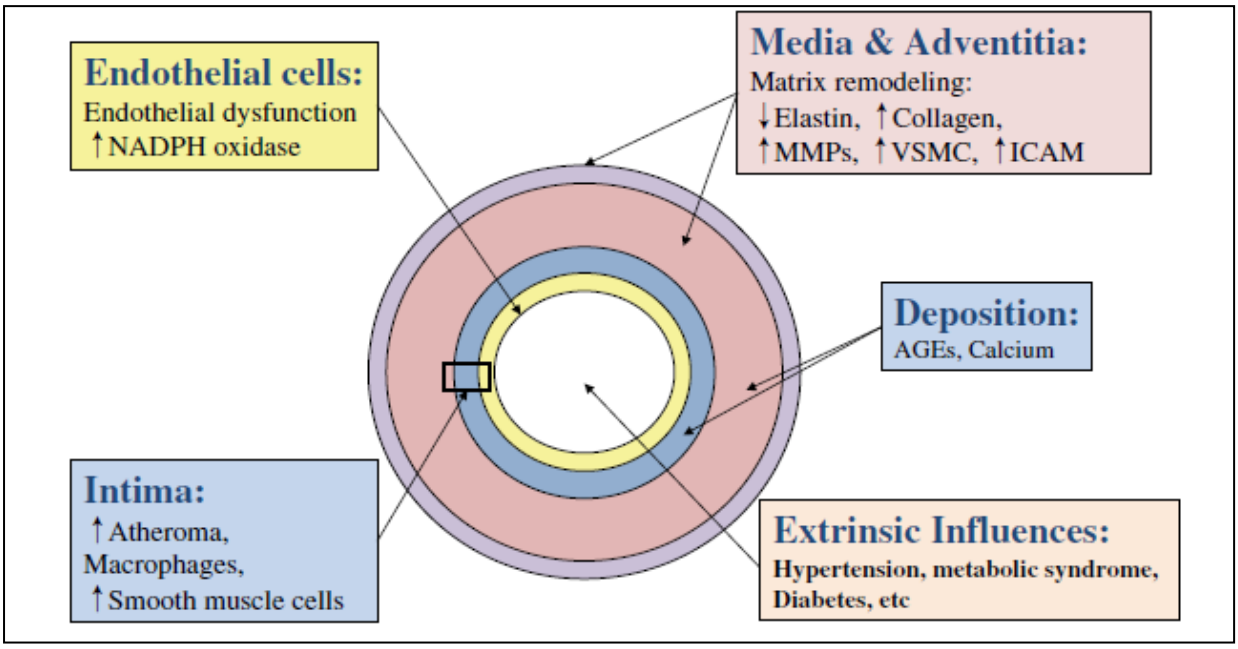

Figure 2: Causes of arterial stiffness (Reference: Lee HY & Oh BH., 2010)

Arterial stiffness also occurs from deposition of advanced glycation end products (AGE) on the proteins leading to alteration in their physical properties. Calcium deposition in the arterial wall might also contribute to reduction in the vascular compliance with age, particularly after the 5th decade (Atkinson J., 2008). The functional change in the arterial system that contributes to stiffness is age associated deterioration in endothelial function (Jin RC & Loscalzo J., 2010). 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). It has been reported that aside from extracellular matrix, increased vascular stiffness with ageing is also attributable to intrinsic changes in vascular smooth muscle cells by increasing the expression of adhesion molecule (Qiu H et al., 2010). Arterial stiffness is a self-supporting and powerful predictor of CV morbidity and mortality among hypertensives with no known cardiovascular diseases (Blacher J et al., 1999; Laurent S et al., 2001) also among well-functioning older individuals (Sutton-Tyrrell K et al., 2005).

Literatures have shown genetic factors which have impact on arterial stiffness. Polymorphic alteration in the fibrillin-1, angiotensin II type-1 receptor and endothelin receptor genes were found associated with vascular stiffness (Medley TL et al., 2002; Lajemi M et al., 2001).

Research evidences mention that the progression of vascular ageing is gender dependent. The gender-associated alteration is because of sex hormones. Novella et al. (2012) state that menopause and ageing both together contribute to arterial stiffness and the hormone estrogen balances vascular response at different stages of life while another study differs about the role of testosterone to the management of

vascular function in old aged males and females(Novella et al., 2012; Lopes et al., 2012).

2.2.Age-associated functional changes in arterial system: vascular endothelial dysfunction

Age also affects the regulation of vascular resistance by vascular endothelium. Age related alteration of functioning of vascular endothelium is the prime regulator of vascular ageing. 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 AR & Kuebler WM., 2006). Structural and functional integrity of endothelial cell are required for various vital CV functions and integrity (Galley HF & Webster NR., 2004). The vasodilator function of endothelium was demonstrated by removing endothelial cells and observing acetylcholine induced dilator response from among isolated arteries (Furchgott RF & Zawadzki JV., 1980). The key factor responsible for arterial relaxation was nitric oxide (NO) which was first discovered as endothelium derived relaxing factor (EDRF) (Vanhoutte PM 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 RC & Loscalzo J., 2010).

A review by El Assar et al. (2012) stated the different processes which alter endothelial function. Also presented the different cellular mechanisms related to vascular ageing and also the preventive measures of those mechanisms that alter normal vascular function (El Assar et al., 2012).

2.3. Functions of 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 WC., 2004; Pries AR & Kuebler WM., 2006). Endothelium by secreting various mediators is involved in both synthetic and metabolic functions .

1. **Vascular homoeostasis**: Vascular endothelium regulates various physiological functions of the blood vessel like vessel dilatation, Intravascular permeability and coagulation properties. Nitric oxide is key determinant of vascular health (Jin RC & Loscalzo J., 2010).

2. **Haemostasis and coagulation**: Vascular endothelium is vital for preventing vascular injury and maintaining blood fluid homeostasis. Vascular endothelium produces a number of substances which regulate haemostasis and coagulation: (a) Prostacyclin and nitric oxide are potent inhibitors of platelet and monocyte activation which leads to vasodilation. Vascular endothelial surface inhibits platelet aggregation. (b) Thrombomodulin acts as a thrombin binding site to activate protein C and heparin-like molecules serve as a antithrombin III cofactor. (c) Fibrinolytic system is activated by tissue plasminogen activator. (d) Platelet adhesion is mediated by von Willebrand factor (Wu KK & Thiagarajan P., 1996).

3. **Vascular tone & blood pressure**: Endothelial cells by secreting a number of vasodilators (NO, prostacyclin) and vasoconstrictors (endothelin, thromboxane A2) regulates vascular tone and BP.

4. **Angiogenesis**: Angiogenesis refers to the growth of new blood vessels (or damaged blood vessels) from pre-existing endothelium. Vascular endothelium produces vascular endothelial growth factor (VEGF) which mediates angiogenesis.

5. **Barrier function**: Tight junction between endothelial cells acts as a 'gate' or semi selective barrier between the blood and surrounding tissue, and controls the passage of substances, leucocytes, ions and water into and out of the blood stream. Increased vascular permeability leads to edema.

6. **Anti-inflammation**: Endothelium produces various inflammatory mediators and prevents inflammation.

2.4. Regulation of blood pressure by vascular endothelial system

Endothelial system is the prime short-term regulator of BP like baroreceptor reflex (Stauss HM & Persson PB., 2000). Basal vascular tone and BP are regulated by normal levels of NO produced by endothelial cells (Jin RC & Loscalzo J., 2010).

The mechanism of regulation of blood pressure by endothelial system is as follows (Figure 3)

i. Elevation in BP increases vascular shear stress.

ii. Vascular shear stress, a mechanical stimulus causes an increase in concentration of cytosolic Ca^{2+} in the endothelial cells.

iii. Ca^{2+} binds with calmodulin and forms a Ca^{2+-} calmodulin complex. This complex increases the activity of endothelial derived of nitric oxide synthase (eNOS).

iv. Nitric oxide produced by eNOS diffuses into the adjacent VSMCs and activates an enzyme guanylyl cyclase (paracrine effect).

v. Activated guanylyl cyclase increases the synthesis of 3,5-cyclic guanosine monophosphate (cGMP).

vi. cGMP reduces the intracellular (VSMCs) Ca2+ concentration.

vii. Ca^{2+} calmodulin myosin light chain kinase complex is inhibited by reduced intracellular Ca^{2+} which inturn inhibits formation in the VSMCs promoting relaxation.

viii. Relaxation of VSMC decreases the vascular resistance, tone and thus reduces BP.

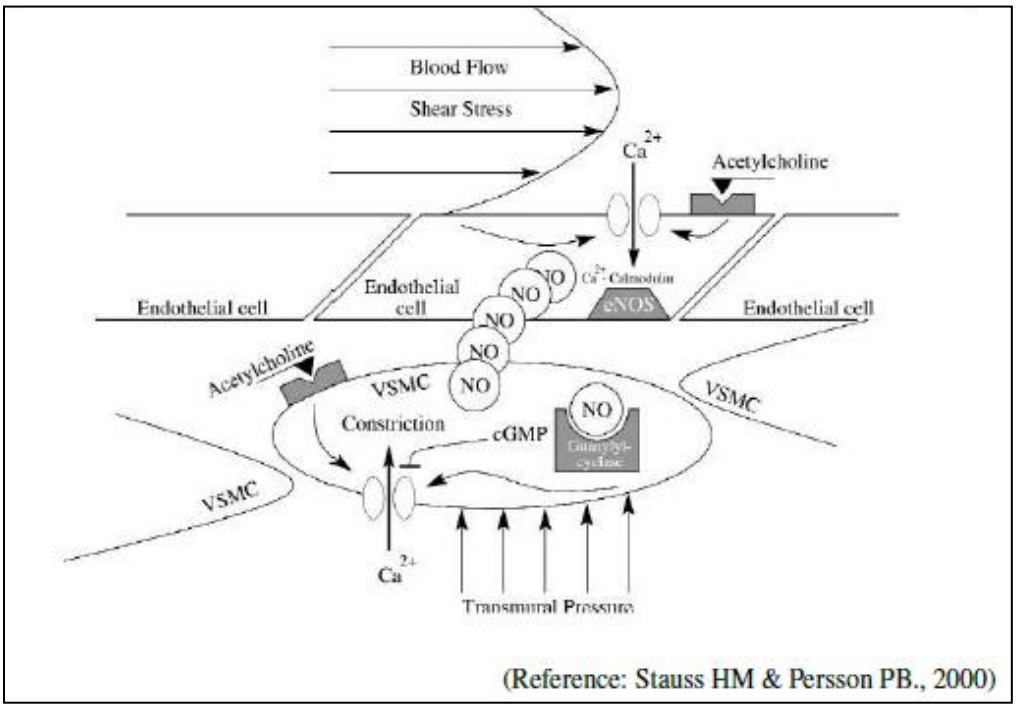

Figure 3: Mechanism of the vascular blood pressure control system

2.4.Ageing and altered endothelial function

Loss of normal function of endothelial cells is characterized by reduced vessel relaxation (Endemann DH & Schiffrin EL., 2004). Loss of age-related endothelial function and decreased bioavailability of NO results in increased vascular tone, arterial stiffness and raised BP (Matz RL et al., 2000; Torregrossa AC et al., 2011; Jin RC & Loscalzo J., 2010). A shift in endothelial function towards the vasoconstrictor dominance increases the peripheral vascular resistance, a pathognomonic characteristic of hypertension in the elderly (Figure 4). Increased arterial stiffness and peripheral vascular resistance with ageing is mainly contributed by impaired NO-mediated vasodilatation (Wilkinson IB et al., 2002; Fitch RM et al., 2001). Coronary endothelial dysfunction is isolated predictor of cardiovascular mortality (Schachinger V et al., 2000; Suwaidi JA et al., 2000). Literatures reported that by blocking NO synthesis and removal of vascular endothelium in animal models leads to increased

local arterial stiffness indicating NO is the prime regulator of large artery stiffness (Wilkinson IB et al., 2002; Boutouyrie P et al., 1997).

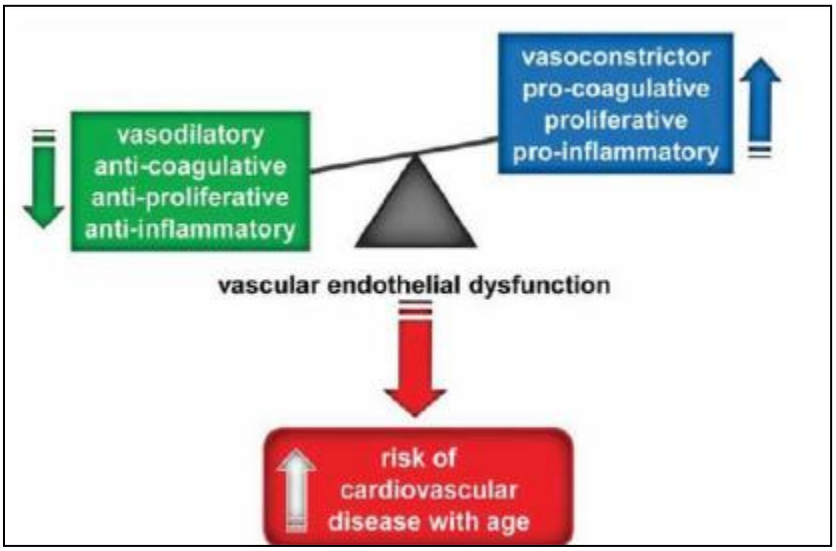

Figure 4: Endothelial dysfunction

3. METHODS FOR ASSESSMENT OF ARTERIAL STIFFNESS

There are many methods to evaluate of arterial stiffness in the human beings.

After each ventricular contraction a wave is formed which travels along the arterial wall when it finds a resistance it is reflected back to the heart as a new wave. The speed of this reflected wave depends primarily on central blood pressure and compliance of arteries (Townsend RR et al., 2015; Vlachopoulos C et al., 2015).

Because of high elastic property of arteries in adults the velocity of reflected wave is slow which reaches to the heart during relaxation. This increases the coronary perfusion. PWV increases with age due to arterial stiffness. Increased PWV reduces blood supply to heart due to reflected wave early reaches to heart during systlole (Nichols W et al., 2011; Safar ME et al., 2003).

The PWV is more reliable and valid measure to evaluate arterial stiffness. Central systolic BP and augmentation index (Figure 5), are less reliable to measure

arterial stiffness because they influenced by some factors like age, medical conditions, drugs and pulse rate (Townsend RR et al., 2015; van Sloten TT et., 2014).

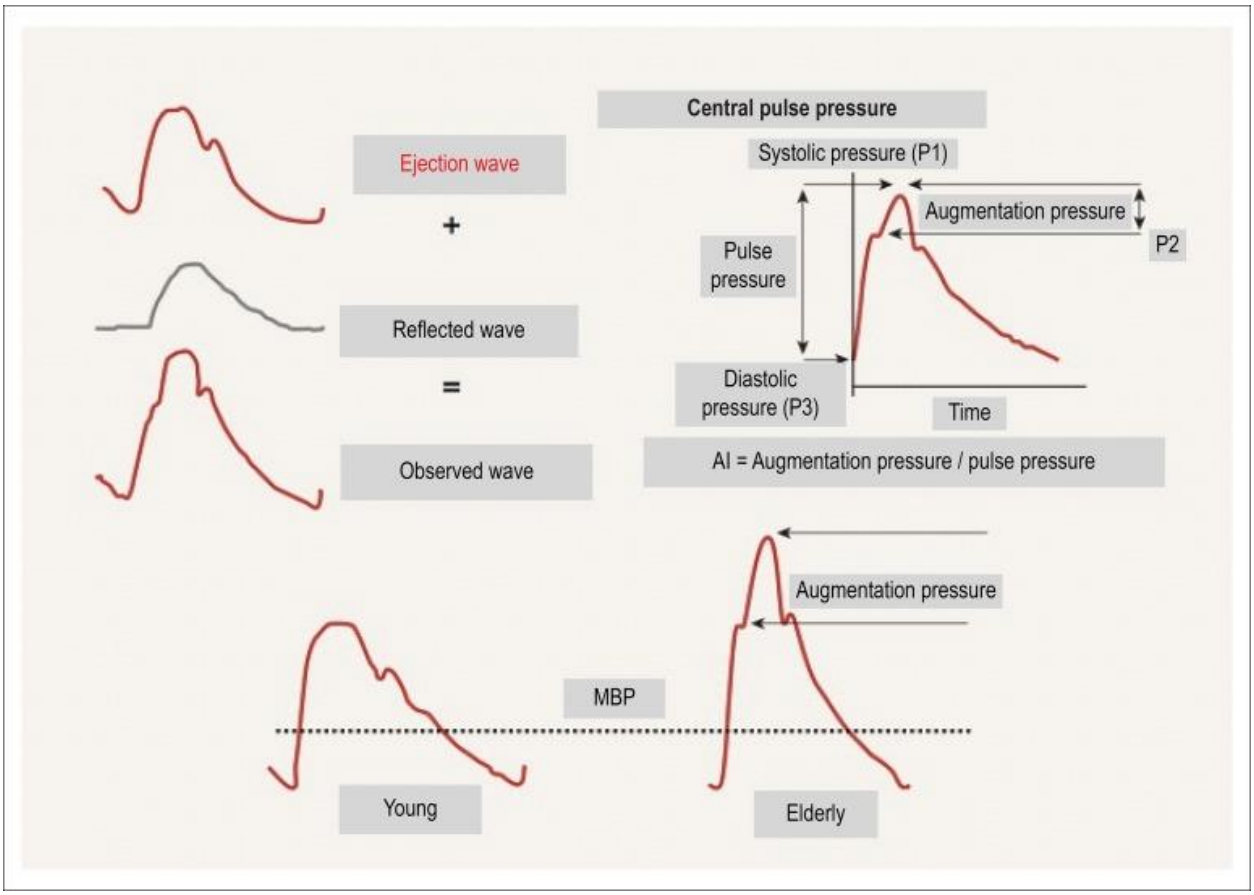

Figure 5: Blood pressure curve with description of its major components. AIx: Augmentation Index; MBP: Mean Blood Pressure (Safar ME., 2010).

Among PWV c-f PWV is more clinically valid measure because it correlates with most of the physiological and pathological effects of arterial stiffness (Townsend RR et al., 2015; Pannier B et al., 2005). The carotid-femoral PWV analysis is gold standard for arterial stiffness assessment, because there is large epidemiological evidence of its predictive value for cardiovascular events, and it requires little technical knowledge to be performed. In addition, PWV can be measured in a point. The method for that has been validated, and consists in calculating, by use of transference with calibration, systolic BP/diastolic BP (SBP/DBP) with mean

BP/DBP (MBP/DBP), being feasible and having better cost-benefit ratio for clinical practice (Townsend RR et al., 2015;Mancia G et al., 2013;Mattace-Raso F ET AL., 2010). In addition, PWV bears a strong correlation to age and BP, in which the elastic properties of the arterial wall are reduced, with consequent increase in vascular stiffness (Greenwald SE et al., 1990; Rizzoni D et al., 2003; Cefalu CA., 2011).

3.1.Pulse pressure

Pulse pressure is an easy, independent and finest instrument for measuring arterial stiffness and a good indicator for cardiovascular hazards with ageing. Studies have shown a strong association between PP and arterial stiffness (Safar ME., 2000; Safar ME et al., 2003; Cecelja M et al 2009). Systolic and diastolic pressure tends to increase with age upto 50-55 years. After 50-55 years, in most of the individuals the diastolic pressure falls and only systolic pressure rises with age thus causing widening of PP. Moreover, measurement of only PP is not adequate to assess arterial stiffness. Since age related stiffness is greater in the aorta (elastic artery) than peripheral arteries, central aortic PP is a good marker than brachial PP to assess arterial stiffness.

Growing evidence suggests that PP is an important predictor of risk in elderly (Franklin SS et al., 2001). A meta-analysis of different studies with data of 8,000 old individuals found that a 10mmHg augment in PP amplified the risk of major CV complications and mortality by nearly twenty percent (Blacher J et al, 2000). Moreover, PP was an independent predictor of stroke and all-cause mortality in the SHEP study (Domanski MJ., 1999).

3.2. Pulse Wave velocity (PWV)

Pulse wave velocity measurement is the most easy, precise and valid technique for the evaluation of local arterial stiffness. PWV is the velocity at which the frontward pulse wave is transmitted throughout the arterial system (Mackenzie IS et al., 2002). It is widely used as an index of large artery elasticity and stiffness. PWV can be measured in any segment of the arteries, provided the pulse waveform at two arterial sites is possible to record and time elapsed between the travels of waves and distance between them can be measured (Deloach SS & Townsend RR., 2008). There are various established methods for measuring PWV. The pulse waves can be recorded in different arteries using various sensors, transducers or probes, among which the most common are pressure sensitive transducers, applanation tonometry (Nelson MR et al., 2002), Doppler ultrasound (Calabia J et al., 2011), piezoelectric transducers (Willum-Hansen T et al., 2006) and photoelectric transducers. The PWV of the given artery can be calculated by measurement of the transit time of the pulse waves (Δt) and the distance between two recording points (d) as follows:

$$PWV\ (cm/m) = \frac{\Delta t}{d}$$

The pulse transit time is the time taken by pulse wave to travel (between points) from peripheral wave to distal wave. The pulse transit time can be calculated by measuring time in seconds elapsed between the peak of the R-wave of ECG and the foot or onset of the pulse waves or between the foot of peripheral and distal wave (foot-foot method).

The PWV can be measured at different regions such as:

o Carotid- femoral PWV

o Carotid-radial PWV

o Femoral-tibial PWV

o Heart-brachial PWV

o Heart-ankle PWV

o Brachial-ankle PWV

Arterial stiffness increases with age due to decrease in elasticity and aorta is the major component of arterial elasticity. The carotid-femoral PWV (c-f PWV) or aortic PWV is the gold standard method for assessment of aortic stiffness. The accurate measurement of path length is an advantage of using MRI, however its usage is limited due to high cost and lack of availability (Mohiaddin RH et al., 1993). Brachial-ankle PWV (b-a PWV) was considered as prime predictor of cardiovascular pathology in old age individuals (Li JY & Zhao YS., 2010). Aortic stiffness is an independent predictor of all cause and CV mortality (Laurent S et al., 2001). A study reported that, the aortic PWV is highly valid than b-a PWV in last stage of renal disease (Pannier B et al., 2005).

Mathematically, Moens-Korteweg defined the PWV as follows (Bramwell JC, Hill AV., 1922):

$$\mathrm{PWV} = \sqrt{Eh/2\rho r}$$

Where E is Young's elastic modulus in the circumferential direction, h is the wall thickness, r is the radius of the vessel and ρ is the blood density.

3.3. Arterial distensibility and compliance

The distensibility and compliance of artery provides a direct measure of arterial stiffness. To evaluate these parameters, the diameter of the artery and its pressure is required to be measured. Ultrasound is usually used imaging technique to measure the arterial stiffness. While evaluating the local arterial stiffness of carotid or

aorta, brachial BP is most frequently used (Rhee MY et al., 2008). There are conflicts on whether local arterial stiffness reflects the stiffness of other arteries.

3.4. Stiffness index and Reflection index

Stiffness index and reflection index (RIx) reflects systemic arterial stiffness. They are usually measured from the digital volume pulse waveform recorded using Finger Photoplethysmograph (Mackenzie IS et al., 2002). Reflection index reflects the peripheral vascular tone.

3.5. Arterial stiffness index

Arterial stiffness index is estimated by quantifying the oscillometric envelopes derived from the oscillations in the respective artery (Naidu MUR et al., 2012).

3.6. Systemic arterial compliance

Arterial compliance is defined as "the relationship between the change in volume and the change in the distending pressure". The simplest method to measure systemic arterial compliance is the ratio of the stroke volume (SV) to the pulse pressure (PP).

$$\text{Compliance} = \frac{SV}{PP}$$

The stroke volume can be measured invasively or non-invasively (Rhee MY et al., 2008). Brachial BP is most frequently used, assuming that the Central PP is similar to the brachial artery. Most of the investigators assess the carotid and aorta BP with applanation tonometry using a transfer function. Other method is 'area method' for measuring systemic arterial compliance.

3.7. Augmentation index (AIx)

The pulse wave generated from left ventricle is composed of a frontward pressure wave and a reflection pressure wave (wave reflection) reflected from the point of impedance mismatch (arterioles). The velocity of pressure wave along the

arterial depends on the elasticity of the vessel wall. More the stiffness (less elasticity), higher is the velocity.

Normally, the wave reflection enters at the aortic root during relaxation of the ventricles which increases the diastolic pressure and enhances the myocardial perfusion. In the stiffened arteries, the pulse wave moves at high speed along the arterial tree and reflected wave arrives earlier during contraction of ventricles, augments the reflected wave to the forward wave resulting in augmentation of the central systolic pressure. Premature entrance of reflected wave during ventricular contraction leads to decrease in diastolic pressure leads to decreased myocardial perfusion. This increased systolic pressure is known as an augmentation pressure. The aortic AIx is the ratio of augmentation pressure to the aortic PP and is expressed in percentage. So, the AIx is a simple method to measure the wave reflection, which reflects the arterial stiffness. More the stiffness, higher is the augmentation index (Rhee MY et al., 2008; Mackenzie IS et al., 2002; Laurent S., 2006).

4.OXIDATIVE STRESS

Oxidative stress is an important factor associated with ageing and disease. Oxidative stress leads to the development of age-related pathogenesis causes arterial dysfunction. 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 RM 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 JS., 2001). This reaction may have some biological significance when the concentration of superoxide molecules rises in blood vessels with advancing age (Faraci FM and Didion SP., 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 TJ et al., 2002; Soucy KG et al., 2006); lower NO levels further increase ROS production. Moreover, peroxynitrite ($ONOO^-$), a highly reactive molecule produced by the reaction of NO with superoxide, has been implicated in impaired EC function and vascular ageing (Zhao H et al., 2016).

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 E et al., 2011; Silva BR et al., 2012).

Reactive oxygen species have got important role in maintaining cardiovascular structure and function by modulating cell growth and inflammatory responses via reduction-oxidation-dependent signaling pathways. Increased vascular oxidative stress damage the endothelium, reduces nitric oxide production by inhibiting eNOS pathways and impairs endothelium-dependent vasodilation with resultant enhanced vascular tone and thus hypertension (Briones AM et al., 2010; Grossman E ., 2008). Further, oxidative stress causes thickening of the vascular media by promoting smooth muscle cell proliferation and hypertrophy with collagen deposition resulting in narrowing of vascular lumen (Grossman E ., 2008; Schulz E et al., 2011). These evidences suggest that oxidative stress may play an important role in the development of hypertension.

Dai et al in their review in respect of the free radical theory of ageing propose that Reactive Oxygen Species (ROS)-induced accumulation of damage to cellular macromolecules is a primary driving force of ageing and a major determinant of lifespan. Although this theory is one of the most popular explanations for the cause of ageing, several experimental rodent models of antioxidant manipulation have failed to affect lifespan. Moreover, antioxidant supplementation in clinical trials has been largely disappointing. The mitochondrial theory of ageing specifies that mitochondria are both the primary sources of ROS as well as the primary targets of ROS damage. In addition to effects on lifespan and ageing, mitochondrial ROS have been shown to play a central role in health span of many vital organ systems (Dai DF et al., 2014).

The findings of Bachschmid et al support the 'free radical theory of ageing' but also show that reactive oxygen and nitrogen species are essential signaling molecules regulating vascular homeostasis. Characteristic morphological and molecular alterations such as vessel wall thickening and reduction of Nitric Oxide occur in

ageing vasculature leading to the gradual loss of vascular homeostasis. Consequently, the risk of developing acute and chronic cardiovascular diseases increases with age. Current research of the underlying molecular mechanisms of endothelial function demonstrates a duality of reactive oxygen and nitrogen species in contributing to vascular homeostasis or leading to detrimental effects when formed in excess. Furthermore, changes in function and redox status of vascular smooth muscle cells contribute to age-related vascular remodeling. The age-dependent increase in free radical formation causes deterioration of the Nitric Oxide signaling cascade alters and activates prostaglandin metabolism and promotes novel oxidative posttranslational protein modifications that interfere with vascular and cell signaling pathways (Bachschmid et al., 2013).

Dato et al by their review suggested that the integration of lifestyle factors (moderate physical activity and healthy nutrition) and genetic background could shift the balance in favor of the antioxidant cellular machinery by activating appropriate defense mechanisms in response to exceeding external and internal stress levels and thus possibly achieving the prospect of living a longer life (Dato et al., 2013).

Thomas Munzell et al in their review, an abnormal production of reactive oxygen species (ROS) and the subsequent decrease in vascular bioavailability of Nitric Oxide (NO) have long been proposed to be the common pathogenetic mechanism of the endothelial dysfunction resulting from diverse cardiovascular risk factors such as hypercholesterolaemia, diabetes mellitus, chronic smoking, metabolic syndrome and hypertension. Superoxide produced by the Nicotinamide Dinucleotide Phosphate (NADPH) Oxidase, mitochondrial sources or xanthine oxidase may react with NO. Thereby, it is resulting in excessive formation of peroxynitrite, a reactive nitrogen species that has been demonstrated to accelerate the atherosclerotic process

by causing direct structural damage as well as further ROS production. Despite this sound biological rationale and a number of pre-clinical and clinical lines of evidence, studies testing the effects of classical antioxidants such as vitamin C, vitamin E or folic acid in combination with vitamin E have been disappointing. Rather, substances such as statins, angiotensin-converting enzyme inhibitors or AT1-receptor blockers which possess indirect antioxidant properties mediated by the stimulation of NO production and simultaneous inhibition of superoxide production (e.g. from NADPH oxidase) have been shown to improve vascular function in pre-clinical and clinical studies and to reduce the incidence of cardiovascular events in patients with cardiovascular disease. Today, oxidative stress remains an attractive target for cardiovascular risk prevention and therapy. However, a deeper understanding of its source and of its role in vascular health is necessary before new trials are attempted (Munzell et al., 2010).

The comparison between middle-aged and older subjects regarding oxidative stress parameters suggests also a progressive and slow decline of antioxidant status in healthy free-living older elderly and underlines the impact on life-style factors on successful ageing (Andriollo-Sanchez M et al., 2005).

5. MOLECULAR MECHANISM OF AGEING (OXYGEN SENSING MECHANISM)

5.1.Erythropoietin (Epo)

Erythropoietin (Epo) is a hormone, so named because of early studies demonstrating that Epo had a singular effect on stimulation of erythropoiesis, the formation of red blood cells. Epo functions by binding to and activating the Epo receptor (EpoR) expressed on the surface of committed erythroid progenitor cells. This in turn induces erythroid progenitor cell survival, proliferation, and

differentiation into circulating enucleated hemoglobin-containing red blood cells (RBCs), which are critical for oxygen transport (Krantz SB., 1991).

Erythropoiesis is stimulated when Epo, a glycoprotein hormone expressed primarily in the kidney, binds and activates the EpoR expressed on the surface of erythroid progenitor cells. The normal level of circulating Epo in humans is approximately 5 pM (~20 mU/mL; 100 pg/mL), substantially below the K_d of the Epo–EpoR interaction (~100 pM), indicating that only a fraction of the EpoR is Epo bound under normal conditions. However, this level of binding is sufficient to sustain erythropoiesis at a rate that will maintain normal RBC levels. Increased Epo concentrations result in an increased rate of erythropoiesis, thereby resulting in an increase in circulating RBCs with a maximal rate of erythropoiesis achieved at Epo concentrations of approximately 0.5–1 U/mL (Eschbach JW et al., 1987; Elliott S et al., 2004; Egrie JC et al., 1986; Wickrema A et al., 1992). Low Epo concentrations, on the other hand, result in apoptosis of precursor cells (Koury MJ and Bondurant MC., 1990). Epo concentrations below the normal circulating concentration therefore result in a decline in RBC numbers in peripheral blood because the rate of loss (~0.8%–1% per day) exceeds the rate of production.

Epo expression increases with decreasing oxygen tension (hypoxia), and this mechanism appears to be the primary driver of erythropoiesis. Hypoxia by itself has little effect on erythropoiesis in vitro (Rogers HM et al., 2008).

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 I and Gerhardt H., 2011).

Erythropoietin (EPO) is the main regulator of red blood-cell production. In the adult, this glycoprotein is synthesized mainly by the kidney, although a small fraction, about 10%, has no renal origin. Erythropoietin production is closely related to the oxygen needs of tissues. Hypoxia stimulates the oxygen sensor in peritubular interstitial cells near the proximal convoluted tubules, where this hormone is produced. Conversely, hyperoxia inhibits the process (Erslev AJ., 1953; Maxwell PH et al., 1993).

A few studies have shown the effects of ageing on erythropoiesis and the factors regulating it. Erythropoietin (Epo) is a specific growth factor for the erythroid line and is mainly produced by the kidney. Malaguarnera M et al studied serum Epo concentrations in a group of apparently healthy subjects divided into age classes in order to evaluate age-related modifications and correlations with hemoglobin (Hb) and red blood cells (RBC). They revealed that Epo values were correlated with age. Epo was higher in the over 65 years age classes than observed in control subjects. They concluded that the elevated Epo values in the latter age class may be required to maintain Hb and RBC within normal range (Malaguarnera M et al., 1996).

Musso CG et al did investigation on 74 healthy volunteers which included 22 adults, 30 old and 22 very old to determine the significant difference in plasma Epo between different age groups. They measured Hematocrit, Hb, plasma creatinine and plasma erythropoietin and creatinine clearance was calculated from serum creatinine using two different formulae. They found among the three groups a significant difference in creatinine clearance ($p< 0.001$), but not in plasma erythropoietin levels. They concluded that normal senescence does not alter plasma erythropoietin levels, even during advanced ageing (Musso CG et al., 2004).

5.2.Vascular endothelial Growth Factor (VEGF)

Vascular endothelial growth factor (VEGF), is a signal protein produced by cells that stimulates the formation of blood vessels (Senger DR 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 N et al., 2003; Yancopoulos GD et al., 2004). VEGF is also implicated in the survival of newly formed blood vessels and blood vessels in tumors (Alon T et al., 1995; Benjamin LE and Keshet E., 1997; Benjamin LE et al., 1999).

Rivard A et al demonstrated that advanced age is associated with a defect in compensatory neovascularization in response to tissue ischemia and they confirmed reduction of VEGF expression in ischemic tissues of old animals (Rivard A et al., 1999). Such impaired angiogenesis in ischemic tissues of old animals was found to be associated with reduced expression of vascular endothelial growth factor (VEGF), an endothelial-specific growth factor that is essential for embryonic and postnatal neovascularization (Carmeliet P et al., 1996; Ferrara N et al., 1996; Ferrara N and Davis-Smyth T., 1997).

Among those factors that have been implicated in the regulation of VEGF expression, hypoxia appears to play a major role, both in vitro and in vivo (Shweiki D et al., 1992; Banai S et al., 1994). The transcriptional and post-transcriptional mechanisms involved in the hypoxic regulation of VEGF are similar to those factors responsible for erythropoietin (Epo) expression.

A study by Malamitsi-Puchner et al concluded that serum VEGF levels are lower in males compared to females and serum VEGF level increased during life periods characterized by enhanced growth and development suggesting increased

rates of angiogenesis (Malamitsi-Puchner et al., 2000). A study on in vitro angiogenesis and the expression of VEGF, P-STAT3, P-CREB and importin-a in neonatal and aged human dermal microvascular endothelial cells (HMVECs) and they showed impaired angiogenesis with reduced VEGF expression is found to be associated with ageing and it is due to impaired nuclear transport of P-STAT3 and P-CREB transcription factors in these cells (Ahluwalia A et al, 2014). Higher VEGF expression in ageing may also be due to greater expression of oxygen sensing gene HIF-1α to combat age associated alteration of VEGF expression (Rivard A et al, 2000).

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CHAPTER III

AIM AND OBJECTIVES OF STUDY

Aim of the study:

The primary goal of the present study was to assess the influence of oxygen tension or oxygen microenvironment *in vivo* including oxidative stress on age and gender associated changes in vascular health among general population of Vijayapur urban area.

Objectives of the study:

1. To evaluate physical anthropometry, cardiovascular and haematological parameters in different age groups of male and female population of Vijayapur city.
2. To find out age associated oxidative and nitrosative stress and antioxidant status in different age groups of male and female population of Vijayapur city.
3. To evaluate vascular functions and oxygen sensing molecular markers like erythropoietin (Epo) and vascular endothelial growth factor (VEGF) in different age groups of male and female population of Vijayapur city.
4. To correlate vascular functions and oxygen sensing molecular markers with different age groups of male and female population of Vijayapur city.
5. Also to compare vascular functions and oxygen sensing molecular markers in male and female of different age groups.

HYPOTHESIS:

1. H0: Null hypothesis:

There is no relationship between oxygen sensing mechanism and ageing in both male and female participants. Further sex has no influences on age related changes in vascular physiology and oxygen sensing cell signaling mechanism.

2. H1: Alternate hypothesis:

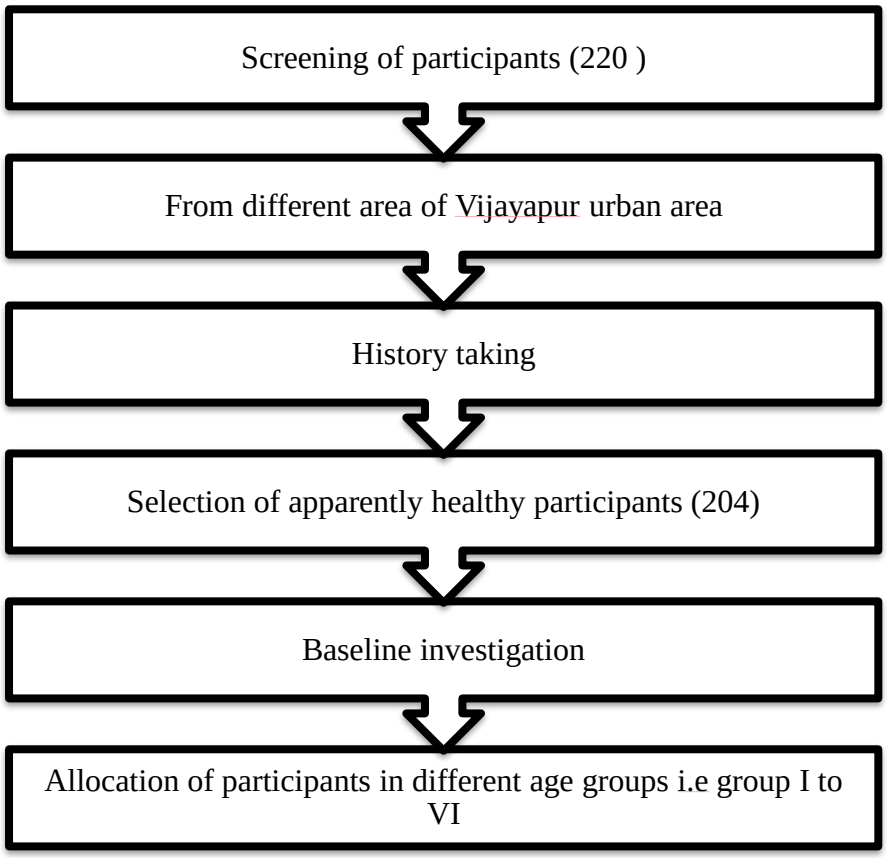
There is relationship between oxygen sensing mechanism and ageing in both male and female participants. Further sex has influences on age related changes in vascular physiology and oxygen sensing cell signaling mechanism.

CHAPTER IV

MATERIALS AND METHODS

1. STUDY DESIGN

A Cross sectional study was on apparently healthy participants between 20 to 95 years from Vijayapur city, Karnataka, India. Volunteers were screened and thorough clinical examination has done. Baseline examination and recordings were done followed by grouping of participants according to age.



2. STUDY POPULATION

2.1. Participants

The study participants were apparently healthy volunteers between 20 to 95 years from Vijayapur city, Karnataka, India.

2.2.Sample size

A total sample size of 204 participants divided into six groups each group contains 34 participants. The probability is 80% (power) that the study will detect a relationship between dependent and independent variables at a two sided 0.05 significant level, if

the true change in the dependent variable (oxygen tension) is o.5mmHg per 1 standard deviation change in independent variable.

- $n = \frac{[(Z_{\alpha/2}+Z_{\beta})^2 \times \{2\ (\sigma)^2\}]}{(\mu)^2}$

Where N= No of sample, Z=Standard normal variate, α=type I error (level of significane)=1.96, β=type II error (1-β=power of test)=0.20, σ=Standard deviation=1, μ=mean difference=0.5

The entire sample size is divided into six groups (Horng WB et al., 2001)

Group I	20 to 29 yrs (n=34)
Group II	30 to 39 yrs (n=34)
Group III	40 to 49 yrs (n=34)
Group IV	50 to 59 yrs (n=34)
Group V	60 to 69 yrs (n=34)
Group VI	70 yrs + (n=34)

3. INCLUSION AND EXCLUSION CRITERIA

3.1.Inclusion criteria:

Participants who have fulfilled the following criteria were selected for study:

- Apparently healthy subject age ranging from 20 to 95 years.
- participants with BMI < 30kg/m^2
- participants with resting blood pressure <140/90mmHg
- Non smokers/ non tobacco chewers, non alcoholics
- participants not taking medications or dietary supplements

3.2.Exclusion criteria:

Any of the following criteria was regarded as an exclusion criterion from the study:

- participants with hypercholesterolemia

- Evidence of hypertension (systolic blood pressure more than 140 and diastolic blood pressure more than 90 mm Hg).
- participants with Diabetes Mellitus and thyroid disorders.
- participants taking medications like statins, antidiabetics, diuretics, antihypertensives, beta blockers, sympathomimetic drugs and vasodilators

4. CRITERIA FOR DISCONTINUATION:

- Participant refusal
- Participant who's fasting blood sugar raised above 110mg/dl and lipid profile raised above normal during biochemical investigation.

5. ETHICS

5.1. Informed consent:

From all participants written informed consent was obtained for participation in the study (Appendix II).

5.2.Institutional approval

Present study obtained Institutional Ethical Certificate by the Institutional Ethical Committee (IEC Ref No-141/2015-16 dated July 20, 2015) Sri B.M. Patil Medical College, Hospital and Research Centre, BLDE (Deemed to be University), India, as per the guidelines (2006) of Indian Council of Medical Research (ICMR ethical guidelines for biomedical research on human participants, 2006).

6. STUDY SUBJECTS SELECTION PROCEDURE:

The participants were screened (n=220) from general population of Vijayapur urban area and volunteers enrolled. Screening and recording for subjects was done from October 2016 to April 2017. Those subjects who are apparently healthy were selected for the study after thorough examination as per our inclusion and exclusion criteria.

7. METHOD OF DATA COLLECTION:

The following parameters were recorded in the morning hours between 9.00hrs to 11.00hrs in the supine position after taking 10 minutes rest.

7.1.Measurement of anthropometric and physiological parameters:

7.1.1. Height

Height was measured using a device (BIOCON^{TM}) mounted on the wall and is expressed in centimeters (cms).

7.1.2. Weight

Weight was measured using a weighing machine and is expressed in Kilograms (Kg).

7.1.3. Body Mass Index (BMI)

Body Mass Index was estimated from weight in Kilograms (Kg) divided by height in meters squared (m^2) and was expressed as Kg/m^2.

7.1.3. Body Surface Area (BSA)

Body Surface area was estimated from weight in Kilograms (Kg) and height in centimeters (cm) and was expressed as m^2 by using Dubois Body Surface Chart.

7.1.4. Heart rate (bpm)

Heart rate was determined by palpating radial artery.

7.1.5. Measurement of blood pressure

- Systolic & Diastolic blood pressure (mmHg): Brachial BP was measured thrice with an interval of one minute in a sitting posture using mercury sphygmomanometer.
- Pulse pressure (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.

- Mean arterial pressure (mmHg): It is an average arterial pressure in an individual during single cardiac cycle. It was estimated by adding 1/3rd of PP (mmHg) to the DBP (mmHg).

7.2.Haematological analysis:

- 1ml of blood was collected in commercial tubes containing about 40 µl potassium EDTA as anticoagulant and the blood cell count was analyzed within 24 hr by automated haematology cell counter (CYSMAX K4500 of transatia). The following parameters were analyzed i.e. RBC, WBC, HB%, PCV, Platelet count and blood indices like MCV, MCH, MCHC (Garcia-Manzano AU et al., 2001).

7.3. Biochemical parameters:

7.3.1. Estimation of blood glucose:

Fasting blood glucose was estimated by Trinder's method (Trinder P., 1969). (Erba diagnostics Mannheim)

Principle

Glucose in sample was oxidized to yield gluconic acid and hydrogen peroxide in the presence of Glucose oxidase. The enzyme peroxidase catalyses the oxidative coupling of 4-aminoantipyrine with phenol to yield a colored quinoneimine complex, the absorbance was proportional to the concentration of glucose in sample.

Reagent

1. Enzyme reagent

Active ingredients	Concentration
Glucose oxidase	≥ 2000 U/L
Peroxidase	≥ 2000 U/L
Phenol	10 mmol/L
Phosphate buffer	200ol/L

2. Glucose standard: 100mg/dl

Procedure

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

	Blank	Standard	Test
Sample	--	--	10 µl
Standard	--	10 µl	--
Enzyme reagent	1.0 ml	1.0 ml	1.0 Ml

2. Mixed well and incubated at 37^0C for 5 minutes.
3. Absorbance of test and standard was read against blank at 505/670 nm.

Calculation

Glucose (mg/dl)

$$= \frac{\text{OD of test}}{\text{OD of standard}} \text{x Concentration of standard (100mg/dl)}$$

Precision of the assay

1. Inter-assay co-efficient of variability (CV): 2.34%

	Level 1	Level 2
No. of samples	10	10
Mean (mg/dl)	68	185
SD	1.9	3.5
CV %	2.79	1.89

2. Intra-assay co-efficient of variability (CV): 2.47 %

	Level 1	Level 2
No. of samples	10	10
Mean (mg/dl)	72	165
SD	1.26	5.24
CV %	1.75	3.18

7.3.2. Estimation of lipid profile

The blood sample was collected in the morning with overnight fasting for estimation of lipid profile (Erba diagnostics Mannheim).

a. Estimation of Serum triglyceride

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

Principle

Triglycerides were enzymatically hydrolyzed 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 catalyzed 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 labeled 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)

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

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 CC et al., 1974; Roeschlau P 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 labeled 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

b. 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 M 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

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.

7.4.Vascular function parameters:

Vascular function was assessed by oscillometric method using a non-invasive automatic device (Periscope, Genesis Medical Systems, India). Periscope is a validated 8-channel real time PC-based simultaneous acquisition (200 samples per second) and analysis system (Naidu MU et al., 2005). According to Nyquist's criterion the minimum sampling rate should be twice the maximum input frequency which is sufficient to avoid aliasing and preserve all the input signal information

(Faulkner EA et al., 1969). The significant frequency content of the pressure as well as ECG waveform was not more than 40 Hz; hence, a sampling rate of 200 samples per second was optimum. This device uses four BP cuffs and two-channel ECG leads to record arterial pressure waveforms and ECG (Lead I & II) simultaneously.

The recordings were made in supine position. BP cuffs were wrapped on both upper arm brachial artery and tibial artery above ankles. ECG electrodes were placed on the ventral surface of both wrists and medial side of the ankles (Figure 9a & b). The BP cuffs were connected to oscillometric pressor sensor and plethysmographic sensor located on the hardware of the system (Periscope) to determine pressure waveforms and volume pulse waveform. The data obtained in 10 seconds was stored in the computer for further analysis and to detect various arterial stiffness parameters. Periscope supports a sophisticated digital-signal algorithm to calculate all the results. As the device is fully automated and does not require any operator for handling any probe to record the waveforms, so it is devoid of any operator bias. Periscope is fully automatic, so once the test is started, the recording completes itself by displaying the results directly.

7.4.1. Estimation of pulse wave velocity

- Brachial-ankle PWV (baPWV), a measure of arterial stiffness (central artery & peripheral semi-muscular arteries) was estimated using arterial pressure waveforms (Brachial and Tibial artery) and ECG recordings (Lead I & II). The pulse transit time (PTT) in seconds elapsed between brachium and respective ankle was calculated as the time difference between the R-wave of ECG and foot of respective pulse wave. The distance between the brachium and ankle was calculated automatically according to the height of the subject. The PWV was calculated by dividing the distance by PTT(Figure 11).

$$ba\ PWV = \frac{Lba}{PTTba}$$

Where b-a PWV= Brachial ankle pulse wave velocity.

Lba = Distance between respective brachium and ankle.

PTTba = PTT between brachium and respective ankle was calculated as the time difference between the feet of respective pulse wave originated from R-wave (QRScomplex) of ECG.

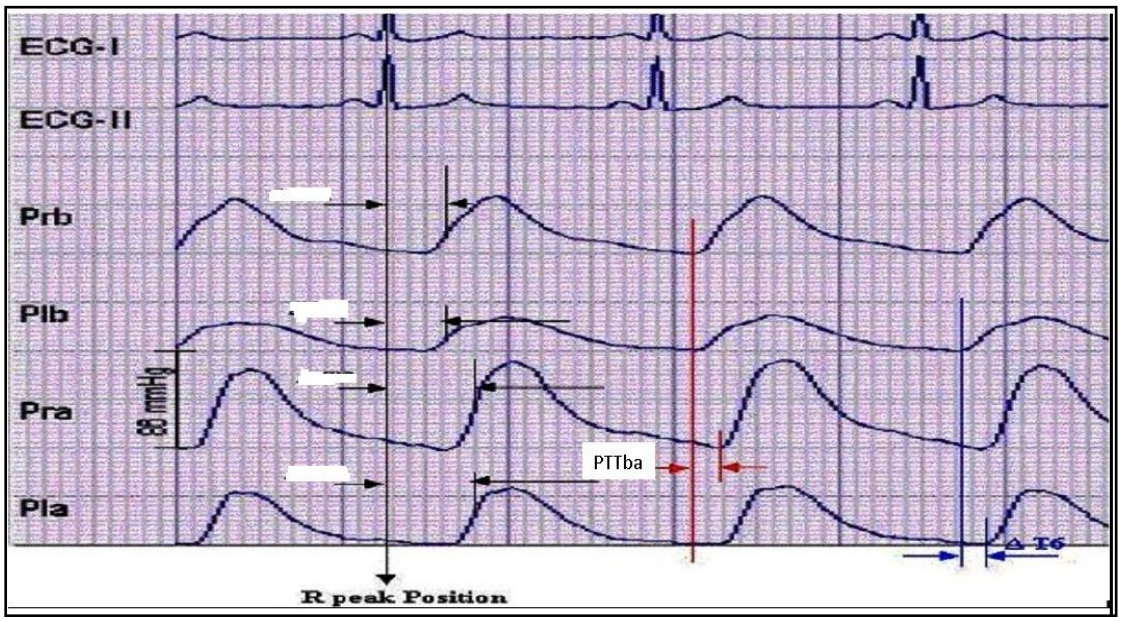


Figure 6. Pulse wave form and ECG and calculation of pulse transit time

- The carotid-femoral PWV (c-f PWV), a measure of central arterial (aortic) stiffness was calculated by the composite baPWV found out by averageing left and right baPWV. Periscope estimates the c-f PWV on the basis of equation (0.8333*Avg.baPWV-233.33) derived by regression analysis between baPWV and c-f PWV from the studies conducted elsewhere (Yamashina A et al., 2002).

$$\boldsymbol{Carotid - femoral\ PWV = 0.8333 * Avg.baPWV - 233.33}$$

7.4.2. Arterial stiffness index (ASI), an another measure of local and peripheral arterial stiffness was estimated at brachial artery (bASI) and tibial artery

(aASI) by quantifying the oscillometric envelopes derived from the oscillations in the respective artery (Naidu MUR et al., 2012).

ASI = (Systolic side Value of cuff pressure at 80% of maximal Oscillation amplitude of cuff) - (Diastolic side Value of cuff pressure at 80% of maximal oscillation amplitude of cuff).

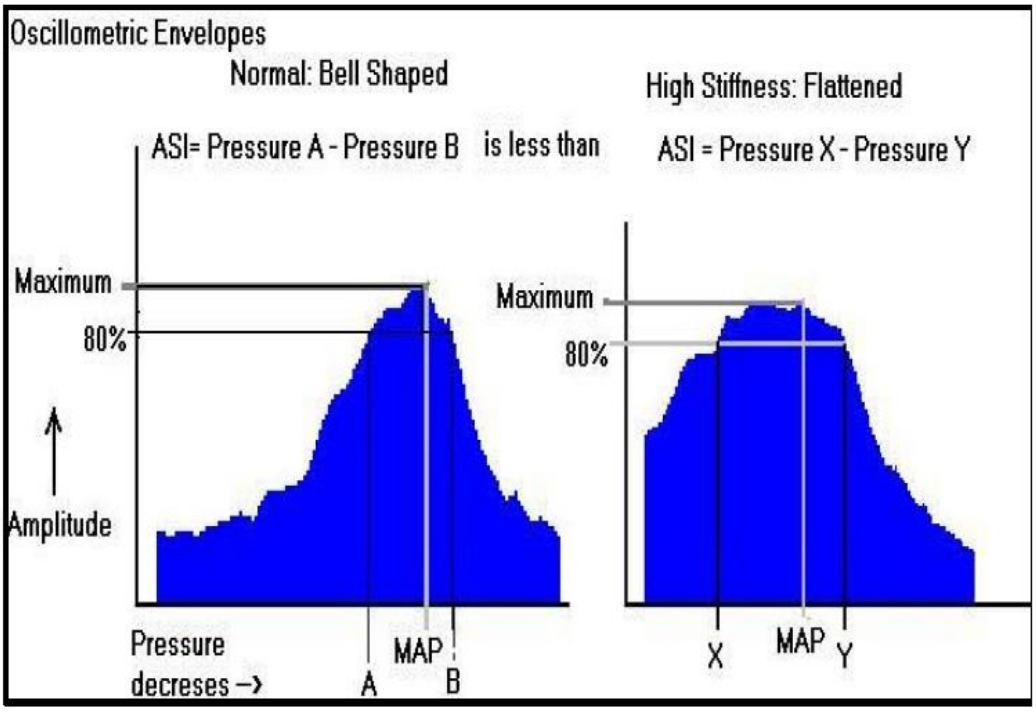


Figure 7. Oscillometric Envelope

Oscillometric envelope

An oscillometric envelope is a graph constructed by mapping the change in arterial pulse amplitude in response to changing cuff pressure (Acton A., 2013) (Figure 13). It is a graphical depiction of compressibility of the artery. It is derived from the oscillations in the artery during the deflation of BP cuffs while recording BP by oscillometric method. The shape of the oscillometric envelope is bell-shaped in normal artery where as it is flattened in stiffened artery. It becomes harder to collapse the stiffened arteries by applying external pressure; hence the oscillometric envelope becomes flatter as the stiffness increases. The ASI value gives a clear indication of

this flattening process (Figure 13). The ASI values increases with an increase in arterial stiffness.

7.4.3. Estimation of augmentation index (AIx)

- Periscope determines aortic pressure by Oscillometric PWV method. It estimates aortic pressure on the basis of regression equation derived by multivariate statistical analysis of invasive aortic pressure values (found by a fluid-filled catheter method) with respect to the brachial pressure and c-f PWV values obtained non-invasively by Periscope (Naidu MUR et al., 2012).

Measurement of aortic pressure by Oscillometric PWV method:

Aortic root pressure gradient is composed of two major components:

I. Systolic Pressure gradient – The rapid rise of pressure at the aortic root is contributed by the left ventricular pressure during systole. As soon as the left ventricle is emptied into the aorta, the aortic pressure falls rapidly. This gradient does not contribute to the aortic root pressure during diastole.

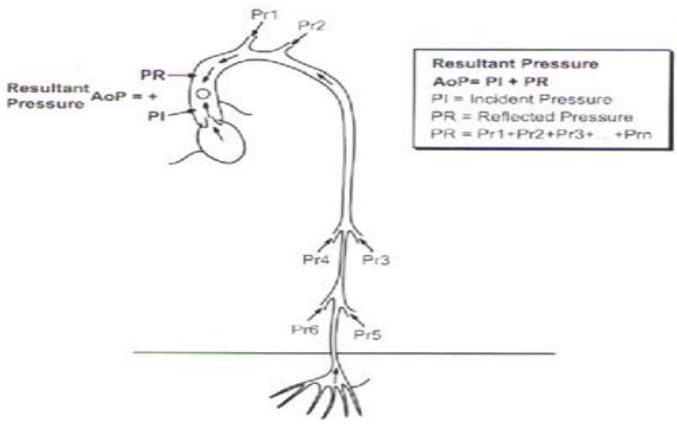


Figure 8. Wave reflection

II. Diastolic Pressure gradient – The pressure wave generated in the aorta during systole is propagated along the arterial tree which is resisted by the systemic vascular resistance from the branches at various points. From this various points of impedance mismatch at different arterial branches, the waves reflect back as a

single wave (wave reflection) to the aorta during diastole and contribute for diastolic pressure gradient (Figure 12).

Thus, the aortic root pressure is mainly dependent on two components: Left ventricular systolic pressure and wave reflection pressure.

The timing of arrival of wave reflection at the aortic root is dependent on the arterial stiffness. The wave reflection arrives earlier during systole in the stiffened arteries due to increase in PWV and contributes to augmentation of aortic systolic pressure. Thus, the resultant aortic root pressure increases in proportion with the arterial stiffness.

Periscope uses brachial BP and c-f PWV to determine the aortic root pressure. It is based on the mathematical analysis of invasive aortic pressure values (Fluid-filled catheter method) with respect to the brachial BP and PWV found non-invasively. Aortic root pressure values are directly proportional to a combination of both the brachial pressure value and c-f PWV. A significant correlation was found in these parameter values when multivariate regression analysis was carried out. Equation relating aortic pressure value, brachial pressure and c-f PWV with respective coefficients was derived from this and added in the Periscope to determine equivalent aortic pressure.

The rise in the systolic pressure is called an augmentation pressure. The augmentation index (AIx) is the ratio of augmentation pressure to the aortic PP and is expressed in percentage. This oscillometric PWV method used for estimation of AIx by periscope has been validated (Naidu MUR et al., 2012). As it was reported that AIx is influenced by heart rate, an index normalized for a heart rate of 75 bpm (AIx@75) was used in this study (Wilkinson IB et al., 2000).

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

The blood sample was collected in the morning with overnight fasting for estimation of biochemical parameters.

7.5.1. Estimation of Serum malondialdehyde (MDA)

Serum malondialdehyde (MDA), a marker of oxidative stress was estimated by Kei Satoh method (Satoh K., 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 colored 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.
4. Sulphuric acid (0.05M)
5. N-butyl alcohol

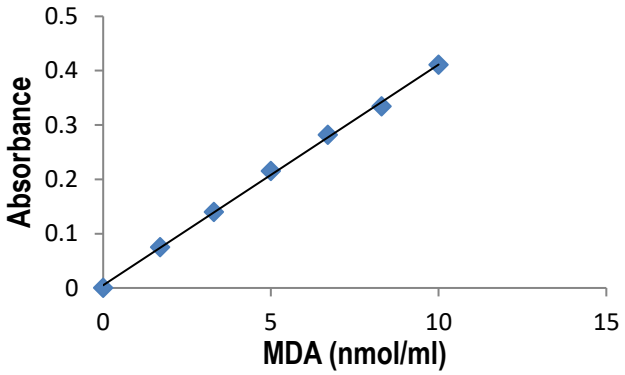
Standards

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

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

Standard Graph



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

$$\text{Concentration of serum MDA (nmol/ml)} = \frac{\text{OD of Test}}{\text{Nano-molar Extinction Co-efficient}} \text{ X } \frac{\text{Total volume of solution in cuvette}}{\text{sample volume}}$$

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

= OD of the Test x 51.28 nmol/ml.

7.5.2. Estimation of Reduced glutathione (GSH)

Blood reduced glutathione (GSH) was estimated by Earnest Beutler method (Beutler E et al. 1963).

Principle

Non-protein sulphydryl groups of red blood cells (RBC) are present in the form of reduced glutathione (GSH). 5, 5'–dithiobis-2-nitrobenzonic acid (DTNB) is a disulphide compound which is readily reduced by sulphydryl compounds, forming a highly colored yellow compound. Optical density was measured at 412 nm and it is directly proportional to GSH concentration.

Reagent

1. Precipitating solution: 1.67gm of glacial metaphosphoric acid, 0.2gm of disodium or dipotassium ethylene diamine tetra acetic acid (EDTA) and 30 gm of sodium chloride was dissolved in 100ml of distilled water.
2. Phosphate solution: 0.3M Na_2HPO_4 (di-sodium hydrogen phosphate) was prepared by dissolving 4.68gm in 100 mL distilled water.

3. 1% Sodium citrate: 1gm of sodium citrate was dissolved in 100ml distilled water.
4. DTNB reagent: 40mg 5, 5'dithiobis– (2-nitrobenzonic acid) was dissolved in 100ml of 1% sodium citrate.
5. Reduced glutathione standard (0.5 mg/ml): Take 5 mg of reduced glutathione and dissolved in 10 ml of distilled water.

Procedure

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

	Blank	Standard	Test
Whole blood	--	--	0.2 mL
Standard	--	0.4 Ml	--
Distilled water	2 mL	1.6Ml	1.8 mL
Mixed well			
Precipitating Solution	3.0 mL	3.0 mL	3.0 mL
Kept for five minutes, centrifuged and 1 ml supernatant was added in a separate labeled test tubes			
Phosphate solution	4.0 mL	4.0 mL	4.0 mL
DTNB Reagent	0.5 mL	0.5 mL	0.5 mL
Mixed and absorbance was read at 412 nm against the blank within 5 minutes			

Calculation

Concentration of Erythrocyte reduced glutathione

$$= \frac{OD\ of\ test}{OD\ of\ Std} X \frac{Conc\ of\ Std}{Volume\ of\ test} X\ 100$$

$$= \frac{OD\ of\ test}{OD\ of\ Std} X \frac{0.04}{0.08} X\ 100$$

$$= \frac{OD\ of\ Test}{OD\ of\ Std} X\ 50$$

= ……. mg/dl

7.5.3. Estimation of superoxide dismutase (SOD)

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

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

$= \frac{C-T}{C \times 50} \times 100 \times \frac{1}{0.1}$

$= \frac{C-T}{C \times 50} \times 1000$

= ---------- U/ml.

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

7.5.4. Estimation of serum vitamin C

Serum vitamin C was estimated by 2, 4-dinitrophenylhydrazine method (Roe JH et al., 1943; Brewster MA., 1996)

Principle

Ascorbic acid was oxidized by copper to form dehydroascorbic acid and diketogluconic acid. These products were treated with 2,4-dinitrophenyl hydrazine (DNPH) to form the derivative bis-2,4-dinitrophenyl hydrazone. This compound in strong sulfuric acid undergoes rearrangement to form a colored product which was measured at 520 nm. The reaction was run in the presence of thiourea to provide a mildly reducing medium which helps to prevent interference from non-ascorbic acid chromogen.

Reagents

1. 10 % Trichloroacetic acid: 10 gm of Trichloroacetic acid (TCA) was dissolved in distilled water to prepare a final volume of 100 ml.

2. DTC reagent: 3.0 gm of 2, 4-dinitrophenyl hydrazine (DNPH), 0.4 gm Thiourea and 0.05 gm copper sulphate were added to 9N sulfuric acid. The final volume of 100 ml was prepared.
3. 65 % sulfuric acid: 65 ml of sulfuric acid was dissolved in 35 mL distilled water.
4. Stock standard: 100 mg ascorbic acid was dissolved in 100 mL of 5 % TCA
5. .Working standard (10µg/mL): 1mL of stock standard was dissolved in 100 mL of 5 % TCA.

Procedure

1. Deproteination

   500 µl of sample was mixed with 500 µl of 10% TCA in an eppendorf tube. Vortexed and then centrifuged. The clear supernatant protein free filtrate was used.
2. 500 µl of sample and standards were taken in a test tube separately to which 100 µl DTC reagent was added.
3. Incubated at 37^0C for 3 hours.
4. 750 µl of 65% sulfuric acid was added to all the test tubes.
5. Vortexed and kept for 30 minutes at room temperature.
6. Absorbance was read at 520 nm.

Calculation

Concentration of Serum Vitamin C

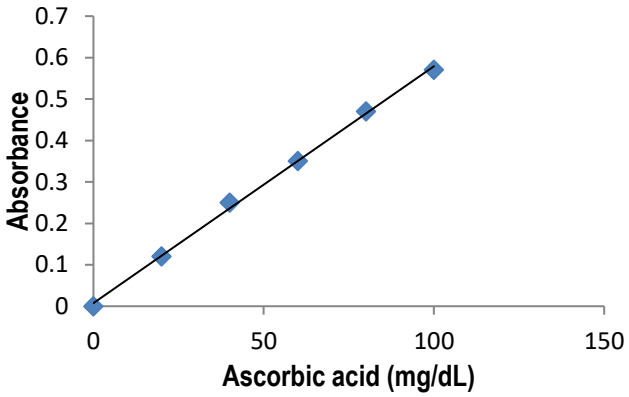
$$= \frac{OD\ of\ test}{OD\ of\ Std} X \frac{Conc\ of\ Std}{Volume\ of\ test} X\ 100$$

$$= \frac{OD\ of\ test}{OD\ of\ Std} X \frac{0.005}{0.25} X\ 100$$

$$= \frac{OD\ of\ Test}{OD\ of\ Std} X\ 2$$

= ……. mg/dl

Standard Graph



7.5.5. 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 JW 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

1. $ZnSO_4$ Solution (75mMol/L)
2. NaOH solution (55mMol/L)
3. Vanadium chloride III
4. Griess reagent
    a. Sulfanilamide
    b. N-Naphthylethylene diamine
5. $NaNO_2$ 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

1.0.ml 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

1.1. 400 µL - Sulfanilamide

1.2. 400 µL - N-Naphthylethylene diamine

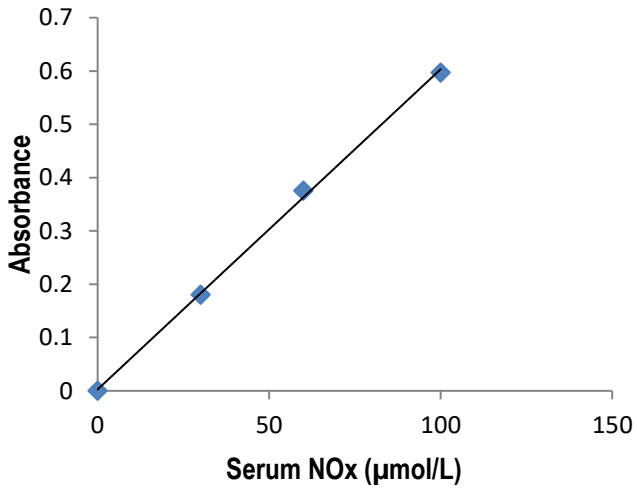
1.3. 200 µL - Vanadium chloride III

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.

Standard graph



Measurement

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

Calculation

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

ii. Slope was determined using linear regression fitting.

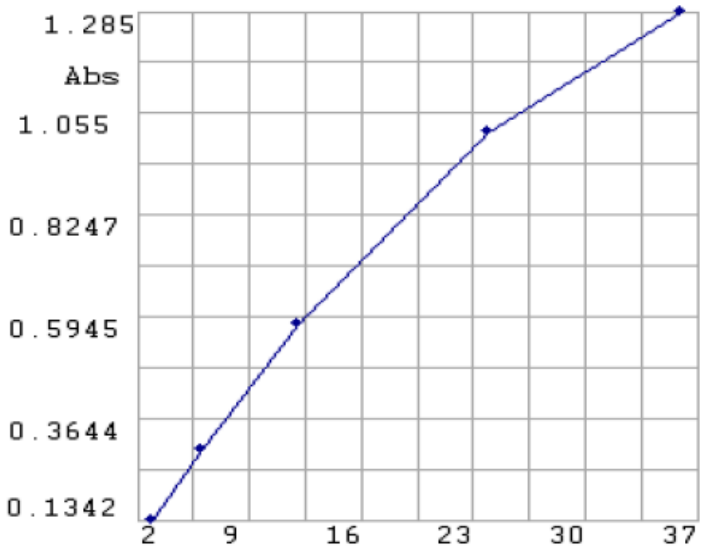
iii. The NO concentration of sample was calculated as

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

7.6. Oxygen sensing molecular markers:

7.6.1 Serum erythropoietin (Epo): based on the principle of a solid phase enzyme-linked immunosorbent assay (ELISA) (Uotila et al., 1981).

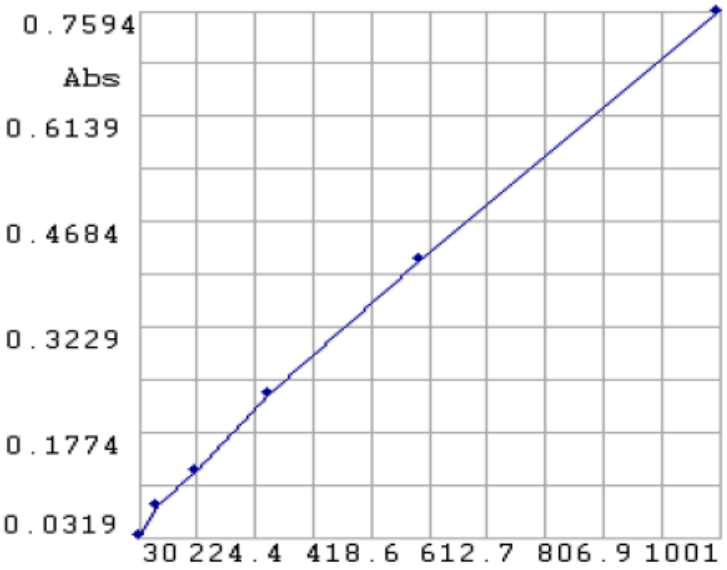
Standard Graph



EPO (in IU/L)

7.6.2. 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 (Hombrey E et al., 2002).

Standard Graph


0.7594
Abs
0.6139
0.4684
0.3229
0.1774
0.0319
30
224.4
418.6
612.7
806.9
1001

VEGF in pg/ml

8. STATISTICAL ANALYSIS:

- Results have been expressed as mean ± Standard Deviation.
- The data have expressed in the form of tables and graphs.
- Data were normally distributed.
- Differences between mean values of parameters between Group I, Group II, Group III, Group IV, Group V and Group VI were evaluated by one way ANOVA followed by Post-Hoc test (Least Significant Difference).
- Analysis of variance (ANOVA) is a statistical technique that is used to check if the means of two or more groups are significantly different from each other. ANOVA checks the impact of one or more factors by comparing the means of different samples. Post hoc tests are run to confirm where the differences occurred between groups, they should only be run when you have a shown an overall statistically significant difference in group means (i.e., a statistically significant one-way ANOVA result).
- We compared mean values for men and women in each age group using the unpaired t- test.
    - The unpaired t-test is used when two separate sets of independent and identically distributed samples are obtained, one from each of the two populations being compared.
    - Correlation was done by Pearson's correlation.
    - Correlation is any of a broad class of statistical relationships involving dependence, though in common usage it most often refers to how close two variables are to having a linear relationship with each other. The most familiar measure of dependence between two quantities is the Pearson product-moment correlation coefficient, or "Pearson's correlation coefficient", commonly called

simply "the correlation coefficient". It is obtained by dividing the covariance of the two variables by the product of their standard deviations.

- P-value <0.05 was taken as significant.
- Statistical analysis was carried out using SPSS version 16.0.

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CHAPTER V

RESULTS AND DISCUSSION

1. Influence of age on anthropometric and physiological parameters

1.1. Results:

The difference between the influence of age and anthropometric and physiological parameters among both male female participants involving six age groups was determined by ANOVA test and difference between each group determined by post-hoc test.

1.1.1. One way ANOVA followed by post-hoc test of male participants involving six age groups

Table 1shows ANOVA results of anthropometric and physiological parameters involving six age groups among male healthy participants.

Table 1: Anthropometric and physiological characteristics of male participants

Parameters	Age groups (years)							
	Group I	Group II	Group III	Group IV	Group V	Group VI	ANOVA	
	20-29 years (n=17)	30-39 years (n=17)	40-49 years (n=17)	50-59years (n=17)	60-69years (n=17)	70 years plus (n=17)	F' value	p' value
Weight, Kg	68.25±9.75	65±4.94	72.37±9.72	65±4.3	68±15.87	58.44±10.15	2.017	0.091
Height, cm	168.5±4.06	167.6±4.20	162.66±6.3	167.7±7.76	167.9±5.52	162.9±6.3	2.086	0.081
BMI, kg/m^2	24.06±2.8	23.33±1.16	25.76±2.44	24.84±0.85	23.87±4.54	21.89±2.31	2.129	0.076
BSA (m^2)	1.77±0.1	1.74±0.7	1.83±0.1	1.71±0.1	1.79±0.17	1.64±0.16	1.867	0.128
PR, bpm	73.58±8.37	74.86±9.86	77.82±9.55	75.54+6.77	72.9±10.7	63.1±7.43^{I,II,III,IV,V}	2.979	0.025*
PP, mmHg	48.5±5.31	51.2±6.3	48.5±3.77	47.8±6.79	57.33±6.9^{I,II,III,IV}	57.77±7.8^{I,II,III,IV}	4.027	0.02*
MAP, mmHg	87.6±6.81	87.9±5.13	89.5±4.73	94.72±5.44	101±8.75^{I,II,III,IV}	102±7.8^{I,II,III,IV}	3.488	0.021*

Data are Mean±SD. Values in the final column represent results of one-way analysis (ANOVA) among different age groups. Post-hoc comparisons were made between each group with LSD method. Superscripts I, II, III, IV, V and VI on each of the group are significantly differ from that group at p<0.05 level. BMI: body mass index, PR: pulse rate, PP: pulse pressure, MAP: mean arterial pressure

ANOVA did not show any significant difference (p<0.05) between age groups in weight (p=0.091), height (p=0.081), BMI (p=0.076), BSA (p=0.128) but showed significant difference in PR (p=0.025), MAP (p=0.02) and PP (p=0.021). Post-hoc

analysis showed significant difference decrease in PR after the age of sixty nine years i.e. in the group VI.

Our study showed statistical increase in MAP after the age of sixty years i.e. in group V (60-69 years) and VI (70 plus years). Our study also showed significant increase ($p<0.05$) in PP after the age of 60 years i.e. in group V (60-69 years) and VI (70 plus years).

1.1.2. One way ANOVA followed by post-hoc test of female participants involving six age groups

Table 2 shows ANOVA results of anthropometric and physiological parameters involving six age groups among female healthy participants.

Table 2: Anthropometric and Physiological parameters of Female participants

Parameters	Age groups (years)							
	Group I	Group II	Group III	Group IV	Group V	Group VI	ANOVA	
	20-29 years (n=17)	30-39 years (n=17)	40-49 years (n=17)	50-59years (n=17)	60-69years (n=17)	70 years plus (n=17)	F' value	p' value
Weight, Kg	56±8.6	55.82±6.73	59.1±5.93	62.2±7.0	57.6±10.39	54.5±5	2.117	0.08
Height, cm	158.29±2.7	152.0±6.3	151.1±3.5	156.6±3.9	150.06±4.77	148.5±3.10	2.091	0.09
BMI, kg/m^2	22.42±3.38	24.02±3.01	25.35±2.3	25±2.6	25.44±3.3	19.72±2.39	2.245	0.078
BSA, m^2	1.59±0.10	1.53±0.10	1.57±0.08	1.62±0.09	1.51±0.16	1.51±0.1	2.599	0.08
PR, bpm	74±9.17	74.13±11.24	71.58±7.77	75.41+4.4	71.93±8.5	65.33±9.09^{I,II,III,IV,V}	2.269	0.031*
PP, mmHg	44.3±8.3	44.58±3.7	46.66±5.2	48.33±5.46	64.84±12.88^{I,II,III,IV}	64.33±10.33^{I,II,III,IV}	7.346	0.000*
MAP, mmHg	82.47±6.76	85.11±8.48	87.83±7.95	91.94±5.44	95.68±8.75^{I,II}	101.43±17.8^{I,II}	3.268	0.013*

Data are Mean±S.D. Values in the final column represent results of one-way analysis (ANOVA) among different age groups. Post-hoc comparisons were made between each group with LSD method. Superscripts I, II, III, IV, V and VI on each of the group are significantly differ from that group at $p<0.05$ level. BMI: body mass index, PP: pulse pressure, MAP: mean arterial pressure

ANOVA did not show any significant difference ($p<0.05$) between age groups in weight ($p=0.08$), height ($p=0.09$), BMI ($p=0.078$), BSA ($p=0.08$) but showed

significant difference in PR (p=0.031), MAP (p=0.000) and PP (p=0.013). Post-hoc analysis showed significant difference decrease in PR after the age of sixty nine years i.e. in the group VI.

Our study showed statistical increase in MAP after the age of sixty years i.e. in group V (60-69 years) and VI (70 plus years). Our study also showed significant increase ($p<0.05$) in PP after the age of 60 years i.e. in group V (60-69 years) and VI (70 plus years).

1.2. Discussion:

Our results from anthropometric parameters in all the age groups in both male and female participants did not corroborate with the study of Dey et al (Dey et al., 2001). They showed variation in anthropometric parameters with age. With ageing body composition alters and there is decrease in body weight, height, and body cell mass. Variations in adult height reflected number of conditions in childhood, including economic status, psychosocial factors education, and upward social mobility. Thus, variation in height is related to the individual's lifetime exposure to certain genetic, environmental, and social factors. On the other hand, variations in body weight are more related to an individual's current health status, physical activity, smoking and dietary habits, and other factors. Such changes may be universal, but their expression and incidence may vary considerably within and between groups of elderly adults.

Our results from PR in different age groups in both male and female participants corroborate with study of Melo RC et al (Melo RC et al., 2005). The resting heart rate is modulated by both branches of the cardiac autonomic nervous system, with a predominance of parasympathetic influence. The vagal activity on sinus node

estimated by multiple heart rate variability (HRV) indices is decreased with age. On the other hand, the literature reports that mean HR at rest does not increase with advancing age presumably due to the decrease in the intrinsic heart rate & the increase in the sympathovagal balance (Franklin SS et al., 1997).

Our results from BP in all the age groups in both male and female corroborate with the study of Franklin et al (Franklin SS et al., 1997). MAP and PP are the two components of arterial blood pressure while MAP is steady and PP is pulsatile component. Cardiac output and vascular resistance determines the MAP. The heart rate, early pulse wave reflection, large artery stiffness and left ventricular ejection influences the variation in pressure around the mean this in turn determines the PP. Increased stiffness with increased resistance elevate SBP while DBP falls with increased stiffness and rises with increased resistance (O'Rourke MF., 1982; Safar ME., 1989; Nichols WW and O'Rourke MF., 1990; Franklin SS and Weber MA., 1994; Franklin SS., 1995). Therefore age-related changes in PP and MAP may predict the relative contributions of vascular resistance and large artery stiffness.

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2. Influence of age on hematological parameters

2.1 .Results:

The difference between the influence of age and hematological parameters among both male female participants involving six age groups was determined by ANOVA test and difference between each group determined by post-hoc test.

2.1.1 One way ANOVA followed by post-hoc test of male participants involving six age groups

Table 3 shows ANOVA results of hematological parameters involving six age groups among male healthy participants.

Table 3: Hematological parameters of male participants

Parameters	Age groups (years)							
	Group I	Group II	Group III	Group IV	Group V	Group VI	ANOVA	
	20-29 years (n=17)	30-39 years (n=17)	40-49 years (n=17)	50-59 years (n=17)	60-69 years (n=17)	70 years plus (n=17)	F' value	p' value
RBC count, millions/mm^3	5.31±0.27	5.21±0.31	4.62±0.62	4.92±0.59	5.02±0.18	4.33±0.6	2.593	0.06
WBC, thousands/mm^3	7310±1524.1	9235±1376.57	6954±1577	6827.5±697.2	7143.3±1928.8	5700±1110	2.663	0.06
Hb, gm%	15.57±1.08	14.67±2.05	14.4±1.24	14.67±0.57	14.7±0.60	13.34±1.16	1.854	0.146
O_2 carrying capacity , ml	20.8±1.44	19.66±2.75	19.29±1.66	19.6±0.75	19.69±0.8	17.87±1.56	1.856	0.145
PCV, %	47.05±3.2	44.9±4.49	44.42±4.03	45.95±2.85	46.16±2.08	41.37±3.5	1.784	0.16
MCV, fl	88.6±6.03	86.37±7.13	96.72±6.91	93.8±6.76	91.9±4.5	96.64±8.7	1.626	0.197
MCH, pg	29.3±1.6	28.2±3.8	31.32±1.8	30.02±2.9	29.26±1.61	31.18±3.18	0.926	0.484
MCHC, %	33.12±0.76	32.55±1.83	32.42±1.04	32±1.39	31.86±0.35	32.2±0.76	0.624	0.683
Platelet Count, lakhs/mm^3	2.72±0.49	2.95±0.28	2.08±0.25	2.79±1.1	2.51±0.51	2.16±0.57	1.713	0.175

Data are Mean±S.D. Values in the final column represent results of one-way analysis (ANOVA) among different age groups. Post-hoc comparisons were made between each group with LSD method. Level of significance 'p' value <0.05. RBC count: red blood cell count, WBC: white blood cell, Hb: Haemoglobin, PCV: packed cell volume, MCV: mean corpuscular volume, MCH: mean corpuscular haemoglobin, MCHC: mean corpuscular haemoglobin concentration

ANOVA did not show any significant difference ($p<0.05$) between age groups in RBC count (p=0.06), WBC count (p=0.06), Hb (p=0.146), O_2 carrying capacity

(p=0.145), PCV (p=0.16), MCV (p=0.197), MCH (p=0.484), MCHC (p=0.683) and platelet count (p=0.175) among male participants.

2.1.2 One way ANOVA followed by post-hoc test of female participants involving six age groups

Table 4 shows ANOVA results of hematological parameters involving six age groups among female healthy participants.

Table 4: Hematological parameters of female participants

Parameters	Age groups (years)							
	Group I	Group II	Group III	Group IV	Group V	Group VI	ANOVA	
	20-29 years (n=17)	30-39 years (n=17)	40-49 years (n=17)	50-59 years (n=17)	60-69 years (n=17)	70 years plus (n=17)	F' value	p' value
RBC count, millions/mm^3	4.32±0.46	4.51±0.45	4.26±0.23	4.93±0.42	4.52±0.30	4.65±0.82	0.982	0.056
WBC, thousands/mm^3	8922±2041	7796±1414	7666.6±681	8136.6±2000	7886±2259	7250±1992	0.383	0.854
Hb, gm%	12.92±1.0	12.7±0.5	12.7±0.5	12.1±2.49	13.16±0.9	12.6±0.9	0.344	0.88
O_2 carrying capacity , ml	17.05±1.4	16.8±0.63	16.8±0.75	15.9±3.29	17.36±1.29	16.6±1.19	0.34	0.882
PCV, %	39.98±3.0	39.78±1.93	40.16±1.98	39.56±5.8	41.98±2.62	40.5±1.11	0.396	0.845
MCV, fl	92.8±4.3	88.68±6.0	94.16±2.8	80.03±6.68	92.88±4.12	89±16.3	1.703	0.185
MCH, pg	30±2.03	28.36±2.4	29.86±1.4	24.4±3.5	29.12±1.37	27.86±5.95	1.715	0.182
MCHC, %	32.32±0.78	31.94±0.6	31.7±0.62	30.4±1.9	31.34±0.55	31.16±1.4	1.756	0.173
Platelet Count, lakhs/mm^3	3.43±0.34	3.27±0.43	2.91±0.89	3.72±0.81	2.76±0.91	2.78±0.19	1.334	0.295

Data are Mean±S.D. Values in the final column represent results of one-way analysis (ANOVA) among different age groups. Post-hoc comparisons were made between each group with LSD method. Level of significance 'p' value <0.05. RBC count: red blood cell count, WBC: white blood cell, Hb: Haemoglobin, PCV: packed cell volume, MCV: mean corpuscular volume, MCH: mean corpuscular haemoglobin, MCHC: mean corpuscular haemoglobin concentration

ANOVA did not show any significant difference ($p<0.05$) between age groups in RBC count (p=0.056), WBC count (p=0.854), Hb (p=0.882), O_2 carrying capacity (p=0.88), PCV (p=0.845), MCV (p=0.185), MCH (p=0.182), MCHC (p=0.173) and platelet count (p=0.295) among male participants.

2.2. Discussion:

With ageing there is progressive declination in the functional reserve of multiple organ systems leading to many diseases. With ageing there is imbalance between blood cells production and destruction. The reason for this is mainly decrease in the bone marrow's ability to respond stimuli like infection, cytotoxic damage and bleeding (Balducci L et al., 2005).

Our results from hematological parameters in all the age groups in both male and female participants did corroborate with the study of Schaan MD et al (2007) to some extent. There study showed no statistically significant differences in hematocrit, hemoglobin, leukocytes and vitamin B_{12} levels between healthy old aged and young aged groups. Mean values of MCV, RDW, eosinophils, folate and ferritin were higher in the healthy old aged than the young aged group. On the other hand, platelets were higher in the young aged group. The comparison between nutritional indicators of anemic and non–anemic apparently healthy elderly people showed statistically significant differences in vitamin B_{12} and protein intake, which were lower in the anemic elderly. The results suggest independent biological differences between hematological parameters of elderly and young individuals (Schaan MD et al., 2007).

Yamada et al. in their showed that with age there is a physiological reduction in normal hematologic values. With adjustments for gender, birth cohort, cigarette smoking and anemia related diseases authors have followed up a group of Japanese individuals for a period of forty–year, measuring hemoglobin levels every two years. They concluded that with age hemoglobin diminishes. Blood hemoglobin (Hb) has, been reported to decline in the elderly, more so among men than among women (Nilsson–Ehle H., 1989; Yamada M et al., 2003).

2.3. References:

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3. Influence of age on Biochemical parameters

3.1. Results:

The difference between the influences of age on biochemical parameters among both male female participants involving six age groups was determined by ANOVA test and difference between each group determined by post-hoc test. Difference between male and female participants in each group is determined by unpaired-t test.

3.1.1 One way ANOVA followed by post-hoc test of male participants involving six age groups

Table 5 shows ANOVA results of biochemical parameters involving six age groups among male healthy participants.

Table 5: Biochemical parameters of male participants

Parameters	Age groups (years)							
	Group I	Group II	Group III	Group IV	Group V	Group VI	ANOVA	
	20-29 years (n=17)	30-39 years (n=17)	40-49 years (n=17)	50-59 years (n=17)	60-69 years (n=17)	70 years plus (n=17)	F' value	p' value
FBS(65-110), mg/dl	87.16±13.5	89.58±12.6	89.23±10.2	99.17±16.55^{I,II,III}	97.6±17.6^{I,II,III}	100.6±16.20^{I,II,III}	5.787	0.000*
TG(60-165), mg/dl	82.06±14.35^{III,IV,V,VI}	90.58±10.5^{III,IV,V,VI}	109±25.2^{I}	110.4±49.3^{I,II}	123.5±28.1^{I,II}	117.9±14.8	5.975	0.000*
TC(150-220), mg/dl	143.1±14.33	147.2±41.3	147.7±41.6	177.9±43.7^{I,II,III}	171.6±43.6^{I,II,III}	171.7±37.43^{I,II,III}	2.715	0.024*
HDLC (35-80), mg/dl	60.7±17.9	59.88±11.3	57.82±20.01	55.76±9.9	55.82±7.8	54.12±7.01	0.688	0.634
LDLC(103-107), mg/dl	66.17±22.4	69.32±20.6	68.12±27.8	100.1±36.4^{I,II,III}	91.1±33.3^{I,II,III}	93.94±31.89^{I,II,III}	2.579	0.031*
VLDLC(12-33), mg/dl	16.47±2.8	18.09±2.10	21.77±5.02	22.1±9.8	41.98±2.62^{I,II,III,IV}	40.5±1.11^{I,II,III,IV}	5.928	0.000*

Data are Mean±S.D. Values in the final column represent results of one-way analysis (ANOVA) among different age groups. Post-hoc comparisons were made between each group with LSD method. Level of significance 'p' value <0.05. FBS-Fasting blood glucose; TG-Triglycerides; TC-Total Cholesterol; HDLC- High density lipoprotein fraction of cholesterol; LDLC-Low density lipoprotein fraction of cholesterol; VLDLC-Very low density lipoprotein fraction of cholesterol.

ANOVA results of our study showed statistical (p=0.000) increase in FBS after the age of fifty years i.e. in group IV (50-59 years), V (60-69 years) and VI (70 plus years) in male participants. ANOVA results of our study showed statistical increase in TG (p=0.000), TC (p=0.024), VLDLC (p=0.000) and LDLC (p=0.031) with ageing in both male and female participants. There is no statistical significant (p=0.634) difference in HDLC with age in male participants.

3.1.2 One way ANOVA followed by post-hoc test of female participants involving six age groups

Table 6 shows ANOVA results of biochemical parameters involving six age groups among female healthy participants.

Table 6: Biochemical parameters of female participants

Parameters	Age groups (years)							
	Group I	Group II	Group III	Group IV	Group V	Group VI	ANOVA	
	20-29 years (n=17)	30-39 years (n=17)	40-49 years (n=17)	50-59 years (n=17)	60-69 years (n=17)	70 years plus (n=17)	F' value	p' value
FBS (65-110), mg/dl	89.6±.10.4	95.3±11.86	91.2±10.26	111.06±22.1^{I,II,III}	110.6±8.63^{I,II,III}	109.4±15.89^{I,II,III}	10.259	0.000*
TG(40-140), mg/dl	79.7±8.96	88.47±15.7	91.8±25.225	125.06±24.6^{I,II,III}	123.8±14.8^{I,I,III}	123.06±16.2^{I,II,III}	21.221	0.000*
TC (150-220), mg/dl	141.18±23	143.3±24.1	145.24±39.9	185.65±37.21^{I,II,III}	206.35±21.66^{I,II,III}	202.24±34.9^{I,II,III}	16.746	0.000*
HDLC (42-88), mg/dl	68.2±10.2	67.82±11.3	63.82±13.7	50.11±9.19^{I,II,III}	50.47±5.9^{I,II,III}	50.47±8.3^{I,II,III}	6.795	0.000*
LDLC(100-104), mg/dl	57.18±9.3	57.8±19.6	63.1±23.3	110.52±34.1^{I,II,III}	131.1±22.4^{I,II,III}	127.2±31.7^{I,II,III}	19.445	0.000*
VLDLC(8-28), mg/dl	15.9±1.7	18.37±5.17	18.4±5.17	25.1±4.9^{I,II,III}	24.7±2.9^{I,II,III}	24.6±3.2^{I,II,III}	21.221	0.000*

Data are Mean±S.D. Values in the final column represent results of one-way analysis (ANOVA) among different age groups. Post-hoc comparisons were made between each group with LSD method. Level of significance 'p' value <0.05. FBS-Fasting blood sugar; TG-Triglycerides; TC-Total Cholesterol; HDLC- High density lipoprotein fraction of cholesterol; LDLC-Low density lipoprotein fraction of cholesterol; VLDLC-Very low density lipoprotein fraction of cholesterol.

ANOVA results of our study showed statistical (p=0.000) increase in FBS after the age of fifty years i.e. in group IV (50-59 years), V (60-69 years) and VI (70 plus years) in female participants. ANOVA results of our study showed statistical increase in TG (p=0.000), TC (p=0.000), VLDLC (p=0.000) and LDLC (p=0.000) with ageing in female participants. HDLC statistically (p=0.000) decreased with age in female participants.

3.1.3 Unpaired 't' test between both male and female participants in each age group

Table 7 shows unpaired't' test results of biochemical parameters between both male and female participants in each age group**.**

Table 7: showing unpaired 't' test

Biochemical parameters					
Parameters	**Age in years**	**Male participants**	**Female participants**	**Unpaired t-test**	
				t' value	**p' value**
FBS	**Group I**	87.16±13.5	89.6±10.4	-1.618	0.115
	Group II	89.58±12.6	95.3±11.86	-1.743	0.091
	Group III	89.23±10.2	91.2±10.26I	-0.668	0.509
	Group IV	99.17±16.55	111.06±22.1	-2.052	0.048*
	Group V	97.6±17.6	109.4±8.63	-2.847	0.008*
	Group VI	100.6±16.20	101.09±15.8	-2.1	0.044*
TG	**Group I**	82.06±14.35	79.7±8.96	0.578	0.57
	Group II	90.58±10.5	88.47±15.7	0.461	0.648
	Group III	109±25.2	91.8±25.8	1.953	0.06
	Group IV	110.4±49.3	125.06±24.6	-1.09	0.284
	Group V	123.5±28.1	123.8±14.8	-0.046	0.964
	Group VI	117.9±14.8	123.06±16.2	-0.961	0.344
TC	**Group I**	143.1±14.33	141.18±23.3	0.328	0.745

	Group II	147.2±41.3	143.3±24.1	0.34	0.736
	Group III	147.7±41.6I	145.24±39.9	0.176	0.861
	Group IV	177.9±43.7	185.65±37.2	-0.553	0.584
	Group V	171.6±43.6	206.35±21.6	-2.941	0.006*
	Group VI	171.7±37.43	202.24±34.9	-2.463	0.019*
HDLC	**Group I**	60.7±17.9	68.2±10.2	-0.951	0.349
	Group II	59.88±11.3	61.82±11.3	-1.051	0.321
	Group III	57.82±20.01	63.82±13.7	-1.021	0.315
	Group IV	55.76±9.9	50.11±9.19	2.235	0.033*
	Group V	55.82±7.8	50.47±5.9	2.249	0.032*
	Group VI	56.12±7.01	50.47±8.3	2.387	0.031*
LDLC	**Group I**	66.17±22.4	57.18±9.28	1.005	0.322
	Group II	69.32±20.6	57.8±19.6	0.991	0.329
	Group III	68.12±27.8	63.1±23.3	0.363	0.719
	Group IV	100.1±36.4	110.52±34	-0.748	0.46
	Group V	91.1±33.3	131.1±22.4	-3.384	0.002*
	Group VI	93.94±31.89	127.2±31.7	-2.457	0.02*
VLDLC	**Group I**	16.47±2.8	15.9±1.7	0.65	0.52
	Group II	18.09±2.10	18.37±5.17	0.435	0.666
	Group III	21.77±5.02	18.4±5.17	1.942	0.061
	Group IV	22.1±9.8	25.1±4.9	-1.09	0.287
	Group V	41.98±2.62	24.7±2.9	-0.046	0.964
	Group VI	40.5±1.11	24.6±3.2	-0.961	0.344

Data are Mean±SD. Values in the final column represent results of unpaired t-test between male and female participants. $p<0.05$, considered as statistically significant.

Unpaired 't' test results of our study showed a more significant increase in plasma glucose level in women than in men after the age of 50 years i.e. in the group IV (50-59 years) (p=0.048), V (60-69 years) (p=0.008) and VI (70 plus years) (p=0.044) in both male and female participants. Unpaired t test showed significantly ($p<0.05$)

higher values of TC, LDLC and HDLC in female participants after the age of 50 years compared to males.

3.2. Discussion:

Ageing is one of the most important factors that impair the different organ functions. With ageing insulin secretion can be decreased as a consequence of progressive impairment of pancreas (Stout RW., 1994; Basu R et al., 2003; Chang AM and Halter JB., 2003). Our study showed statistical increase in FBS after the age of fifty years i.e. in group IV (50-59 years), V (60-69 years) and VI (70 plus years) in both male and female participants. Our results from FBS in all the age groups in both male and female corroborate with the study of Gary TC Ko et al. Gary TC Ko et al observed very clear and significant increase in plasma glucose levels with age. They also observed more significant increase in plasma glucose level per decade in women than in men. Women in the random plasma glucose group had 80% higher increase in plasma glucose level per decade than men (Gary TC Ko et al., 2006). This Similar to their finding, we observed a more significant increase in plasma glucose level in women than in men. This may be accounted by a rapid deterioration in insulin resistance-in women after menopause (Kutty VR et al., 2002). With ageing the increase of plasma glucose is believed to be multi-factorial (Wolever TM et al., 1988).

Triacylglycerols, cholesterol and its esters, and phospholipids are circulating lipoproteins. The low-density lipoproteins (LDL) carry cholesterol from liver to peripheral cells, while high-density lipoproteins (HDL) carry cholesterol in the reverse direction. So, HDL is a vessel-protective agent which prevents the formation of atherosclerotic changes while increased levels of LDL augments the risk of cardiovascular diseases (Hertzberg G.R., 2004). Mainly, an increased risk of

cardiovascular diseases is associated with augmented levels of LDL and triacylglycerols (Austin M.A., 1998). Serum lipid levels are related to age. In previous cross-sectional (Carroll MD et a., 2005; Heiss G., 1980; Schaefer EJ., 1994; Abbott RD et al., 1983; Kuzuya M et al., 2002) and prospective (Anderson KM et al., 1987; Yamada M et al.,1986; Wilson PW et al., 1994; Ferrara A et al., 1997; Newschaffer CJ et al., 1992) studies, total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-c) gradually increase after adolescence until the age of 60–65 in men and 70–75 in women, and thereafter start to decline. High-density lipoprotein cholesterol (HDL-c) and triglycerides change less during adulthood [Carroll MD et a., 2005], but in cross-sectional reports HDL-c tends to be higher in old age-groups (Abbott RD et al., 1983; Ettinger WH et a., 1992). Existing data on the longitudinal changes of HDL-c in the elderly is rather inconsistent (Wilson PW et al., 1994; Ferrara A.,1997; Weijenberg MP., 1996; Abbott R.,1993). With advanced age, the role of serum lipids, in determining the risk of cardiovascular and total mortality becomes more complex due to multiple illnesses and frailty (Kronmal RA et al., 1993; Corti MC et al., 1997; Brescianini S et al., 2003).

Our study showed statistical increase in TG, TC, VLDLC and LDLC with ageing in both male and female participants. HDLC statistically decreased with age in female participants our results corroborated with Deepti et al (Deepti et al., 2014). Significantly TC and LDLC are high and HDLC is low in female participants after the age of 50 years compared to males may be due to hormonal effect on the lipid metabolism with age in females. Deepti et al showed increase in total cholesterol, triglyceride and LDL-Cholesterol and decrease in HDL-Cholesterol in women in perimenopausal period as compared to women in the younger age group. It can be concluded that serum lipid profile changes can possibly be mediated by changing

hormonal profile, especially estrogen which has role in lipid metabolism and indirectly on coronary artery disease (Deepti et al., 2014). Another study concluded that LDL increases significantly from reproductive period to postmenopausal period. The rise in LDL can also be attributed to age but the significant raise after menopause is also attributed to hormone levels (Stevenson JC et al., 1993).

The data of Marhoum TA et al suggest that the ageing of apparently healthy Sudanese individuals over 55 years is likely to increase both total cholesterol and LDL-cholesterol. Also observed HDL-cholesterol levels were significantly higher in elderly females compared with the elderly males (Marhoum TA et al., 2013).

Present study showed significant difference in lipid profile in both male and participants with age. Present study also showed significant difference in cholesterol and lipoproteins in women below 50 years and women in the menopausal period. Hence regular monitoring of men and women in menopausal period with lipid profile would be helpful to prevent the age related risk of coronary heart disease.

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4 . Influence of age on oxidative and nitrosative stress and antioxidant parameters

4.1. Results:

The difference between the influence of age on oxidative and nitrosative stress and antioxidant parameters among both male and female participants involving six age groups was determined by ANOVA test and difference between each group determined by post-hoc test. Difference between male and female participants in each group is determined by unpaired-t test. Correlation of oxidative and nitrosative stress and antioxidant parameters with ageing have done by Pearson's correlation.

4.1.1 One way ANOVA followed by post-hoc test of male participants involving six age groups

Table 8 shows ANOVA results of oxidative and nitrosative stress and antioxidant parameters involving six age groups among male healthy participants.

Table 8: Oxidative and nitrosative stress and antioxidative parameters of male participants

Parameters	Age groups (years)							
	Group I	Group II	Group III	Group IV	Group V	Group VI	ANOVA	
	20-29 years (n=17)	30-39 years (n=17)	40-49 years (n=17)	50-59 years (n=17)	60-69 years (n=17)	70 years plus (n=17)	F' value	p' value
MDA, nmol/L	0.913±0.12^{III,IV,V,VI}	1.003±0.14^{IV,V,VI}	1.3006±0.21^{I}	1.405±0.33^{I,II}	1.457±0.34^{I,II}	1.520±0.23^{I,II}	4.03	0.002*
SOD, U/ml	2.415±0.42^{IV,V,VI}	2.28±0.51^{IV,V,VI}	2.082±0.43^{V,VI}	1.823±0.5^{I,II}	1.621±0.5^{I,II,III}	1.625±0.3^{I,II,III}	5.166	0.000*
GSH, mg/dl	21.015±2.6^{III,IV,V,VI}	20.284±1.9^{V,VI}	19.29±2.26^{I,VI}	18.73±2.0^{I}	17.7±2.3^{I,II}	17.18±2.4^{I,II,III}	6.931	0.000*
Vit C, mg/dl	1.28±0.32^{III,IV,V,VI}	1.214±0.21^{III,IV,V,VI}	1.0±0.2^{I,II,IV,V,VI}	0.833±0.2^{I,II,III}	0.749±0.2^{I,II,III}	0.73±0.13^{I,II,III}	18.201	0.000*
Nox, µmol/L	7.59±1.34^{IV,V,VI}	7.32±1.32^{V,VI}	6.57±1.14^{V,VI}	6.34±1.2^{I,V,VI}	5.16±1.43^{I,II,III,IV}	5.03±1.63^{I,II,III,IV,}	6.333	0.000*

Data are Mean±S.D. Values in the final column represent results of one-way analysis (ANOVA) among different age groups. Post-hoc comparisons were made between each group with LSD method. Level of significance 'p' value <0.05.MDA-Serum malondialdehyde; SOD-Serum superoxide dismutase; GSH-Erythrocyte reduced glutathione; Vit C- serum vitamen C; LDLC-Low density lipoprotein fraction of cholesterol; VLDLC-Very low density lipoprotein fraction of cholesterol.

ANOVA results of our study showed statistically significant increase in MDA (p=0.002) and decrease in SOD (p=0.000), GSH (p=0.000), Vit C (p=0.000) and NOx (p=0.000) levels in male participants with age.

4.1.2 One way ANOVA followed by post-hoc test of female participants involving six age groups

Table 9 shows ANOVA results of oxidative stress and nitrosative stress and antioxidant parameters involving six age groups among female healthy participants.

Table 9: Oxidative and nitrosative stress and antioxidative parameters of female participants

Parameters	Age groups (years)							
	Group I	Group II	Group III	Group IV	Group V	Group VI	ANOVA	
	20-29 years (n=17)	30-39 years (n=17)	40-49 years (n=17)	50-59 years (n=17)	60-69 years (n=17)	70 years plus (n=17)	F' value	p' value
MDA, nmol/L	0.675±0.12^{II,III,IV,V,VI}	0.706±0.14^{III,IV,V,VI}	1.071±0.21^{I,II,III}	1.338±0.33^{I,II,III}	1.414±0.34^{I,II,III}	1.496±0.23^{I,II,III}	20.049	0.000*
SOD, U/ml	2.4047±0.42^{IV,V,VI}	2.24±0.51^{IV,V,VI}	2.03±0.43^{V,VI}	1.73±0.5^{I,II}	1.528±0.5^{I,II,III}	1.488±0.3^{I,II,III}	7.129	0.000*
GSH, mg/dl	20.194±2.6^{IV,V,VI}	19.99±1.6^{IV,V,VI}	19.18±2.22^{V,VI}	18.42±1.79^{I,II}	17.386±2.^{I,II}	17.13±2.4^{I,II,III}	6.08	0.000*
Vit C, mg/dl	1.25±0.31^{III,IV,V,VI}	1.204±0.191^{III,IV,V,VI}	1.0±0.2^{I,II,IV,V,VI}	0.819±0.2^{I,II,III}	0.738±0.17^{I,II,III}	0.685±0.13^{I,II,III}	20.229	0.000*
Nox, µmol/L	7.9±1.25^{III,IV,V,VI}	6.79±1.77^{IV,VI}	6.27±1.07^{I}	5.66±1.41^{I}	5.25±1.33^{I,II}	5.14±1.66^{I,II}	5.382	0.000*

Data are Mean±S.D. Values in the final column represent results of one-way analysis (ANOVA) among different age groups. Post-hoc comparisons were made between each group with LSD method. Level of significance 'p' value <0.05.MDA-Serum malondialdehyde; SOD-Serum superoxide dismutase; GSH-Erythrocyte reduced glutathione; Vit C-serum vitamen C; LDLC-Low density lipoprotein fraction of cholesterol; VLDLC-Very low density lipoprotein fraction of cholesterol.

ANOVA results of our study showed statistically significant increase in MDA (p=0.000) and decrease in SOD (p=0.000), GSH (p=0.000), Vit C (p=0.000) and NOx (p=0.000) levels in female participants with age.

4.1.3 Unpaired 't' test between both male and female participants in each age group

Table 10 shows unpaired 't' test results of oxidative stress and nitrosative stress and antioxidant parameters between both male and female participants in each age group**.**

Table 10: showing unpaired 't' test

Oxidative parameters					
Parameters	**Age in years**	**Male participants**	**Female participants**	**Unpaired t-test**	
				t' value	**p' value**
MDA, nmol/L	**Group I**	0.913±0.12	0.675±0.12	2.877	0.007
	Group II	1.003±0.14	0.706±0.14	2.08	0.046
	Group III	1.3006±0.21	1.071±0.21	0.967	0.341
	Group IV	1.405±0.33	1.338±0.33	0.533	0.598
	Group V	1.457±0.34	1.414±0.34	0.369	0.715
	Group VI	1.520±0.23	1.496±0.23	0.17	0.866
SOD, U/ml	**Group I**	2.415±0.42	2.4047±0.42	0.048	0.962
	Group II	2.28±0.51	2.24±0.51	0.789	0.436
	Group III	2.082±0.43	2.03±0.43	0.251	0.803
	Group IV	1.823±0.5	1.73±0.5	0.482	0.633
	Group V	1.621±0.5	1.528±0.5	0.433	0.668
	Group VI	1.625±0.3	1.488±0.3	0.918	0.365
GSH, mg/dl	**Group I**	21.015±2.6	20.194±2.6	0.903	0.373
	Group II	20.284±1.9	19.99±1.6	0.466	0.645
	Group III	19.29±2.26	19.18±2.22	0.137	0.892
	Group IV	18.73±2.0	18.42±1.79	0.477	0.636
	Group V	17.7±2.3	17.386±2	0.464	0.646
	Group VI	17.18±2.4	17.13±2.4	0.052	0.959
Vit C, mg/dl	**Group I**	1.28±0.32	1.25±0.31	0.209	0.836
	Group II	1.214±0.21	1.204±0.191	0.137	0.892
	Group III	1.0±0.19	1.0±0.2	0.042	0.967
	Group IV	0.833±0.2	0.819±0.2	0.161	0.873
	Group V	0.749±0.2	0.738±0.17	0.26	0.797
	Group VI	0.73±0.13	0.685±0.13	1.061	0.297
Data are Mean±SD. Values in the final column represent results of unpaired t-test between male and female participants. $p<0.05$, considered as statistically significant.					

Unpaired t test did not show any significant ($p>0.05$) difference of oxidative stress and nitrosative stress and antioxidant parameters between both male and female participants in each age group.

4.1.4 Pearson's Correlation between ageing and oxidative stress and nitrosative stress and antioxidant parameters in both male and female participants involving six age groups

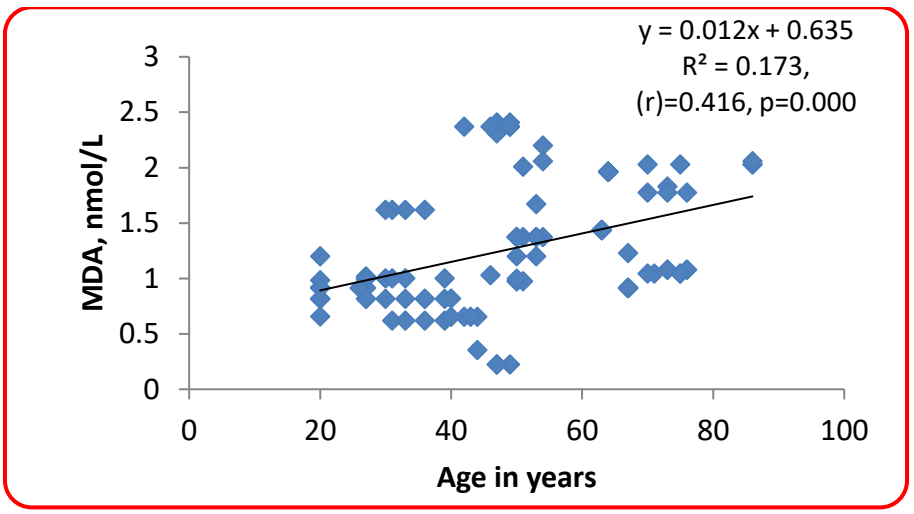

Figure 9: Pearson's Correlation between MDA and ageing among male participants in different Age Groups.

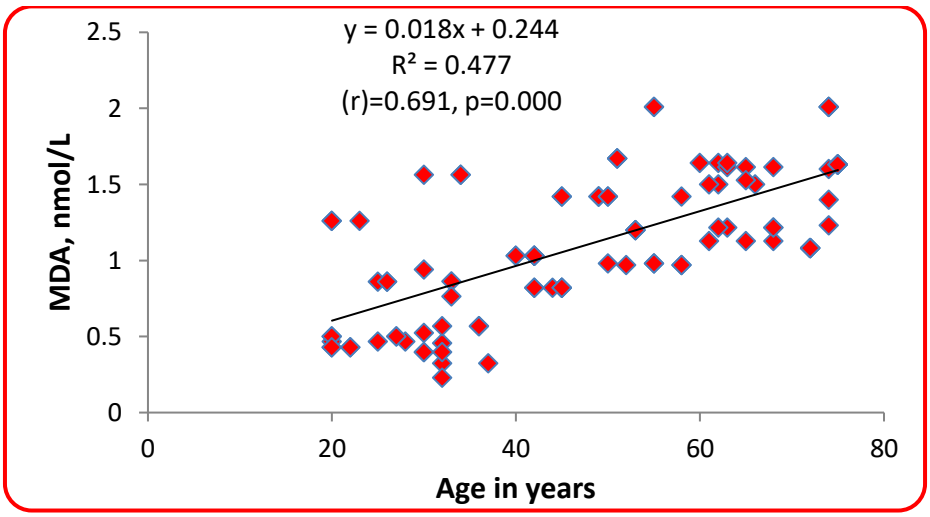

Figure 10: Pearson's Correlation between MDA and ageing among female participants in different Age Groups.

Our results showed statistically significant positive correlation of MDA with age in both male and female participants.

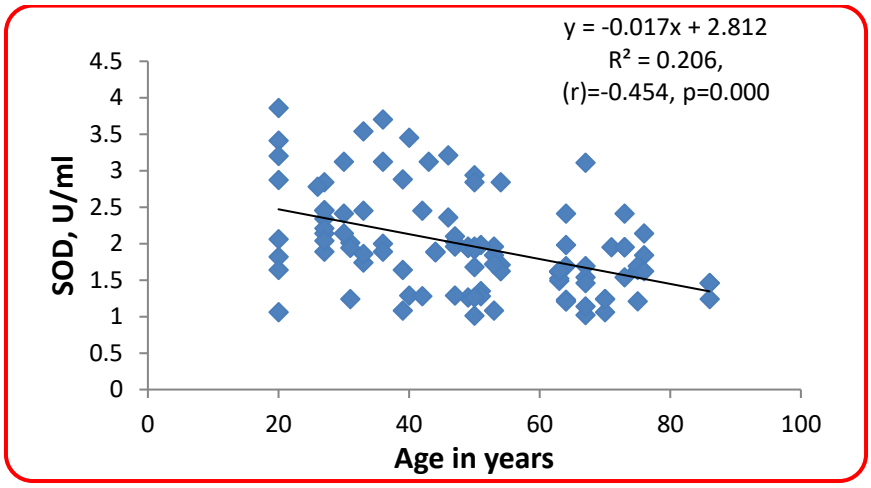

Figure 11: Pearson's Correlation between SOD and ageing among male participants in different Age Groups.

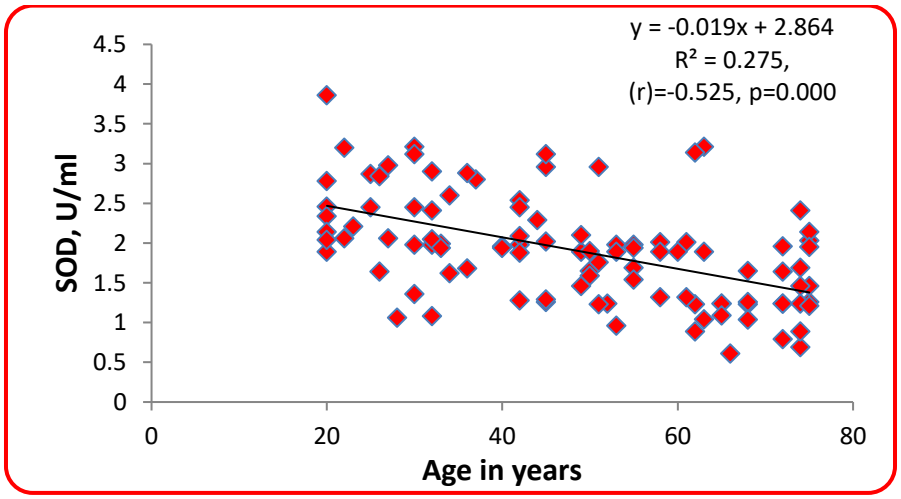

Figure 12: Pearson's Correlation between SOD and ageing among female participants in different Age Groups.

Our results showed statistically significant negative correlation of SOD with age in both male and female participants.

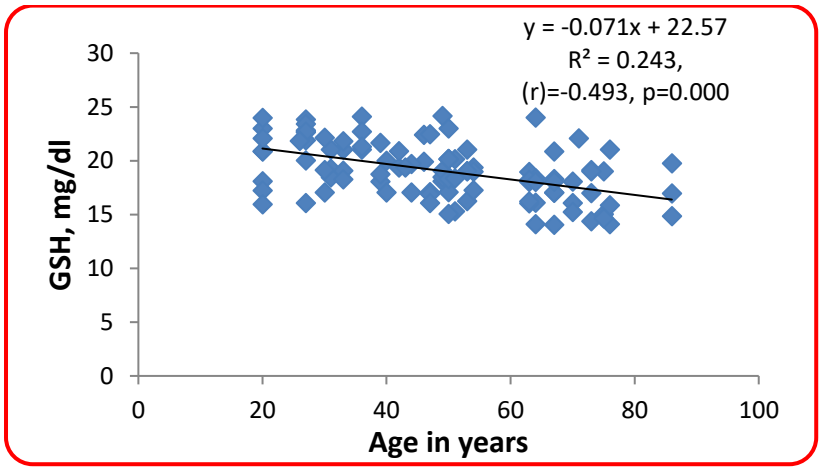

Figure 13: Pearson's Correlation between GSH and ageing among male participants in different Age Groups.

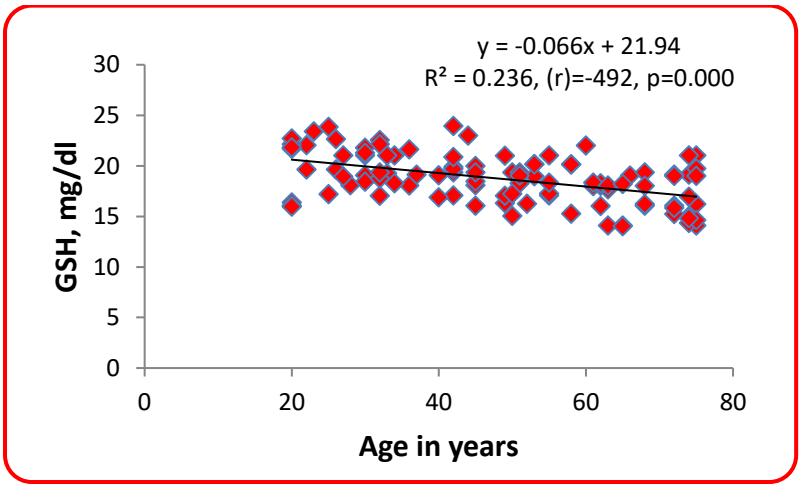

Figure 14: Pearson's Correlation between GSH and ageing among female participants in different Age Groups

Our results showed statistically significant negative correlation of GSH with age in both male and female participants

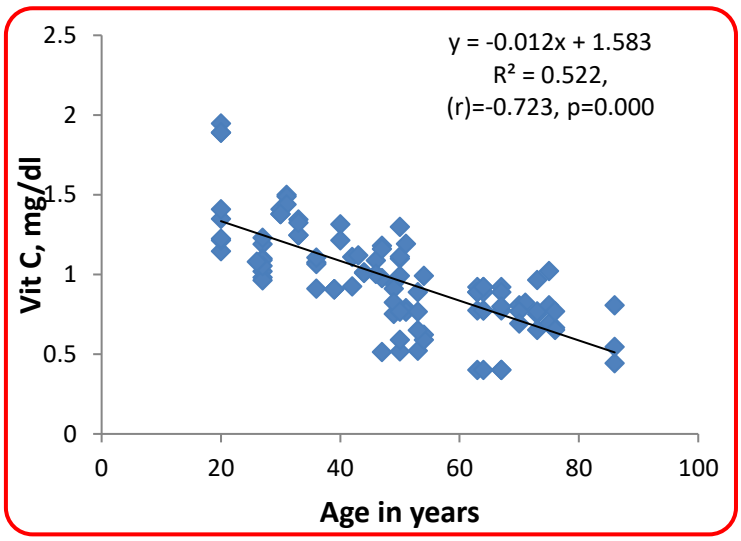

Figure 15: Pearson's Correlation between Vit C and ageing among Male participants in different Age Groups

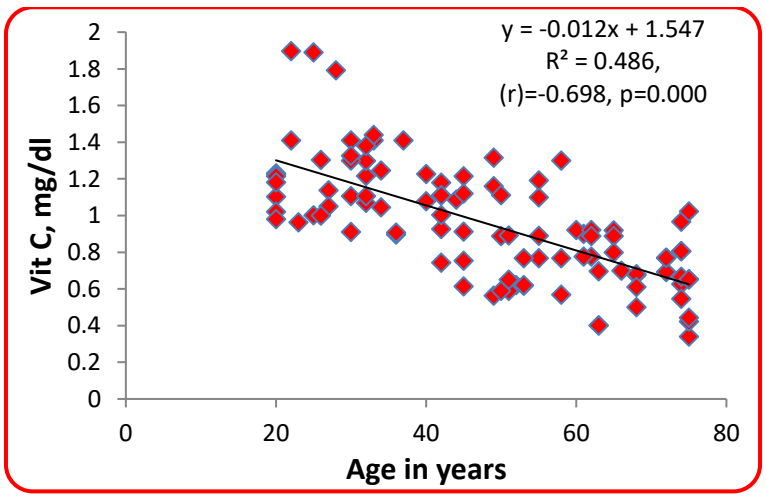

Figure 16: Pearson's Correlation between Vit C and ageing among Female participants in different Age Groups

Our results showed statistically significant negative correlation of Vit C with age in both male and female participants

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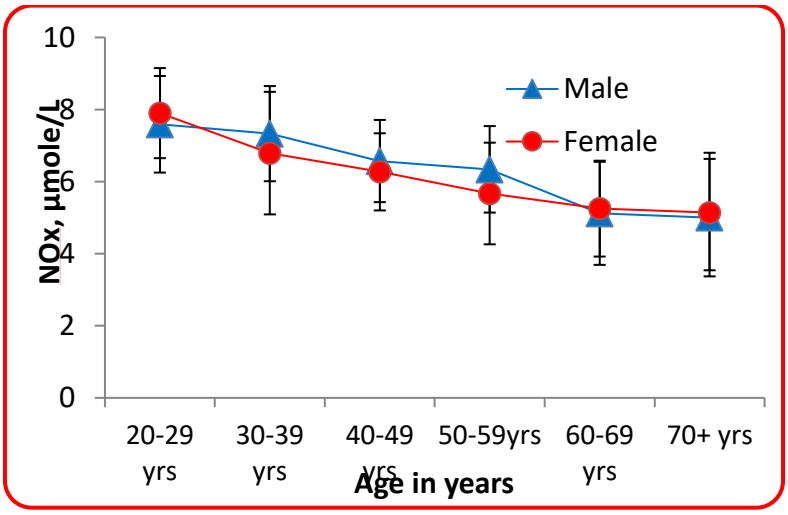

Figure 17: Serum Nitric Oxide (NOx) Level between Males and Females from Different Age Groups. Values are Mean ± SD of Each Age Group

Our results did not show any significant difference of NOx in both male and female participants with age.

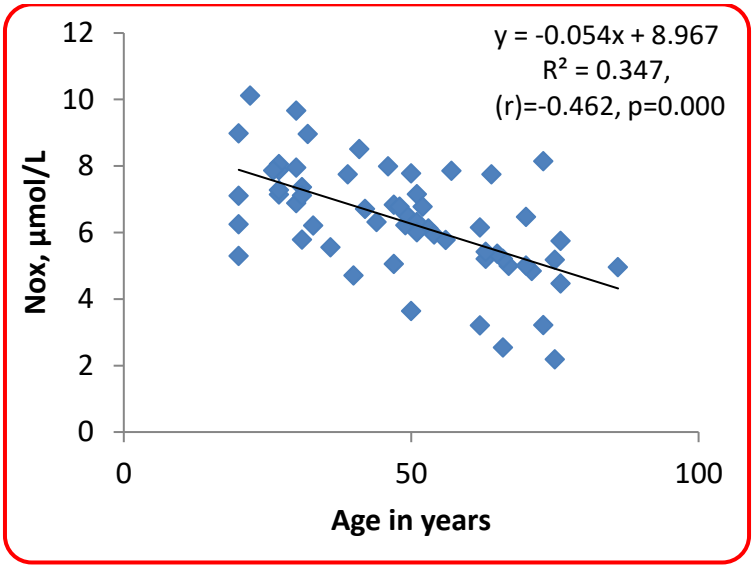

Figure 18: Pearson's Correlation between sNox and ageing among male participants in different age groups.

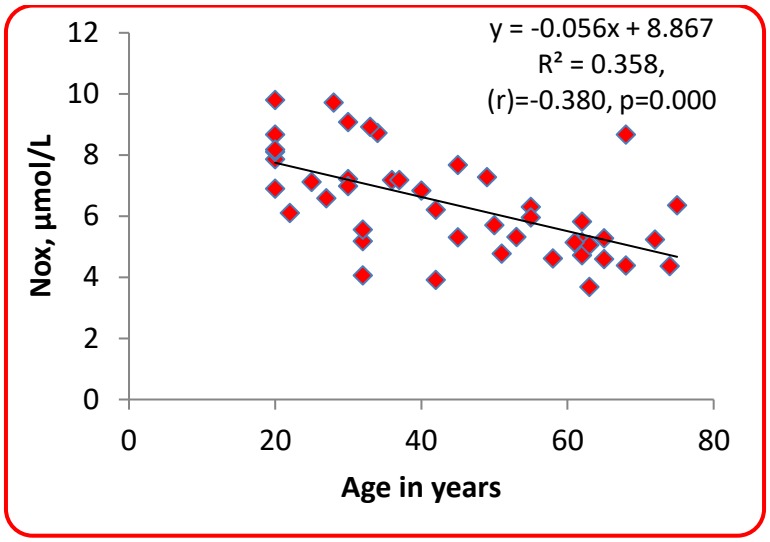

Figure 19: Pearson's Correlation between sNox and ageing among female participants in different age groups.

Our results showed statistically significant negative correlation of NOx with age in both male and female participants

4.2 .Discussion:

MDA is an indicator of oxidative stress. MDA is generated due to reactive oxygen species induced oxidative stress damage the membrane polyunsaturated fatty acids. Increase in MDA with age has been demonstrated (Massudi et al., 2012). In the present study demonstrated statistically significant increase in MDA in both male and female participants with age and results corroborate with Massudi et al (Massudi et al., 2012). This suggests with age lipid peroxidation increases.

Superoxide dismutase (SOD) catalase, glutathione, vitamins C and E are important for antioxidative defense. A decrease in the activity of these antioxidants may contribute to oxidative stress (Ceriello A., 2008). Antioxidants such as SOD, catalase and glutathione act as a primary line of defense against the toxic effects of ROS. Superoxide radicals are detoxified by SOD to produce hydrogen peroxide (H_2O_2) which is further converted to water by catalase and glutathione peroxidase (GSPx). Glutathione peroxidase requires GSH as a coenzyme to convert H_2O_2 to

water (Li H et al., 2013). A negative correlation between age and antioxidants has been found in some studies (Lang et al, 1992; De La Paz et al, 1996). Our results also showed statistical decrease in SOD (Lang et al, 1992), GSH (De La Paz et al, 1996) and Vit C levels in both male and female participants suggests increased oxidative stress with ageing.

Increased oxidative damage with age leads to production of free radicals which include superoxide (O_2^-), hydrogen peroxide (H_2O_2), nitric oxide (NO), and peroxynitrite ($ONOO^-$) (Peinado MA.,1998). Nitric oxide, a potent vasodilator produced by the vascular endothelial cells is a simple molecule that regulates vascular tone, vascular permeability and antithrombotic properties (Jin RC & Loscalzo J., 2010). The endothelial-dependent vasodilator function is reduced with ageing and this impaired NO-mediated vasodilatation is a potential contributor to the age-related increase in arterial stiffness (Wilkinson IB et al., 2002; Fitch RM et al., 2001). According to Peinado MA, free radicals do not have deleterious effects on the human body in a general environment; the enhanced production of NO aggravates ageing process in the CNS (Peinado MA., 1998). The results from the present study on age associated gradual decrease of serum NOx in both male and female participants indicate a reduction of bioavailability of NOx as age advances (Massimo et al, 2006). Interestingly our results differ from another observation where increase of serum NOx was found as age advances from 50 years onwards in both male and female participants (Ahimastos et al, 2003). Also found there is no significant difference in sNOx level in males and females and our results corroborates with study by Ahimastos et al (Ahimastos et al, 2003). As NO plays a significant role in normal ageing, some other studies also showed increased NO production during ageing (Law A et al., 2001; McCann SM et al., 1988; Calabrese V et al., 2000).

There is a strong association between oxidative stress and endothelial dysfunction. Age-associated increase in vascular oxidative stress damages the endothelium and reduces its NO production. It also contributes to inactivation of NO. Thus, finally resulting in reduction in bioavailability of NO and endothelial dysfunction with ageing (Schulz E et al., 2011; Silva BR et al., 2012).

4.3. References:

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5 . Influence of age on vascular functions

5.1 Results:

The difference between the influences of age on vascular functions among both male female participants involving six age groups was determined by ANOVA test and difference between each group determined by post-hoc test. Difference between male and female participants in each group is determined by unpaired-t test. Correlation of vascular functions with ageing has done by Pearson's correlation.

5.1.1 Brachial-ankle pulse wave velocity (b-a PWV) and Carotid-Femoral pulse wave velocity (c-f PWV) between males and females from different age groups

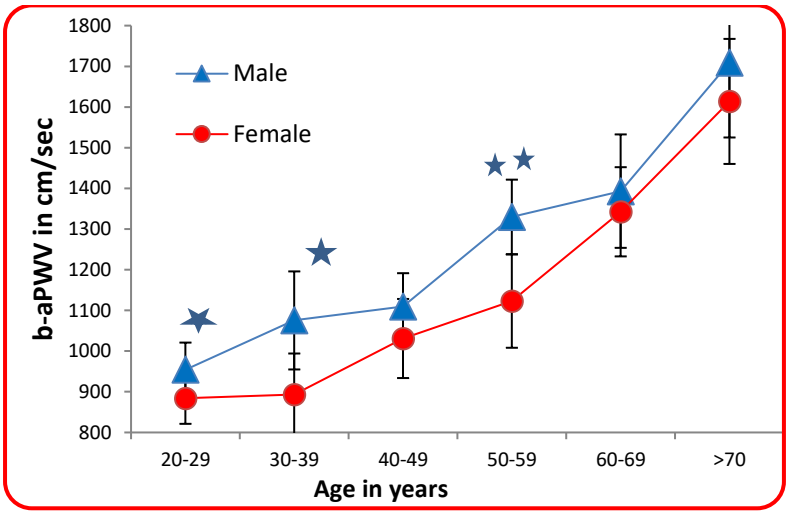

Figure 20: Brachial-ankle pulse wave velocity (b-a PWV) between males and females from different age groups. Values are mean±SD of each age group. *p<0.05, ** p<0.01 while Comparing Male and Female Values

Figure 20 shows values of mean and SD of b-a PWV among both male and female participants in different age groups. We observed that age dependent increase in b-a PWV among both male and female participants. Also we observed that significant (*p<0.05, ** p<0.01) higher values of b-a PWV in males compared to females till group IV after that we observed higher values in males but not significant (p>0.05).

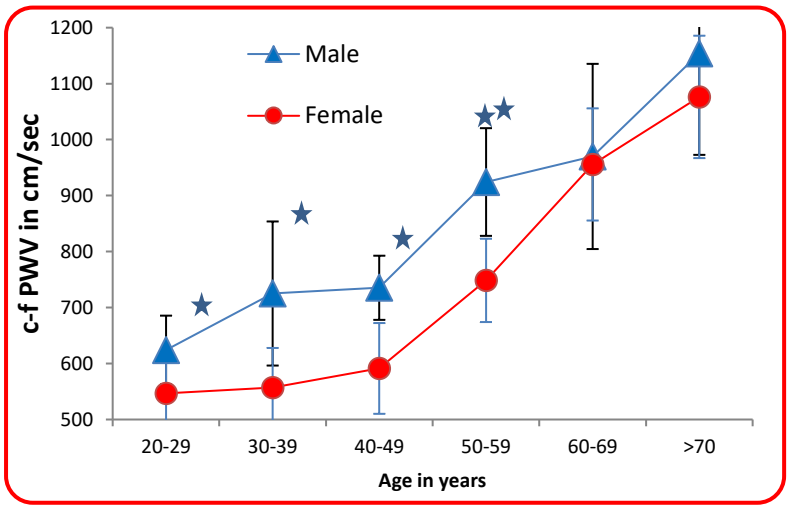

Figure 21: Carotid-femoral pulse wave velocity (c-f PWV) between males and females from different age groups. Values are mean±SD of each age group. *p<0.05, ** p<0.01 while Comparing Male and Female Values

Figure 21 shows values of mean and SD of c-f PWV among both male and female participants in different age groups. We observed that age dependent increase in c-f PWV among both male and female participants. Also we observed that significant (*p<0.05, ** p<0.01) higher values of c-f PWV in males compared to females till group IV after that we observed higher values in males but not significant (p>0.05)

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5.1.2 Pearson's Correlation between b-a PWV and c-f PWV with ageing among male and female participants in different age groups

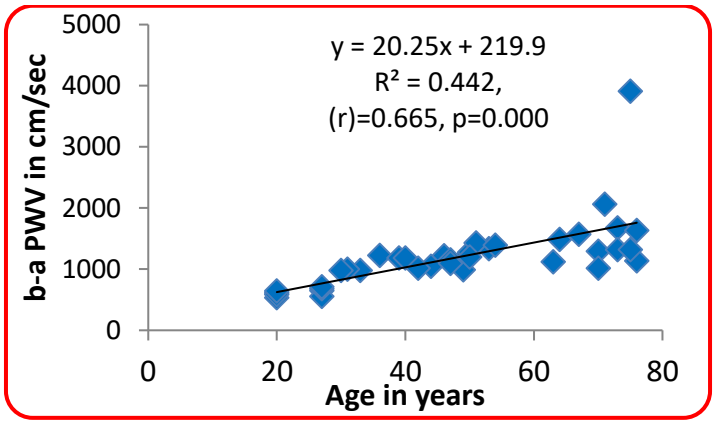

Figure 22: Pearson's Correlation between b-a PWV and ageing among male participants in different age groups.

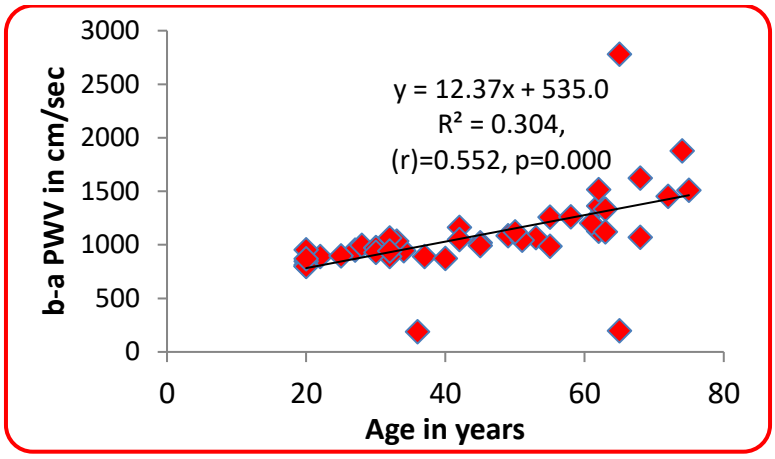

Figure 23: Pearson's Correlation between b-a PWV and ageing among female participants in different age groups.

Figure 22 and figure 23 showed statistically significant positive correlation between b-a PWV with age among both male (r=0.665, p=0.000) and female (r=0.552, p=0.000). We observed with age b-a PWV increases.

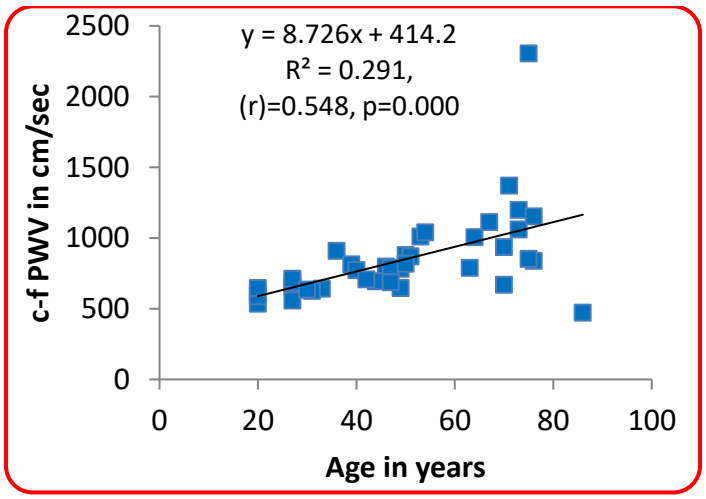

Figure 24: Pearson's Correlation between c-f PWV and ageing among male participants in different age groups.

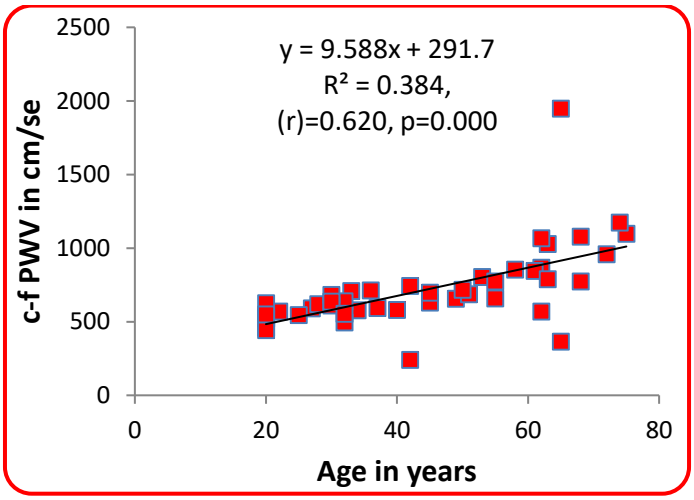

Figure 25: Pearson's Correlation between c-f PWV and ageing among female participants in different age groups.

Figure 24 and figure 25 showed statistically significant positive correlation between c-f PWV with age among both male (r=0.548, p=0.000) and female (r=0.620, p=0.000). We observed with age c-f PWV increases.

5.1.3 Brachial arterial stiffness index (bASI) and Ankle arterial stiffness index (aASI) between male and female participants from different age groups.

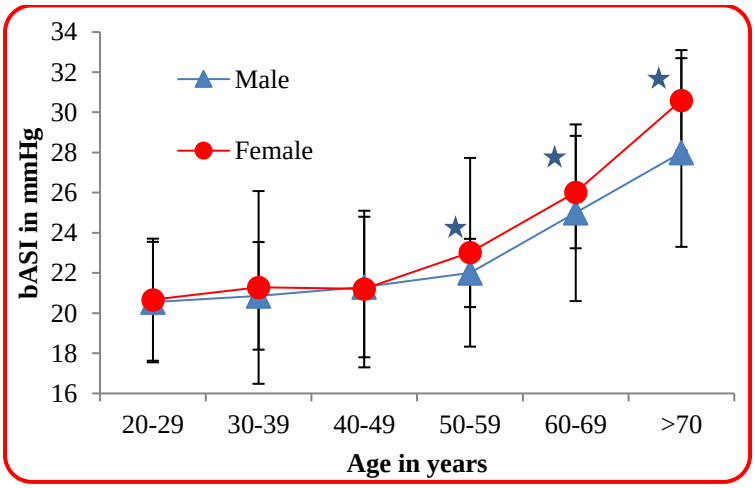

Figure 26: Brachial arterial stiffness index (bASI) between male and female participants from different age groups. Values are mean±SD of each age group in male and female participants. . *p<0.05, ** p<0.01 while comparing Male and Female Values

Figure 26 shows values of mean and SD of b-ASI among both male and female participants in different age groups. We observed that age dependent increase in bASI among both male and female participants.

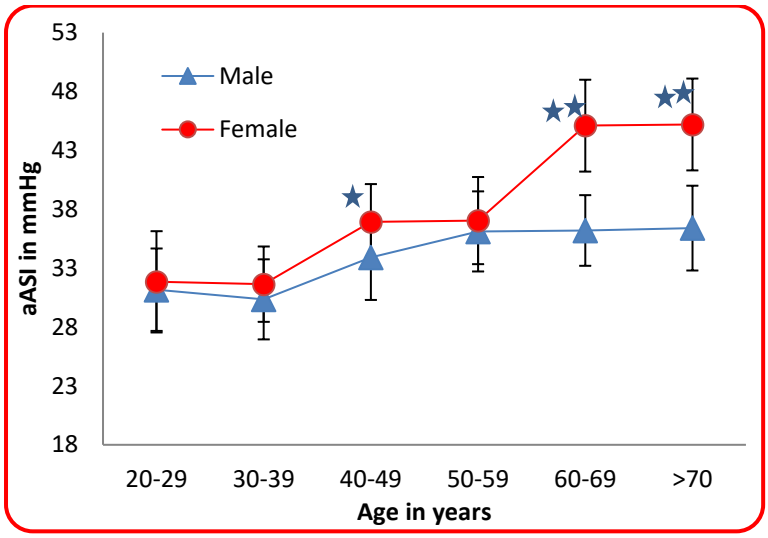

Figure 27: Ankle arterial stiffness index (aASI) between male and female participants from different age groups. Values are mean±SD of each age group in male and female participants. . *p<0.05, ** p<0.01 while Comparing Male and Female Values

Figure 27 shows values of mean and SD of aASI among both male and female participants in different age groups. We observed that age dependent increase in aASI among both male and female participants.

5.1.4 Augmentation index heart rate @ 75 (Aix @75, %) between male and female participants from different age groups.

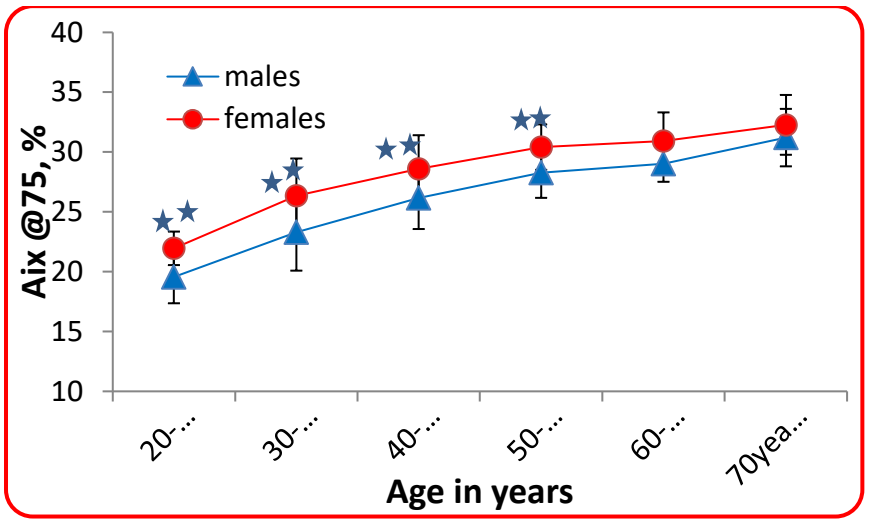

Figure 28: Augmentation index heart rate @ 75 (Aix @75, %) between male and female participants from different age groups. Values are mean±SD of each age group in male and female participants. . *p<0.05, ** p<0.01 while Comparing Male and Female Values

Figure 28 shows values of mean and SD of Aix @75 among both male and female participants in different age groups. We observed that age dependent increase in Aix @75 among both male and female participants.

5.1.5 Pearson's Correlation between Aix@75 and ageing among male and female participants in different age groups

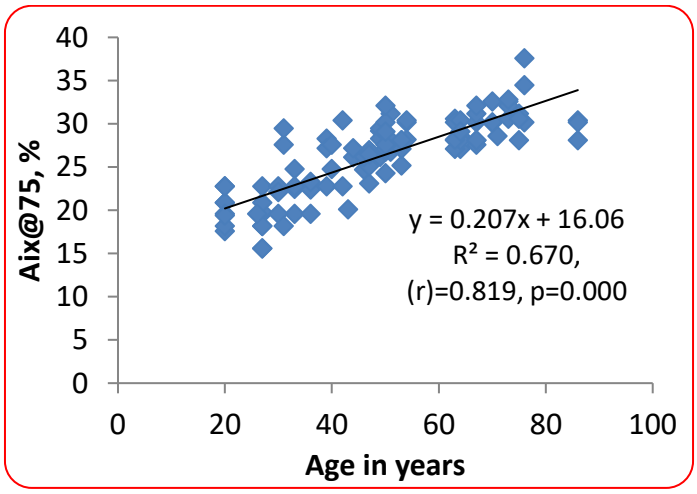

Figure 29: Pearson's Correlation between Aix@75 and ageing among male participants in different age groups.

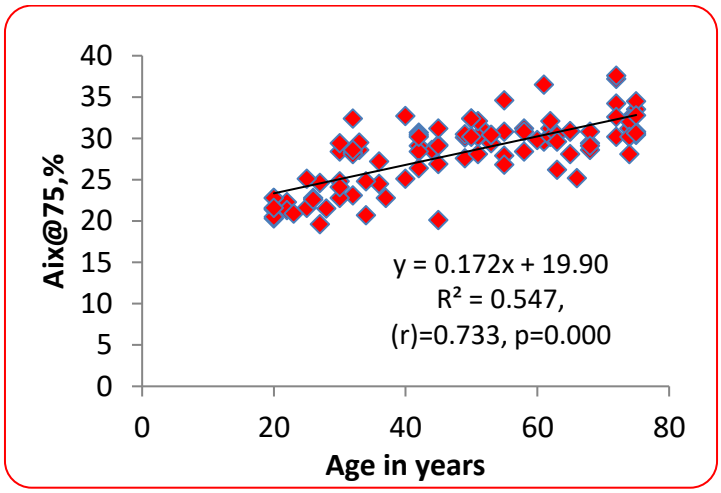

Figure 30: Pearson's Correlation between Aix@75 and ageing among female participants in different age groups.

Figure 29 and figure 30 showed statistically significant positive correlation between Aix@75 with among both male (r=0.819, p=0.000) and female (r=0.547, p=0.000) participants. We observed with age Aix@75 increases.

5.2 Discussion:

Age is one of the most powerful determinants of cardiovascular risk and is associated with a number of deleterious changes in the cardiovascular system (Lakatta E.G and Levy D., 2003). Arterial stiffness has become an increasingly important biomarker in the evaluation of CV risk. Pulse wave velocity (PWV) and the augmentation index (AIx) are the 2 major non-invasive methods of assessing arterial stiffness. PWV reflects the elasticity of the segmental artery. Cardiac contraction generates a pulse wave, which is propagated distally to the extremities. PWV is calculated as the distance traveled by the pulse wave divided by the time taken to travel the distance (Laurent S et al., 2006). Increased arterial stiffness results in increased speed of the pulse wave in the artery. PWV can be measured in any arterial segment between 2 regions. The b-a PWV is a measure of central elastic and muscular

arterial stiffness and is strongly correlated with c-f PWV, a measure of aortic stiffness (Yamashina A et al., 2002). A number of studies have investigated the effects of age on aortic PWV and AIx (Avolio AP et al., 1985; Hayward CS and Kelly RP., 1997; Smulyan H et al., 2001; Mitchell GF et al., 2004). Most suggest a linear, age-related increase in both indexes across a variety of different populations. Results from our study showed age dependent increase in both b-a PWV and c-f PWV of males and females which correlate with the study of McEniery and Hall (McEniery and Hall., 2005). Our study consistently showed a higher b-a PWV and c-f PWV in males as compare to females in all the age groups but some studies showed a differential changes of PWV in males and females due to post menopausal physiology in females (Alecu C et al., 2006; Liu H et al., 2005). One possible explanation for this is elastin fatigue fracture and degradation, with a consequent increased loading on stiffer collagen fibers (O'Rourke MF., 1976). In addition, there is a marked increase in calcification of the aortic media with age, particularly after the fifth decade, that might also to contribute to a loss of arterial distensibility (Yu SY and Blumenthal HT., 1963).

Age related increase in bASI and aASI of both male and female participants in present study reflect a possible brachial and tibial artery stiffness (Patil SG et al., 2015). Age related increase in arterial stiffness of the present study further indicate the rigidity of the vascular wall possibly due to altered biochemical and histopathological architecture in arterial wall (Munakata M et al., 2014). Disarrangement of elastic laminae along with increased in collagen fibers and connective tissues may be other reasons behind increase in arterial stiffness (Wolinsky H et al., 1985).

Significant increase of bASI and aASI in females after fifty years (group V and group VI) in the present study may be due to post menopausal hormonal profile (Tomiyama H et al., 2003). It has further been observed that vessel diameter increased due to arterial stiffness could lead to generate an increase in tensile stress and make the vessel more susceptible to risk factors for increased inflammation (Wildman RP et al., 2008). It has also been reported that PWV and aortic stiffness index of women are good indicators to assess age dependent cardiovascular risk factors (Kim JY et al., 2014).

The augmentation index (AIx) is an indirect measure of arterial stiffness and increases with age. Our results showed age dependent statistical (p=0.000) increase in Aix in both male and participants and our results corroborate with study Chung et al (Chung et al., 2010). Our results showed consistently high values of Aix in female participants compared to male participants in all the age groups (Chung et al., 2010).

5.3 References:

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6 . Influence of age on oxygen sensing molecular markers

6.1 Results:

The difference between the influences of age on oxygen sensing molecular markers among both male female participants involving six age groups was determined by ANOVA test and difference between each group determined by post-hoc test.

6.1.1 One way ANOVA followed by post-hoc test of male participants involving six age groups

Table 11 shows ANOVA results of oxygen sensing molecular markers involving six age groups among male healthy participants.

Table 11: oxygen sensing molecular markers of male participants

Parameters	Age groups (years), in males							
	Group I	**Group II**	**Group III**	**Group IV**	**Group V**	**Group VI**	**ANOVA**	
	20-29 years (n=17)	**30-39 years (n=17)**	**40-49 years (n=17)**	**50-59 years (n=17)**	**60-69 years (n=17)**	**70 years plus (n=17)**	**F' value**	**p' value**
Epo, IU/L	8.51±0.6^{IV,V,VI}	8.15±0.9^{V,VI}	8.07±0.2^{V,VI}	7.86±0.8^{I,V,VI}	6.07±0.69^{I,II,III,IV}	6.17±0.66^{I,II,III,IV}	23.332	0.000*
VEGF, pg/ml	277.8±30.8^{II,III,IV,V,VI}	328.41±32.54^{I,III,IV,V,VI}	400.6±68.5^{I,II,,V,VI}	430.0±58.21^{I,II,III,V,VI}	485.7±14.92^{I,II,III,IV,VI}	626.6±47.2^{I,II,III,IV,V}	122.53	0.000*

Data are Mean±S.D. Values in the final column represent results of one-way analysis (ANOVA) among different age groups. Post-hoc comparisons were made between each group with LSD method. Level of significance 'p' value <0.05. Epo- Erythropoeitin; VEGF-Vascular endothelial growth factor

ANOVA results of our study showed statistically significant decrease in Epo (p=0.000) and increase in VEGF (p=0.000) levels in male participants with age.

6.1.2 One way ANOVA followed by post-hoc test of female participants involving six age groups

Table 12 shows ANOVA results of oxygen sensing molecular markers involving six age groups among female healthy participants.

Table 12: oxygen sensing molecular markers of female participants

Parameters	Age groups (years), in females							
	Group I	**Group II**	**Group III**	**Group IV**	**Group V**	**Group VI**	**ANOVA**	
	20-29 years (n=17)	**30-39 years (n=17)**	**40-49 years (n=17)**	**50-59 years (n=17)**	**60-69 years (n=17)**	**70 years plus (n=17)**	**F' value**	**p' value**
Epo, IU/L	7.66±0.9^{IV,V,VI}	7.48±0.3^{V,VI}	7.38+0.2^{V,VI}	7.2±0.2^{V,VI}	6.05±0.5^{I,II,III,IV}	6.01±0.4^{I,II,III,IV}	21.02	0.000*
VEGF, pg/ml	429.78±26.828^{II,III,IV,V,VI}	555.4±33^{I,III,IV,V,VI}	597.64±20.2^{I,II,,IV,V,VI}	648.92±22.1^{I,II,III,VI}	675.74±30.3^{I,II,III,VI}	722.2±76.7^{I,II,III,IV,V}	115.49	0.000*

Data are Mean±S.D. Values in the final column represent results of one-way analysis (ANOVA) among different age groups. Post-hoc comparisons were made between each group with LSD method. Level of significance 'p' value <0.05. Epo- Erythropoeitin; VEGF-Vascular endothelial growth factor

ANOVA results of our study showed statistically significant decrease in Epo (p=0.000) and increase in VEGF (p=0.000) levels in female participants with age.

Table 13: ANOVA in male participants among different age groups

		Sum of Squares	**df**	**Mean Square**	**F**	**Sig.**
EPO, IU/L	**Between Groups**	96.71	5	19.342	23.332	0.000*
	Within Groups	79.584	96	0.829		
	Total	176.294	101			
VEGF, pg/ml	**Between Groups**	1281451	5	256290.3	122.537	0.000*
	Within Groups	200786.6	96	2091.527		
	Total	1482238	101			

Table 14: ANOVA in female participants among different age groups

		Sum of Squares	df	Mean Square	F	Sig.
EPO, IU/L	**Between Groups**	22.735	5	4.547	21.02	0.000*
	Within Groups	20.766	96	0.216		
	Total	43.501	101			
VEGF, pg/ml	**Between Groups**	915945.58	5	183189.1	115.486	0.000*
	Within Groups	152279.94	96	1586.249		
	Total	1068225.5	101			

6.1.3 Serum erythropoietin (Epo) level between males and females from different age groups

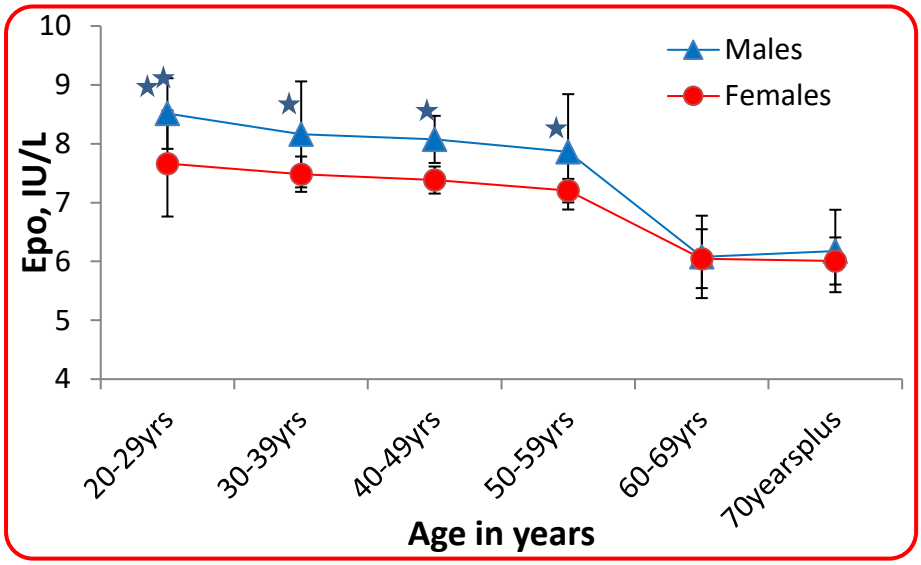

Figure 31: Serum erythropoietin (Epo) level between male and female participants from different age groups. Values are mean±SD of each age group in male and female subject. *p<0.05, ** p<0.01 while Comparing Male and Female Values

Figure 31 shows values of mean and SD of Epo among both male and female participants in different age groups. We observed that age dependent decrease in Epo among both male and female participants. We observed consistent high value of Epo among male participants compare to female participants till 60 years after that Epo level becomes almost same in both male and female participants.

6.1.4 Pearson's Correlation between Epo and ageing among male and female participants in different age groups

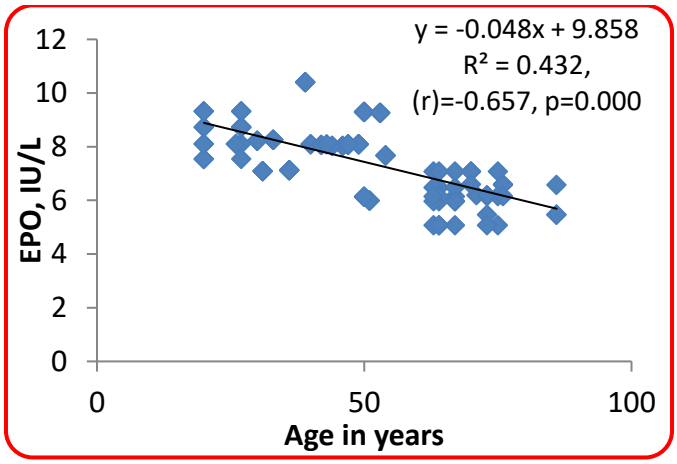

Figure 32: Pearson's Correlation between EPO and ageing among male and participants in different age groups.

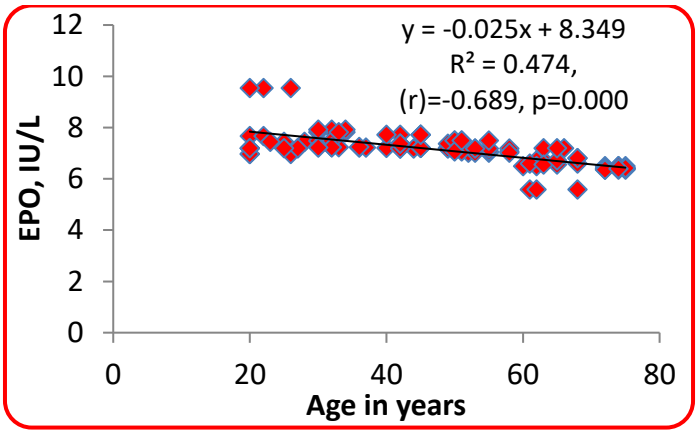

Figure 33: Pearson's Correlation between EPO and ageing among female and participants in different age groups.

Figure 32 and figure 33 showed statistically significant negative correlation between Epo with among both male (r=-0.657, p=0.000) and female (r=-0.689, p=0.000). We observed with age Epo decreases.

6.1.5 Serum vascular endothelial growth factor (VEGF) level among males and females from different age groups.

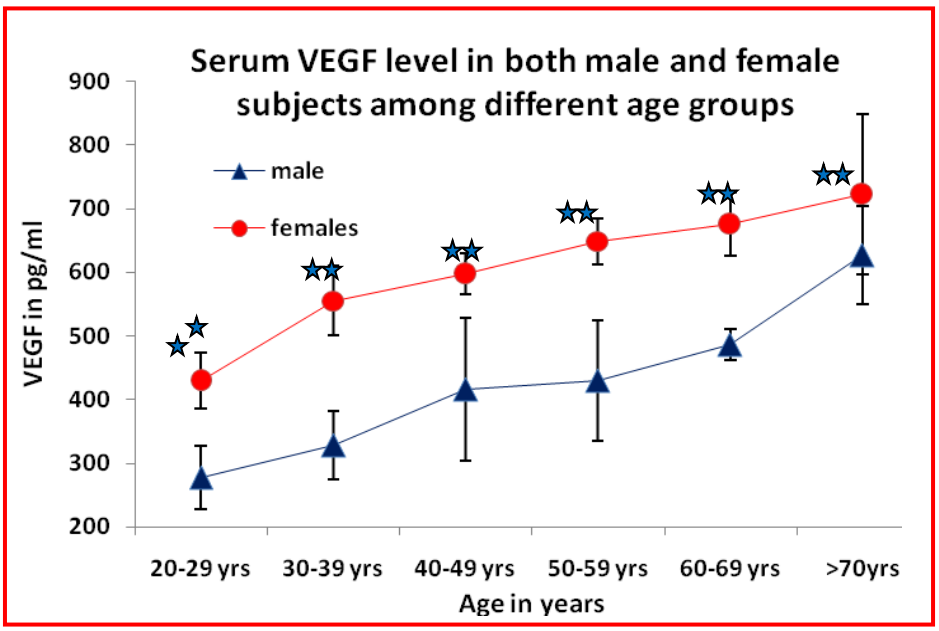

Figure 34: Serum vascular endothelial growth factor (VEGF) level between males and females from different age groups. Values are mean±SD of each age group. *p<0.05, ** p<0.01 while Comparing Male and Female Values

Figure 34 shows values of mean and SD of VEGF among both male and female participants in different age groups. We observed that age dependent increase in VEGF among both male and female participants. We observed consistent high value of VEGF in all the groups among male participants compare to female participants.

6.1.6 Pearson's Correlation between VEGF and ageing among male and female participants in different age groups

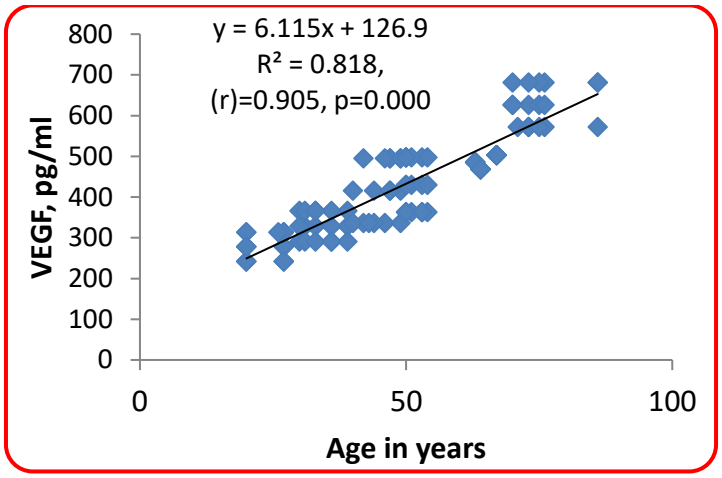

Figure 35: Pearson's Correlation between VEGF and ageing among male participants in different age groups.

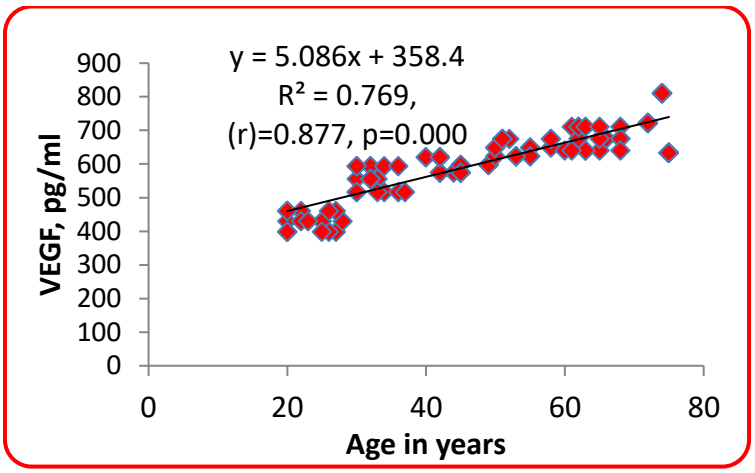

Figure 36: Pearson's Correlation between VEGF and ageing among male participants in different age groups.

Figure 35 and figure 36 showed statistically significant positive correlation between VEGF with age among both male (r=0.905, p=0.000) and female (r=0.769, p=0.000). We observed with age VEGF increases.

6.2. Discussion:

Epo is the principal physiological mediator of hypoxic induction of erythropoiesis. The Epo production is regulated by an oxygen sensor which measures oxygen supply and that it stimulates erythropoiesis (Jacobson et al., 1957). Significant decrease of serum Epo level in present study in higher age groups i.e. group V(60-69 years) and VI(70 years plus) of both males and females indicate a possibility of vasoconstriction and angiogenesis (Elliott S and Sinclair AM., 2012; Hosseini-Zare MS et al., 2012). The results from the present study on serum erythropoietin differs from the study of Musso et al (2004) where unaltered Epo levels were noticed in relation to ageing and the study of Ershler et al (2005) where increased Epo in relation to ageing were found (Musso et al., 2004; Ershler et al., 2005). In our study age associated with low Epo but unchanged erythrocyte count and Hb concentration indicate no serious pathophysiological impact in haematopoiesis on ageing.

Rivard A et al, have demonstrated that advanced age is associated with a defect in compensatory neovascularization in response to tissue ischemia (Rivard A et al., 1999). Such impaired angiogenesis in ischemic tissues of old animals was found to be associated with reduced expression of vascular endothelial growth factor (VEGF), an endothelial-specific growth factor that is essential for embryonic (Carmeliet P et al., 1996; Ferrara N et al., 1996) and postnatal (Ferrara N et al., 1997) neovascularization. The molecular alterations responsible for this age-dependent decline in VEGF expression, however, have not been elucidated. Among those factors that have been implicated in the regulation of VEGF expression, hypoxia appears to play a major role, both *in vitro* (Shweiki D et al., 1992) and *in vivo* (Banai S et al., 1994). The transcriptional and post-transcriptional mechanisms involved in the

hypoxic regulation of VEGF are similar to those factors responsible for erythropoietin (Epo) expression.

Our results showed an increase in VEGF as age increases in both male and female participants where as in case of females the concentration of VEGF remained consistently higher in all the age groups (20-70 plus years) as compared to their male counterparts. The results indicate a greater angiogenesis in females in all the age groups which may be considered as greater protection against vascular ageing due to VEGF induced angiogenesis. Our results corroborated with study by Malamitsi-Puchner et al (Malamitsi-Puchner et al.,2000). Impaired angiogenesis with reduced VEGF expression is found to be associated with ageing (Ahluwalia A et al, 2014). Higher VEGF expression in ageing may also be due to greater expression of oxygen sensing gene HIF-1α to combat age associated alteration of VEGF expression (Rivard A et al, 2000). Hence the results from our study clearly indicate age associated greater vascular stability in female as compared to male counterparts. Decreased VEGF secretion among male participants in all the age groups as compared to females clearly indicate lower angiogenesis or there may be possibly greater impairment of oxygen sensing mechanism in vascular system which leads to vascular integrity.

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7 . Impact of ageing on vascular function and oxygen sensing mechanism.

7.1 Impact of ageing on vascular function and oxygen sensing mechanism among male participants from different age groups

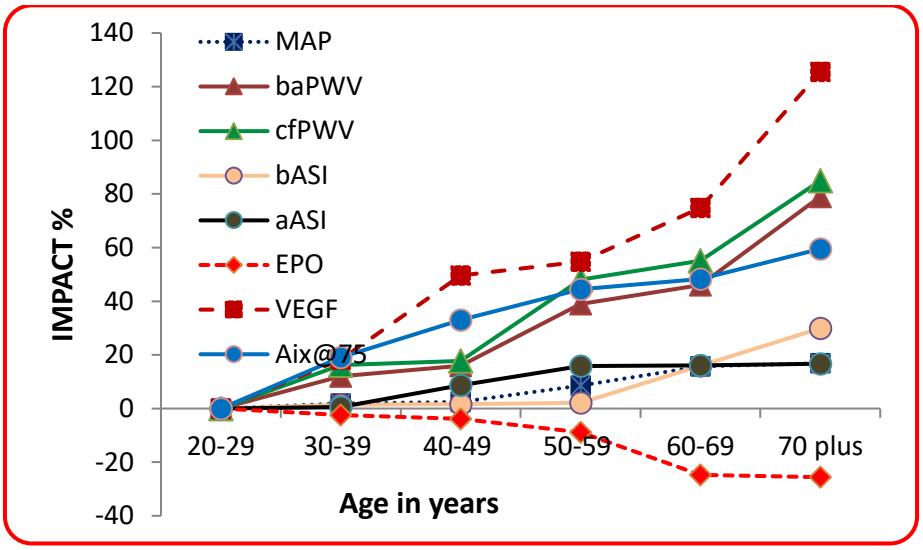


Figure 37: Indicate Impact of ageing on vascular function and oxygen sensing mechanism among male participants from different age groups. Values are percentage (%) of each parameter of each age group in male participants

Figure 37 shows the impact of ageing on vascular functions and oxygen sensing molecular markers. We observed percent increase in VEGF, b-a PWV and c-f PWV is more with age than ASI and MAP. Also observed percent decrease in Epo with age in male participants.

7.2 Impact of ageing on vascular function and oxygen sensing mechanism among female participants from different age groups.

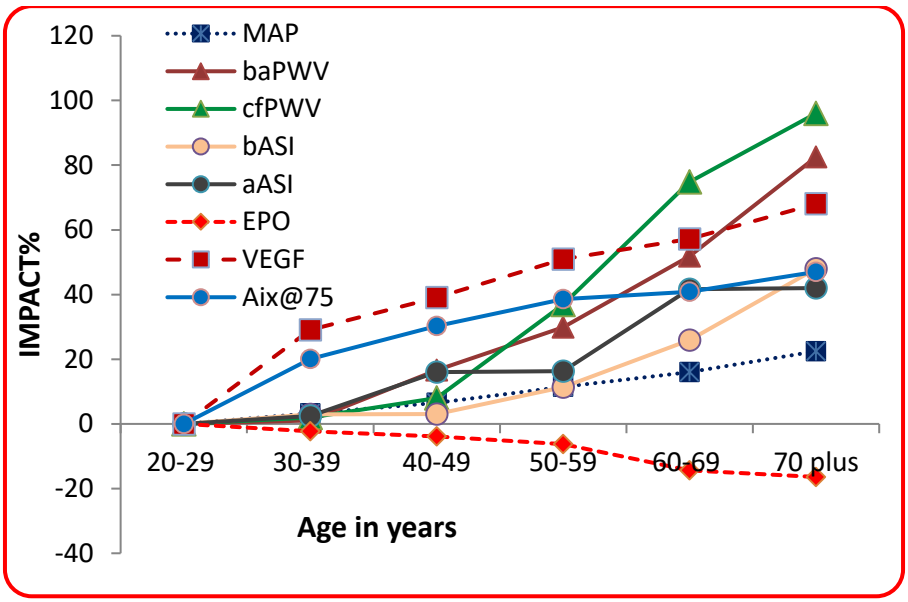


Figure 38: Indicate Impact of ageing on vascular function and oxygen sensing mechanism among male participants from different age groups. Values are percentage (%) of each parameter of each age group in female participants

Figure 38 shows the impact of ageing on vascular functions and oxygen sensing molecular markers. We observed percent increase in VEGF, b-a PWV and c-f PWV is more with age than ASI and MAP. Also observed percent decrease in Epo with age in male participants.

7.3 Discussion:

PWV may be considered as more reliable marker than MAP to evaluate age associated arterial stiffness (Yamashina A et al., 2002). Higher serum VEGF levels with ageing both in females and males indicate increased rates of angiogenesis. Oxygen sensitive Epo might be playing a crucial homeostatic role in ageing.

7.4 References:

- Yamashina A, Tomiyama H, Takeda K, Tsuda H, Arai T, Hirose K, Koji Y, Hori S, Yamamoto Y. Validity, reproducibility, and clinical significance of noninvasive brachial-ankle pulse wave velocity measurement. Hypertens Res. 2002;25(3):359-64.

CHAPTER VI

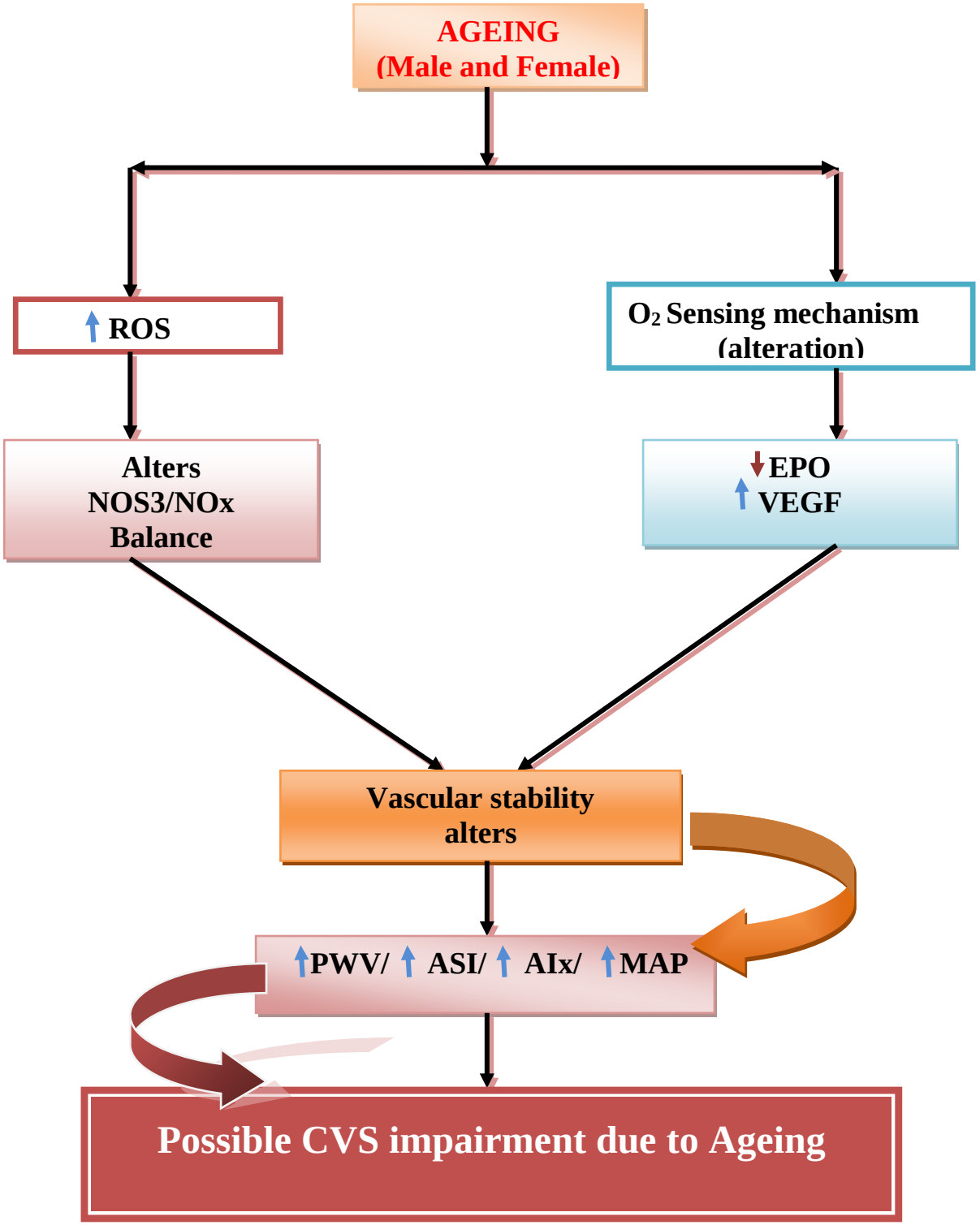
SUMMARY AND CONCLUSION

Summary and Conclusion:

- The purpose of the present study was to assess the influence of oxygen tension or oxygen microenvironment *in vivo* including oxidative stress on age and gender associated changes in vascular health among general population of Vijayapur urban area.
- We hypothesized that there is relationship between oxygen sensing mechanism and ageing in both male and female participants. Further sex has influences on age related changes in vascular physiology and oxygen sensing cell signaling mechanism.
- The following parameters were tested: Anthropometric parameters: height (cms), weight (kg), 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; biochemical parameters: fasting blood glucose (FBS), serum triglyceride, serum cholesterol, serum HDL, serum LDL, serum VLDL; Arterial stiffness parameters: Brachial-ankle pulse wave velocity (baPWV), carotid-femoral pulse wave velocity (c-f PWV), augmentation index (AIx@75), arterial stiffness index at brachial (bASI) and tibial arteries (aASI); Oxidative and nitrosative stress measure: serum malondialdehyde (MDA), serum nitric oxide (sNOx) concentration; and Antioxidant capacity: serum superoxide dismutase (SOD) activity, erythrocyte reduced glutathione (GSH), serum ascorbic acid or vitamin C; Oxygen sensing molecular markers: serum erythropoietin (Epo) and vascular endothelial growth factor (VEGF).

- Possibly this study is one of the latest research on assessing the relationship between oxygen sensing mechanism and ageing among urban population in India.
- Age associated increased PWV, ASI, AIx and MAP in both male and females in the present study clearly showed altered vascular functions in ageing. PWV may be considered as more reliable marker than MAP to evaluate age associated arterial stiffness.
- Increased MDA and decreased SOD, GSH and Vit C levels in both males and females in association with age shows increased oxidative stress.
- Decreased serum NOx level in both males and females in association with age shows possible functional alterations of vascular homeostasis.
- Decreased Epo level with ageing among both male and female participants indicates oxygen sensitive Epo might be playing a crucial homeostatic role in ageing.
- Ageing alters serum VEGF and causes vascular dysfunction. Higher serum VEGF levels with ageing both in females and males indicate increased rates of angiogenesis. Females have an augmented protection against age related alteration of vascular pathophysiology due to greater VEGF concentration as compared to their male counterparts.
- Understanding of these mechanisms may support greater pharmaco-physiological understanding of arterial stiffness which may possibly improve cardiovascular health of an individual irrespective to their sex.
- Endothelial function is not the final protection for arterial function as role of VEGF and its expression in arterial smooth muscles is very important for vascular stability.

GRAPHICAL ABSTRACT

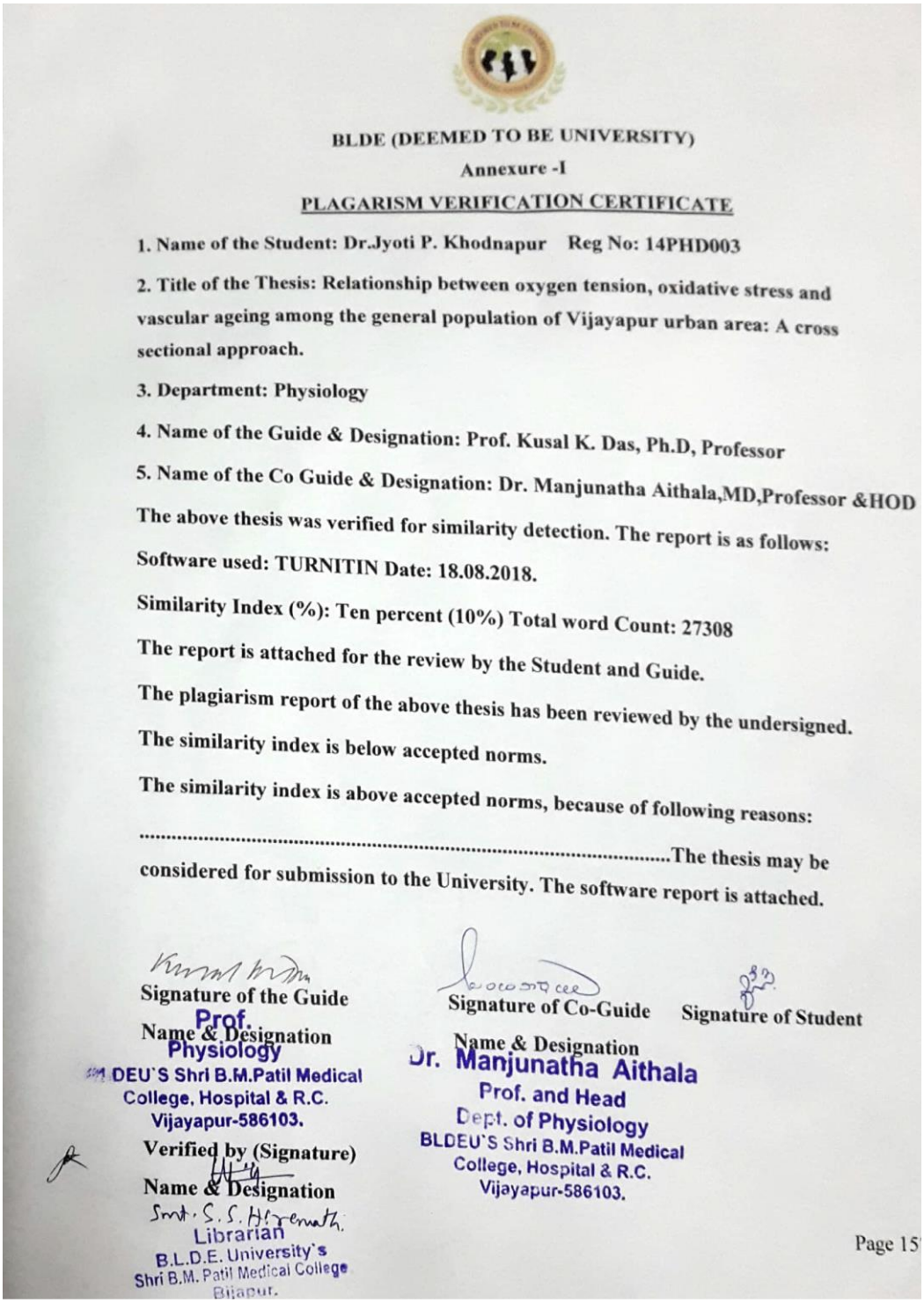


Limitations and future perspectives of the study:

- We could not evaluate serum VEGF and serum Epo proteins expression by Western blotting. Further studies are needed to assess oxygen sensing protein like HIf 1α and VEGF expression by Western blotting and analysis of DNA sequencing of these proteins in association with ageing.

ANNEXURES

ANNEXURE –I



BLDE (DEEMED TO BE UNIVERSITY)

Annexure -I

PLAGARISM VERIFICATION CERTIFICATE

1. Name of the Student: Dr.Jyoti P. Khodnapur Reg No: 14PHD003

2. Title of the Thesis: Relationship between oxygen tension, oxidative stress and vascular ageing among the general population of Vijayapur urban area: A cross sectional approach.

3. Department: Physiology

4. Name of the Guide & Designation: Prof. Kusal K. Das, Ph.D, Professor

5. Name of the Co Guide & Designation: Dr. Manjunatha Aithala,MD,Professor &HOD

The above thesis was verified for similarity detection. The report is as follows:

Software used: TURNITIN Date: 18.08.2018.

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The report is attached for the review by the Student and Guide.

The plagiarism report of the above thesis has been reviewed by the undersigned.

The similarity index is below accepted norms.

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Signature of Co-Guide
Name & Designation
Dr. Manjunatha Aithala
Prof. and Head
Dept. of Physiology
BLDEU`S Shri B.M.Patil Medical College, Hospital & R.C. Vijayapur-586103.

Signature of Student

Verified by (Signature)
Name & Designation
Smt. S. S. Hiremath.
Librarian
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ANNEXURE – II

INSTITUTIONAL ETHICAL CLEARANCE CERTIFICATE

B.L.D.E. UNIVERSITY

(Declared vide notification No. F.9-37/2007-U.3 (A) Dated. 29-2-2008 of the MHRD, Government of India under Section 3 of the UGC Act,1956)

The Constituent College

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

IEC Ref No-141/2015-16 July 20, 2015.

INSTITUTIONAL ETHICAL CLEARANCE CERTIFICATE

The Ethical Committee of this University met on 23rd June 2015 at 10.30 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 – "Relationship between oxygen tension, oxidative stress and vascular ageing among the general population of Vijayapura Urban Area: A cross sectional approach."

Name of Ph.D./ P. G. / U. G. Student / Faculty member. Dr. Jyoti Khodnapur .Asst.Professor Department of Physiology.

Name of Guide : Prof Kusal K Das. Professor, Department of Physiology.

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

Institutional Ethical Committee ★ BLDE University, BIJAPUR ★

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.

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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, VIJAYAPUR

CONSENT FORM

TITLE OF THE PROJECT : "Oxygen Tension, Oxidative stress and vascular ageing among general population of Vijayapur urban area: A cross sectional approach"

PRINCIPAL INVESTIGATOR : Dr.Jyoti Khodnapur
Ph D student

GUIDE'S NAME : Prof Kusal K Das
Professor, Dept Of Physiology

CO GUIDE'S NAME : Dr Manjunatha Aithala
Professor & HOD, Dept Of Physiology

1: PURPOSE OF RESEARCH: I have been informed that this study will assess the Oxygen Tension, oxidative stress and vascular ageing. This study will be useful academically as well as for 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 Oxygen Tension, Oxidative stress and vascular ageing 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 field of ageing in 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.)

ANNEXURE –IV

PRESENTATIONS AND AWARDS

NATIONAL CONFERENCE:

1. "Arterial stiffness and its relationship with oxygen tension among different age groups (20-75yrs)- A preliminary study" has been presented at-"3rd Annual conference of association of Physiologists of India, ASSOPICON 2016, during 14th to 17th September, 2016 held at Department of Physiology, BLDE University's, Shri. B.M.Patil Medical college and Research centre, Vijayapur.

   - Received **"BEST PAPER AWARD"**

INTERNATIONAL CONFERENCE

2. "Age and vascular health-A study on male and female participants (age 20 to 70 plus years) of Vijayapur city, India with special reference to erythropoietin." has been presented at-FIPSPHYSIOCON-2017, during 5th to 7th November, 2017 held at DRDO, New Delhi.

ANNEXURE – V

PUBLICATIONS

1. Khodnapur JP, Aithala MR, Das KK. Ageing and Pulse Wave Velocity in Relation to Serum Nitric Oxide. JKIMSU. 2018 Jan 1;7(1):25-38.

   (Indexed in scopus)

2. Khodnapur JP, Aithala MR, Das KK. Serum Vascular Endothelial Growth Factor and Aging: Study among Urban Population of Vijayapur, Karnataka, India. Journal of Clinical & Diagnostic Research. 2018 Mar 1;12(3):5-8.

   (Indexed in pubmed)

3. 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)

DOI: 10.7860/JCDR/2018/35354.11320

Original Article

Physiology Section

Serum Vascular Endothelial Growth Factor and Aging: Study among Urban Population of Vijayapur, Karnataka, India

JYOTI P KHODNAPUR[1], MANJUNATHA R AITHALA[2], KUSAL K DAS[3]

ABSTRACT

Introduction: It is known that angiogenesis delays in aging. Vascular Endothelial Growth Factor (VEGF) is the most potent angiogenic factor. But the influence of aging on VEGF is still unclear among healthy population.

Aim: To determine the relationship between aging and VEGF among different age groups in both male and female subjects of Vijayapur city, Karnataka, India.

Materials and Methods: The present cross-sectional study conducted in Sri B.M. Patil Medical College (October 2016 to April 2017) on 196 healthy subjects male (n= 98) and female (n=98) subjects (20-95 years) were randomly selected among general population of Vijayapur city, Karnataka, India. Subjects were divided into six group: Group I (20-29 years), II (30-39 years), III (40-49 years), IV (50-59 years), V (60-69 years) and VI (>70 years). Anthropometric and physiological parameters like height (cms), weight (kg), Body Mass Index (BMI in kg/m^2), Body Surface Area (BSA in m^2), Pulse Rate (PR in bpm) and Blood Pressure (BP) were assessed. The VEGF was assessed by Enzyme-Linked Immunosorbent Assay (ELISA) method. Statistical analysis was done by using one-way ANOVA followed by post-hoc t-test and unpaired t-test by using SPSS software.

Results: Group I to Group VI showed significant ($p<0.001$) steady increase of VEGF in both male and female subjects. There was significant difference ($p<0.001$) of VEGF between male and female subjects.

Conclusion: Aging alters serum VEGF and causes vascular dysfunction. Females are greater protected against age related alteration of vascular pathophysiology due to greater VEGF concentration as compared to male counterparts.

Keywords: Angiogenesis, Body mass index, Vasculogenesis

INTRODUCTION

With aging, there is a progressive decline almost all physiological functions including vascular function [1]. Aging is an important risk factor for arterial aging and most forms of Cardiovascular Disease (CVD) [2]. Angiogenesis is not only an endogenous repair mechanism after ischaemic injury but also an essential adaptive response to physiological stress [3]. With aging, impaired angiogenesis and endothelial dysfunction likely contribute to the increased prevalence of CVD [3].

Angiogenesis acts as a major process in the development and maintenance of an individual health. Angiogenesis, the development of new vessels from pre-existing vasculature, is delayed in aging [4-8]. To proceed normally, the formation of new vessels requires endothelial cell activation, degradation of basement membrane, migration, and proliferation. These steps are regulated by interactions among cells, growth factors, and matrix proteins [9-11]. Growth factors, e.g., basic Fibroblast Growth Factor (b-FGF), VEGF, and Insulin-Like Growth Factor-1 (IGF-1), support the proliferation and migration of endothelial cells [12-17].

The VEGF is the most potent angiogenic factor. Matrix proteins, such as fibronectin, laminin, and Type 1 collagen provide the scaffold on which angiogenesis occurs [18-20]. In contrast, Secreted Protein Acidic and Rich in Cysteine; Osteonectin (SPARC), Thrombospondin-1 (TSP-1), and Thrombospondin-2 (TSP-2), are termed “matricellular” because they do not function as structural proteins but act as modulators of the angiogenic response [21-25]. The local balance among these competing factors is critical in determining if blood vessels will develop within a tissue or not.

For ischaemic diseases, induction of angiogenesis acts as a promising therapeutic approach [3]. Although, much is known about angiogenesis in general, the changes that occur during angiogenesis with aging are not well defined. So, to understand and manage CVD it is important to understand the basis of age related impairment of endothelial function and angiogenesis. So, the present study was undertaken to know the influence of ageing on VEGF among the healthy general population of Vijayapur, Karnataka, India.

MATERIALS AND METHODS

The present cross-sectional study was started after approved by the Institutional Ethical Committee (IEC Ref No-141/2015-16 dated July 20, 2015) Sri B.M. Patil Medical College, Hospital and Research Centre, (BLDE Deemed to be University) as per the ICMR guidelines 2006. Screenings were performed from October 2016 to April 2017 and included 192 apparently healthy subjects of age ranging from 20-95 years from Vijayapur city, Karnataka, India. Informed consent was obtained for participation in the study. Subjects from both sexes with resting BP <140/90 mmHg, BMI <30 kg/m^2 and subjects not taking medications or dietary supplements were included. Subjects with alcohol intake, smoking, tobacco consumption in any form, suffering from mental disorders, hypercholesterolemia, hypertension, diabetes mellitus and taking medications like statins, antidiabetics, diuretics, antihypertensives, beta blockers, vasodilators etc., were excluded from the study. All the recordings were done in the morning between 9-11 am at room temperature following supine rest for 10 minutes.

Anthropometric parameters like height in centimetres (cms), weight in kilograms (kg), BMI in kilograms per square meter (kg/m^2) and BSA in square meter (m^2) and physiological parameters like pulse rate in (beats/minute), Systolic Blood Pressure (SBP) in millimetre of mercury (mmHg), Diastolic Blood Pressure (DBP) (mmHg), Pulse

Pressure (PP) (mmHg) and Mean Arterial Pressure (MAP) (mmHg) were recorded by using standard procedures. Total serum VEGF was measured as an index of endothelial function. Serum VEGF was estimated based on the principle of a solid phase ELISA by using a commercially available kit [26].

Sample Size Calculation

We screened 210 subjects, among them 192 subjects were included in the study. A total of 96 male subjects and 96 female subjects were sufficient to detect a clinically important difference of 0.8 between groups in detecting change assuming a standard deviation of 2 using a two tailed z-test of means between groups with 80% power and a 5% level of significance.

The entire sample was divided into six groups by age decades including male and female together

- Group I: between 20 and 29 years (n=32)
- Group II: between 30 and 39 years (n=32)
- Group III: between 40 and 49 years (n=32)
- Group IV: between 50 and 59 years (n=32)
- Group V: between 60 and 69 years (n=32)
- Group VI: 70 plus years (n=32)

STATISTICAL ANALYSIS

Data was expressed as mean±Standard Deviation (mean±SD). The data have been expressed in the form of tables and graphs. Differences between mean values of parameters between Group I, Group II, Group III, Group IV, Group V and Group VI were evaluated by one-way ANOVA followed by Post-hoc test (Least significant difference). We compared mean values for male and female in each age group using the unpaired t-test. Correlation of VEGF with age was done by Pearson's correlation. The level of statistical significance was observed at $p<0.05$, $p<0.01$ using SPSS software 16.0.

RESULTS

The anthropometric and physiological characteristics among males divided into six groups by age [Table/Fig-1]. There were no significant difference in weight, height, BMI, BSA, PP and MAP between the observed groups. In case of PR ($p<0.05$) ANOVA showed significant results. The anthropometric and physiological characteristics among female subjects divided into six groups by age decades [Table/Fig-2]. In case of height ($p<0.01$), weight ($p<0.001$), BMI ($p<0.01$), BSA ($p<0.01$), PR ($p<0.01$), PP ($p<0.001$), and MAP ($p<0.01$), ANOVA shows significance.

The serum VEGF between male and female subjects in all the six age matched groups [Table/Fig-3]. Results from unpaired t-test showed significant difference ($p<0.001$) between male and female subjects in all the age groups. Results reflect that there was a steady increase of VEGF in both male and female as age progressed. Interestingly VEGF concentration in female, in all the age matched groups with male was found to remain significantly higher.

Parameters	Age groups (years)							
	Group I	Group II	Group III	Group IV	Group V	Group VI	ANOVA	
	20-29 years (n=16) (mean±SD)	30-39 years (n=16) (mean±SD)	40-49 years (n=16) (mean±SD)	50-59 years (n=16) (mean±SD)	60-69 years (n=16) (mean±SD)	70 years plus (n=16) (mean±SD)	f-value	p-value
Weight (Kg)	66.2±8.5	65.5±4.64	71.7±9.2	64.9±4.1	66±4.87	59.44±9.6	2.010	0.089
Height (cm)	167.5±4.0	167.7±4.2	161.55±5.34	166.7±7.66	167.0±5.5	161.9±6.0	2.085	0.081
BMI (kg/m^2)	24.0±2.8	23.3±1.2	25.8±2.5	24.8±0.9	23.9±4.54	21.9±2.3	2.130	0.080
BSA (m^2)	1.76±0.12	1.75±0.74	1.84±0.14	1.72±0.2	1.80±0.16	1.63±0.15	1.868	0.127
PR (bpm)	73.6±8.43^{I,VI}	74.9±9.92^{VI}	77.8±9.60^{VI}	75.5±6.81^{VI}	73.0±10.66^{VI}	63.0±7.39^{I,II,III,IV,V}	2.978	0.026
PP (mmHg)	56.31±5.4	56.39±6.28	47.59±3.69	47.79±6.81	57.42±6.8	57.67±7.7	2.028	0.101
MAP (mmHg)	87.39±6.79	88.89±5.21	88.42±4.69	94.69±5.39	100.9±8.69	101.9±7.79	1.489	0.222

[Table/Fig-1]: Anthropometric and physiological characteristics of male subjects.
Data are Mean+SD. Values in the final column represent results of one-way analysis (ANOVA) among different age groups. Post-hoc comparisons were made between each group with LSD method. Superscripts I, II, III, IV, V and VI on each of the group are significantly differ from that group at p<0.05 level. BMI: Body mass index, BSA: Body surface area, PR: Pulse rate, PP: Pulse pressure, MAP: Mean arterial pressure

Parameters	Age groups (years)							
	Group I	Group II	Group III	Group IV	Group V	Group VI	ANOVA	
	20-29 years (n=16) (mean±SD)	30-39 years (n=16) (mean±SD)	40-49 years (n=16) (mean±SD)	50-59 years (n=16) (mean±SD)	60-69 years (n=16) (mean±SD)	70 years plus (n=16) (mean±SD)	f-value	p-value
Weight (Kg)	56.1±8.59^{VI}	55.79±6.69^{VI}	59.19±6^{VI}	61.9±7.0^{VI}	56.9±9.39^{VI}	44.1±4.9^{I,II,III,IV,V}	3.430	0.008
Height (cm)	158.31±2.69	151.9±6.29	151.2±3.49	157.1±3.89	150.10±4.81	149.1±3.92	8.130	0
BMI (kg/m^2)	22.39±3.49	24.10±2.90	25.40±2.28	25.01±2.56	25.43±3.41	19.81±2.41	4.030	0.003
BSA (m^2)	1.60±0.10^{VI}	1.50±0.10^{VI}	1.60±0.08^{VI}	1.63±0.09^{VI}	1.52±0.16^{VI}	1.32±0.1^{I,II,III,IV,V}	3.560	0.009
PR (bpm)	73.9±8.8	74.2±10.9	72.1±8.1	75.4±5.0	71.91±8.5	65.31±9.21	2.310	0.030
PP (mmHg)	44.4±7.9^{V,VI}	44.6±3.67^{V,VI}	46.71±5.2^{V,VI}	48.4±5.5^{V,VI}	64.39±12.78^{I,II,III,IV}	64.32±9.9^{I,II,III,IV}	7.350	0
MAP (mmHg)	82.50±6.8^{V,VI}	85.2±8.5V,VI	88.1±7.89	91.89±5.38	95.7±8.69^{I,II}	101.36±17.74^{I,II}	3.267	0.012

[Table/Fig-2]: Anthropometric and physiological characteristics among female subjects.
Data are Mean+S.D. Values in the final column represent results of one-way analysis (ANOVA) among different age groups. Post-hoc comparisons were made between each group with LSD method. Superscripts I, II, III, IV, V and VI on each of the group are significantly differ from that group at p<0.05 level. BMI: Body mass index, BSA: Body surface area, PP: Pulse pressure, MAP: Mean arterial pressure

VEGF in pg/mL					
Age groups	Age in years	Male subjects (mean±SD)	Female subjects (mean±SD)	Unpaired t-test	
				t-value	p-value
Group I	20-29 years (n=16)	277.8±30.06	429.78±26.17	-14.758	≤0.001
Group II	30-39 years (n=16)	328.4±31.75	555.43±32.50	-19.349	≤0.001
Group III	40-49 years (n=16)	415.68±66.91	597.64±19.67	-10.105	≤0.001
Group IV	50-59 years (n=16)	430±56.81	648.92±21.55	-13.953	≤0.001
Group V	60-69 years (n=16)	485.73±14.56	675.74±29.55	-22.332	≤0.001
Group VI	70 years plus (n=16)	626.6±46.06	722.19±74.81	-4.214	≤0.001

[Table/Fig-3]: VEGF in pg/mL among both male and female subjects.
Data are Mean±SD. Values in the final column represent results of unpaired t-test between male and female subjects. p<0.05, considered as statistically significant

The ANOVA for all six groups in males which found to be statistically significant (p-value=0.001) [Table/Fig-4]. Similarly, significant difference in female subjects in all the age groups by ANOVA [Table/Fig-5].

ANOVA					
VEGF in pg/mL in male subjects					
	Sum of squares	Degrees of freedom	Mean square	f-value	p-value
Between groups	1130701.206	5	226140.24	113.528	0.031597
Within groups	167322.187	90	1991.931		
Total	1298023.394	95			

[Table/Fig-4]: VEGF in pg/mL in male subjects.
Values in the final column represent results of ANOVA between group I, II, III, IV, V and VI of male subjects. p<0.05, considered as statistically significant

ANOVA					
VEGF in pg/mL in female subjects					
	Sum of squares	Degrees of freedom	Mean square	f-value	p-value
Between groups	808190.837	5	161638.167	106.995	0.036093
Within groups	126899.951	90	1510.714		
Total	935090.788	95			

[Table/Fig-5]: VEGF in pg/mL in female subjects.
Values in the final column represent results of ANOVA between group I, II, III, IV, V and VI of female subjects. p<0.05, considered as statistically significant

DISCUSSION

Angiogenesis is fundamental for many physiological and pathological processes. In the present study, we assessed VEGF in relation to ageing in apparently healthy males and females among different age groups (20-95 years).

The present study showed a statistically significant (p<0.05) decrease in PR after the age of 70 years i.e., in Group VI (70 plus years) in both male and female subjects. The present study also showed significant increase (p<0.001) in PP after the age of 60 years in females i.e., in Group V (60-69 years) and VI (70 plus years). A linear rise in SBP from age 30-84 years with initial increase in DBP were reported earlier by Franklin SS et al., [27]. The study further reported a decline of DBP after the age of 50 years with concomitant increase of PP and MAP [27]. The present results from BP in all the age groups in female subjects corroborate with the study of Franklin SS et al., [27].

The present results showed an increase in VEGF as age increases in both male and female subjects where as in case of females the concentration of VEGF remained consistently higher in all the age groups (20-70 plus years) as compared to their male counterparts. The results indicate a greater angiogenesis in females in all the age groups which may be considered as greater protection against vascular aging due to VEGF induced angiogenesis [Table/Fig-3]. The present results corroborated with study by Malamitsi-Puchner A et al., [28]. Impaired angiogenesis with reduced VEGF expression is found to be associated with aging [29]. Higher VEGF expression in aging may also be due to greater expression of oxygen sensing gene HIF-1α to combat age associated alteration of VEGF expression [30]. The relationship between distinct ocular aging and VEGF is well established [31]. Age related altered angiogenesis indicates endothelial dysfunction which may even lead to cerebral death [14,32]. Angiogenesis in aging is not merely delayed, but is altered due to multiple factors like altered inflammatory response, reduced expression of proangiogenic factors, decreased vessel density, less newly deposited collagen, and increased expression of TSP-2, an inhibitor of angiogenesis. The expression of VEGF was decreased in sponges from the mice aged at 14 days and 19 days compared to young tissue at the same time points which indicated an impaired angiogenesis [33]. The same study also observed a moderate increase in VEGF expression in aged tissue from 14-19 days [33]. Hence, results from the present study clearly indicate age associated greater vascular stability in female as compared to male counterparts. Decreased VEGF secretion among male subjects in all the age groups as compared to females clearly indicate lower angiogenesis or there may be possibly greater impairment of oxygen sensing mechanism in vascular system which leads to vascular integrity.

Some studies have demonstrated that administration of angiogenic growth factors as in recombinant protein therapy or gene transfer may facilitate angiogenesis in animal models of myocardial and limb ischaemia [34,35]. Such therapeutic strategies in older patients may help to manage the CVD due to impaired angiogenesis with aging.

LIMITATION

We could not evaluate serum VEGF expression by Western blotting. Further studies are needed to assess oxygen sensing protein like HIF 1α and VEGF expression by Western blotting.

CONCLUSION

Aging alters serum VEGF and causes vascular dysfunction. Higher serum VEGF levels with aging both in females and males indicates increased rates of angiogenesis. Females have an augmented protection against age related alteration of vascular pathophysiology due to greater VEGF concentration as compared to their male counterparts.

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ORIGINAL ARTICLE

Ageing and Pulse Wave Velocity in Relation to Serum Nitric Oxide

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Abstract:

Background: The Pulse Wave Velocity (PWV) is an important marker of arterial stiffness. Age related changes of arterial stiffness in relation to PWV and endothelial derived Nitric Oxide (NOx) are least explored. *Aim and Objectives:* The present study was aimed to assess a relationship between age associated vascular stiffness and endothelial derived nitric oxide in both males and females. *Materials and Methods:* One hundred twenty healthy subjects male (n= 60) and female (n=60) subjects (20 to 95 years) were randomly selected among general population of Vijayapur city, Karnataka. Subjects were divided into group I (20-29 years), II (30-39 years), III (40-49 years), IV (50-59 years), V (60-69 years) and VI (>70 years). Physiological parameters like blood pressure and endothelial derived NOx were assessed. Vascular stiffness parameter like brachial-ankle PWV (b-aPWV) and carotid femoral PWV (c-fPWV) were also evaluated. Statistical analysis was done by using one way ANOVA and post hoc t test by using SPSS software. *Results:* Group I to group VI showed significant steady increase of b-a PWV and c-f PWV with concomitant significant decrease of serum NOx in both male and female subjects. Further a significant negative correlation between b-aPWV and c-f PWV with NOx in both male and female subjects were also observed. *Conclusion:* Results suggested possible influences of ageing on vascular stiffness which may be due to alteration of endothelial derived NOx.

Keywords: Pulse Wave Velocity, Vascular Stiffness, Nitric Oxide, Gender, Ageing.

Introduction:

Achievement of ageing is a privilege, at the same time it is also a challenge which will impact on all aspects of 21st century society [1]. In 2000, there were 600 million people aged 60 years and above and it will be 1.2 billion by 2025 or 2 billion by 2050 [2].

Age is one of the most powerful determinants of cardiovascular risk and is associated with a number of deleterious changes in the cardiovascular system [3]. Large arteries stiffening and dilatation are the more prominent changes with ageing which has been documented worldwide.

Arterial stiffness is an independent marker of Cardiovascular (CV) risk that increases with age [4]. Pulse Wave Velocity (PWV) and Arterial Stiffness Index (ASI) are widely accepted and recommended for measure of arterial stiffness [5-6]. High PWV indicates either decrease in vascular compliance or an increase in arterial stiffness.

Measurement of PWV and wave reflection have now recognized as an important prognostic indicator than Blood Pressure (BP) to assess the CV risk [7-8]. Brachial-ankle PWV (b-a PWV) and carotid-femoral PWV (c-f PWV) are considered as index of arterial stiffness [4,9]. PWV reflects the stiffness of both the aorta and peripheral arteries in an arm and a leg, and would be more applicable to general practice since its measurement, which uses a separate cuff for each

limb, is automated and easier to perform [10-12]. Several cross-sectional studies showed PWV could be a good predictor of Cardiovascular Events (CVE) including coronary artery disease and myocardial injury [13-16]. Further to note that central elastic artery and peripheral muscular arteries functions are assessed by c-f-PWV. It is an independent predictor of carotid atherosclerosis in the elderly and simple measure of arterial stiffness [17].

As Nitric Oxide (NOx) is in gaseous form so it acts as an ideal paracrine and autocrine signaling molecule to diffuses freely across membranes [18]. NOx along with its anti-atherogenic property it also influences vascular tone. Decreased bioavailability of NOx, in resistance and conduit arteries is characterized as an endothelial dysfunction, is a predictor of cardiovascular risk and outcome [19-21].

The present study was aimed to assess the vascular health through ageing and PWV in relation to endothelial functions among the general healthy population (age from 20-70+ years) of Vijayapur city, Karnataka, India.

Material and Methods:

The present cross sectional study was conducted in Laboratory of Vascular Physiology and Medicine, Department of Physiology, BLDE University's, Shri. B. M. Patil Medical College and Research Centre, Vijayapur, Karnataka, India. This study was approved by the Institutional Ethics Committee (IEC Ref No-141/2015-16 dated July 20, 2015) of Sri B.M. Patil Medical College, Hospital and Research Centre, BLDE University as per the ICMR guidelines 2006. The study was conducted on 120 apparently healthy subjects of age ranging from 20 to 95 years. Subjects from both sexes were included in the study.

Sample size calculation:

A total sample size of 120 subjects included in the study. The probability is 80% (power) that the study detected a relationship between dependent and independent variables at a two sided 0.05 significant level, if the true change in the dependent variable (NOx) is 0.5µmole/L per 1 standard deviation change in independent variable. Calculated sample size by using following formula.

$$n= \frac{[(Z_{\alpha/2}+Z\beta)^2*\{2(\sigma)^2]}{(\mu)^2}$$

Where N=No of sample, Z=Standard normal variate, α=type I error (level of significance)=1.96 β=type II error (1-β=power of test)=0.842, σ=Standard deviation=1, µ=mean difference=0.5 After calculation we got 60 subjects, so we selected 60 subjects in each gender so total 120 subjects we have included.

The inclusion and exclusion criteria are as follows:

Inclusion criteria:

1. Apparently healthy subject age ranging from 20 to 95 years.
2. Subjects with BMI < 30kg/m^2
3. Subjects with resting blood pressure <140/90mmHg
4. Non smokers
5. Subjects not taking medications or dietary supplements

Exclusion criteria:

1. Subjects with hypercholesterolemia
2. Evidence of hypertension (systolic blood pressure more than 140 and diastolic blood pressure more than 90 mm Hg).
3. Subjects with diabetes mellitus
4. Subjects taking medications like statins, antidiabetics, diuretics, antihypertensives,

beta blockers, sympathomimetic drugs and vasodilators

5. Subjects with history of tobacco consumption in any form.
6. Subjects with history of alcohol intake.

Informed consent was obtained for participation in the study. A detailed history was taken from all the subjects. All the recordings were done in the morning between 9 am to 11 am at room temperature following supine rest for 10 minutes. The entire sample is divided into six groups by age decades [22].

I. Measurement of anthropometric and physiological parameters:

All subjects underwent recording of anthropometric parameters like height (cms), weight (kg), Body Mass Index (BMI) (kg/m^2) and Body Surface Area (BSA) (m^2) and physiological parameters like pulse rate in (beats/min), Systolic Blood Pressure (SBP) (mmHg), Diastolic Blood Pressure (DBP) (mmHg), Pulse Pressure (PP) (mmHg) and Mean Arterial Pressure (MAP) (mmHg) by using standard procedures.

II. Vascular function parameters: Arterial stiffness was assessed by using a non-invasive automatic device based on Oscillometric method (Periscope, Genesis Medical Systems, India). Periscope uses two channel Electrocardiography (ECG) leads to record ECG and four BP cuffs to record arterial pressure waveforms [23]. This device is a validated 8 channel real time based simultaneous acquisition and analysis system while acquisition rate was 200 samples/ second. All recordings were made in supine position while BP cuffs were wrapped on both upper arms and above ankles and ECG electrodes applied on ventral surface of both wrists and medial side of ankles. BP volume waveforms were measured by an oscillometric pressure sensor connected by BP cuffs. Volume pulse form were determined from brachial and tibial arteries by plethysmographic sensor. The data was recorded for 10 seconds. For further analysis the data was stored in computer. The procedure is devoid of any operator bias because the device is fully automated and does not require any operator. As the periscope is automatic the recording completes itself by displaying the results. PWV and ASI are calculated by periscope as follows:

Pulse wave velocity:

a. Brachial-ankle PWV (b-a PWV): This reflects stiffness of central elastic artery & peripheral semi-muscular arteries. Periscope uses brachial and tibial artery pressure waveforms and ECG recordings (Lead I & II) to estimates b-a PWV. Pulse Transit Time (PTT) between brachium and respective ankle was calculated as the time difference between the feet of respective pulse wave which originates from R-wave (QRS complex) of ECG. The device calculated automatically the distance between the sampling points of b-a PWV according to the height of the subject. The formula is used to calculate b-a PWV.

$$ba\,PWV = \frac{Lba}{PTTba}$$

Where b-a PWV= Brachial ankle pulse wave velocity.

Lba = Distance between respective brachium and ankle.

PTTba = PTT between brachium and respective ankle was calculated as the time difference between the feet of respective pulse wave originated from R-wave (QRS complex) of ECG.

a. **The carotid-femoral PWV (c-f PWV):** A measure of aortic stiffness was calculated by the composite b-a PWV found out by averaging left and right b-a PWV. Studies conducted elsewhere [11] estimate the c-f PWV on the basis of equation (0.8333*Avg. b-aPWV-233.33) derived by regression analysis between b-a PWV and c-f PWV by using periscope.

I. **Serum Nitric oxide (NOx) level:** Total serum NOx concentration 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™ Nitric Oxide Assay Kit: D2NO-100, BioAssay Systems, USA).

Statistical Analysis:

Statistical analysis was carried out using SPSS version 16.0. Results are expressed as mean ± standard deviation. The data have expressed in the form of tables and graphs. Differences between mean values of parameters between Group I, Group II, Group III, Group IV, Group V and Group VI were evaluated by one way ANOVA followed by Post-Hoc test (LSD). We compared mean values for men and women in each age group using the unpaired t- test. Correlation b-a PWV, c-f PWV and NOx was done by Pearson's correlation. Further correlation between aging and b-a PWV, c-f PWV in both male and female were also done. P-value <0.05 was taken as significant.

Results:

Among males, there was no significant difference in means of weight, height, BMI, BSA, DBP and PP between different age groups observed. However, mean SBP (p=0.005) and mean MAP (p=0.021) differed significantly among the different age groups (Table 1).

Among female participants, there was no significant difference in means of height, BMI and DBP between different groups. However means of weight (p=0.000), BSA (p=0.008), SBP (p=0.019), PP (p=0.001) and MAP (0.002) differed significantly among the different age groups (Table 2).

It was found that both b-a PWV and c-f PWV increased with age among both male and female subjects. Our results showed both b-a PWV and c-f PWV in females were significantly lower as compared to males in all the respective age groups except group V (60-69 yrs) and VI (70 yrs plus). Further it was observed that a greater magnitude of steady rise in b-a PWV and c-f PWV in females from age forty onwards (group III) as compared to males. Further it was observed that from 60 years age (group V) onwards there were hardly any differences between male and female b-a PWV and c-f PWV (Fig.1 & 2).

It was found that NOx was decreased with age among both male and female subjects in our study. There was no significant difference in serum NOx levels between male and female subjects in all the age groups. It was observed that from 60 yrs (group V) onwards decrease of NOx concentration between male and female subjects remained near similar (Fig. 3).

Results showed a significant negative correlation between NOx and b-a PWV in males (r = -0.344, P= 0.032) (Fig. 4) and in females (r = -0.322, P= 0.031) (Fig. 5). In case of c-f PWV a similar negative correlation (r = -0.402, P= 0.011) in male (Fig. 6) and (r = -0.344, P= 0.021) in female were noticed (Fig. 7).

Results showed a significant positive correlation between age and b-a PWV in males (r=0.665, P=0.000) (Fig. 8) and in females (r = 0.552, P= 0.000) (Fig. 9). In case of c-f PWV and aging a similar positive correlation (r = 0.548, P= 0.000) in male (Fig. 10) and (r = 0.620, P= 0.000) in female were noticed (Fig. 11).

Table 1: Anthropometric and Physiological Characteristics of Male Subjects

Parameters	Age groups (years)							
	Group I 20-29 years (n=10)	Group II 30-39 years (n=10)	Group III 40-49 years (n=10)	Group IV 50-59years (n=10)	Group V 60-69years (n=10)	Group VI 70 years plus (n=10)	ANOVA	
							F' value	p' value
Weight (Kg)	68.25±9.75	65±4.94	72.37±9.72	65±4.3	68±15.87	58.44±10.15	2.04	0.099
Height (cm)	168.5±4.89	166.6±3.20	167.63±8.73	164.2±5.71	169±1.73	162.78±7.03	1.045	0.409
BMI (kg/m^2)	24.06±2.8	23.33±1.16	25.76±2.44	24.84±0.85	23.87±4.54	21.89±2.31	2.38	0.061
BSA (m^2)	1.77±0.13	1.74±0.73	1.83±0.15	1.71±0.1	1.79±0.17	1.64±0.16	1.867	0.128
SBP (mmHg)	120±7.7^{V,VI}	118.2±10.2^{V,VI}	122.5±5.1^{V,VI}	122+14.6^{V,VI}	132.67±13.6^{I,II,III,IV}	136.88±10.2^{I,II,III,IV}	4.075	0.005
DBP (mmHg)	71.5±7.76	69±7.61	73.25±6.58	76.8±9.23	82±14.4	79.33±5.19	2.103	0.09
PP (mmHg)	48.5±6.11	49.2±11.6	49.25±7.99	45.2±5.76	50.67±7.15	57.55±6.8	2.333	0.06
MAP (mmHg)	87.66±7.2^{V,VI}	85.4±6.59^{V,VI}	89.67±4.83^{VI}	91.86±10.9	98.88±14.1^{I,II,III}	98.52±6.5^{I,II,III}	3.111	0.021

Data are Mean ± S.D. Values in the final column represent results of one-way analysis (ANOVA) among different age groups. Post-hoc comparisons were made between each group with LSD method. Superscripts I, II, III, IV, V and VI on each of the group are significantly differ from that group at p<0.05 level. BMI: body mass index, BSA: body surface area, SBP: systolic blood pressure, DBP: diastolic blood pressure, PP: pulse pressure, MAP: mean arterial pressure

Table 2: Anthropometric and Physiological Characteristics among Female Subjects

Parameters	Group I 20-29 years (n=10)	Group II 30-39 years (n=10)	Group III 40-49 years (n=10)	Group IV 50-59 years (n=10)	Group V 60-69 years (n=10)	Group VI 70 years plus (n=10)	ANOVA	
							F' value	P' value
Weight (Kg)	57.42±8.3^{VI}	57.33±6.13^{VI}	59.16±6.5^{VI}	61.4±7.8^{VI}	56.6±11.54^{VI}	45±5^{I,II,III,IV,V}	2.04	0
Height (cm)	158.64±2.76	150.25±5.81	152.83±3.9	156.2±3.56	149.3±5.16	148.33±3.7	1.045	0.173
BMI (kg/m^2)	22.9±3.33	25.00±2.03	24.9±2.9	24.7±3.15	25.07±3.8	24.43±2.3	2.38	0.084
BSA (m^2)	1.59±0.10^{VI}	1.53±0.10^{VI}	1.57±0.08^{VI}	1.62±0.09^{VI}	1.51±0.16^{VI}	1.31±0.1^{I,II,III,IV,V}	1.867	0.008
SBP (mmHg)	112.78±10.8^{V,VI}	110.58±11.07^{V,VI}	118.33±17.08	118.8+9.01	132.67±21.14^{I,II}	130.6±25.48^{I,II}	4.075	0.019
DBP (mmHg)	68.64±5.63	70.08±9.6	75.33±14.58	72±6	75±9.7	67.33±11	2.103	0.463
PP (mmHg)	44.14±9.2^{V,VI}	40.5±3.08^{V,VI}	43±4.73^{V,VI}	46.8±7.56^{VI}	57±14.8^{I,II,III}	62±15.6^{I,II,III,IV}	2.333	0.001
MAP (mmHg)	83.35±6.43^{V,VI}	83.58±10.01^{V,VI}	89.66±15.3^{VI}	87.59±15.3^{VI}	93.99±12.78^{I,II,VI}	113±22.6^{I,II,III,IV,V}	3.111	0.002

Data are Mean ± S.D. Values in the final column represent results of one-way analysis (ANOVA) among different age groups. Post-hoc comparisons were made between each group with LSD method. Superscripts I, II, III, IV, V and VI on each of the group are significantly differ from that group at p<0.05 level. BMI: body mass index, BSA: body surface area, SBP: systolic blood pressure, DBP: diastolic blood pressure, PP: pulse pressure, MAP: mean arterial pressure

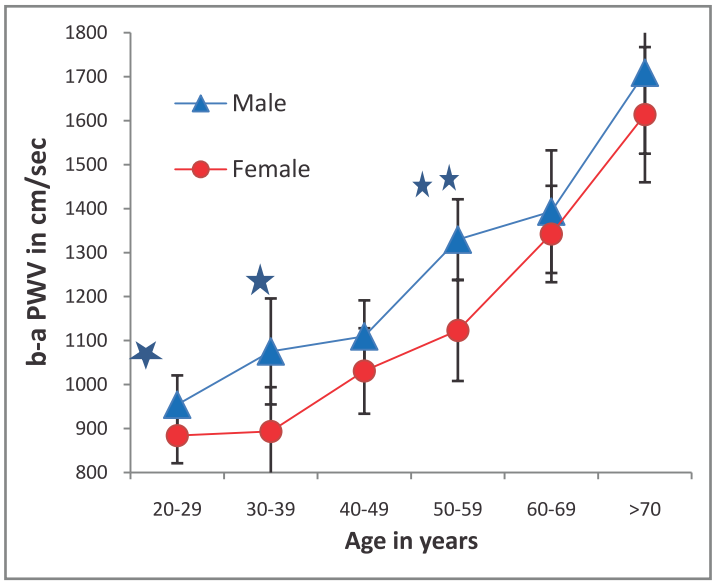

Fig. 1: Brachial-ankle Pulse Wave Velocity (b-a PWV) between Males and Females from Different Age Groups. Values are mean ± SD of each age group. *p<0.05, ** p<0.01 while Comparing Male and Female Values

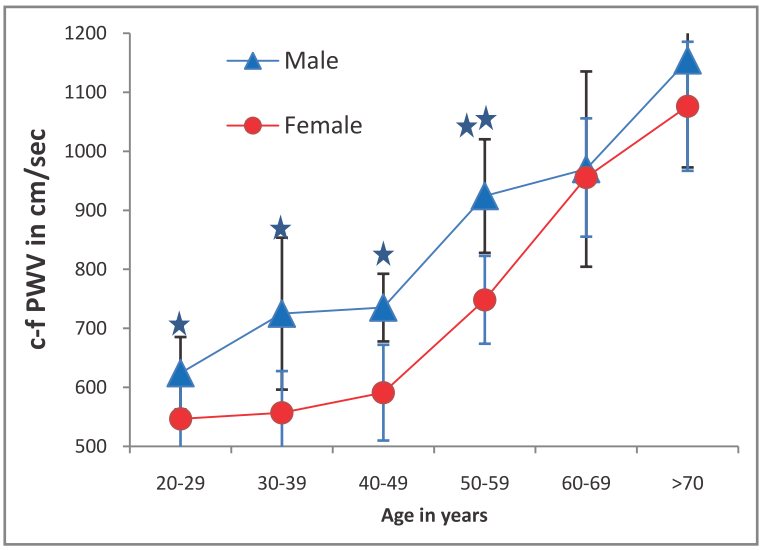

Fig. 2: Carotid-Femoral Pulse Wave Velocity (C-F PWV) Between Males and Females from Different Age Groups. Values are Mean ± SD of Each Age Group. *P<0.05, ** P<0.01 While Comparing Male and Female Values

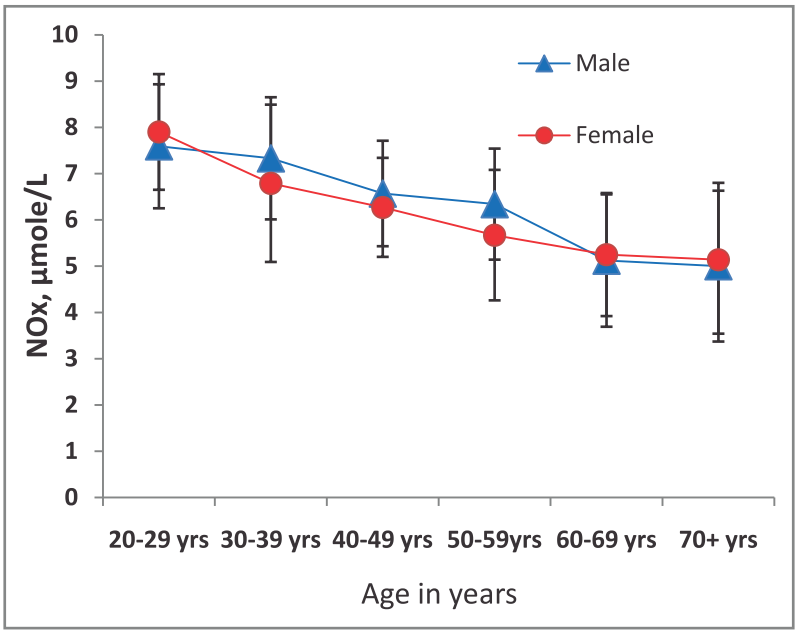

Fig. 3: Serum Nitric Oxide (NOx) Level between Males and Females from Different Age Groups. Values are Mean ± SD of Each Age Group.

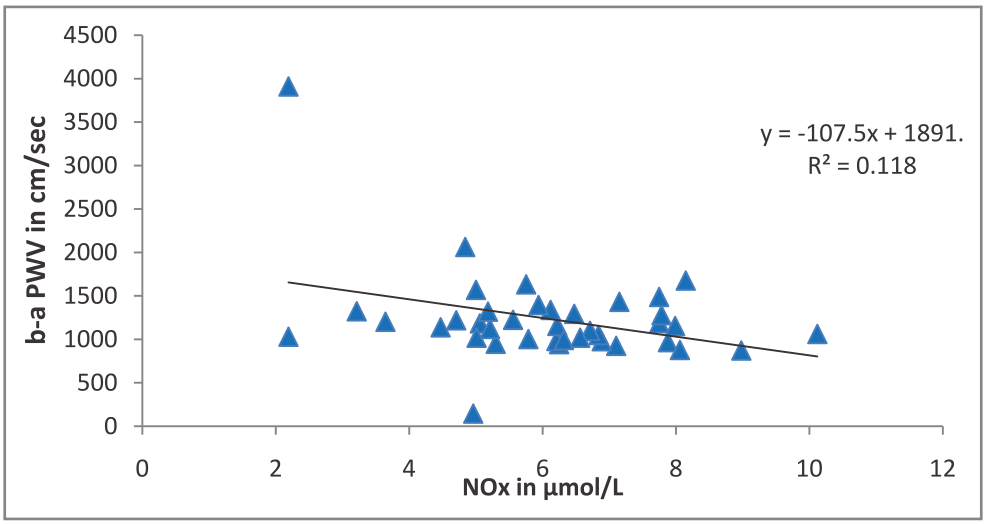

Fig. 4: Pearson's Correlation between B-A PWV and Nox among Male Subjects in Different Age Groups. Correlation (R) =-0.344; P=0.032

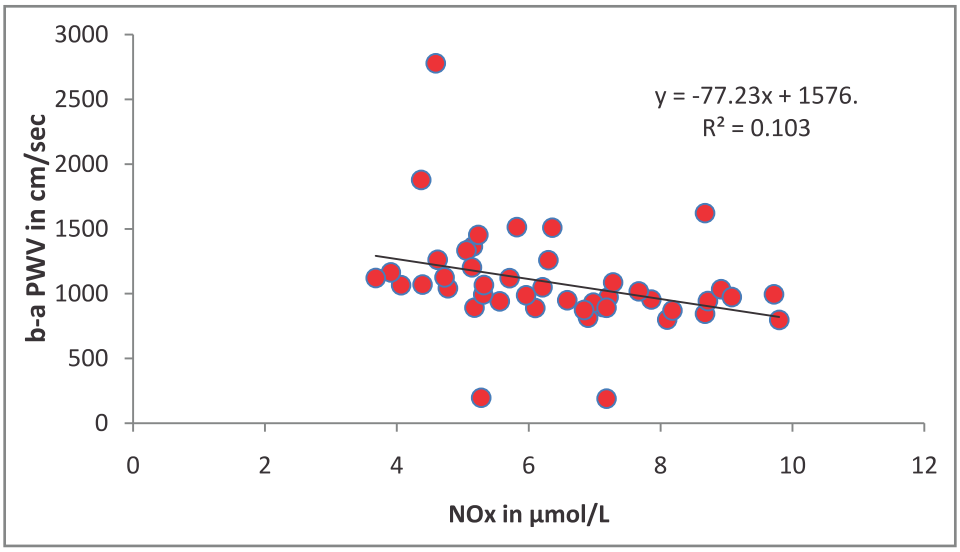

Fig. 5: Pearson's Correlation between b-a PWV and NOx among Female Subjects in Different Age Groups. Correlation (r) = -0.322; P= 0.031

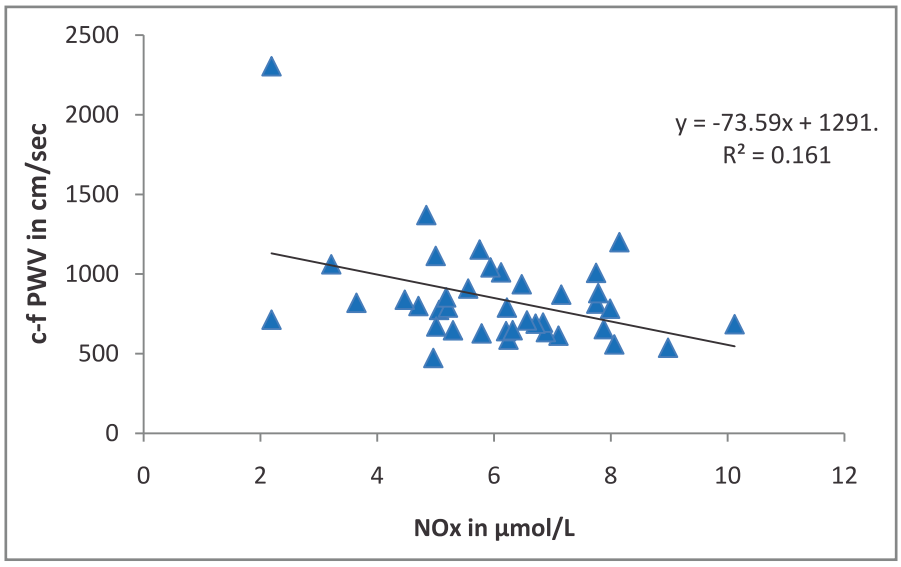

Fig. 6: Pearson's Correlation between C-F PWV and Nox among Male Subjects in Different Age Groups. Correlation (R) = -0.402; P= 0.011

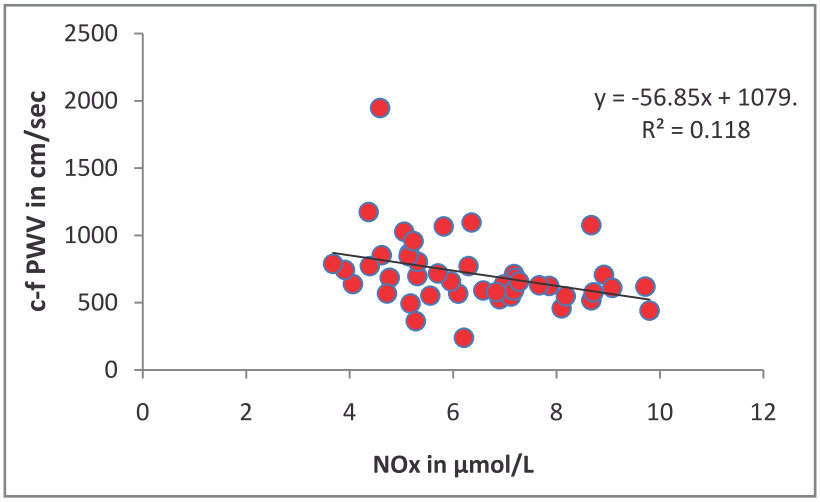

Fig. 7: Pearson's Correlation between c-f PWV and NOx among Female Subjects in Different Age Groups. Correlation (r) = -0.344; P= 0.021

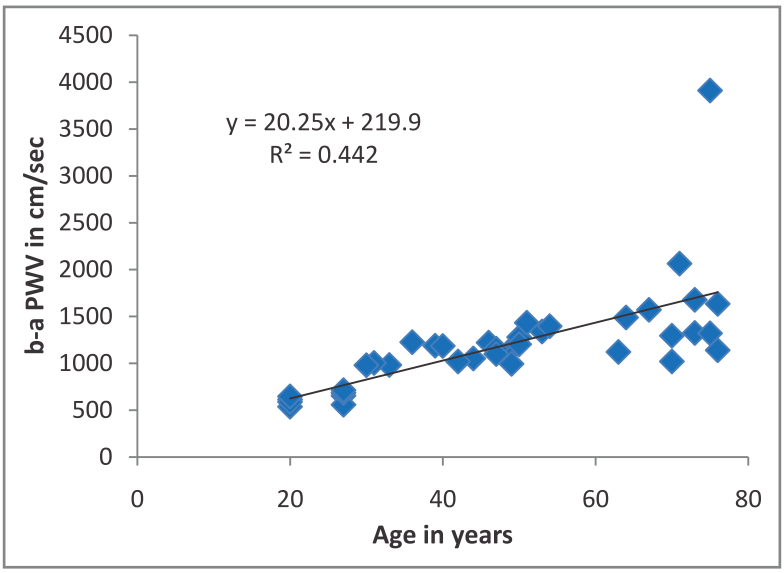

Fig. 8: Pearson's Correlation between b-a PWV and Aging among Male Subjects in Different Age Groups. Correlation (r) = 0.665; P= 0.000

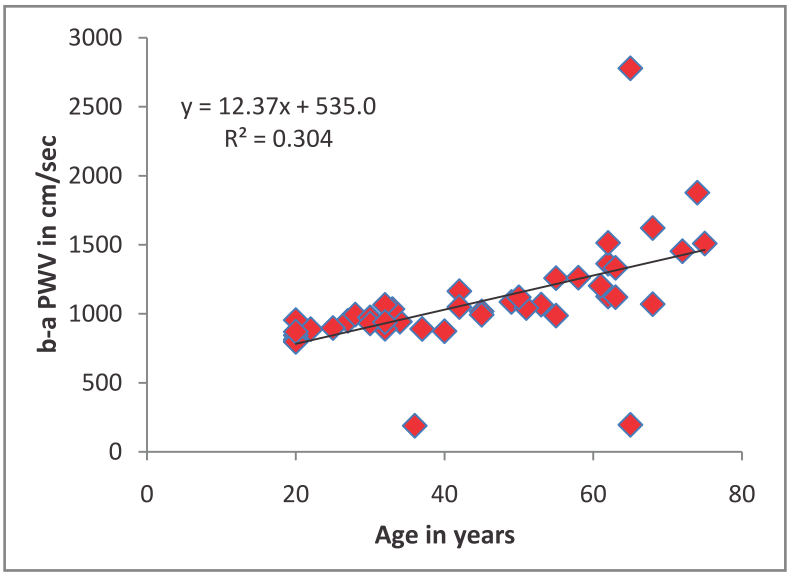

Fig. 9: Pearson's Correlation between b-a PWV and Aging among Female Subjects in Different Age Groups. Correlation (r) = 0.552; P= 0.000

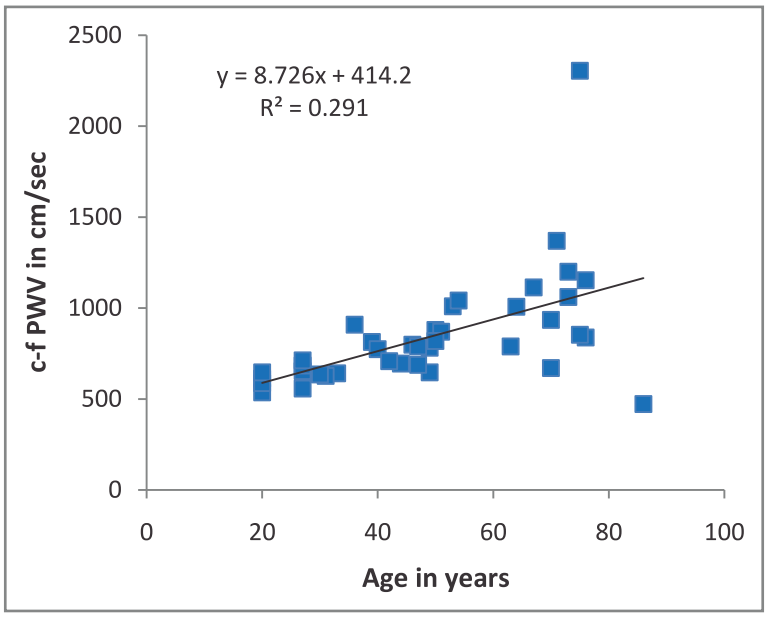

Fig.10: Pearson's Correlation between c-f PWV and Aging among Male Subjects in Different Age Groups. Correlation (r) = 0.548; P= 0.000

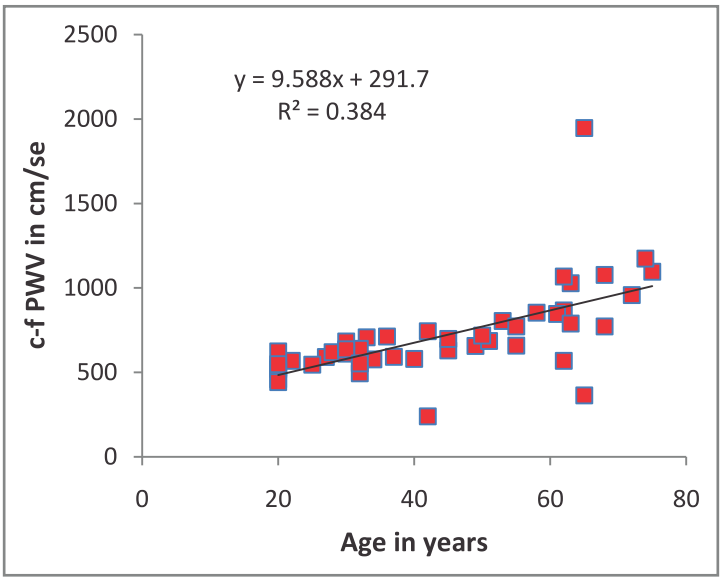

Fig.11: Pearson's Correlation between b-a PWV and Aging among Female Subjects in Different Age Groups. Correlation (r) = 0.552; P= 0.000

Discussion:

In the present study, we assessed arterial stiffness (PWV) and serum NOx in relation to ageing in apparently healthy males and females among different age groups (20-95 years). In this study involving six age groups among both male and female healthy subjects did not show any significant difference ($p<0.05$) between age groups in anthropometric, physiological parameters except SBP and MAP. Our study showed statistically significant ($p<0.05$) increase in SBP and MAP after the age of sixty years i.e. in group V (60-69 years) and VI (70 plus years) in both male and female subjects. Our study also showed significant increase ($p<0.05$) in PP after the age of 60 years in females i.e. in group V (60-69 years) and VI (70 plus years). A linear rise in SBP from age 30 to 84 years with initial increase in DBP were also reported earlier [24]. Study further reported a decline of DBP after age of 50 years with concomitant increase of PP and MAP [24]. Our results from BP in all the age groups in both male and female corroborate with this study [24].

To evaluate arterial stiffness, PWV is considered useful marker. The PWV indicates the speed at which the arterial pulsation produced due to ejection of blood from the heart propagates to the periphery. The PWV is also known to be proportional to the rigidity of the arterial wall through which it propagates and inversely proportional to the vessel diameter [25]. Age dependent increase in b-a PWV and c-f PWV of males and females in our study corroborate with the study of McEniery and Hall (2005) [26]. There are no any cut off value of Brachial-Ankle and Carotid-Femoral Pulse Wave Velocity Index to confirm arterial stiffness among normal individuals with aging in India. PWV increased linearly with aging with 6–8% with each decade of life; this tendency is more pronounced after 50 years. A significant increase of PWV over 60 years

in our study is supported by Diaz *et al.* (2014) [27]. Kawai *et al.* (2013) showed 1750.0 cm/sec could be a useful cut-off value for baPWV to predict cardiovascular prognosis in hypertensive individuals [28]. Some studies indicated differential changes of PWV in females due to post menopausal physiology and the results from our study in females support these observations [29-30].

The results from the present study on age associated gradual decrease of serum NOx in both male and female subjects indicate a reduction of bioavailability of NOx as age advances [31]. Interestingly our results differ from another observation where increase of serum NOx was found as age advances from 50 years onwards in both male and female subjects [32]. A negative correlation between NOx with b-a PWV and c-f PWV in both male and female subjects in our studies reflect that the changes of PWV are dependent on NOx in any age group. In our study significant difference in PWV in both male and females with age but there is no significant difference in sNOx level in males and females may be due to hormonal influence in female subjects and our results corroborates with study by Ahimastos *et al.* (2003) [33].

Age associated increased PWV and MAP in both male and females in the present study clearly showed altered vascular functions in ageing. PWV may be considered as more reliable marker than MAP to evaluate age associated arterial stiffness. Decreased serum NOx level in both males and females in association with age shows possible functional alterations of vascular homeostasis. Conditions associated with endothelial dysfunction may also be associated with increased arterial stiffness which may be partially counteracted through improved Nitric Oxide Synthase 3 (NOS3) pathways by generating greater NOx that improve endothelial stability and reduce arterial stiffness [34-37].

Conclusion:

Arterial stiffness is a major indicator of altered vascular functions and it is age dependent. PWV may be considered as potential marker in age associated alteration of vascular stiffness. Serum NOx probably plays as an important endothelial derived mediator to influence vascular stiffness in both males and females in the process of ageing. Understanding of these mechanisms in depth may help to explore new avenues on our knowledge in vascular sciences.

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