In the name of ALLAH, the most merciful and the mighty

Effects of different intensities of exercise on concentrations of Endostatin and VEGF in the plasma of healthy volunteers

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Abstract

The balance between angiogenesis and angiostasis is important in growth and development processes in the body. However, this balance is disturbed in pathological conditions such as atherosclerosis and can cause harmful events like increase in size and rupture of the plaque, thrombosis and MI. Angiogenic and angiostatic mediators control this balance.

Endostatin is one of the prominent angiostatic mediators. It is 22 kDa factor, attached to the COOH terminal of collagen XVIII and XV and requires a catalytic release for its action. The marked angiostatic effects of endostatin includes inhibiting endothelial cell migration, proliferation and apoptosis mainly VEGF induced. Ranges of endostatin concentration in health and diseased populations are reported by different research groups.

Physical activity decreases the risk and development of many angiogenesis related health problems including atherosclerosis and numerous cancers. WHO and ACSM recommend carrying out more than 30 minutes of moderate intensity physical activity on most of the days for all healthy individuals. Physiological influences of different physical activities on plasma endostatin concentration are controversial and not completely clear. The extent of changes in endostatin and its subsequent effects on the concentration of VEGF as a result of exercise are equally indistinct and vague. Moreover, influence of different physical characteristics and metabolic factors on circulating concentration of endostatin is also not clear and poorly speculated.

The work reported in this thesis, aimed to investigate circulatory endostatin concentration in plasma and the effects of different intensities of exercise ranging from low to maximum on the distribution of endostatin and VEGF concentrations in plasma. Additionally, correlations of anthropometric characteristics, metabolic profiles and exercise parameters with endostatin and VEGF were investigated.

The first study aimed to investigate the bioavailability of endostatin in the plasma of healthy volunteers and to look for any gender, physical characteristics or ethnic based differences. 50 healthy volunteers including 34 males (age = 28.5

± 8.5 years) and 16 females (age = 23.1 ± 4.4 years) were recruited to the study. Based on their origin, they were divided into three ethnic groups, European (n = 25), Middle Eastern (n = 10) and South Asian (n = 15). Single blood samples were collected through venepuncture. Plasma was separated and subsequently analysed for endostatin concentration using ELISA. The mean plasma endostatin concentration measured was 105 ng/ml ± 12 ng/ml (ranging 81 to 132 ng/ml). No significant differences were observed on the basis of gender or ethnic group. The endostatin concentrations were not correlated with different anthropometric characteristics. This study provided basal endostatin concentration as a reference point for the subsequent studies, which aimed to determine the influence of physical activities on endostatin concentrations in plasma.

The second study consists of two phases; the first phase aimed to determine the effects of mild, moderate and vigorous exercise on the concentration of endostatin in plasma. The duration and work rate for each participant was determined through sub-maximal exercise one week before. 22 participants, 16 males (age = 30.6 ± 7.8 years) and 6 females (age = 26.5 ± 5 years) were recruited to the study. Based on their fitness to run on the treadmill, participants were divided into walking and running group. One pre exercise and two post exercise samples were taken at intervals of 10 and 60 minutes. Low intensity exercise decreases the endostatin concentration in plasma, while moderate and vigorous intensities increased the endostatin concentrations. The effects were never strongly influenced by gender or exercise mode.

The second phase was designed due to different in influence of low and high intensity exercises on endostatin. As endostatin antagonises the VEGF induced angiogenesis, it was aimed to investigate the VEGF concentration at these intensities. The left over samples were analysed for VEGF. Both the exercise intensities increased the VEGF concentration. The increase was more after vigorous activity. No correlation between the endostatin and the change in endostatin concentration with VEGF or the change in it was observed.

During this experiment, it was observed that VEGF concentration dropped back at 1hour interval despite high endostatin concentration.

Therefore, a third study with maximal exercise intensity (VO_{2max}) to volitional exhaustion was designed. 8 male healthy volunteers (age = 31 ± 6.1 years) were recruited. Body fat percentages, blood glucose and metabolic profiles were also included to check for any correlation with endostatin concentration or the change in it after physical exercise. Blood samples were taken up to 24 hours after exercise. Significant induction in endostatin concentration was observed up to 2 hours after exercise. VEGF concentration was also increased by exercise but significant only at 30 minutes interval. No significant correlations of endostatin or VEGF were observed.

In conclusion, low intensity exercises did not influence endostatin concentration. Moderate to high and maximum intensity exercises, however, significantly increase endostatin concentration for up to 2 hours. VEGF concentration is influenced by all exercise intensities transiently and less pronounced. Moderate to high intensity exercises favours angiostatic phenotype and therefore, help to decreases the progression of atherosclerosis.

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Dedicated to my parents, wife Hina Inayat and Son Rayaan A Shah

Who always prayed for my success and never let me down
in the diverse situations of my life. I am greatly indebted
and pay gratitude to them for their endless love.

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Author's Declaration

I declare that the work presented in this thesis is my own, unless otherwise stated by reference to published literature or acknowledgment. This work has not been presented at any other institution for any degree.

Dr. Inayat Shah

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May 2015

The more I know, the more I realise, I don't know.

(Aristotle)

Definitions/Abbreviations

µg	microgram
ACSM	American College of Sports Medicine
ANOVA	Analysis of variance
BMI	Body Mass Index
CAD	Coronary artery diseases
CI	Confidence interval
CV	Coefficient of Variance
CVD	Cardiovascular disease
EDTA	Ethylene Diamine Tetra acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
FGF	Fibroblast growth factor
H_2SO_4	Sulphuric Acid
HIV	Human immunodeficiency virus
HR_{max}	Maximum predicted heart rate
HSP	Heat Shock Protein
IHD	Ischemic heart disease
IL	Interleukin
KD	Kilo Dalton
kg	Kilogram
ml	Millilitre
mmol	millimol
mRNA	Messenger Ribonucleic acid
ng	nanograms
NO	Nitric oxide
NOS	Nitric Oxide synthase
OD	Optical density
PDA	Patent Ductus Arteriosis
pg	picograms
RHD	Rheumatic heart disease
TMB	Tetra methyl benzidine
VSD	Ventricular septal defect
VEGF	Vascular endothelial growth factor
VO_{2max}	Maximum oxygen uptake
WHO	World Health Organisation

1 Introduction and literature review

Chapter: 1

1.1 Introduction

Cardiovascular disease (CVD) especially atherosclerosis, is a major health problem globally. Atherosclerosis is the process of building of fibro-fatty lesions in the intimal layer of the muscular arteries especially coronary arteries. This process of plaque formation takes years and narrows the lumen of the vessels, leading to thrombosis and fatal heart attacks (Griffin, 1999).

CVD accounts for more than 30% deaths across the globe. In Europe it causes 46% mortality every year, more in females (51%) than males (42%) (Nichols et al., 2013). In the UK, CVDs are responsible for 180,000 deaths annually making it the leading cause of death (British Heart Foundation, 2012).

Atherosclerosis has a complex aetiology with many non-modifiable (hereditary) and modifiable (life style) factors. However, it is probable that a sedentary life style and a lack of physical activity play a significant role in the progression of atherosclerosis.

Scientific and clinical research has focused intensely on this problem over recent decades. The link between physical activity and decreased risk of CVD was established in early 1950s (Morris et al., 1953). Over time low physical activity was established as a risk factor for developing CVD (Williams, 2001). Increased physical activity has been extensively investigated as a possible solution for primary and secondary prevention of atherosclerosis (Thompson et al., 2003).The level of risk conferred by physical inactivity is similar to that of other major risk factors including high blood pressure, dyslipidaemia and smoking (Berlin and Colditz, 1990).

Physical activity stops the progression of atherosclerosis directly by modifying many cellular mechanisms identified as beneficial in preventing the development and progression of atherosclerosis. These mechanisms include production of heat shock proteins, increase in antioxidant capacity, expression of stress proteins and adaptations in mitochondria (Golbidi and Laher, 2011). Physical activity also exerts its beneficial effects indirectly by improving glucose tolerance, increasing insulin sensitivity and reducing high blood pressure, hyperlipidaemia, obesity and platelet aggregation (Gill and Cooper, 2008,

Francis, 1998). However, a detailed understanding of physical activity and its interactions with these mechanisms is not yet completely clear.

Studies suggests that angiogenesis is one of the stronger determinants in the progression of atherosclerosis (Isner, 1999, O'Brien et al., 1994). It is a marker of ongoing diseases and may predict the plaques with risk of expansion and thrombosis (Falk, 2006). The process of angiogenesis is controlled by the balance between angiogenic and angiostatic mediators. Regulation of angiogenesis is very important since poor control can lead to impaired angiogenesis resulting in progression of atherosclerosis (O'Brien et al., 1994). However, angiogenesis itself is a complex process and many mediators affecting it have been identified (Hanahan and Folkman, 1996). VEGF and endostatin have been identified as main mediators with angiogenic and angiostatic properties respectively (Kurz, 2000, Ferrara et al., 2003, Distler et al., 2003, Fu et al., 2009).

Exercise and physical activity have been found to affect the process of angiogenesis by increasing or decreasing the circulatory concentrations of these mediators (Suhr et al., 2010, Rehman et al., 2004, Gu et al., 2004). However, information on changes in circulatory concentration of angiogenic mediators especially endostatin and VEGF by different exercise intensities is limited and sometimes confusing.

Therefore, it is timely to investigate the relationship of exercise and angiogenesis with the hope to find facts which might enhance our understandings. It was hypothesized that single bouts of exercise might increase endostatin concentrations in the circulation of healthy individuals and by promoting angiogenic phenotype could protect the body against atherosclerosis.

It was also hypothesized that the effects of different intensities of exercises (mild, moderate, vigorous and maximum) on plasma endostatin and VEGF would be different and might be affected by anthropometric and physical characteristics, gender and metabolic profiles of the individuals.

1.2 Literature review

This section contains background knowledge of cardiovascular disease especially atherosclerosis and angiogenesis. This is followed by an in depth review of VEGF and endostatin. It also provides brief information on how physical activities influence different cardioprotective mechanisms in the body. Finally, scientific rationales for the experimental studies in this thesis are described.

1.3 An Overview of Cardiovascular Diseases

1.3.1 Definitions

Disease of the heart and blood vessels, whether acquired or genetic are referred to as cardiovascular disease (Walker et al., 2013). Acquired heart disease usually manifests later in life and is classically divided to three broad groups:

i. Diseases manifested by atherosclerotic processes like coronary artery disease (CAD) also called as ischemic heart disease (IHD) or coronary heart disease (CHD), stroke and peripheral vascular disease.

ii. Diseases manifested by chronic inflammatory processes like rheumatic heart disease (RHD), cardiomyopathies, myocarditis, endocarditis etc.

iii. Diseases of the heart caused by hypertension called as hypertensive heart disease.

CAD and stroke are the most important and prevalent types of cardiovascular disease and both are caused by the atherosclerotic processes (World Health Organization, 2014a). Subsequent paragraphs will focus on the epidemiology, pathogenesis and risk factors of CAD and stroke.

1.3.2 Epidemiology of Cardiovascular Diseases

Cardiovascular disease remains one of the leading global health problems, causing 30% deaths due to all causes (Gaziano, 2008, Deaton et al., 2011, World Health Organization, 2014a). This causes three times more deaths than infectious diseases including HIV/AIDS, Tuberculosis and Malaria. Thus, in 2012,

17.5 million deaths out of 58 million were due to CVD. Coronary heart diseases claimed 7.3 million lives and another 6.2 million by stroke, as shown in figure 1-1.

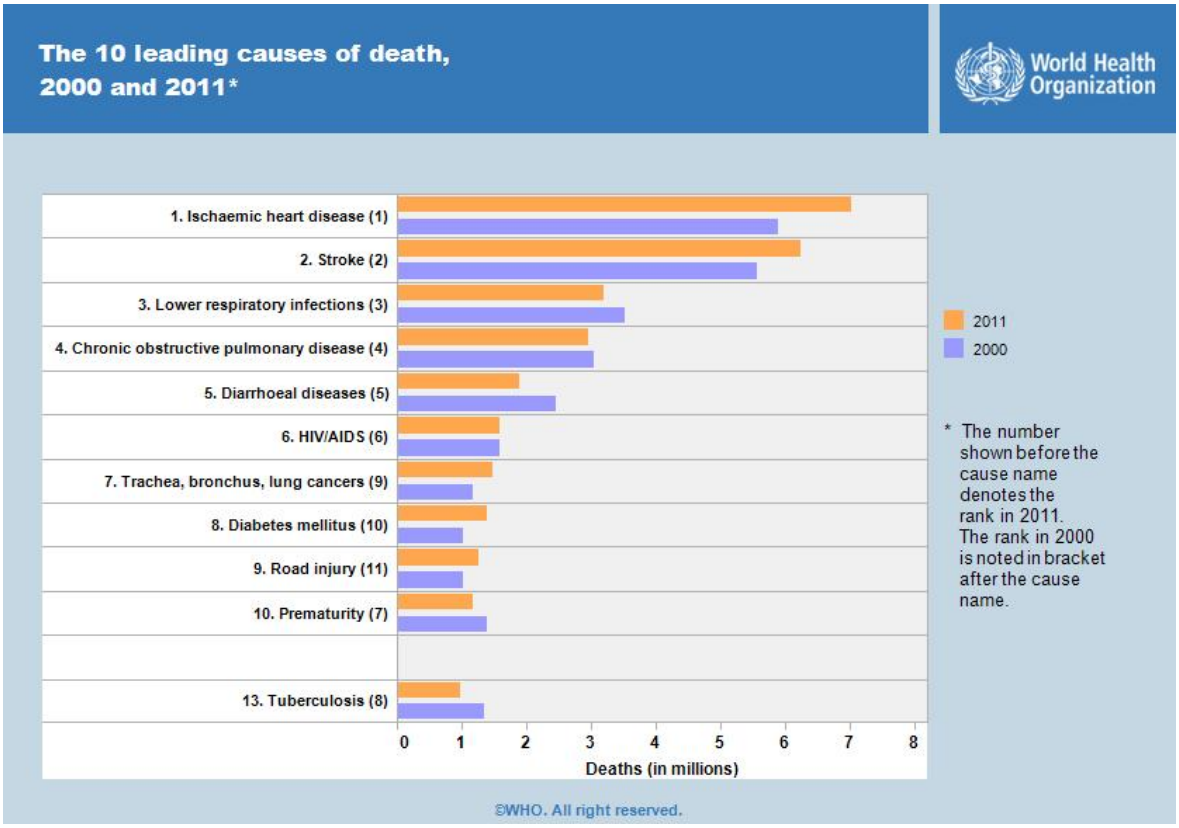


Figure 1-1; Worldwide mortality caused by the leading health problems

It can be seen that IHD and stroke are the leading causes of mortality worldwide and more than infectious diseases. The Rise in CVD from 2000 to 2011 and decrease in communicable diseases can be seen. (Picture taken from WHO website)

The incidence of the cardiovascular disease is on the rise, equally affecting both genders and it currently causes more than 80% deaths in low and middle socio economic groups (World Health Organization, 2014a). It is predicted that deaths due to CVD will be around 23.3 million by the year 2030 (World Health Organization, 2014).

CVD is a global health problem with no geographic, ethnic or socioeconomic barriers (World Health Organization, 2014a). The developing world is more affected due to factors such as lack of professional expertise as well as financial support (Deaton et al., 2011). The lower socioeconomic groups in the developed

countries have a higher incidence of CVD due to higher prevalence of the risk factors (Yusuf et al., 2001). This shifts the diseases burden to the lower socio economic group as a result of maturation of the CVD epidemics (World Health Organization, 2014a).

Deaths due to CVDs affect the different regions in the world differently. Low income countries have more mortality and morbidity due to CVD than economically stable countries. For example the mortality rate in Russia and Egypt is more than 500 per 100,000 inhabitants, while it is between 100 and 200 in Australia, France and United States. China, Brazil, South Africa and Saudi Arab lies in the middle range of 300 to 450 per 100,000 inhabitants (Fuster and Kelly, 2010).

CVDs also kill people at younger ages in the developing world. For example, in USA 9% deaths between 34-61 years age can be attributed to CVD. while in countries like South Africa and India this proportion is as high as 41% and 35% respectively (Raymond et al., 2006).

The number of deaths due to CVD varies considerably from country to country and over time. There are differences observed between the countries with in the same geographical regions and sharing the same cultural values. Three different trends in the mortality from CVD since 1950 have been reported (Beaglehole, 1990, Mirzaei et al., 2009).

i. A rise and fall pattern: In this pattern the mortality rises to its peak value showing an ongoing epidemic of the disease followed by significant fall. This pattern is observed in the developed nations including United States, Australia and some European countries, where the decline in the mortality is more than 50% in the last few decades.

ii. A steady pattern: This is also called as a flat pattern. In this pattern, a relatively low and stable mortality rate is observed. This pattern has been mostly observed in Mediterranean countries and Japan.

iii. A rising pattern: This pattern shows an ongoing epidemic of the CVD by a steady increase in the mortality rate. The countries affected by this

pattern have shown an alarming increase in the mortality rate and include the former Soviet Union, Indian subcontinent and some of the countries in Eastern Europe.

CVD causes more than 4 million deaths in Europe each year. This is 46% of deaths due to all causes. CHD and stroke causes more deaths in central and Eastern Europe than other parts of Europe (Nichols et al., 2014).

The CVD is one of the biggest killing diseases in United Kingdom. In England and Wales in 2013, CHD caused approximately 64,000 deaths and stroke caused approximately 35,000 deaths (Office for National Statistics UK, 2013a). The burden of the disease is concentrated in the North of England and Scotland. More than 1 in 4 person dies prematurely i.e. before the age of 75 years. The incidence of angina is higher in Scotland (British Heart Foundation, 2012). Around 7.1% of men and 5.3% of women live with CHD (Information Service Division Scotland, 2015). Obesity and high cholesterol levels are also on the rise leading in the Scots and this is likely to increase the incidence of CVD (Information Service Division Scotland, 2014).

This imposes a huge burden on the economy of United Kingdom. The current cost is around 30 billion pounds each year. This includes; issuing approximately 266 million prescriptions and carrying out 90,000 per cutaneous interventions for CVD annually (British Heart Foundation, 2010).

1.3.2.1 CVD in Indian subcontinent and Pakistan;

The epidemiology of Indian subcontinent especially Pakistan is reported here due to 2 reasons;

i. The incidence and prevalence of CVD in Indian subcontinent is very high due to genetic vulnerability of the South Asian population

ii. The author belongs to Pakistan and will return to Khyber Medical University Peshawar, Pakistan, where he is a member of faculty. The intentions are that the research and reported information about

epidemiological data in this thesis may be helpful for future research in Pakistan.

The clinical picture of CVD in the Indian subcontinent is even worse than Europe. The Indian subcontinent including Pakistan, India, Sri Lanka, Nepal and Bangladesh accommodates more than 20% of the total world population and may be one of the regions in the world with most concentrated CVD burden (Goyal and Yusuf, 2006). Studies done in western countries have also demonstrated a high prevalence of the disease in the immigrants from the Indian subcontinent (McKeigue et al., 1989, Enas et al., 1992, Balarajan, 1991). The mortality rate in immigrants from Indian subcontinent was highest when studied retrospectively and it has showed little improvement in the past few decades. This can be explained by the genetic vulnerability to some co-morbid conditions like Insulin resistance and the environmental factors leading to disease onset at comparatively younger age (Balarajan, 1991).

In author's opinion, the data from the Indian subcontinent needs to be revised as most of the data is under reported. In 2003, 3- 4% prevalence in rural areas and 8 to 10% in urban areas were estimated for CVD. Though, these were 3 fold higher for the same regions than 40 years ago, but still are much less than actual statistics. In 1990 estimated deaths due to CVD in India were around 1.17 million with a predictive rise to 2.3 million by year 2010. CVD manifestation starts around 10 years earlier in Indians, causing a high premature death rate (below the age of 70 years) of 52% compared to the western countries where it is 23% (Goyal and Yusuf, 2006).

Reports available on the burden of CVD in Pakistan are not representative of the whole population as there is no national data base with exact figures and statistics. The studies done by different investigators are mostly estimates, indicating a high prevalence and disease burden of CVD but they are limited to self reporting people without the knowledge of their existing health conditions.

For example, one study reported the CVD prevalence to be 1.9% in wealthy population and 0.6% in non wealthy population of Karachi. Main limitation of the study was mere reliance on the questionnaire (Hameed et al., 1995).

Subsequent studies used better approaches (Jafar et al., 2005, Nishtar et al., 2004). They reported a of 26.9% prevalence of CVD in males and 30% in females. The prevalence was high in females and young adults. The premature prevalence reported was 23.6%, which points to a massive disease burden in Pakistan. High rates of dyslipidmia, high blood pressure and diabetes in Pakistani population, counterbalance their lower rate of smoking and alcohol consumption (Ministry of Health Pakistan, 2004).

1.3.3 Risk factors for cardiovascular diseases

This section will focus on different risk factors for atherosclerosis in general population. Physical inactivity, as risk factor for cardiovascular disease is discussed separately in section 1.3.9, of this chapter.

Risk factors are defined as any behaviour, practice or exposure of an individual that leads to the development of a particular health problem (Deaton et al., 2011). The risk factors for CVD can be 'non modifiable' for example age, sex, family history or 'modifiable' for example cigarette smoking, obesity, high blood pressure, abnormal lipid profile, diabetes and lack of physical activity (Susan et al., 2009).

When multiple risk factors are present they combine to increase the risk of incidence, mortality and morbidity (Kannel and Gordon, 1968). Though single risk factors influence the overall outcome for the CVD, risk factors often tends to occur in clusters and accelerate the disease process (Webber et al., 1979). Recent research attributes 75 percent of all CVD to smoking, high blood pressure, deranged blood glucose levels, high blood cholesterol and sedentary life style (Yusuf and Ôunpuu, 2003). A study by Berenson correlating the extent of atherosclerosis at post mortem in 204 young individuals who died of some other cause, with the ante mortem history on the risk factors revealed a significant aggregating effects of risk factors on atherosclerosis (Berenson et al., 1998). Subjects with 3 or more risk factors had 35% streak in the intimal layer of aorta compared to 19% for those with a single risk factor. The coronary arteries showed similar changes.

A study conducted in 52 different countries involving 27098 cases and controls revealed that abnormal lipids, high blood pressure, physical inactivity, smoking, diabetes, non healthy diet, obesity, alcohol consumption and stress were the 9 modifiable risk factors in 90 percent of the CVD related events in both males and females of all ages (Yusuf et al., 2004). However, poor awareness of the modifiable risk factors due to lack of proper education also contribute to the increase mortality and morbidity due to CVD in developing world (Rosengren et al., 2009). The INTERHEART study reported that despite the fact that CVD causes more deaths than any other disease for both gender, males develop CVD generally a decade earlier than females (Anand et al., 2008). The study reported that the ratio of males to females suffering from CVD was 3 to 2 before the age of 60. 4 out of 5 males with early onset of the disease had an aggregation of the risk factors.

Change in diet with higher consumption of saturated fat, meats and dairy product has increases obesity in adults (Friel et al., 2007), as well as in children and the incidence of high cases of IHD in adolescence is associated with childhood obesity (Baker et al., 2007).

Smoking is one of the dangerous risk factors leading to CVD with a peculiar property of multiplying the risk due to other factors (Deaton et al., 2011). The rate of smoking in the developed countries is decreasing compared to developing world due to effective enforcement of law, anti smoking campaigns and policies (Ezzati et al., 2005).

However, lack of implementing such policies along with cheap availability of the cigarettes in developing world increase the incidence, mortality and morbidity due to CVD.

1.3.3.1 Risk factors in South Asians

Apart from South Asian ethnicity, as an independent risk factor, regional and traditional risk factors contributing to the enormous disease load, do exist (Goyal and Yusuf, 2006). Despite apparent low levels of risk factors, south Asians have more CVD than European population. An overseas study on South Asians showed 5 times higher incidence of diabetes (Bhopal et al., 1999).

High blood pressure was responsible for 1 out of 4 deaths due to CVD in India (Gupta, 2004). Similarly, smoking with 56% prevalence rate in men between the ages 12 to 60 years and abnormal lipids levels affecting 37.5% of adults are other dominant risk factors (Goyal and Yusuf, 2006).

The data available on the risk factors leading to CVD in Pakistan is underreported, outdated and biased due to lack of central data base system. Most of the data available are based on cross sectional surveys, population based studies with small sample size or estimated numbers of the individuals with attributed risk. Contrary to this, studies done on overseas Pakistani population are more reliable, systematic and organised.

High blood pressure and diabetes are considered major risk factors. The national health survey conducted 20 years ago, reported 17.9% and 33% prevalence of high blood pressure below and above 45 years age (Ministry of Health Pakistan, 2004). Similar prevalence rates were mentioned in other studies (Jafar et al., 2003, Shah et al., 2001).

Obesity in Pakistan is also on the rise and more prevalent in women than men. 40% of females in urban area above the age of 45 years are overweight or obese. Similarly, 12.6% of the population have abnormal cholesterol or lipids levels. Dyslipidaemia at young age requiring dietary interventions (62% of school going girls and 54% of boys) were reported in one of study (Ministry of Health Pakistan, 2004).

In summary, the data from Ministry of Health in Pakistan shows that the risk factors for developing CVD are more concentrated in urban areas than rural areas. This can be attributed to the fact that urban population consume more western diet and are involved in less physical activity. The population in the rural area depends mostly on farming, consume more fresh vegetables and fruits and perform more physical work. Women in general and especially overweight women in urban area are at a high risk of developing CVD. It is probably due to their reduced physical activity. The social, cultural and religious discouragement also plays a huge role in condensing risk factors in females (Ministry of Health Pakistan, 2004). The data from national health survey provided by the Pakistan Medical Research Council (PMRC) are tabulated in table 1-1.

		Rural area %	Urban area%
Coronary artery disease	Male	--	26.9
	Female	--	30
	Overall	--	--
Hypertension	Male	18-25	28-37
	Female	15-28	25-43
	Overall	16.2	21.5
Diabetes	Male	10.3	11.1
	Female	4.8	10.6
	TOTAL	6.39-13.5	10.8-16.5
Obesity	Male	9	22
	Female	14	37
	Overall	--	--
Smoking	Male	39.13	30.51
	Female	--	--
	Overall	--	--
Abnormal lipids	Male	--	--
	Female	--	--
	Overall	12.7	12.7

Table 1-1; Prevalence of CVD and risk factors in Urban and rural area of Pakistan

It can be seen that even the national level data is not completed for most of the risk factors (Modified from Ministry of health Pakistan report).

1.3.4 Pathogenesis of Atherosclerosis

Atherosclerosis is regarded as the basis of ischemic heart diseases having a complex and diverse mechanism based on multiple factors (Berliner et al., 1995, Falk, 2006). Atherosclerosis was initially regarded as an age dependent degenerative disease, but the advances in science have shown it to be a multifocal, immuno-inflammatory disease of medium and large arteries, which is initiated by the concentration dependent transport of lipoprotein to the artery wall (Young and Parthasarathy, 1994, Falk, 2006).

High cholesterol levels are a risk factor for the development of the atherosclerosis and are capable of forming atheroma on the susceptible sites alone. Other risk factors including smoking, diabetes, high blood pressure and inflammation play their role in accelerating the process. However, their role is uncertain and could be partially attributed to the rise in the susceptibility of the arterial wall or increase in the LDL atherogenicity by different mechanisms, like increasing particle size and changing composition. In addition, smooth muscle cells, endothelial cells and white blood cells play important roles in development of atherosclerosis (Falk, 2006).

Atherosclerosis begins in the susceptible area of the endothelium, which is intact but non-functional and permeable to many plasma molecules. The endothelium is lost from this area leaving sub endothelial tissue exposed (Davies et al., 1988), which is followed by transport of lipoprotein to the sub endothelial space and formation of the fatty streak by oxidation of the lipoprotein (Berliner et al., 1995, Navab et al., 1995). This modification of the lipoprotein to fatty acid is very complex and the precise mechanism is unknown, but Nitric Oxide synthase (NOS) produced by macrophages, myloperoxidase and lipoxygenase are thought to play important roles in oxidation (Glass and Witztum, 2001). Fatty streak molecules induce a chronic inflammatory process and initiate the migration of different cells to the sub endothelial space. Due to the inflammatory stimulus, the endothelium and platelets enhances the expression of different adhesion molecules including vascular cellular adhesion molecule-1 (VCAM-1), inter-cellular adhesion molecule-1 (ICAM-1), E selectin and P selectin and chemokines such as monocyte chemotactic protein 1 (MCP-1). These lead to the migration of white blood cells to the atherogenic area (Quillard and Croce, 2015). Circulating monocytes and B lymphocytes are the first to respond and their presence in the affected area accelerates the ongoing disease process. Recruitment and migration of monocytes into the sub-endothelial space is essential for the process of atherosclerosis (Rolin and Maghazachi, 2014). Plaque development has been reported to be reduced after depletion of monocytes from the circulation. A high monocyte count has been identified as one of the independent risk factors for development of atherosclerosis (Afiune Neto et al., 2006). The process of recruitment is very complex. Initially MCP1 expression was considered to play an important role. However, recent work has shown that large number of

chemokines and their receptors are involved in this process (Rolin and Maghazachi, 2014). The most important include: CCR2, CCR5, CXCR2, CX_3CR1, CX3CL1 (Fractalkine), CCR1 and CCR2. In addition, to chemotaxis of monocytes they also perform additional functions during the disease process. For example, adhesion molecule CX_3CR1 is expressed on the surface of monocytes. Cross talk with CX3CL1 (Fractalkine) on the surface of smooth muscles cells increases the expression of other patherogenic and inflammatory mediators including TNF-α, MMP-2, IL-6, IL-1β (Butoi et al., 2011). B lymphocytes and plasma cells are mostly present in later stage of the disease and mostly in the adventitial layer of the vessels. Mast cells are mostly present in the non benign plaque (Kaartinen et al., 1998) and neutrophils are usually seen in thrombosed or ruptured plaques (Naruko et al., 2002).

The disease progresses by conversion of monocytes to macrophages which start digesting the fatty streak. This leads to the formation of lipid loaded macrophages called foam cells. They are considered to be characteristic of primitive and recent atherosclerotic activity. The activity of macrophages continues till their death which leads to the formation of a soft and destabilized lipid core (Glass and Witztum, 2001). Macrophages also contribute to matrix degradation by up regulating enzymes like matrix metalloproteinase (MMP) (Libby et al., 2002). The immune response due to different mediators discussed above, modified LDL and heat shock protein goes side by side throughout all the process and modulates the disease (Hansson, 2005). Interaction between foam cells and lymphocytes causes the release of inflammatory mediators such as IL-4, IL-10 and IL-14 which in turn causes smooth muscle cells to migrate into the area (Quillard and Croce, 2015). Smooth muscle cells mediate the fibro-proliferative response for healing the artery. Both the inflammatory and healing process continue in parallel and in most cases, leaving a massive effect on the vessel wall. The lumen is reduced in the process leading to compromised blood flow and may precede an ischemic event (Patel, 2014). At the same time, the formation of a collagen rich matrix produced by the smooth muscle, is beneficial as it can stabilise the plaque and reduce the devastating consequences of rupture and thrombosis (Schwartz et al., 2000).

Cell death due to apoptosis and necrosis in the area of the plaque can occur, during the process of atherosclerotic plaque formation (Falk, 2006). This can be

followed by calcification of the necrotic area which may increase with age (Hoffmann et al., 2003). As this process of deposition of lipid and cholesterol continues along with proliferation and migration of smooth muscle cells, the plaque grows in size and becomes unstable. MMPs secreted by macrophages degrade the fibrous plaque and many other mechanisms including angiogenesis, plaque haemorrhage and infiltration of inflammatory cells, makes the plaque vulnerable to rupture (Quillard and Croce, 2015).

In advanced atherosclerotic plaque there is a greater chance of plaque rupture. The review by Falk, reported that globally 76% of the mortality due to heart attack is because of rupture and/or thrombosis of the plaque (Falk et al., 2004). Erosion of the plaque and other ill defined events were responsible for the remaining of 24% of the mortality. Plaque rupture was more common in males (~80%) than in females (~60%). Coronary artery thrombosis was most frequently due to rupture of a plaque.

1.3.4.1 Angiogenesis in malignant plaque and rupture of the plaque

Angiogenesis also called neovascularisation (see section 1.3.5 for details), is frequently present in the advance stages of atherosclerosis and is probably one of the markers distinguishing a malignant plaque from a benign one (O'Brien et al., 1994). Under normal physiological conditions angiogenesis is rare in arteries. It is thought that endothelial dysfunction initiates the process of angiogenesis following plaque formation (Moreno et al., 2006). Atherosclerosis stimulates angiogenesis by switching on the angiogenic switch through changing the concentrations of angiogenic and angiostatic mediators (Matsunaga et al., 2005). In the initial stages as the plaque size increases, intimal neovascularisation as well as hyperplasia of the vasa vasorum in the adventitial layer occurs to provide nutrition to the plaque cells and medial arterial walls. In the later stages, however, the ectopic neovascularisation is initiated by vessels in vasa vasorum (Patel, 2014). The intimal neovascularisation is derived mostly from adventitial vessels in most cases (96.5%) as compared to arterial lumen (3.5%). Typically, angiogenesis originates in the adventitial layer and erodes into the base of the plaque. Increased blood pressure in the atherosclerotic area causes the adventitial vessels to collapse and impairs the local blood flow which in turns produces local hypoxia and stimulates angiogenesis (Fleiner et al., 2004).

Adventitial angiogenesis continues as the disease progresses. It is thought to be an adaptive mechanism to provide nutrients to the medial wall and prevent local ischemia. However, despite its protective role, it contributes to the increase in plaque size and leads to adverse cardiovascular events. Vessel density in the vasa vasorum in advanced stages of atherosclerosis has been reported to be higher than early stage (Fleiner et al., 2004). In the later stages of the disease the sub-intimal space and media decreases and become sandwiched between intima and adventitial, leading to sprouting of the neo-vessels to plaque in the intima (Herrmann and Lerman, 2007).

Vessels formed as a result of the angiogenesis lead to local influx of the inflammatory cells, red blood cells and plasma protein, which may play an important role in the rapid progression of atherosclerosis (Virmani et al., 2005). These neo-vessels are leaky, more delicate and prone to micro and gross haemorrhage into the plaque area (Armstrong et al., 2011). The leaky neo-vessels cause more influx of inflammatory cells and RBCs into the plaque. Lyses of the RBCs adds to increase lipid load and plaque size and hence increase the plaque vulnerability to rupture (Moreno et al., 2004).

In summary, angiogenesis plays an important role in the early stage of atherosclerosis by providing nutrition to the medial wall of the diseased artery. In the later stages, angiogenesis provides a secondary route for transfer of many inflammatory mediators and lipid deposition into the plaque. This in turn adds to the vulnerability of the plaque to rupture. However, angiogenesis is not the only process increasing the malignancy of atherosclerotic plaque. Many other mechanisms are thought to play important roles as shown by morphological characteristics of malignant plaques i.e. thin fibrous cap, angiogenesis, large lipid core, necrosis, intra plaque haemorrhage and excessive inflammatory cells infiltration (Nighoghossian et al., 2005, Patel, 2014).

1.3.5 Angiogenesis

Angiogenesis is defined as the formation of new vessels from the capillary beds already present (O'Brien et al., 1994). The word is derived from ancient Greek. “Angio” means vessel and “genesis” means birth, collectively meaning the production of new vessels (Korivi et al., 2010). However, vasculogenesis is also a

process of formation of vessels from the primitive vascular plexus and can be differentiated from angiogenesis. The latter arises from the pre-existing vessels. Vasculogenesis starts during embryonic life and give rises to heart and first vascular web. Subsequent remodelling and expansion of this primary network to secondary network is brought about by angiogenesis (Distler et al., 2003, Korivi et al., 2010).

The term angiogenesis was formerly used to describe the formation of vessels after terminal capillaries. The most recent studies use angiogenesis to describe the conversion and development of simple vascular networks into a more complex ones (Carmeliet, 2000). Angiogenesis occurs under normal physiological conditions in processes like wound healing, body development, damaged tissue repair, female reproductive cycle and pregnancy. However, progressive angiogenesis can be dangerous, for example in the presence of primary plaque, it can expand the size of plaque causing significant complications like vascular thrombosis and rupture of the plaque etc (O'Brien et al., 1994, Isner, 1999, Kahlon et al., 1992). Moreover, impaired regulation of angiogenesis can also lead to other pathological conditions like tumour progression and metastasis etc ((Boehm et al., 1997) (Distler et al., 2003), delayed wound healing (Charifi et al., 2004), age related macular degeneration and diabetic retinopathy (Korivi et al., 2010).

Angiogenesis is regulated through a balance of endogenous angiogenic (activators) and angiostatic (inhibitors) factors (Hanahan and Folkman, 1996) with a precise mutual interactions (Distler et al., 2003). Any shift in favour of positive regulators will lead to angiogenesis and a shift towards negative regulators will lead to angiostasis. Under normal physiological conditions, the angiostatic factors outweigh the angiogenic factors, not allowing the angiogenesis to occur. It is only under pathological circumstances like wound healing the angiogenic factors overcome the angiostatic factors leading to angiogenesis (Distler et al., 2003, Hall et al., 2006).

To date about 30 angiogenic and angiostatic factors are known. Angiostatic mediators include Endostatin, Angiostatin, Thrombospondin, Pigment epithelium derived factor (PEDF) etc (Distler et al., 2003).

Angiogenic factors are divided to 3 different categories based on their effects at different levels and target cells. These categories include;

i. Factors affecting degradation of basement membrane and consist mostly of proteolytic enzymes like plasminogen activator (PA) and matrix metalloproteinases (MMPs) (Liekens et al., 2001).

ii. Factors affecting endothelial cell proliferation and migration and these include; VEGF, Angiopoietin, Fibroblast growth factor (FGF) and other cytokines, chemokines and enzymes. This group also includes non directly related mediators such as tumor necrosis factor-alpha (TNF-α) and transforming growth factor-beta (TGF-ß) (Klagsbrun and Moses, 1999).

iii. Factors which help in migration, cell to cell and cell to basement membrane interactions called adhesion molecules. These include; different Immunoglobulins, selectins, integrins and cadherins (Bischoff, 1997).

The process of angiogenesis can be activated readily through various mechanisms and factors; such as metabolic stress, physical stress, inflammation. Metabolic stresses include hypoxia, decreased pH and low calcium levels (Carmeliet and Jain, 2000, Korivi et al., 2010).

1.3.5.1 Mechanism of angiogenesis

Angiogenesis starts with dilatation of the vessels mediated by Nitric Oxide (NO), released by the vascular endothelial growth factor VEGF (see section 1.3.7) through its action on NOS. VEGF also increases the permeability of endothelial cells with formation of vacuoles from central to peripheral part. The transport of plasma protein and plasminogen to the surrounding space rises and serves as base for the migration of endothelial cells (Kohn et al., 1992, Dvorak et al., 1995). Before this migration, removal of the mural cells, reshaping of the stroma and degradation of the basement membrane is mediated by angiopoietin along with different mediators including plasminogen, proteases, chymase and matrix metalloproteinase (Moses, 1997).

This is followed by VEGF, FGF and angiopoietin dependent migration of endothelial cells through the degraded basement membrane and proliferation in the reshaped perivascular space. They form a tube like structure by arranging themselves in a monolayer (Distler et al., 2003). Transport of mesenchymal cells and their proliferation and conversion into pericytes and smooth muscle cells at the anti-luminal surface occurs. These cells stabilises the vascular tube (Kurz, 2000).

1.3.5.2 Different angiogenic and angiostatic mediators and their role in control of angiogenesis

As mentioned in section 1.3.5, angiogenesis is controlled and regulated by many endogenous angiogenic and angiostatic mediators. This section will briefly discuss some of the most important angiogenic and angiostatic mediators and their role in angiogenesis.

Angiogenic mediators

Vascular endothelial growth factor (VEGF)

VEGF is probably the most important angiogenic factor and it initiates the process of angiogenesis. It has been shown that disruption of a single allele of the VEGF gene in mice causes severe vascular abnormalities and intra uterine death (Ferrara et al., 1998). The specific actions of VEGF include: endothelial cell proliferations and migration, increasing permeability of the endothelial cells, induction of the MMP and plasminogen and increasing endothelial cell survival by decreasing apoptosis (Distler et al., 2003). This is further discussed in section 1.3.7.

Fibroblast growth factor (FGF)

There are 22 different types of fibroblast growth factors identified so far in human body. They are heparin sulphate binding proteins. FGF were amongst the first molecules shown to initiate angiogenesis (Ornitz and Itoh, 2001). They act on endothelial cells and are involved in proliferation of endothelial cells. They work in close contact with VEGF as they increase the expression of VEGF and plasminogen activator (Distler et al., 2003). They have four different types of

receptors (FGFR1, FGFR2, FGFR3, and FGFR4) which are expressed by different cells and tissues (Ornitz and Itoh, 2001). FGF are expressed by almost all tissues in the body. They are involved in a variety of functions including embryonic development, organogenesis, tissue maintenance, repair and regeneration. They are also involved in phosphate, bile acid, carbohydrate and lipid metabolism (Ornitz and Itoh, 2015).

Angiopoietin

These are paracrine growth factors which act on endothelial cells. There are 2 types of these factors angiopoietin 1 and 2. Angiopoietin 1 is involved in the vessel stabilization (Ikeoka et al., 2014) while angiopoietin 2 in the presence of VEGF causes migration and proliferation of endothelial cells while in the absence of VEGF; it causes apoptosis and destabilizes the blood vessel (Distler et al., 2003).

Platelet derived growth factor (PDGF)

This growth factor is involved in mitosis of cells of mesenchymal origin including smooth muscle cells and glial cells. There are 4 different types of PDGF (PDGF A – D) and 2 different types of receptors (Distler et al., 2003). They are involved in smooth muscle cells and pericytes proliferation during angiogenesis (Carmeliet and Jain, 2000, Shah et al., 2014).

Transforming growth factor-β (TGF-β)

TGF-β receptors are expressed by pericytes and endothelial cells. TGF-β has been reported to have both pro-angiogenic and anti-angiogenic properties. An in vitro study has reported angiogenic effects (increased proliferation and migration of endothelial cells) of TGF-β while in higher dose the effects are opposite (increased proliferation and migration of endothelial cells) (Pepper, 1997).

Tumor necrosis factor-α (TNF-α)

(TNF-α) is an inflammatory mediator which has similar effects on angiogenesis as TGF-α. It also promotes mitosis of endothelial cells and formation of vessel tubes in low doses while in high doses it inhibits the FGF induced proliferation of endothelial cells (Distler et al., 2003).

Chemokines

Chemokines such as CXCR2 and CXCR4 are involved in angiogenesis. They act as chemotactic proteins for the migration of endothelial cells (Kanzler et al., 2013).

Integrins

Integrins work as adhesion receptors for many angiogenic and angiostatic factors and play important role in the control of angiogenesis. They promote and control endothelial cells adhesion, migration and survival (Foubert and Varner, 2012).

Cadherin

They are trans-membrane molecules and are involved mainly in cellular signalling mechanisms. VE- Cadherin is specifically involved in angiogenesis and works closely with ß- catenin and maintains the cell to cell adhesion (Bentley et al., 2014).

Other angiogenic factors

Beside the molecules discussed above many other molecules has been shown to have angiogenic effects. These include MMPs, plasminogen, erythropoietin, NO, platelet endothelial cell adhesion molecule, insulin like growth factor and epidermal growth factor. Their roles are in the later stages of angiogenesis and most of them are initiated in response to VEGF action (Distler et al., 2003).

Angiostatic factors

Many endogenous angiostatic factors have been discovered. The most important include:

Endostatin

It is discussed in detail in section 1.3.6.

Angiostatin

Angiostatin is derived from plasminogen. It was first purified from urine of mice (O'Reilly et al., 1994). It requires the action of MMPs to produce it from plasminogen. It inhibits proliferation and migration of endothelial cells and tube formation (Eriksson et al., 2003). It has also been reported to inhibit smooth muscle cells migration and proliferation (Walter and Sane, 1999).

Thrombospondin

These are glycosylated extracellular proteins with angiostatic effects on endothelial cells. TSP-1 and TSP-2 are mainly involved in decreasing endothelial cells migration and survival by enhancing apoptosis (Carmeliet, 2000).

Other angiostatic factors

Beside the factors discussed above many other angiostatic factors have also been reported. These include pigment epithelium derived growth factor, TGF-ß, MMPs, TNF – α, restin, tumstatin. Interestingly, some of these mediators, such as TGF- ß, TNF – α, MMPs posses both angiogenic and angiogenic properties (Distler et al., 2003).

The VEGF family plays the most important role in angiogenesis, whereas the most important angiostatic inhibitors include Angiostatin, Endostatin and Thrombospondin (Korivi et al., 2010, Dameron et al., 1994, O'Reilly et al., 1997). However, endostatin has been identified as the main potent inhibitor of angiogenesis and is one of the major topics of research in recent times.

1.3.6 Endostatin

Endostatin is one of the potent angiostatic mediators (O'Reilly et al., 1997, Abdollahi et al., 2004, Yamaguchi et al., 1999). Endostatin halts angiogenesis by specifically inhibiting the proliferation of endothelial cells (O'Reilly et al., 1997). It was identified for the first time in conditioned media of haemangioendothelioma cells of mice (O'Reilly et al., 1997).

1.3.6.1 Structure of the Endostatin

Endostatin is a 20 kDa, C-terminal fragment of collagen XVIII and collagen XV (Sasaki et al., 2000, O'Reilly et al., 1997). There are 28 types of collagens identified in the mammals (Ricard-Blum, 2011). They are involved in the formation of the extracellular fibrillar network. Collagen XVIII and Collagen XV are found in the basement membrane (Sasaki et al., 2000). Collagen XVIII is also found in the intestine, choroid plexus, skin, liver and kidneys (Halfter et al., 1998).

They are classified as part of the multiplexins family with a central collagen triple helix sequence and terminal C domain called Non Collagenous or NC1, to which endostatin is attached (Marneros and Olsen, 2001).

Figure 1-2 shows the difference between collagen XV and XVIII. The N terminal of collagen XVIII shows three different variants as a result of alternative splicing (Muragaki et al., 1995). Both collagen XV and XVIII are proteoglycans which differs from other members due to prominent modification by chondroitin sulphate in collagen XV (Li et al., 2000) and by heparin sulphate in collagen XVIII (Halfter et al., 1998). They are thought to play a significant role in the organization of basement membrane and other extracellular structure, as well as a source of endostatin release (Sasaki et al., 2002).

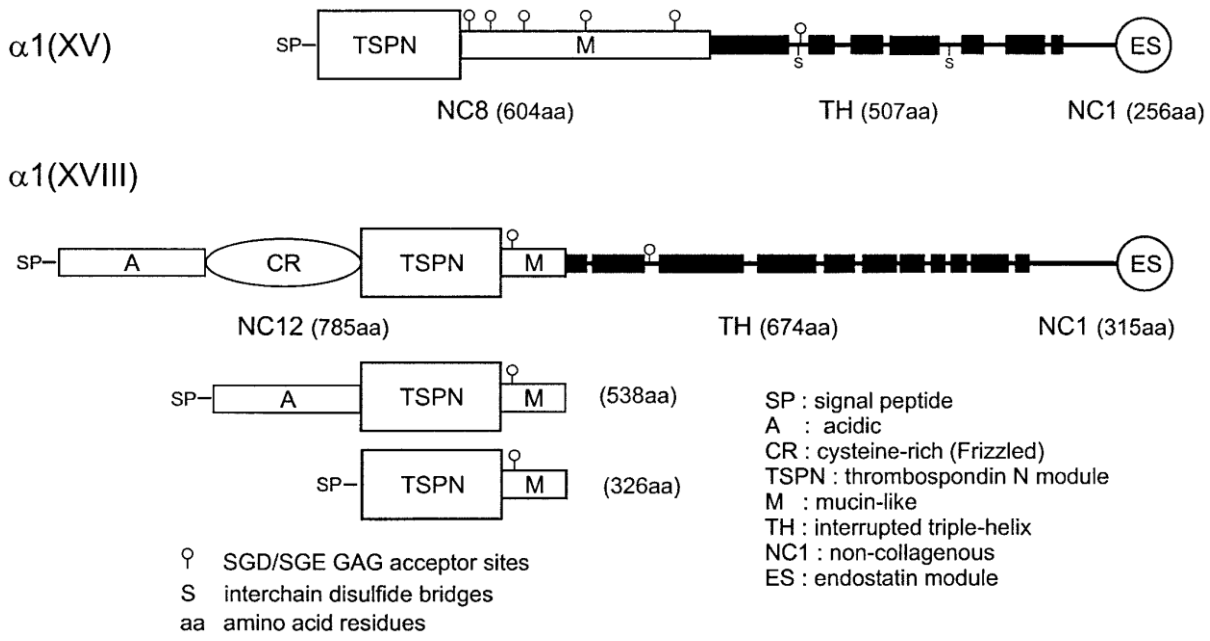


Figure 1-2; Structure of collagen XV and XVIII

Collagen XV and collagen XVIII domain structure showing that Collagen XVIII has three different splice variants. The endostatin is attached to the NC1 terminal. Figure reproduced with permission from (Sasaki et al., 2002).

The structure and function of endostatin have been studied by many researchers (Hohenester et al., 1998, Sasaki et al., 2002). Both endostatin XV and XVIII have been isolated from many species including murine cells (O'Reilly et al., 1997), bacteria (Ramchandran et al., 1999) yeast (Dhanabal et al., 1999a) and mammalian cells (Yamaguchi et al., 1999).

The form of the endostatin obtained from bacteria was found to be highly insoluble with improper folding due to disulfide linked oligomers (Boehm et al., 1998). However, endostatin in monomeric forms were obtained from yeast and mammalian cells with proper folding and high solubility (Sasaki et al., 2002).

NC1 domain of collagen XVIII consists of 312 residues in human, which comprises of N terminal region, a middle hinge part and a C terminal part, to which endostatin is attached. The middle part or the hinge contains proteolytic sites sensitive to many endogenous proteases, as shown in figure 1-3. These proteases initiate the release of endostatin from basement membrane to soluble endocrine form (Sasaki et al., 1998).

The hinge is flexible and is longer in collagen XVIII with more proteolytic sites than in collagen XV. That is why collagen XVIII possesses high susceptibility for the proteolytic cleavage and most available form (Sasaki et al., 1998, Felbor et al., 2000).

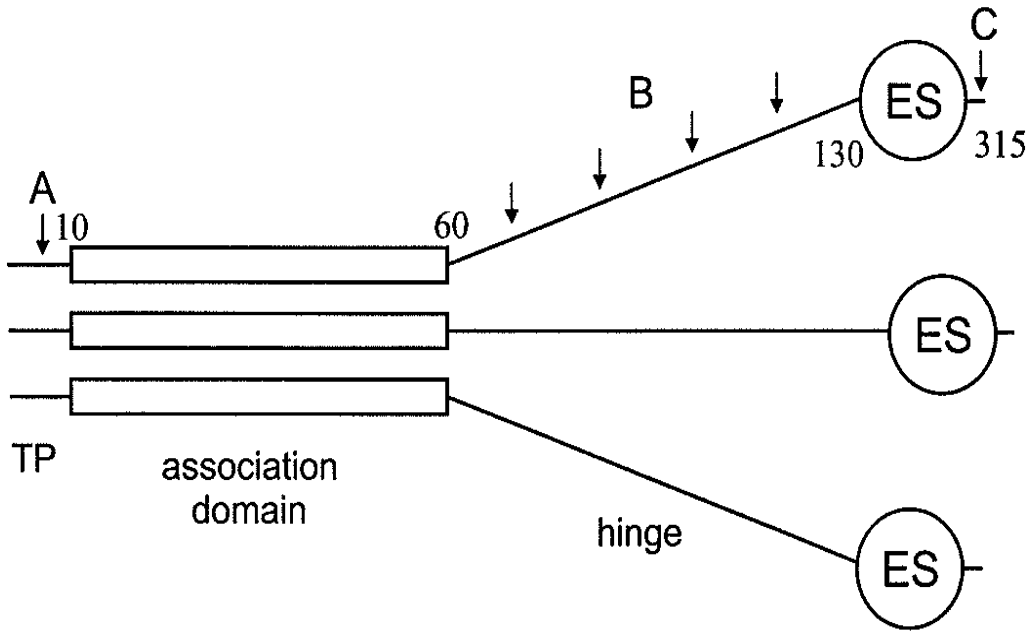


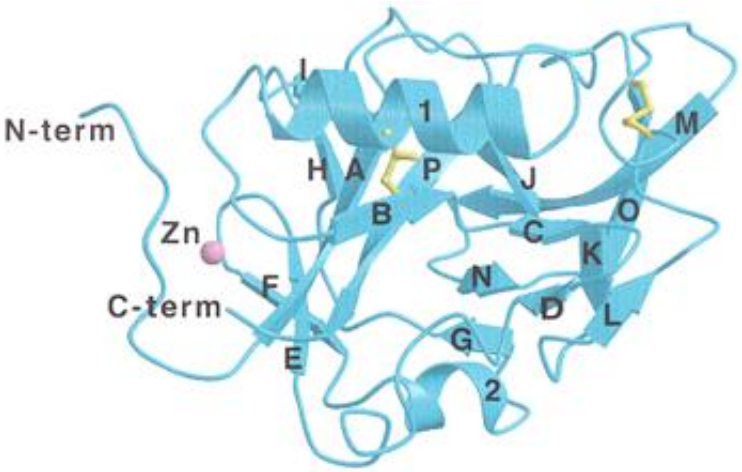
Figure 1-3; NC1 part of collagen XVIII

Schematic structure of the NC1 part of collagen XVIII is shown. TP is the region of the N terminal which shows the area of attachment with collagen XVIII, while ES shows the C terminal area to which endostatin is attached. Arrows shows the proteolytic sites and the number represents the approximate sequence positions within NC1. Reproduced with permission from the author given in appendix "G" (Sasaki et al., 1998). Note that NC1 for collagen XV is identical to this with a smaller hinge and less proteolytic sites.

When the structure of endostatin was determined using high resolution X-Rays crystallography, it is globular, about 3 nm in diameter and has 16 beta strands, 2 alpha helices and 2 disulfide bridges, as shown in figure 1-4 A & B (Hohenester et al., 1998, Sasaki et al., 2002).

The same studies also reported the presence of a patch containing 11 arginine residues and a site for zinc binding in collagen XVIII. Arginine residues give endostatin XVIII a much higher affinity for binding with heparin than endostatin XV, which lacks both. Zinc, however, is thought to provide structural strength to the shape of endostatin. Removal of the zinc from recombinant mouse endostatin did not affect the inhibitory activity of endostatin (Sasaki et al., 1999).

A



B

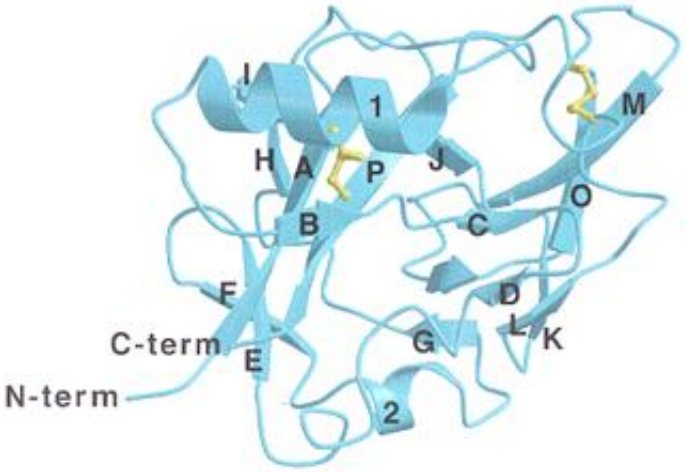


Figure 1-4; Atomic structures of Endostatin for (A) collagen XVIII and (B) collagen XV

Collagen XVIII showing 16 beta strands indicated as A-P, 2 alpha helix indicated as 1 & 2, disulphide bridges are in Yellow and Zn attachment sight in Pink. Note that there is no zinc attachment sight in collagen XV. Figure reproduced with permission from Review Article by (Sasaki et al., 2002).

1.3.6.2 Release of endostatin and molecular mechanisms for endostatin actions

As discussed earlier, release of endostatin requires a proteolytic cleavage. Many endogenous enzymes groups such as protinases including matrix metalloproteinases (MMP 3, 7,9, 12,14, 20) (Ferreras et al., 2000), elastase (Wen et al., 1999) and cathapsin L (Felbor et al., 2000) have been shown to increase the endostatin release.

Endostatin has been shown to inhibit angiogenesis through many mechanisms. Some of them are listed below and shown in figure 1-5.

Endostatin binds to the α5β1 on the surface of the endothelial cells. After binding with the help of cavolin-1, a cascade of molecular events starts leading to activation of actin stress fibres. These in turn decrease the migration of endothelial cells (Wickström et al., 2003).

Endostatin has also been reported to bind directly to VEGF receptors and to block the VEGF induced phosphorylation of KDR/FLK-1. This stops the signalling mechanism of VEGF (Kim et al., 2002). The inhibition of Wnt/ß-catenin signalling pathway at the level of ß-catenin has also been suggested as one of the possible mechanisms of endostatin (Hanai et al., 2002).

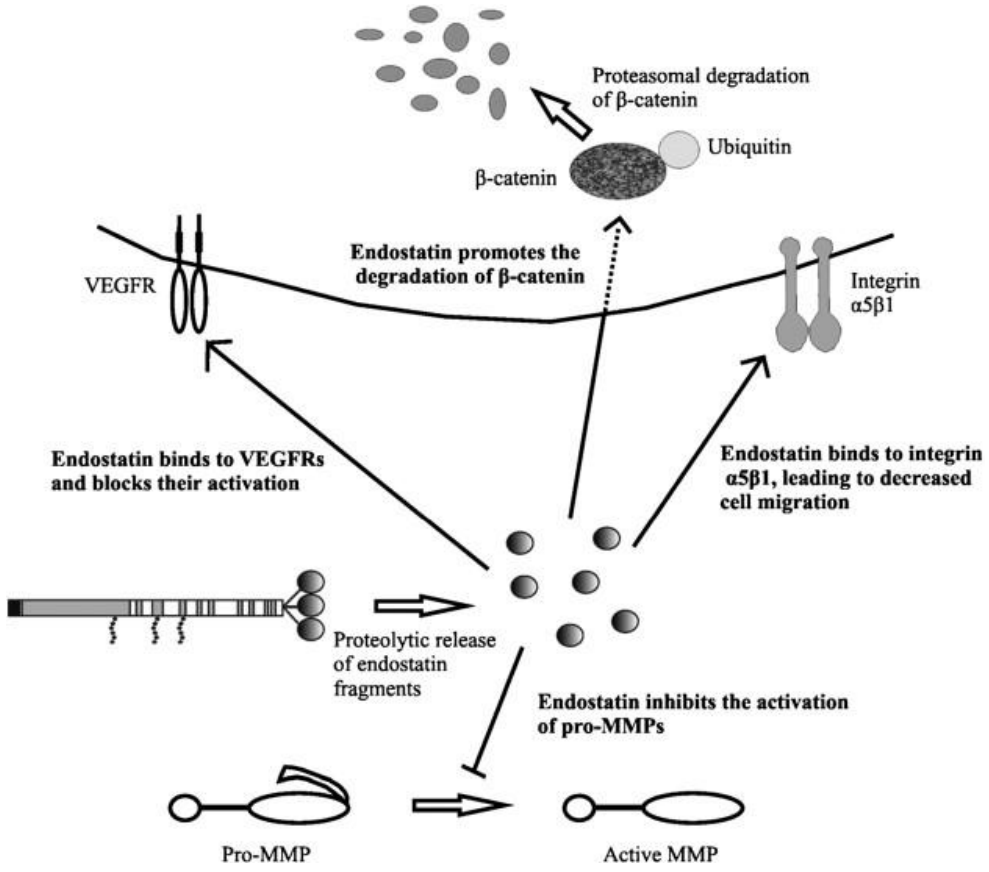


Figure 1-5; Mechanisms for release and different actions of endostatin

The possible different mechanisms of release and actions of endostatin on VEGF and other receptors are shown. Reproduced with permission from the author given in appendix "G" (Seppinen and Pihlajaniemi, 2011)

1.3.6.3 Effects of endostatin

The physiological role and the different cellular responses by endostatin are diverse and research is still underway to explore its precise role. Some of the established facts are listed below;

1. A comprehensive and novel in vitro study revealed, endostatin for the first time as an inhibitor of endothelial cell proliferation in low concentration (O'Reilly et al., 1997).

2. Endostatin has been shown to disrupt various cellular events in angiogenesis including endothelial cell migration induced by VEGF (Yamaguchi et al., 1999) and FGF (Eriksson et al., 2003). It also induces apoptosis (Dhanabal et al., 1999b) and affects cellular adhesions (Dixelius

et al., 2002). Endostatin also inhibits the conversion of pro MMP2 to MMp2, which reduces the endothelial cellular invasion (Kim et al., 2000).

3. Endostatin also inhibits the release of circulatory endothelial progenitor cells into the blood stream in mice treated with VEGF (Schuch et al., 2003).

4. Endostatin also changes the genetic regulation of angiogenesis. The effect is bifocal; including up-regulation of angiostatic genes and mediators and down-regulating the angiogenic genes (Abdollahi et al., 2004).

5. Endostatin also inhibits proliferation and migration of lymphatic endothelial cells. Mice with skin cancers and high expression of endostatin were found with decreased lymph vessels density and metastasis through it (Brideau et al., 2007).

6. Endostatin has been used to stop angiogenesis in laser induced ruptured Bruch's membrane of the choroid (Mori et al., 2001). Similarly, formation of new vessels in retina and retinal detachment was slowed, when mice were given long release intraocular endostatin. No toxicity was reported for long term expression and the overall results were reduction in vascular permeability, macular oedema and degenerative changes (Takahashi et al., 2003).

7. Endostatin has also shown good effects in reducing the markers and onset of inflammatory diseases such as inflammatory arthritis (Yin et al., 2002), cystitis (Beecken et al., 2004) and asthma in mouse models (Suzaki et al., 2005).

8. The progression of nephropathy in streptozocin induced type 1 diabetic mouse was delayed by endostatin, which suppressed glomerular hypertrophy and albuminuria. The hyperglycaemia was however, not affected by high endostatin concentrations (Ichinose et al., 2005).

1.3.6.4 Endostatin and tumour suppression

Angiogenesis around many tumour cells has been widely and extensively studied (Seppinen and Pihlajaniemi, 2011). As reviewed by Folkamn, endostatin has been reported to inhibit 65 different types of cancers including; ovarian, prostate, pancreatic, small cell lung tumors and leukaemia. It also affects many tumors genetically, leading to down regulation of the angiogenesis (Folkman, 2006). The review also reported endostatin to be active against cancers both of human and animal origin, with no toxicity reported, in trials as longer as 3 and half years.

Evidence about endostatin, as endogenous inhibitor of tumour in genetically knocked out mice exists (Sund et al., 2005). Clinical evidence of this effect in humans can be correlated to the fact that individuals with trisomy 21 (Down's syndrome) have low incidence of solid tumours. They possess an extra copy of collagen XVIII gene and have high concentrations of circulatory endostatin (Zorick et al., 2001). Increasing the concentration of endostatin in plasma by 1.6 fold (almost equal to that of individuals with Down's syndrome), resulted in 3 fold slower growth of tumour in mice than wild type (Li and Olsen, 2004).

Endostatin has been used in phase 1 (Eder et al., 2002), phase 2 (Whitworth, 2006) and phase 3 (Sun et al., 2005) clinical trials with less than expected results. However, the mechanism of release of endostatin, type, route of administration and dosage is still debatable and research has not unified on single treatment strategy (Abdollahi et al., 2005).

Recent research aims to find alternative ways to extract recombinant endostatin with high potency, effective dosage and administration mode (Fu et al., 2009, Whitworth, 2006). These efforts may see the beneficial effects in near future.

1.3.6.5 Endostatin and cardiac protection

Being a potent angiogenic inhibitor, endostatin plays its role in the cardioprotective mechanism of the body (Golbidi and Laher, 2011). Increase in angiogenic activity signifies healing of the ischemic area (Isner and Losordo, 1999). However, persistent angiogenesis in cholesterol fed mice, increased the plaque expansion significantly up to 14 folds (Celletti et al., 2001). The experiment was repeated in rabbits to address question of species difference,

with similar results. The progressive angiogenesis also increases plaque vulnerability with significant morbid outcomes such as plaque rupture and vascular thrombosis (Kahlon et al., 1991).

These findings signifies the presence of an efficient control mechanism and free availability of endostatin in circulation has been suggested to control progressive angiogenesis in diseased conditions (Miosge et al., 1999). Endostatin provides cardiac protection mainly by halting the angiogenic process and stimulating apoptosis (Taddei et al., 1999, Shichiri and Hirata, 2001). Decreases in size of atherosclerotic plaque in mice has been reported after treating with recombinant endostatin at 20 $mg.kg^{-1}.day^{-1}$ for 16 weeks (Moulton et al., 1999).

As discussed earlier in section 1.3.4, formation of the foam cells by macrophages after retaining the LDL, is one of the initial steps in atherosclerosis (Williams and Tabas, 1998). The blood vessels with the atherosclerotic plaque have been found with less endostatin. Based on these observations, Zeng and his colleagues demonstrated in vitro, that in the presence of recombinant human endostatin, the retaining of LDL decreases significantly (Zeng et al., 2005).

Angiogenesis is a favourable sign for recovery after myocardial infarction (MI). However, using anti endostatin antibody treatment in rats after MI, resulted in significantly higher mortalities and morbidities than control group (Isobe et al., 2010), which also shows its importance in the overall control of the process.

In conclusion, on the one hand endostatin is involved in the cardio protective mechanism of the body against progression of atherosclerosis. The different anti atherosclerotic actions of endostatin include inhibition of neovascularisation, suppressing the endothelial cell proliferation, reducing the macrophages in the plaque area, interfering with the low density lipoprotein retention mechanism and antagonising the vascular endothelial growth factor (VEGF) signalling. On the other hand, it is required for remodelling of the left ventricle after acute myocardial infarction (Isobe et al., 2010, Maemura, 2010).

1.3.6.6 Tissue forms and processing of endostatin

Immuno-staining of the basement membrane of blood vessels has shown expression of both types of collagen. Based on this, endothelial and smooth muscle cells were assumed to be involved in production of collagen XV and XVIII (Marneros and Olsen, 2001). Similarly, human vasculature medium was shown with more expression of endostatin than other tissues such as brain and myocardial tissues (Sasaki et al., 1998). Out of the two isoforms, endostatin derived from collagen XVIII is more frequently available form (Sasaki et al., 2000).

Collagen XVIII has been studied in liver cells, especially hepatocytes. They revealed a strong expression of collagen XVIII and are thought to be a source of endostatin into circulation (Schuppan et al., 1998).

1.3.6.7 Plasma endostatin concentration in healthy and clinical populations

The concentration of endostatin in plasma of healthy volunteers is discussed in depth in chapter 3 sections 3.2.

Briefly, endostatin is freely available in circulation. It was first detected by using radioimmunoassay methods (Ständker et al., 1997). The concentrations of endostatin measured in plasma and serum were in the range of 0.1-300 ng/ml. Lower concentrations were found in urine using the same methods (Sasaki et al., 1998).

The basal concentration of circulating endostatin may be generated as a result of physiological collagen turnover (O'Reilly et al., 1997). It may play a vital role in “Angiogenic Switch”, a homeostatic mechanism controlling angiogenesis (Boehm et al., 1997) (Distler et al., 2003).

Literature has frequently reported higher endostatin concentrations in circulation of patients with neoplastic conditions such as stage IV Clear Cell Renal carcinoma (Feldman et al., 2000), Colorectal carcinoma (Feldman et al., 2001a) and non neoplastic conditions with peculiar regulations of angiogenesis, such as systemic sclerosis (Hebbar et al., 2000) and diabetes (Sponder et al., 2011). The specific mechanisms for these differences are not completely clear.

However, as mentioned in section 1.3.6 endostatin was purified from conditioned media of the cancer cells for the first time and it is possible that cancer cells produce both VEGF and endostatin simultaneously (O'Reilly et al., 1997, Liu et al., 2011, Senger et al., 1983).

1.3.7 Vascular endothelial growth factor (VEGF)

Vascular endothelial growth factor (VEGF) is an angiogenic factor and a key regulator of vasculogenesis and angiogenesis (Ferrara et al., 2003, Neufeld et al., 1999). It is a homodimeric glycoprotein having high binding affinity with heparin and heparin sulphate in the extracellular matrix (Ferrara and Henzel, 1989). It is in brain, kidney, heart, spleen, pituitary follicular cells and many other cells (Veikkola and Alitalo, 1999). Its production can be induced in different types of cells including skeletal muscle cells, myocardial cells, vascular and lymphatic endothelial cells, stem cells, monocytes and cancer cells when they are exposed to hypoxic conditions (Holmes et al., 2007, Ferrara et al., 2003, Carmeliet, 2004). It has a molecular weight of 45 kDa.

The VEGF family consists of 6 isoforms, VEGF (also referred as VEGFA), PlGF (Placental growth factor), VEGFB, VEGFC, VEGFD and VEGFE (Veikkola and Alitalo, 1999). These isoforms differ from each other in molecular weights and functional abilities, such as attachment capability to cell receptors and proteoglycans.

VEGF was discovered in 1983 by Senger et al. He characterised it as a growth factor with a high affinity for heparin causing mitosis of the endothelial cells, during the study of vascular patterns in pituitary cells (Senger et al., 1983). Purification and cloning of VEGF was done by Ferrara and colleagues (Ferrara and Henzel, 1989).

It is of great importance both during physiological and pathological angiogenesis (Ferrara et al., 2003). VEGF specifically causes division of endothelial cells, one of the basic pre-requisites for angiogenesis (Neufeld et al., 1999).

VEGF A is the prototype of all these factors and is simply referred as VEGF due to its important and rate limiting role in the process of angiogenesis (Ferrara et

al., 2003). Therefore, this study aims to conduct the research on this VEGF A isoform.

1.3.7.1 Variants of VEGF

Based on the amino acid sequence and alternative splicing of the VEGF mRNA, 4 different variants of VEGF are reported. They are $VEGF_{121,}$ $VEGF_{165,}$ $VEGF_{189,}$ and $VEGF_{206}$ (Leung et al., 1989, Tischer et al., 1989). Less frequently available variants $VEGF_{145}$ and $VEGF_{183}$ were discovered later on (Neufeld et al., 1999). The ability of these isoforms to bind with heparin and heparin sulphate differs from each other (Park et al., 1993). The $VEGF_{165}$ has optimum binding capability with heparin and heparin sulphate and is closely similar to the native VEGF in properties (Houck et al., 1992). Due to easy cleavage of the $VEGF_{165}$ at the C terminus in the extra cellular matrix, this isoform possess optimal biological properties and bioavailability, which makes it the predominant VEGF (Keyt et al., 1996).

1.3.7.2 Regulating factors for VEGF

The regulation of VEGF is carried out by many internal and external stimuli. These stimuli/factors can also be called indirect stimuli/factors for the process of angiogenesis and vasculogenesis.

Hypoxia

Cell hypoxia is the most potent stimulator of VEGF expression and the mechanism is identical to the process, by which hypoxia induces expression of erythropoietin (Goldberg and Schneider, 1994). Hypoxia mediates the transcription of VEGF mRNA by binding hypoxia inducible factor 1 (HIF-1) to the HIF-1 site on VEGF promoter gene, leading ultimately to over expression of the VEGF (Levy et al., 1995).

Cytokines and other growth factors

A plethora of stimuli are involved indirectly in regulating the expression of VEGF mRNA in different cells. These stimuli include cytokines such as Interleukin1-beta (IL-1ß) (Li et al., 1995), Interleukin6 (IL-6) (Cohen et al., 1996), growth

factors such as, fibroblast growth factor 4 (FGF-4) (Deroanne et al., 1997) and transforming growth factor beta (TGF-ß) (Pertovaara et al., 1994).

1.3.7.3 VEGF Receptors

There are two main subtypes of VEGF receptor. VEGF receptor 1 (VEGFR-1) and VEGF receptor 2 (VEGFR-2), which are members of the receptor tyrosine kinases (RTKs) family (Ferrara et al., 2003). VEGFR-1 and VEGFR-2 are expressed mostly by endothelial cells (Neufeld et al., 1999) and some other cells such as monocytes (Barleon et al., 1996) and stem cells in bone marrow (Katoh et al., 1995). Though the activity of VEGF is limited to VEGFR-1 and VEGFR-2, but these receptors are capable of transducing the signals from all other members of VEGF family (Takahashi and Shibuya, 1997). Just like VEGF activation, hypoxia can regulate the expression of both receptors directly (Gerber et al., 1997) and indirectly (Wilting et al., 1996).

Activation of these receptors initiates different types of responses. For example activation of VEGFR-1 leads to migration of the endothelial cells (Barleon et al., 1996). Activation of VEGFR-2 receptors leads to the proliferation of the endothelial cells (Ferrara et al., 2003). These differences in signalling cascade caused by the activation of these receptors are not completely clear. Briefly, activation of VEGF receptors cause breakdown of the basement membrane through production of proteases like phospolipase C-γ in the first phase. In the later phase, these receptors up regulate the expression of integrins required for angiogenesis and ultimately take part in the proliferation and migration of endothelial cells (Ferrara et al., 2003). $VEGF_{165}$ also interact with other receptors such as neuropilin-1 (NP-1), located on endothelial cells surface (Soker et al., 1998).

1.3.7.4 Functions of VEGF

VEGF plays central role in the process of angiogenesis and vasculogenesis and is involved in many physiological and pathological process, in developmental and adult life.

During development of the embryo, VEGF induced by hypoxia causes angiogenesis accompanied by organ development. The best example is the

development of retina and its vascular system. Some of the studies reported that reduced VEGF supply can lead to non development of organs like corpus luteum in rat models (Ferrara et al., 1998).

Early neonatal development needs optimal activity of VEGF. It was showed that partial inhibition of VEGF leads to early neonatal death and stunted nourishment in mouse models (Gerber et al., 1999).

Optimum concentration and activity of VEGF is required for the development of cardiovascular system. VEGF Knockout mice show premature deaths due incomplete cardiovascular system development (Carmeliet et al., 1996).

VEGF production by tumour cells is essential for their growth (Hanahan and Folkman, 1996, Folkman, 1990). Inhibiting the VEGF signalling mechanism in tumor cells has shown impaired and reduced growth of cancer (Kim et al., 2000). Moreover, chemotherapies which includes anti VEGF receptor drugs gives a better prognosis (Klement et al., 2000) and reduction of tumor metastasis (Skobe et al., 1997). These findings suggest the development of anti VEGF drugs as a treatment option for many cancers.

VEGF plays an important role in ovarian angiogenesis during follicular stage. The inhibition of VEGF during this stage has shown delayed follicular development (Ferrara et al., 2003).

VEGF also promotes production of surfactants in alveoli and tends to stop apoptosis by over expressing anti apoptotic proteins and hence increase endothelial cells survival (Gerber et al., 1998).

1.3.8 Techniques for measurement Endostatin and VEGF

Different techniques such as ELISA, Western blotting, Mass spectrometry etc, have been used to measure circulatory endostatin and VEGF concentrations. This is reviewed in chapter 3, table 3-1. ELISA has been used frequently due to its peculiar abilities of measuring single proteins in a sample and one of the most validated tools for biomedical research across the globe on cytokines and other protein derived factors (Leng et al., 2008).

1.3.8.1 Enzyme Linked Immunosorbent Assay (ELISA) technique

Over the last few decades the improvement in the field of research is enormous and older techniques are widely replaced by much better and sophisticated ones.

One such technique is ELISA, introduced in 1971 by Engvall, which take over the older isotopes labelling technique. The latter technique had a greater chance of contamination (Butler et al., 1978). ELISA is the best validated techniques used in clinical and research laboratories (Leng et al., 2008).

1.3.8.2 Principle of ELISA

The principle of ELISA is based on the antigen antibody interaction where samples with unknown quantity of the antigens interact with pre-coated antibodies on to the well of ELISA plate. After immobilization of the antigen, detection antibody is added to the wells, which form antigen antibody complex. An enzyme is attached to this complex. The ELISA plate is washed between the steps with wash buffer to remove unbound antibodies. In the final step a substrate solution is added to the well which produces signals on reaction with enzymes, in form of developing a colour. A stop solution, usually sulphuric acid (H_2SO_4) is added in the final stage to shut off the activity of enzymes. The colour intensity can be quantified by reading with a multichannel spectrophotometer (Crowther, 2008).

There are three different types of ELISA.

Direct ELISA

In this type of ELISA, antigen is incubated to the plastic wells, followed by a direct application of antibody with enzymes.

Indirect ELISA

In this type of ELISA, antigen is incubated into the plastic wells, followed by a primary antibody. After incubation, a secondary antibody with enzymes is introduced against primary antibody. The secondary antibody is usually referred as anti species conjugate.

Sandwich ELISA

This is one of the more specialized forms of ELISA, used for antigens in low quantity or unrefined forms. In this type of ELISA the antigen does not attach directly to the wells. The principle of this type of ELISA is based on two monoclonal antibodies. The first antibody is added directly to the wells (Pre coated) to which the antigen is incubated. The second antibody is added after the incubation of the antigen to the first antibody, as illustrated in the figure 1-6. The second antibody is usually conjugated with enzymes like peroxidase. The sandwich ELISA can be direct or indirect. More complex forms of Sandwich ELISA also include competition or inhibition ELISA, which work on the same principles with pre-titration labelled antibodies and antigen (Crowther, 2008).

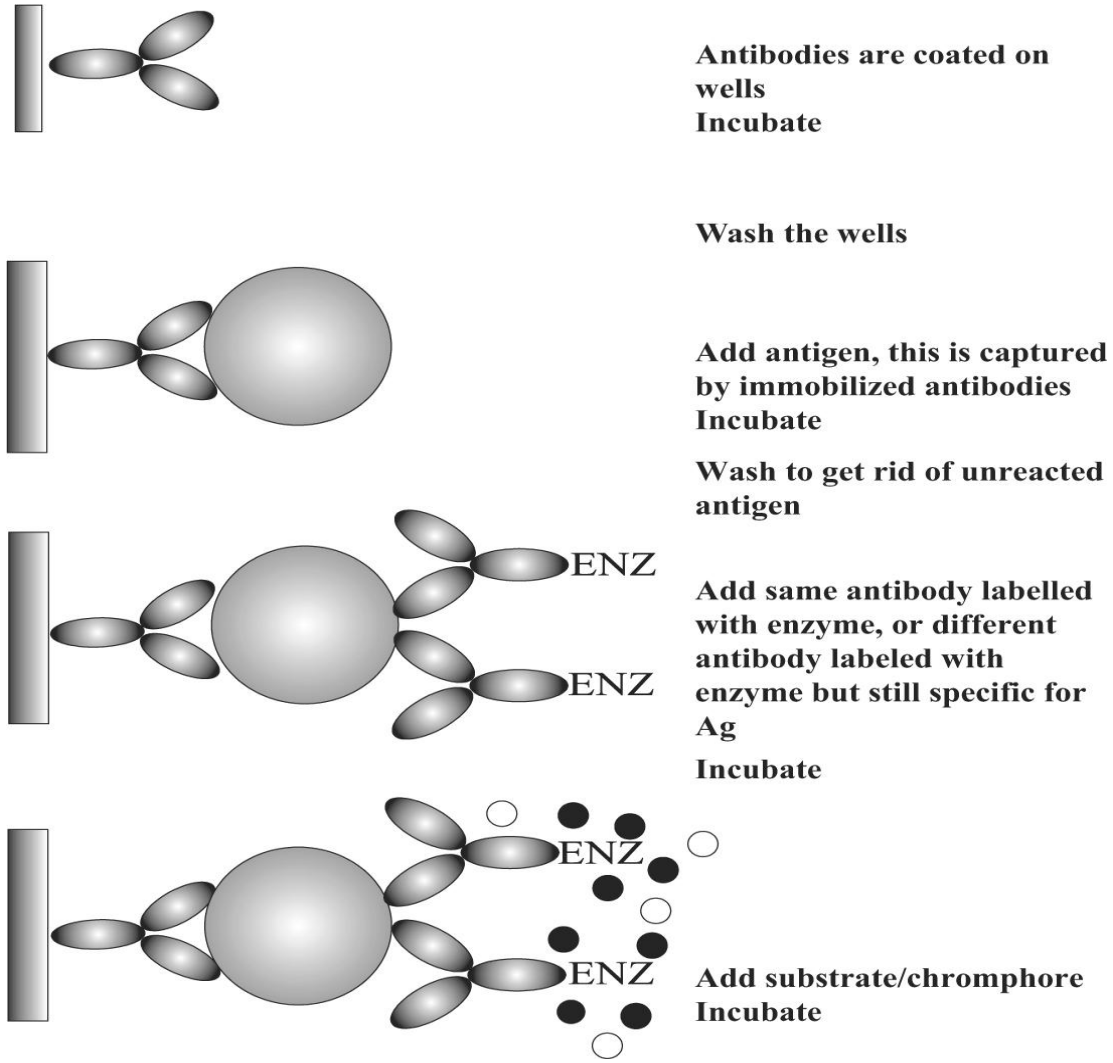


Figure 1-6; Sandwich ELISA technique

Two antibodies have been used. First one is pre coated and the second one is used in the conjugated step with enzyme. This type could also be referred as direct Sandwich ELISA technique. Reprinted with permission from (Crowther, 2008).

1.3.9 Physical Activity

As the research in this thesis focuses on effects of physical activity on angiogenesis mediators, the importance of physical activities in decreasing CVD related events along with different effects on endogenous cardioprotective mechanisms, are outlined in the following sections.

Physical activity is defined as the production of movement of the human body as a result of contraction and relaxation of the skeletal muscles, consuming more

energy than the body at rest (Pate et al., 1995, World Health Organization, 2014b).

However, physical activity should not be confused with exercise. Exercise is a form of physical activity which is more robust, well ordered, controlled and recurring movements of the body muscles with the aim to improve or maintain physical fitness (Thompson et al., 2003). According to the American College of Sports Medicine, exercise is defined as "*Any and all activities involving generation of force by the activated muscle(s) that result in disruption of homeostatic state*".

Exercise is categorised into endurance exercise, resistance exercise and sprint exercise based on the mode, duration and intensity of the physical activity (ACSM, 2013).

Endurance exercise is performed against relatively low resistance for relatively long periods. It involves many repetitions of muscle contraction. Resistance exercise is performed against higher resistance for shorter periods of time. Sprint exercise refers to short periods of exercise against low resistance. The activity usually is maximal and intense (Ghosh et al., 2010). Endurance or resistance exercise can be combined with sprint exercise like running with extra weight (Ghosh et al., 2010).

1.3.9.1 Intensity of exercise

Intensity refers to the rate at which the energy is consumed during physical activity (Pate et al., 1995). The intensity of exercise can be expressed in absolute or relative terms. Absolute intensity indicates the rate of energy consumption during exercise. It is denoted directly in watts, or METs or metabolic equivalents. It can also be described indirectly in terms of oxygen consumption. One METs is equal to basal metabolic rate which is ≈3.5ml of oxygen/kg/min. Relative intensities refer to the individual's capacity of performing exercise. This is usually expressed as percentage of the predicted maximal heart rate (HR_{max}) or predicted maximal oxygen consumption (VO_{2max}) (Fletcher et al., 2001, ACSM, 2013). Different Sub-maximal intensities of exercise based on ranges of HR_{max} and VO_{2max} are given in table 1-2. Near

maximal to maximal exercise refers to maximum tolerable effort of individuals and they are determined by various criteria discussed in chapter 6.

Exercise intensity	HR_{max}%	$VO_{2\ max}$%
Light	57 to < 64	37 to < 45
Moderate	64 to < 76	46 to < 64
Vigorous	76 to < 96	64 to < 91

Table 1-2; Different types of exercise intensities based on percentages of predicted maximum heart rate (HR_{max}) and predicted maximum oxygen consumption (VO_{2max}). Table adapted from ACSM

1.3.9.2 Physical activity and risk of cardiovascular disease

In addition to the risk factors listed in section 1.3.3, lack of physical activity is one of the basic modifiable risk factor for CVD. According to the World Health Organisation, lack of physical activity causes 6% of deaths due to all causes worldwide, making it the 4th leading cause of mortality and is responsible for approximately 30% of the ischemic heart diseases and 27% of type II diabetes (World Health Organization, 2014b). Regular physical activity substantially reduces other risk factors for cardiovascular diseases like obesity (Wing and Hill, 2001), diabetes (Gill and Cooper, 2008), high blood pressure and elevated triglycerides and lipoproteins (Waxman, 2005). Moreover, physical activity can help to control bodyweight by balancing the energy consumption to intake (DeLany et al., 2014).

The importance of physical activity in decreasing the mortality and morbidity due to cardiovascular disease is widely accepted (Sofi et al., 2008). A recent large scale study has shown that regular physical activity can modify the genetic predisposition of individuals by 40% to health problems like obesity which is one of the major risk factor for CVD (Li et al., 2010). In addition, post MI survival rate is greater in individuals involved in physical activity then those with a more sedentary life style (Berlin and Colditz, 1990). A meta- analysis of 33 studies involving 883,372 participants showed that there was a significant reduction of 33% in mortality due to all causes amongst the participants, who were actively

involved in physical activities. The meta-analysis also revealed a 35% risk reduction in cardiovascular mortality showing the inverse relationship of physical activity to cardiovascular mortality and morbidity (Nocon et al., 2008).

Over the last few decades decrease in the low incidence of CVD in active population has been reported extensively, in reviews and meta analysis of prospective and retrospective research studies (Lee et al., 1997, Williams, 2001). These finding are consistent with other studies and provides sufficient data to identify physical inactivity, as a cause of cardiovascular disease (Powell et al., 1987). Physical activity increases the life span by postponing mortality as the risk of CVD decrease significantly with each percentile increase in it (Lee et al., 1997). This risk reduction ratio is even more pronounced in fit and physically active participants (Williams, 2001).

Modern lifestyles have led to increased numbers of inactive people. This in turn increases the obesity due to disturbances of energy balance (DeLany et al., 2014). Consequently, a cascade of other risk factors like insulin resistance, obesity and high blood pressure is initiated with onset of many diseases (Yusuf and Ôunpuu, 2003).Thus increasing physical activity remains one of the strongest areas for primary prevention of the CVD. Beside these, physical activity is also beneficial socially in providing and improving physical and mental health (Penedo and Dahn, 2005). The application of increased physical activity as preventive measure requires a more multi-dimensional and culturally accepted approach (World Health Organization, 2014b).

1.3.9.3 Physical activity for prevention of CVD

The World Health Organization recommends that populations should adopt a healthy diet and perform the recommended minimum physical activity in order to decrease the risk of CVD. The global recommendations are summarised in table 1-3;

Age	Recommendations
5-17 years	1 hour of moderate physical activity per week is a must. For further benefits physical activity which strengthens muscles and bone should be incorporated.
18-64 years	Minimum 2 and half hours of moderate or 1 and half hour of strenuous activity per week is a must. To gain further benefits the duration can be extended and aerobic exercise can be incorporated.
65 years and old	Minimum 2 and half hours of moderate physical activity with aerobic exercise is a must. They can increase the duration for better outcomes and muscles strength. However, exercise for this age group is limited to the ability and degree of mobility of the individual. It is recommended to perform physical activity on 3 days of the week at least.

Table 1-3; Minimal recommendations of physical activities for different age groups by WHO

The American College of Sports Medicine (ACSM) and the Centre for Disease Control (CDC) recommends that individuals should do moderate physical activity on most days of the week for about 30 minutes per day (Pate et al., 1995). This suggestion is supported by the American Heart Association (Thompson et al., 2003) and the Ministry of Health in Pakistan (Ministry of Health Pakistan, 2004).

1.3.9.4 Physical Activity and Cardioprotective Mechanisms

It is quite clear that exercise and physical activity enhances cardiac protection but the detailed cellular mechanism is not clear. Particular attention has been given to the influence of physical activity on super oxide dismutase (SOD) and inducible heat shock protein (Yamashita et al., 1999). However, the recent development of new techniques (In-situ hybridization, immunohistochemistry, proteomics etc) have identified other cardioprotective mechanisms within coronary muscles (Golbidi and Laher, 2011).

Heat Shock Proteins (HSPs)

Heat shock response or the production of heat shock protein, also referred as "cellular stress response", is the usual response of cells to a number of exogenous stimuli including ischemia, oxidative stress, increased intracellular

calcium concentrations, hypoxia, acidosis and protein degradation (Marber et al., 1995, Golbidi and Laher, 2011). Cardiac HSPs is over expressed after physical activity. An extensive study revealed, over expression of myocardial HSPs 30 to 60 minutes after an exercise bout in rat myocardium (Powers et al., 2008). The mechanism of over expression of the myocardial HSP after exercise is unclear. However, other associated changes such as the reductions of glucose and glycogen concentrations, formation of reactive oxygen species, reduction of intracellular pH and increased intracellular calcium concentration all take place in parallel which might cause HSP over expression (Powers et al., 2008).

There are different types of HSP including HSP10, HSP40, HSP60, HSP 90 and HSP 70. All are considered to have cardioprotective qualities (Latchman, 2001). HSP 70 has been studied in detail and HSP72 a member of the HSP70 family has been shown to increase myocardial antioxidant activity. During reperfusion after an ischemic episode this protects cells from death by stabilizing the mitochondrial complex (Steel et al., 2004), and enable cells to recover quickly (Benjamin and McMillan, 1998, Golbidi and Laher, 2011).

In contrast, other studies reported that during ischemic reperfusion, the over expression of HSPs does not stimulate the cardiac protection (Hamilton et al., 2001).They maintained that superoxide dismutase was responsible for the cardioprotective mechanism in response to exercise (Quindry et al., 2007). It is possible that there could be convergence of several different mechanisms onto one single mechanism, leading to the initiation of responses necessary for the body defence (Golbidi and Laher, 2011).

Myocardial antioxidant capacity

Another cellular mechanism against free radicals is the antioxidant system of the cells with enzymes such as glutathione, peroxidise, catalase and superoxide dismutase (SOD). It is complemented by other components like vitamin E and vitamin C which bind with the reactive oxidants and convert them to less reactive species (Golbidi and Laher, 2011). Both these enzymatic and non enzymatic systems work simultaneously to protect cells (Golbidi and Laher, 2009). For example, superoxide dismutase neutralizes superoxide to form peroxide which is reduced by glutathione peroxidase (Cox et al., 2010).

The effect of exercise on the antioxidant capacity of the myocardium has remained an important area for research and it has been widely investigated. Where, some researchers reported an increase in myocardial glutathione peroxidise after exercise (Gunduz et al., 2004), others disagree (Hamilton et al., 2003). Similar contradictory results about increase and decrease in catalase expression after exercise has also been documented (Demirel et al., 2001, Lennon et al., 2004).

However, the best evidence about the increase in antioxidant capacity after short term endurance exercise concerns manganese superoxide dismutase (MnSOD), which provides protection against heart attacks (Yamashita et al., 1999).

Cyclooxygenase II (COX-2) and inducible nitric oxide synthase (iNOS)

Cyclooxygenase II and inducible nitric oxide synthase play an important role in the later phase of ischemic preconditioning. They provide protection to myocardium against reperfusion (I/R) injuries after ischemia (Bolli, 2000). Sub maximal ischemic stress can activate many complicated stress related mechanisms and exercise can produce and activate these pathways in the same manner as ischemic stress (Bolli, 2000).

Nitric oxide (NO) plays a major role in the late phase of ischemic preconditioning (Stein et al., 2004). The existing nitric oxide are used and the iNOS is up-regulated to increase the production of more NO (Stein et al., 2004).This triggers many mechanisms involved in cardiac protection such as reductions in cardiac oxygen consumption (Shen et al., 1995) and inhibition of the calcium transport by the cells (Mery et al., 1993). In addition, NO also produces other effects like reversible inhibition of programmed cell death and mitochondrial respiration. These mechanisms deliver prolonged protection of heart muscles (Loke et al., 1999).

Exercise plays a central role in regulation of nitric oxide production and even a short sessions of physical activity provides a better and prolonged protection to the heart muscles in dogs (Babai et al., 2002).

Cardiac mitochondria

Cardiac mitochondria play an important role during the ischemic insult (Golbidi and Laher, 2011). Regular exercise brings significant changes in the myocardial mitochondria and leads to the adaptations that are more of cardioprotective phenotype. For example, endurance exercise reduces the production of reactive oxygen species in cardiac mitochondria and this in turn results in high tolerance to calcium (Marcil et al., 2006). Moreover exercise also enhances the production of antioxidants which in turn increase the anti-apoptotic property of mitochondria (Kavazis et al., 2008). Exercise also enhances the level of enzymes involved in energy expenditure during exercise, like acyl-CoA, which enhance the ß-oxidation of fatty acids and in turns improves the bioenergetics (Kavazis, 2009).

Autophagy

Autophagy is the process of replacement of all old eukaryotic cells and their organelles with new cells (Debnath et al., 2005).The process is essential to keep the balance between the new generating cells and old cells. The target cells are engulfed by the phagosome and transferred to lysosome where they are degraded by the lysosomal enzymes (Brady et al., 2007). Autophagy is very important during the turnover of amino acids and organelles where it causes protein degradation (Tsukada and Ohsumi, 1993). Protein degradation increases during exercise and so is the apoptotic activity of the cardiac cells. Exercise exerts its cardioprotective effects by up-regulating autophagy during the actual endurance physical activity (Dohm et al., 1987),as well as down regulating necrosis and apoptosis (French et al., 2008). Autophagy protects cells from apoptosis during ischemic injury, as the later causes inflammatory responses (Golbidi and Laher, 2011).

Coronary circulation, arteriogenesis and angiogenesis

Physical activity enhances the coronary collateral circulation by arteriogenesis and angiogenesis (Leung et al., 2008). By widening diameter of coronary arteries and arterioles, regular physical activity leads to formation of vascular systems with improved blood supply, compensating for the loss occurring through occlusion of others arteries (Brown, 2003). 11 to 15 weeks of endurance exercise in patients with coronary artery diseases and angina increased thresholds for

angina, which suggest improved blood supply to heart muscles (Sim and Neill, 1974).

Exercise facilitates angiogenesis either by increasing division of already existing endothelial cells or through the mobilization of the progenitor endothelial and macrophage cells from the bone marrow (Rehman et al., 2004). There are also suggestions of a rise in the mobility of the progenitor cells after exercise in healthy individuals (Laufs et al., 2005) and cardiac patients (Steiner et al., 2005).

Physical activity affects angiogenesis through over expression of the main angiogenic mediator, vascular endothelial growth factor (VEGF) (Isner and Losordo, 1999) . However, impaired regulation of angiogenesis can lead to angiogenesis dependent diseases like atherosclerosis (Golbidi and Laher, 2011). In that case, the angiostatic mediators tend to stop the process of angiogenesis. Therefore a fine balance between the pro angiogenic and pro angiostatic factors always exist and exercise also plays a key role by in maintain this balance by affecting angiostatic regulators like endostatin, leading to inhibition of plaque expansion (Gu et al., 2004).

1.3.10 Conclusion and aims

It is evident from the preceding sections, that cardiovascular disease remains as one of the major health problems across the globe. Research has also established many mechanisms in the body which tries to protect the body from the development of atherosclerosis. These mechanisms include local coronary adaptations as well distant muscular, nervous and blood related events.

Angiogenesis plays a vital role in the development of atherosclerotic plaque. Angiogenesis is regulated by many endogenous angiogenic and angiostatic mediators. Vascular endothelial growth factor (VEGF) and endostatin are the most important mediators in the process of angiogenesis with opposing actions.

Evidence also suggests that exercise and regular physical activities are beneficial in the primary and secondary prevention of cardiovascular and many other metabolic diseases.

Exercise affects the angiogenic process overall but the precise effects of different intensities of structured exercise on these mediators; especially endostatin is not completely understood. Moreover, the protocols of exercises and the timings for sample collection to measure these mediators are sometime not appropriate. In addition, most of the studies professional athletes with better coronary adaptations have been used as volunteers. Population with sedentary behaviour are at higher risk of developing atherosclerosis and the effects of exercise in such individuals may be different.

The hypotheses to be tested are;

i. Different intensities of exercise would acutely affect endostatin and VEGF concentrations differently with inverse relationship.

ii. It is possible that the effects of the same intensity of exercise might be influenced by anthropometric and physical characteristics of the individuals as well as gender and exercise mode.

Therefore, the aim of this thesis is to find out the effects of different intensities (mild to maximum) of exercise on plasma endostatin and vascular endothelial growth factor concentrations. This project includes 3 studies with separate aims highlighted as follow:

The first study was a pilot project with the following aims;

i. Establish a basal plasma endostatin concentration within a population of healthy individuals

ii. Investigate whether endostatin concentrations differs between genders

iii. Investigate whether concentrations differs across anthropometric factors

iv. Evaluate the ELISA technique for measuring the endostatin concentration

The first part of second study aimed to investigate the impacts of 3 different intensities of exercise (mild, moderate and vigorous) with different durations and equal amount of energy expenditure on the plasma endostatin

concentration. The second part aims to determine the effects of low and high intensities of exercise on plasma VEGF concentration.

The third study was aimed to investigate the impact of single bout of high intensity exercise to the level of volitional exhaustion, on both plasma endostatin and VEGF concentrations for up to 24 hours after exercise.

2 Material and methods

All the experimental work related to participants and plasma samples were carried out in laboratories of the West Medical Building, University of Glasgow, between May 2012 and September 2014.

2.1 Contribution of author and other staff members to research discussed in this thesis

The research in this thesis was designed by the author with advice from his supervisor Dr RH Baxendale.

The author was solely responsible for recruitment of volunteers, anthropometric measurements, air displacement plethysmography, blood pressure measurements, phlebotomy, plasma separation and ELISA analyses. Mrs. Josephine Cooney (Department of Biochemistry, Western infirmary Glasgow), provided advice when required about the quality control, reliability and the pipetting techniques.

The exercise tests were carried out with the help of two lab technicians: Mr John Wilson and Miss Lynsey Johnston. Mr John Wilson helped me in the exercises protocol described in chapter 4 & 5 (sub-maximal exercise), while Miss Lynsey Johnston helped in the maximal exercise discussed in chapter 6.

Plasma analyses for lipid profile were carried out in the Biochemistry Department of Western Infirmary, Glasgow by Mrs. Josephine Cooney and Miss Khloud Ghafouri.

All calculations including measurements of different components of exercises, IPAQ questionnaire analyses, and statistical analyses were carried out by the author.

2.2 Ethical review process and approvals

Ethical approvals were granted by the College of Medical Veterinary and Life Sciences Ethics Committee for Non-Clinical Research. The committee approved 3 different applications for phases of the study reported in this thesis .All the ethical approvals are attached in appendix “A”. It is worth noting that approval for the first study took a longer time. The application to the committee was made in December 2011 and the final approval came in May 2012, after some minor changes. This delayed the start of the experimental work.

Volunteers in all 3 experiments were given a chance to discuss the complete experimental procedures and were provided with the Participant Information Sheets before their recruitment into the study. After their agreement, they were asked to sign two consent forms, one each for researcher and participant. Information sheets and sample of consent form are shown in appendices “B” and “C”.

The participants were informed that participation is entirely voluntary and they can stop the experiment at any point of time, if they do not want to participate.

2.3 Participants

Young healthy volunteers were recruited for all the 3 experiments. They were mostly students from the University of Glasgow and contacted through posters displayed around the main campus and in the School of Life Sciences. In some cases, personal contacts to fellow students and friends were also made.

After showing interest to take part in the study, each volunteer was contacted and the experimental procedure was explained. The information sheet was given to them and any question asked, was answered.

Once they agree to participate, they were asked to complete confidential health screening questionnaires to confirm their good health. Participants with potential health problems, on medication or smokers were not recruited to the study. Few such cases were encountered and they are mentioned in material and methods section of each study, with respective causes for non recruitment.

Three different experiments involving volunteers were performed at different times. A short 4th experiment was also carried out on the leftover plasma samples obtained from volunteers in experiment 2. A separate approval from ethical committee was obtained for that.

The first experiment had 50 volunteers including 16 females and 34 males. Their anthropometric and physical characteristics are given in chapter 3.

The second experiment included 22 volunteers, 6 females and 16 males. Their mean age and anthropometric measurements are given in chapter 4. Each volunteer visited the laboratory 4 times, one week apart. The first session included familiarisation with equipments and a sub maximal $\dot{V}O_2$ test to elicit intensities and durations for the exercises in main trial. The main trial included; exercise at 3 different intensities for different durations followed by taking blood samples at different time intervals up to 1 hour.

In the final study 8 male volunteers were recruited. The participants attended the laboratory on 2 consecutive days. The first day was exercise day followed by serial blood samples up to 6 hours after exercise. On the second day the participants attended the lab for a final 24 hours blood sample. Methods and techniques specific to the studies are discussed in the relevant chapter.

2.4 Inclusion and exclusion criteria

The inclusion criteria for recruitment into the first study were as follow;

Healthy male/female volunteers aged 18 to 60 years,
Not on any medication,
Non smokers,
Have not performed any physical vigorous activity/ exercise in the last 24 hours

The exclusion criteria were;

Participants with history of known cardiovascular disease or abnormalities,
If the participant was anaemic on general physical examination,

Current illness,
Lactation,
Pregnancy,
Taking part in another research study

The inclusion criteria for second and third study were the same except the age of participants were between 18 and 40 years. The volunteers were asked to refrain from physical activity for 24 hours before the main trial on all occasions.

2.5 General plan of the experiments

The design of first study was simple. All the participants attended the lab on only one occasion. During this visit the nature of the research was explained to them. After their agreement, they were asked to fill consent forms and two questionnaires to assess their general health and physical activity behaviour. These questionnaires are attached in Appendix "D2" & "D3". After measuring their weight, height, heart rate and blood pressure, a single sample of 10 ml blood was taken.

In the second experiment the participants, after showing interest were asked to attend the lab for familiarization session during which, the use of the equipments and nature of the experiment were explained. After the participant agreed to take part and signing the consent forms, their health status was assessed using health screening questionnaires. The participants wore sports clothes. Their height, weight, blood pressure and pulse rate were measured. The participants in study 2 were asked to perform a sub maximal exercise test. Data from this test was used to predict the intensities and durations of the exercises in the main trial. The time and dates for the further 3 visits were arranged and they were asked to refrain from physical activity for 24 hours before each main trial. The order of the exercise on subsequent visits was randomly arranged. On each visit, participants performed exercise at certain intensity for certain duration, elicited from the sub maximal exercise test on the first visit.

On the main trial day, their height, weight, blood pressure and pulse were measured again. A cannula was passed in an ante-cubital vein. Blood samples

were taken immediately before and at 10 and 60 minutes after completion of exercise. The cannula was then removed.

The volunteers in 3rd experiment attend the lab on 2 consecutive days. After showing interest the nature of the experiment was explained to the participants. Written informed consent was taken and questionnaires assessing the health status of the volunteers were completed. Their height, weight, waist & hip circumference, blood pressure, heart rate and body fat percentages were measured. Cannula was put in and basal blood samples were taken. Participant exercised on treadmill till exhaustion. Serial blood samples were taken at 10, 30, 60, 120 and 240 minutes after exercise. The cannula was removed. The participant attended the lab on next day for a final 24 hours blood sample. The details of the cannulation are given in section 2.10. No infection or adverse effects were encountered during any cannulation.

2.6 Baseline measurements

2.6.1 Weight

The weight of each volunteer was recorded using a digital balance (Seca 213, Germany). The weight was recorded to 0.1kg. Volunteers stood barefoot with minimal possible clothing during the weighing. The scale was checked for zero error each day and adjusted accordingly. Volunteers were weighed, with both feet on the scale in flat position and arms on the respective lateral sides of the body.

2.6.2 Height

A fixed stadiometer (Seca, Germany) was used to measure heights of the volunteers. The volunteers stood barefoot with the back positioned against the scale. They assumed the lateral anatomical position with chin up, and arms relaxed on the respective lateral sides. They were instructed to take a deep breath and movable head board was pressed on to the top of the head, with pressure enough to compress the hair. The height was measured to the nearest 0.1cm.

2.6.3 Body mass index

Body mass index (BMI) was calculated using the weight in Kilograms (kg) divided by square of the height in meters.

$BMI = Weight\ (kg)/\ (Height\ (m))^2$

2.6.4 Measurements of Blood Pressure

Blood pressures were measured using the electronic blood pressure monitor (Boso Medicus, Jungingen, Germany). Blood pressure was checked after the individual rested for 5 minutes. Most of the time, right arm was used for checking the blood pressure and in case of any ambiguity contra-lateral arm was used to confirm the readings are correct. 139/89 mm Hg was considered as the upper limit of the normal range (NICE Clinical Guidelines, 2011). Volunteers with blood pressure levels more than permitted were excluded from the study with the instructions to have an appointment with General Practitioner. During the whole recruitment, two such cases were encountered.

2.6.5 Waist and hip circumference

An inelastic and flexible measuring tape was used to measure the waist and hip circumference (Seca, Germany). The waist circumference was measured at the level of navel, midway between the costal margin and the superior iliac crest in a horizontal plane. The hip circumference was taken around the hips at the maximum circumferential points in a horizontal plane. These measurements were carried out by the same sex investigator and providing full privacy to the subjects. The values presented are mean of 3 values.

2.6.6 Body composition

A Bod Pod (Bod Pod®, Version 1.69, CA, USA) shown in figure 2-1, was used to estimate body fat percentages in the final study. The machine was calibrated daily using cylinder of known volume as recommended by the manufacturer. The details of the volunteers with height, age and sex were entered using desktop computer attached to the Bod pod.

The volunteer wore minimal clothing whilst they sat in the Bod pod quiet and relaxed with their hands in the lap. Two or three measurements were made. Based on the calibration, the Bod pod uses the air pressure to estimate body density and fat percentage. Each measurement takes about 90 seconds and a mean of two or three measurements is displayed on the screen at the end. A complete analyses of body composition including; body mass, fat mass, fat percentage, lean mass, density and volume of the body is displayed. The device is shown in Fig 2-1.

The principle of Bod Pod (Manufacture information) is based on determining the body density from body volume, predicted through air pressure using Boyle's law (COSMED LMI, 2011);

$$P_1V_1 = P_2V_2 = \text{Constant}$$

The pressure increases with the decrease in volume and vice versa. Based on this, Bod Pod software calculates the pressure changes with volume changes during the calibration. The software uses this method to calculate the volume of the chamber without the participant and then with the participant. From this the volume of the participant is measured as follows;

> Volume of the participant = volume of the empty chamber – volume of the chamber with participant

Density of the body of the participant is then calculated by using density and weight of the participant as follow;

$$\text{Density (d) (kg/l)} = \text{Weight (kg)} / \text{Volume (l)}$$

The participant breathes normally during this process and the volume of the body is corrected by the Bod pod using the relaxed tidal breathing volume. From the density of the body, lean body fat weight is measured using the Siri equation;

$$\text{Percent Fat} = [495/\text{density}] - 450$$

Knowing the percent fat lean body mass percentage can be determined as follow;

Percent lean weight = 100 – percent fat

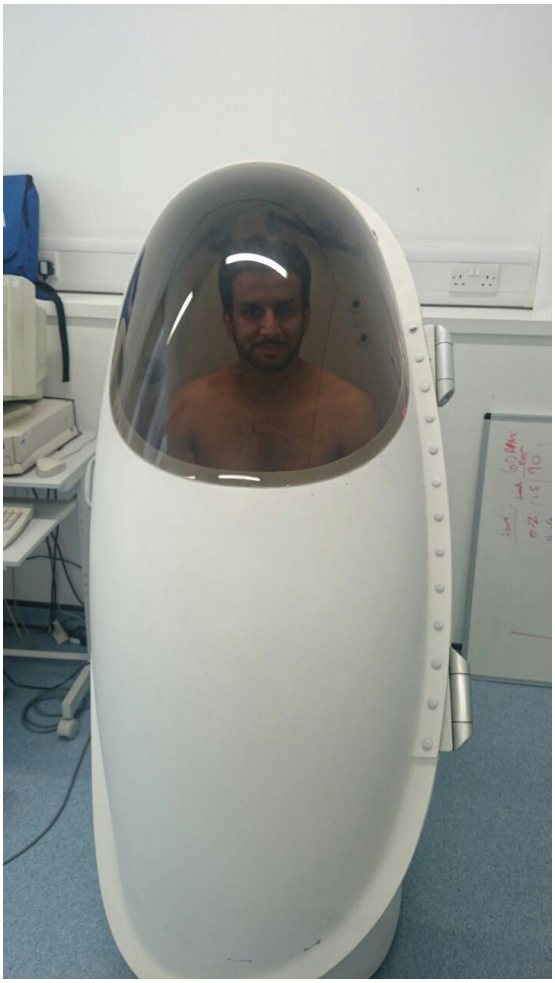


Figure 2-1; Bod pod measurements

Picture shows one of the participants in Bod Pod chamber for measuring body fat percentage.

2.7 Exercise

Different intensities of exercise were used in different studies described in the subsequent chapters of this thesis. All of the exercises were carried out on the same calibrated automated treadmill (Woodway Ergo ES2, Germany). The volunteers were familiarised with the treadmill speeds and controls before test.

In study 2, the aims for the participants were to exercise at 60%, 70% and 80% of their predicted maximum heart rates. For this reason, all the participants undertook a sub-maximal exercise test to estimate the speed necessary to elicit these intensities. The test consisted of 4 stages to determine the relationship between oxygen consumption and increasing speed. The primary idea was to use the same exercise protocol for all participants during sub-maximal test. This protocol asked the volunteers to start running at 6 km/hr in the initial stage for 5 minutes. Thereafter, speed was increased by 1 km/hr, every 5 minutes. In total 4 stages were measured for each participant. The expired air was collected in Douglas bags during the last minute of each stage.

However, some of the volunteers were not able to run at this speed. For all such volunteers, the speed was adjusted according to their comfort. In order to get higher heart rates during the sub-maximal tests, the inclination angle of the treadmill was increased every 5 minutes. Same speed for each volunteer was employed in the main trials.

2.7.1 Sub-maximal oxygen consumption test

During the sub-maximal test volunteers walked or run on a treadmill (Woodway Ergo ES2, Germany), according to their comfort, using standard Douglas bag technique for collection of expired gases.

The volunteers wore head sets to support the apparatus for collecting expired air gases. Volunteers breathed through a mouth piece (Medgraphics, Minnesota, US) attached to two-way respiratory valve (Hans Rudolph, Two-Way Non-Rebreathing valve, Kansas, US), while wearing a nose clip to stop nasal breathing. The mouth piece was connected to light weight tube of 150cm length and 30mm width, which terminates on a two way valve at the junction with the Douglas bags (Medgraphics, Minnesota US). The expired air gases were collected during the last minute of each stage. During this period, the heart rate was monitored continuously and recorded every 15 seconds. The average heart rate during collection time was used in the final calculations.

The temperature of the room was kept at 21^{o}C for all sub-maximal tests. At the start of the test, all volunteers stood on the treadmill at rest for 5 minutes.

After this, the first stage of the test was started based on selecting a comfortable speed (4 to 5 km/hr for walking group and 6 km/hr for running group) for each participant. If it was a walking protocol, the gradient of the treadmill was increased in each subsequent stage by 3%. In case of a running protocol, speed was increased by 1 km/hr. The test was stopped at the end of the stage, if the heart rate reached 85% of maximum predicted. Usually the test comprised 4 stages excluding the rest stage, as shown schematically figure 2-2. Predicted maximum heart rate was calculated using the formula;

Maximum heart rate (HR max) beats.min^{-1} =220 - age in years

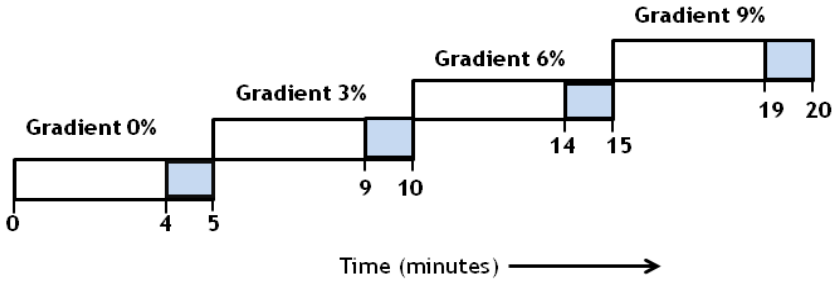


Figure 2-2; Multi stage sub-maximal exercise test

Schematic diagram of 4 stage sub-maximal exercise test performed by the participants in chapter 4. It is presented with gradient % which was used for walking group. The test was the same for running group except they started at higher speed (6 km/hr) which was increased by 1 km/hr instead of gradient%. The light blue blocks represent the expired air and heart rate measurement periods.

2.7.2 Measurement of Oxygen and Carbon dioxide in the expired air gases during sub maximal test

A Servomex 1440 Dual Gas Analyser (Servomex Limited, Sussex, England) was used to measure the concentration of oxygen (O_2) and carbon dioxide (CO_2), as fractions of the collected gases. It was calibrated with standard gases of known concentration each day before test. The flow rate of gases through the machine

were determined and added to the remaining volume in the Douglas bags at the end. The flow rate used was 0.5litre/minute.

The remaining volumes of gases in the Douglas bags were measured using Harvard Dry Gas Meter (Harvard Apparatus Limited, Kent England). The temperature of the expired air was measured by a digital thermometer connected to the outlet of dry gas meter. The barometric pressure was recorded using the Fortin barometer (FD, Watford UK).

2.7.3 Calculation of oxygen uptake ($\dot{V}O_2$), carbon dioxide production ($\dot{V}CO_2$)

The expired air gases were collected at ambient temperature and pressure. A correction factor was used to convert the recorded volumes to standard temperature and pressure. This process is based on the universal gas equation. Ambient pressure and temperature refers to the pressure and temperature during test conditions, while standard pressure refers to 760mm of Hg and standard temperature refers to 273K (0°C). The expired air contains water vapours that exert pressure which was corrected using the following equations:

$$\dot{V}E_{STPD} = (\dot{V}E_{ATPS} \times (BP - SWVP)/760) \times 273/ (273 + t)$$

Where STPD refers to standard temperature and pressure, ATPS refers to ambient temperature and pressure with water vapours, BP is barometric pressure and t refers to temperature of the gas in degrees Celsius.

SWVP refers to the saturated water vapour pressure at ambient temp and was calculated as follow:

$$SWVP = (1.1001 \times t) - 4.19$$

SWVP is measured in mm of Hg and t is temperature in degrees Celsius.

Oxygen uptake and carbon dioxide production were calculated as follows:

$$\text{Oxygen uptake} = \text{Volume of } O_2 \text{ inspired} - \text{Volume of } O_2 \text{ expired}$$

$$\dot{V}O_2 = \dot{V}I \times F_IO_2\%/100 - \dot{V}E \times F_E\,O_2\%/100$$

$$\text{Carbon dioxide production} = \text{Volume of } CO_2 \text{ expired} - \text{Volume of } CO_2 \text{ Inspired}$$

$$\dot{V}CO_2 = \dot{V}E \times F_E\,CO_2\%/100 - \dot{V}I \times F_ICO_2\%/100$$

Where $\dot{V}O_2$ refers to the oxygen uptake and is expressed (l/min). To get the $\dot{V}O_2$ in ml/kg/min, we multiplied the volume in litres by 1000 and divided it by the weight in kg.

$\dot{V}CO_2$ refers to the carbon dioxide production and is expressed (l/min). To get the $\dot{V}CO_2$ in ml/kg/min, the volume in litres was multiplied by 1000 and divided by weight in kg. $\dot{V}I$ refer to the inspired air volume per minute and $\dot{V}E$ refers to the measured expired volume per minute. $F_IO_2\%$ is the percentage of oxygen in atmospheric air taken as 20.93% and $F_EO_2\%$ is the percentage of oxygen measured in the expired air. $F_ECO_2\%$ is the percentage of carbon dioxide in the expired air and $F_ICO_2\%$ is the percentage of carbon dioxide in the atmospheric air which is 0.03%.

Inspired air gas ($\dot{V}I$) is an unknown value and Haldane transformation was used to determine the inspired air volume (Consolazio et al., 1963). The Haldane transformation equation is as follow:

$$\dot{V}I = \dot{V}E \times F_EN_2\%/F_IN_2\%$$

Where $F_EN_2\%$ is the percentage of nitrogen in the expired air and $F_IN_2\%$ is the percentage of nitrogen in the atmospheric air.

Once the volumes of inspired gases are known; oxygen uptake, carbon dioxide production, respiratory exchange ratio and minute ventilation values were determined.

2.7.4 Calculation of energy expenditure

The second study required the participants to use 200 kcal of energy during each session of the exercises based on different intensities. Therefore, the energy expenditure per minute at that specific intensity was calculated to find out durations required for one session of exercise. For this reason, respiratory exchange ratio was calculated during each stage of the sub maximal exercise from $\dot{V}O_2$ and $\dot{V}CO_2$.

$$\text{Respiratory exchange ratio (RER)} = \dot{V}CO_2 / \dot{V}O_2$$

Where $\dot{V}CO_2$ is the volume of carbon dioxide produced and $\dot{V}O_2$ is the volume of oxygen consumed.

The rate of carbohydrate oxidation and rate of fat oxidation were calculated, using the indirect calorimetric method described by (Frayn, 1983).

$$\text{Rate of carbohydrate oxidation (CHO)} = (1.4 \times \dot{V}CO_2 - \dot{V}O_2)/0.3$$

$$\text{Rat of fat oxidation} = (\dot{V}O_2 - \dot{V}CO_2)/0.57$$

The rates of oxidation of fat and carbohydrate are expressed in gram/minute.

The rate of energy expenditure was then calculated using the following formula:

Energy expenditure EE (kJ/min) = (Rate of carbohydrate oxidation x15.6) + (Rate of fat oxidation x 39)

This rate of energy expenditure is in kilojoules (kJ) per minute. Values of 15.6 and 39 refer to the density of energy in kJ per 1gm of carbohydrate and fat molecules respectively. To convert the energy expenditure to kcal/min the values were divided by conversion factor as follow;

$$\text{EE kcal/min} = \text{EE kJ.min}^{-1} \times 0.239$$

2.7.5 Measurement of predicted maximal Oxygen Consumption ($\dot{V}O_{2\ max}$), intensities and durations for main trials

In the second study, participants had to perform exercises at 60%, 70% and 80% of their predicted maximum heart rate. For this reason predicted maximal oxygen consumption was calculated ($\dot{V}O_{2\ max}$), by plotting average heart rate at each stage against the oxygen consumption ($\dot{V}O_2$). The slope of the graph was extrapolated to the predicted maximum heart rate, as shown in figure 2-3. The unknown values were calculated with the formula given in the figure, where x refers to the value at specific time point.

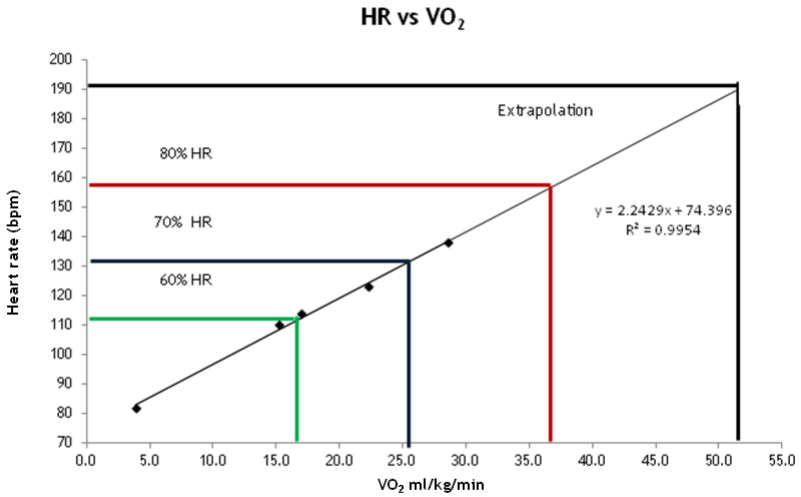


Figure 2-3; Prediction of maximal oxygen consumption ($\dot{V}O_{2max}$) from sub-maximal oxygen uptake ($\dot{V}O_2$)

The graph represents data of one subject, showing oxygen consumption in ml/kg/min and heart rate in beats per minute during different stages of sub-maximal test. Five samples (including rest sample) were taken during the test. After plotting the data a linear trend line was added with extrapolating it to the maximum predicted heart rate of 190 beats per minute. Participant was 30 years old. The line is fitting the data properly, as shown by R^2=0.9954.

Once the $\dot{V}O_{2\ max}$ value is established, it is straightforward to calculate the $\dot{V}O_2$ at 60%, 70% & 80% of the heart rate, which are shown by the green, blue and red lines. The estimated $\dot{V}O_{2max}$ in this case was 52 ml/kg/min and the values were 17 ml/kg/min, 26 ml/kg/min and 38 ml/kg/min respectively, for exercise at 60%, 70% and 80% of the predicted maximum heart rate.

Graphs for each individual were plotted for determining work rate (speed or gradient) and energy expenditure per minute at each respective intensity. Finally, 200 kilocalories were divided by the amount of energy expenditure per minute at respective specific speed to find out the duration of exercise for each subject.

2.8 Heart rate

Heart rate of the volunteers was monitored during all trials mentioned in this thesis through short range telemetry system (Polar FT1, Finland).

2.9 Maximal exercise

For the study discussed in chapter 6, the volunteers performed maximal exercise test on a treadmill (Wood way, Germany). The test was carried out according to protocol designed by Taylor (Taylor et al., 1955), with slight modifications. The initial warm up stage of the test started at speed of 5.6 km/hr and a gradient of 10% which was equal to 230W. After this, speed was kept constant at 8 km/hr, for all volunteers with an initial gradient of 0. The gradient was then increased at a rate of 2.5% every three minute, till volitional exhaustion. After the first test was conducted, it was found out that the work load of 230W in the warm up stage is more than the work load of 211W in stage 1. So in the subsequent tests, the speed of the warm up was decreased to 4.6 km/hr with 10% gradient. This was equal to a work load of 193W.

Volunteers wore a head set to support the mouth piece attached to the one way valve, as described in section 2.6.1, for analysing the expired air. The expired air passed through a breath by breath analyser (Medgraphics, Braunfels,

Germany), which was calibrated with the known concentrations of O_2 and CO_2 before every single test, as shown in figure 2-4.

To ensure volunteers are not hyperventilating, they were asked to stand calm on the treadmill for at least three minutes until RER of $\leq$ 0.85. Heart rates were recorded throughout the test and perceived rates of exertion were recorded at the end of each stage, using Borg Scale (Borg, 1973).

The machine analyses every single breath in real time and displays the results on the screen attached to it. These include; oxygen uptake ($\dot{V}O_2$), Carbon dioxide production ($\dot{V}CO_2$), respiratory exchange ratio (RER) and respiratory rate.

Each stage was 3 minutes long. The calculations are based on data collected between 1 minute 45 seconds and 2 minutes 45 seconds of this period. The data from the last minute of the last stage was taken as the maximal effort and oxygen uptake was considered as ($\dot{V}O_{2max}$).

A

B

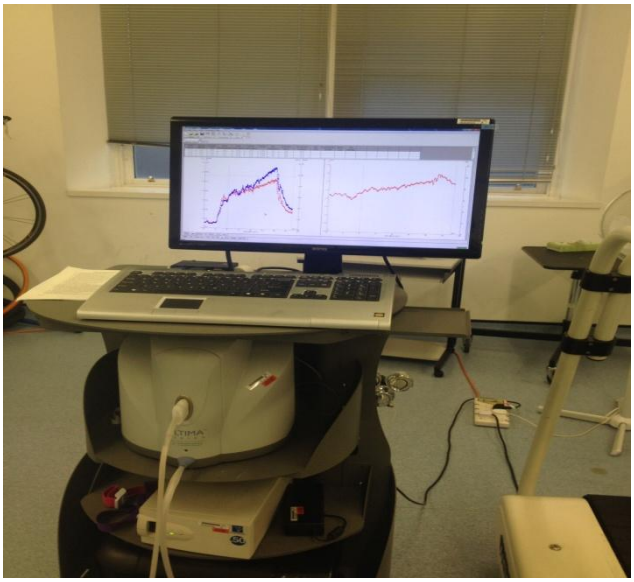


Figure 2-4; (A) Maximal oxygen consumption test and (B) breath by breath analyser

Panel (A) shows picture of one participant during maximal exercise test, breathing through one way value while wearing nasal clip. Panel (B) shows the breath by breath analyser with participant data. Picture presented with written permission of the participant and laboratory assistant.

2.10 Post exercise lactate concentration

In chapter 5, one of the required criteria of the maximal exercise effort was to determine blood lactate concentrations at 1 min, 3 min and 5 minutes intervals post exercise. The thumbs of the volunteers were pricked with disposable lancet and small amount of blood was collected in short capillary tube (Analox, London, UK). The tubes are heparinised to stop blood clotting. This was then transferred through a small pipette directly to the blood lactate analyser (Analox, London, UK), which read the sample within 30 seconds and display result.

2.11 Blood sampling

In all the three sets of experiments serial blood samples were collected by insertion of a 20G cannula (Versatus-W, Leuven, Belgium) in the ante-cubital vein. Standard aseptic procedures were used. The cannula was connected to 3 way valve connector (BD Connecta^{TM}, Sweden) after removal of needle. The connection was kept in place by applying transparent I.V line dressing (EASI-V, Unimedical,UK), as shown in figure 2-5. Blood samples were taken by attachment of the holder to the three way connector and collecting the blood in 6ml EDTA (ethylene di-amine tetra acetic acid) tubes (BD Vacutainer, Plymouth, UK). These tubes were selected to meet requirements of the ELISA kits. After taking blood samples, the cannula was flushed with non-heparinised normal saline solution (0.9% NaCl) to prevent blocking of the cannula.

Where single blood samples were required, they were taken aseptically with 21G butterfly (Nipro Corporation, Zaventem, Belgium) from anti-cubital vein. All blood samples were immediately kept on ice before centrifugation.

Plasma from the blood was separated using centrifuge machine (Universal 320R, Tuttlingen, Germany) at 1000 revolutions per minute for 15 minutes at 4°C. 3 aliquots of plasma were separated in 0.5ml of Eppendorf tubes and kept in freezer at -80^{o}C prior to analyses. The storage times were kept minimal. In most of the analyses, the storage time was less than 2 months except the second part of study 2, where analyses of the VEGF were done after 6 months. These storage times are acceptable. An extensive study on storage timings and VEGF concentration has shown that the VEGF protein concentration is affected by

repeated freeze thaw cycles and extended storage time before centrifugation. Once the centrifugation is done in time (2 to 4 hrs), the protein concentration can be accurately determined up to 2 years (Azimi-Nezhad et al., 2012). The samples used in our studies were always centrifuged within 30 minutes of collection and thawed only once.

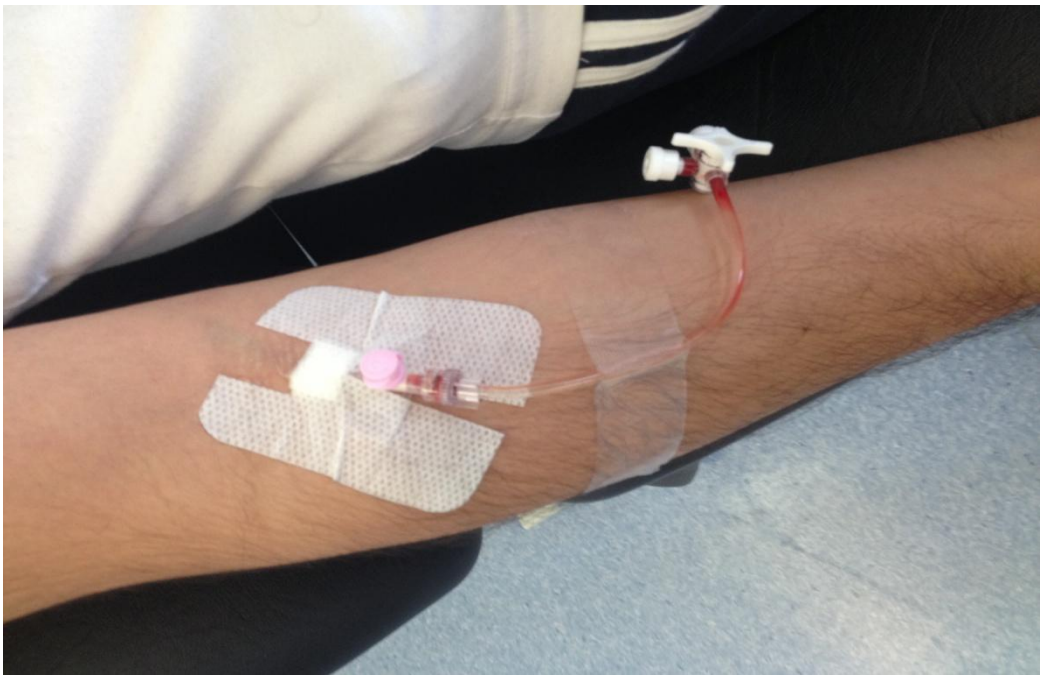


Figure 2-5; Cannula in ante cubital-vein

Picture showing the cannula in the anti-cubital vein of a participant attached to a 3 way connector filled with normal saline to keep the tube patent.

2.12 ELISA

The plasma samples were analysed for endostatin, VEGF and insulin through ELISA techniques. All the ELISA performed in these studies used quantitative sandwich ELISA technique, which works on the principal of two antigenic determinants directed against two monoclonal antibodies. The first monoclonal antibody is fixed to the walls of micro titration well on the ELISA plate to which the antigen in the samples attaches. The second one is in conjugate solution attached to peroxidase enzyme. Unbound antibodies are washed away in the washing process and substrate solution is added to the wells. The substrate solution contains Tetramethyl benzidine (TMB) and hydrogen peroxide which

produces a colour on reacting with the enzyme labelled antibodies. After incubation, the density of the colour corresponds to the concentration of antigen in the sample. At the end, stop solution (1M H_2SO_4) is added and the end point colour density is read with a spectrophotometer.

2.12.1 Endostatin ELISA

The concentration of endostatin was determined in all the blood samples using Quantikin® ELISA human endostatin kits (R&D systems for Europe, Abingdon, UK).

2.12.1.1 Sample preparation and dilutions

All the solutions in the kit were brought to room temperature as per description of the kit. The plasma samples liquefy when kept at room temperature for 20 to 30 minutes. All solutions were prepared and diluted as per instructions included with the kit. A copy of the full instructions was downloaded from the manufacture website (R & D system, 2015a) and is attached in appendix “F1”. Standards were prepared in a series of dilutions.

Plasma samples required 50 fold dilutions, for which 20µl of plasma were added to 980µl of calibrator diluting solution, provided with the kit. Care was taken to follow the exact steps and technical hints provided by the kit manufacturer. Face masks were worn all the time to avoid cross contamination with saliva, as saliva also expresses endostatin.

2.12.1.2 Test procedure

Exact procedures were followed as mentioned in the guide book provided with the kit. Controls provided separately by the same manufacturer were also tested (Quantikin® ELISA kit controls QC81). All the samples, controls and standards were analysed in duplicate.

Assay diluent was added to all the wells before 50µl of samples, controls and standards were added. The plate was kept for 2 hours incubation on a plate shaker at 450 rpm before washing it 3 times with a buffer solution in an automated plate washer (Dynex MRW AM60, USA). In the next step 200µl of

human endostatin conjugate solution was added to each cell, followed by 2 hours incubation. After repeating the washing step, 200µl of substrate solution was added to the plate followed by 30 minutes incubation in dark.

Finally, stop solution containing 1M sulphuric acid was added to the plate and end point reading was taken at 450 nm and 570 nm by spectrophotometer (Spectra Max M2, CA, USA).

The concentrations of the standards were plotted against the absorbance by using the software (Softmax pro 5.4). A specimen curve is shown in the figure 2-6. Based on the linearity of the curve, concentrations of the samples were calculated by the software, from their respective absorbance.

2.12.1.3 Precision of the ELISA

The consistency and reliability of the actual samples were checked through the results of the controls. Inter and intra assays controls were carried out in all trials. The coefficients of variance (CV%) recorded were less than 5% for inter and intra assays precision.

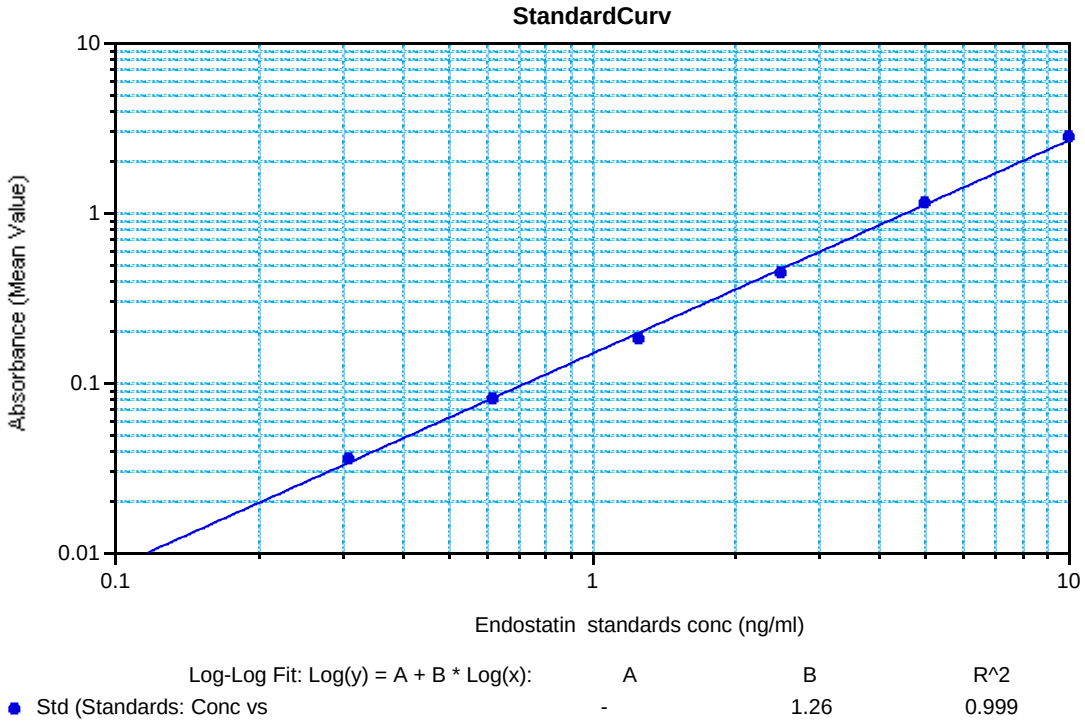


Figure 2-6; Computer generated standards curve

An example of computer generated standard logarithmic curve used by the Soft Max Pro 5.4 software for reading the Plasma endostatin concentration. Linearity is shown by $R^2 = 0.999$

2.12.2 VEGF

VEGF in plasma samples were also determined through Quantikin® ELISA human VEGF kits (R&D systems for Europe, Abingdon UK).

The principle of the Kit is sandwich ELISA which is the same as described for endostatin. The tests were performed according to the standard protocols of the kit and the samples were run in duplicate. The precision of assays were checked during each trial through repeated measures of controls (Quantikin® immunoassay control group 1) provided with the kit. A complete detail of the procedure downloaded from the manufacture website (R & D system, 2015b) is given in appendix “F2”.

2.12.3 Insulin

Concentrations of insulin in plasma were determined using commercially available ELISA kit (Mercodia, Uppsala, Sweden). It is a solid phase two site enzyme immunoassay kit working on direct sandwich technique principle. High and low control of the same company were used for inter assay precision. The CV for the test was less than 3%. Complete details are downloaded from the commercial website of the manufacture (Mercodia diagnostics, 2015) and given in appendix "F3".

2.13 Lipid profile

2.13.1 Spectrophotometric assays

The analyses were carried out by Mrs. Josephine Cooney and Kholoud Ghaffori (Department of Biochemistry, Western infirmary Glasgow) using clinical chemistry analyser (ILAB^{TM} 600, Instrumentation Laboratory, USA).

Commercially available enzymatic kits were used for the analyses of plasma glucose (Glucose hexokinase, Randox laboratories, UK), total and high density lipoprotein cholesterol (HDL) (CHOL and HDL-C, Roche diagnostic, UK), triglyceride (TG) (Randox Laboratories, Crumlin, UK) and non- esterified fatty acid (NEFA) (Wako chemicals, USA).

Friedewald equation was used for the determination of low density lipoprotein (LDL), as follow (Friedewald et al., 1972);

LDL mmol.l^{-1} = Total cholesterol – HDL cholesterol – (TG /2.2)

For small density lipoprotein SDLDL, enzymatic kit (Denka Seiken, Tokyo, Japan) was used.

2.14 Statistical analyses

Statistical analyses were performed using statistical software SPSS (IBM SPSS statistics for Windows, Version 17, Armonk, NY) and Minitab for windows (Version 16, MINITAB Inc., State college, PA). Normality of the data was checked

using Kolmogorov- Smirinov and Shapiro-Wilk tests of normalities. Log transformations of the data were done where appropriate. Descriptive statistics were carried out and expressed as mean ± SD (standard deviation). Time trends were measured using one-way ANOVA with repeated measures. Independent sample t-test was used to differentiate between males and females or walking and running group. Relationships between basal endostatin concentrations, VEGF and the change in these mediators with basal anthropometric measure, metabolic profiles and exercise parameters were determined through Pearson correlations. Linear regressions were used for determining dependency of endostatin on different variables. P values of < 0.05 were accepted significant.

3 Measurement of endostatin concentration in plasma of human volunteers

Chapter: 3

3.1 Introduction

As discussed in detail, in chapter 1 section 1.3.5, angiogenesis is essential to many normal development processes in the human body. These include embryonic development, organogenesis, the healing process and tissue regeneration. On the other hand, it also contributes to the progression and maturity of many cardiovascular and cancerous diseases including atherosclerosis (O'Brien et al., 1994).

There is a fine balance between the angiogenic and angiostatic mediators in the overall process of angiogenesis and shift in favour of any particular phenotype determines the outcome of angiogenesis (Boehm et al., 1997). The angiogenic mediators like VEGF and FGF etc favour angiogenesis, by binding to the endothelial surface cell receptors and initiating a cascade of intracellular signalling pathways. However, endogenous angiostatic mediators, including angiostatin and endostatin tend to stop the angiogenic process by mechanisms involving antagonising the actions of angiogenic mediators (Eriksson et al., 2003).

Endostatin, discovered by O'Reilly et al in 1997, has come to the front as one of the main angiostatic mediators (O'Reilly et al., 1997). Endostatin plays an essential role in control of the angiogenesis. By inhibiting angiogenesis, endostatin prevents progression of atherosclerosis (Zeng et al., 2005), primary growth of tumors and metastasis (O'Reilly et al., 1997).

Endostatin is a small globular protein attached through a hinge to the C terminus of collagen XV and XVIII in the basement membrane of endothelial cells (Sasaki et al., 2002). It is also found in many other body tissues including heart, skeletal muscles, liver, placenta, ovaries, prostate and kidneys (Deininger et al., 2003). Release of the endostatin requires a proteolytic cleavage (Sasaki et al., 2002).

The release of endostatin and its up-regulation in an in vitro setup were enhanced after the endothelial cell lines were subjected to cellular stress, hypoxia and interferon-γ challenge. Moreover, nitric oxide synthase inhibitors reduced the release of endostatin (Deininger et al., 2003).

3.2 Plasma endostatin concentration in healthy population

The available literature contains several studies reporting the concentration of endostatin in the plasma. These are summarised in table 3-1. The data in this table shows that the sample populations are often small and drawn from patients with a range of pathologies. In addition, a variety of analytical techniques have been used. Thus the current literature does not provide the average plasma concentration in normal population.

Some studies have reported the plasma endostatin concentration as low as 1.0 ng/ml while others have reported this level to be in the range of 300ng/ml (Refer to table 3.1). These huge differences in means and ranges are not completely clear.

It is clear from the table, that for the determination of endostatin concentration in plasma, different methods like mass spectrometry, radioimmunoassay, western blots and ELISA have been used frequently. The difference in the reference range could possibly be due to use of different techniques. However, even the same techniques used in different studies have reported significant differences in the basal plasma endostatin concentrations (Zorick et al., 2001, Suhr et al., 2007).

Reference	Mean [ES] Plasma	Mean [ES} Serum	Mean Age years	Assay Method	Comments
(Ständker et al., 1997)	10^{-10}M			Mass spectrometry	Human plasma hemofiltrate was used for isolation of endostatin using mass spectrometry
(Hebbar et al., 2000)		Healthy volunteers = 9.9 ± 9.7 ng/ml Sclerosis patients 63.2 ± 20.2 ng/ml	48.5 ± 12.7 54.1 ± 14.6	Enzyme immune essay	50 patients with different levels of established sclerosis were evaluated for plasma endostatin concentration.
(Feldman et al., 2000)		Controls =14.1 ng/ml (1.0-19.3 ng/ml) Renal cancer patient = 24.6 (15.1- 54ng/ml)	Patients=48 Controls= age matched	Competitive enzyme immunoassay	Serum samples of at pre- nephrectomy stage of 15 renal cancer patients were analysed against fresh samples from 18 healthy controls
(Zorick et al., 2001)		Downs patients (38.6 ± 20.1 ng/ml) Healthy controls (20.3 ± 11.5 ng/ml)		ELISA	Endostatin concentrations of Down syndrome patients were almost double to that of normal healthy volunteers.
(Feldman et al., 2001a)	colorectal cancer patients (71.6 ± 28.6 ng/ml) Healthy controls (43.2 ± 15.1 ng/ml)		60 years 56 years	Competitive enzyme immunoassay	High endostatin concentrations were found in plasma of 30 colorectal cancer patients. Elevated levels were associated with disease progression.
(Feldman et al., 2001b)		controls = 25.8 ng/ml Sarcoma patients = 43 ng/ml	Patients=44 yrs, Age matched for controls	Competitive enzyme immunoassay	Serum samples from 25 soft tissue cancer patients in pre surgery states were analysed for endostatin and compared to controls.

(Gu et al., 2004)	20.3 ± 3.2 ng/ml	volunteers = 33 ± 13	ELISA	7 healthy volunteers performed high intensity exercise. Their baseline concentrations were checked before exercise.
(Teh et al., 2004)	controls (34.97 ± 3.76 ng/ml Patients (30.62 ± 4.54 ng/ml)		ELISA	Post operative breast cancer patients had a higher endostatin concentrations in plasma compared to controls.
(Suhr et al., 2007)	87.3 ± 5.7 ng/ml	27.8 ± 5.4	ELISA Western Blot	In this study 12 male cyclist perform exercise with and without hypoxia and vibration, and their effect on angiogenesis.
(Sponder et al., 2011)	Smoker elderly (103.3 ± 16 ng/ml)	47.12 (39-62)	ELISA	Impact of exercise was checked on the plasma endostatin concentration in 17 elderly smokers, 20 non elderly smokers, 19 young healthy non smokers and 14 type 2 diabetics. Endostatin concentration was measured before and after exercise.
	Non smoker elderly (116.3 ± 15 ng/ml)	51.55(36-70)		
	Young Non smoker (93.4 ± 15 ng/ml)	23.16 (18-34)		
	Diabetic (108.5 ± 17 ng/ml)	57.86 (42-70)		
(Sponder et al., 2013)	Female athletes (129 ± 21 ng/ml)	26 ± 5	ELISA	Impact of bicycle exercise in athletes and sedentary males and females was checked. Baseline endostatin concentration was observed to be high in athletes than sedentary volunteers. Exercise increased the endostatin concentration in both groups.
	Female controls (89 ± 15 ng/ml)	23 ± 4		
	Male athletes (148 ± 28 ng/ml)	24± 4		
	Male controls (93 ± 15 ng/ml)	23 ± 4		

(Makey et al., 2013)	111 ± 5 ng/ml	24 ± 0.8	ELISA	Low intensity exercise was performed in females belonging to African American (n = 35, mean age 26.1 ± 1.21) and Caucasian (n = 37 mean age 22.1 ± 0.84) ethnicities. No effect of exercise on plasma endostatin concentration was observed.

Table 3-1; Evidence table of previous studies with different endostatin concentrations

A chronological arrangement of the different studies reporting the endostatin concentrations in healthy and diseased population is shown. Endostatin concentrations are outlined as mean ± SD with ranges, where possible. Age of the participants and techniques used for analyses of endostatin are also given in separate columns.

3.3 Rationale of the study

The ultimate aim of this project was to investigate if exercise has an effect on concentration of endostatin in plasma. The available literature provides a broad range of concentrations reported in relatively small samples of healthy controls. Thus, it was essential to use one standard technique to measure endostatin concentrations, as reference point following studies. Moreover, as healthy and sedentary individuals are the major proportion of western population, this study seeks to determine the basal endostatin concentration in plasma of healthy volunteers.

The hypothesis for this study was;

I. The concentration of endostatin in plasma will be different in males and females.

II. Increasing age, weight and BMI, and poor physical fitness will affect endostatin concentrations in plasma.

3.4 Aims of the study

The aims of the study are as follow;

a. To measure the basal concentration of endostatin in plasma of healthy adult population. This will be used, as a reference concentration for further studies.

b. To determine if the basal concentration of endostatin in plasma differs between genders.

c. To determine the difference in basal endostatin concentration with age, height, weight, and physical activity behaviour of the individuals.

3.5 Material and methods

The experimental protocol was approved by the University of Glasgow, College of Medical, Veterinary and Life Sciences Ethics committee. The ethical approval is attached in appendix “A1”. All recruitments and experimental procedures were carried out in the different labs of school of Life Sciences.

3.5.1 Volunteers recruitment

The volunteers were recruited through poster presentation in the public places of the University of Glasgow and advertising through social media. In some cases, volunteers were also approached through personal contacts. Mostly, students of Glasgow University were recruited to the study.

Volunteers were enrolled to the study, after initial screening by asking questions about the inclusion and exclusion criteria. One male participant was dropped out of the study due to high blood pressure reading on three consecutive occasions. Three females were not included. One of them was suffering with Pernicious anaemia. Another was taking a course of oral antibiotics. The third one was enrolled in a regular swimming practice sessions. The participants belonged to 3 main geographical regions, Europe, Asia and Middle East.

The purpose and potential risks of the experiment were explained to each subject and any specific questions asked, were answered. After their agreement to participate, the time and day of sample collection was agreed. Participants were advised not to participate in any physical activity on the day before the blood sample. In total, 50 healthy adult volunteers were recruited to the study.

The characteristics of the volunteers are summarised in table 3-2. This subdivides the data by gender to allow comparison. Another table 3-3 shows these data by geographical origin of the volunteers.

	Total	Female	Male	P-Value
Sample size (n)	50	16	34	
Age (Years)	26.7 ± 7.80	23.1 ± 4.4	28.5 ± 8.5	0.021
Height (m)	1.71 ± 0.07	1.66 ± 0.1	1.74 ± 0.05	<0.001
Weight (kg)	73.6 ± 10.4	67.8 ± 8.01	76.3 ± 10.4	0.006
BMI (kg/m^2)	24.89 ± 2.77	24.65 ± 2.33	25.0 ± 2.98	0.676
Heart Rate (bpm)	75 ± 5	77 ± 6	73 ± 5	0.090
SBP (mm Hg)	123 ± 9	118 ± 12	125 ± 7	0.059
DBP (mm Hg)	74 ± 8	73 ± 8	74 ± 8	0.378
$MET.min.week^{-1}$	2471 ± 1627	2572 ± 1536	2424 ± 1689	0.768
PA (High)%	38	50	32.4	0.370
PA (Moderate)%	52	37.5	58.8	
PA (Low)%	10	12.5	8.8	

Table 3-2; Characteristics of the participants

Characteristics of the all individuals are presented as pooled data and divided by gender. The right hand column shows the results of independent sample t-test. P-value <0.05 shows significant difference between male and female volunteers. Male participants were significantly older, taller and heavier than females. The lower panels of the table show the outcome of Chi Square test. It can be seen that most of the volunteers reported a moderate physical activity. BMI; Body Mass index, SBP; Systolic blood pressure DBP; Diastolic blood pressure (values are presented as mean± SD), MET are the metabolic equivalent minutes of basal metabolic rate

3.5.2 Protocol of the study

After giving informed consent, the volunteers filled out the International Physical Activity Questionnaire (IPAQ short form), as shown in appendix "D1". This was used to assess their physical activity. Height, weight, heart rate and blood pressure of the volunteers were recorded, as discussed in section 2.5.

A tourniquet was applied to the arm of the volunteers to make the anti-cubital veins visible. Blood was taken with 21G butterfly (Nipro Corporation, Zaventum, Belgium) using standard aseptic technique. The blood was collected in 6 ml EDTA tubes (BD vacutainer, Plymouth, UK). Pressure was applied at the site of venepuncture for 30 seconds to 1 minute, to stop bleeding followed by applying sterile adhesive plaster.

3.5.3 Sample storage

The blood samples were put on ice immediately after collection and centrifuged within 30 minutes of collection. A Universal centrifuge (Universal 320R, Germany) spun the samples at 4°C and 1000 RPM for 15 minutes. Each sample of plasma was divided into 3 aliquots and transferred into 0.5 ml Eppendorf tubes. After exact labelling the plasma samples were stored at -80°C in an ultra low temperature freezer (New Brunswick Scientific, U725-86, US), for up to one month before analyses.

3.5.4 Physical activity measurements from IPAQ questionnaires

The weekly physical activities of the participants were determined from IPAQ questionnaires and were categorised in low, moderate and high activity groups. The activity in MET.min.week^{-1} was also calculated numerically, according to the data processing rules given in the guide book (IPAQ, 2005). These are given in table 3.2 and discussed in section 3.7. The rules for measuring these activities in categorical and numerical form are given in appendix "E".

3.5.5 Sample analysis

All the samples, standards and controls were thawed and brought to room temperature before analyses. The samples were analysed for plasma endostatin concentration, using Quantikin^{R} ELISA kit bought from R&D systems. Details are given in section 2.11.1. The samples were run in duplicates after dilution following the instructions of the manufacturer. A series of standards and controls were provided with each kit and these were used to generate calibration plots. Figure 2-6, shows a typical standard curve for the analysis. The minimum concentration of endostatin in standards was 0.313 ng/ml. The minimum concentration of endostatin in analysed sample was 81 ng/ml, which exceeds the minimum detectable standard. Similarly the maximum concentration detected was 132 ng/ml, which was also within the range of detection of the kit.

3.5.6 Statistical analyses of the data

Statistical analyses were carried out using SPSS 17 statistical software. The normality of the data was tested using Kolmogorov- Smirinov and Shapiro-Wilk tests. Where it was important, not normally distributed data was subjected to log transformation. Histograms and Q-Q plots showing the normalities of some variables are given in figure 3-1 & 3-2.

Summary statistics were calculated. Independent sample T-tests were used to investigate difference between means for males and females. ANOVA was applied to check the difference between means of participants from different ethnic groups. Box plots were produced to demonstrate the difference between the mean endostatin concentrations for different data sets.

Pearson correlations tests were used to investigate the correlations of endostatin with anthropometric and physical characteristics of the volunteers. Scatter plots with fitted lines were produced to show, the relationship between plasma endostatin concentration and different anthropometric variables. Simple linear regressions were carried out for uni-variate and multivariate analysis to investigate association of endostatin concentrations with other variables. Categorical data of physical activity was analysed using Chi-square tests. P value less than 0.05 was accepted as significant.

3.6 Results

3.6.1 Distribution of the data

Results were obtained for all 50 volunteers. Kolmogorov- Smirinov and Shapiro-Wilk tests were used to investigate the normality of the data sets. The data were normally distributed. The analyses were easier with normal distribution of the data. This might be expected for the height and age but even variables like MET minutes showed a normal distribution which would have been harder to predict, as shown in figure 3.1.

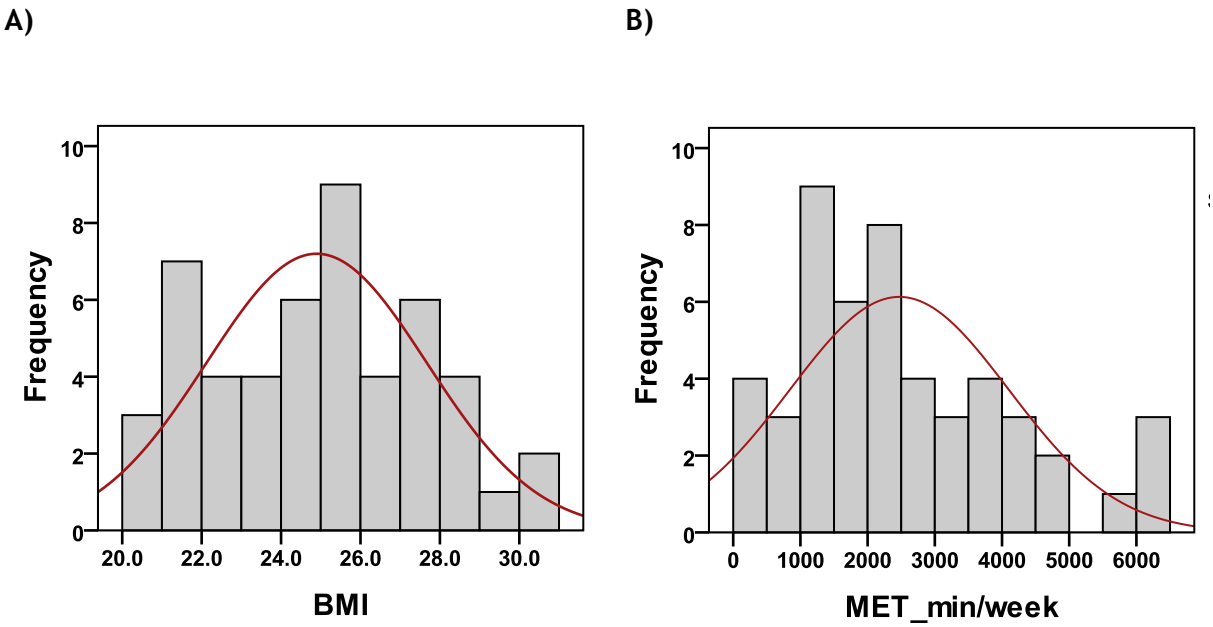


Figure 3-1; Normality test for (A) BMI and (B) MET- minutes per week of the volunteers showing normal distributions.

3.6.2 Difference in characteristics of males and females

The data shown in table 3-2 were subjected to independent sample T-tests. These revealed that males were significantly older (P = 0.021), taller (P <0.001) and heavier (P = 0.006) than females. No statistical differences were observed for other variables.

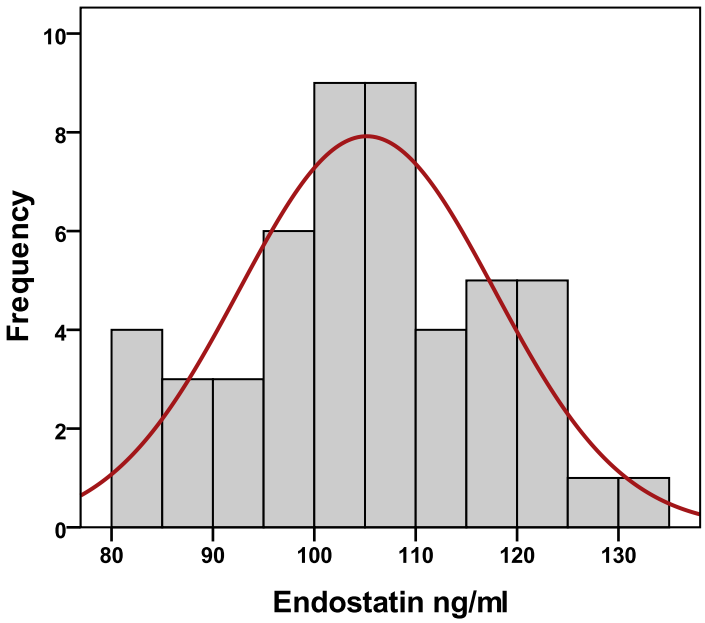
The data collated from IPAQ questionnaires were analysed according to the provided guidelines for data processing (IPAQ, 2005), attached in appendix "E". The data was analysed for different types of physical activity both in categorical (high, moderate and low physical activity) and continuous forms (MET-min per week).

Chi square test showed no significant difference in physical activity levels between male and female volunteers (P = 0.370). Most of the participants had a moderate level of physical activity, as shown in table 3-2.

3.6.3 Endostatin concentration in plasma for all volunteers

The concentration of Endostatin in plasma was normally distributed, as shown by the histogram and Q-Q plot in Figure 3.2. The mean value for plasma endostatin in all samples was 105 ± 12 ng/ml (Mean ± SD). The range of concentration observed in this data set was from 81 to132 ng/ml.

A)



B)

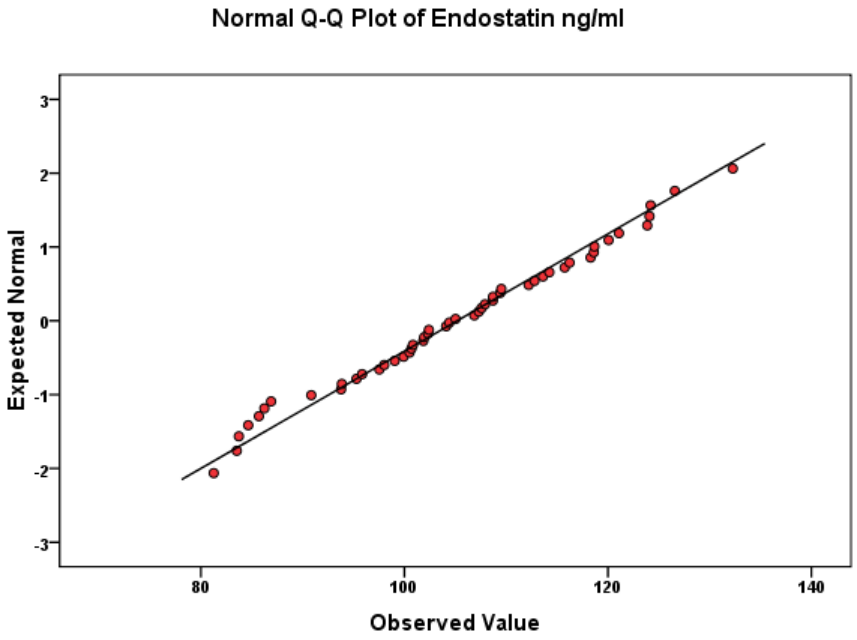


Figure 3-2; Normality for endostatin concentrations of all participants

Panel (A) shows a histogram of the endostatin concentrations in plasma with a normal distribution curve fitted to the data. Panel (B) shows the same data re-plotted as Q-Q plot. The data was normally distributed as shown by Kolmogorov- Smirinov and Shapiro-Wilk tests (P = 0.2 & 0.6) and can be easily appreciated with naked eye.

3.6.4 Comparison of endostatin concentration in plasma between males and females

Endostatin concentration for males and females were found normally distributed (P = 0.20 & 0.5), as shown by Q-Q plots in figure 3-3. The individual data points for males and females are plotted in figure 3.4A. Box plots of data for males and females are shown in figure 3.4B.

The mean endostatin concentration for male was 107 ± 13 ng/ml, while for females; it was 102 ± 12 ng/ml. When mean concentrations for both groups were compared, no significant difference was observed (P = 0.21).

A) **B)**

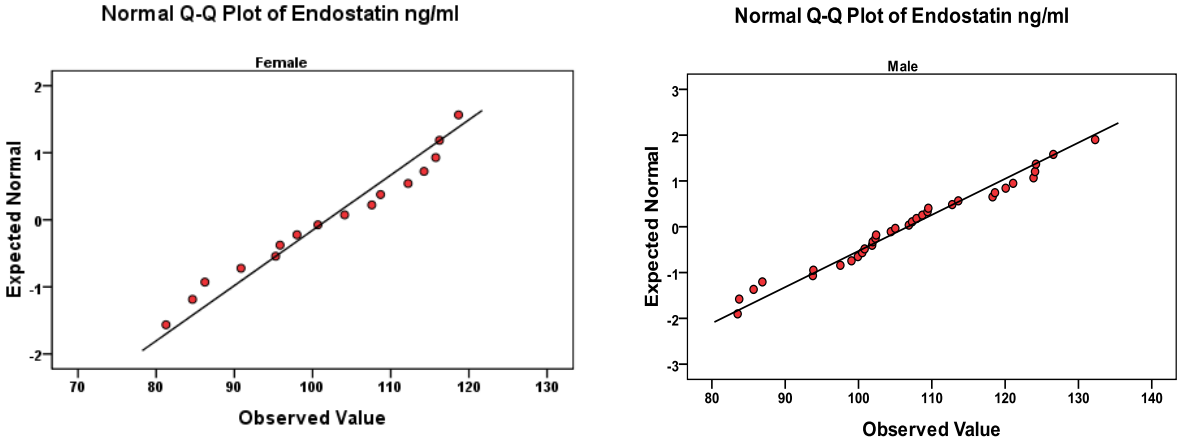
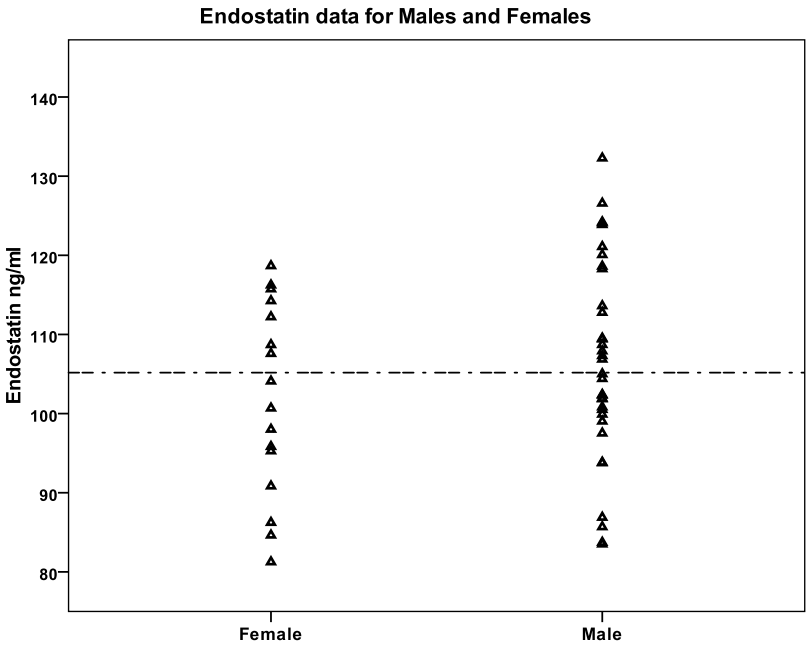


Figure 3-3; Q-Q plots for normality of endostatin concentrations in females (left) and males (right)

Normality for endostatin concentrations in females and males are shown by Q-Q plot. The data was normally distributed. The observed values are lying closely to the expected normal values and can be seen by the distribution of the data close to the expected fit line.

A)



B)

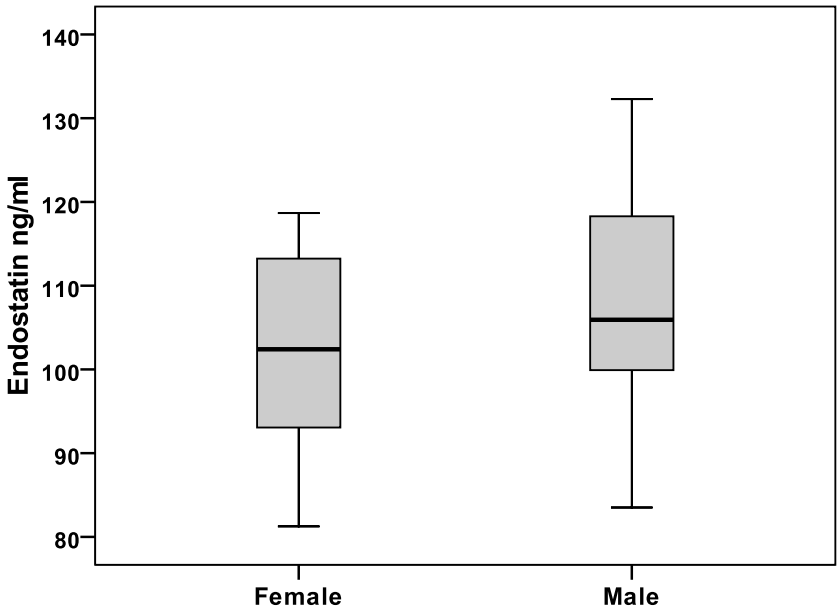


Figure 3-4; A) Endostatin concentrations in males and females (B) Box plot showing group data for males and females

Endostatin concentrations in plasma for males and females are shown. Panel (A) shows the data for males and females with mean endostatin concentration (dotted line) for the pooled data, while Panel (B) shows Box plots with median value and range for the both groups. No significant difference between the mean endostatin concentration of both groups was observed (P = 0.21).

3.6.5 Endostatin concentration in different geographical groups

The participants came from three main geographical regions: Europe, The Middle East and South Asia. This distribution reflects the proportion of international students in the university campus. The difference in their physical characteristics are summarised in table 3-3. As most of the volunteers were university students, they share many similar characteristics.

There was no significant difference between the ages of male participants. European females were significantly younger than Middle Eastern females (P = 0.009). There were no females in South Asian group. European males and females were comparatively more active than volunteers in other groups. Middle Eastern females reported the lowest physical activity. In addition, they had higher pulse rates and low systolic blood pressures, as shown in table 3-3.

Individual concentrations of Endostatin for participants in each regional group are plotted, in figure 3-5A. The data were normally distributed. The concentrations of endostatin from Middle Eastern participants lie within a narrower range (95 -119 ng/ml), and this could be a consequence of low numbers of participants in this group.

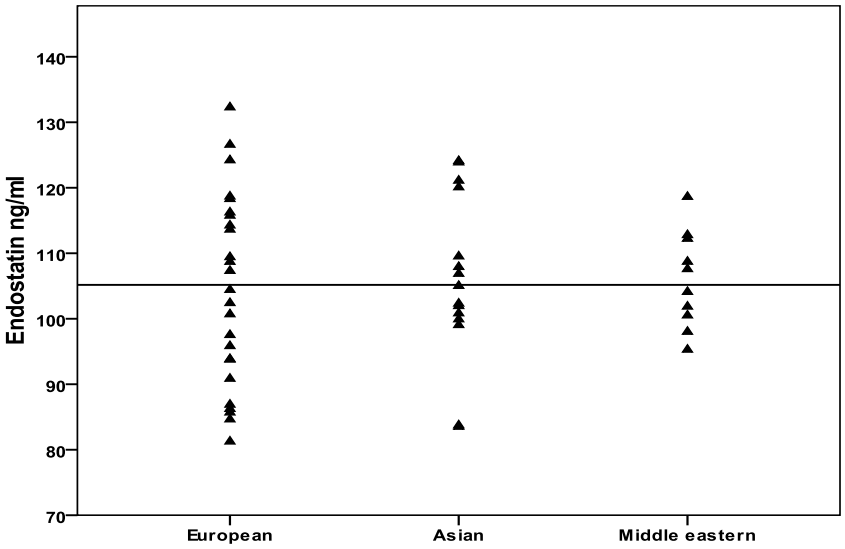
A one-way ANOVA was used to investigate differences in endostatin concentrations between the groups. The mean endostatin concentrations for all the groups are shown, in table 3-3. Box plot shows median values with range of data for males and females, in figure 3-5B. No significant difference in endostatin concentration was found.

		Europeans	Asians	Middle Eastern	P- Annova
Sample size (n)	M	14	15	5	
	F	11	-	5	
	Total	25	15	10	
Age (years)	M	25 ± 10.5	32 ± 6.06	27.2 ± 6.02	0.081
	F	21.2 ± 1.6	-	27.6 ± 4.5	0.009
	Total	23.3 ± 8.1	32 ± 6.06	27.4 ± 5.04	0.004
Height (m)	M	1.75 ± 0.04	1.74 ± 0.05	1.74 ± 0.04	0.60
	F	1.67 ± 0.1	-	1.62 ± 0.1	0.24
	Total	1.72 ± 0.1	1.73 ± 0.05	1.68 ± 0.08	0.08
Weight (kg)	M	76.3 ± 11.6	76.2 ± 10.8	76.9 ± 2.9	0.99
	F	68.9 ± 8.3	-	65.6 ± 7.8	0.46
	Total	73 ± 10.7	76 ± 10.8	71.2 ± 9	0.15
HR	M	74 ± 6	74 ± 5	71 ± 3	0.54
	F	76 ± 6	-	78 ± 5	0.60
	Total	75 ± 6	74 ± 5	74 ± 5	0.97
SBP	M	127 ± 5	122 ± 9	127 ± 2	0.11
	F	120 ± 12	-	116 ± 11	0.54
	Total	124 ± 10	122 ± 9	121 ± 9	0.16
DBP	M	77 ± 9	72 ± 7	78 ± 5	0.18
	F	73 ± 8	-	73 ± 10	0.96
	Total	75 ± 9	72 ± 7	75 ± 8	0.48
Met.min.week^{-1}	M	2803 ± 1704	2065 ± 1695	2441 ± 1747	0.50
	F	2850 ± 1031	-	1958 ± 2346	0.30
	Total	2824 ± 1420	2065 ± 1695	2200 ± 1967	0.48
Endostatin (ng/ml)	M	107 ± 15	106 ± 13	108 ± 7	0.90
	F	101 ± 14	-	103 ± 7	0.70
	Total	105 ± 14	106 ± 13	106 ± 7	0.80

Table 3-3; Anthropometric and physical characteristics of volunteers from different regional groups

Differences in characteristics of the individuals from different regional groups are shown. As expected, no significant difference in variables with the exception of age can be seen. The lower portion contains the mean endostatin concentrations in different groups. No significant difference was observed, as shown by P values.

A)



B)

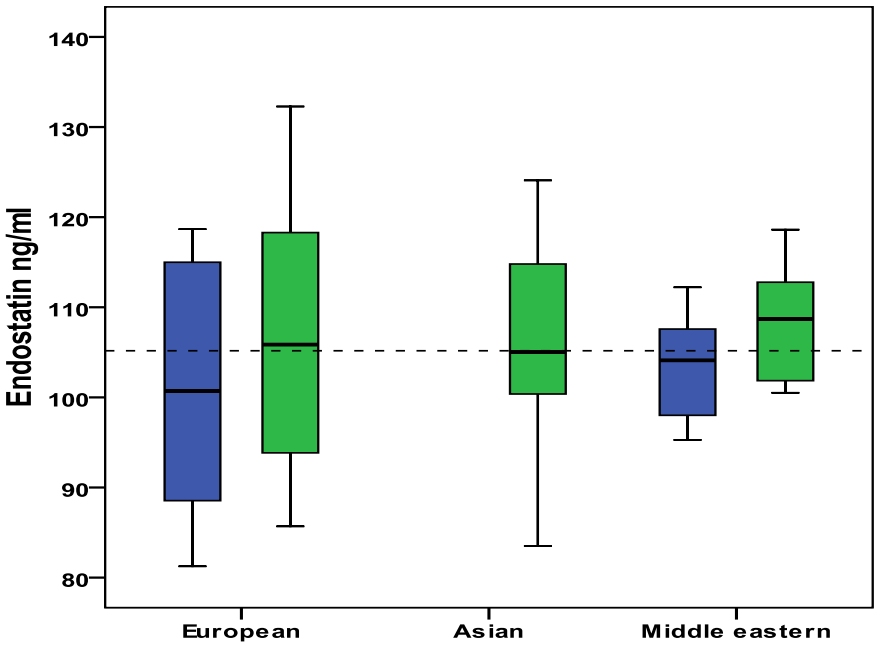


Figure 3-5; Endostatin concentrations for different ethnic groups (A) individual values & (B) Group data

Panel (A) represent the dot plot with individual values for different ethnic groups. Panel (B) represents the median and range for males and females in each group. Dotted line represents the mean concentration for all data. European participants show bigger range than others. Blue colour represent female and Green colour represent male groups.

3.6.6 Correlation of endostatin with different anthropometric and physical variables

The third aim of research in this chapter was to investigate any correlation of endostatin concentration with different anthropometric and physical characteristics of the participants. This was done using Pearson correlation tests. As the data was normally distributed using Pearson correlations will provide strength of linearity between the endostatin and other variables.

Table 3-4 shows the correlation values (r) and significance (P) for all variables as pooled data set, while table 3-5 represent the same data for different regional groups. No correlations of statistical significance were found between most variables and plasma endostatin concentration, as shown in table 3-4. However, the correlation between basal heart rate and plasma endostatin concentration was trending significance (P = 0.053) for the data set of all individuals. Further analyses of the data by gender revealed, that males had a negative correlation between heart rate and plasma endostatin concentration, as shown in figure 3-8A.

Secondary analyses (splitting data by ethnic group and gender) revealed that Middle Eastern females also showed a negative correlation between resting heart rates and plasma endostatin concentration (P= 0.005, r = -0.973) i.e. participant with low basal heart rate tend to have high endostatin concentration. On the other hand, in South Asian volunteers, there was a positively correlation for age (P = 0.049, r = 0.51) and diastolic blood pressure (P = 0.037, r = 0.54). These findings are tentative. This was not a primary aim of the study. The sample size is very small. A larger study would be required before drawing a confident conclusion.

However, the regression analyses were inconclusive in providing any association between different anthropometric variables and plasma endostatin concentrations, as shown in table 3-6. Uni-variate as well as multi-variate analyses were carried out. In Uni-variate analysis heart rate and physical activity was approaching significance (highlighted area) which is lost by inclusion of

other variables, as shown in multi-variate analyses. The overall value for R^2 in multi-variate analyses was 0.182.

	n		Age	Height	Weight	BMI	SBP	DBP	HR	MET.min.week^{-1}
Male	34	r	.114	-.093	-.152	-.152	-.029	.279	-.351*	-.265
		P	.520	.600	.390	.392	.872	.110	.042	.130
Female	16	r	-.081	-.106	.047	.165	.301	-.107	-.023	-.048
		P	.766	.696	.863	.541	.257	.692	.932	.860
Total	50	r	127	.026	-.023	-.059	.156	.173	-.275	-.208
		P	0.379	.857	.873	.686	.279	.230	.053	.148

Table 3-4; Pearson correlation of endostatin with anthropometric and physical variables for all individuals

A trend in correlation between basal heart rate and endostatin concentration can be observed. Male individual had a significant negative correlation between heart rate and endostatin concentration. r; Pearson coefficient value, P; Significance of the correlation, SBP; Systolic blood pressure, DBP; Diastolic blood pressure

Ethnicity		n		Age	Height	Weight	BMI	SBP	DBP	HR	MET.min.week^{-1}
European	Male	14	r	-.116	-.192	-.328	-.321	-.080	.040	-.395	-.254
			P	.694	.512	.253	.263	.786	.892	.162	.381
	Female	11	r	-.409	-.036	.167	.238	.298	-.234	.114	.020
			P	.211	.916	.623	.481	.373	.488	.739	.954
	Total	25	r	-.085	.032	-.073	-.110	.206	-.023	-.198	-.166
			P	.686	.881	.727	.600	.323	.913	.342	.429
South Asian	Male	15	r	.516	-.108	-.053	-.033	-.037	.542	-.302	-.395
			P	.049	.701	.851	.908	.897	.037	.274	.145
Middle Eastern	Male	5	r	.552	.496	.815	.813	-.037	.673	-.203	.170
			P	.335	.396	.093	.094	.953	.213	.744	.784
	Female	5	r	-.001	-.352	-.474	-.304	.510	.426	-.973	-.514
			P	.999	.561	.420	.619	.380	.474	.005	.129
	Total	10	r	.253	.265	.340	.182	.451	.551	-.685	-.145
			P	.480	.460	.337	.615	.191	.099	.029	.816

Table 3-5; Pearson correlations of endostatin with anthropometric and physical variables for individuals in different regional groups

No correlations were found for height, weight, SBP, DBP, BMI and MET minutes with endostatin concentration. Strong negative correlation was observed in Middle Eastern females for heart rate. South Asian males were positively correlated for age and DBP. r; Pearson correlation coefficient value P; Significance of the correlation, SBP; Systolic blood pressure, DBP; Diastolic blood pressure

Endostatin	Uni-variate		Multi-variate	
	Beta (CI)	P	Beta (CI)	P
Age	0.13 (-18.1 - 46.7)	0.38	-0.05 (-50 - 39.0)	0.81
Height	0.03 (-46 - .355.4)	0.86	-0.55 (-6920 - 499.4)	0.75
Weight	-0.02 (-0.4 - 0.3)	0.87	0.78 (-6 - 7.8)	0.78
Sex	0.18 (-2.8 - -12.5)	0.21	0.10 (-9 - 14.1)	0.65
BMI	-0.06 (-1.6 - 1.1)	0.69	-0.65 (-24 - 17.7)	0.77
SBP	0.16 (-0.2 - 0.6)	0.28	0.17 (0 - 0.8)	0.38
DBP	0.17 (-0.2 - 0.7)	0.23	0.04 (-1 - 0.6)	0.81
HR	-0.27 (-1.3 - 0.009)	0.05	-0.29 (-1 - 0.1)	0.10
MET_min.week^{-1}	-0.21(-.004 - .005)	0.15	0.06 (-.004 - 0.005)	0.84
PA Level	-0.23 (-10.2 - 0.9)	0.10	-0.27 (-16 - 5.5)	0.32

Table 3-6; Regression analysis for endostatin with anthropometric and physical variables

Uni-variate and multi- variate regression analyses for all variables were carried out, as shown by Beta coefficient (Beta) with confidence intervals (CI) and significance (P). It can be seen that variables which were trending significance in uni-variate analyses (Highlighted area) lost its significance in multivariate model. The overall dependency of endostatin on the different anthropometric characters were only 18% in multivariate analyses (R^2=0.182).

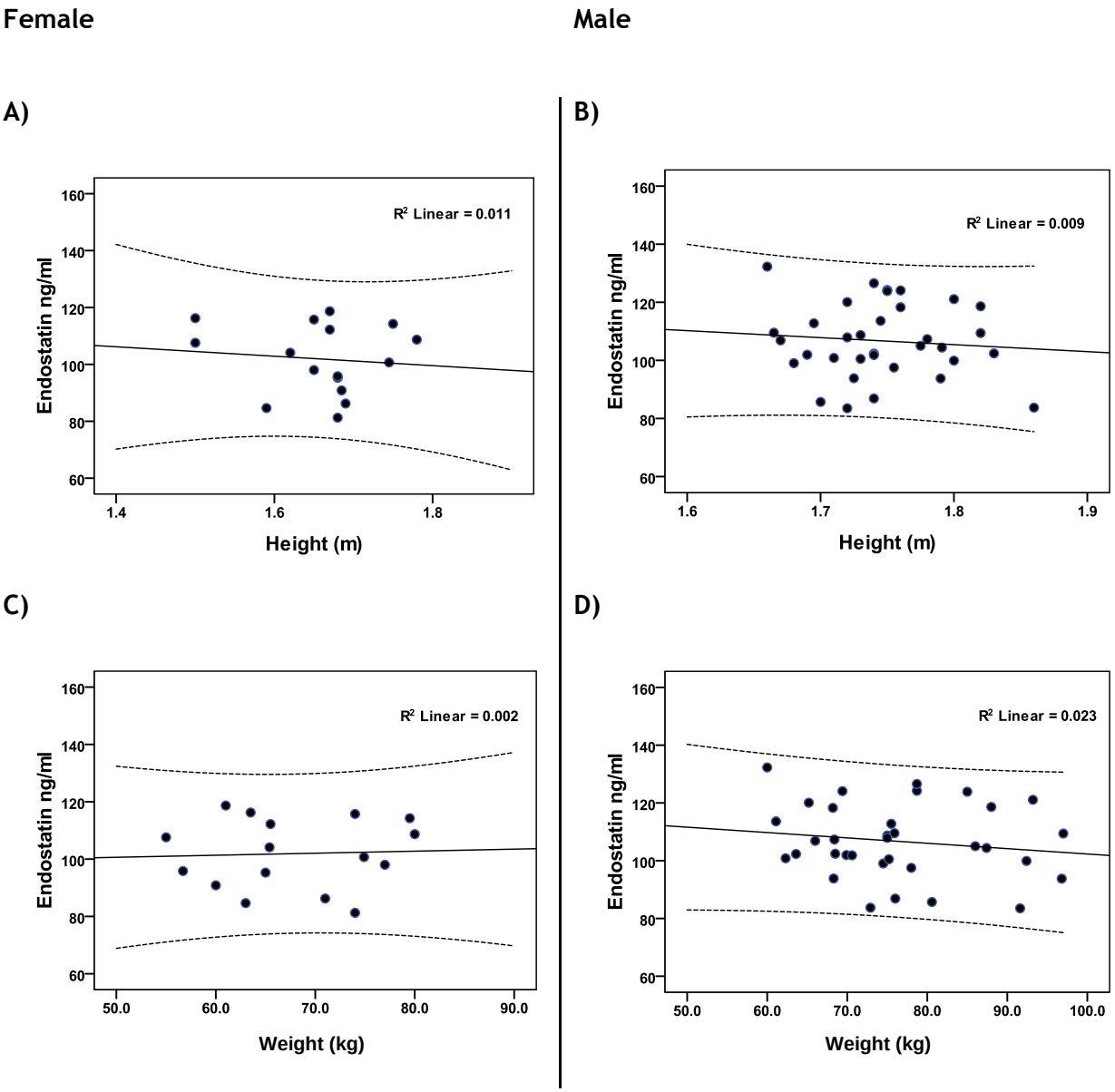


Figure 3-6; Fitted line plots for height and weight with regression lines females and males

Fitted line plots showing the regression lines (solid lines) and 95% CI (dotted lines) lines for height and weight with endostatin. The wide CI and low R^2 values clearly show no significant association of endostatin with weight and height in both female (A & C) and male volunteers (B & D).

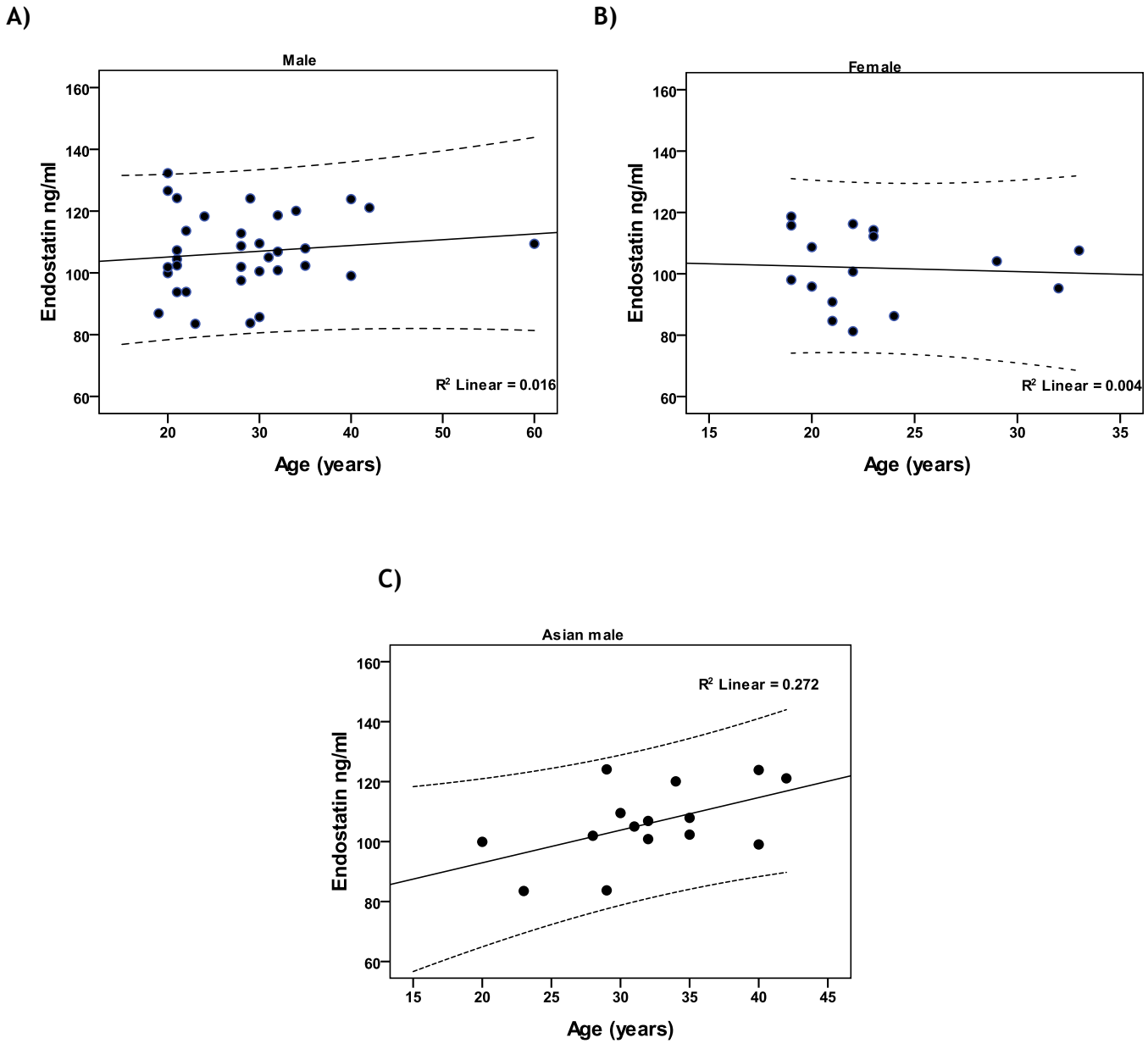


Figure 3-7; Fitted line plots for age with regression lines for (A) males, (B) females from pooled data and (C) Asian males

Fitted line plots showing the regression lines (solid lines) and 95% CI (dotted lines) lines for age with endostatin. Section C has comparatively shorter CI for South Asian males. The R^2 value shows weak link between the age and endostatin.

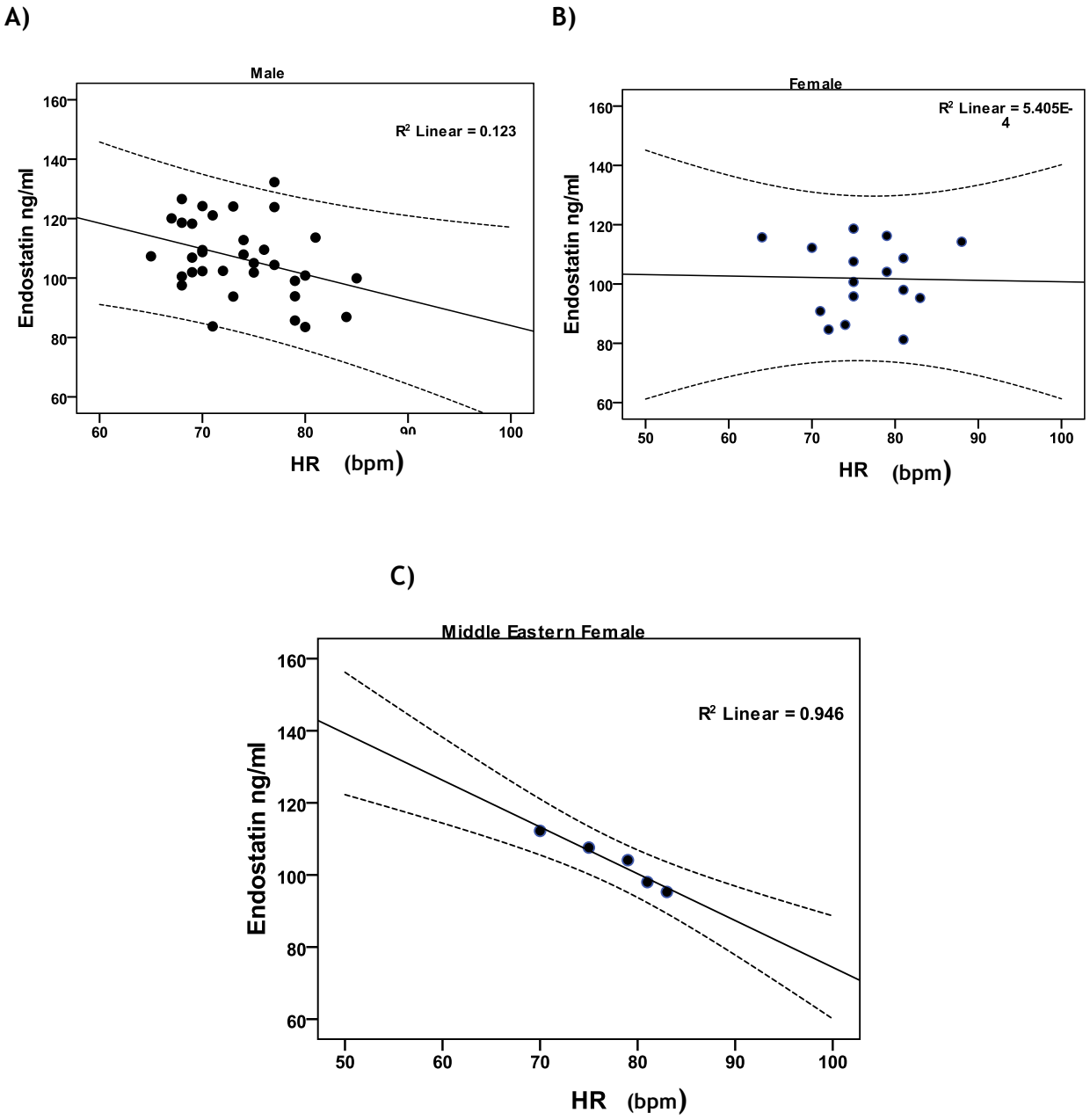


Figure 3-8; Fitted line plots for HR (heart rate) with regression lines for (A) males, (B) females from pooled data and (C) Middle Eastern females

Fitted line plots showing the regression lines (solid lines) and 95% CI (dotted lines) lines for heart rate with endostatin. Middle Eastern females have very short confidence interval and high R^2 value, which shows a strong relationship of basal heart rate and endostatin, as shown in panel C (n=5).

3.7 Discussion

Since the discovery of endostatin, in the last few years a large number of studies have pointed towards its anti-angiogenic effects and potential to protect individuals from progression of atherosclerosis and cancers. However, to fully understand its physiological role, it is essential to know the range of its concentration in plasma. To the best of author's knowledge, there is no study to demonstrate the baseline endostatin concentration in healthy volunteers with the aim to find a mean concentration with reference range. That's why this study measured the concentration of endostatin in plasma samples from healthy volunteers. The study achieved the aims outlined in section 3.4. Mean endostatin concentration for all individuals was measured and difference in mean for males and females was compared. In addition, the association of different anthropometric and physical variables with endostatin concentration were also checked.

The analyses undertaken in this study showed that mean plasma endostatin concentration was 105 ng/ml with a range of 81 to 132ng/ ml. These results are consistent with the results published by (Sponder et al., 2011), who reported a mean concentration of 93.4 ± 15.3 ng/ml and (Suhr et al., 2007), who found a mean concentration of 87.3 ± 4.5 ng/ml. The studies carried out by Sponder included 13 male healthy volunteers while the latter study had a sample size of 88. Another study published recently, reported mean endostatin concentration of 111 ± 5 ng/ml, in 72 healthy females (Makey et al., 2013).

However, these results are significantly different from the mean plasma endostatin concentration of 20.3 ng/ml reported by (Gu et al., 2004), 14.1 ng/ml by (Feldman et al., 2000) and 43.2 ng/ml by (Teh et al., 2004), for healthy volunteers of different ages in their studies. These differences in concentrations could be attributed to the different techniques (ELISA, Western blot, Mass spectrometry etc) used for analyses. It should be noted from the studies listed in table 3-1, that more recent studies have reported comparatively high endostatin concentrations, which is pointing towards more sophistication and maturity of techniques with time.

However, the sensitivity of the ELISA kits from different manufactures might be a factor. Different studies using the same ELISA kits (Accucyte, Cytimmune Sciences Inc., MD, USA) have reported a low endostatin concentration (Gu et al., 2004, Zorick et al., 2001), which could possibly be the reason for reporting low endostatin concentration. Moreover, the media used for analyses (plasma, serum, haemofiltrate) may have different sensitivity and specificity for the same techniques. Nonetheless, these differences are not completely clear because concentrations reported by the same author in different studies using the same techniques and media, show significant difference for healthy volunteers (e.g. see Feldman et al., 2000 and 2001a in Table 3.1). One might speculate about the causes of these differences. Serum concentrations of endostatin have been reported to be affected by circadian rhythms (Glenjen et al., 2002), age (Bruserud et al., 2005, Brixius et al., 2008) and physical activity (Gu et al., 2004, Bruserud et al., 2005). It was not possible to address these factors in this project.

The results in our study seems more reliable due to the larger sample size, good quality control indicators and the results lying within the range provided by the kit supplier. It is worth noting, that even this larger sample does not find volunteers with endostatin concentrations as low as those reported elsewhere (Feldman et al., 2001a, Gu et al., 2004). It is also unlikely that geographical variations could explain these differences. Figure 3-5B, compares European, Middle Eastern and South Asian populations, with only minor differences observed.

The second aim of the study was to investigate the difference in concentration of endostatin between males and females. The mean plasma endostatin concentration appears higher in male than female volunteers. This is shown in figure 3-4B. However, the difference is not statistically significant ($P = 0.21$). These results are in agreement with the result from other studies, where the male shows more mean concentration than female (Feldman et al., 2001b, Zorick et al., 2001). Though these studies have not mentioned any direct comparison between the two groups, the difference looks non significant. In addition, another study which compared the concentration on gender basis also reported a slightly higher but non-significant concentration in males (Sponder et al., 2014).

The hypothesis tested in this study was that females might have lower concentrations of plasma endostatin. The basis of this expectation lies in the lower concentration of many biological molecules such as haemoglobin in females (Walters et al., 1973). Gu speculated that endostatin in circulation might be due to physiological collagen turnover (Gu, 2004).Based on this supposition, it can be assumed that more muscle mass in males may cause higher physiological collagen turnover which consequently might increase circulatory endostatin.

Plasma endostatin concentrations in volunteers of different ages were also investigated. The volunteers had a broad range (19-60 years) of age. However, age of 96% of the participants were 40 years and below. The oldest participant in the study was 60 years old male and his plasma concentration was 109 ng/ml, which is very near to the mean concentration. No significance correlation of plasma endostatin with age was found when the data from all volunteers was investigated, as shown in table 3-5 and figure 3-7C. The data from the South Asian volunteers showed a positive correlation with age (P = 0.049). Strong conclusion from this correlation cannot be drawn because; firstly the P value is border line to significance, secondly sample size is small (n = 15) and thirdly by including the male participants from Middle Eastern and European group, significance is lost (P = 0.52), as shown in table 3-4.

However, significantly higher endostatin concentration in older participants than young adults has been reported previously (Sponder et al., 2011, Bruserud et al., 2005). With advancing age the chances for angiogenic dependent diseases increases. It is possible that the circulatory endostatin concentration rises as an adaptive protective mechanism.

It was important to know that variations in physical and anthropometric characterises might affect the endostatin concentration in plasma. But the plasma endostatin concentration seemed uncorrelated with most of physical and anthropometric parameters such as height, weight, BMI and blood pressure, as shown in table 3-4 and figure 3-6. The author is unaware of any previous published data reporting these observations. However, when the data was split on ethnic and gender basis, the results suggest that endostatin concentration in plasma was correlated with some characteristics. For e.g. a positive correlation

with DBP was observed in South Asian participants. These observations must be treated with caution; firstly, due to small number of participants in the respective groups and secondly, the volunteers included in the study were healthy with normal ranges of blood pressure.

Similarly, resting heart rate was found to be negatively correlated with plasma endostatin concentrations, as shown in table 3-4, 3-5 and figure 3-8. Middle Eastern females showed a strong negative correlation. This is notable but inconclusive finding, as this group has only 5 female participants. Heart rates are usually unstable and can be easily influenced by many stimuli including time of the day (Massin et al., 2000) and fear response due to release of adrenaline (Fellows et al., 1985).

Physical activity has been widely accepted to decrease the risk of cardiovascular related mortality and morbidity (Nocon et al., 2008). The physical activities were assessed using the short form of International Physical Activity Questionnaire. They were categorised in low, moderate and high activity groups. The activity in MET.min.week^{-1} was also calculated numerically, according to the data processing rules given in the guide book (IPAQ, 2005). METs refer to multiples of resting metabolic rate during an activity. MET-minutes are the MET score of an activity during a specific time period. The analysis revealed no significant relationship between plasma endostatin concentration and reported physical activity ($P = 0.816$). This is shown in tables 3-4 and 3-5.

Higher concentrations of plasma endostatin were reported by Sponder in male and female athletes than in matched controls (Sponder et al., 2013). This difference in results could be due to several factors. Perhaps habitual physical activity has different effects from shorter periods of intense athletic training. It was assumed before the study, that regular physical activity might have an effect on the basal endostatin concentration. As participants were mostly students of the University of Glasgow, their involvement in physical activities was expected to be more than population in general. To minimise this effect, all volunteers were asked to abstain from physical exercise for 24 hours before the trial.

However, under or over reporting of physical activities, unclear definitions of different intensities of activities, and the presence of the option “I don’t know” in the questionnaire, could be the possible reasons for the data of physical activity being unreliable. More sophisticated approaches such as using accelerometers and GPS devices, to monitor the physical activity behaviour of the participants prior to blood sampling, could have provided more accurate information. Due to large sample size, cost of the GPS devices was one of the barriers in implementations.

The distribution of plasma endostatin concentration seems normal in volunteers from different ethnicities, as shown figure 3-5A & B. To our knowledge this is a new finding.

The strengths of the study include; a wide sample size and inclusion of both male and female participants from different ethnic groups with wide range of age and other parameters including height, weight, BMI and heart rate.

There were some limitations to the study such as more males (n = 34) than females (n = 16). This was not intentional and volunteers were recruited randomly. Moreover, 3 female participants were not recruited to the study due to different reasons, given in section 3.5.1. The results imply no difference on basis of gender and the imbalance between the number male and female participants may not have a profound influence on the results. However, the strength given to the results in setting the trends with a more evenly gender distribution, could not be rejected completely.

Plasma samples were run in duplicate on ELISA kits to calculate mean values for each subject as compared to the classical triplicate pattern used in many studies. There are two reasons for this: firstly the ELISA manufacturer instruction says the plasma samples to be run in duplicate and secondly time and financial restraints. Triplicate results might have produced more accurate mean result but the good quality control indicators (inter and intra assays CV% <10 & 5% respectively) show that handling technique of the plasma samples, controls and standards, including pipetting and different steps in the analyses, were carried out accurately. The differences in mean endostatin concentrations were small and triplicates would have minimal effects on the overall results.

The experiment was not planned to investigate the difference in concentration in different regional groups. The regional groups were included only, when the initial statistics revealed no difference between the male and female participants and their demographic relationship with plasma endostatin concentration. This is also the reasons of the uneven distribution of participants in different ethnic groups. The results could be more reliable, had there been more participants in each group and study planned on more ethnic bases in the initial stage.

3.8 Conclusion

In conclusion all the aims of the study were achieved.

I. The mean endostatin concentration in healthy adult population was measured. This will allow a suitable sample size to be estimated for further research, which aims to determine the effects of exercise on the plasma endostatin concentration.

II. The endostatin concentration was found to be slightly lower in female than male participants and the difference was insignificant. Moreover, the basal plasma concentrations were found to be independent of age and other demographic characteristics.

III. Moreover, the distribution of basal endostatin among individuals from different regions was normal and comparable.

Based on this study's results, further research in to the effects of exercise on plasma endostatin concentration could be carried out without any specific gender or anthropometric requirements.

4 The effects of different intensities of exercise on plasma endostatin concentrations

4.1 Overview

The research in chapters 4 & 5 represents same study in two parts. The results are reported separately for clarity. The first part consists of the effects of short periods of different exercise intensities on the concentration of endostatin in plasma. The initial plan of the study was to investigate endostatin, but during the experiments secondary analyses were conducted to examine the effects of some exercise intensities on VEGF. The sections are written in the order, the research was carried out.

4.2 Introduction

The importance of physical activity for decreasing the risk of many chronic diseases including cardiovascular disease and diabetes is established worldwide and discussed already in section 1.3.9. Enhanced physical activity decreases the risk of these diseases through different mechanisms including local cardiovascular adaptations such as production of heat shock proteins and increasing anti oxidant capacity (Golbidi and Laher, 2011). It also modifies other risk factors like obesity and diabetes through increasing insulin sensitivity and changing the lipid profile (Francis, 1998, Gill and Cooper, 2008). However, the molecular basis for the effects and interactions of exercise and atherosclerosis is not completely clear (Golbidi and Laher, 2011).

Extension of collateral circulation is one of the important determinants in CVDs. Controlled angiogenesis expands the vascular network by formation of new vessels (Distler et al., 2003). Uncontrolled angiogenesis is dangerous in atherosclerotic plaque, as it increases the size of plaque and can make it vulnerable to rupture (O'Brien et al., 1994). During such conditions, the roles of angiostatic mediators become important, as they tend to halt the process.

Exercise also affects the angiogenesis by increasing or decreasing the circulating levels of angiogenic mediators. The over expression of circulating VEGF (key angiogenic mediator) in myocardium (Isner and Losordo, 1999), skeletal muscles (Olenich et al., 2013) and plasma (Suhr et al., 2007) after exercise has been reported. Similarly, influence of exercise on endostatin (Key angiostatic mediator) has also been shown (Sponder et al., 2014).

Endostatin has emerged as one of the potent inhibitors of angiogenesis and has been extensively studied for its different properties as mentioned previously in section 1.3.6. In a pilot study, exercise has been reported to increase the endostatin concentration by 73% in young healthy male participants (Gu et al., 2004). Later on, another study also reported 23% increase in endostatin concentration after cycling. Additionally, increase was more pronounced in young healthy volunteers than diabetics and smokers (Sponder et al., 2011).

However, Brixius and his colleagues reported a decrease in endostatin concentration after 6 months of endurance exercise in middle to old age individuals (Brixius et al., 2008). Although the exact mechanism, by which exercise influence endostatin is not completely, understood, the differences in exercise protocols, durations and physical status of the participants could be considered for these variations in results.

4.3 Rationale of the present study

The effect of exercise on circulatory endostatin concentration is still poorly understood. The studies which have reported the effects of exercise on plasma endostatin have used different intensities of exercise with different modes (treadmill, cycling, climbing etc.). The durations of the exercise has also not been related properly to changes in circulatory endostatin. In order to have a better understanding of how exercise influences the circulatory endostatin concentrations, it was proposed to use 3 exercise protocols with different intensities. The purpose of using a range of intensities is to replicate the dose response relationship between health benefits and physical activity. The over load principal of exercise suggests that exercise below certain threshold intensity will not affect the physiological parameters of the body (ACSM, 2013).

The proposed types of exercises are as follow;

i. Light intensity exercise at 60% of the predicted maximum heart rate
ii. Moderate intensity exercise at 70% of the predicted maximum heart rate
iii. Vigorous exercise at 80% of the predicted maximum heart rate

The energy consumption during each session of exercise was kept constant at 200 kcal and the duration for each session was adjusted to keep the same energy expenditure in each individual.
In this study the hypotheses to be tested were:

i. Exercise will increase the endostatin concentration in plasma.
ii. Different intensities of the exercise will have different effects on the endostatin concentration in plasma.
iii. Different intensities of exercise will have different effects in males and females.

4.4 Aims of the study

This study has the following aims;

I. To determine the effects of different intensities of exercise on the endostatin concentrations in plasma.
II. To determine if the change in endostatin concentration after exercise is affected by gender or exercise mode (walking and running).
III. To determine the correlation between change in endostatin and different exercises parameters.
IV. To determine if the basal endostatin concentration corresponds to study in chapter 3.

4.5 Material and methods

Approval for this experimental study was granted by the University of Glasgow, College of Medical, Veterinary and Life Sciences Ethics Committee. The ethical approval is attached in appendix “A2”. The recruitment of volunteers and the exercise sessions were carried out in different exercise laboratories of School of Life Sciences. For the analyses of the blood samples metabolic suites in School of Life Sciences were used.

4.5.1 Volunteer recruitment

Volunteers were recruited through posters around the University of Glasgow and public places in the west end of Glasgow. In some cases, personal contacts were also made. Most of the volunteers were students of the Glasgow University.

The purpose and potential risks during the participation were explained to the participants and any questions asked, were answered. The volunteers were told that they are entirely free to drop out of the trial at any point, if they do not want to participate. Volunteers were screened before enrolment into the study. 2 pre-participation health screening questionnaires were completed by each participant; the Physical Activity Readiness Questionnaire (PAR-Q) and the adopted form of health fitness facility pre- participation health screening questionnaire of the American College of Sports Medicine (ACSM) (ACSM, 2013). Both of these questionnaires are given in appendix "D2" & "D3". In addition, blood pressure and basal heart rates of the participants were recorded. Volunteers in the age group 18-45 years, non smokers with no relevant cardiac history, not on medication, not pregnant or lactating and asymptomatic otherwise were enrolled into the study. 22 male and female volunteers were recruited. Their characteristics are given in table 4-1.

	Total	Male	Female	P-value
Number (n)	22	16	6	
Age (Yrs)	29.4 ± 7.2	30.6 ± 7.8	26.5 ± 5.0	0.30
Height (m)	1.69 ± 0.10	1.73 ± 0.07	1.58 ± 0.01	<0.001
Weight (kg)	69.7 ± 14.2	75.7 ± 10.8	53.6 ± 8.5	<0.001
BMI	24.2 ± 3.33	25.3 ± 2.8	21.34 ± 2.9	0.02
SBP (mm of Hg)	115 ± 0	118 ± 9	107 ± 10	0.04
DBP (mm of Hg)	77 ± 7	78 ± 6	74 ± 7	0.20
HR (bpm)	77 ± 5	77 ± 6	80 ± 3	0.10

Table 4-1; Characteristics of the participants

The differences in variables for males and females were determined with independent sample t-tests. P-value <0.05 shows significant difference. Significant difference between males and female were observed for height, weight, BMI and systolic blood pressure. BMI; Body Mass index, SBP; Systolic blood pressure DBP; Diastolic blood pressure, HR; Heart rate (values are presented as mean ± SD)

4.5.2 Protocol of the study

The participants visited the lab on 4 occasions. 7 days elapsed between visits. The day by day lab activities are discussed in the following sections and a schematic presentation of protocols is shown in figure 4-1. The order of visits 2, 3 and 4 were randomised, so that exercise intensities did not come in an ascending or descending sequence. The participants were paid £40 each, after completing the trials.

4.5.2.1 Visit 1

The participants attended the lab. After completing the consent forms and questionnaires about health status, their blood pressure, heart rate, height and weight were recorded, as described in chapter 2 sections 2.5. Each volunteer performed a sub maximal exercise test, using a walking or running protocol. This choice was based on their individual fitness levels, as described earlier in chapter 2 section 2.6. In the walking protocol, the speed was kept constant and the gradient was increased every 5 minutes. In the running protocol, the speed was increased by 1km/hr every 5 minutes while keeping the gradient at 0%. The maximum duration of the test was 20 minutes but stopped if the volunteer's heart rate reached 85% of predicted maximum heart rate. This happened with only one volunteer.

During each test, expired air was collected using Douglas bags and analysed to elicit the speeds and durations to be used in the subsequent 3 exercise protocols. The methods for calculating expired air gases and determining the exact speed and duration at specific heart rate are outlined, in chapter 2 section 2.6.3.

The dates and timings for the next visits were discussed with the participants and they were asked to abstain from physical activity for 24 hours before each trial.

4.5.2.2 Visit 2

On the 2nd visit, the measurements for weight, height, blood pressure and heart rate were repeated. A cannula was passed in the ante-cubital vein and a pre-exercise blood sample was taken. The participants initially stood quietly on treadmill for 5 minutes and their expired air was collected in the last minute. This was used to calculate their oxygen consumption and substrate utilization at rest and to confirm that participants are not hyperventilating. After this, the participants walked on the treadmill on pre-calculated speed capable of eliciting 60% of the expected maximum heart rate. The duration of the exercise was set so, that the predicted energy expenditure of each participant was 200 kcal. This prediction was confirmed by expired gas collection in Douglas bags, after 5 minutes of exercise. Where participants were consuming less or more energy, the work rates were increased or decreased. The maximum increase or decrease for a participant was 0.4 km/hr. A second measurement of expired air gas was taken 5 minutes after any adjustment made. The rate of perceived exertion was measured using Borg scale (Borg, 1973).

After finishing exercise, participants relaxed in a chair and blood samples were taken at 10 and 60 minutes intervals. They were only offered water during this time. After the final blood sample, cannula was removed and participants left the lab.

4.5.2.3 Visit 3

The sequences of events were the same, as on visit 2, except that participants ran at speed eliciting 70% of the predicted maximum heart rate. The duration of the exercise period was shorter than visit 2.

4.5.2.4 Visit 4

Participants repeat the same sequence of events as in visits 2 & 3. During this visit the participant ran at 80% of their predicted maximum heart rate. Again, the duration of the exercise was shorter.

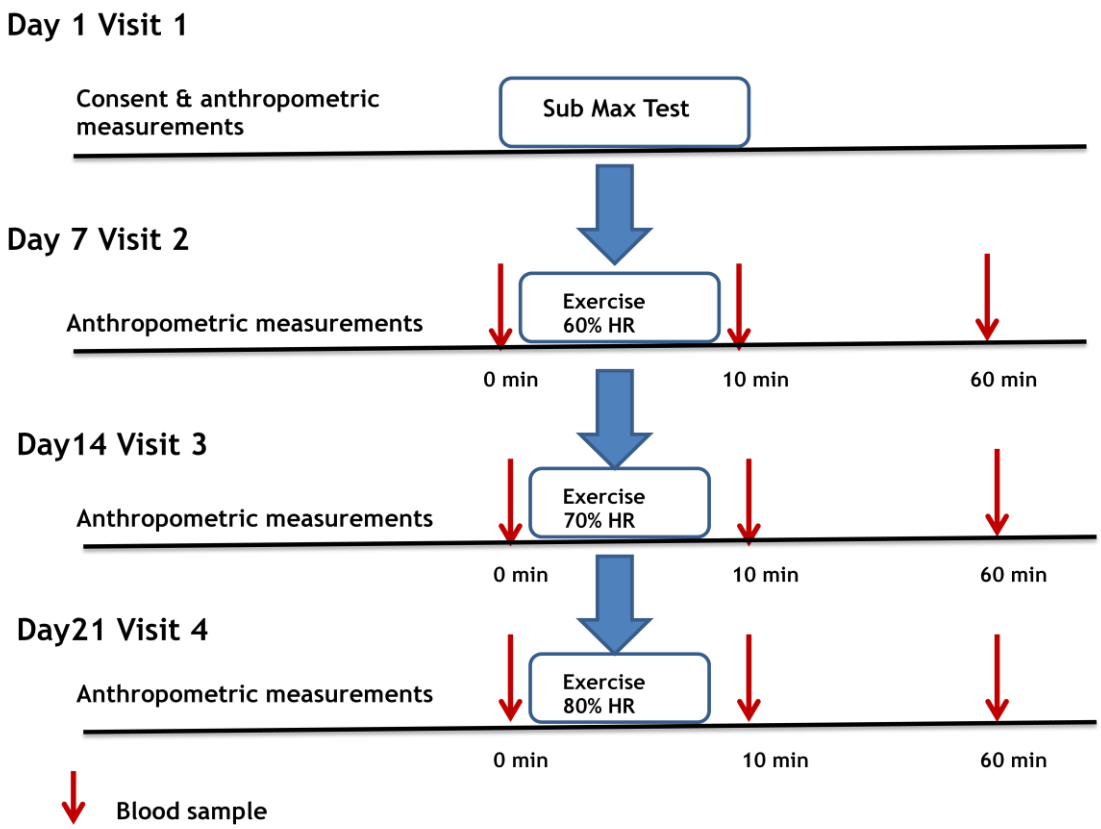


Figure 4-1; Schematic diagram of study protocol

Protocol of the study is schematically presented. Sub maximal oxygen consumption test is done on day 1 to measure the intensities and duration for the main trial. The volunteers then performed 3 exercises at 60%, 70% and 80% of predicted maximum heart rate. The sequence of visit 2, 3 & 4 were randomised. Blood samples were taken before and 10 and 60 minutes after exercise on visit 2, 3 & 4.

4.5.3 Measurements made during exercise

Different features of the response to exercise at each intensity are given in tables 4-2, 4-4, 4-6 and other tables in this chapter. They are:

Speed

The speed of the treadmill belt was measured in km/hr. The intensity of exercise was adjusted by changing the speed.

Duration

This is the duration for which participant actually exercised on the treadmill. It is given in minutes.

Distance

The distance covered during each exercise session was recorded and is given in kilometres. It does not include the distance covered during warm-up and recovery periods of the exercise.

Oxygen consumption (VO_2)

Values for oxygen consumption at rest (BVO_2) and at exercise (VO_2_60, VO_2_70, VO_2_80,) were determined from sub-maximal values, as discussed in section 2.7. The difference in oxygen consumption was calculated by subtracting the oxygen consumption at rest (BVO_2) from oxygen consumption at exercise and is denoted as ΔVO_2. All these values are given in ml/kg/min.

Carbon dioxide production (VCO_2)

This denotes the amount of carbon dioxide produced during the exercise. It is also measured in ml/kg/min.

RER

This is the ratio of carbon dioxide produced and oxygen consumed during one breath. It is called as respiratory exchange ratio and is determined as follows;

$$RER = VCO_2 / VO_2$$

The RER values in table 4-2, 4-4 and 4-6 refers to RER values at respective exercise intensity.

Rate of energy expenditure (EE) measured in kcal/min, rate of carbohydrate oxidation (RCHO) and rate of fat oxidation (RFO)

All these values are derived from RER during the exercise stage and are discussed in section 2.7.4.

Rate of perceived exertion (RPE)

This refers to the exercise intensity as perceived by the participants after performing exercise at different intensities. These were determined by asking participant reaction to exercise intensity on Borg scale (Borg, 1973).

4.5.4 Blood sample collection

A 20G cannula (Versatus W, Leuven, Belgium) was passed before the exercise and pre exercise blood sample was collected into 6ml EDTA tube. The blood samples were put on ice immediately after collection. They were spun at 1000 RPM for 15 minutes, using a Universal 320 centrifuge (Universal 320R, Tuttlingen, Germany), within 30 minutes of collection. The plasma was transferred into 1 ml Eppendorf tube. 3 aliquots of plasma were made and stored at -80°C in an ultra low temperature freezer (New Brunswick Scientific, U725-86, US) for up to one month before being analysed.

4.5.5 Sample analysis

The frozen samples were thawed and analysed for plasma endostatin concentration using Quantikin^{R} ELISA kit of the R&D systems, as discussed in detail in section 2.11.1.

4.5.6 Statistical analyses of the data

Statistical analyses were carried out using SPSS 17 and MINITAB 16 statistical software. The distributions of all the variables were checked using Kolmogorov-Smirinov and Shapiro-Wilk tests of normality. Log transformations of the data were done where appropriate.

Descriptive statistics were carried out for all the variables and are presented as mean ± standard deviation (SD). Independent sample T-tests were applied to find out the differences between the means for male/female and running/walking groups. One-way ANOVA with Bonferroni post hoc analyses with repeated measures was applied to determine the changes over time, before and after exercise.

Box plots and independent line plots were produced for graphical demonstration of the data. Correlations of different variables with plasma endostatin concentration were done using Pearson correlation tests. Simple linear regressions were carried out for uni-variate and multivariate analyses to check any association of change in mean endostatin concentration with different exercise indices.

4.6 Results

4.6.1 Overview of the results

As this study involved 3 different exercise interventions, which were carried out at 3 different intensities i.e. light, moderate and vigorous, as discussed in section 4.3. For convenience we will refer these interventions as follow:

I. Protocol 1 refers to exercise at intensity of 60% of the maximum heart rate
II. Protocol 2 refers to exercise at intensity of 70% of the maximum heart rate
III. Protocol 3 refers to exercise at intensity of 80% of the maximum heart rate

Data were analysed separately for each protocol and where appropriate collective analyses were carried.

4.6.2 Distributions of the anthropometric data of all individuals

The anthropometric data of all the participants were found normally distributed. Blood pressures and heart rates were recorded on all visits. Analysis with repeated measure ANOVA showed no difference between their means. Independent sample T- tests were applied to check the difference in means of different variables for males and females. Unsurprisingly, men were significantly taller ($P < 0.001$) & heavier ($P < 0.001$). In addition, males had higher systolic blood pressure ($P = 0.04$). These data are summarised in table 4-1.

4.6.3 Exercise at (Protocol 1)

4.6.3.1 Distribution of the data for protocol 1

The different features of exercise like speed, gradient, duration, heart rate etc of exercise at 60% of the predicted maximum heart rate were calculated and checked for normality. The differences in means for male and female were determined through independent sample T-test. Table 4-2; summarize the different components of the exercise in protocol 1 for all individuals as well as for males and females.

In summary, all the volunteers walked or ran on the treadmill to deliver low intensity exercise. Their mean speed was 5.01 ± 0.1 km/hr and the mean duration of exercise was 42 ± 15 minutes. The mean distance covered at this exercise was 3.5 ± 0.8 km.

During exercise the mean heart rate of female participants was significantly higher than males (118 ± 5 Vs 111 ± 5, P = 0.1). However, the change in the heart rate between resting and exercise condition was not different in two groups. This is because females had a higher resting heart rate, as shown in table 4-2.

As all females chose gradient protocol, their mean speed was less than males. Females spent significantly more time on the treadmill and walked for longer distance. Oxygen consumption and rate of energy expenditure (kcal/min) was significantly higher in males and so was the rate of carbohydrate and fat oxidation. The exercise was perceived as “very light” by both sexes.

	Total	Male	Female	P-value
Sample size (n)	22	16	6	
BHR_60 (bpm)	77 ± 5	77 ± 6	80 ± 3	0.10
HR_60 (bpm)	112 ± 5	111 ± 5	118 ± 5	0.01
ΔHR_60 (bpm)	36 ± 7	35 ± 8	38 ± 5	0.36
Speed (Km/hr)	5.01 ± 0.1	5.3 ± 0.7	4.2 ± 0.3	0.002
Duration (minutes)	42 ± 15	35 ± 9	61 ± 11	<0.001
Distance (km)	3.5 ± 0.8	3.1 ± 0.6	4.4 ± 0.7	0.001
BVo_2_60 (ml/kg/min)	4 ± 0.4	4.1 ± 0.4	3.7 ± 0.3	0.020
VO_2_60 (ml/kg/min)	16 ± 4	17 ± 4	13 ± 2	0.009
ΔVO_2_60 (ml/kg/min)	12 ± 4	13 ± 4	9 ± 2	0.021
$VO_{2\ max}$ % (ml/kg/min)	39 ± 6	39 ± 7	41 ± 3	0.350
VCO_2 (ml/kg/min)	14 ± 4	15 ± 5	11 ± 2	0.020
RER	0.86 ± 0.04	0.86 ± 0.05	0.88 ± 0.03	0.520
EE (kcal/min)	5.4 ± 2.1	6.2 ± 1.9	3.4 ± 0.7	0.002
RCHO_60 (gm/min)	0.82 ± .5	0.92 ± 0.6	0.53 ± 0.14	0.020
RFO_60 (gm/min)	0.25 ± .1	0.3 ± 0.1	0.15 ± 0.05	0.005
RPE	8 ± 2	8 ± 2	8 ± 1	0.50

Table 4-2; Different components of exercise and their mean difference

Table shows the different components of the exercise for all individuals and the difference between the means for males and females. BHR; Resting heart rate, HR_60; Heart rate during exercise, ΔHR; Difference between resting and exercise heart rate, BVO2; Resting oxygen consumption, Δ VO_2; Difference in oxygen consumption at exercise and rest, EE; Rate of energy expenditure (kcal/min), RCHO; Rate of carbohydrate oxidation, RFO; Rate of fat oxidation, RPE; Rate of perceived exertion

4.6.3.2 Endostatin concentration in Plasma before and after exercise at protocol 1

The concentrations of endostatin in plasma was measured before and 10 and 60 minutes after exercise. The normality tests for the plasma endostatin revealed a non normal distribution for concentration at 10 minutes (P = 0.024 & 0.005) and 60 minutes (P = 0.012 & 0.05) samples as shown by Kolmogorov- Smirinov and Shapiro-Wilk tests. The data were subjected to log transformations (P = 0.15 & 0.12).

Line plots for all individuals on different times points are illustrated in figure 4-2. It is obvious that the individual values lie in close ranges to each other. One participant can be seen with higher basal endostatin concentration.

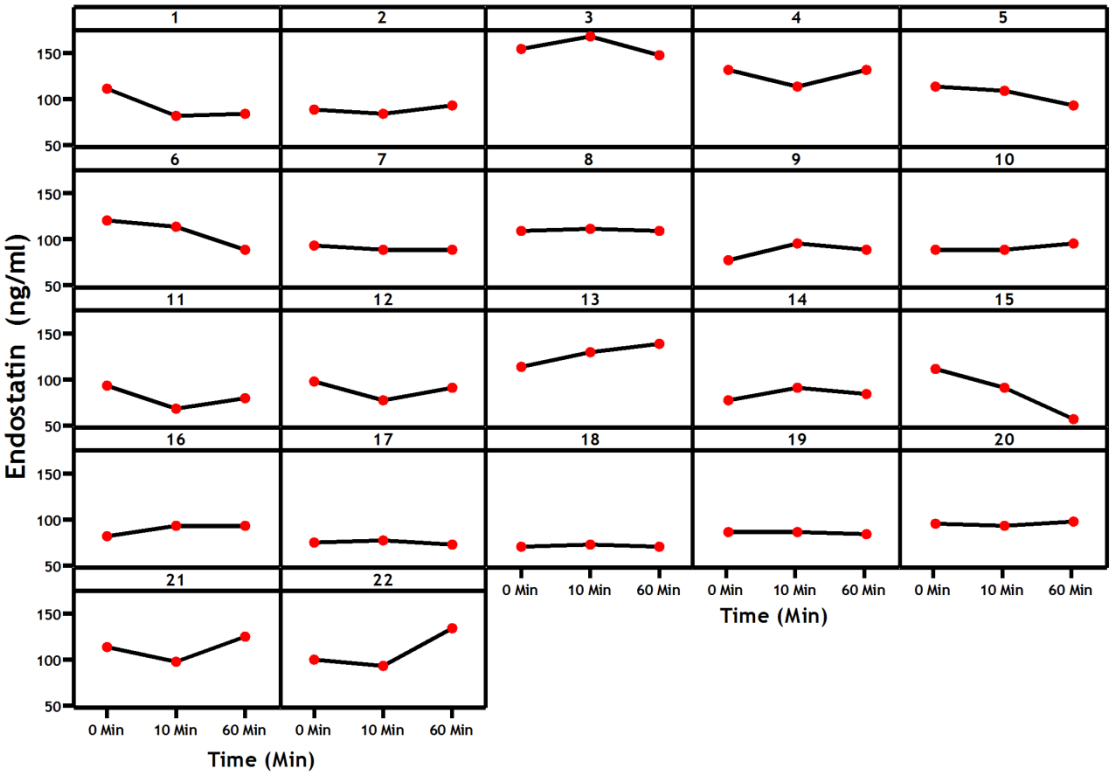


Figure 4-2; line plots for all participants

Individual line plots for all participants showing the plasma endostatin concentrations before (0 Min) and different intervals (10 Min & 60 Min) after exercise at protocol 1. Participant 3 can be seen clearly with high values.

Some of the participants showed a reduction in endostatin concentration after the exercise, while others had a positive change. The line plots for all participants based on the increase or decrease in the endostatin concentration after the exercise were created, as shown in figure 4-3. It was calculated, that 36% of the participants showed a positive change while 64% showed a negative change after exercise.

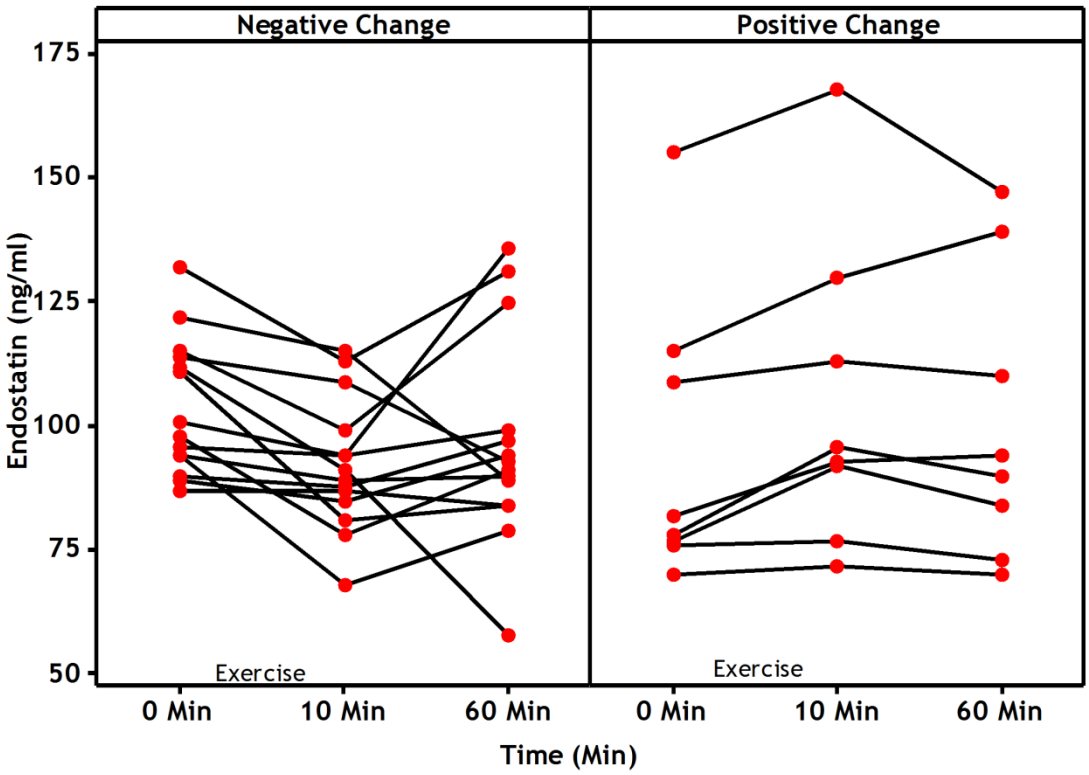


Figure 4-3; Line plots illustrating the endostatin concentration in plasma before exercise (0 Min) and different intervals (10 Min & 60 Min) after exercise at protocol 1.

It is obvious that 14 out of 22 volunteers showed immediate decrease in basal concentration after the exercise.

The basal mean endostatin concentration in plasma was determined as 101 ± 20 ng/ml. ANOVA with repeated measures revealed, no significant change in endostatin concentrations after exercise (P = 0.5). The mean endostatin concentrations were 97 ± 22 ng/ml and 98 ± 23 ng/ml, 10 and 60 minutes after exercise respectively. This is equal to 4% and 3% decrease in mean Endostatin concentration. These changes were not significant (P = 0.5 & 0.8), as shown in figure 4-4.

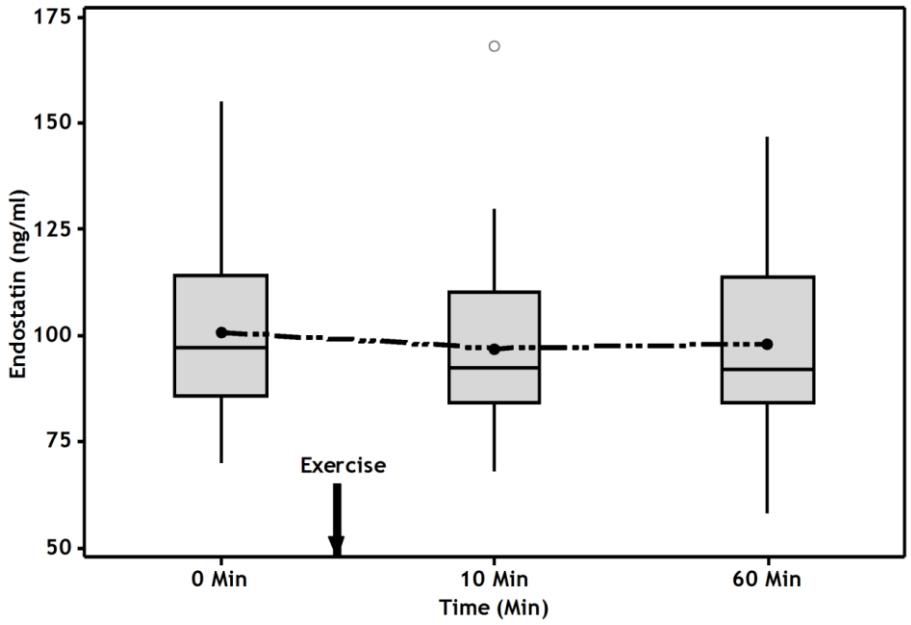


Figure 4-4; Endostatin concentrations before and different intervals after exercise at protocol 1

Box plots illustrating the mean, median, quartile range and range of endostatin concentration in plasma before and 10 and 60 minutes after exercise at protocol 1. The clear circle represents outlier. ANOVA with repeated measures show no significant changes in mean endostatin concentrations at any time point (P = 0.5).

4.6.3.3 Mean endostatin concentrations in males and females

Figure 4-5 shows interaction line plots for male and female participants. It can be seen that the effect of exercise on endostatin concentrations is mixed in both groups.

The basal mean endostatin concentrations were 105 ± 21 ng/ml and 90 ± 13 ng/ml for males and females respectively. The mean endostatin concentration for males, 10 minutes after exercise was 99 ± 25 ng/ml and 60 minutes after exercise was 98 ± 26 ng/ml. The mean endostatin concentration for females,10 minutes after exercise was 90 ± 6 ng/ml and 60 minutes after exercise was 97 ± 14 ng/ml. Independent sample T-tests was applied to investigate the difference in mean endostatin concentration. No significant difference between the means in males and females at any time point was observed.

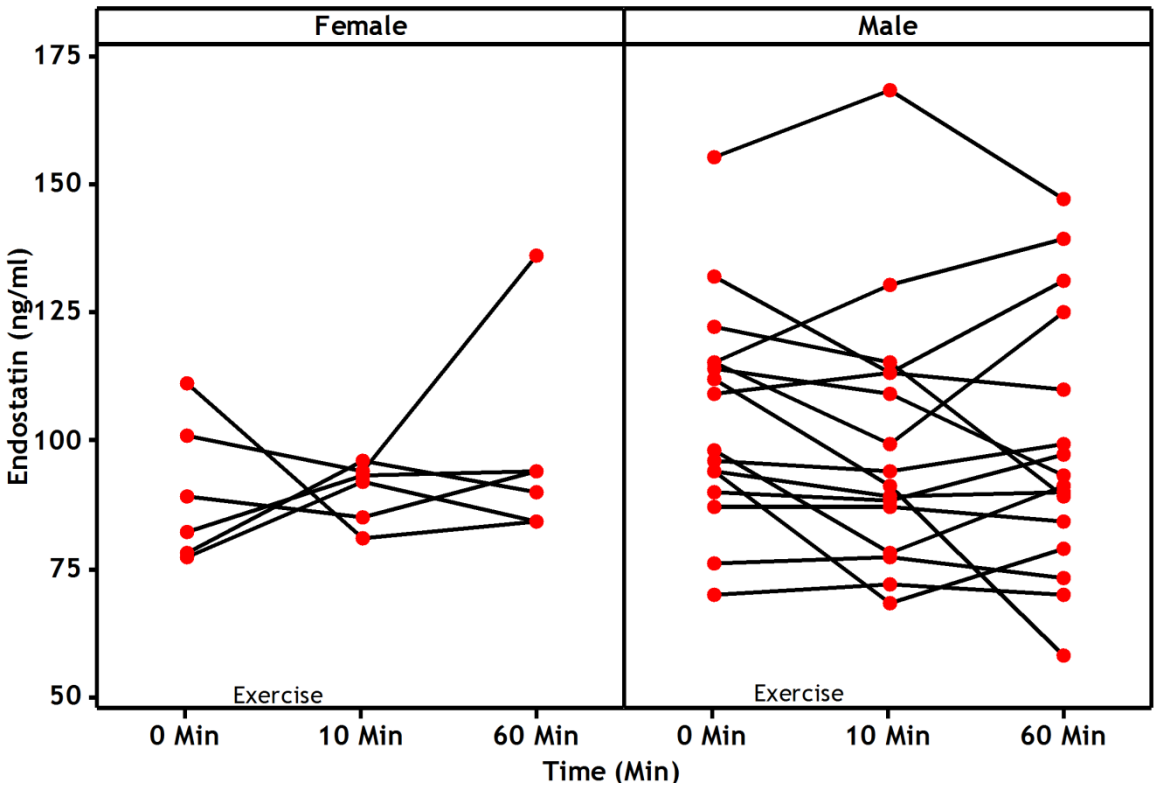


Figure 4-5; Interaction line plots showing the individual values for male and female participants before and after exercise at protocol 1

Repeated measure ANOVA revealed no significant changes in mean endostatin concentration before and after the exercise for males (P = 0.2) and females (P = 0.5). The decrease in endostatin for the male group was 6% and 7% at 10 minutes and 60 minutes interval. No change in mean endostatin concentration for females was observed at 10 minutes interval. However, 7% increase at 60 minutes interval was observed, as shown in figure 4-6.

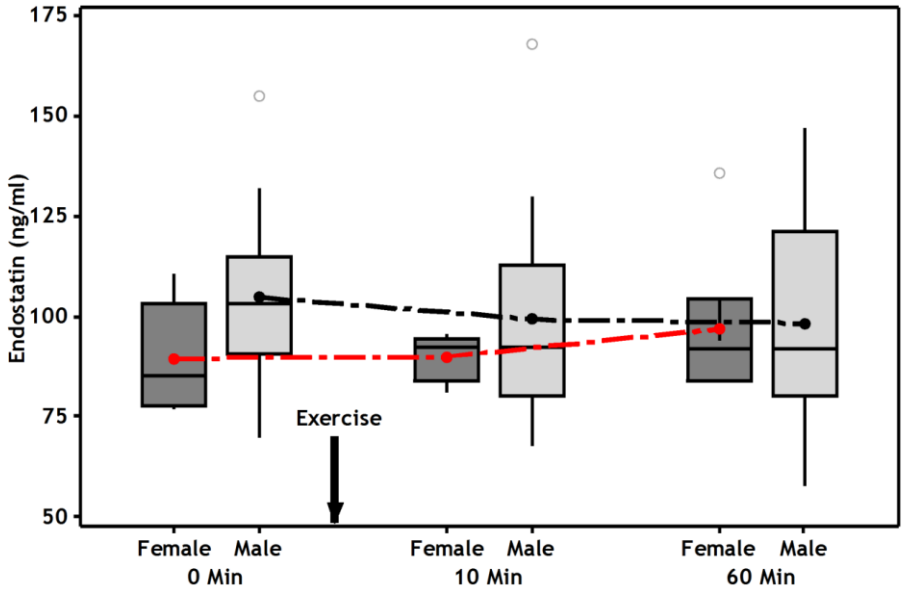


Figure 4-6; Endostatin concentration in males and females before and after exercise at protocol 1

Box plots presenting the mean, median, inter quartile range and range of endostatin for male and female participants before and at different intervals after exercise at protocol 1. No significant differences were observed at any time point between the two groups. Clear circles represent outliers.

4.6.3.4 Mean endostatin concentrations in running and walking group

As described in section 4.5.2.1, the volunteers followed walking or running protocol as determined by their fitness levels. Interaction line graphs were created which illustrate the individual values for participants in each group and shown in figure 4-7. No clear difference, in term of influence of exercise on endostatin concentration is visible and both groups show a mixed effect of increase and decrease in endostatin concentrations after exercise.

The mean endostatin concentration at rest was 100 ± 24 ng/ml for walking group and 101 ± 18 ng/ml for running group. Independent sample T-test revealed no difference between these values.

ANOVA with repeated measure showed no significant change in any group.

The individual and group data are shown in figures 4-7 and 4-8. One of the volunteers was a clear outlier in the walking group. No significant difference was observed even after removal of outlier's data from analysis.

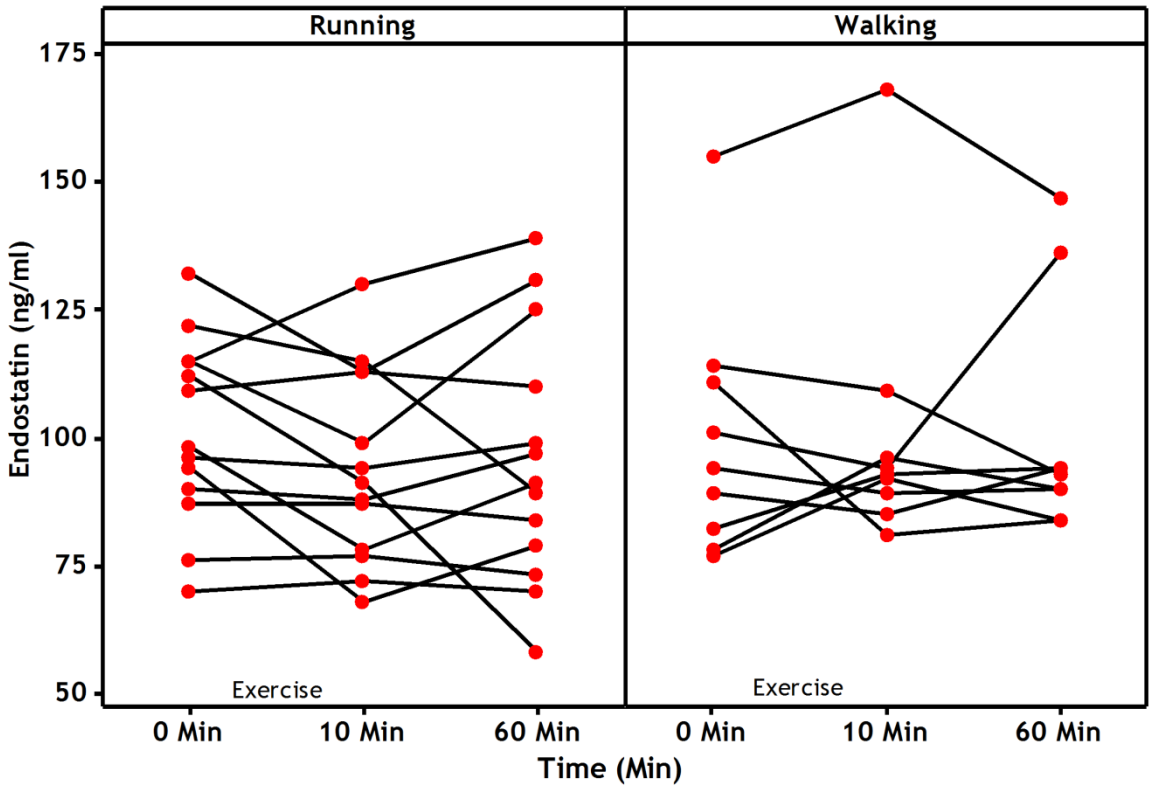


Figure 4-7; Interaction line plots for participants in walking and running group shows the individual values for all participants. Mix effect of exercise in both groups can be seen.

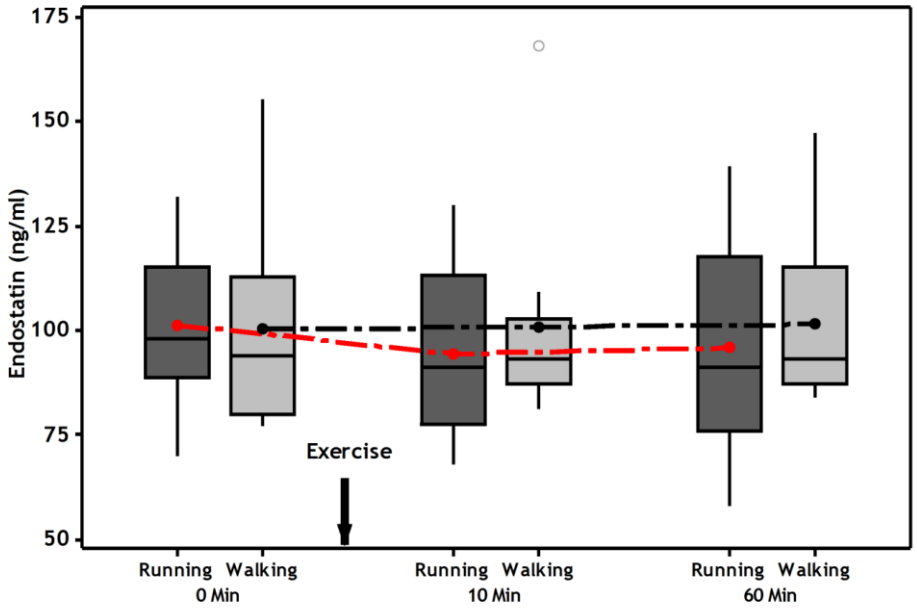


Figure 4-8; Endostatin concentrations in running and walking groups before and after exercise at protocol 1

Box plots presenting the mean, median, inter quartile range and range of endostatin for participants in running and walking groups, before and at different intervals after exercise at protocol 1. No significant differences were observed at any time point between the two groups. Clear circles represent outliers.

4.6.4 Exercise at 70% heart rate (Protocol 2)

4.6.4.1 Distribution of the data

The normalities of data generated from different exercise indices in protocol 2 were checked using Kolmogorov- Smirinov and Shapiro-Wilk tests. Where not normal, it was subjected to log transformation. Descriptive statistics were done for all individuals and difference between the means for males and females were determined using independent T-test, as shown in the table 4-3.

In this protocol the volunteers exercised at a moderate intensity. The mean speed on the treadmill was 5.8 ± 1.2 km/hr. Female volunteers exercised only with the gradient protocol, so their speed was less than males. The mean duration for the exercise was 30 ± 11 minutes during which the volunteers covered a distance of 2.7 ± 0.6 km. The exercise at protocol 2 was carried out at 55% ± 5% (mean ± SD) of the predicted maximal oxygen consumption ($\dot{V}O_{2\ max}$).

Significant differences in means for some variables between male and female participants were observed, as shown by the P values in table 4-3. There was no difference in resting heart rate. Female volunteers ran slower than males but had significantly higher heart rates during exercise. The female volunteers also had lower rates of oxygen consumption and energy expenditures per minute. As a result, females exercised for longer durations than males. Males had higher rates of carbohydrate and fat oxidation. The exercise was perceived as 'light' by both sexes.

	Total	Male	Female	P-value
Sample size (n)	22	16	6	
BHR_70 (bpm)	78 ± 5	78 ± 5	78 ± 5	0.8
HR_70 (bpm)	133 ± 7	131 ± 7	138 ± 5	0.04
ΔHR_70 (bpm)	55 ± 8	53 ± 7	60 ± 7	0.05
Speed (Km/hr)	5.8 ± 1.2	6.3 ± 1	4.2 ± 0.3	<0.001
Duration (minutes)	30 ± 11	24 ± 5	45 ± 8	<0.001
Distance (km)	2.7 ± 0.6	2.5 ± 0.6	3.2 ± 0.6	0.005
$B\dot{V}O_2$_70 (ml/kg/min)	3.7 ± 0.3	3.7 ± 0.3	3.7 ± 0.4	0.6
$\dot{V}O_2$_70 (ml/kg/min)	22 ± 5	24 ± 5	18 ± 2	0.001
$\Delta\dot{V}O_2$_70 (ml/kg/min)	18 ± 5	20 ± 5	14 ± 3	0.003
$\dot{V}O_{2\ max}$ % (ml/kg/min)	55 ± 5	54 ± 5	56 ± 2	0.34
$\dot{V}CO_2$ (ml/kg/min)	20 ± 5	21 ± 5	16 ± 2	0.016
RER	0.9 ± 0.04	0.9 ± 0.03	0.89 ± 0.04	0.6
EE (kcal/min)	7.6 ± 2.7	8.7 ± 2.2	4.6 ± 1	<0.001
RCHO_70 (gm/min)	1.4 ± 0.6	1.6 ± 0.6	0.8 ± 0.3	0.001
RFO_70 (gm/min)	0.27 ± .1	0.3 ± 0.1	0.18 ± 0.29	0.027
RPE	11 ± 2	10 ± 2	11 ± 2	0.4

Table 4-3; Different exercise indices (protocol 2) and their mean differences between males and females

Table shows the different exercise indices for all individuals and for males and females at protocol 2.BHR; Resting heart rate, HR_70; Heart rate during exercise, ΔHR; Difference between resting and exercise heart rate, $B\dot{V}O_2$; Resting oxygen consumption, $\Delta\dot{V}O_2$; Difference in oxygen consumption at exercise and rest, EE; Rate of energy expenditure (kcal/min), RCHO; Rate of carbohydrate oxidation, RFO; Rate of fat oxidation, RPE; Rate of perceived exertion.

4.6.4.2 Endostatin concentrations in Plasma before and after exercise at protocol 2

The distribution of endostatin concentrations at rest and different time intervals post exercise were examined using Kolmogorov- Smirinov and Shapiro-Wilk tests of normality. The basal and 60 minutes endostatin data were not normally distributed (P = 0.007 &0.032). Therefore, all endostatin samples were subjected to log transformations to obtain normal distributions (P = 0.28 & 0.12).

The concentrations of endostatin for each volunteer before and after exercise are illustrated in figure 4-9. The basal endostatin concentration for all participants can be seen in close ranges except 2. (Panel 19 and 22 shows higher basal endostatin concentration of 132 ng/ml and 155 ng/ml respectively). After exercise, some of the volunteers had an increase in their endostatin concentrations while others had decrease. It was plotted in figure 4-10, which illustrates that 77% of the individuals exhibit a positive change after exercise and 23% exhibit a negative change. However, it can be seen that at 60 minutes interval, the concentration raises for all participants.

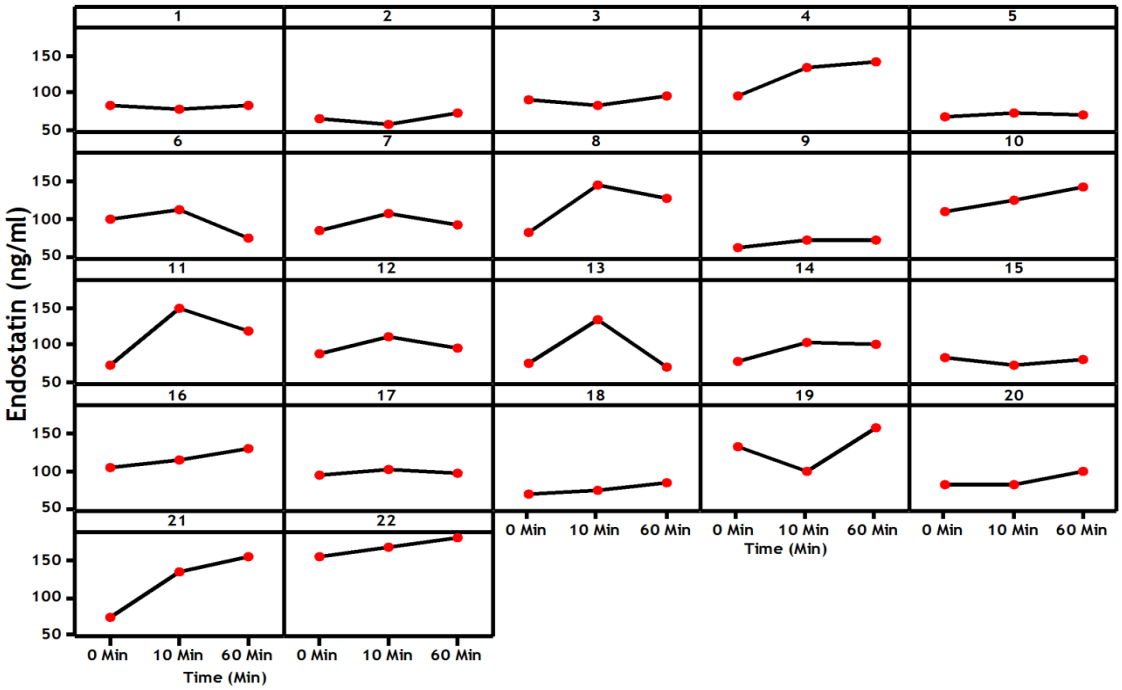


Figure 4-9; Individual line plots for all the volunteers showing the plasma endostatin concentrations before exercise (0 Min), and at different intervals (10 Min & 60 Min) after exercise at protocol 2.

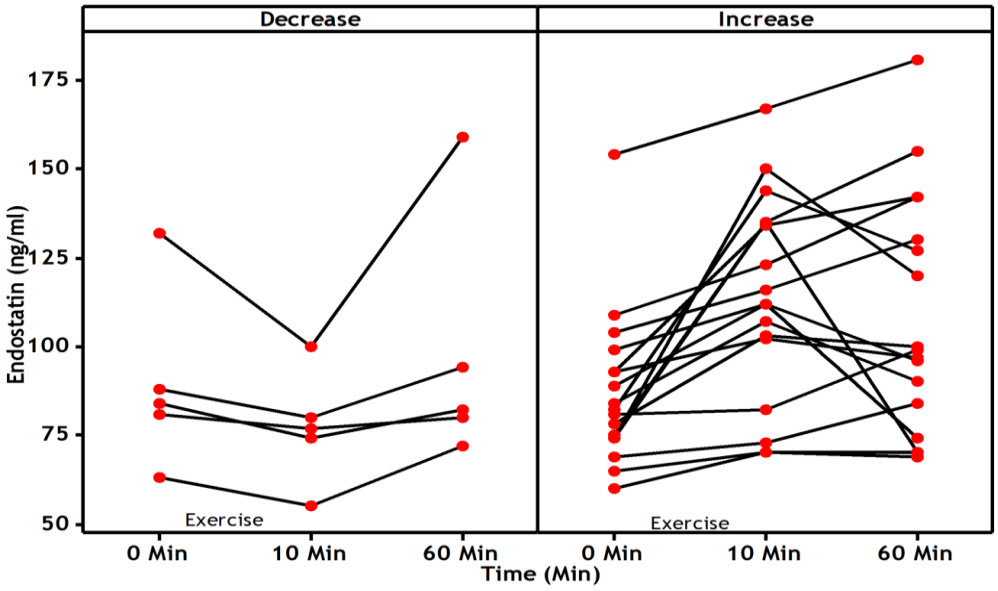


Figure 4-10; Line plots illustrating the endostatin concentration in plasma before exercise (0 Min) and at different intervals (10 Min & 60 Min) after exercise at protocol 2.

17 out of 22 participants exhibited immediate increase as shown by the right panel. On the left panel, the initial decrease followed by an increase can be seen.

Mean endostatin concentration in plasma at rest was 88 ± 22 ng/ml. Mean endostatin concentration was 105 ± 30 ng/ml at 10 minutes interval and 106 ± 33ng/ml at 60 minutes interval. One-way ANOVA with repeated measures showed a significant difference in the mean endostatin concentration at rest and subsequent time points (P = 0.002). Bonferroni post hoc analyses revealed significant rise in plasma endostatin concentration at both time points after exercise (P = 0.02 & 0.004). This increase was 18% and 19% respectively, as shown in figure 4-11.

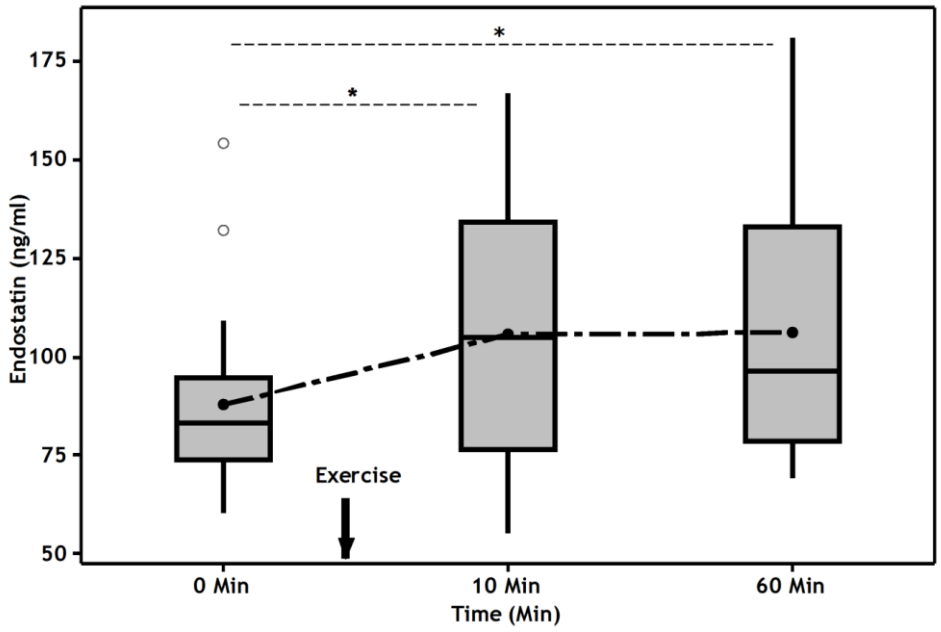


Figure 4-11; Endostatin concentration before and different intervals after exercise at protocol 2

Box plots illustrating the mean, median, quartile range and range of endostatin for all participants before and at 10 and 60 minutes after exercise at protocol 2. Two participants presented with high endostatin concentrations in plasma at rest and are shown as outlier by clear circles. The mean endostatin concentrations increase by 18% (P = 0.02) and 19% (P = 0.004) respectively, at 10 and 60 minutes after exercise and shown by the dotted line with *.

4.6.4.3 Endostatin concentration in males and females

Plots illustrating the individual values for male and female volunteers are shown in figure 4-12. The mixed effect of exercise in both groups can be seen.

Independent sample T-test showed no difference in means for both groups at any time point. The mean resting endostatin concentrations were 87 ± 43 ng/ml for males and 90 ± 35 ng/ml for females. Two participants with unusually high resting endostatin concentration of 155 ng/ml and 132 ng/ml were found, one in each group. There was no obvious explanation for these observations. Omitting them from the analysis did not change the difference between the two groups.

The mean endostatin concentration for males, 10 minutes after exercise was 108 ± 29 ng/ml and 60 minutes after exercise, it was 106 ± 30 ng/ml. The mean endostatin concentration for females 10 minutes after exercise was 98 ± 40 ng/ml and 60 minutes after exercise it was 106 ± 43 ng/ml.

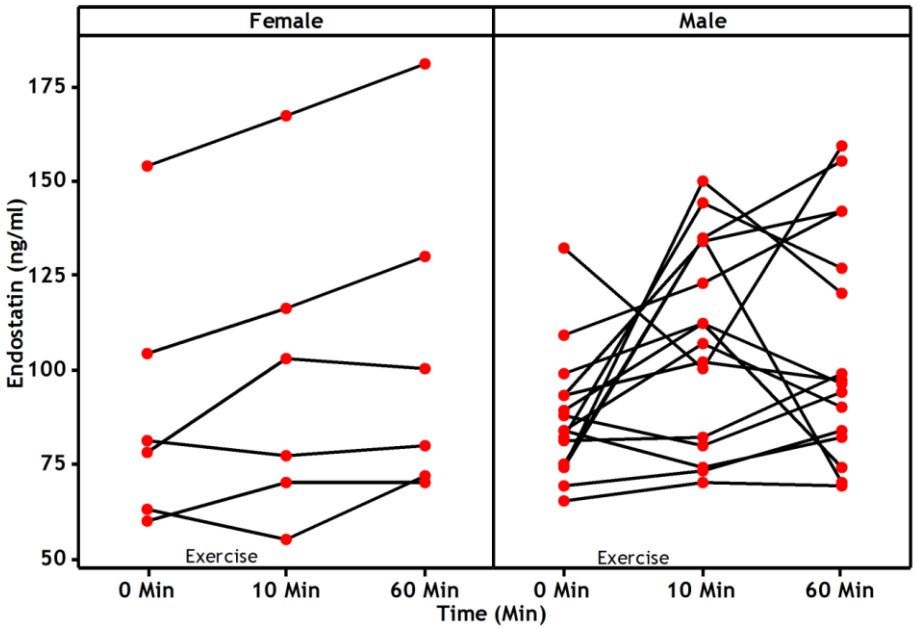


Figure 4-12; Interaction line plots showing the individual values for male and female participants before and at 10 and 60 minutes after exercise at protocol 2.

One-way ANOVA with repeated measures with Bonferroni corrections were carried out to investigate the significance of the differences between the endostatin concentrations at the different time points. In males, a significant increase of 24% (P = 0.04) was observed 10 minutes after exercise. After 60 minutes the rise in mean endostatin concentration was 22%. This was also statistically significant (P = 0.03). In females, the endostatin concentration increased by 9% (P = 0.2) at 10 minutes and by 17% (P = 0.05) at 60 minutes interval after exercise. These data are illustrated in figure 4-13.

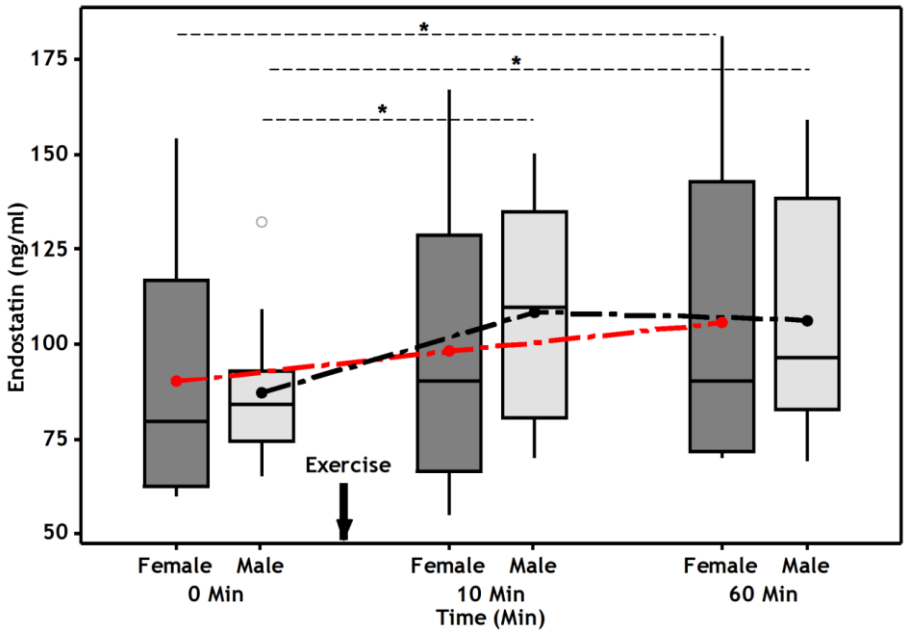


Figure 4-13; Endostatin concentration in males and females before and after exercise at protocol 2

Box plots presenting the mean, median, inter quartile range and range of endostatin concentration for male and female participants before and different intervals after exercise at protocol 2. Dotted line with * represent the significant difference at different time points after exercise with basal endostatin concentration.

4.6.4.4 Endostatin concentration in running and walking group

Independent sample T-test revealed no significant difference between the mean endostatin concentrations of the running and walking group at any time point. Interaction line plot for all individuals in different groups were plotted, as shown in figure 4-14. Resting mean endostatin concentration was 89 ± 17 ng/ml for running cohort and 86 ± 29 ng/ml for walking cohort.

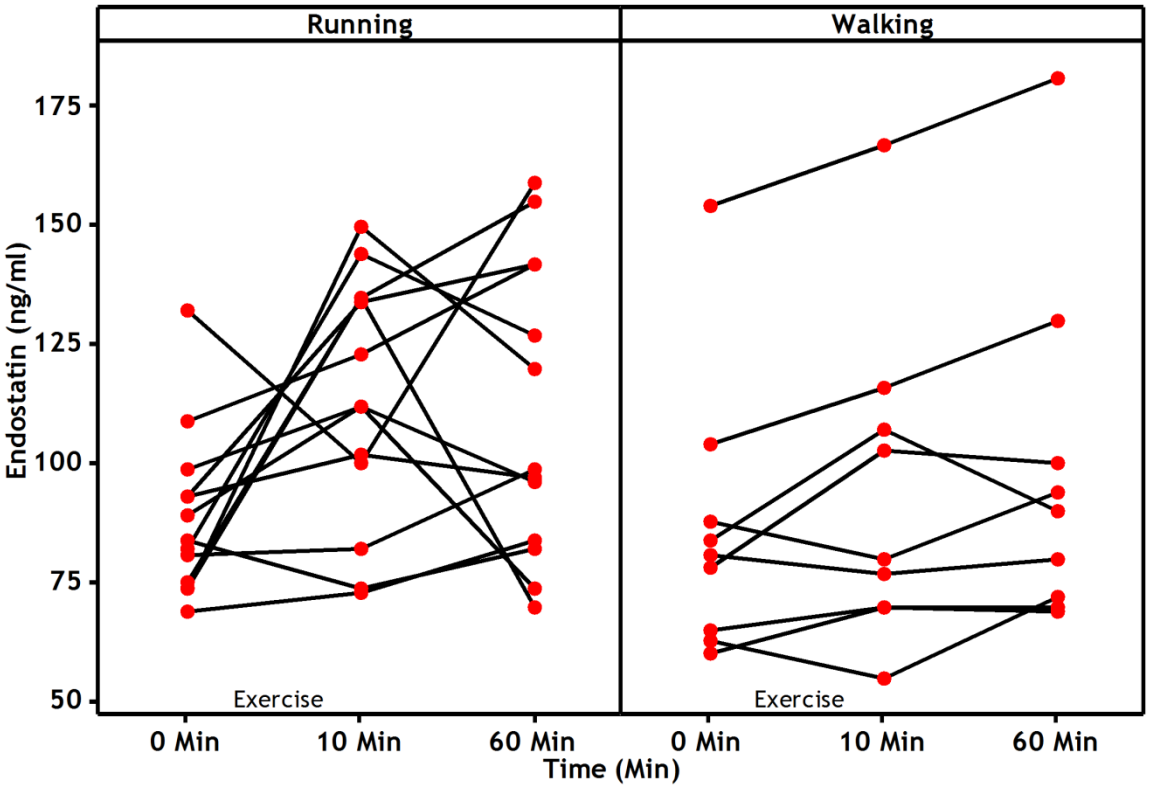


Figure 4-14; Interaction line plots for participants in walking and running group showing the individual values for all participants before exercise (0 Min) and at different intervals (10 Min & 60 Min) after exercise at protocol 2.

ANOVA with repeated measures were carried out for both the groups. The exercise increased the plasma endostatin concentration significantly in both groups (P = 0.014 & 0.017) but the increase in the running group was more pronounced. In the running group an increase of 28% (P = 0.05) at 10 minutes interval and 26% (P = 0.04) at 60 minutes interval was observed. However, in the walking group, the rise in endostatin concentration was 9% (P = 0.31) at 10 minutes interval and 14% (P = 0.02) after exercise, as represented in figure 4-15.

Walking group contain all females, therefore the pattern of change in endostatin in this group is almost similar to female group.

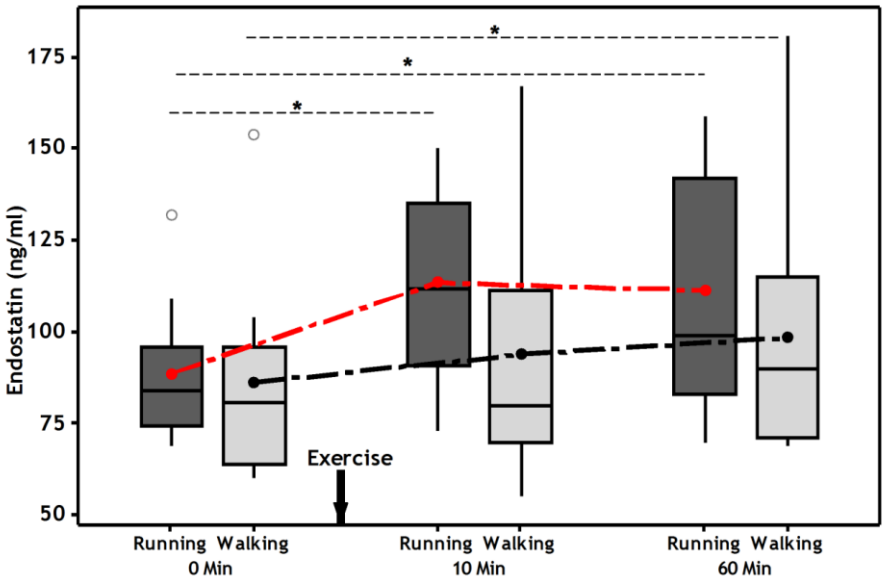


Figure 4-15; Endostatin concentration in running and walking group before and after exercise at protocol 2

Box plots showing the mean, median, inter quartile range and range of endostatin concentration for participants in the running and walking groups. Data are shown for pre exercise and different time intervals after exercise at protocol 2. Clear circles represent outlier. A more significant change was observed in the running cohort than walking. Dotted line with (*) represent the significant difference.

4.6.5 Exercise at 80% heart rate (Protocol 3)

4.6.5.1 Distribution of the data for protocol 3

Different variables of the exercise were calculated and checked for normalities. The differences in means for the male and female volunteers were determined using independent T-test. Table 4-4 summarize the different exercise indices in protocol 3 for pooled data and for male and female groups.

	Total	Male	Female	P-value
Sample size (n)	22	16	6	
BHR_80 (bpm)	78± 5	77 ± 5	80 ± 5	0.3
HR_80 (bpm)	153 ± 6	152 ± 7	156 ± 4	0.2
ΔHR_80 (bpm)	75 ± 8	75 ± 9	77 ± 8	0.6
Speed (Km/hr)	6.4 ± 1.9	7.2 ± 1.6	4.2 ± 0.3	<0.001
Duration (minutes)	23 ± 8	19 ± 4	34 ± 7	0.002
Distance (km)	2.4 ± 0.4	2.3 ± 0.4	2.6 ± 0.5	0.2
BVO_2_80 (ml/kg/min)	3.7 ± 0.4	3.6 ± 0.3	3.8 ± 0.5	0.3
VO_2_80 (ml/kg/min)	28 ± 6	30 ± 6	22 ± 3	0.001
ΔVO_2_80 (ml/kg/min)	25 ± 7	27 ± 6	18 ± 4	0.004
$VO_{2\ max}$ % (ml/kg/min)	69 ± 3	69 ± 4	71 ± 2	0.3
RER	0.92 ± 0.04	0.92 ± 0.03	0.91 ± 0.05	0.3
EE (kcal/min)	9.8 ± 3.3	11.2 ± 2.5	5.9 ± 1.3	<0.001
RCHO_80 (gm/min)	1.98 ± 0.8	2.25 ± 0.6	1.09 ± 0.41	<0.001
RFO_80 (gm/min)	0.27 ± .1	0.3 ± 0.1	0.15 ± 0.05	0.2
RPE	14 ± 2	13 ± 2	15 ± 2	0.2

Table 4-4; Different components of exercise and their mean difference

Table shows the different components of the exercise for all individuals and the difference between the means for males and females. BHR; Resting heart rate, HR; Heart rate, ΔHR; Difference between resting and exercise heart rate, BVO_2; Resting oxygen consumption, Δ VO_2; Difference in oxygen consumption at exercise and rest, EE; Rate of energy expenditure (kcal/min), RCHO; Rate of carbohydrate oxidation, RFO; Rate of fat oxidation, RPE; Rate of perceived exertion.

During this protocol, the volunteers exercised at a higher intensity on the treadmill. The mean speed was 6.5 ± 1.9 km/hr. It should be noted that female volunteers only exercised with the gradient protocol, so their speed was less but the gradient was steeper. The mean duration of exercise was 23 ± 8 minutes during which the volunteers covered a distance of 2.4 ± 0.4 km. This exercise was performed at 69% ± 3 of their predicted maximum oxygen consumption ($\dot{V}O_{2\,max}$).

Male participants exercised at significantly higher speed with high rates of energy expenditure for shorter periods. Males also showed a significantly higher rate of substrate metabolism. Respiratory exchange ratio for both the sexes was almost equal. Females perceived the exercise as 'hard' while males perceived it as 'somewhat hard'.

4.6.5.2 Endostatin concentration in Plasma before and after exercise at protocol 3

The data for endostatin concentration at different time points were found to be normally distributed (P = 0.2, 0.2 & 0.1).

The endostatin concentrations at different time intervals for all participants are plotted in figure 4-16. It can be seen that all of the volunteers exhibited a positive change after exercise at this intensity. One Volunteer (Panel 3) can be seen with higher basal endostatin concentration. The same volunteer showed a higher basal endostatin concentration on one previous occasion as well. The reasons for high baseline values are not completely clear.

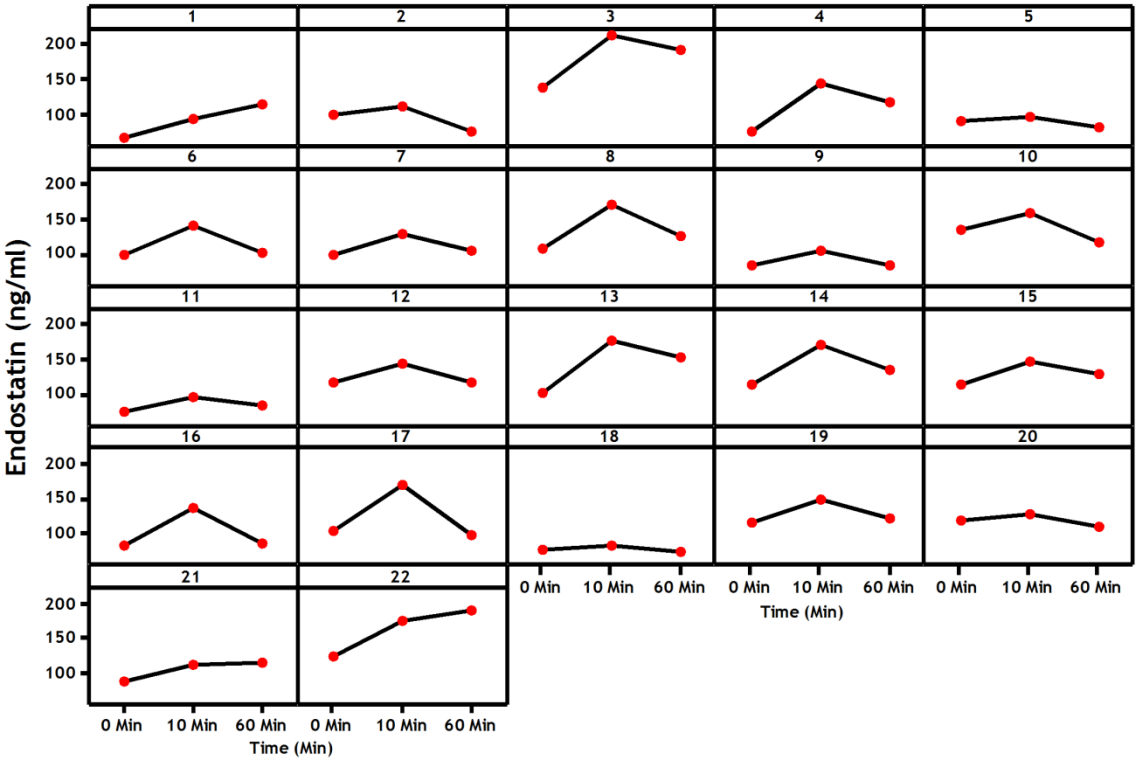


Figure 4-16; Individual line plots for all participants showing the plasma endostatin concentrations before exercise (0 Min), and at different intervals (10 Min & 60 Min) after exercise at protocol 3. Participant 3 can be seen clearly with high baseline concentration.

The mean endostatin concentration at rest was 100 ± 20 ng/ml. One-way ANOVA with repeated measures showed a significant increase in mean endostatin concentration after exercise (P <0.001). The mean endostatin concentrations at 10 and 60 minutes intervals were 138 ± 34 ng/ml and 114 ± 34 ng/m respectively. These increase in means are equal to 38% (P <0.001) and 14% (P= 0.021). Box plots of the pooled data are shown in figure 4-17.

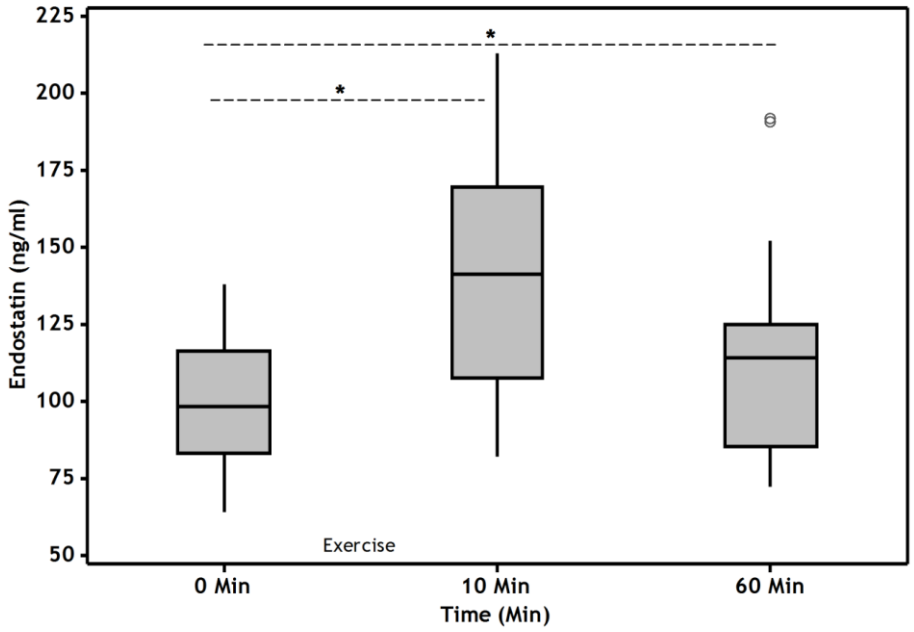


Figure 4-17; Endostatin concentration before and at different intervals after exercise at protocol 3

Box plots illustrating median, quartile range and range of endostatin concentration in plasma before and at 10 and 60 minutes after exercise at protocol 3. Significant changes in endostatin concentrations were observed at 10 minutes (P<0.001) and 60 minutes (P = 0.021). Clear circles represent outlier.

4.6.5.3 Endostatin concentration in males and females

Individual values at different time intervals were plotted using interactive line plots and shown in figure 4-18. Mean endostatin concentrations at baseline were 102 ± 20 ng/ml for males and 94 ± 22 ng/ml for females. The mean endostatin concentration for males raised to 140 ± 34 ng/ml (P < 0.001) at 10 minutes and was 114 ± 28 ng/ml (P = 0.06) at 60 minutes after exercise. The mean endostatin concentration for females, at 10 minutes after exercise was 131 ± 35 ng/ml (P = 0.018) and at 60 minutes was 113 ± 44 ng/ml (P = 0.22).

No significant differences were observed between the means of both groups at any time point. Exercise changed the endostatin concentration significantly in both groups, as shown in the figure 4-19.

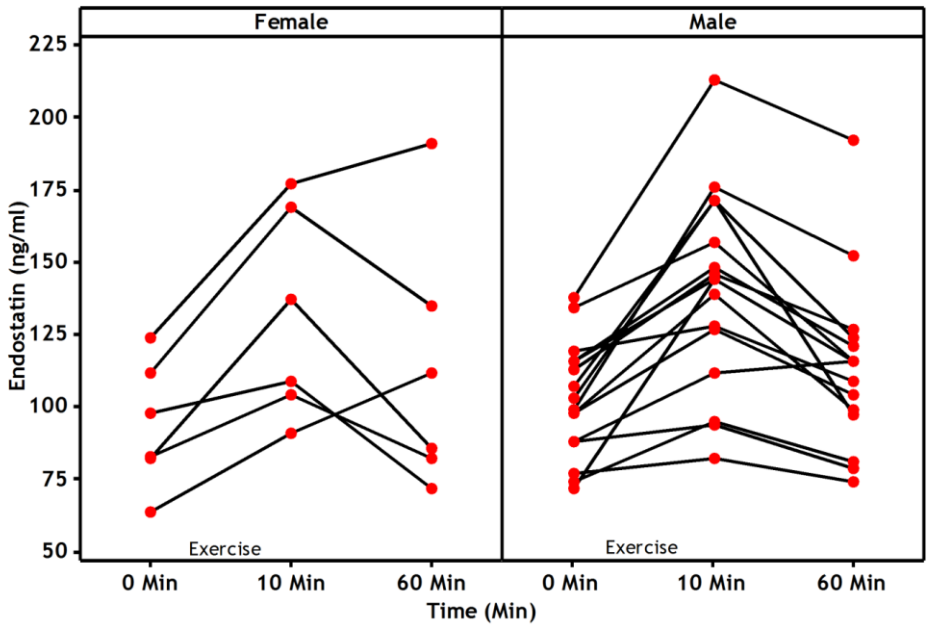


Figure 4-18; Interaction line plots showing the individual values for male and female participants before and at 10 and 60 minutes after exercise at protocol 3. All participants showed a positive change after exercise.

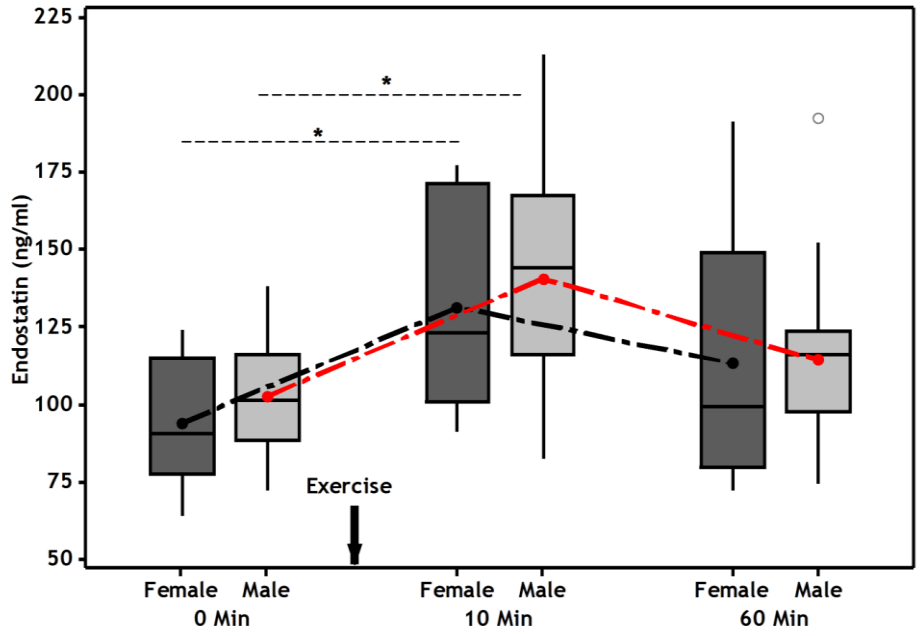


Figure 4-19; Endostatin concentrations in males and females before and after exercise at protocol3

Box plots presenting the mean, median, Inter quartile range and range of endostatin concentration for male and female participants before and different intervals after exercise at protocol3. Significant difference in endostatin concentration can be seen at 10 minutes interval for both groups. Clear circle represents outlier.

4.6.5.4 Endostatin concentration in running and walking group

The data for the participants in the walking and running group was also analysed. Interaction line plots showing the individual values for all participants can be seen, in figure 4-20. Independent sample t-test reported no difference in the mean endostatin concentrations in both groups at different time points. Mean basal endostatin concentration for running group was 101 ± 19 ng/ml and for walking group was 99 ± 23 ng/ml.

The mean endostatin concentration for running group raised to 139 ± 29 ng/ml at 10 minutes after exercise (P < 0.001) and dropped back to 111 ± 20 ng/ml at 60 minutes interval (P = 0.3 with basal and 0.001 with 10 minutes sample). The mean endostatin concentration for walking group, 10 minutes after exercise was 136 ± 42 ng/ml and 117 ± 46 ng/ml at 60 minutes (P <0.001 and 0.3 respectively), as shown in figure 4-21.

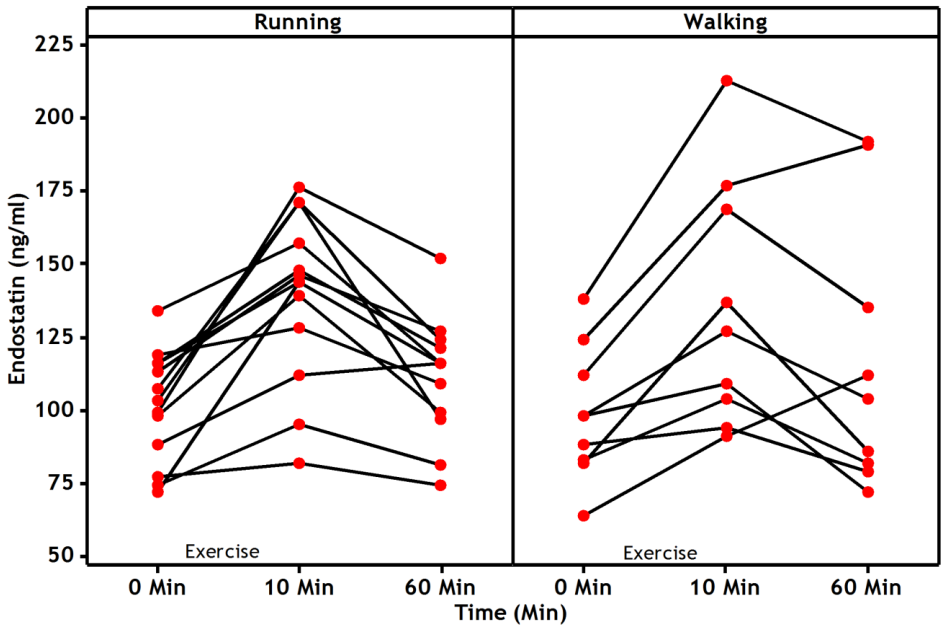


Figure 4-20; Interaction line plots for participants in walking and running group showing the individual values for all participants in each group before and after exercise at protocol 3.

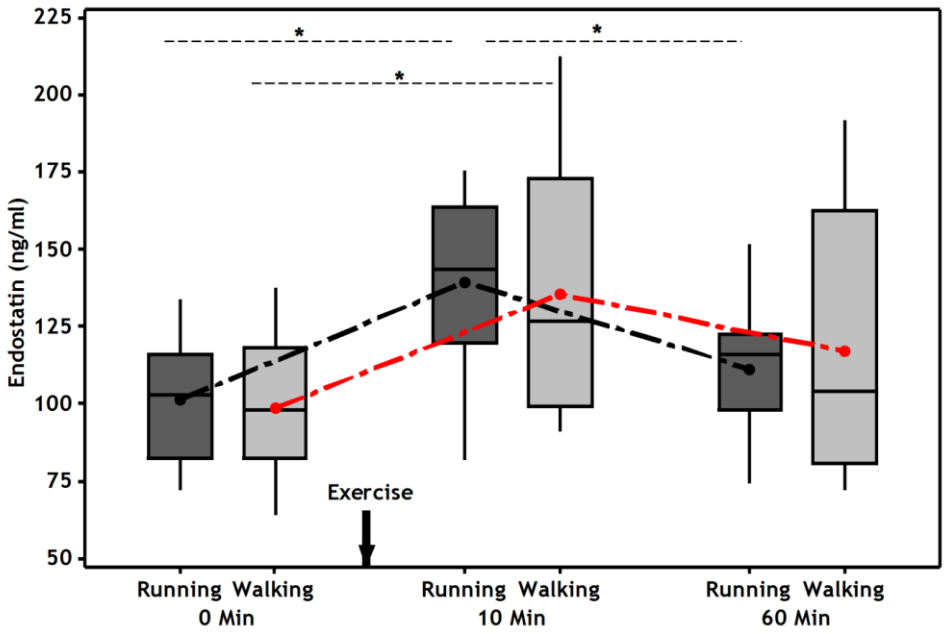


Figure 4-21; Endostatin concentration in walking and running group before and after exercise at protocol 3

Box plots presenting the mean, median, inter quartile range and range of endostatin for participants in running and walking group, before and different intervals after exercise in protocol 3. Significant increase is observed immediately in both groups (P <0.001 for walking and running group, respectively) at 10 minutes interval which decreases in both groups at 60 minutes interval (P= 0.001 & 0.13 between 10 and 60 minutes for running and walking respectively).

4.6.6 Endostatin concentration and BMI at different intensities

Within the volunteers recruited two groups could be identified on the basis of their BMI, 11 overweight and 11 normal. Normal individuals had a BMI less than 25 kg/m^2 and overweight individuals had a BMI of more than 25 kg/m^2.

The data from these groups were analysed to compare differences in endostatin concentration after exercise at all intensities. At protocol 1, no difference between basal mean endostatin concentrations for both groups were observed (102 ± 25 Vs 99 ± 16 ng/ml for normal vs. overweight). Both groups showed an insignificant decrease in mean endostatin concentration (P = 0.8 & 0.2, for normal and overweight respectively).

At protocol 2, again no difference between the basal endostatin concentration for both the groups was observed (P = 0.3). Significant increase in mean

endostatin concentration was observed for both groups after exercise at protocol 2 (ANOVA P = 0.03 & 0.05 for normal and overweight)

At protocol 3, there was no difference in basal endostatin concentration for both the groups. The exercise at protocol 3 significantly increased the endostatin concentration in plasma for both groups (ANOVA P <0.001 for both groups).

In conclusion, the effects of exercise on endostatin concentrations in both groups were the same and not much different from the analyses done already.

4.6.7 Correlations of changes in endostatin concentrations and the different parameters of the exercise at all intensities

As the different exercise intensities changed the endostatin concentration in plasma differently, it was thought that some features of these exercises might influence the changes directly and more than others. For this reason, the difference in the endostatin concentration, (ΔES1) baseline and 10 minutes after the exercise and (ΔES2) baseline and 60 minutes were calculated for all the 3 exercise intensities. Further statistical analyses were carried out to examine the Pearson correlation between features of the exercise and ΔES1 & ΔES2 at all intensities. The results are shown in the table 4-5.

It is interesting that no particular pattern of correlation between any feature of exercise and the change in endostatin concentration was observed. Some of correlations were of statistical significance at one time point but not at others. For example; significant positive correlation was found between respiratory exchange ratio and the ΔES1 at protocol 1 exercise but not at other time points. Similarly, significant negative correlations were found between the rate of fat oxidation and ΔES1 & ΔES2 at protocol 1 but not at protocol 2 or 3. It might be possible that these correlations are by chance and not true.

However, other components of the exercise like change in heart rate, duration of the exercise, distance covered, oxygen consumption, change in oxygen consumption, energy expenditure, rate of carbohydrate oxidation and rate of perceived exertion showed no significant correlations with the changes in

endostatin concentration after exercise at different intensities. Some of the variables and their correlation values with linear regression lines are shown in appendix "I".

Variables	ΔES1-60	ΔES2-60	ΔES1-70	ΔES2-70	ΔES1-80	ΔES2-80
Heart Rate (bpm)	0.216	0.386	-0.158	-0.120	-0.143	-0.088
	0.334	0.076	0.483	0.595	0.525	0.696
Δ Heart rate (bpm)	0.156	0.109	-0.261	-0.131	0.083	-0.014
	0.489	0.630	0.241	0.562	0.713	0.952
Speed (km/h)	-0.038	-0.090	0.312	0.061	0.142	-0.008
	0.868	0.690	0.157	0.787	0.528	0.972
Gradient (%)	-0.151	-0.146	**-0.687**	-0.505	-0.116	0.130
	0.698	0.708	**0.041**	0.165	0.766	0.738
Duration (Min)	0.170	0.211	-0.090	0.142	-0.070	0.124
	0.448	0.347	0.690	0.529	0.757	0.581
Distance (km)	0.102	0.187	0.116	0.378	0.157	0.262
	0.651	0.404	0.608	0.082	0.484	0.240
Basal VO_2	0.052	-0.227	-0.333	-0.134	-0.114	-0.117
	0.818	0.310	0.130	0.551	0.614	0.603
Actual VO_2 (ml/kg/min)	0.048	-0.020	0.059	-0.195	0.144	0.154
	0.830	0.931	0.793	0.385	0.522	0.493
ΔVO_2 (ml/kg/min)	0.044	0.002	0.077	-0.184	0.149	0.159
	0.844	0.992	0.734	0.412	0.508	0.479
VO_2 Max (%)	0.330	0.232	0.005	-0.243	-0.099	0.128
	0.133	0.299	0.981	0.276	0.663	0.570
Resp. Exch. Ratio	**0.535**	0.396	0.076	0.035	-0.108	0.184
	0.010	0.068	0.735	0.876	0.632	0.413
EE (kcal/min)	0.051	-0.024	0.122	-0.280	0.159	-0.044
	0.823	0.914	0.588	0.207	0.480	0.847
Rate of Fat Oxidation	**-0.528**	**-0.466**	-0.032	-0.234	0.154	-0.196
	0.012	**0.029**	0.888	0.295	0.495	0.381
Total Fat Oxidation (g)	**-0.531**	-0.390	-0.086	-0.037	0.087	-0.175
	0.011	0.073	0.705	0.870	0.702	0.436
Rate of Carb. Oxidation	0.332	0.228	0.160	-0.193	0.118	0.042
	0.131	0.307	0.476	0.391	0.601	0.853
Total Carbohydrate (g)	0.522	0.413	0.048	0.021	-0.124	0.190
	0.013	0.056	0.830	0.927	0.583	0.397
Rate of Perceived	0.193	0.235	-0.053	-0.067	0.128	0.095
Exertion	0.389	0.293	0.815	0.766	0.571	0.673

Table 4-5; Pearson Correlation of change in plasma endostatin concentration at 10 (ΔES1) and 60 minutes (ΔES2) of exercise at all protocols with different components of exercises at all intensities

Pearson correlation values (Upper values) and P value (Lower values) for change in endostatin at 10 minutes and 60 minutes interval at each exercise to the different components of exercise at respective intensities. Δ Heart rate; Change in heart rate, VO_2; (Oxygen consumption), ΔVO_2; change in oxygen consumption from resting to exercise, Resp. Exch. Ratio; Respiratory exchange ratio, Rate of Carb Oxidation; Rate of carbohydrate oxidation. EE; Rate of energy expenditure (kcal/minute)

4.6.8 Regression analyses

Linear regressions were carried out to determine any association of different anthropometric and exercise components with the change in endostatin concentration (Δ ES1) at 10 minutes and (Δ ES2) at 60 minutes during all exercise intensities. Uni-variate analyses were carried out for all the predictors including anthropometric characteristics and exercise parameters at all intensities- Beta Coefficient values, P values and R^2 adjusted values were calculated. No association in uni-variate analyses were found between any predictors and Δ ES1 and Δ ES2 at protocol 3. However, the variables with P value less than 0.1 in uni-variate analyses at protocol 1 and 2 were identified as predictors of interest and included in the table 4-6.

The predictors of interest were further analysed in multivariate linear regression model, as shown in table 4-7. Distance covered during the exercise and diastolic blood pressure at protocol 2 were found to be significantly associated (P = 0.053 & 0.043) with the Δ ES2 for the pooled data. When adjusted for gender and walking/running group, the distance covered during the exercise was significantly associated with the Δ ES2 for both the adjusted groups (P = 0.011 & 0.022). However, association between diastolic blood pressure and change in endostatin concentration Δ ES2 was approaching significance (P = 0.069 & 0.081), as shown in the table 4-7. These are likely to be chance findings as they could not be confirmed for the data set at other protocols.

Predictors	ΔES1-60			ΔES2-60			ΔES1-70			ΔES2-70		
	ß-coef	p	R^2-adj	ß-coef	p	R^2-adj	ß-coef	p	R^2-adj	ß-coef	p	R^2-adj
Age (years)												
Weight (kg)												
Height (m)												
BMI (kg/m^2)												
Systolic BP (mmHg)							1.648	0.018	21.30			
Diastolic BP (mmHg)							1.419	0.109	08.00	1.206	0.105	08.30
Heart Rate (bpm)												
Work Rate (km/h)												
Gradient (%)							-3.715	0.041	39.70			
Duration (Min)												
Distance (km)										14.91	0.082	10.00
VO_2 Max (%)												
Resp. Exch. Ratio	154.6	0.010	25.00	159.52	0.068	11.40						
Rate of energy exp. (kcal/min)												
Rate of Fat Oxidation	-65.99	0.012	24.20	-81.25	0.029	17.80						
Total Fat Oxidation (g)	-2.047	0.011	24.60	-02.10	0.073	11.00						
Rate of Carb. Oxidation												
Total Carb. (g)	0.819	0.013	23.60	0.903	0.056	12.90						
Rate of Perceived Exertion												

Table 4-6; Uni-variate regression analysis of change in endostatin concentration with anthropometric and exercise predictors

ΔES; change in endostatin levels between resting and 10 minutes (called as ΔES1) or 60 minutes (called as ΔES2) after exercise, B-coef; beta coefficient, p; p-value, R^2-adj; R^2 adjusted, BMI; body mass index expressed as kg/m^2, VO_2; oxygen consumption, Resp. Exch. Ratio; respiratory exchange ratio, Energy Exp.; rate of energy expenditure expressed as kcal/min, carb.; carbohydrate in g

	ΔES1-60			ΔES2-60			ΔES1-70			ΔES2-70		
	ß-coef	**p**	**R^2-adj**	**ß-coef**	**p**	**R^2-adj**	**ß-coef**	**p**	**R^2-adj**	**ß-coef**	**p**	**R^2-adj**
Unadjusted multivariate analysis												
Systolic BP (mmHg)							0.418	0.433	49.32%			
Diastolic BP (mmHg)							0.501	0.245		1.35	**0.053**	22.60%
Gradient (%)							-3.210	0.070				
Distance (km)										16.491	**0.043**	
Resp. Exch. Ratio	2555.8	0.301	23.97%	-4220.8	0.251	14.20%						
Rate of Fat Oxidation	-31.62	0.379		-75.16	0.166							
Total Fat Oxidation (g)	17.17	0.422		-29.87	0.348							
Total Carb. (g)	-6.350	0.256		11.03	0.186							
Adjusted multivariate analysis for Gender												
Systolic BP (mmHg)							0.698	0.258	52.82%			
Diastolic BP (mmHg)							0.399	0.350		1.218	**0.069**	29.80%
Gradient (%)							-3.723	0.058				
Distance (km)										25.129	**0.011**	
Resp. Exch. Ratio	2732.6	0.325	19.36%	-3796.8	0.355	9.260%						
Rate of Fat Oxidation	-25.25	0.638		-59.65	0.454							
Total Fat Oxidation (g)	18.72	0.436		-26.11	0.462							
Total Carb. (g)	-06.64	0.272		10.32	0.249							
Adjusted multivariate analysis for Walking/Running												
Systolic BP (mmHg)							0.418	0.433	49.32%			
Diastolic BP (mmHg)							0.501	0.245		1.192	0.081	26.92%
Gradient (%)							-3.209	0.070				
Distance (km)										19.102	0.022	
Resp. Exch. Ratio	2562.7	0.315	19.45%	-4222.7	0.266	8.850%						
Rate of Fat Oxidation	-25.62	0.582		-76.79	0.274							
Total Fat Oxidation (g)	17.68	0.425		-30.01	0.363							
Total Carb. (g)	-06.15	0.292		10.97	0.209							

Table 4-7; Multivariate regression analyses of change in endostatin concentration with anthropometric and exercise predictors

Predictors which had a p≤0.1 in uni-variate analysis were analysed in multivariate analysis. ΔES; change in endostatin levels between resting and 10 minutes (called as ΔES1) or 60 minutes (called as ΔES2) after exercise, B-coef; beta coefficient, p; p-value, R^2-adj; R2 adjusted, Resp. Exch. Ratio; respiratory exchange ratio, carb.; carbohydrate in g.

4.6.9 Baseline endostatin concentration on different days during the trial

The baseline endostatin concentrations on each of the 3 lab visits were analysed to investigate the consistency of the data. Independent sample T-test revealed no significant difference between mean endostatin concentration for males and females on each experimental day. These data are shown in table 4-8.

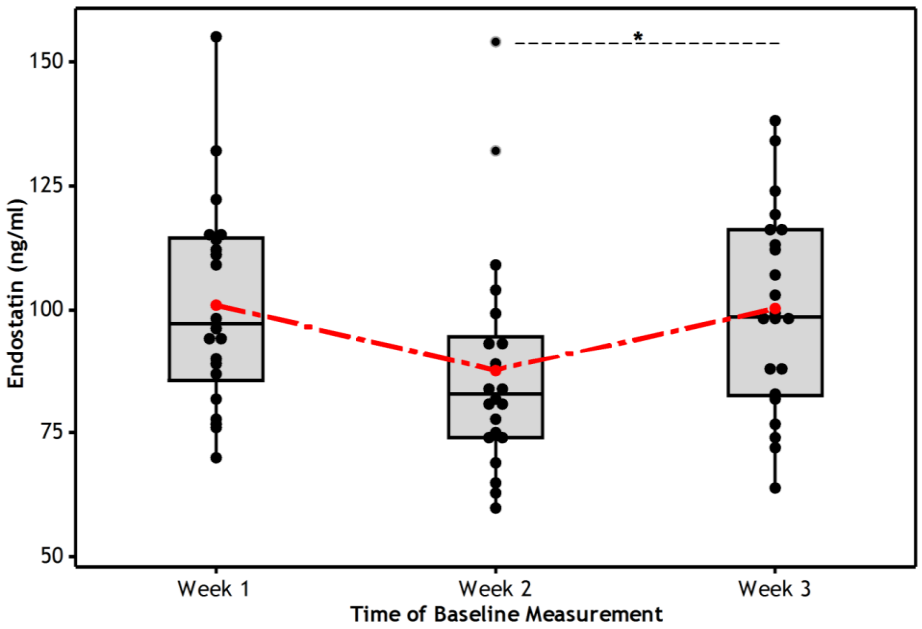
	Gender	Mean Endostatin(ng/ml) ± SD	P Value
week 1	Female	90 ± 14	0.068
	Male	105 ± 21	
	All	101 ± 20	
week 2	Female	90 ± 35	0.844
	Male	87 ± 17	
	All	88 ± 22	
week 3	Female	94 ± 22	0.421
	Male	102 ± 20	
	All	100 ± 20	

Table 4-8; Baseline endostatin concentrations for male, female and all individuals on different weeks

Mean basal endostatin concentrations in plasma of male and female individuals on all 3 weeks. The data for males and females were not significantly different on 3 weeks. SD; Standard deviation

The differences in the mean on the 3 weeks were determined using repeated measures ANOVA with Bonferroni post hoc analysis. No significant difference between the endostatin concentration at week 1 with week 2 or week 3 were observed (P = 1.0 and 0.16 respectively). However, the Difference between week 2 and 3 was borderline significant (P = 0.049).

Box plots showing the mean, median, individual values and the difference between the means on all visits are given in figure 4-22.

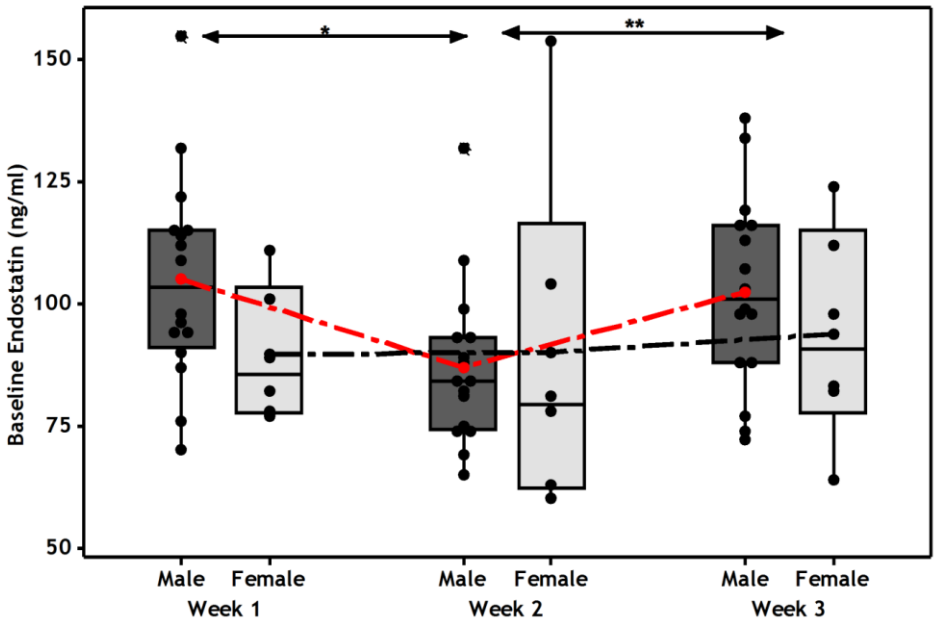


4-22; Baseline endostatin concentration on 3 visits

Box plots presenting basal endostatin concentration on all 3 weeks with mean, median and individual values. Dotted line above the box plot shows the significant difference between week 2 & week 3 concentration (P value 0.049).

In males, the difference in mean endostatin concentrations between week 2 and week 3 were significant (P = 0.014). There was no significant difference between week 1 and week 2 (P = 0.068) and week 1 and week 3 (P = 1.0).

However, no differences in basal endostatin concentrations were observed for females on all 3 weeks (Anova P = 0.93). The data is shown in figure 4-23.



4-23; Baseline endostatin concentrations in males and females on all 3 visits

Box plots presenting the mean, median, inter quartile range, individual values and significant difference in the basal endostatin concentration on different weeks for males and females. Female participants can be seen with almost the same mean concentrations on all the three weeks. Solid arrows with * shows the difference for males participants. (* = P value 0.068, ** = P value 0.014)

4.6.10 Correlations of basal endostatin concentration with anthropometric and resting variables

Basal endostatin concentrations on all 3 weeks and the different anthropometric and other baseline variables were checked for any correlation between them. Moreover, mean endostatin values for all individual from the basal endostatin concentrations on 3 weeks were determined and correlated as well. These correlations are shown in table 4-9. Age show significantly positive correlation (P = 0.008, r = 0.55) with basal endostatin concentration only at week 1 but not week 2 or week 3. However, when correlated with the mean data of all weeks, it was approaching statistical significance (P = 0.053, r = 0.24).

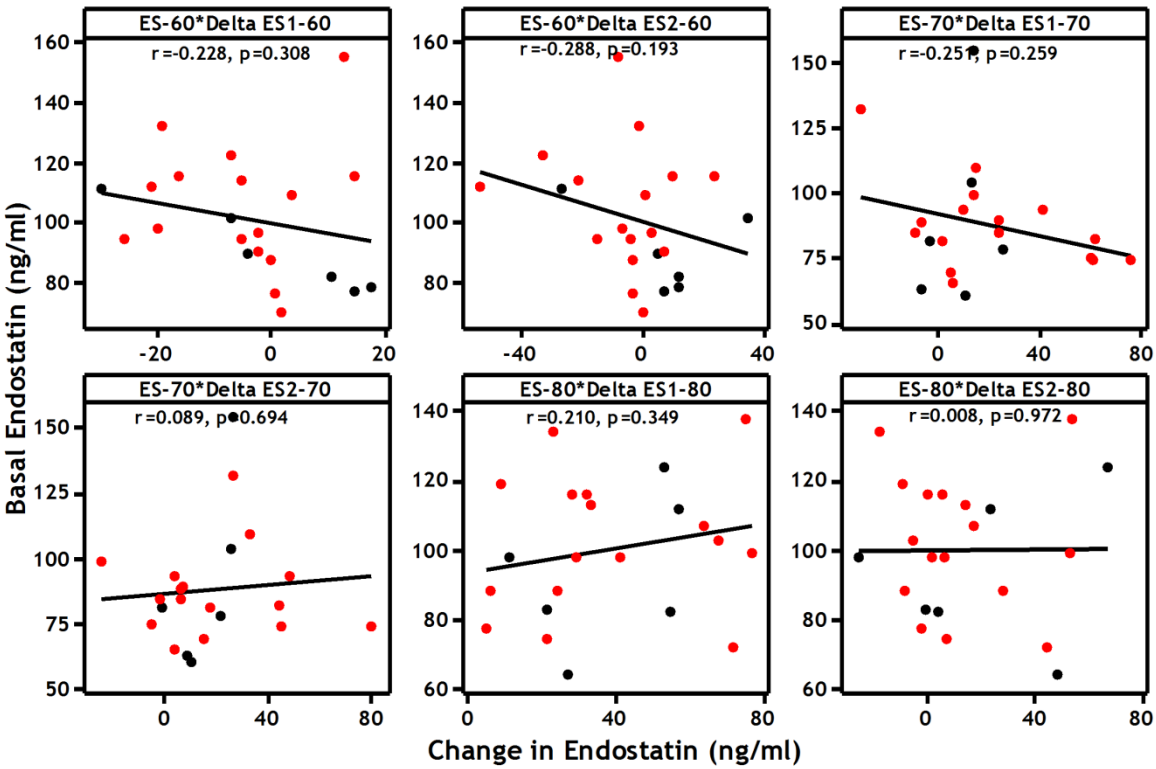
Variables	ES-Week 1		ES-Week 2		ES-Week 3		ES-All weeks	
	r	p	r	p	r	p	r	p
Age (years)	0.553	0.008	0.009	0.967	0.207	0.356	0.240	0.053
Weight (kg)	0.114	0.615	0.015	0.946	0.312	0.157	0.137	0.272
Height (m)	0.017	0.939	0.047	0.836	0.348	0.113	0.129	0.303
BMI (kg/m^2)	0.185	0.410	-0.028	0.901	0.202	0.367	0.111	0.376
Systolic BP	0.273	0.220	0.170	0.450	0.142	0.530	0.186	0.134
Diastolic BP	-0.053	0.815	0.226	0.312	0.209	0.350	0.124	0.319
Basal HR	-0.389	0.074	-0.024	0.917	-0.172	0.444	-0.207	0.095
Basal VO_2	-0.091	0.689	0.128	0.569	-0.218	0.329	-0.015	0.903
ΔES1	-0.228	0.308	-0.251	0.259	0.210	0.349	-0.074	0.556
ΔES2	-0.288	0.193	0.089	0.694	0.008	0.972	-0.114	0.360

4-9; Correlations of baseline endostatin concentration on all visits with anthropometric and physical characteristics

Pearson correlations for different anthropometric measures and basal endostatin concentration on different weeks are shown. ES; basal endostatin concentration, r; Pearson correlation value, P; P value, ΔES1; difference in endostatin concentration between basal and 10 minutes sample, ΔES2; difference in endostatin concentration between basal and 60 minutes sample, BP; blood pressure as mmHg, HR; heart rate as bpm, VO_2; oxygen consumption in ml/kg/min

4.6.11 Correlation of the baseline endostatin concentration with the changes in endostatin concentration on respective days

It was thought that basal endostatin concentration might affect the change in it after exercise on respective trials. To answer this, Pearson correlation between the basal endostatin concentrations on different days with respective changes (ΔES1 & ES2) were carried out, as shown in the table 4-9. No correlations of statistical significance were observed between basal endostatin concentration and ΔES1 & ES2 on all weeks, as illustrated in the figure 4-24.

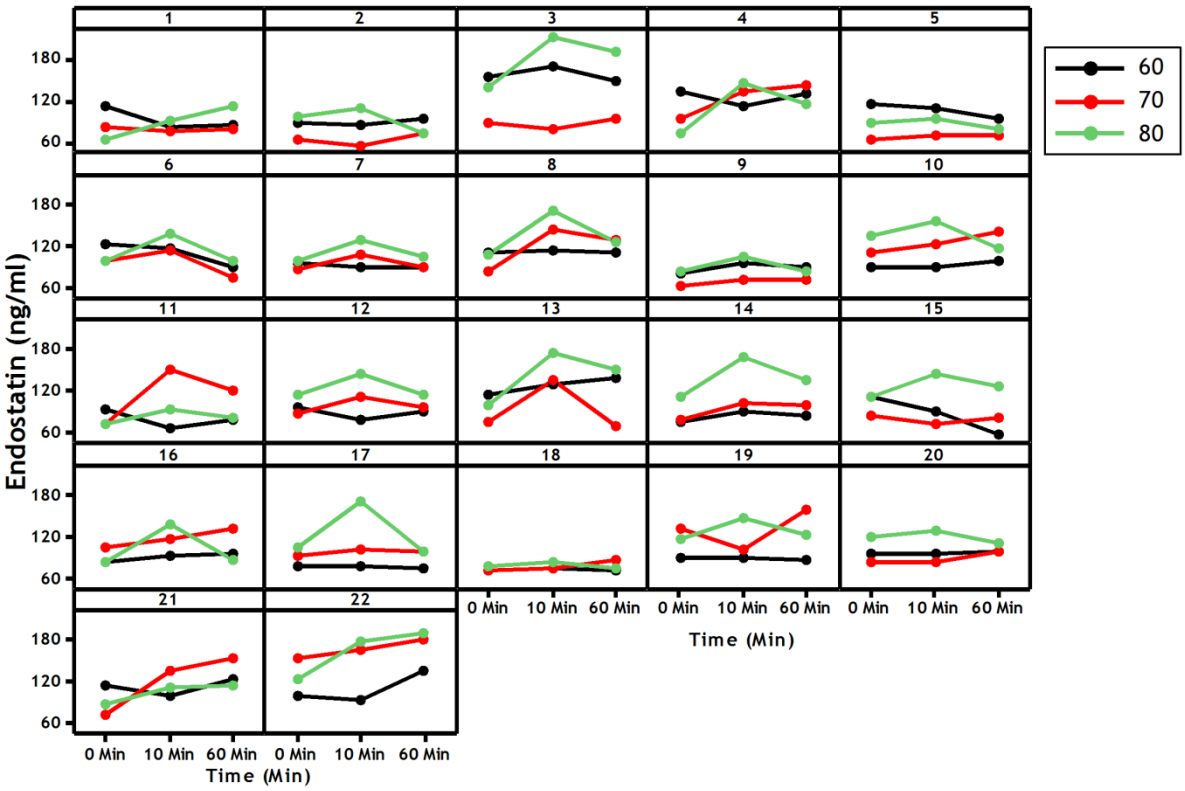


4-24; Correlations with linear regression lines of basal endostatin concentration on all weeks with changes in endostatin concentration on respective days

No significant correlation can be appreciated. Delta ES1; difference between basal ES concentration and 10 minutes sample, Delta ES2; difference between basal ES concentration and 60 minutes sample. ES; Endostatin, values in red; male volunteers, values in black; female volunteers

4.6.12 Collective data of all individuals on all visits

Finally the data for all individuals were plotted in the same interactive line graphs, as shown in figure 4-25, to observe any specific trend with naked eye. Mixed pattern of increase and decrease in endostatin concentration after exercises were observed for all individuals except; participant no 3 who had high basal endostatin concentrations on 2 occasions and participants 18, who showed no changes in endostatin concentration after exercise on all 3 occasions.



4-25; Individual line graphs for all participants on all days. Participant 3 presented with a clear outlier data for basal endostatin on week 1 and week 3. Participant 18 can be seen with almost no change after the exercise on all occasions.

4.7 Discussion

Prevalence of cardiovascular disease is on the rise in the recent era and certain ethnicities are at higher risk than others. For example, the South Asian population have a higher incidence of premature onset of the CVDs as well as increased mortality and morbidity (Gholap et al., 2011). Research has established the beneficial effects of exercise on decreasing the development, mortality and morbidity due to cardiovascular diseases, although the mechanisms for these beneficial effects are poorly understood (Thompson et al., 2003, Golbidi and Laher, 2012). Therefore, health organizations such as WHO has suggested a minimal recommendation of quantitative and qualitative physical activity to diminish the risk of CVD (World Health Organization, 2011). In some cases these physical activities are structured while in other cases vigorous.

In general, this study was designed to investigate the impact of structured exercise activities on the endostatin concentration which is one of the key regulators of angiogenesis. In more specific terms, this study examined the impact of 3 different exercise intensities (mild, moderate and vigorous) on the concentration of endostatin in plasma of young healthy adult males and females. It was proposed that different exercise intensities might influence the endostatin concentration in plasma differently. Moreover, it was also proposed, that gender and variations in physical characteristics might be differently influenced.

4.7.1 Summary of findings of the study

22 young healthy volunteers completed the exercise within the time schedule without any dropout. It includes 16 males and 6 females. No difference in anthropometric characteristics and age between males and females were observed, as shown in table 4-1.

The results in this chapter reported different effects of exercise on endostatin concentration in plasma. The changes in the circulatory endostatin with different intensities were different. With the mild intensity exercise a non significant negative change was observed, while with higher intensities the increase in endostatin was significantly high. The moderate intensity exercise

reported mixture of effects but all the participants showed an increase in endostatin concentration at 60 minutes interval.

The exercise in protocol 1 was of light intensity i.e. at 60% of the predicted maximum heart rate. After exercise, the plasma endostatin concentration was reduced by ~5%, as shown in figure 4-4. The effect was however not significant.

The exercise in protocol 2 was of moderate intensity i.e. at 70% of the predicted maximum heart rate. The mean endostatin concentration after exercise changed significantly by 18% at 10 minutes after the exercise and 19% at 60 minutes after the exercise, as shown in figure 4-11.

The exercise in protocol 3 was of high intensity i.e. at 80% of the predicted maximum heart rate. The mean endostatin concentration changed significantly ($P < 0.001$) at 10 minutes and 60 minutes ($P = 0.021$) after the exercise, as shown in figure 4-17. This change was equal to 38% and 14% respectively.

Changes in endostatin concentration at these respective exercises were never strongly influenced by gender, exercise mode (walking/running) and BMI of the individuals.

Moreover, changes in endostatin concentration could not be correlated with different anthropometric characteristics and different exercise features.

4.7.2 Study design

To the best of author's knowledge, this is the first study to demonstrate the effect of 3 different structured physical activities of different intensities on the endostatin concentration in plasma of young healthy volunteers.

The main aim of the study was to investigate the impact of different intensities of exercise on the endostatin concentration in plasma with the hypothesis that different intensities will affect endostatin concentration differently. The intensities of the exercise, according to the American College of Sports Medicine (ACSM, 2013) were equivalent to light, moderate and vigorous. The participants were divided in to 3 groups and the orders of the exercise were randomised.

Male participants were significantly heavier and taller than females, as shown in table 4-1. The participants were asked to refrain from any physical activity for 24 hours before the main trial. It served two aims; firstly the university students are more active than common individuals due to their involvement in different sports and secondly, prior exercise might interfere with the actual effects.

Recruitment of the participants was not easy due to number of visits. However, it was encouraging that all the participants after recruitment followed the full protocol and no drop outs were observed.

For standardization of the experiment, the exercise activities of all the participants at all the visits were monitored and recorded by the same researcher with the help of the same lab technician. Moreover, the same treadmill was used for all participants in the same lab. Same heart rate monitor and Douglas bags were used for participant on all occasions. Only one type of gas analyser was used for all measurements. The lab temperature was kept constant during all trials. However, the researcher was unable to control the barometric pressure. Moreover, due to very high time consumption of the ELISA (6 to 7 hours), only one plate per day was analysed.

The sample size of the study was 22. It was calculated based on the results of the study in chapter 3. Using the mean and standard deviation of endostatin concentration, it was assumed that exercise will change the mean endostatin concentration by 10 ng/ml. Applying this data to the nomogram designed by Altman (Altman, 1990) and Eng (Eng, 2003), it was predicted that recruiting 18-22 individuals will provide a chance of 90% success. This study sample was bigger than that by Gu (Gu et al., 2004) who investigated 7 individuals, Suhr who studied twelve and thirteen participants (Suhr et al., 2007, Suhr et al., 2010) and Brixius, who recruited 21 individuals (Brixius et al., 2008). However, the sample size was smaller than those used by Sponder (Sponder et al., 2013) who studied 42 Type 2 diabetic patients and 45 controls in his study, and Makey who tested 72 female breast cancer patients (Makey et al., 2013). Nonetheless, their study protocol was different in 2 respects; firstly they use single interventional exercise and secondly their studies involved cohort of patients.

4.7.3 Comparison with published studies

This study investigates the impact of 3 different exercise intensities on endostatin concentrations in young healthy individuals. In simple terms, the intensities corresponded to walking, jogging and running at a moderate speed. Very high intensities i.e. more than 80% predicted maximum heart rate, were not selected due to 3 main reasons; firstly it would be reasonably impractical for sedentary population to run at high intensity as individual tends to adhere to lower intensity programme (Duncan et al., 2005), secondly low intensities are easy to incorporate with long term compliance into one's life style (Choi et al., 2007) and thirdly low intensity activities could be implied clinically with ease in patients (Johnson et al., 2009).

4.7.3.1 Exercise at protocol 1

This study found that endostatin concentration was negatively but not significantly altered with low intensity exercise. During this exercise the participants lightly walked for relatively longer distance over reasonably longer durations. Though exercise minimally decreases the mean endostatin concentration but 64% of the individuals showed a transient decrease after the intervention, as shown in the figure 4-3. The decrease in endostatin concentration acutely after exercise has not been reported previously.

However, Brixuis reported a significant decrease in the basal endostatin concentration after 6 months of endurance training in overweight men (Brixius et al., 2008). This 6 month interventional exercise programme included 3 episodes of walking for 90 minutes at 2-4 mmol/l lactate levels or biking for 60 minutes at the 2-4 mmol/l per week. Nonetheless, this study fails to report the time duration between the last exercise session and the blood samples taken for endostatin analyses. Moreover, Bruserud and his colleagues also reported no difference in endostatin concentrations for healthy adults after high mountain climbing (Bruserud et al., 2005). A more recent study reported a non significant and minimal increase in the plasma endostatin concentration after 30 minutes of walking on treadmill at 55-59% maximum heart rate intensity (Makey et al., 2013). The decrease in endostatin concentration in the skeletal muscles of the

mice, after 4 weeks of moderate exercise for 30 minutes a day for 7 days a week was reported by Gu (Gu et al., 2006).

The exact mechanism of why endostatin concentration decreases after low intensity exercise is, however, poorly defined. Gu speculated that because endostatin is inversely correlated with the capillary density in the skeletal muscles and that exercise tends to improve the capillary density, in turn decreases the endostatin concentration in the skeletal muscles (Gu et al., 2006). Whereas, Brixius postulated that long term exercise decrease the circulating endostatin with two possible effects; firstly with the decrease in endostatin concentration a more angiogenic phenotype in body muscles is enhanced and secondly in case of high intensity physical activity, a rapid increase in the circulatory endostatin provides the beneficial effects against atherosclerosis (Brixius et al., 2008). However, no evidence is there to back this hypothesis and further experimental work would be required to prove this.

In Bruserud's study no significant difference in endostatin concentration in healthy adults was reported after mountain climbing. It could possibly be due to many reasons including age of the participants (Mean age 74 years), co-morbid conditions, intensity of the exercise and timing of the blood samples. Moreover, it is hard to know the extent of change in endostatin concentration in his study, as only P values are reported. However, when manually calculated from the graph, 40% of the individuals showed a decrease in endostatin concentration after climbing. Though the design of the study differs to our study (Unstructured physical activity, 30 minutes break during climbing), the results are in line with our study (Bruserud et al., 2005).

Based on the results in this chapter, we can say that a threshold mechanism required for the release of endostatin after exercise, does exist and low intensity does not affect that threshold. Person to person variation in this threshold mechanism might explain the difference in influence by the same intensity of exercise. Moreover, it is also possible that decrease in venous endostatin concentration after exercise may be due to uptake of endostatin by tissue from circulation.

This study also finds that the change in males and females is not uniform after the exercise. In fact, the males showed a negative change at 10 and 60 minutes after the exercise while females showed no change in the mean concentration, 10 minutes after the exercise and positive change of 7% at 60 minutes. This finding is interesting, because male participants exercised comparatively at higher speed with higher oxygen consumption and metabolic variables, as shown in table 4-2.

In a recent study, endostatin concentration at base line as well as after exercise was shown to be higher in females and a hormonal influence was suggested as the possible cause (Sponder et al., 2013). Menstrual history of the female participants in our study was not taken and the hormonal influence on endostatin concentration or the change in it could not be excluded completely. However, it could be implied that females can benefit even from low intensity exercises.

4.7.3.2 Exercise at protocol 2 and protocol 3

A steady and significant increase in endostatin concentration in plasma was observed after the exercise at protocol 2. The increase in the mean endostatin was 18% and 19%, 10 minutes and 60 minutes after the exercise respectively (P = 0.02 & 0.001). There was no sex specific difference in increase, although the pattern of increase between males and females was slightly different. Male showed an initial significant increase of 24% followed by a slight decrease to 22% at 60 minutes interval while females showed a steep increase of 9% initially which increased to 17% at 60 minutes. Contrasting to the results in previous section, 77% of the participants exhibited a positive change in endostatin concentrations after exercise while 23% showed a negative change, as illustrated in figure 4-10. However, it can be seen that after initial decrease, all participants exhibited positive changes.

Similar results were obtained when the volunteers performed the 3rd physical activity at higher intensity (protocol 3). The increase in mean endostatin was 38% at 10 minutes interval which remained higher even after 60 minutes of the exercise. There was no sex specific difference in mean concentrations.

Concurrently, no differences between the running and walking groups were observed.

The results in this section of the study confirm and expand the earlier observations of increase in plasma endostatin concentration after a moderate to high intensity exercise by different research groups (Sponder et al., 2013, Sponder et al., 2011, Gu et al., 2004, Suhr et al., 2007). Gu and his colleagues were the first to demonstrate significant increase in plasma endostatin concentration, when 7 healthy individuals performed exercise at 80-93% predicted maximum heart rate on treadmill for 4 to 10 minutes. The mean endostatin concentration raised by 43%, 73% and 33% at 30 minutes, 120 minutes and 360 minutes after exercise while in our study the maximum increase after the high intensity exercise was 38% at 10 minutes. The difference could possibly be due to the small sample size by Gu (n = 7) and only male participants in the study. If male to male comparison is carried out, the highest increase for males in our study was 38% at 10 minutes interval.

In a recent study, when a cohort of twelve healthy males undertook exercise protocols under different conditions including normoxic and normobaric hypoxic conditions with or without vibrations, the significant raise in endostatin concentration was observed in all groups up to 1 hours post exercise (Suhr et al., 2007). The participants in this study were males and most of them were national levels cycling athletes.

In a better structured study with both healthy & type 2 diabetic male and female volunteers, an increase in endostatin concentration after performing bicycle stress test was also shown (Sponder et al., 2013). Our results can be compared to this study, because this study also reported slightly higher increase in males than females. However, the highest increase reported by Sponder was only 15% observed in healthy males.

Results in this study are different from the study done by Brixius (Brixius et al., 2008),who reported a decrease in plasma endostatin concentration after 6 months of exercise training and Makey who did not find any increase in endostatin concentration after exercise at 55-59% heart rate in females (Makey

et al., 2013). The difference in design, intensity and timings of the samples taken could possibly explain the difference in results.

Just as the walking and running groups, analyses were also carried out on the bases of BMI. Participants were divided into normal (BMI < 25) and overweight (BMI > 25) groups. No differences in endostatin concentration of plasma were found between the two groups before or after the exercise at any time point during all the trials. Only one previous study has compared the increase in endostatin concentration for normal and obese females without any difference (Sponder et al., 2013).

Moreover, as the change in endostatin with high intensities of exercise was significant, correlations of the different components with the change at different intervals after exercise were carried out, as shown in table 4-5. No prominent pattern of correlation was observed. Though, respiratory exchange ratio and rate of fat oxidation showed significant negative correlation with change in endostatin concentration at low intensity exercise. But these could not be reproduced for other exercise intensities. It seems that these are only chance findings. However, Gu find a strong positive significant correlation (R^2 = 0.9388) between peak oxygen consumption and % change in endostatin consumption (Gu et al., 2004), a finding which is in dispute with this study.

The data was further analysed through linear regressions, to find any association between the change in endostatin concentration and different anthropometric and exercise variables, as shown in table 4-6. In order to find a true association all the prominent predictors in uni-variate analysis (P <0.1) were put in multiple regression models, as shown in table 4-7. The association was checked un-adjusted as well as adjusted for gender and walking/running. It is not clear, that why despite a significant change in endostatin concentration, no correlations or associations of statistical importance were found in the analyses. It might be possible that exercise, through other mechanisms like increasing MMP concentrations, influence the endostatin concentration (Suhr et al., 2010).

The last aim of our study was to find, if the basal endostatin concentrations on different weeks are relevant and consistent with the results in chapter 3. The mean basal endostatin on week 1 and week 3 are nearly the same i.e. 101 ng/ml

and 100 ng/ml, which are almost replicating the mean endostatin concentration of 105 ng/ml in chapter 3. However, these were different from mean endostatin concentration of 87 ng/ml on week 2. The reason for this difference is not clear, but one participant with a distinctly high endostatin concentration (treated as outlier on week1 and week 3) had less concentration by almost 70 ng/ml on week 2.

In order, to get a better picture, the individual values on all occasions for all participants were plotted, as shown in figure 4-25. Individual 3 can be seen clearly with high basal endostatin concentration. His health questionnaires and anthropometric variables were checked again to find any issue that could potentially cause the unexplained high basal endostatin concentration. No discrepancy was found in his health questionnaire or anthropometric measures. However, when contacted recently, to take part in another study given in chapter 6 of this thesis, the participant reported a health problem of serious nature for which he is under investigation. It is possible that during participation in our study, the participant was in subclinical stage.

The basal endostatin concentration was also correlated to the anthropometric characteristics of all the volunteers, as shown in table 4-9. The results were consistent with chapter 3 and no significant correlation was found for any variable except age. Age showed strong positive correlation with endostatin concentration only at week 1. Final analyses were carried out by correlating the average of basal endostatin concentration with the different variables. As expected no significant correlations were found.

Due to the difference in basal endostatin concentration on week 2, the degree of changes in endostatin concentrations were correlated with the basal concentrations on respective days, to check if basal endostatin concentration is affecting the change. The changes in endostatin concentration were found independent of basal concentrations, as shown in figure 4-24.

4.7.4 Limitations of the study

The data was anonymised by using serial numbers for a participant’s data, but as all the experimental procedures including recruitment, laboratory and statistical

analyses were carried out by the author, complete blinding of the data was not possible.

5 Effects of different exercise intensities on concentration of VEGF in plasma of healthy volunteers

Introduction

It has been established that endostatin inhibits angiogenic signals by VEGF (Yamaguchi et al., 1999, Ling et al., 2007, Eriksson et al., 2003, Kim et al., 2002). It has also been reported that exercise induces an angiostatic phenotype by increasing the endostatin concentration and by decreasing the VEGF concentration in circulation of humans (Gu et al., 2004), as well as mice (Gu et al., 2006). The same study also correlated high endostatin concentration with low VEGF concentration in plasma and vice versa.

The data reported in the previous chapter of this thesis, showed different and opposite effects of low and high intensity exercise on plasma endostatin concentration. This was unexpected. It raised the possibility that low and high intensities of exercise might also influence the VEGF concentration in plasma differently. To investigate this, a small scale study was carried out using the remaining volume of plasma samples from the experiments reported before. Samples of all participants at low (protocol 1) and high (protocol 3) intensities exercise were analysed for VEGF.

5.1 Rationale of the study

It was proposed that, as endostatin opposes VEGF by interrupting the angiogenic signalling mechanism, the effects of exercise on VEGF concentration in plasma may be opposite to that of endostatin. The intensities of exercise used in previous section revealed contrasting influence on endostatin concentration. Therefore, it was hypothesized that

i. Exercise at protocol 1 will increase the VEGF concentration in plasma.

ii. Exercise at protocol 3 will decrease the VEGF concentration in plasma.

5.2 Aims of the study

The aims of this study were:

i. To determine the effects of two different intensities of the exercise on the VEGF concentration in plasma.

ii. To determine if there is any gender difference in effects of the exercise on VEGF.

iii. To determine if there is any difference between the effects of exercise on VEGF for walking and running group.

iv. To determine whether the changes in VEGF concentration are correlated or associated with respective changes in endostatin.

5.3 Material and methods

The Research Ethics Committee of the College of Medical, Veterinary and Life Science was requested to grant permission to use the archived samples from the previous experiment. Permission was approved and it is shown in appendix "A3".

5.3.1 Sample analysis

Unused frozen samples from the previous trial were thawed. The samples were analysed for VEGF concentration using Quantikin^{R} ELISA kit for human VEGF, of the R&D systems, as discussed in detail in section 2.11.2. All analyses were carried out in the lab at West Medical Building, University of Glasgow. The time between the samples collection and analyses were roughly 6 months.

5.3.2 Statistical analyses of the data

Statistical analyses were carried out using SPSS 17 and Minitab 16 statistical software. Normalities for distribution were carried out.

Descriptive statistics were determined for VEGF as mean ± SD. Independent sample T-tests were applied to find out the difference between the means for male /female and walking/ running groups. One-way ANOVA with repeated

measures were applied to determine the difference between the means before and at different intervals after exercises.

Box plots were produced to demonstrate the difference between the mean VEGF concentrations. Pearson correlations between different variables and plasma VEGF concentration were determined. Simple linear regressions were carried out for uni-variate and multivariate analyses, to check the association of change in VEGF concentrations with respective change in endostatin concentrations.

5.4 Results

5.4.1 VEGF after exercise at protocol 1

VEGF concentrations at rest and different intervals after exercise were checked for normalities using Kolmogorov- Smirinov and Shapiro-Wilk tests and found normally distributed. Individual line graphs in a single panel are shown for all participants in figure 5-1. It can be seen that most of the participants exhibited an increase in the VEGF concentration after exercise.

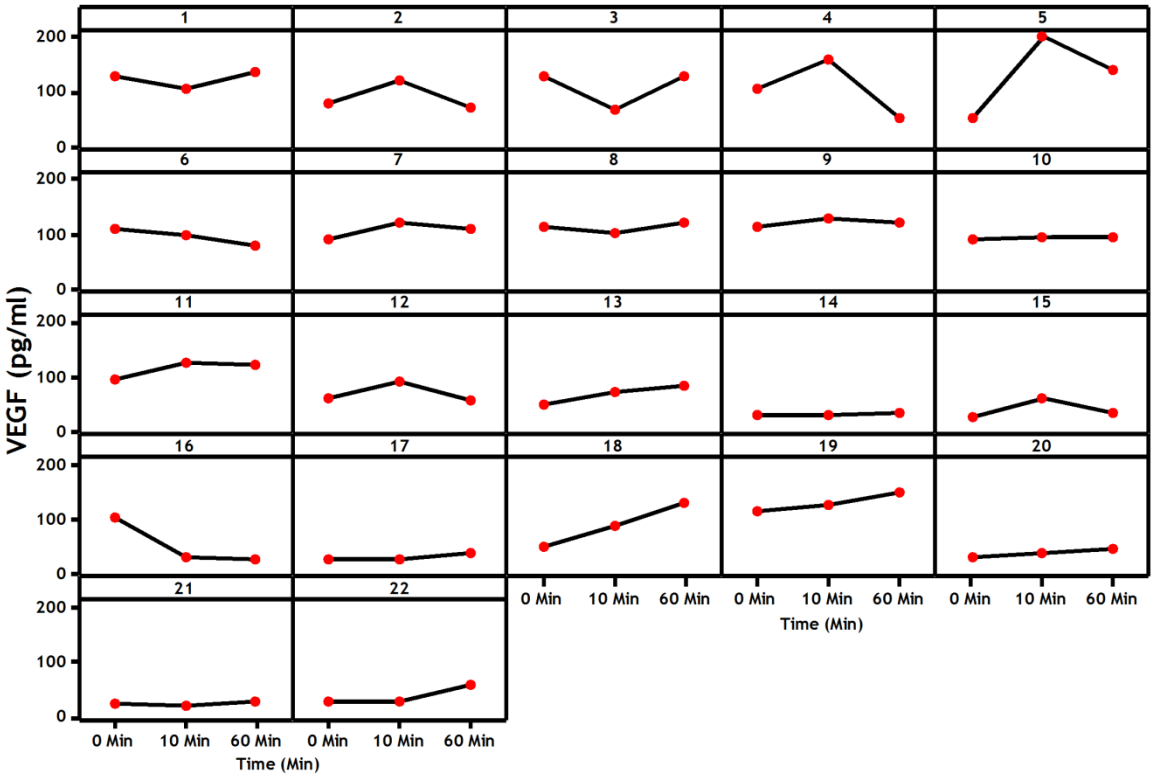


Figure 5-1; Individual line plots for all the volunteers showing

VEGF concentrations before (0 Min) and different intervals (10 Min & 60 Min) after exercise at protocol 1 are shown. The effect of the exercise is mixed but most of the participants can be seen with an immediate increase in plasma VEGF.

The basal VEGF concentration was 75 ± 36 pg/ml. It rise to 89 ± 47 pg/ml at 10 minutes and was 84 ± 41 pg/ml at 60 minutes interval after the exercise. A one-way ANOVA was applied to test the difference in means before and after

exercise. No significant difference (P = 0.28) was observed. Box plot showing the mean and median concentrations of VEGF before and different intervals after exercise are shown in figure 5-2.

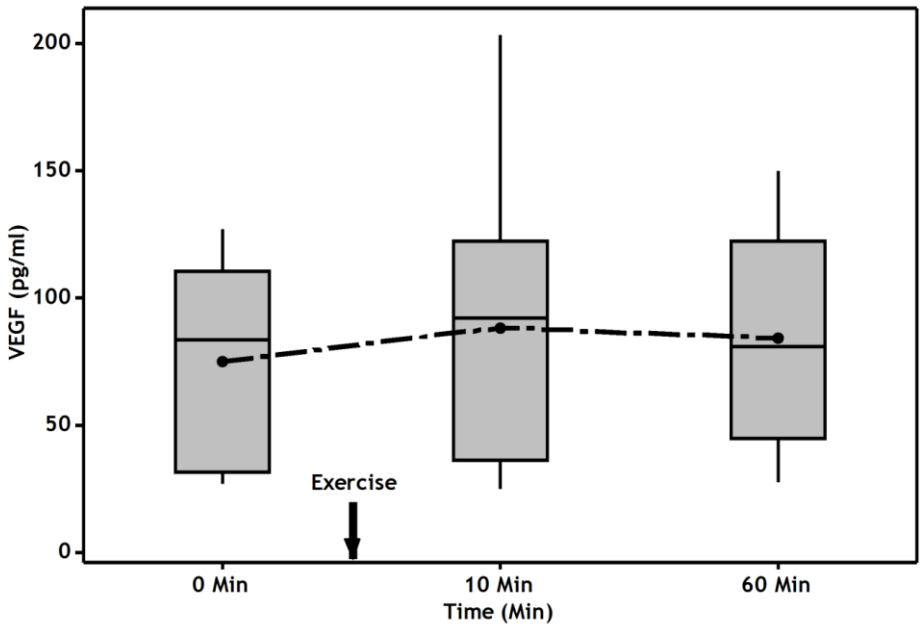


Figure 5-2; VEGF concentration before and different intervals after exercise at protocol 1

Box plot illustrating the mean, median, quartile range and range of VEGF data for all individuals, before and at 10 and 60 minutes after exercise at protocol1. No significant difference between means was observed.

5.4.1.1 VEGF concentration in males and females

The individual values for males and females VEGF concentrations, before and after exercise are plotted in figure 5-3.The VEGF concentrations before and after exercise in both groups can be seen with no obvious difference, which was confirmed statistically.

Resting mean VEGF concentration for males was 73 ± 36 pg/ml. Mean concentrations after exercise were 94 ± 47 pg/ml and 88 ± 41 pg/ml, at 10 and 60 minutes respectively.

The mean VEGF concentration for females at rest was 81 ± 42 pg/ml. The mean concentrations ,10 minutes and 60 minutes after exercise were 74 ± 48 pg/ml and 88 ± 41 pg/ml respectively.

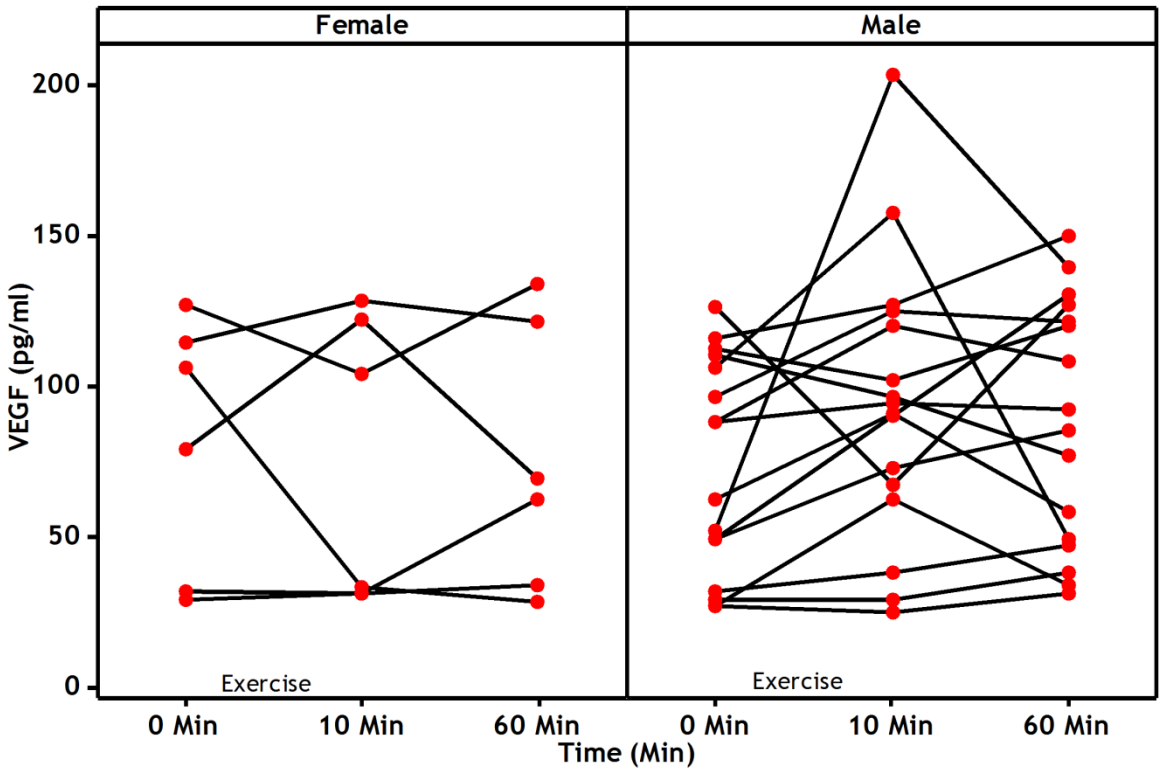


Figure 5-3; Interaction line plots showing the individual values of VEGF for male and female participants before and at 10 and 60 minutes after exercise at protocol 1.

One-way ANOVA with Bonferroni post hoc analyses revealed no significant difference between the mean VEGF concentration at different time points before and after exercise for both groups (P = 0.12 & 0.8 for males and females respectively), as shown in the figure 5-4.

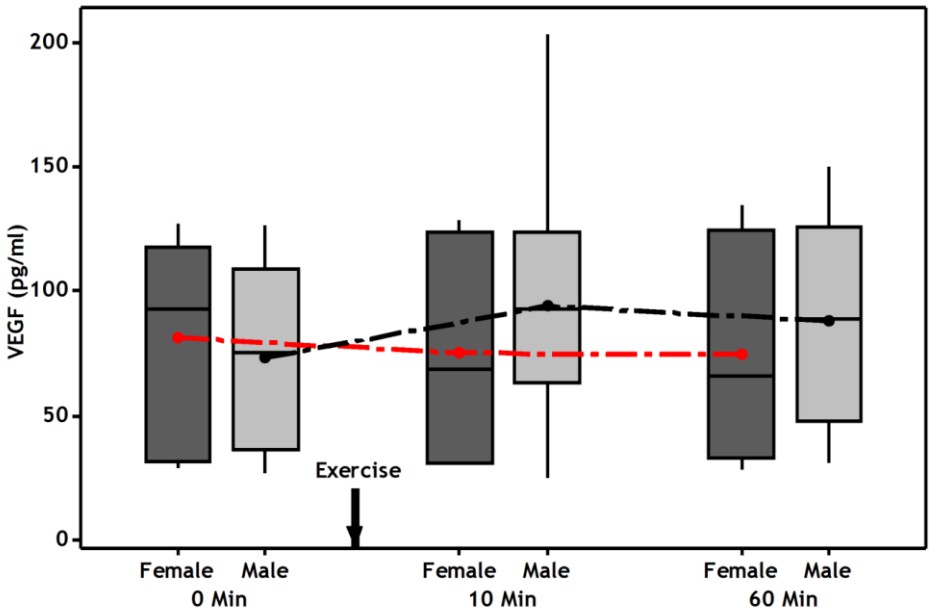


Figure 5-4; VEGF concentration in males and females before and at different intervals after exercise at protocol 1

Box plot presenting the mean, median, inter quartile range and range of VEGF concentrations for male and female participants before and at different intervals (10 & 60 minutes) after exercise at protocol 1. No significant differences were found between the means of the groups or time points.

5.4.1.2 VEGF concentrations in running and walking groups

Interaction line graphs showing the individual values for VEGF are plotted in figure 5-5. Mean basal VEGF concentrations were 69 ± 36 pg/ml and 84 ± 38 pg/ml for running and walking group respectively. No significant differences were found in the means between the groups (P = 0.2) or time points (P = 0.8). This is shown in figure 5-6.

In summary; the running group showed a non significant 23% increase in mean VEGF concentration at 10 minutes interval after exercise. The difference remained only 14% at 60 minutes interval after the exercise. On the other hand an increase of 10% and 8% in mean VEGF concentration was observed in the walking group.

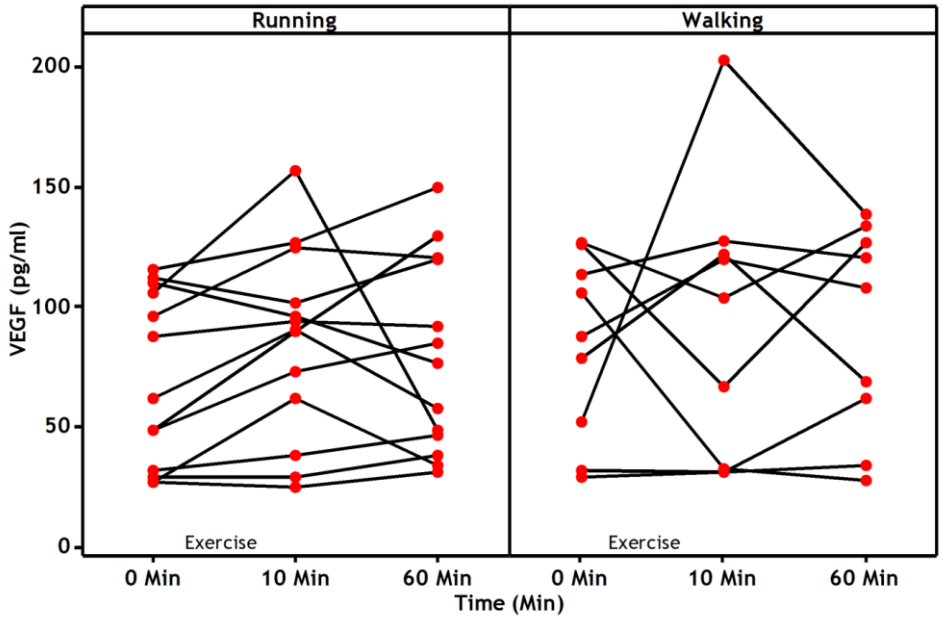


Figure 5-5; Interaction line plots showing the individual values of VEGF for participants in running and walking group before and at 10 and 60 minutes after exercise at protocol 1

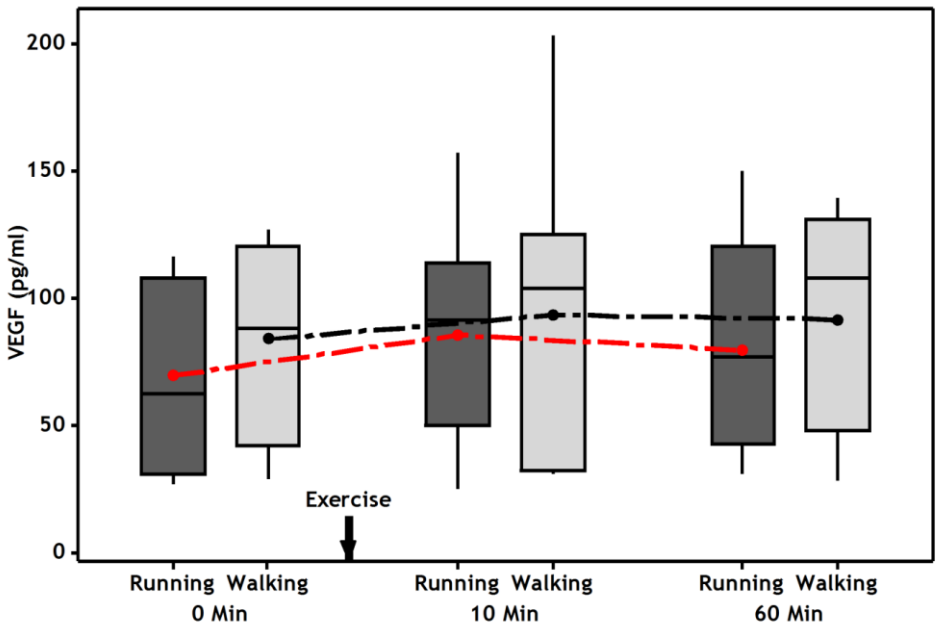


Figure 5-6; VEGF concentrations in running and walking groups before and after exercise at protocol 1

Box plots presenting the mean, median, Inter quartile range and range of VEGF concentration in running and walking groups before and at different intervals after exercise at protocol1. No significant change was observed at any time point.

5.4.2 VEGF after exercise at protocol 3

The VEGF samples at rest and different intervals after exercise at protocol 3 were found to be normally distributed. Interaction line plots for all individuals are illustrated in figure 5-7. Intra-personal variations in the basal VEGF concentration and initial rise after exercise can be seen, in most of the samples.

The mean VEGF concentration at rest was 77 ± 33 pg/ml. Mean VEGF concentration 10 minutes after exercise was 100 ± 45 pg/ml, while at 60 minutes, it was 85 ± 38 pg/ml. One-way ANOVA showed that the change after exercise was significant (P = 0.017). The mean VEGF concentration increased significantly by 30% after10 minutes (P = 0.02). At 60 minutes, the increase above baseline was 10% with no statistical significance (P = 0.53). Figure 5-8 shows box plots for this data.

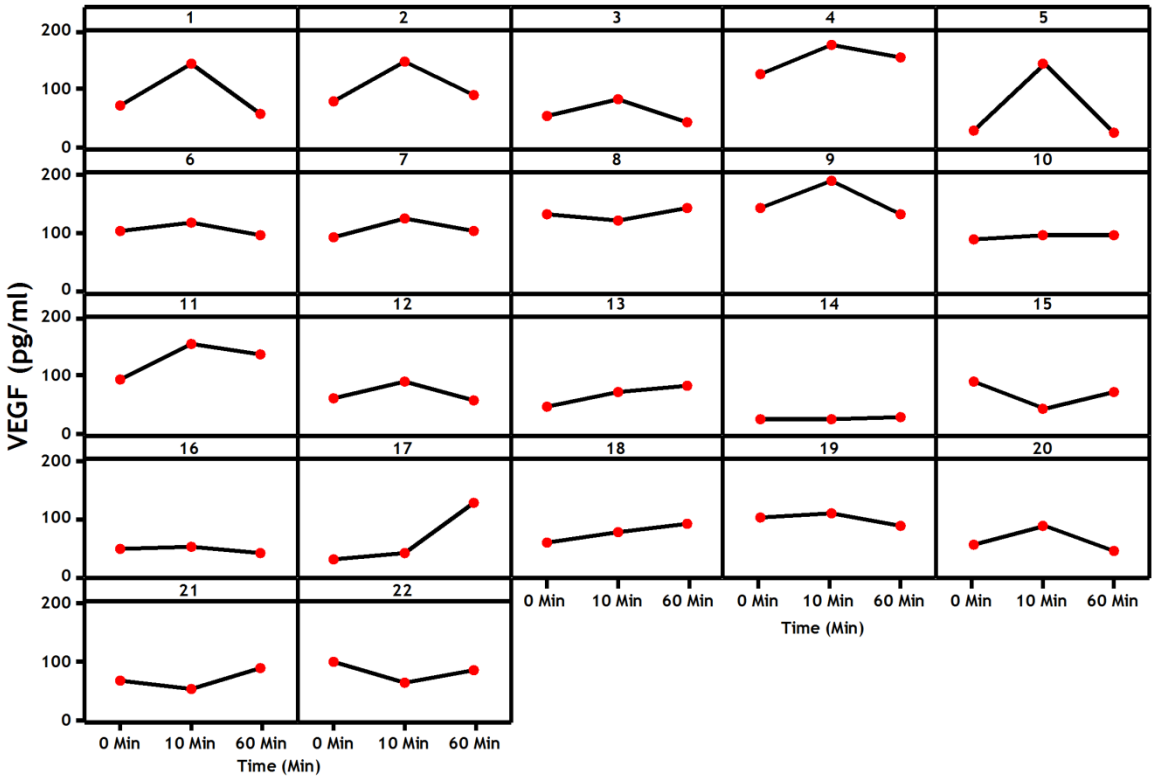


Figure 5-7; Individual line plots for all volunteers showing the plasma VEGF concentrations before (0 min) and at different intervals (10 min & 60 min) after exercise at protocol 3. It can be seen that some participants have low basal VEGF concentration compared to others.

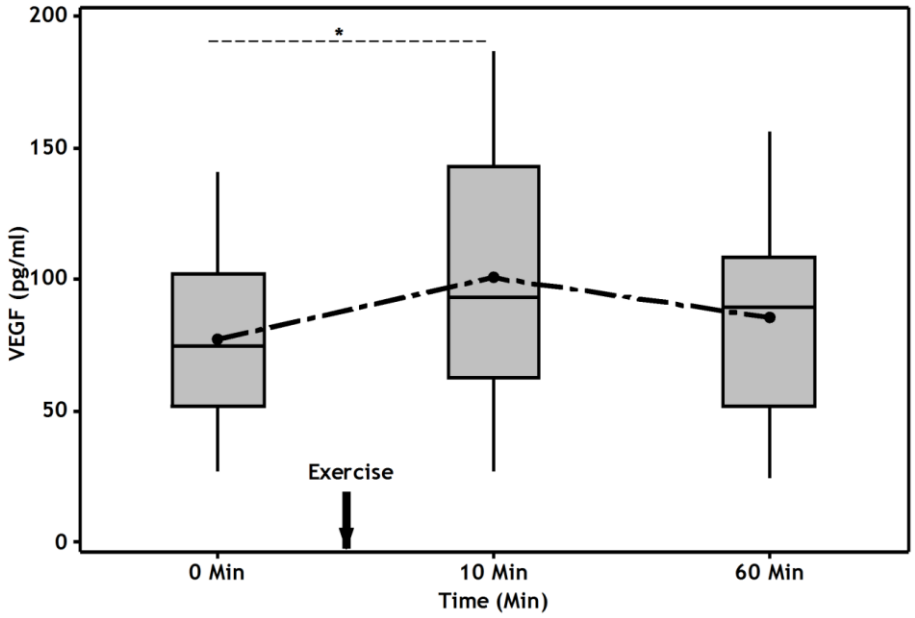


Figure 5-8; VEGF concentration before and after exercise at protocol 3

Box plots illustrating the mean, median, quartile range and range of pool data before and at 10 and 60 minutes after exercise at protocol 3. Significant difference for mean VEGF between baseline and 10 minutes samples were observed, as shown by the dotted line with * (P = 0.02).

5.4.2.1 VEGF concentrations in males and females

The individual values for males and females are plotted in figure 5-9. The basal VEGF concentration for males was 77 ± 31 pg/ml. It was 99 ± 39 pg/ml and 91 ± 38 pg/ml at 10 and 60 minutes respectively. The mean VEGF concentration for females at rest was 78 ± 40 pg/ml. The mean VEGF concentrations 10 and 60 minutes after exercise were 103 ± 64 pg/ml and 71 ± 38 pg/ml respectively. The SD of 64pg/ml for female participants is high at 10 minutes interval. It is due to less participants with variations in basal concentrations and high increase by some of the participants acutely after exercise, as shown in figure 5-9 & 5-10. A one-way ANOVA was carried out. No significant differences were observed between the means at basal and different intervals after exercise for females. However, a significant increase in mean VEGF concentration in males was observed at 10 minutes (P = 0.024).

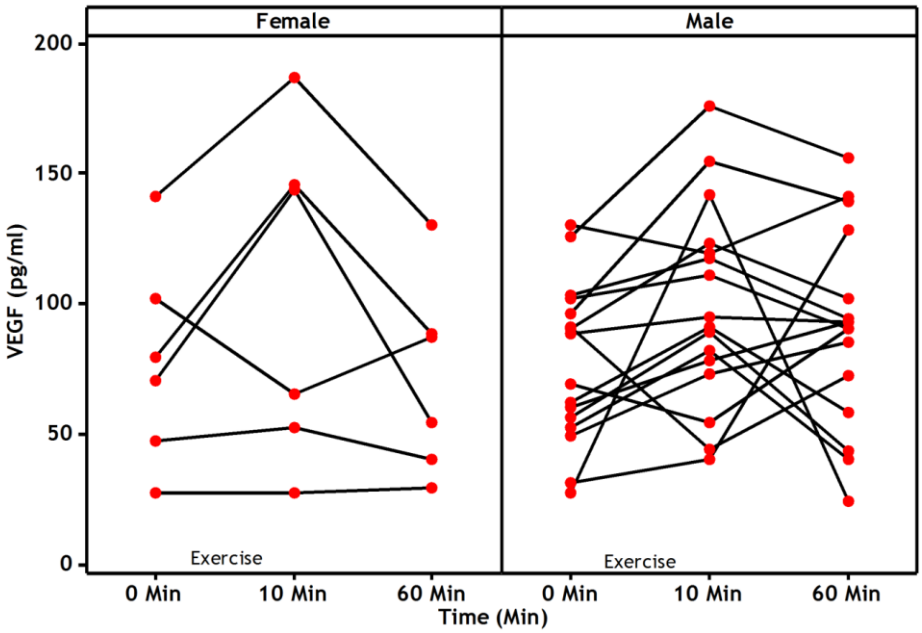


Figure 5-9; Interaction line plots showing the individual values of VEGF for male and female participants before and at 10 and 60 minutes after exercise at protocol 3. Variations in basal VEGF concentration and high increase in some of the participants immediately after exercise is visible.

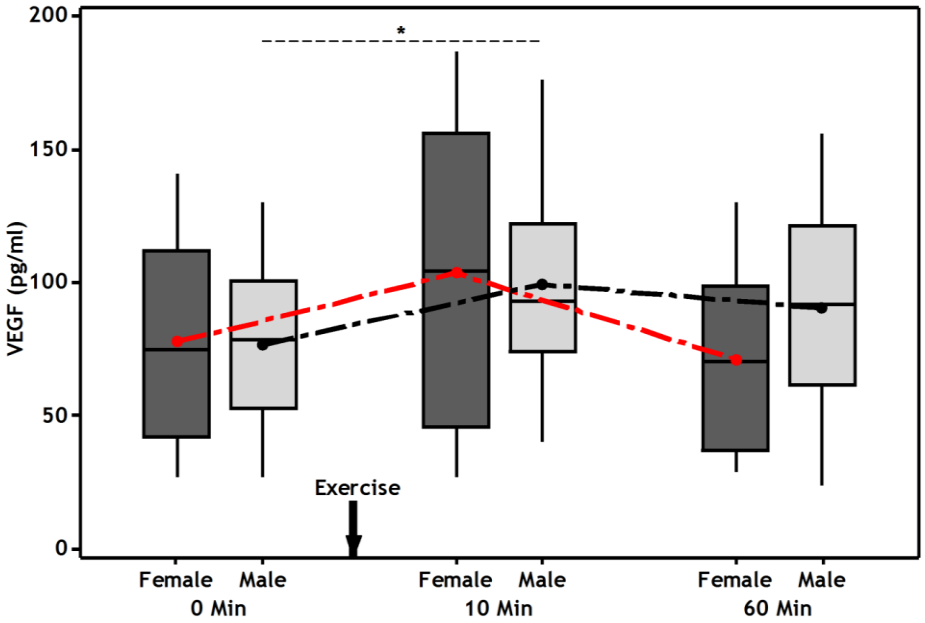


Figure 5-10; VEGF concentrations in males and females before and after exercise at protocol 3

Box plots presenting the mean, median, Inter quartile range and range of VEGF concentrations for males and females before and different intervals after exercise at protocol 3. Males showed a significant increase at 10 minutes interval after exercise (P = 0.024).

5.4.2.2 VEGF concentrations in running and walking groups

Individual values of VEGF concentration at rest and different intervals after exercise are shown in figure 5-11. The basal VEGF concentrations were 82 ± 30 pg/ml and 71 ± 37 pg/ml for running and walking group respectively.

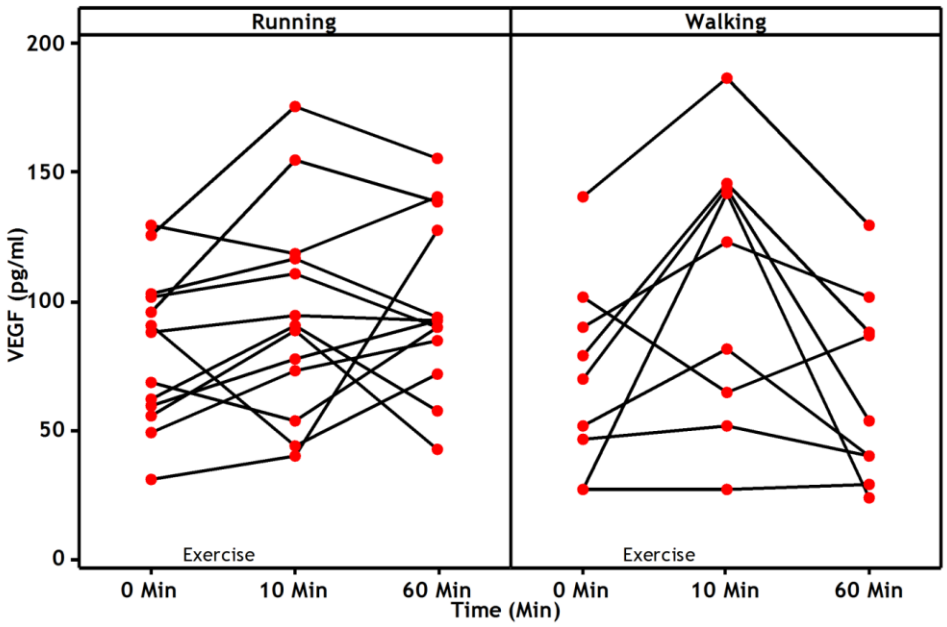


Figure 5-11; Interaction line plots showing the individual values of VEGF for participants in running and walking groups before and at 10 and 60 minutes after exercise at protocol 3.

Briefly; the running group showed 17% increased in mean VEGF concentration at 10 minutes interval after exercise. The difference increased further to 21% at 60 minutes interval after the exercise. However, the walking group showed a significant increase by 52% in mean VEGF concentration at 10 minutes interval (P = 0.04). The mean VEGF concentration dropped down significantly, and at 60 minutes interval it was higher by only 7% from the basal mean (P = 0.66). Figure 5-12 demonstrate this data graphically.

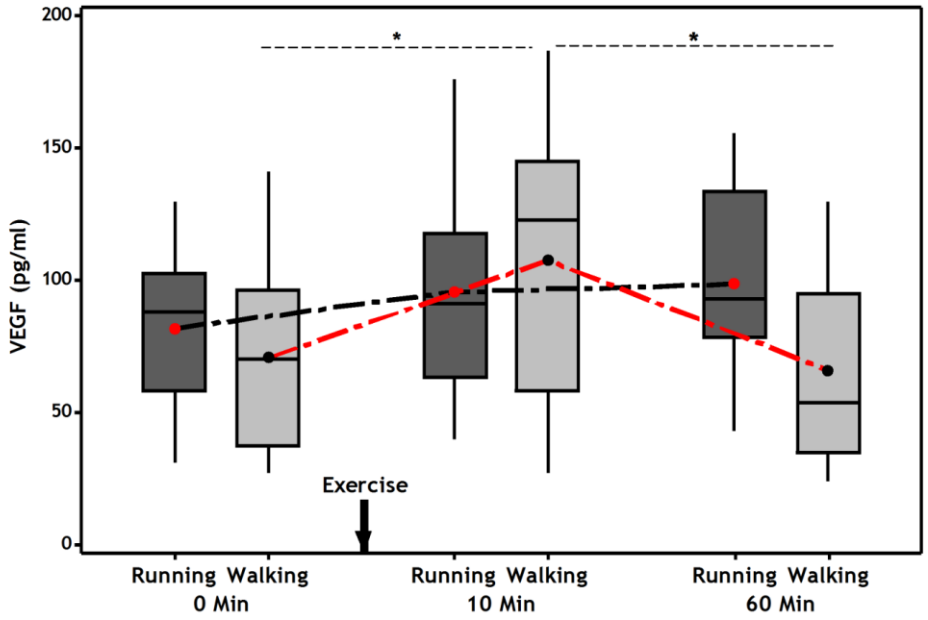


Figure 5-12; VEGF concentrations in males and females before and after exercise at protocol 3

Box plots presenting the mean, median, inter quartile range and range of VEGF concentrations in running and walking groups before and at different intervals after exercise at protocol 3. Significant increase was observed in the walking group at 10 minutes interval which dropped down significantly at 60 minutes samples, as shown by the dotted line with * (P =0.04) .

5.4.3 Correlation of changes in VEGF concentrations with the changes in Endostatin concentrations

Changes in VEGF at different intervals after both intensities of exercise were determined and correlated with respective changes in endostatin. Scatter plots with linear regression lines were plotted and shown in figure 5-13. No significant correlations between the two mediators were observed, as shown by the linear regression coefficient (r) and significance of the correlation (P) values in the respective panels.

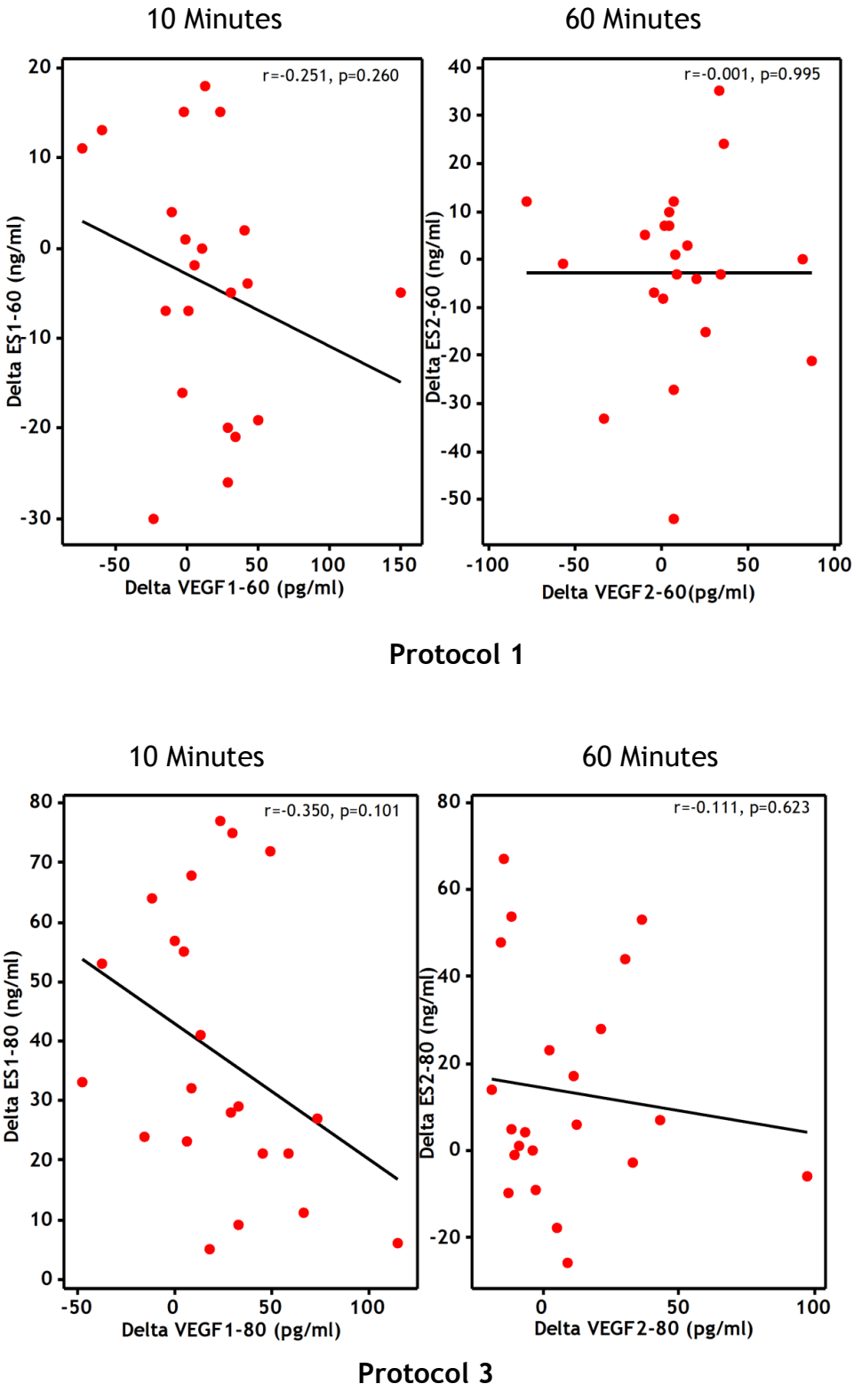


Figure 5-13; Pearson correlations for changes in endostatin with changes in VEGF

Scatter plots illustrates the correlations between the changes in endostatin from baseline (shown as delta ES1-60, delta ES2-60, delta ES1-80, delta ES2-80) with respective changes in VEGF from baseline (shown as delta VEGF1-60, delta VEGF2-60, delta VEGF1-80, delta VEGF2-80). The linear regression (r) and significance of the correlation (P) are given in each figure. No correlation of statistical significance was observed at any time point.

5.5 Discussion

The main aim of the study was to determine the influence of low and high intensities of exercise on concentrations of VEGF in plasma and to correlate it with endostatin concentrations.

5.5.1 Findings of the study

The plasma samples of all participants were analysed using the ELISA technique. The plasma VEGF concentrations for all participants were successfully determined. On the day of light exercise, mean VEGF concentration for all volunteers was 75 ± 36 pg/ml. The mean VEGF concentration in this study is in line with the concentrations of 91 pg/ml in healthy volunteers (Czarkowska-Paczek et al., 2006). However, huge person to person variability in plasma VEGF concentrations exist (Gunga et al., 1999). Concentrations as low as 10 pg/ml (Hiscock et al., 2003) to as high as 485 pg/ml have been reported (Gunga et al., 1999). It could be assumed that quantifying influence of exercise on VEGF concentrations in plasma would be difficult due to inter and intra personal variations.

A non significant increase of 19% and 14% in mean VEGF was observed at 10 and 60 minutes interval after light intensity exercise, as shown in figure 5-2.

High intensity exercise increased the mean VEGF concentration of 77 ± 33 pg/ml significantly by 30% which dropped down to 10% at 60 minutes interval, as shown in figure 5-8. There was not a huge difference between the patterns of influence of exercise on plasma VEGF, in male/female or walking/running groups.

Findings in this study are in line with the published data from many research groups. For example, an increase of 80% in the serum VEGF was observed immediately, when 14 young healthy individuals performed a high intensity exercise to exhaustion on graded cycling. The rise in mean VEGF concentration remained 49% at 2 hours interval after the exercise (Czarkowska-Paczek et al., 2006). Similarly, significant increase in VEGF, after 50% VO_{2max} test immediately

and 2 hours after exercise in endurance athletes has been reported. However, the same study showed no increase in sedentary group subjected to same exercise protocol (Kraus et al., 2004). Another study reported a 2.4 fold increase in VEGF concentration in marathon runners, after 67 km marathon at moderate altitude of 2300 meters. The immediate significant increase in VEGF concentration after the exercise remained persistent until 5 days after exercise (Schobersberger et al., 2000). Low activity exercise for a longer duration has also been reported with increased expression of VEGF mRNA in skeletal muscles (Hiscock et al., 2003).

However, the results in this study are different from other published studies reporting a decrease in VEGF concentrations after exercise. A decrease in arterial and venous plasma VEGF concentrations by 12% and 15% have been reported after short bouts of exercise for 10 days (7 sessions of 45 minutes one legged knee extensions) (Gustafsson et al., 2002). Gu and his colleagues also reported a significant decrease in VEGF concentration after high intensity exercise lasting 4 -10 minutes. The VEGF concentration was on decline consistently up to 6 hours after exercise and the maximum 53% decrease was observed at 2 hours interval (Gu et al., 2004). A transient decrease in VEGF has also been reported in hypobaric hypoxic condition in marathon runners (Gunga et al., 1999). In line with these, no influence of the endurance exercise on plasma VEGF was seen after 6 months exercise training programme in middle aged individuals (Brixius et al., 2008) and in females after 30 minutes exercise at 55-59% predicted maximum heart rate (Makey et al., 2013). The difference in these results were attributed to many factors including the physical fitness of the individuals, modes and intensities of exercise, environmental conditions , sampling time and techniques.

Another aim of this study was to correlate the changes in VEGF concentrations with respective changes in endostatin after exercise. The hypothesis was that exercise will increase the endostatin concentration in plasma as a result of enhanced catalytic activity and subsequent release into the circulation. Consequently, VEGF concentration will decrease partly due to uptake by the skeletal muscles and partly by the inhibition of endostatin. However, the results in this study are in contrast to what was anticipated and outlined in section 5.2 of this study. It can be seen that at high intensity exercise the VEGF

concentration in plasma increased along with an increase in endostatin concentration. VEGF is produced locally in skeletal muscles as a result of hypoxia and mechanical stretch (Hiscock et al., 2003, Czarkowska-Paczek et al., 2006) and is subsequently released into the circulation (Rullman et al., 2007). This seems plausible as skeletal muscles are the main site of action for VEGF. However, as mentioned in section 1.3.7, VEGF is produced by many other cells and tissues including platelets and a local release from these cells and tissue can possibly cause the increase in VEGF concentration in plasma after exercise.

No information about the half life of VEGF in plasma could be found. It has been shown that VEGF mRNA contains at least 2 destabilising elements due to which the VEGF mRNA is intrinsically labile (Levy et al., 1995). However, under hypoxic conditions the mRNA becomes much more stable which shows the complicated gene regulatory mechanism (Dibbens et al., 1999). It suggests that due to exercise the VEGF concentration in skeletal muscle would increase and favours its release to circulation which is then taken by other tissue not actively involved in VEGF production.

As shown in figure 5-13, no correlations of statistical significance between the endostatin concentration and VEGF were observed. The results reported here differ from that published by Gu, who found significant negative correlations between VEGF and endostatin concentrations (Gu et al., 2004).

Though the contrasting differences in VEGF concentrations after exercise in the published literature is not completely clear yet they are possibly due to the difference in the timings of the samples. Brixius and his colleagues reported no influence of long term exercise programme on plasma VEGF, but samples taken for final VEGF analyses were a day later than the last exercise session. From the results in this study and published literature, we can say safely that exercise influence the VEGF concentration transiently and 24 hours sample after exercise would be more or less the same as baseline VEGF concentration.

Moreover, in this study though a transient increases is observed at 10 minutes after the exercise for both intensities. However, the initial increase in all cases follows a decrease towards the base line at 60 minutes interval. The initial increase in VEGF could be attributed to the compensatory mechanisms by the

skeletal muscles cells to exercise, by enhancing its production and subsequent release into circulation (Rullman et al., 2007). It is possible that the decrease in VEGF concentration becomes significant at later time after the exercise, as shown by Gu and his colleagues, who observed the decrease consistently up to 6 hours after exercise.

5.5.2 Strengths

Sample size of the study was comparable to most of the published work. The study includes both males and females. No dropout in the study was observed and the experiment was completed strictly according to the protocol. The quality control indicators for ELISA were within the limit and samples with high CV values were repeated.

5.5.3 Limitations

Limitations of the study include uneven distribution of males and females. More female participants might have brought more information and would have given more balance to the study. Participants were asked to abstain from the physical activity but they may not have followed this advice. After passing the cannula, baseline samples were taken immediately and the acute action of endothelial injury with needle, on the release of angiogenic mediators were not considered.

Moreover, samples which were analysed for VEGF were in the freezer for a longer period of time (around 6 months). It may have an effect on the plasma VEGF concentration, as during ELISA analyses some of the samples with high values (beyond the reading capability of the machines) were encountered. Secondary ELISA analyses of these samples produced completely different results compared to primary analyses. Results of these secondary analyses were taken as actual values used in the results section of the chapter. It is unknown why the primary analyses yield high concentrations of VEGF in plasma.

6 The effects of high intensity exercise to exhaustion on the concentrations of endostatin and VEGF in plasma

6.1 Introduction

It is clear from the study in chapter 4, that short periods of moderate to high intensity exercise significantly increase the concentration of endostatin in plasma. The changes are different at different intensities of exercise. Moreover, change in VEGF concentration is not correlated with change in endostatin concentration. However, during those experiments samples were taken up to one hour after exercise during which endostatin and VEGF concentrations did not return to baseline. The possibility of later changes in VEGF or endostatin concentration could not be ruled out completely, as significant changes in endostatin and VEGF concentrations up to 6 hours after exercise has been reported (Gu et al., 2004).

Moreover, in the previous 2 studies it has been established that basal concentration of endostatin could not be associated with any anthropometric or physical characteristics of the individuals. It was thought that factors important in atherosclerosis and endothelial functions should be explored to check for any possible association with endostatin. These factors include; body fat composition, fasting lipid profile, fasting blood glucose concentration and insulin sensitivity.

Endostatin is a part of collagen VXIII in the basement membrane. It is in close proximity to the endothelium and other vascular structures. It is known that internal milieu strongly influences the endothelium and other vascular structure and their functions. For example, an increase in body fat is associated with activation of the renin angiotensin aldosterone system (RAAS), and subsequent increases in angiotensin II affects the vascular stiffness (Engeli et al., 2000). Moreover, increase in body fat increases the risk of type 2 diabetes (Chan et al., 1994) which in turn increases the risk of atherosclerosis (Folsom et al., 1999).

Similarly, insulin resistance is central to many metabolic disorders including diabetes and obesity. Insulin resistance is also associated with endothelial dysfunction and impaired vascular relaxation which in turn contributes to atherosclerosis and other cardiovascular events (Kim et al., 2006).

In addition, deranged lipid profile and hyperglycaemias are important determinants of endothelial dysfunction derived health problems including atherosclerosis and peripheral vascular diseases (Deaton et al., 2011). Thus, the possibility of an association of endostatin with these metabolic factors could not be ruled out.

Therefore, this final experiment was designed to look for the influence of high intensity exercise on endostatin and VEGF concentration in plasma for a longer duration (24 hours) after exercise and to investigate their association with metabolic factors discussed in the preceding paragraphs.

6.2 Aims of the study

The aims of the study were as follow;

i. To determine the correlations of basal endostatin concentration and the change in it after exercise, with anthropometric and metabolic factors of young healthy volunteers.

ii. To determine the effect of a single bout of high intensity exercise on the plasma endostatin concentration up to 24 hours.

iii. To determine the effect of single bout of high intensity exercise on the plasma VEGF concentration up to 24 hours.

iv. To determine the correlations between endostatin and VEGF concentrations in plasma and between their respective changes at pre and post exercise intervals.

6.3 Material and methods

The Research Ethics Committee of the College of Medical, Veterinary and Life Sciences granted the approval for this study. The permission is attached in appendix “A4”. All the experiments and subsequent analyses were carried out in the laboratories of the School of Life Sciences, University of Glasgow.

6.3.1 Recruitment of the participants

The volunteers were recruited through advertisements placed around the University campus. Personal contacts with participants of the previous study were also made. The inclusion criteria for participants were healthy male/female of 18 to 45 years age, non smokers, not had known heart problems, not on medications for any acute/ chronic illnesses and non pregnant or lactating.

After indicating their interest to participate, the volunteers were contacted at a convenient time and taken through two pre- participation health screening questionnaires, to assess their physical fitness and ability to perform maximal exercise to volitional exhaustion. The first questionnaire was PAR-Q and the second was adapted form of the ACSM Health Fitness Facility Pre- Participation Health Screening Questionnaire (ACSM, 2013), as shown in appendix “D2” & “D3”. Participants meeting the inclusion criteria were recruited to the study and dates for attending the laboratory were identified.

8 healthy male volunteers were recruited to the study. The purpose and nature of the experiment and potential risks during it, were completely explained. They were told about their right to stop the experiment at any time. Their anthropometric data are shown in the table 6-1 below.

S. no	Age (Yrs)	Ht. (m)	Wt. (kg)	BMI (kg/m^2)	SBP (mmHg)	DBP (mmHg)	WC (cm)	HC (cm)	W:H ratio
1	34	1.68	75.1	26.6	112	76	79	93	0.85
2	25	1.85	69.9	20.4	116	76	80	99	0.81
3	36	1.61	61.3	23.6	123	76	75	79	0.95
4	27	1.82	72.2	21.8	123	82	88	99	0.89
5	30	1.91	70.1	19.2	118	81	74	95	0.78
6	34	1.71	67.2	22.9	130	80	83	99	0.84
7	22	1.75	69.7	22.8	118	62	81	96	0.84
8	40	1.80	88.9	27.4	126	76	85	94	0.90
Mean ± SD	31 ± 6.1	1.77 ± 0.1	71.8 ± 7.9	23.1 ± 2.8	121 ± 6	76 ± 6	81 ± 5	94 ± 7	0.86 ±0.05

Table 6-1; Anthropometric and physical characteristics of the participants

Different characteristics of all participants with mean ± SD are illustrated. Ht; Height in m, Wt; Weight in kg, BMI; Body mass index, SBP; Systolic blood pressure, DBP; Diastolic blood pressure, WC; Waist circumference, HC; Hip circumference, W:H ratio; Waist to hip circumference ratio

6.3.2 Study protocol

All the volunteers attend the lab on 2 consecutive days. The total duration of their stay, was 6 to 7 hours on the first day and about 30 minutes on the second day. The activities on each day are discussed in the subsequent sections and schematically presented in figure 6-2.

6.3.2.1 Day 1

The participants were requested to visit the lab fasted. After signing consent forms their height, weight, blood pressure, heart rate, waist and hip circumference were measured, as described in chapter 2 sections 2.5. Their body fat percentages were estimated using air displacement plethysmography in a Bod Pod, as discussed in section 2.5.6.

The fasting blood glucose concentrations of the participants were checked using glucometer (Accu-Chek Aviva, Manheim, Germany). A cannula was put in an ante cubital vein and flushed with saline, as discussed in chapter 2 section 2.10. The participants then rested for 20 minutes in a quiet room. This was to

counterbalance the effects of needle injury and physical effort on baseline endostatin and VEGF. After rest 3 blood samples of 5 ml were taken, one each for endostatin, VEGF and fasting blood lipid profile and insulin concentration. The participants were then given isotonic drinking solution to restore some energy. Hydration status of the participants was not checked at this stage which might have an effect on the distribution of endostatin and VEGF concentration in plasma prior to and after exercise. This has been discussed in the limitations (section 6.5.6) of this chapter.

The participants performed maximal exercise test on the treadmill using the modified Taylor protocol (Taylor et al., 1955). During the test, maximal oxygen uptake of the participant was measured with breath by breath analyser (Medical Graphics Corporation, Borngasse, Germany), as discussed in chapter 2 section 2.8. The test was completed by all the volunteers and they were encouraged to make a maximum effort. The effort was considered maximum, if most of the following criteria were observed, as per ACSM guidelines (ACSM, 2013).

1. Achieved heart rate during the test was in the range of age predicted maximum heart rate ± 10 bpm.

2. A respiratory exchange ratio of more than 1.10.

3. A post exercise lactate concentrations of 8 mmol.

4. A plateau in oxygen consumption (VO_2) or failure in oxygen uptake by 150ml.min^{-1}, beside increase in work rate, as shown in the figure 6-1 from the data of one participant.

5. A rating of > 17 on Borg scale of perceived exertion.

At the end of the test all the participants walked slowly for 5 minutes till the heart rate dropped to 120 bpm. Blood was taken by a finger prick at 0, 3 and 5 minutes at the end of running to measure the lactate concentrations.

The participants then relaxed in a quiet room and more blood samples were taken at 10, 30, 60, 120 and 240 minutes after the exercise. During this time

they watched movies or videos as per their choice, while sitting on a chair. They were given biscuits and isotonic drinks during this time. After the final blood sample, cannula was removed and a full meal was served. They were asked to attend the lab on next day for a 24 hours sample.

6.3.2.2 Day 2

On day 2 the participants attended the lab for two blood samples, one each for VEGF and endostatin.

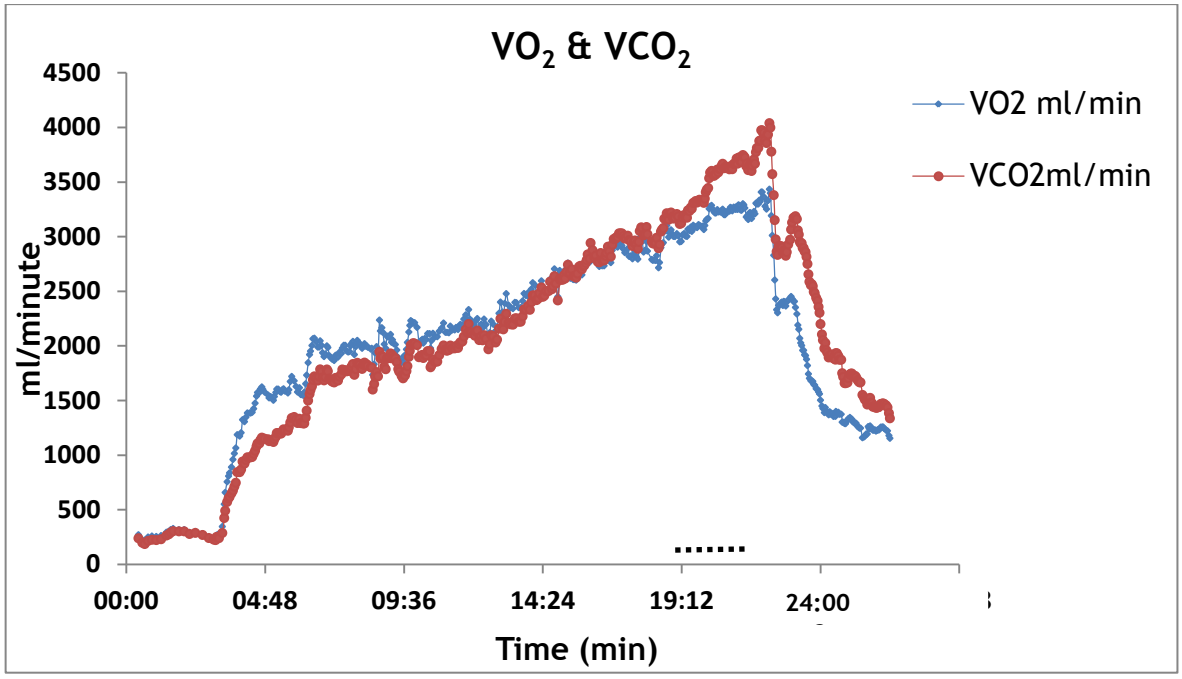


Figure 6-1; Breath by breath oxygen consumption and carbon dioxide production

Shows oxygen consumption (VO_2) and carbon dioxide production (VCO_2) from one of the participants during exercise. The initial 3 minutes are the resting period and the maximum effort was noticed at 21 minutes. The dotted line on the horizontal axis shows the maximum effort period during which the plateau in oxygen consumption can be seen.

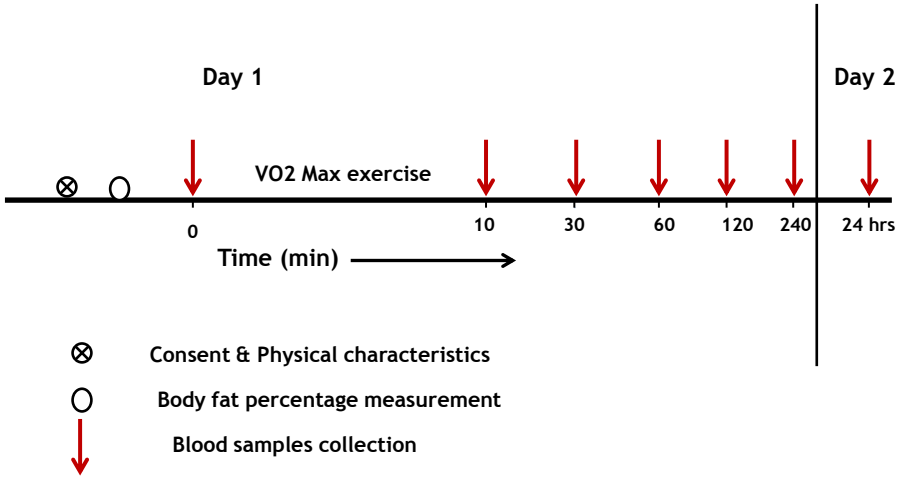


Figure 6-2; Schematic presentation of the study protocol

The protocol of the study is presented schematically. Physical characteristics include height, weight, waist & hip circumference, blood pressure and pulse rate measurements. Volunteers performed a running exercise according to the modified Taylor protocol on treadmill till voluntary exhaustion. Blood samples were collected after exercise at different intervals on the same day and 24 hours later on next day.

6.3.3 Collection of blood samples

The blood was collected by putting 20G cannula (Versatus-W, Terumo®, Belgium) in an ante-cubital vein. The cannula was attached to 3 way valve (BD Connecta™, Sweden) and flushed with normal saline to keep it clear. Blood samples were collected into 6ml EDTA tubes and put on ice. They were spun in centrifuge within 15 minutes of the collection. The blood was centrifuged at 1000 RPM for 15 minutes for endostatin and VEGF and at 3000 RPM for 15 minutes for lipid profiles and insulin. Plasma was separated into 3 aliquots each for endostatin, VEGF and lipid profile into 1ml Eppendorf tube using disposable plastic pipettes (Wilford Ind. Nottingham, UK). The plasma samples were stored at -80°C in an ultra low temperature freezer (New Brunswick Scientific, U725-86, US) for less than 1 month before analyses.

6.3.4 Samples analyses

After thawing, the frozen samples were analysed for endostatin and VEGF concentrations using Quantikin^{R} ELISA kit of the R&D systems. Insulin was measured using different types of Elisa kits (Mercodia, Uppsala Sweden). The procedures for ELISA analyses are given in chapter 2, section 2.11.

6.3.5 Lipid profiles

Plasma samples for lipid profiles were analysed in another laboratory of the University of Glasgow, as described in chapter 2 sections 2.1 and 2.13. Plasma glucose, total and high density lipoprotein, triglyceride and non- esterified fatty acid (NEFA) were determined using commercially available enzymatic kits.

Friedewald equation was used for the determination of low density lipoprotein (LDL) as follows (Friedewald et al., 1972);

LDL mmol.l^{-1} = (Total cholesterol – HDL cholesterol) – (TG /2.2)

6.3.6 Insulin resistance

Insulin resistance was determined using the QUICKI equation (Perseghin et al., 2001) as follow;

QUICKI (I.R) = 1/ (log G_0 + logI_0)

Where G_0 is the fasting blood glucose concentration in mg/dl, I_0 is the fasting insulin concentration in Mu/l.

6.3.7 Statistical analyses

SPSS version 17 and Minitab version 16 were used for the statistical analyses. The normalities of all variables were determined and where necessary log transformations were carried out. Summary statistics were carried out for data and presented as mean ± SD.

ANOVA with repeated measures was used for checking the difference between the mean in pre and post exercise blood samples. Correlations between

endostatin, VEGF and other variables were determined using Pearson correlation. Box plots were produced to graphically present the data, where necessary.

6.4 Results

6.4.1 Distributions of the different anthropometric variables and lipid profiles

The anthropometric characteristics, metabolic parameters, exercise components and angiogenic mediators at all time points were checked for normality using Kolmogorov- Smirinov and Shapiro-Wilk tests. All but 3 variables showed a normal distribution which were subjected to log transformation. These include NEFA, IR and the maximum gradients achieved during the test. As the sample size is small, the non normal distributions of these factors are less likely to affect the overall results.

Data for the body fat composition and lipid profile of all individuals with their mean ± SD is tabulated in table 6-2 and 6-3 respectively.

S. no	Weight	Body Fat	Body Fat%	Lean weight	Body density
1	75.1	16.8	22.4	58.3	1.04
2	69.9	5.8	8.2	64.1	1.09
3	61.3	11.1	18.2	50.1	1.05
4	72.2	12.4	17.0	60.5	1.06
5	70.1	5.9	8.4	64.2	1.08
6	67.2	8.9	19.8	58.2	1.07
7	69.7	6.5	9.3	63.2	1.07
8	88.9	21.5	24.1	67.4	1.04
Mean ± SD	71.8 ± 7.9	11.1 ± 5.6	15.9 ± 6.4	60.8 ± 5.3	1.06 ± 0.01

Table 6-2; Body composition of participants

The body fat compositions after Bod Pod measurements are shown for all participants with their mean and SD. Weight, body fat and lean weight are expressed in kg.

S. no	FBG	TG	Chol	HDL	SDLDL	NEFA	Insulin	IR
1	5.0	1.06	5.61	1.12	11.30	0.79	8.97	0.34
2	4.7	0.59	3.33	1.14	17.00	0.65	4.24	0.39
3	4.5	2.04	6.40	0.65	22.50	0.77	12.50	0.33
4	4.9	1.13	5.35	1.29	25.00	0.72	6.98	0.36
5	4.8	0.59	3.31	1.18	16.70	0.69	4.34	0.39
6	5.1	1.54	5.72	0.78	32.50	0.81	9.71	0.34
7	4.9	0.60	3.12	0.90	22.90	1.43	4.65	0.39
8	5.2	1.07	5.24	1.08	23.30	0.74	9.76	0.34
Mean ± SD	4.9 ± 0.2	1.08 ± 0.5	4.76 ± 1.3	1.02 ± 0.2	21.4 ± 6.4	0.82 ± 0.2	7.64 ± 3.07	0.36 ± 0.02

Table 6-3; Fasting blood glucose, lipid profiles and insulin of participants

Fasting lipid profile and insulin for all individuals with their mean and SD are tabulated. FBG; Fasting blood glucose (mmol), TG; Triglycerides (mmol), Chol; Cholesterol (mmol), HDL; High density lipoprotein (mmol), SDLDL; Small dense low density lipoprotein (mg/dl), NEFA; Non-esterified fatty acids, IR; Insulin resistance.

6.4.2 Response to exercise

All the volunteers performed exercise without any complications. The mean duration for the test was 17.04 ± 2.6 minutes (ranging 12 to 19 minutes) during which the participants covered a mean distance of 2.1 ± 0.34 Km. All the participants run at fixed speed of 8 km/ hr with a mean maximum gradient of 11 ± 2%. The mean maximum heart rate achieved during the test was 191 ± 10 bpm which increased by 167% from the mean basal heart rate of 72 bpm ($p < 0.001$). A mean increase of 38 ml/kg/min in oxygen consumption (VO_2) was observed with the mean maximum oxygen consumption (VO_{2max}) of 42 ml/kg/min achieved during the test ($P < 0.001$). Mean lactate concentration post exercise was 7.4 mmol (ranging 5.4 to 9.8 mmol). The individual values and mean ± standard deviations of the parameters of exercise are given in table below 6-4.

In summary; all of the participants gave a maximum effort which was confirmed by achieving most of parameters indicated by ACSM guidelines. It can be seen, in

table that most participants were aerobically fit and delivered their maximal effort.

S. no	BHR	Max HR	Duration	RR	distance	Max Grad	BVO2	VO_{2Max}	RER	Lactate
1	68	180	12.04	62	1.45	7.5	3.3	33	1.20	9.8
2	71	194	19.25	48	2.40	12.5	4.3	45	1.44	7.9
3	68	184	18.04	39	2.20	10.0	4.8	44	1.29	7
4	70	210	18.16	49	2.25	12.5	4.3	39	1.59	8.8
5	82	184	19.10	35	2.38	12.5	3.9	45	1.17	5.4
6	82	194	15.07	48	1.84	10.0	4.8	39	1.23	6.9
7	76	196	19.28	47	2.43	12.5	5.4	47	1.34	6.9
8	63	184	15.44	41	1.93	10.0	4.0	44	1.30	6.5
Mean	72	191	17.04	46	2.1	11	4.3	42	1.3	7.4
± SD	± 9	± 10	± 2.6	± 8	± 0.34	± 1.9	± 0.6	± 5	± 0.6	± 1.39

Table 6-4; Different parameters of exercise at base levels and during exercise

The individual values for components of exercise and mean ± standard deviation are shown. These indicate a maximal effort by all participants according to ACSM guidelines. BHR; Basal heart rate (beats per minute), Max HR; Maximum heart rate, Max Grad; Maximum gradient (%), $BVO_{2;}$ Resting oxygen consumption (ml/kg/min), VO_{2Max}; Maximum oxygen consumption (ml/kg/min), RER; Respiratory exchange ratio, RR; Respiratory rate (breaths per minutes). lactate concentration in mmol/l.

6.4.3 Baseline endostatin concentration

The individual line plots for endostatin concentration before and different intervals after exercise are plotted in figure 6-3. The entire group showed rapid increase in endostatin concentration after exercise. Mean endostatin concentration at rest for all participants was determined as 104 ± 6 ng/ml. The mean endostatin concentration at rest was consistent with the results in chapter 3 and chapter 4.

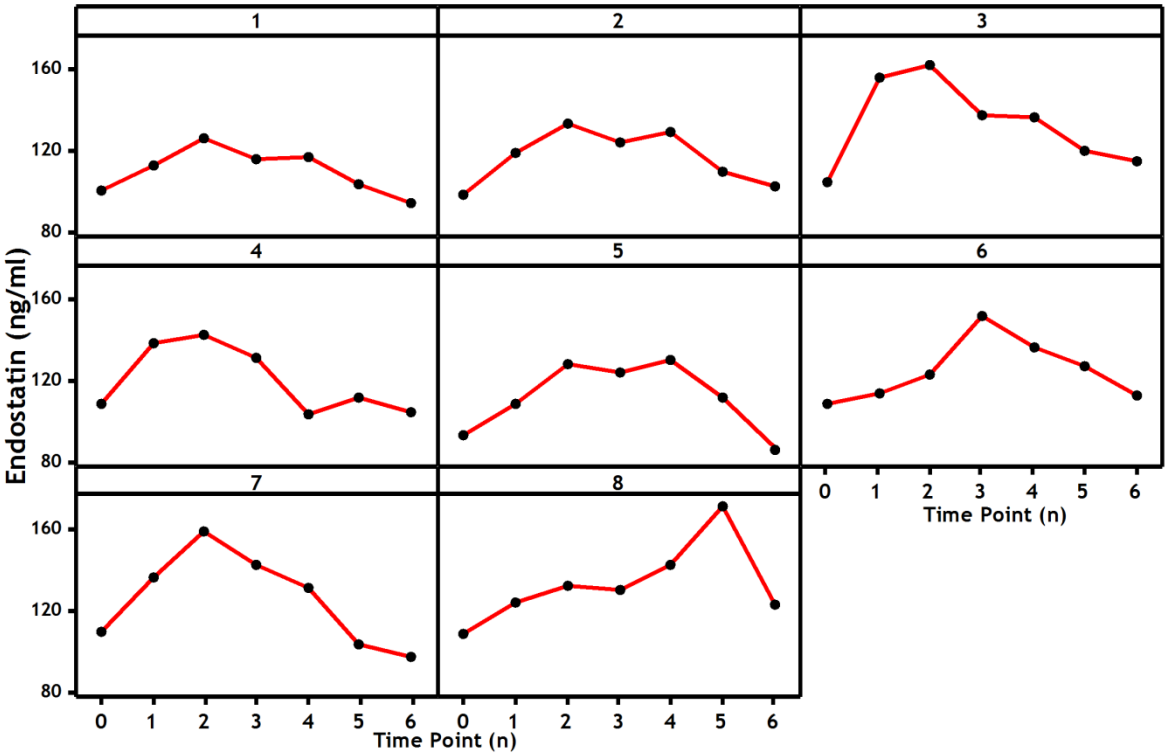


Figure 6-3; line plots showing the endostatin concentrations for all participants before and after maximum intensity exercise

Individual line plots of endostatin concentration for all participants in the study are shown. Time points on the horizontal axis shows the time points for blood sampling (0 = before exercise, 1= 10 minutes, 2 = 30 minutes, 3 = 1 hour, 4 = 2 hours, 5 = 4 hours and 6 = 24 hours after exercise). The rapid increase in endostatin concentration after exercise for all individuals can be seen.

6.4.3.1 Effect of exercise on mean endostatin concentration

Mean concentrations of endostatin in plasma before and after exercise are shown in figure 6-4. It is clear, that single bout of short period of high intensity exercise increased the mean endostatin concentration as confirmed by ANOVA with Bonferroni corrections ($P < 0.001$). The mean values were: 126 ± 16, 139 ± 15, 132 ± 12, 129 ± 12, 120± 23 and 107 ± 15 ng/ml respectively, at 10 minutes, 30 minutes, 1 hour, 2 hours, 4 hours and 24 hours after the exercise. These changes corresponds to increases of 21% ($P = 0.003$), 34% ($P < 0.001$), 27% ($P < 0.001$), 24% ($P = 0.001$), 15% ($P = 0.07$), and 3% ($P = 0.883$) compared to resting endostatin concentration. The changes at points 1-4 i.e. up to 2 hours post exercise were statistically significant. The highest increase was observed at 30 minutes interval after the exercise.

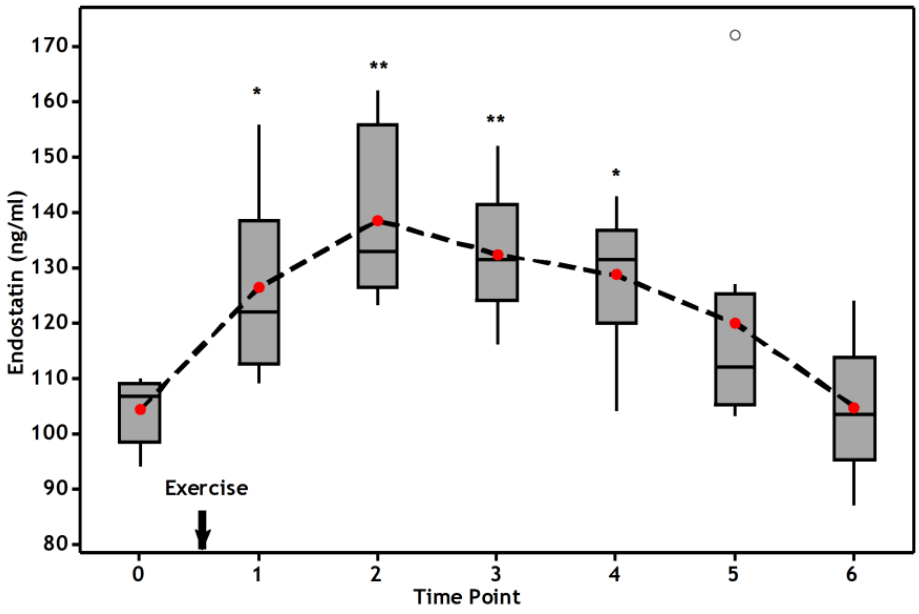


Figure 6-4; Endostatin concentration before and at different intervals after maximal exercise

Endostatin concentration before and at different intervals after exercise (0 = before exercise, 1= 10 minutes, 2 = 30 minutes, 3 = 1 hour, 4 = 2 hours, 5 = 4 hours and 6 = 24 hours after exercise). Significant changes in mean endostatin concentrations were observed after exercise at 10 minutes, 30 minutes, 1 hour and 2 hour intervals. (* = P value <0.05 between basal and time point, ** = P value <0.001 between basal and time point). Clear circle represent outlier.

6.4.3.2 Correlations of endostatin concentration and the changes in endostatin concentration at different intervals with anthropometric, metabolic and exercise parameters

Extensive Pearson correlation tests were done to investigate any link between endostatin concentration and the changes in it at different intervals after exercise with different physical and metabolic factors in detail. No conclusive or significant correlations of importance were observed. They are presented in appendix “H” in tabular forms.

6.4.4 Baseline VEGF concentration

Line plots showing the plasma VEGF concentration for all volunteers are illustrated in figure 6-5. No specific pattern of increase or decrease in VEGF

concentration after exercise was observed. Three out of eight participants exhibited a decrease immediately after exercise. One of the volunteers barely showed any effect of exercise on VEGF at different time points, while 4 showed an initial increase followed by a decrease at some points. The mean VEGF concentration determined at rest was 91 ± 35 pg/ml (ranging 59 -168 pg/ml).This is slightly higher than the VEGF concentration of 75 pg/ml in chapter 4.

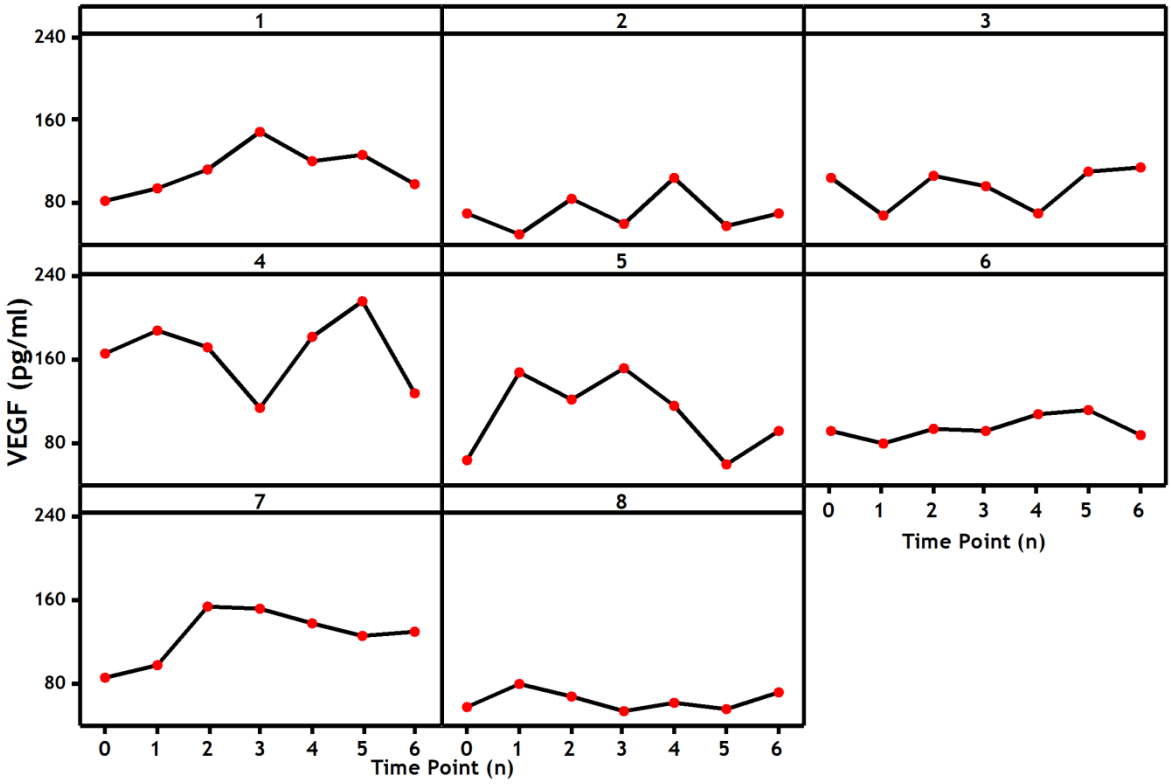


Figure 6-5; Line plots with VEGF concentration before and at different intervals after maximum intensity exercise for all participants

VEGF concentrations for all participants are shown in the individual line plots. Time points on the horizontal axis shows the time points for blood sampling (0 = before exercise, 1= 10 minutes, 2 = 30 minutes, 3 = 1 hour, 4 = 2 hours, 5 = 4 hours and 6 = 24 hours after exercise). Panel 8 can be clearly seen with no specific changes or pattern.

6.4.4.1 Effect of exercise on mean VEGF concentration

The effect of exercise on VEGF concentration was also investigated. The mean VEGF concentrations were: 102 ± 46, 115 ± 35, 109 ± 40, 113 ± 38, 108 ± 53 and 99 ± 23 pg/ml at 10 minutes, 30 minutes, 1hour, 2 hours, 4 hours and 24 hours respectively, after the exercise. These changes are equal to 12% (P = 0.43), 26%

(P = 0.04), 19% (P = 0.32), 24% (P = 0.067), 19% (P = 0.077) and 9% (P = 0.36), respectively. The increase in VEGF concentration was only significant at 30 minutes interval (P = 0.04). The VEGF concentration remained higher than basal at all time points up to 24 hours after exercise, as shown in figure 6-6.

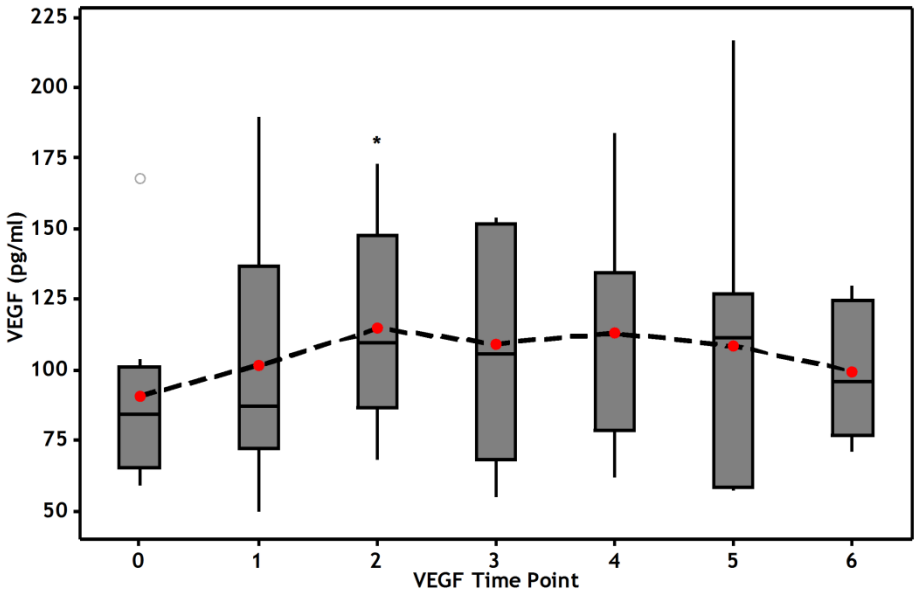


Figure 6-6; VEGF concentrations before and at different intervals after maximal exercise test

Figure shows the VEGF concentrations before and at different time points after exercise (0 = before exercise, 1= 10 minutes, 2 = 30 minutes, 3 = 1 hour, 4 = 2 hours, 5 = 4 hours and 6 = 24 hours after exercise). The significant change in VEGF concentration was only observed at 30 minutes after exercise (P = 0.04). Clear circle shows outlier in basal VEGF.

6.4.4.2 Correlations of VEGF concentration and the change in VEGF at different intervals with anthropometric, metabolic and exercise factors

Similar to endostatin, correlations for VEGF were also determined. No solid conclusion can be drawn from it and therefore, they are included in the appendix “H” in tabulated form.

6.4.5 Correlations of changes in endostatin concentration with changes in VEGF

Finally, the changes in endostatin concentrations at different time intervals after exercise with the respective changes in VEGF were carried out. No significant correlations of statistical importance were observed, as shown by the

Pearson correlation coefficient (r) and significance of the correlation (P) values in table 6-5.

		ΔVEGF10	ΔVEGF 30	ΔVEGF 60	ΔVEGF 120	ΔVEGF 240	ΔVEGF 24hr
ΔES10	r	-0.391	-0.136	-0.321	-0.588	0.004	-0.071
	P	0.339	0.747	0.438	0.125	0.993	0.867
ΔES30	r	-0.208	0.295	0.070	-0.221	-0.005	0.304
	P	0.620	0.478	0.869	0.599	0.990	0.463
ΔES60	r	-0.223	0.005	-0.015	-0.142	-0.178	0.108
	P	0.596	0.990	0.973	0.738	0.673	0.799
ΔES120	r	0.021	0.155	0.393	-0.065	-0.834	0.637
	P	0.961	0.713	0.336	0.878	0.010	0.090
ΔES240	r	0.110	-0.377	-0.219	-0.404	-0.583	0.017
	P	0.796	0.357	0.602	0.321	0.129	0.968
ΔES24	r	-0.463	-0.761	-0.585	-0.790	-0.590	-0.252
	P	0.248	0.028	0.128	0.137	0.123	0.547

Table 6-5; Correlations of the changes in Endostatin with changes in VEGF

Pearson correlation shows the association between changes in VEGF (ΔVEGF) with changes in endostatin concentration (ΔES) at respective times and different time intervals. As shown by the P values, no correlations of statistical significance were determined. r; Coefficient value of Pearson correlation

6.5 Discussion

This study was aimed to determine the effect of maximal intensity exercise to volitional exhaustion (VO_{2max}) on plasma concentration of endostatin and VEGF in young healthy participants. Additional aims include the correlation of basal endostatin and VEGF with anthropometric, physical and metabolic characteristics of the individuals. Moreover, the effects of changes in endostatin concentration on the respective changes in VEGF were also investigated. This experiment followed the same approach, as in chapter 4 with some changes. It was hoped, that this study would provide more comprehensive results, in terms of bigger changes in concentration of endostatin and VEGF for longer durations after maximal exercise test and its association with metabolic factors. The following changes were made to the protocol;

i. Exercise in this study was maximal to volitional exhaustion.

ii. Endostatin and VEGF were measured simultaneously.

iii. Blood samples were taken more frequently and for a longer duration.

iv. Metabolic and lipid profiles were taken into consideration to check for the possible correlation with endostatin and VEGF.

6.5.1 Major findings of the study

This study provided more conclusive results in terms of effect of exercise on endostatin concentration. The change in endostatin concentration was clear and more pronounced than studies in chapter 4. Moreover, this study also verified the results in the previous studies i.e. the basal endostatin concentration remained consistent and higher intensity of exercise significantly increased the endostatin concentration for up to 2 hours. Additionally, no correlations of statistical significance between anthropometric characteristics including body fat percentages and metabolic parameters including fasting lipid profile, fasting blood glucose and insulin with endostatin and VEGF were observed.

Exercise also influenced VEGF concentration transiently and only at 30 minutes interval increase in VEGF was statistically significant. The participants showed

the increase in VEGF concentration at different time points after exercise. Due to this variation in person to person response after exercise, quantification of VEGF is difficult. However, it is worth noting that even those participants who showed an immediate decrease in VEGF after exercise, later on exhibited a concentration higher than basal.

6.5.2 Study design

All the experiments were concluded safely. The risks associated with maximal exercise were managed successfully by the protocol adopted. Standardised protocols were followed to deliver the maximum effort of the participants. The sample size calculated for this study was 16. Based on the results in previous chapter, it was anticipated that high intensity exercise will change the endostatin concentration by 20%. However, the experiment was stopped after recruiting 8 participants; partly due to financial and partly due to time constraints. From the results obtained, the influence of exercise on endostatin is very clear and more than anticipated (38%). It is unlikely, that including more participants would drastically change the results for endostatin.

However, the influence of exercise on VEGF was not clear and participants exhibited a variable pattern of response to exercise, as discussed earlier. The VEGF concentration increased for all participants but at different time points. It would require a larger study with more participants. Completing this project is one of the potential future work areas.

6.5.3 Discussion of the results and comparisons with the published literature

6.5.3.1 Endostatin and exercise

High intensity exercise showed significant increase in endostatin. Although the endostatin concentration after exercise in plasma was higher than basal, up to 4 hours but significantly high concentrations were observed up to 2 hours. The results in this study confirm the previous results in chapter 4, when the volunteers exercised at 70% and 80% predicted maximum heart rate. Moreover, these results are also consistent with previous published studies carried out by many researchers (Gu et al., 2004, Suhr et al., 2007, Sponder et al., 2011,

Sponder et al., 2013, Bruserud et al., 2005). It seems that short bouts of relatively high intensity exercise increase the endostatin concentration transiently for duration up to 2 hours.

The extent of change in endostatin concentration was, however, different and can be attributed to the difference in mode, intensity and physical characteristics of the participants. Physical fitness seems to affect the degree of increase in endostatin concentration after exercising (Sponder et al., 2013). Gu and his colleagues have reported a significant increase up to 6 hours after exercise (Gu et al., 2004). It could be implied that many other reasons, such as easy fatigability, presences of co-morbid conditions and age of the participants may contribute to the extent of difference as well. This could be true as one study reported increase in endostatin concentration only in healthy and young individuals and not in old age individuals (Bruserud et al., 2005). However, our results can be compared with study done by Suhr, who also found significant increase lasting for two hours after exercise (Suhr et al., 2007).

The effects of long term physical activity on the basal endostatin concentration are contradictory. Sponder and his colleagues reported significantly high basal endostatin concentration in athlete males and females compared to their controls. It was assumed, that high basal endostatin concentration is due to the regular physical training of the athletes (Sponder et al., 2013). In contrast, decrease in basal endostatin concentration after 6 months of regular physical training in 50 – 60 years old individuals (Brixius et al., 2008), as well as in long and short track elite runners have been reported (Suhr et al., 2010). The mechanism behind these differences is not clear and needs further studies.

It can be seen in section 6.4.3.1 that the maximum increase of 34% was observed at 30 minutes interval. This finding is interesting because in the studies outlined in chapter 4, the concentration of endostatin was determined at 10 and 60 minutes intervals after exercise. It was not intentional to take only at 10 and 60 minutes intervals. Adding more time points in this study shows the development of the research hypothesis. It is possible that the interval at which exercise had the more pronounced effect might have been missed. However, it is suggested for future studies that multiple blood samples at different intervals especially at 30 minutes should be taken.

6.5.3.2 Correlation of endostatin concentration with metabolic profile and anthropometric characteristics

The other aim of the study was to correlate the basal endostatin concentration with anthropometric and metabolic parameters of the participants. Good metabolic profile indicates healthy endothelial status and well being of the individual. It was hypothesised, that they might affect basal endostatin concentration, as well as the change in it after exercise. No correlations of significant importance were observed either with the basal endostatin concentration or the change in it at any time point after the exercise, as shown in tabular form in appendix "H". To the author's knowledge this is a complete novel finding. It is interesting due to the fact that deranged lipid profile, glucose intolerance or insulin insufficiency play important roles in early endothelial events leading to atherosclerosis. Despite endostatin being present in blood and basement membrane, it did not show any interactions with these parameters. However, sample size is small to appreciate any such associations and study with a bigger sample size will be required to draw solid conclusion.

Finally, no correlation of statistical significance was observed between endostatin concentration and anthropometric measurements, which are in line with previous results in chapter 3 and 4.

6.5.3.3 Correlations of endostatin concentrations with exercise parameters

The exercise intensity in this series of experiments was higher than used previously. The duration of near maximal exercise was shorter and the use of breath by breath measurement allowed the gas exchange to be monitored in real time. Different features of exercise showed no statistically significant correlations with the changes in endostatin concentrations, at different time points after the exercise. These results are different from Gu who reported a strong linear correlation between change in exercise and peak oxygen consumption (Gu et al., 2004). In author's opinion, the results in our study are more reliable because the values for the peak oxygen consumption, in Gu's study, were estimated by calculation rather than measured. Moreover, Gu's findings could not be confirmed in later studies (Sponder et al., 2013, Sponder et al., 2014). It was observed consistently that moderate to high intensity exercise increase the endostatin concentration without direct correlations with any

exercise feature. From this it can be assumed, that exercise does change the endostatin concentration by altering different mechanisms indirectly including increasing expression of enzymes involved in endostatin release (Suhr et al., 2010).

6.5.4 VEGF and exercise

Mean basal VEGF concentration of 91 + 34 pg/ml in this study group is higher than that seen chapter 4, which was 75 + 36 pg/ml. However this difference is not significant. It is clear from the broad ranges, shown in figure 6-6, that there is substantial variation in basal VEGF concentrations. A wide range of basal concentrations, between 98 to 485 pg/ml, has been reported and this is attributed to the differences in genetic regulation and makeup of individuals (Gunga et al., 1999). Such variations make it difficult to quantify the increase in VEGF after exercise.

The mean VEGF concentration increased initially after exercise until 30 minutes. After this time point, a pattern of increase and decrease can be observed, as shown in figure 6-6. It can also be seen that mean VEGF concentration was never found to be lower than basal during this experiment. These results confirm our earlier results in chapter 4, of increase in mean VEGF concentration after exercise with no specific pattern.

The literature about exercise as a regulator of VEGF is conflicting and limited. On one hand, increases in VEGF concentrations after exercise have been reported (Czarkowska-Paczek et al., 2006, Hiscock et al., 2003, Kraus et al., 2004, Schobersberger et al., 2000, Rullman et al., 2007). The extent of increase in mean VEGF after exercise reported, varies from as low as 30% (Kraus et al., 2004) to as high as 240% (Schobersberger et al., 2000) and as acutely as 30 minutes post exercise to as long as 5 days post exercise. These changes may reflect different exercise modalities.

On the other hand, a decrease in mean VEGF concentration after acute exercise has also been reported by (Gustafsson et al., 2002, Gu et al., 2004). Again the modes of exercise in the above studies were completely different.

In Gustafsson study, the participants performed one knee extension for 7 sessions of 45 minutes over a period of 10 days. Timings of the blood sample collection are crucial, as the first sample was taken before 1st exercise session and the last sample was taken 24 hours after the 7th exercise session. In our study it was observed that exercise affect VEGF concentration transiently. The VEGF concentration tends to drop back towards normal at about 1 hour after exercise. So it is possible, that VEGF concentration 24 hours after exercise may be more or less the same as basal VEGF concentration.

Similar results to Gustafsson have also been reported in another study, where long term endurance exercise programme for 6 months, showed no alteration in VEGF concentration. Interestingly, post intervention plasma samples were taken, on next day after the last exercise session (Brixius et al., 2008).

However, the study done by Gu, has shown the decrease in mean VEGF concentration significantly up to 6 hours, a finding which could not be confirmed by published literature or this study.

There is convincing evidence in the literature, that acute exercise bouts increase VEGF concentration in skeletal muscles and its subsequent release to venous circulation (Kraus et al., 2004, Hiscock et al., 2003, Höffner et al., 2003) but not to arterial circulation(Hiscock et al., 2003). It is plausible that after release from the stretched muscles during exercise into the venous system, the VEGF is taken by other tissues, not directly involved in the exercise. However, the possibility of increase in VEGF from cells like platelets and myocytes cannot be overruled, as electrical stimulation of cardiac myocytes has been shown to increase VEGF release (Seko et al., 1999).

6.5.4.1 Correlation of endostatin and VEGF concentrations before and after exercise

Finally correlations between endostatin and VEGF concentrations before and at all time points after exercise were carried out. Concurrently the correlations between the changes in both mediators at each time point were also checked, as shown in table 6-5. No correlations of statistical importance at any time point were observed. These results are in line with our earlier results in chapter 4.

It is established from the literature that endostatin antagonises the signalling mechanisms of VEGF (Eriksson et al., 2003, Yamaguchi et al., 1999). However, it is clear from the results, that increase in endostatin concentration after high intensity exercise has no negative effect on the plasma VEGF concentration, a finding which is in dispute with the results published by Gu and his co-workers (Gu et al., 2004). An increase in VEGF concentration after exercise seems more logical as exercise increase the expression of mRNA in skeletal muscles (Hiscock et al., 2003), which enhances the production of VEGF in skeletal muscles and favours the release of VEGF from tissue to circulation (Rullman et al., 2007).

In conclusion, the high intensity exercise increases the mean endostatin more prominently and for a longer duration than VEGF without any important interaction between the two mediators.

6.5.5 Strengths of the study

This study investigated both the mediators at the same time with no delay in analyses compared to the previous study. Additionally, body fat compositions and fasting metabolic profiles of the participants were also investigated for correlation with endostatin and VEGF. Similarly, blood samples were taken more frequently for longer period after exercise, which provided more clear trends in distribution of these two mediators in circulation after exercise. Moreover, provisions of resting time between cannulation and basal sample and calculating the exercise parameters with breath by breath analyser were done to get more accurate results.

6.5.6 Limitations of the study

Sample size of this study is low and the results could be more reliable with the inclusions of more participants, especially females. However, it was difficult to recruit sedentary females for high intensity exercise to volitional exhaustion. Non invasive techniques like flow mediated dilatation percentage (FMD) or reactive hyperaemia index (RHI), which give a more accurate status of endothelial insufficiency than metabolic profiles, should have been considered.

FMD and RHI are the more sophisticated non invasive techniques in research and diagnostics to assess the endothelial insufficiency. In these techniques

vasodilator responses of the peripheral arteries are measured with ultrasound before and after applying pressure through a blood pressure cuff. The shear stress causes endothelium dependent dilatation. Median dilatation of more than 10.7% signifies a normal endothelial function. Participants with less than 10.7% dilatation are more prone to CVD development. The tests by these methods correlate with invasive methods and are suitable for normal and healthy subjects (Raitakari and Celermajer, 2000).

The exercise is of high intensity and relatively short duration. Thus sweat loss is unlikely to exceed 0.25-1% of body weight. This was offset by giving the participants fluids before and after exercise. The time course of changes in water balance is impossible to predict. The author is unaware of any potential effect of dehydration on endostatin concentration after exercise.

It could be possible that endostatin correlates with the hydration status of the participants. Hydration status (plasma osmolailty electrolytes and haematocrit, urine osmolailty and specific gravity) of the participants should have been included and checked for any correlation with endostatin in this or the previous studies.

7 General Discussion

7.1 Overview

In recent era, cardiovascular disease has remained a major health problem causing more mortality and morbidity than infectious disease. The incidence and prevalence of cardiovascular disease is on the rise and has placed a huge burden on the economy of developing and developed world equally; through cost of the treatment and wasting productive years of life. Cardiovascular diseases are associated with building up of plaque of fatty and fibrous streaks in the intimal layer of muscular arteries, especially coronary arteries. The process is called atherosclerosis which narrows the lumen of the arteries, compromises the blood supply to the area and causes thrombosis and major ischemic events (Griffin, 1999). The major risk factors for CVD include; sedentary life style, smoking, dyslipidaemia, high blood pressure and obesity.

Uncontrolled angiogenesis in the atherosclerotic plaque can increase the size of the plaque with rupture or complete occlusion of the vessel leading to major cardiovascular event (O'Brien et al., 1994). Angiogenesis is controlled by endogenous mediators, of which “VEGF” as angiogenic and “endostatin” as angiostatic mediators have been recognised as key players controlling angiogenesis.

Sedentary life style remains one of the isolated and leading risk factors for CVD. The importance of physical activity in decreasing the morbidity and mortality due to CVD is accepted globally (Sofi et al., 2008). Regular physical activity decreases the CVD related events directly, through improving and activating different cardio protective mechanisms and indirectly through modifying other risk factors for CVD; including diabetes, dyslipidaemia, obesity and blood pressure (Golbidi and Laher, 2011). Cardioprotective mechanisms include; heat shock protein production, improving myocardial antioxidant capacity and coronary collateral circulation etc.

The world has never witnessed as many advances in medical sciences as these days in the human history. Thanks to the research in science that has enabled humans to live longer and joyful life than ever before. Particular advances have been made in medicine and public health which have resulted many more people living longer (UNDP, 2001). In UK over the last 5 decades average life expectancy

has increased by 10 years for males and 8 years for females (office for National Statistics UK, 2010). In addition to increase in work force, the elderly population also faces a great risk of contracting health problems regularly and mortality of in this age group is 9 times higher than adults (Office for National Statistics UK, 2013b). This problem is further complicated by lack of regular physical activity which is one of the major issues in this age group and needs to be addressed. Despite one of the best health services in world, the National Health Service Scotland (NHS) faces a great challenge of improving the health of poorest sections of society. The difference in life expectancy between affluent and non-affluent societies in Scotland is 10 years and inappropriate diet and physical inactivity have been considered as some of the main root causes. The Scottish Government is keen in taking initiatives like healthy eating, increasing physical activity and tackling obesity (The Scottish Government, 2008).

Like every other walk of life, research has extensively focused on exercise and the cardioprotective mechanisms. The role of physical activity and regular exercise on decreasing the incidence, prevalence, mortality and morbidity has been established. That is the reason, qualitative and quantitative regular physical activities are recommended by the health organisations, as preventive measures for CVD and to enhance quality of life. However, the mechanisms by which physical activities provide cardiac protection are not completely clear. It might be due to several causes. Exercise modifies many local cardio-protective mechanisms as discussed in section 1.3.9.4. In addition, it simultaneously affects various systemic features such as blood pressure control, cardio-respiratory fitness, and humoral features such as better glycaemic control, improving insulin sensitivity and lipid metabolism.

Considering the problems associated with the understanding of structured physical activities and cardiac adaptability mechanisms, research presented in this thesis is aimed at, understanding and evaluating the effects of different intensities of exercise activities on endostatin and VEGF concentration in plasma in young healthy human volunteers. More precisely, acute effects of exercises of 4 different intensities equivalent to mild, moderate, vigorous and maximal effort were investigated. Moreover, the association of these mediators with different anthropometric characteristics and exercise parameters were also determined. To the best of author’s knowledge, this is the first study to demonstrate not only

the effects structured physical activities of different intensities on endostatin and VEGF but also correlating them to different variables discussed, throughout chapter 3, 4 & 5. It also includes first study of its kind evaluating the bioavailability of endostatin concentration in males and females from different ethnic groups.

7.2 Summary of the findings in this thesis

This section outlines the research questions in chapter 1 of this thesis and the results that were obtained in the subsequent chapters (3, 4 & 5), with some discussions.

7.2.1 Bioavailability of basal endostatin concentration

Endostatin was detected in plasma of all volunteers. In chapter 3, the mean endostatin concentration measured for all volunteers were 105 ± 12 ng/ml. The concentration measured in the subsequent experiments in chapter 4 & 5 showed almost the same results. Considering time to time and person to person variations, we can safely say that basal endostatin concentration ranges between 80 and 120 ng/ml.

The bioavailability of endostatin in plasma is not completely clear. Different mechanisms are proposed to be responsible for the endostatin concentration in plasma. Some of the possibilities are as under;

Endostatin associated with collagen XVIII is the most frequently available form in plasma. Collagen XVIII is released by fibroblasts and hepatocytes (Seppinen and Pihlajaniemi, 2011). It might be possible that same cells are involved in the release of endostatin into the circulation.

Endostatin has been reported to be present within the platelets and are released from the platelets when treated with thrombin (Ma et al., 2005) and ticlopidine (Ma et al., 2001).

Similarly, collagen turn over in different tissues occurs on regular basis, which varies with a mean rate of 3 to 5 % per day (Laurent, 1987). Basal levels of

circulating endostatin may be generated as a result of physiological collagen turnover, suggesting its importance in angiogenesis regulation during normal conditions (Gu et al., 2004).

The release of endostatin from endothelial cell lines by exposing it to hypoxic stimuli and reactive oxygen species has also been reported. The release of endostatin was also enhanced by increased nitric oxide concentration. This assumes that NO signalling pathway (nitric oxide/cGMP pathway) and enhanced production of NO may augment the release of endostatin into circulation (Deininger et al., 2003).

As mentioned earlier, the origin of endostatin is not completely understood, but based on the published literature; we could say that fibroblasts and hepatocytes cells, under general and other mechanisms listed above, under special circumstances, appear to be the major sources of endostatin in the circulation. This is because fibroblasts are the main source that synthesizes the components of extracellular matrix and many collagens including collagen XVIII. Additionally, high circulatory endostatin concentration in patients with fibrotic lung diseases with various degrees of fibrosis, has been reported (Hebbar et al., 2000, Seko et al., 1999). On the other hand, all other mechanisms including release from platelets, collagen turnover and NO signalling pathway could be responsible for release under special circumstances like physical activity, cell stress and hypoxia.

7.2.2 Difference between basal endostatin concentration in males and females

It was hypothesized, that females might possess less basal endostatin concentration than males. Results in chapter 3, show that females have slightly less mean endostatin concentration of 102 ng/ml than males 107 ng/ml, but the difference is not significant.

Results in chapter 3 and most of the published literature agree that females have non-significantly less endostatin concentration than males. Increased number of platelets and increased collagen turn over in males might be possible reasons to their higher basal endostatin concentrations. It could be true because

collagen is the most abundant protein present in the body and makes 20- 25% of the total body protein (Ricard-Blum, 2011, Kjaer et al., 2005). Furthermore it has been reported that the collagen turn over increases with physical activity, enhanced blood flow and mechanical loading (Kjaer et al., 2005). Based on our observation in table 4-1 males participants were significantly heavier than females ($P<0.001$) and might have more collagen mass and turn over than females. Enzyme like MMPs in the extracellular matrix are directly involved in release of endostatin (Sasaki et al., 2002).

Only the study carried out by Sponder reported a higher basal endostatin concentration in females than males and a hormonal influence was suggested for it(Sponder et al., 2014). Estradiol has been shown to increase endostatin concentration along with MMP- 2 and MMP 9 (Nilsson et al., 2007, Nilsson and Dabrosin, 2006). Diurnal variations (Glenjen et al., 2002) and influence of age (Bruserud et al., 2005) and regular physical activity on plasma concentration of endostatin has also been reported (Sponder et al., 2013). It is plausible that difference in baseline endostatin concentration is due to simple biological variations during the course of the day.

7.2.3 Difference in basal endostatin concentration with age and other anthropometric characteristics

It was also anticipated, that age and other anthropometric characteristics might influence the basal endostatin concentration. No significant differences in endostatin concentration were observed for different variables including age, weight, height, BMI, blood pressure and previous physical activity levels of the volunteers. The Pearson correlation analyses did not show any correlations between the factors mentioned above and endostatin concentration as shown in the result sections of chapter 3, 4 & 5. More over no difference in mean endostatin concentration was observed in volunteers belonging to different ethnic groups including European, Middle Eastern and South Asians.

These findings are really interesting as during the whole research, we thoroughly investigated the correlations of endostatin with the different anthropometric characteristics. Age and blood pressure were the two variables, which in some

set of experiments showed significant positive and negative correlations respectively. The possibility of these findings by chance could not be over ruled because; firstly the findings were only determined in some data sets. For example in chapter 3, age showed a negative correlation for males especially South Asians and not for females or males belonging to other ethnic groups. Secondly, the patterns in correlations observed in chapter 4 were not uniform and despite the same participants in trials, correlation between age and endostatin concentration could not be observed on different days. Thirdly, as individual variations in endostatin concentration were observed in chapter 4, it would be very difficult to report any such correlations in small data set. In fact, experimental models with large sample size, different ages and longitudinal observations would be required to safely speculate about these correlations.

7.2.4 Effect of mild intensity exercise (60% heart rate) on endostatin

The results of chapter 4 show that low intensity exercise decreases the mean endostatin concentrations by 4% and 3% at 10 minutes and 60 minutes interval after exercise. These changes were not significant but unexpected. Individually 64% of the participants showed an acute negative change after exercise. No differences in change on gender basis and exercise mode were observed.

This finding is interesting, because it was hoped that exercise will increase the mean endostatin concentration as most of the published literature, at the time this study was carried out, reported increase in endostatin concentrations with physical activities. Study carried out by Makey and her colleagues recently, reported almost the same results in females after performing exercise at 55-59% heart rate on treadmill for 30 minutes (Makey et al., 2013).

As discussed in chapter 4 and 6, no correlations between exercise parameters and change in endostatin concentration were observed, it could be assumed that exercise indirectly modifies the different mechanisms concerned with endostatin release, such as MMP, collagen turn over, release from platelets etc. After looking at the effect of higher intensities of exercise on endostatin in the subsequent experiments, we can safely assume that low intensity of exercise

was not enough to initiate the release of endostatin. However, even on that low intensity exercise 36% of the participants showed an increase in endostatin concentration. Therefore, it can be assumed, that intensity induced activation of the above mechanisms may have different thresholds for sensitivity which varies from person to person.

7.2.5 Effect of moderate intensity exercise (70% heart rate) on endostatin

Results in chapter 4 shows that exercise at intensity of 70% predicted maximum heart rate, increased the mean endostatin concentration significantly, by 18% and 19% at 10 minutes and 60 minutes intervals, respectively.

7.2.6 Effect of high intensity exercise (80% heart rate) on endostatin

High intensity exercise increases the mean endostatin concentration by 38% and 14%, at 10 minutes and 60 minutes intervals respectively, as shown in chapter 4. No difference was observed between the degree and pattern of change for males and females.

7.2.7 Effect of maximal intensity exercise (100% heart rate) on endostatin

Exercise at maximum intensity increased the endostatin concentration significantly up to 2 hours after exercise. The highest increase in mean was 34% at 30 minutes interval after exercise. These results are shown in chapter 5 of this thesis.

From the results in chapter 4,5 &6, it is clear that high intensity exercise increase the mean endostatin concentration in plasma up to 2 hours after exercise. The results in this chapter also suggest that degree of change in endostatin concentration is independent of gender or exercise mode (walking/running). However, it is still not clear why endostatin concentration

increases after exercise and what physiological functions it performs. Some of the possible explanations are as follows;

Endostatin is proteolytically released from collagen XVIII by the action of many enzymes including cathapsin L (Felbor et al., 2000) and MMPS especially MMP2 & 9 (Ferreras et al., 2000). Exercise has been reported to increase the expression of these enzymes which in turn enhance the generation of endostatin (Suhr et al., 2010).

Exercise has been shown to influence collagen turn over (Kovanen and Suominen, 1989) which is thought to be one of the sources for direct increase in endostatin production (Gu et al., 2004). However, indirect effects of collagen turn over on the enzymes involved in the release of endostatin could also possibly increase the endostatin in circulation.

Exercise increases the metabolic demands of the cells for oxygen (O_2) for energy. It has also been established, that inhibition of NO synthase reduces the endostatin concentration (Deininger et al., 2003) and that endostatin causes vasorelaxation by up regulating production of NO (Wenzel et al., 2006, Sunshine et al., 2012). It is possible that the increase in endostatin concentration may be one of the mechanisms for vasorelaxation during exercise, to ensure optimal oxygen and nutrients supply to the body. It can be suggested, that besides its role in angiogenesis, increased endostatin concentration help in maintaining optimal vascular tone during stress time for effective nutrients supply.

7.2.8 Correlations of change in endostatin concentrations with anthropometric and metabolic factors

Results in chapter 4 & 5 showed, that the changes in endostatin after different exercises were independent of anthropometric, physical and metabolic characteristics.

To understand what are the possible anthropometric and metabolic factors that can potentially affect the physiological change in endostatin, correlations were carried out. No correlations of statistical importance were observed. Metabolic factors were also taken into consideration, due to its active involvement in the

early events in endothelium leading to atherosclerosis. It might be possible that because recruitment of only healthy volunteers with not much difference in their metabolic, endothelial and vascular status were carried, results are inconclusive. Because it was not the initial aim and these factors were included during the maturation of research process over the time, the sample size selected might not be enough to draw a solid conclusion. Large scale studies with the inclusion of volunteers with known endothelial and vascular status would be required.

7.2.9 Effects of exercise on VEGF

On all exercise intensities used in our experiments, increase in VEGF concentration in plasma was observed. In some cases, this was minimal and insignificant, while in other cases it was statistically significant. The impacts of exercise on VEGF increase were transient and for short duration without any specific pattern. Moreover, the change in VEGF after exercise was not much different in males/females or walking/running group.

The increase in VEGF concentration acutely after exercise has been reported extensively and discussed in chapter 4 & 5. The possible mechanisms for this increase and subsequent physiological action after increase could be;

I. Local up-regulations in skeletal muscles as a result of tissue hypoxia by exercise (Kraus et al., 2004).

II. mRNA expression and VEGF protein concentration has been shown to increase in skeletal muscles (Rullman et al., 2007) and interstitial spaces (Höffner et al., 2003), which in turn can enhance the production and release of VEGF in to blood.

III. VEGF is also found in platelets (Ma et al., 2005) and local release from these cells could not be excluded.

IV. It was suggested, that mechanical stress in the exercising leg could possibly cause the increased production of VEGF by myocytes which

accounts for local angiogenesis as well elevated plasma concentration (Hiscock et al., 2003).

V. Additionally, it is also possible that the increased production of VEGF not only occurs in the skeletal muscles but in many other cells and tissues, which collectively increase the release of VEGF into blood and consequently the plasma concentration raises.

Just like endostatin, the mechanism for the increase in VEGF concentration after exercise, is still not completely clear due to existence of multiple possible release mechanisms and the ability of exercise to influence these mechanisms equally. However, increase in VEGF after low intensity exercise shows that even this intensity is beneficial by enhancing the VEGF production and promoting angiogenesis at tissue levels. Hence, it can be suggested, that as hypoxia is the main stimulator of VEGF release, and exercise by producing hypoxia in skeletal muscles are responsible for production and subsequent release of VEGF into circulation.

The consequential decrease in VEGF after initial increase could be due to simultaneous increase in binding capacity of VEGF to its receptors. Subsequently, they can also be used at micro-vascular levels to initiate angiogenesis. However, it is also possible that exercise may increase the number of binding sites for VEGF. Moreover, as the promotion of angiogenesis at tissue levels after exercise along with increase VEGF concentrations is established, it is equally possible that exercise induces the VEGF release from cells like platelets within the circulation which are then taken up by the tissues.

7.2.10 Correlation of endostatin and VEGF

No correlations of statistical importance were observed between plasma VEGF and endostatin concentrations, before or after exercise in chapter 4 & 5. The correlations between the extents of changes were also insignificant.

It has been established, that endostatin inhibits VEGF induced endothelial cell proliferation and migration. It was thought, that as endostatin possesses anti VEGF properties, exercise induced increase in its concentration will conversely

affect VEGF concentration as reported (Gu et al., 2004). Exercise induced changes in endostatin concentrations were found unrelated to VEGF concentrations. It is possible, that exercise affects the concentration of endostatin and VEGF through completely different mechanisms.

7.3 General considerations

Though the effects of exercise on these mediators were transient, they were important and not trivial. Single bouts of moderate to high exercise brought about 20% to 40% changes in endostatin concentration with no gender difference. It is important to note, that the intensities of exercise were managed comfortably by relatively untrained individuals. As the exercises were carried out according to the comfort of the individuals, the least fit volunteers did not perform as well as fit volunteers in the trial, but even for them, the influence of exercise on these mediators was substantial and prominent. In fact, 21 out of 22 volunteers showed a significant increase in endostatin concentration with exercise and the latter, as a beneficial tool for increasing circulatory endostatin concentration, can be suggested.

The data from this study suggest that plasma concentration of endostatin is more profoundly influenced by exercise than VEGF. However, as angiogenesis in skeletal muscles as a result of exercise mediated by VEGF is an established fact, it can be speculated, that exercise might increase the uptake of VEGF into the tissue along with enhanced local production. Further research involving kinetic tracers may be helpful in elucidating these speculations. Kinetic tracers are used widely in research and mainly deal with kinetics of different substances, their production and transport into cells, biochemical transformation and utilization. For this purpose tracers like dye, labelled radioactive or stable isotopes are used (Wolfe, 1992). It is also worth mentioning that other mechanisms, which are not yet discovered, may be involved in changing the concentrations of these mediators after moderate to high exercise. The clarification of these mechanisms, though necessary, clearly would require specialised equipments and expertise. These factors might be an area for future research, but even without the knowledge of exact mechanisms, it is clear that moderate to high intensity exercise clearly affects endostatin in different individuals with diverse

characteristics. Therefore, moderate to high intensity exercise can be implemented as preventive measure for atherosclerosis.

However, the question of incorporating structured physical activity in life style to the intensity levels, where it influences the endostatin concentration, is a matter of debate. The answer to this question based on the research in this thesis, is “yes”; because the exercise intensity of 70% predicted maximum heart rate was easy to incorporate into the relatively sedentary populations, due to the fact that no drop outs were observed during the experimental work and the exercise was perceived as “light” by most of the participants. Moreover, as the pattern of change in endostatin was rarely influenced by mode of exercise (walking/running); participants with potential inability to run can also achieve the required intensity easily by walking uphill.

With advances in technology in recent times, life style factors especially physical activity and diet has acquired a crucial role in determining the health of general population. The scientific evidence of physical activity on improving health indicators is undeniable and certain amount of different physical activities including cardio-respiratory fitness exercises beyond daily activities, are recommended and vital for every individual’s health. However, the physical activity of individuals can be modified according to the needs, comfort and health status. Physical activity less than recommended by the health organizations would undoubtedly prove beneficial in some respects. In ongoing un-published research by another group in this department, the preliminary results show that even interrupting long sitting intervals during work by mere standing periodically, improves the risk associated with CVD (Personnel communication September 2014).

As CVD is a multi-factorial complex health problem. Interventions implied for prevention should also be multi-factorial and multi-faceted. This would include encouraging physical activity to the minimum recommended levels, diet modifications, public awareness through print and electronic media and addressing the cultural and religious aspect of the problem.

Individual compliance to adhere to regular physical activity has remained one of the problems in implementing and prescribing physical activity. As per WHO

statistics, 60% of the world population do not achieve the required physical activity partly due to less engagement in physical activity and more work burden. This would require making exercise a joyful and pleasant event. However, behavioural orientation towards decreasing the risk of cardiovascular diseases by exercise, regular risk evaluations for CVD and gradual increase in exercise intensity over certain period may equally enhance the compliance and safety of exercise.

7.4 Implications

It is very premature to suggest clinical implications in the management of atherosclerosis, from the results obtained in this thesis. However, potentially many implications of this study can be taken into consideration.

Endostatin has been found to be one of the key regulators in angiogenesis and its beneficial role in angiogenic dependent diseases, provide it with the advantage to be considered seriously. As shown in this thesis, plasma bioavailability of endostatin coupled with the increase in its concentration, after physical exercise also points potential application of exercise with certain intensities (70% heart rate and above) necessary into life style. Exercise intensity of 70% predicted maximum heart rate was relatively low, easily applicable and perceived as light by both males and females. However, due to the fact that studies were carried out in healthy individuals, competent enough to perform the exercise, these intensities might not be easy to incorporate in life style of people with different ages and co-morbid conditions.

Moreover, young adults performing more than 3 sessions of endurance exercise (45 minutes) per week for 6 months have shown an increase in the basal endostatin concentration compared to sedentary controls (Sponder et al., 2014). It can be assumed that incorporation of regular physical activities would increase the basal endostatin concentration to levels, enough to counteract atherosclerosis. Chances of rupture of plaque remain high due to intra- plaque angiogenesis and endostatin has been shown to stabilize the plaque by inhibiting this process. The results in this thesis contribute to the understanding of one of the links between physically active life style and decrease risk of atherosclerosis.

Endostatin is the first angiogstatic mediator to reach clinical trials. It was reported with no toxicity and well tolerated drug showing less than expected results on stopping angiogenesis (Eder et al., 2002). Many reasons, including active form, mode of administration and optimal dose, were used to account for the possibilities of not getting expected results and in such cases exercise induced increase can be used as an alternative way of getting optimum results.

Due to enhancing production of the NO and vaso-relaxation of the vessels, endostatin has been used as an alternative to Avastin (anti VEGF drug) in ocular retinal changes in mice (Sunshine et al., 2012). Again exercise induced increase could be alternatively used in humans with retinal changes and could be one of the potential areas for future research.

Additionally, patients with Down syndrome have been reported with high circulatory endostatin concentration and low incidence of solid tumors (Zorick et al., 2001). Though the cause for this increase is mutations in the gene responsible for expression of collagen XVIII, it is speculative to mention that in healthy humans, endostatin can be increased with regular physical activities, which might conversely decrease the incidence of solid tumors.

Despite the fact, that endostatin and VEGF play important roles in angiogenesis, many other mediators and molecules are also involved in angiogenesis. It is a matter of debate, whether these two are sufficient enough to decide the progression of disease. Further research is needed to quantify the effect of these two in the overall angiogenic process, which might help in overcoming the difficulties in therapeutic use of these mediators.

It is evident from this thesis that most of the volunteers were sedentary and short bouts of exercises were easy to perform. So incorporating exercise programme of moderate to high intensities are not difficult, as part of interventional programmes for sedentary populations. Another striking feature of relative importance was, sticking of the participants to all exercises especially second study without any drop outs. It is clear that slight motivation is necessary to deliver a practical life style in sedentary populations.

Prophylactic changes in life style are beneficial than waiting for the disease onset and active treatment. However, in cases of increasing age, where diseases are expected, active treatment may be vital in improving quality of health.

7.5 Conclusion

In conclusion, angiogenesis occurs as one of the adaptive mechanisms in the body to compensate the increasing metabolic requirements of the body. Angiogenesis is regulated with a fine balance between angiogenic and angiostatic mediators and shift in favours of any mediators predicts the phenotype of angiogenesis. However, progressive angiogenesis in presence of pathologic conditions can be dangerous with life threatening outcomes.

Endostatin is one of the main angiostatic factors in the body and can be increased in circulation with moderate to high intensity exercises. The origin of increase in endostatin concentration is not completely clear, and many organs and mechanisms may be involved in its release. However, regardless of the origin, results in this thesis show that exercise promotes endostatin concentration in plasma more than VEGF and may effectively influence pathological processes like atherosclerosis under pathological conditions. Under non pathological conditions it may be more helpful in maintaining vascular tone.

Exercise has also shown a positive increase in VEGF less than endostatin. As the muscles VEGF concentrations were not measured, possibility of local increase cannot be excluded. However, tissue VEGF concentration seems more important for the increasing metabolic requirements than systemic VEGF concentration.

7.6 Limitations

Throughout the research described in this thesis, there were some challenges and limitations in conducting the different studies.

i. Ethics approval for the first study was granted after 5 months due to some unexplained circumstances. The application to the committee was made in December 2011 and approval was granted on 25th May 2012.The delay was attributed to shortage of administrative staff and problems with the

implementation of a new electronic system. When the committee decided to grant the approval, the project was approved without any ethical reservations. This delay had a huge impact on the overall progress, as by the end of first year of research, we were unable to finish one simple study. Ethics approval for the subsequent studies, however, was straight forward and easy.

ii. Reliability of ELISA technique was another issue. Due to non availability of skilled technical support, the reliability of the technique was questioned in the annual review process, although my supervisor regularly checked the results with me after I had learned the technique from another department. The review committee asked me to have more sessions on the technique and to cross check all results with specialised technical expert in other departments. Though the quality control indicators showed a reliable technique, special sessions with skilled technical staff were taken to ensure quality control. Later on all the results after each analysis were cross checked with Mrs Josephine Cooney. Consistency in results shows the accuracy of handling. Whereas, in the initial experiment coefficient of variance (CV) of less than 10% for results were accepted as reliable, in the last experiment obtained CV for all samples were less than 4%.

iii. Recruitment of the healthy individuals was difficult for all experiments especially second one, where individual has to attend the lab 4 times. It was always a time consuming process. Many potential participants refused to participate after initial agreement.

iv. Recruitment mainly from students of Glasgow University is really a limitation of this study. Results would have been truer representative of population with recruitments of non students. Similarly females were less in numbers than males. Control groups were not considered in any of the study.

v. Samples for VEGF in chapter 4 were analysed more than 6 months after collection.

7.7 Future directions

Based on the research in this thesis, future research should be focused on;

1. Besides elucidating the mechanism of release and interactions between these two mediators, effective intensity with shorter duration should be investigated. Different individuals exercised for different duration in this study and successfully completed the trials, but still a large population will not exercise at these intensities regularly. It is therefore, required to design practical studies with short bouts of higher intensities to gain health benefits.

2. Establishing public health recommendations of optimum physical activity. This could have two elements: to establish the best practice in physical activity to prevent future CVD and secondly to identify the optimum activity for patients who have developed progressive angiogenesis.

3. Validating basal endostatin concentration as predictive marker for CVD and cancer with prospective studies. This may include people from normal and diseased population especially with cardiovascular and cancer problems. The model of the study would be prospective and may require longer duration.

4. A longitudinal study evaluating long term effects of physical activities on the basal plasma endostatin and VEGF concentrations in healthy and non healthy population of both gender and different age groups.

5. A prospective study model on mice, whereby comparing the effect of exercise induced rise in endostatin concentration against a group with regular high endostatin concentration (could be done through slow release endostatin depot), in respect of onset of atherosclerosis.

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Appendices

Appendix- A; Ethics approval for all studies:

A1- Study 1 (Chapter 3)

University of Glasgow | College of Medical, Veterinary & Life Sciences

25 May 2012

Dear INAYAT SHAH

MVLS College Ethics Committee

Project Title: Measurement of human Endostatin concentrations in the plasma of healthy volunteers
Project No: 2012038

The College Ethics Committee has reviewed your application and has agreed that there is no objection on ethical grounds to the proposed study. They are happy therefore to approve the project, subject to the following conditions

- The research should be carried out only on the sites, and/or with the groups defined in the application.
- Any proposed changes in the protocol should be submitted for reassessment, except when it is necessary to change the protocol to eliminate hazard to the subjects or where the change involves only the administrative aspects of the project. The Ethics Committee should be informed of any such changes.
- If the study does not start within three years of the date of this letter, the project should be resubmitted.
- You should submit a short end of study report to the Ethics Committee within 3 months of completion.

Yours sincerely

Dorothy McKeegan

Dr Dorothy McKeegan
College Ethics Officer

Dr Dorothy McKeegan

Senior Lecturer

R303 Level 3
Institute of Biodiversity Animal Health and Comparative Medicine
Jarrett Building
Glasgow G61 1QH Tel: 0141 330 5712
E-mail: Dorothy.McKeegan@glasgow.ac.uk

A2- Study 2 (Chapter 4)

University of Glasgow College of Medical, Veterinary & Life Sciences

Dr Inayat Shah
Dr Ronald Baxendale
Room 208
School of Life Sciences
West Medical Building
Glasgow G12 8QQ

25 March, 2013

Dear Dr Shah and Dr Baxendale

MVLS College Ethics Committee

Project Title: Effects of different exercise protocols on plasma Endostatin concentrations of healthy volunteers
Project No: 200120019

The College Ethics Committee has reviewed your application and has agreed that there is no objection on ethical grounds to the proposed study. They are happy therefore to approve the project, subject to the following conditions:

- The research should be carried out only on the sites, and/or with the groups defined in the application.
- Any proposed changes in the protocol should be submitted for reassessment, except when it is necessary to change the protocol to eliminate hazard to the subjects or where the change involves only the administrative aspects of the project. The Ethics Committee should be informed of any such changes.
- If the study does not start within three years of the date of this letter, the project should be resubmitted.
- You should submit a short end of study report to the Ethics Committee within 3 months of completion.

Yours sincerely

Dorothy McKeegan

Dr Dorothy McKeegan
College Ethics Officer

Dr Dorothy McKeegan
Senior Lecturer
Institute of Biodiversity Animal Health and Comparative Medicine
R303 Level 3
Jarrett Building
Glasgow G61 1QH
Tel: 0141 330 5712
E-mail: Dorothy.McKeegan@glasgow.ac.uk

A3- Study 2 (Chapter 5)

University of Glasgow College of Medical, Veterinary & Life Sciences

13th February 2014

Dear Dr Baxendale

MVLS College Ethics Committee

Project Title: Effects of different exercise protocols on plasma Endostatin concentrations of healthy volunteers
Project No: 200120019

The College Ethics Committee has reviewed your request for a minor amendment to the above project and has granted this in full. Specifically, you now have permission to use remaining plasma samples for further assays. Your request was treated as an amendment to application 200120019; a new application is not necessary. This is because in your original application you recognised the future potential value of the samples and took steps to secure permission for further analysis. Thus, the original study subjects have already given their written, informed consent to further use of their samples (unless they opted out).

This permission is subject to the conditions detailed below:

- The research should be carried out only on the sites, and/or with the groups defined in the application.
- Any proposed changes in the protocol should be submitted for reassessment, except when it is necessary to change the protocol to eliminate hazard to the subjects or where the change involves only the administrative aspects of the project. The Ethics Committee should be informed of any such changes.
- You should submit a short end of study report to the Ethics Committee within 3 months of completion.

Yours sincerely

Dorothy McKeegan

Dr Dorothy McKeegan
College Ethics Officer

Dr Dorothy McKeegan

Senior Lecturer

R303 Level 3
Institute of Biodiversity Animal Health and Comparative Medicine
Jarrett Building
Glasgow G61 1QH Tel: 0141 330 5712
E-mail: Dorothy.McKeegan@glasgow.ac.uk

A4- Study 3 (Chapter 5)

University of Glasgow | College of Medical, Veterinary & Life Sciences

30th July 2014

Dear Dr Inayat Shah and Dr Ronald Baxendale

MVLS College Ethics Committee

Project Title: Effects of single bout of high intensity exercise on plasma Endostatin and vascular endothelial growth factor concentrations in healthy volunteers
Project No: 200130151

The College Ethics Committee has reviewed your application and has agreed that there is no objection on ethical grounds to the proposed study. They are happy therefore to approve the project, subject to the following conditions

- Project end date: October 2014
- The research should be carried out only on the sites, and/or with the groups defined in the application.
- Any proposed changes in the protocol should be submitted for reassessment, except when it is necessary to change the protocol to eliminate hazard to the subjects or where the change involves only the administrative aspects of the project. The Ethics Committee should be informed of any such changes.
- You should submit a short end of study report to the Ethics Committee within 3 months of completion.

Yours sincerely

Prof. Andrew C. Rankin
Deputy Chair, College Ethics Committee

Andrew C. Rankin
Professor of Medical Cardiology
BHF Glasgow Cardiovascular Research Centre
College of Medical, Veterinary & Life Sciences
University of Glasgow, G12 8TA
Tel: 0141 211 4833
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Appendix- B; Information sheets

B1- Information sheet for study 1

Title of study

Measurement of human Endostatin concentrations in the plasma of healthy volunteers

Invitation to take part

Thank you for reading this information sheet.

This is an invitation for you to take part in a research study. It is important for you to understand why this study is being conducted and what it would involve before you decide to take part in this study. Please take time to read the following information carefully and discuss it with others if you wish. If you are not clear about anything or would like to have more information, feels free to ask. Take time to decide whether or not you wish to take part.

What is the purpose of the study?

Endostatin is normally present in human blood. It is of interest because of its potential role in cardiac protection. This study aims to measure the concentration in healthy people.

Why have I been chosen?

Because you are:

- A healthy normal adult individual
- A non smoker
- Not suffering from any chronic illness
- Not pregnant or lactating
- Have not performed any strenuous physical activity in last 24 hours

Do I have to take part?

It is entirely your own decision to take part or not. You will be given this information sheet in case you decided to take part. In case of withdrawal from the study after you decided to take part and giving consent, you are free to do so without providing any reason.

What will happen to me if I take part?

If you agree to take part, a member of the team conducting the study will explain the study at your convenient place and time.

What do I have to do?

On taking the decision to be a part of the study and signing the consent one of the team members will make an appointment with you. You have to fill in a questionnaire regarding your health status, physical activity and smoking habits. Your weight, height, Body Mass Index, waist circumference and blood pressure will be recorded. One sample of 5 ml blood will be taken from a vein according to a standard protocol by an experienced Physician in the laboratory of Glasgow University.

What are the possible disadvantages and risks of taking part?

There are very minimal risks or disadvantages associated with this study other than time loss. These are needle prick and slight bleeding.

What are the possible benefits of taking part?
There are no direct benefits to the participants. However, it will provide a baseline about the concentrations of Endostatin in people.

Will my taking part in this study be kept confidential?
All the information will be kept confidential, available only to the researcher. Your data will be stored anonymously. Your name and address will not be stored.

What will happen to the results of the research study?
Your data will be included in a thesis, published in scientific journals and may be discussed in meeting and conferences. This is so that other people may know about the results of the study. Results can be discussed with you as well if you are interested to know about that.

Who is organising and funding the research?
This project is being organised by the School of Life Sciences, University of Glasgow. It is funded by a scholarship from Khyber Medical University, Peshawar, Pakistan.

Who has reviewed the study?
This project has been reviewed by the College of Medical, Veterinary & Life Sciences Ethics Committee for Non Clinical Research Involving Human Subjects.

Contact for further information
If you require further information please contact

- Dr Inayat Shah by telephone no 07438334455 or via e-mail at i.shah.1@research.gla.ac.uk

- Dr Ronald Baxendale by telephone at 0141 330 5344 or via e-mail at Ronald.Baxendale@glasgow.ac.uk

Thank you for reading this information sheet.

B2- Information sheet for study 2

Title of study
The effect of different exercise protocols on human plasma endostatin level

Invitation to take part
Thank you for reading this information sheet.
This is an invitation for you to take part in a research study. It is important for you to understand why this study is being conducted and what it would involve before you decide to take part in this study. Please take time to read the following information carefully and discuss it with others if you wish. If you are not clear about anything or would like to have more information, feel free to ask. Take time to decide whether or not you wish to take part.

What is the purpose of the study?
Endostatin is normally present in human blood. It is of interest because of its potential role in cardiac protection. Heart diseases are quite common across the globe and are a leading cause of death in Scotland. Studies suggest that exercise enables the body to protect it from the heart problems, but we do not understand this mechanism completely. This study will help to increase that understanding. This is important because we can find exercise as treatment for the heart problems.

Why have I been chosen?
Because you are:

- A healthy normal adult individual
- A non smoker
- Not suffering from any chronic illness
- Not pregnant or lactating

Do I have to take part?
It is entirely your own decision to take part or not. You will be given this information sheet to help you understand what is involved. You can stop the experiment at any time without providing any reason.
What will happen to me if I take part?
If you agree to take part, a member of the team conducting the study will explain the study at your convenient place and time.

What do I have to do?
You will be requested to attend the lab at 4 times. Each visit will last up to 2 hours. In the first visit you will be asked to sign a consent form followed by filling a complete confidential questionnaire regarding your health and physical ability. We will record your height, weight, and blood pressure and you will be familiarized with the whole procedure and equipments. You will be asked to perform a preliminary exercise test on treadmill which will help us predicting the exercise protocols in the subsequent visits. The test will last for about 30 minutes and will be at speed suitable to you. On later visits you will be asked to walk or run on a treadmill for less than 45 minutes. Blood samples will be taken before and after the walk.

What are the possible disadvantages and risks of taking part?
There are very minimal risks or disadvantages associated with this study other

than time loss.

You will give three blood samples of 5ml and these require a needle. The

walking is a low to moderate intensity exercise and caries a low risk of changes

in blood pressure or abnormal heart beat.

What are the possible benefits of taking part?
The study will give you an idea and information about your physical and health fitness. Moreover, this study will help us to determine the effects of exercise on the body own ability to protect it from heart problems.

What if something goes wrong?
The chances of something going wrong are extremely small. All of the procedures involved in this study are low risk and our screening tests are designed to ensure that you will only participate if it is safe for you to do so. In the unlikely event that you are harmed due to someone's negligence, then you may have grounds for a legal action.

Will my taking part in this study be kept confidential?
All the information will be kept confidential, available only to the researcher. Your data will be stored anonymously. Your name and address will not be stored.
What will happen to my samples after the study has finished?
The blood samples that you provide for this study may be useful for future research into the prevention and treatment of heart disease; this may involve investigating new biochemical markers that may or may not yet identified. Samples will be analysed anonymously and will require a new ethics application before they would be used for future research. If you do not wish your samples to be used for future research, please indicate this on the consent form.

What will happen to the results of the research study?
Your data will be included in a thesis, published in scientific journals and may be discussed in meeting and conferences. This is so that other people may know about the results of the study. Results can be discussed with you as well if you are interested to know about that.

Who is organising and funding the research?
This project is being organised by the School of Life Sciences, University of Glasgow. It is funded by a scholarship from Khyber Medical University, Peshawar, Pakistan.

Who has reviewed the study?
This project has been reviewed by the College of Medical, Veterinary & Life Sciences Ethics Committee for Non Clinical Research Involving Human Subjects.

Contact for further information

If you require further information please contact

- Dr Inayat Shah by telephone no 07438334455 or via e-mail at i.shah.1@research.gla.ac.uk

- Dr Ronald Baxendale by telephone at 0141 330 5344 or via e-mail at Ronald.Baxendale@glasgow.ac.uk

Thank you for reading this information sheet.

B3- Information sheet for study 3

Title of study

The effect of graded intensity exercise on human plasma endostatin and vascular endothelial growth factor level

Invitation to take part
Thank you for reading this information sheet.
This is an invitation for you to take part in a research study. It is important for you to understand why this study is being conducted and what it would involve before you decide to take part in this study. Please take time to read the following information carefully and discuss it with others if you wish. If you are not clear about anything or would like to have more information, feel free to ask. Take time to decide whether or not you wish to take part.

What is the purpose of the study?
Endostatin and vascular endothelial growth factor are normally present in human blood. It is of interest because of its potential role in cardiac protection. Heart diseases are quite common across the globe and are a leading cause of death in Scotland. Studies suggest that exercise enables the body to protect it from the heart problems, but we do not understand this mechanism completely. This study will help to increase that understanding. This is important because we can find exercise as treatment for the heart problems.

Why have I been chosen?
Because you are:

- A healthy normal adult individual
- A non smoker
- Not suffering from any chronic illness
- Not pregnant or lactating

Do I have to take part?
It is entirely your own decision to take part or not. You will be given this information sheet to help you understand what is involved. You can stop the experiment at any time without providing any reason.

What will happen to me if I take part?
If you agree to take part, a member of the team conducting the study will explain the study at your convenient place and time.
What do I have to do?
You will be requested to attend the lab twice on 2 consecutive days. On day first, you will be asked to sign a consent form followed by filling a complete confidential questionnaire regarding your health and physical ability. We will record your height, weight, body fat percentage, blood pressure and you will be familiarized with the whole procedure and equipments. A cannula will be passed onto your vein to take first sample of blood before exercise, and you will be asked to walk on the treadmill. The speed/gradient of the treadmill will be increased periodically till you reach your maximum effort/ exhaustion. The

duration of your exercise will be less than 20 minutes. The exercise will start at lower speed and gradient and will be increased periodically after 3 minutes each till your exhaustion. After completion you will set in the lab for 4 hours and Blood samples will be taken at 10, 30, 60,120 and 240 minutes after exercise. This visit will take approximately 6 hours and you will be served with soft drinks/water during that time. Facilities like watching movies; internet will also be available to you during this time. Cannula will be removed after the last sample and you will be asked to attend the lab next day after 24 hours for 1 more blood sample. The second visit will be for 30 minutes only. You will give 7 samples each of 10 ml during the whole trial, 6 samples on day first and a final sample on second day of your trial. All the samples on day first will be collected through the cannula and final sample will need only through a small venous puncture.

What are the possible disadvantages and risks of taking part?
There are very minimal risks or disadvantages associated with this study other

than time loss. During your exercise 2 staff members competent in first aid will

always be available to you. Moreover, your heart rate will be monitored all the

time. You will give 7 blood samples of 10 ml each which require a needle.

What are the possible benefits of taking part?
The study will give you an idea and information about your physical fitness and health. Moreover, this study will help us to determine the effects of exercise on the body own ability to protect it from heart problems.

What if something goes wrong?
The chances of something going wrong are extremely small. All of the procedures involved in this study are low risk and our screening tests are designed to ensure that you will only participate if it is safe for you to do so. In the unlikely event that you are harmed due to someone's negligence, then you may have grounds for a legal action.

Will my taking part in this study be kept confidential?
All the information will be kept confidential, available only to the researcher. Your data will be stored anonymously. Your name and address will not be stored.
What will happen to my samples after the study has finished?
The blood samples that you provide for this study may be useful for future research into the prevention and treatment of heart disease; this may involve investigating new biochemical markers that may or may not yet identified. Samples will be analysed anonymously and will require a new ethics application before they would be used for future research. If you do not wish your samples to be used for future research, please indicate this on the consent form.

What will happen to the results of the research study?
Your data will be included in a thesis, published in scientific journals and may be discussed in meeting and conferences. This is so that other people may know

about the results of the study. Results can be discussed with you as well if you are interested to know about that.

Who is organising and funding the research?
This project is being organised by the School of Life Sciences, University of Glasgow. It is funded by a scholarship from Khyber Medical University, Peshawar, Pakistan.

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This project has been reviewed by the College of Medical, Veterinary & Life Sciences Ethics Committee for Non Clinical Research Involving Human Subjects.

Contact for further information
If you require further information please contact

- Dr Inayat Shah by telephone no 07438334455 or via e-mail at i.shah.1@research.gla.ac.uk

- Dr Ronald Baxendale by telephone at 0141 330 5344 or via e-mail at Ronald.Baxendale@glasgow.ac.uk

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Thank you for reading this information sheet.

Appendix- C; Consent form (specimen)

CONSENT FORM

Title of Project:
Effects of single bout of high intensity exercise on plasma Endostatin and vascular endothelial growth factor concentrations in healthy volunteers

Name of Researcher:

Please initial box

1. I confirm that I have read and understand the information sheet dated. ..... (version.....) for the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason. ☐

3. I agree to my samples (Blood) being stored and used for further analysis to look at biochemical compound endostatin and VEGF (but not human DNA or RNA). ☐

4. I agree to take part in the above study. ☐

Name of subject	Date	Signature

1 for subject; 1 for researcher

Appendix- D; Questionnaires

D1- International Physical Activity Questionnaire (IPAQ)

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the **last 7 days**. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the **vigorous** activities that you did in the **last 7 days**. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. Think *only* about those physical activities that you did for at least 10 minutes at a time.

1. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, digging, aerobics, or fast bicycling?

   _____ **days per week**

   ☐ No vigorous physical activities → ***Skip to question 3***

2. How much time did you usually spend doing **vigorous** physical activities on one of those days?

   _____ **hours per day**

   _____ **minutes per day**

   ☐ Don't know/Not sure

Think about all the **moderate** activities that you did in the **last 7 days**. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

3. During the **last 7 days**, on how many days did you do **moderate** physical activities like carrying light loads, bicycling at a regular pace, or doubles tennis? Do not include walking.

   _____ **days per week**

   ☐ No moderate physical activities → ***Skip to question 5***

4. How much time did you usually spend doing **moderate** physical activities on one of those days?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

Think about the time you spent **walking** in the **last 7 days**. This includes at work and at home, walking to travel from place to place, and any other walking that you might do solely for recreation, sport, exercise, or leisure.

5. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time?

_____ **days per week**

☐ No walking → ***Skip to question 7***

6. How much time did you usually spend **walking** on one of those days?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

The last question is about the time you spent **sitting** on weekdays during the **last 7 days**. Include time spent at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading, or sitting or lying down to watch television.

7. During the **last 7 days**, how much time did you spend **sitting** on a **week day**?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

D2- Physical Activity Readiness Questionnaire (PAR-Q)

Name: **D O B:**

Address: **Postcode:**

Email: **Mobile:**

Physical Activity Readiness Questionnaire (PAR-Q)

If you are between the ages of 15 and 69, the PAR-Q will tell you if you should check with your doctor before you significantly change your physical activity patterns. If you are over 69 years of age and are not used to being very active, check with your doctor. Common sense is your best guide when answering these questions. Please read carefully and answer each one honestly: check YES or NO.

1. Has your doctor ever said you have a heart condition and that you should only do physical activity recommended by a doctor?	Yes ☐	No ☐
2. Do you feel pain in your chest when you do physical activity?	Yes ☐	No ☐
3. In the past month, have you had a chest pain when you were not doing physical activity?	Yes ☐	No ☐
4. Do you lose you balance because of dizziness or do you ever lose conciousness?	Yes ☐	No ☐
5. Do you have a bone or joint problem (for example, back, knee, or hip) that could be made worse by a change in your physical activity?	Yes ☐	No ☐
6. Is your doctor currently prescribing medication for your blood pressure or heart condition?	Yes ☐	No ☐
7. Do you know of any other reason why you should not do physical activity?	Yes ☐	No ☐

If yes, please comment: _____

YES to one or more questions:
You should consult with your doctor to clarify that it is safe for you to become physically active at this current time and in your current state of health.

NO to all questions:
It is reasonably safe for you to participate in physical activity, gradually building up from your current ability level. A fitness appraisal can help determine your ability levels.

I have read, understood and accurately completed this questionnaire. I confirm that I am voluntarily engaging in an acceptable level of exercise, and my participation involves a risk of injury.

Signature _____

Print name _____

Date _____

Having answered YES to one of the above, I have sought medical advice and my GP has agreed that I may exercise.

Signature _____

Date _____

Note: This physical activity clearance is valid for a maximum of 12 months from the date it is completed and becomes invalid if your condition changes so that you would answer YES to any of the 7 questions.

D-3 Health Screening Questionnaire Adapted from ACSM

HEALTH SCREEN FOR STUDY VOLUNTEERS

Volunteer No: __

It is important that volunteers participating in research studies are currently in good health and have had no significant medical problems in the past. This is to ensure (*i*) their own continuing well-being and (*ii*) to avoid the possibility of individual health issues confounding study outcomes.

Please complete this brief questionnaire to confirm fitness to participate:

1. **At present**, do you have any health problem for which you are:
   (a) on medication, prescribed or otherwise yes [ ] no [ ]
   (b) attending your general practitioner yes [ ] no [ ]
   (c) on a hospital waiting list yes [ ] no [ ]
2. **In the past two years,** have you had any illness which required you to:
   (a) consult your GP yes [ ] no [ ]
   (b) attend a hospital outpatient department yes [ ] no [ ]
   (c) be admitted to hospital yes [ ] no [ ]
3. **Have you ever** had any of the following:
   (a) Convulsions/epilepsy yes [ ] no [ ]
   (b) Asthma yes [ ] no [ ]
   (c) Diabetes yes [ ] no [ ]
   (d) A blood disorder yes [ ] no [ ]
   (e) Digestive problems yes [ ] no [ ]
   (f) Disturbance of balance/co-ordination yes [ ] no [ ]
   (g) Numbness in hands or feet yes [ ] no [ ]
   (h) Disturbance of vision yes [ ] no [ ]
   (i) Thyroid problems yes [ ] no [ ]
   (j) Kidney or liver problems yes [ ] no [ ]
   (k) Chest pain or heart problems yes [ ] no [ ]
   (l) Any other health problems yes [ ] no [ ]
   (m) An allergy to soya protein or eggs yes [ ] no [ ]
4. **For female volunteers only**
   (a) Are you pregnant or think that you might be pregnant yes [ ] no [ ]

5. **Have any of your family (parents, grandparents, brothers, sisters, children, aunts, uncles, cousins)** ever had any of the following: (if yes please give details including age of first diagnosis)

(a) Any heart problems	yes [ ]	no [ ]
(b) Diabetes	yes [ ]	no [ ]
(c) Stroke	yes [ ]	no [ ]
(d) Any other family illnesses	yes [ ]	no [ ]

6. Do you currently **smoke** yes [ ] no [ ]
   Have you ever smoked yes [ ] no [ ]
   If so, for how long did you smoke and when did you stop? ……

7. How many units of alcohol do you typically drink in a week? …….

8. Have you taken part in a research study in the last 3 months? yes [ ] no [ ]

If YES to any question, please describe briefly if you wish (e.g. to confirm whether problem was short-lived, insignificant or well controlled.) (Use a separate sheet if necessary)

Appendix- E; IPAQ data processing guidelines

Guidelines for Data Processing and Analysis of the International Physical Activity Questionnaire (IPAQ)

– Short and Long Forms

November 2005

Contents

1. Introduction

This document describes recommended methods of scoring the data derived from the telephone / interview administered and self-administered IPAQ short and long form instruments. The methods outlined provide a revision to earlier scoring protocols for the IPAQ short form and provide for the first time a comparable scoring method for IPAQ long form. Latest versions of IPAQ instruments are available from
www.ipaq.ki.se.
Although there are many different ways to analyse physical activity data, to date there is no formal consensus on a 'correct' method for defining or describing levels of physical activity based on self–report population surveys. The use of different scoring protocols makes it very difficult to compare within and between countries, even when the same instrument has been used. Use of these scoring methods will enhance the comparability between surveys, provided identical sampling and survey methods have been used.

2. Uses of IPAQ Instruments

IPAQ short form is an instrument designed primarily for population surveillance of physical activity among adults. It has been developed and tested for use in adults (age range of 15-69 years) and until further development and testing is undertaken the use of IPAQ with older and younger age groups is not recommended.
IPAQ short and long forms are sometimes being used as an evaluation tool in intervention studies, but this was not the intended purpose of IPAQ. Users should carefully note the range of domains and types of activities included in IPAQ before

using it in this context. Use as an outcome measure in small scale intervention studies is not recommended.

3. Summary Characteristics of IPAQ Short and Long Forms

1. IPAQ assesses physical activity undertaken across a comprehensive set of domains including:

a. leisure time physical activity
b. domestic and gardening (yard) activities
c. work-related physical activity
d. transport-related physical activity;

2. The IPAQ **short** form asks about three specific types of activity undertaken in the four domains introduced above. The specific types of activity that are assessed are walking, moderate-intensity activities and vigorous-intensity activities.
3. The items in the **short** IPAQ form were structured to provide separate scores on walking, moderate-intensity and vigorous-intensity activity. Computation of the total score for the short form requires summation of the duration (in minutes) and frequency (days) of walking, moderate-intensity and vigorous-intensity activities. Domain specific estimates cannot be estimated.

4. The IPAQ **long** form asks details about the specific types of activities undertaken within each of the four domains. Examples include walking for transportation and moderate-intensity leisure-time activity.
5. The items in the **long** IPAQ form were structured to provide separate domain specific scores for walking, moderate-intensity and vigorous-intensity activity within each of the work, transportation, domestic chores and gardening (yard) and leisure-time domains. Computation of the total scores for the long form requires summation of the duration (in minutes) and frequency (days) for all the types of activities in all domains. Domain specific scores or activity specific subscores may be calculated. Domain specific scores require summation of the scores for walking, moderate-intensity and vigorous-intensity activities within the specific domain, whereas activity-specific scores require summation of the scores for the specific type of activity across domains.

4. Overview of Continuous and Categorical Analyses of IPAQ

Both categorical and continuous indicators of physical activity are possible from both
IPAQ forms. However, given the non-normal distribution of energy expenditure in many populations, it is suggested that the continuous indicator be presented as median minutes/week or median MET–minutes/week rather than means (such asmean minutes/week or mean MET-minutes/week).

4.1 Continuous Variables

Data collected with IPAQ can be reported as a continuous measure. One measure of the volume of activity can be computed by weighting each type of activity by its energy requirements defined in METs to yield a score in MET–minutes. METs are multiples of the resting metabolic rate and a MET-minute is computed by

multiplying the MET score of an activity by the minutes performed. MET-minute scores are equivalent to kilocalories for a 60 kilogram person. Kilocalories may be computed from MET-minutes using the following equation: MET-min x (weight in kilograms/60 kilograms). MET-minutes/day or MET-minutes/week can be presented although the latter is more frequently used and is thus suggested.
Details for the computation for summary variables from IPAQ short and long forms are detailed below. As there are no established thresholds for presenting METminutes, the IPAQ Research Committee propose that these data are reported as comparisons of median values and interquartile ranges for different populations.

4.2 Categorical Variable: Rationale for Cut Point Values

There are three levels of physical activity proposed to classify populations:
1. Low
2. Moderate
3. High

The algorithms for the short and long forms are defined in more detail in Sections 5.3 and 6.3, respectively. Rules for data cleaning and processing prior to computing the algorithms appear in Section 7.

Regular participation is a key concept included in current public health guidelines for physical activity. Therefore, both the total volume and the number of days/sessions are included in the IPAQ analysis algorithms.
The criteria for these levels have been set taking into account that IPAQ asks questions in all domains of daily life, resulting in higher median MET-minutes estimates than would have been estimated from leisure-time participation alone. The criteria for these three levels are shown below.
Given that measures such as IPAQ assess total physical activity in all domains, the “leisure time physical activity” based public health recommendation of 30 minutes on most days will be achieved by most adults in a population. Although widely accepted as a goal, in absolute terms 30 minutes of moderate-intensity activity is low and broadly equivalent to the background or basal levels of activity adult individuals would accumulate in a day. Therefore a new, higher cutpoint is needed to describe the levels of physical activity associated with health benefits for measures such as
IPAQ, which report on a broad range of domains of physical activity.
‘High’

This category was developed to describe higher levels of participation. Although it is known that greater health benefits are associated with increased levels of activity there is no consensus on the exact amount of activity for maximal benefit. In the absence of any established criteria, the IPAQ Research Committee proposes a measure which equates to approximately at least one hour per day or more, of at least moderate-intensity activity above the basal level of physical activity Considering that basal activity may be considered to be equivalent to approximately 5000 steps per day, it is proposed that “high active” category be considered as those who move at least 12,500 steps per day, or the equivalent in moderate and vigorous activities.
This represents at least an hour more moderate-intensity activity over and above the basal level of activity, or half an hour of vigorous-intensity activity over and

above basal levels daily. These calculations were based on emerging results of pedometers studies.
This category provides a higher threshold of measures of total physical activity and is a useful mechanism to distinguish variation in population groups. Also it could be used to set population targets for health-enhancing physical activity when multidomain instruments, such as IPAQ are used.

'Moderate'

This category is defined as doing some activity, more than the low active category. It is proposed that it is a level of activity equivalent to "half an hour of at least moderate-intensity PA on most days", the former leisure time-based physical activity population health recommendation.

'Low'

This category is simply defined as not meeting any of the criteria for either of the previous categories.

5. Protocol for IPAQ Short Form

5.1 Continuous Scores

Median values and interquartile ranges can be computed for walking (W), moderate intensity activities (M), vigorous-intensity activities (V) and a combined total physical activity score. All continuous scores are expressed in MET-minutes/week as defined below.

5.2 MET Values and Formula for Computation of MET-minutes/week

The selected MET values were derived from work undertaken during the IPAQ Reliability Study undertaken in 2000-2001[3]. Using the Ainsworth et al. Compendium
(*Med Sci Sports Med* 2000) an average MET score was derived for each type of activity. For example; all types of walking were included and an average MET value for walking was created. The same procedure was undertaken for moderate-intensity activities and vigorous-intensity activities. The following values continue to be used for the analysis of IPAQ data: Walking = 3.3 METs, Moderate PA = 4.0 METs and
Vigorous PA = 8.0 METs. Using these values, four continuous scores are defined:

*Walking MET-minutes/week = 3.3 * walking minutes * walking days*
*Moderate MET-minutes/week = 4.0 * moderate-intensity activity minutes * moderate days*
*Vigorous MET-minutes/week = 8.0 * vigorous-intensity activity minutes * vigorous-intensity days*
Total physical activity MET-minutes/week = sum of Walking + Moderate + Vigorous METminutes/ week scores.

5.3 Categorical Score

Category 1 Low

This is the lowest level of physical activity. Those individuals who not meet criteria for Categories 2 or 3 are considered to have a ‘low’ physical activity level.

Category 2 Moderate

The pattern of activity to be classified as ‘moderate’ is either of the following criteria:
a) 3 or more days of vigorous-intensity activity of at least 20 minutes per day

OR

b) 5 or more days of moderate-intensity activity and/or walking of at least 30 minutes per day

OR

c) 5 or more days of any combination of walking, moderate-intensity or vigorous intensity activities achieving a minimum Total physical activity of at least 600 MET-minutes/week.
Individuals meeting at least one of the above criteria would be defined as accumulating a minimum level of activity and therefore be classified as ‘moderate’.
See Section 7.5 for information about combining days across categories.

Category 3 High

A separate category labelled ‘high’ can be computed to describe higher levels of participation.
The two criteria for classification as ‘high’ are:
a) vigorous-intensity activity on at least 3 days achieving a minimum Total physical activity of at least 1500 MET-minutes/week

OR

b) 7 or more days of any combination of walking, moderate-intensity or vigorous-intensity activities achieving a minimum Total physical activity of at least 3000 MET-minutes/week.
See Section 7.5 for information about combining days across categories.

5.4 Sitting Question in IPAQ Short Form

The IPAQ sitting question is an additional indicator variable of time spent in sedentary activity and is not included as part of any summary score of physical activity. Data on sitting should be reported as median values and interquartile ranges.
To-date there are few data on sedentary (sitting) behaviours and no well-accepted thresholds for data presented as categorical levels.

7. Data Processing Rules

In addition to a standardized approach to computing categorical and continuous measures of physical activity, it is necessary to undertake standard methods for

the cleaning and treatment of IPAQ datasets. The use of different approaches and rules would introduce variability and reduce the comparability of data.
There are no established rules for data cleaning and processing on physical activity.
Thus, to allow more accurate comparisons across studies IPAQ Research Committee has established and recommends the following guidelines:

7.1 Data Cleaning

I. Any responses to duration (time) provided in the hours and minutes response option should be converted from hours and minutes into minutes.

II. To ensure that responses in 'minutes' were not entered in the 'hours' column by mistake during self-completion or during data entry process, values of '15', '30', '45', '60' and '90' in the 'hours' column should be converted to '15', '30', '45', '60' and '90' minutes, respectively, in the minutes column.

III. In some cases duration (time) will be reported as weekly (not daily) e.g., VWHRS, VWMINS. These data should be converted into an average daily time by dividing by 7.

IV. If 'don't know' or 'refused ' or data are missing for time or days then that case is removed from analysis.

Note: Both the number of days *and* daily time are required for the creation of categorical and continuous summary variables

7.2 Maximum Values for Excluding Outliers

This rule is to exclude data which are unreasonably high; these data are to be considered outliers and thus are excluded from analysis. All cases in which the sum total of all Walking, Moderate and Vigorous time variables is greater than 960 minutes (16 hours) should be excluded from the analysis. This assumes that on average an individual of 8 hours per day is spent sleeping.
The 'days' variables can take the range 0-7 days, or 8, 9 (don't know or refused); values greater than 9 should not be allowed and those cases excluded from analysis.

7.3 Minimum Values for Duration of Activity

Only values of 10 or more minutes of activity should be included in the calculation of summary scores. The rationale being that the scientific evidence indicates that episodes or bouts of at least 10 minutes are required to achieve health benefits.
Responses of less than 10 minutes [and their associated days] should be re-coded to 'zero'.

7.4 Truncation of Data Rules

This rule attempts to normalize the distribution of levels of activity which are usually skewed in national or large population data sets.
In IPAQ short - it is recommended that all Walking, Moderate and Vigorous time variables exceeding ' 3 hours' or '180 minutes' are truncated (that is re-coded) to

be equal to ‘180 minutes’ in a new variable. This rule permits a maximum of 21 hours of activity in a week to be reported for each category (3 hours * 7 days).
In IPAQ long – the truncation process is more complicated, but to be consistent with the approach for IPAQ short requires that the variables total Walking, total Moderateintensity and total Vigorous-intensity activity are calculated and then, for each of these summed behaviours, the total value should be truncated to 3 hours (180 minutes).

When analysing the data as categorical variable or presenting median and interquartile ranges of the MET-minute scores, the application of the truncation rule will not affect the results. This rule does have the important effect of preventing misclassification in the ‘high’ category. For example, an individual who reports walking for 10 minutes on 6 days and 12 hours of moderate activity on one day could be coded as ‘high’ because this pattern meets the ‘7 day” and “3000 MET-min” criteria for ‘high’. However, this uncommon pattern of activity is unlikely to yield the health benefits that the ‘high’ category is intended to represent.

Although using median is recommended due to the skewed distribution of scores, if
IPAQ data are analysed and presented as a continuous variable using mean values, the application of the truncation rule will produce slightly lower mean values than would otherwise be obtained.

7.5 Calculating MET-minute/week Scores

Data processing rules 7.2, 7.3, and 7.4 deals first with excluding outlier data, then secondly, with recoding minimum values and then finally dealing with high values.
These rules will ensure that highly active people remain classified as ‘high’, while decreasing the chances that less active individuals are misclassified and coded as ‘high’.
Using the resulting variables, convert time and days to MET-minute/week scores [see above Sections 5.2 and 6.2; METS x days x daily time].

7.6 Calculating Total Days for Presenting Categorical Data on Moderate and High Levels

Presenting IPAQ data using categorical variables requires the total number of ‘days’ on which all physical activity was undertaken to be assessed. This is difficult because frequency in ‘days’ is asked separately for walking, moderate-intensity and vigorous intensity activities, thus allowing the total number of ‘days’ to range from a minimum of 0 to a maximum of 21’days’ per week in IPAQ short and higher in IPAQ long. The IPAQ instrument does not record if different types of activity are undertaken on the same day.

In calculating ‘moderately active’, the primary requirement is to identify those individuals who undertake activity on at least ‘5 days’/week [see Sections 4.2 and 5.3]. Individuals who meet this criterion should be coded in a new variable called “*at least five days”* and this variable should be used to identify those meeting criterion b) at least 30 minutes of moderate-intensity activity and/or walking; and those meeting criterion c) any combination of walking, moderate-intensity or vigorous-intensity activities achieving a minimum of 600 MET-minutes/week.

Below are two examples showing this coding in practice:

i) an individual who reports ‘2 days of moderate-intensity’ and ‘3 days of walking’ should be coded as a value indicating “*at least five days”*;
ii) an individual reporting ‘2 days of vigorous-intensity’, ‘2 days of moderate intensity’ and ‘2 days of walking should be coded as a value to indicate “*at least five days”* [even though the actual total is 6].
The original frequency of ‘days’ for each type of activity should remain in the data file for use in the other calculations.
The same approach as described above is used to calculate total days for computing the ‘high’ category. The primary requirement according to the stated criteria is to identify those individuals who undertake a combination of walking, moderate intensity and or vigorous-intensity activity on at least 7 days/week [See section 4.2].
Individuals who meet this criterion should be coded as a value in a new variable to reflect *“at least 7 days*”.

Below are two examples showing this coding in practice:
i) an individual who reports ‘4 days of moderate-intensity’ and ‘3 days of walking’ should be coded as the new variable *“at least 7 days*”.
ii) an individual reporting ‘3 days of vigorous-intensity’, ‘3 days moderateintensity’ and ‘3 days walking’ should be coded as *“at least 7 days*” [even though the total adds to 9] .

8. Summary algorithms

The algorithms in Appendix 1 and Appendix 2 to this document show how these rules work in an analysis plan, to develop the categories 1 [Low], 2 [Moderate], and 3
[High] levels of activity.

At A Glance

IPAQ Scoring Protocol (Short Forms)

Continuous Score

Expressed as MET-min per week: MET level x minutes of activity/day x days per week
Sample Calculation

MET levels MET-minutes/week for 30 min/day, 5 days

Walking = 3.3 METs 3.3*30*5 = 495 MET-minutes/week
Moderate Intensity = 4.0 METs 4.0*30*5 = 600 MET-minutes/week
Vigorous Intensity = 8.0 METs 8.0*30*5 = 1,200 MET-minutes/week

_____
TOTAL = 2,295 MET-minutes/week
Total MET-minutes/week = Walk (METs*min*days) + Mod (METs*min*days) + Vig (METs*min*days)

Categorical Score- three levels of physical activity are proposed

1. Low

• No activity is reported **OR**
• Some activity is reported but not enough to meet Categories 2 or 3.

2. Moderate

Either of the following 3 criteria

• 3 or more days of vigorous activity of at least 20 minutes per day **OR**
• 5 or more days of moderate-intensity activity and/or walking of at least 30 minutes per day **OR**
• 5 or more days of any combination of walking, moderate-intensity or vigorous intensity activities achieving a minimum of at least 600 MET-minutes/week.

3. High

Any one of the following 2 criteria
• Vigorous-intensity activity on at least 3 days and accumulating at least 1500 MET-minutes/week **OR**
• 7 or more days of any combination of walking, moderate- or vigorous-intensity activities accumulating at least 3000 MET-minutes/week

Please review the full document "Guidelines for the data processing and analysis of the International Physical Activity Questionnaire" for more detailed description of IPAQ analysis and recommendations for data cleaning and processing [www.ipaq.ki.se].

Appendix- F; ELISA

F1- Endostatin

Catalogue Number DNST0

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human Endostatin has been pre-coated onto a microplate. Standards, controls and samples are pipetted into the wells and any Endostatin present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for human Endostatin is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of Endostatin bound in the initial step. The color development is stopped and the intensity of the color is measured.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*

Saliva - Collect saliva into a polypropylene tube. Centrifuge for 5 minutes at 10,000 x g in microcentrifuge tubes. Collect the aqueous layer (no pellet) and assay immediately or aliquot and store samples at 2-8 °C.

Note: *Saliva collector must not have any protein binding or filtering capabilities.*

SAMPLE PREPARATION

Serum and plasma samples require a 50-fold dilution. A suggested 50-fold dilution is 20 µL of sample + 980 µL of Calibrator Diluent RD5P (diluted 1:5).

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Note: *High concentrations of Endostatin are found in saliva. It is recommended that a face mask and gloves are used to protect kit reagents from contamination.*

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Calibrator Diluent RD5P (diluted 1:5) - Add 20mL of Calibrator Diluent RD5P Concentrate to 80mL of deionized or distilled water to prepare 100mL of Calibrator Diluent RD5P (diluted 1:5).

Human Endostatin Standard - **Refer to the vial label for reconstitution volume.**
Reconstitute the Human Endostatin Standard with deionized or distilled water. This reconstitution produces a stock solution of 100 ng/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. Pipette 900 µL of Calibrator Diluent RD5P (diluted 1:5) into the 10 ng/mL tube. Pipette 500 µL into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 10 ng/mL standard serves as the high standard. Calibrator Diluent RD5P (diluted 1:5) serves as the zero standard (0 ng/mL).

ASSAY PROCEDURE
Bring all reagents and samples to room temperature before use. It is recommended that

All standards, samples, and controls be assayed in duplicate.
Note: *High concentrations of Endostatin are found in saliva. It is recommended that a face mask and gloves are used to protect kit reagents from contamination.*

1. Prepare all reagents, working standards, and samples as directed in the previous sections.

2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.

3. Add 100 µL of Assay Diluent RD1W to each well.

4. Add 50 µL of Standard, control, or sample* per well. Cover with the adhesive strip provided.
Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker
(0.12" orbit) set at 500 ¤} 50 rpm. A plate layout is provided to record standards and samples assayed.

5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any

remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

6. Add 200 µL of Human Endostatin Conjugate to each well. Cover with a new adhesive strip.
Incubate for 2 hours at room temperature on the shaker.

7. Repeat the aspiration/wash as in step 5.

8. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**

9. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.

10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings madedirectly at 450 nm without correction may be higher and less accurate.

CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a log/log curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on a log/log graph. The data may be linearized by plotting the log of the human Endostatin concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

LIMITATIONS OF THE PROCEDURE

• FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
• The kit should not be used beyond the expiration date on the kit label.
• Do not mix or substitute reagents with those from other lots or sources.
• If samples generate values higher than the highest standard, further dilute the samples with Calibrator Diluent and repeat the assay.
• Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
• Variations in sample collection, processing, and storage may cause sample value differences.

F2- VEGF ELISA

Catalogue Number DVE00

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for VEGF has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any VEGF present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for VEGF is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of VEGF bound in the initial step. The color development is stopped and the intensity of the color is measured.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Cell culture supernates should contain at least 1% fetal calf serum for stability of the VEGF. Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA, heparin, or citrate as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

VEGF Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the VEGF Standard with Calibrator Diluent RD5K (*for cell culture supernate samples*) or Calibrator Diluent RD6U (*for serum/plasma samples*). *Calibrator Diluent RD6U may contain a precipitate. Mix well before and during*

use. This reconstitution produces a stock solution of 2000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

For Cell Culture Supernate Samples: Use polypropylene tubes. Pipette 500 µL of Calibrator Diluent RD5K into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 1000 pg/mL dilution serves as the high standard. Calibrator Diluent RD5K serves as the zero standards (0 pg/mL).

For Serum/Plasma Samples: Use polypropylene tubes. Pipette 500µL of Calibrator Diluent RD6U into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 2000 pg/mL standard serves as the high standard. Calibrator Diluent RD6U serves as the zero standard (0 pg/mL).

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, samples, and controls be assayed in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.

3. **For Cell Culture Supernate Samples:** Add 50 µL of Assay Diluent RD1W to each well.
For Serum/Plasma Samples: Add 100 µL of Assay Diluent RD1W to each well.

4. **For Cell Culture Supernate Samples:** Add 200 µL of Standard, control, or sample per well.
For Serum/Plasma Samples: Add 100 µL of Standard, control, or sample per well.
Cover with the adhesive strip provided and incubate for 2 hours at room temperature.
A plate layout is provided to record the standards and samples assayed.

5. Aspirate each well and wash, repeating the process twice for a total of three washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance.
After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

6. Add 200 µL of VEGF Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.

7. Repeat the aspiration/wash as in step 5.

8. Add 200 µL of Substrate Solution to each well. **Protect from light.**

For Cell Culture Supernate Samples: Incubate for 20 minutes at room temperature.
For Serum/Plasma Samples: Incubate for 25 minutes at room temperature.

9. Add 50 µL of Stop Solution to each well. If color change does not appear uniform, gently tap the plate to ensure thorough mixing. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.

10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the VEGF concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- It is important that the Calibrator Diluent selected for the standard curve be consistent with the samples being assayed.
- If samples generate values higher than the highest standard, dilute the samples with the appropriate Calibrator Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

F3- Insulin ELISA (Mercodia Kit)

Catalogue no 10-1113-01

PRINCIPLE OF THE PROCEDURE

Mercodia Insulin ELISA is a solid phase two-site enzyme immunoassay. It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation insulin in the sample reacts with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to microplate welles. A simple washing step removes unbound enzyme labelled antibody. The bound conjugate is detected by reaction with 3,3',5,5'-tetramethylbenzidine (TMB). The reaction is stopped by adding acid to give a colorimetric endpoint that is read spectrophotometrically.

REAGENTS

Each Mercodia Insulin ELISA kit (10-1113-01) contains reagents for 96 wells, sufficient for 42 samples and one calibrator curve in duplicate. For larger series of assays, use pooled reagents from packages bearing identical lot numbers. The expiry date for the complete kit is stated on the outer label. The recommended storage temperature is 2–8°C.

Coated Plate with **Mouse monoclonal anti-insulin 8-well strips**
For unused microplate wells completely reseal the bag using adhesive tape and use within two months.
Calibrators 1, 2, 3, 4, 5
(recombinant human insulin) Color coded yellow
Enzyme Conjugate
Peroxidase conjugated mouse monoclonal anti-insulin.
Enzyme Conjugate Buffer
Substrate TMB 1
Stop Solution 0.5 M H_2SO_4

SPECIMEN COLLECTION AND HANDLING

Serum
Collect blood by venipuncture, allow clotting and separating the serum by centrifugation. Samples can be stored at 2–8°C up to 24 hours. For longer p eriods, store samples at –20°C. Avoid repeated freezing and thawing.

Plasma
Collect blood by venipuncture into tubes containing heparin or EDTA as anticoagulant, and separate the plasma fraction. Samples can be stored at 2–8°C up to 24 hours. For longerperiods store samples at –20°C. Avoid repeated free zing and thawing.

Preparation of samples
No dilution is normally required, however, samples containing >200 mU/L should be diluted 1+9 v/v with Calibrator 0.

TEST PROCEDURE

All reagents and samples must be brought to room temperature before use.
Prepare a calibrator curve for each assay run.
1. Prepare enzyme conjugate 1X solution and wash buffer 1X solution.

2. Prepare sufficient microplate wells to accomodate Calibrators and samples in duplicate.
3. Pipette 25 µL each of Calibrators and samples into appropriate wells.
4. Add 100 µL of enzyme conjugate 1X solution to each well.
5. Incubate on a plate shaker (700-900 rpm) for 1 hour at room temperature (18–25°C).
6. Wash 6 times with 700µL wash buffer 1X solution per well using an automatic plate washer with overflow-wash function. Do not include soak step in washing procedure. Or manually, discard the reaction volume by inverting the microplate over a sink. Add 350µL wash buffer 1X solution to each well. Discard the wash solution, tap firmly several times against absorbent paper to remove excess liquid. Repeat 5 times. Avoid prolonged soaking during washing. paper to remove excess liquid. Repeat 5 times. Avoid prolonged soaking during washing.
7. Add 200µL Substrate TMB into each well
8. Incubate for 15 minutes at room temperature (18–25°C).
9. Add 50µL Stop Solution to each well. Place plate on a shaker for approximately 5 seconds to ensure mixing.
10. Read optical density at 450 nm and calculate results. Read within 30 minutes.
Note! To prevent contamination between the conjugate and substrate, separate pipettes are recommended.

CALCULATION OF RESULTS
Computerized calculation
The concentration of insulin is obtained by computerized data reduction of the absorbance for the Calibrators, except for Calibrator 0, versus the concentration.

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• For in vitro diagnostic use.
• The contents of this kit and their residues must not be allowed to come into contact with ruminating animals or swine.
• The Stop solution in this kit contains 0.5 M H2SO4. Follow routine precautions for handling hazardous chemicals.
• All samples should be handled as of capable of transmitting infections.
• Each well can only be used once.

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Appendix- H; Tables for correlations in chapter 6

1-Change in VEGF with Physical and metabolic characteristics of volunteers

Variable	0 Min	ΔVEGF10	ΔVEGF 30	ΔVEGF 60	ΔVEGF 120	ΔVEGF 240	ΔVEGF 24hr
Age (years)	-0.239	-0.097	-0.536	-0.146	-0.634	-0.270	-0.058
	0.569	0.820	0.171	0.730	0.092	0.518	0.891
Height (cm)	-0.167	0.698	0.323	0.101	0.591	-0.335	-0.047
	0.693	0.054	0.435	0.812	0.123	0.417	0.912
Weight (kg)	-0.306	0.349	-0.041	-0.022	0.128	-0.058	0.036
	0.461	0.397	0.924	0.959	0.763	0.892	0.932
Body Fat (Kg)	-0.006	-0.037	-0.421	-0.234	-0.408	0.155	-0.165
	0.988	0.931	0.300	0.577	0.315	0.714	0.696
Body Fat (%)	0.132	-0.257	-0.609	-0.324	-0.568	0.249	-0.301
	0.756	0.539	0.109	0.433	0.142	0.552	0.468
Lean weight (kg)	-0.409	0.569	0.372	0.188	0.619	-0.226	0.193
	0.314	0.141	0.364	0.656	0.102	0.591	0.647
Body density	-0.119	0.125	0.305	0.099	0.493	-0.354	0.040
	0.779	0.768	0.463	0.815	0.215	0.390	0.925
body volume	-0.383	0.662	-0.061	-0.027	0.171	-0.200	0.022
	0.397	0.105	0.897	0.954	0.714	0.667	0.962
BMI (kg/m^2)	-0.143	-0.269	-0.292	-0.065	-0.368	0.244	0.090
	0.735	0.519	0.483	0.878	0.370	0.560	0.833
SBP (mmHg)	0.220	-0.199	-0.545	-0.555	-0.567	-0.131	-0.379
	0.600	0.637	0.162	0.153	0.143	0.757	0.354
DBP (mmHg)	0.268	0.198	-0.584	-0.390	-0.270	-0.213	-0.678
	0.521	0.638	0.129	0.340	0.517	0.613	0.065
WC (cm)	0.665	0.702	-0.090	-0.432	0.360	0.518	-0.640
	0.150	0.120	0.866	0.392	0.483	0.293	0.171
HC (cm)	0.061	0.569	0.147	-0.073	0.748	0.219	-0.281
	0.908	0.239	0.782	0.891	0.087	0.677	0.590
W/H ratio	0.512	-0.265	-0.333	-0.315	-0.839	0.098	-0.171
	0.299	0.612	0.519	0.543	0.037	0.853	0.747
FBG (mmol)	-0.154	0.278	-0.034	0.046	0.252	0.265	-0.037
	0.716	0.504	0.935	0.914	0.547	0.526	0.931
TG (mmol)	0.351	-0.582	-0.683	-0.464	-0.894	0.064	-0.343
	0.394	0.130	0.062	0.247	0.003	0.880	0.405
Cholesterol (mmol)	0.395	-0.459	-0.750	-0.482	-0.800	0.237	-0.474
	0.333	0.252	0.032	0.226	0.017	0.572	0.235
HDL (mmol)	0.123	0.609	0.154	0.003	0.538	0.107	-0.293
	0.771	0.109	0.716	0.994	0.169	0.800	0.481
Insulin resistance	0.414	-0.443	-0.673	-0.492	-0.627	0.321	-0.480
	0.308	0.272	0.067	0.216	0.096	0.438	0.229
SDLDL (mg/dl)	0.352	-0.278	-0.389	-0.517	-0.392	0.082	-0.356
	0.393	0.506	0.341	0.190	0.336	0.847	0.387
NEFA (mg/dl)	-0.025	-0.040	0.638	0.403	0.341	0.468	0.561
	0.953	0.925	0.089	0.322	0.408	0.243	0.148

2-Change in VEGF with different exercise parameters of the participants

Variable	ΔVEGF10	ΔVEGF 30	ΔVEGF 60	ΔVEGF 120	ΔVEGF 240	ΔVEGF 24hr
Basal HR	0.348	0.432	0.422	0.496	-0.016	0.192
	0.398	0.285	0.297	0.212	0.970	0.649
Max HR	-0.087	-0.148	-0.567	0.095	0.428	-0.632
	0.839	0.727	0.143	0.824	0.290	0.093
ΔHR	-0.294	-0.401	**-0.767**	-0.225	0.390	-0.680
	0.480	0.325	**0.026**	0.592	0.340	0.063
Change HR %	-0.337	-0.498	**-0.700**	-0.428	0.210	-0.517
	0.415	0.210	**0.053**	0.290	0.618	0.190
Duration	0.123	0.312	-0.087	0.115	-0.321	0.071
	0.771	0.452	0.838	0.786	0.437	0.866
distance	0.130	0.327	-0.079	0.120	-0.322	0.094
	0.759	0.430	0.853	0.776	0.437	0.824
Max Gradient	0.299	0.339	-0.107	0.339	-0.191	-0.061
	0.473	0.411	0.802	0.412	0.651	0.887
Basal VO_2	-0.393	0.126	-0.165	-0.153	0.075	0.138
	0.335	0.767	0.697	0.717	0.861	0.744
VO_2-Max	0.025	0.340	0.071	-0.007	-0.568	0.450
	0.953	0.409	0.868	0.987	0.142	0.263
Δvo2 max	0.085	0.350	0.101	0.015	-0.627	0.468
	0.841	0.395	0.812	0.972	0.096	0.242
ΔVO_2 %	0.549	0.220	0.326	0.220	-0.637	0.311
	0.159	0.601	0.430	0.600	0.089	0.453
Basal-VCO_2	-0.048	0.074	-0.327	-0.046	0.213	-0.064
	0.910	0.862	0.429	0.915	0.612	0.880
VCO_2-max	0.100	0.022	-0.332	0.063	-0.173	-0.057
	0.814	0.959	0.422	0.881	0.682	0.892
ΔVCO_2	0.109	0.017	-0.320	0.070	-0.199	-0.055
	0.798	0.968	0.440	0.869	0.636	0.897
ΔVCO_2%	0.156	0.004	0.097	0.203	-0.353	0.053
	0.712	0.993	0.819	0.629	0.392	0.901
Basal RER	0.263	0.136	-0.328	-0.003	0.349	-0.322
	0.529	0.748	0.428	0.995	0.397	0.437
RER-max	-0.243	-0.320	**-0.732**	-0.122	0.256	-0.670
	0.563	0.440	**0.039**	0.773	0.540	0.069
ΔRER	-0.520	-0.533	**-0.761**	-0.165	0.098	-0.681
	0.187	0.174	**0.028**	0.697	0.817	0.063
ΔRER%	-0.616	-0.566	-0.661	-0.151	-0.027	-0.572
	0.104	0.144	0.074	0.721	0.950	0.139
Basal RR	-0.399	-0.392	-0.327	-0.595	-0.073	0.049
	0.328	0.337	0.429	0.120	0.863	0.908
RR-max	-0.296	-0.097	0.008	0.255	0.654	-0.207
	0.476	0.820	0.984	0.542	0.079	0.623
ΔRR	-0.113	0.069	0.140	0.477	0.635	-0.211
	0.790	0.871	0.741	0.232	0.091	0.615
ΔRR %	0.178	0.228	0.261	0.652	0.447	-0.189
	0.673	0.588	0.532	0.080	0.267	0.655

3- Change in Endostatin with Physical and metabolic characteristics of volunteers

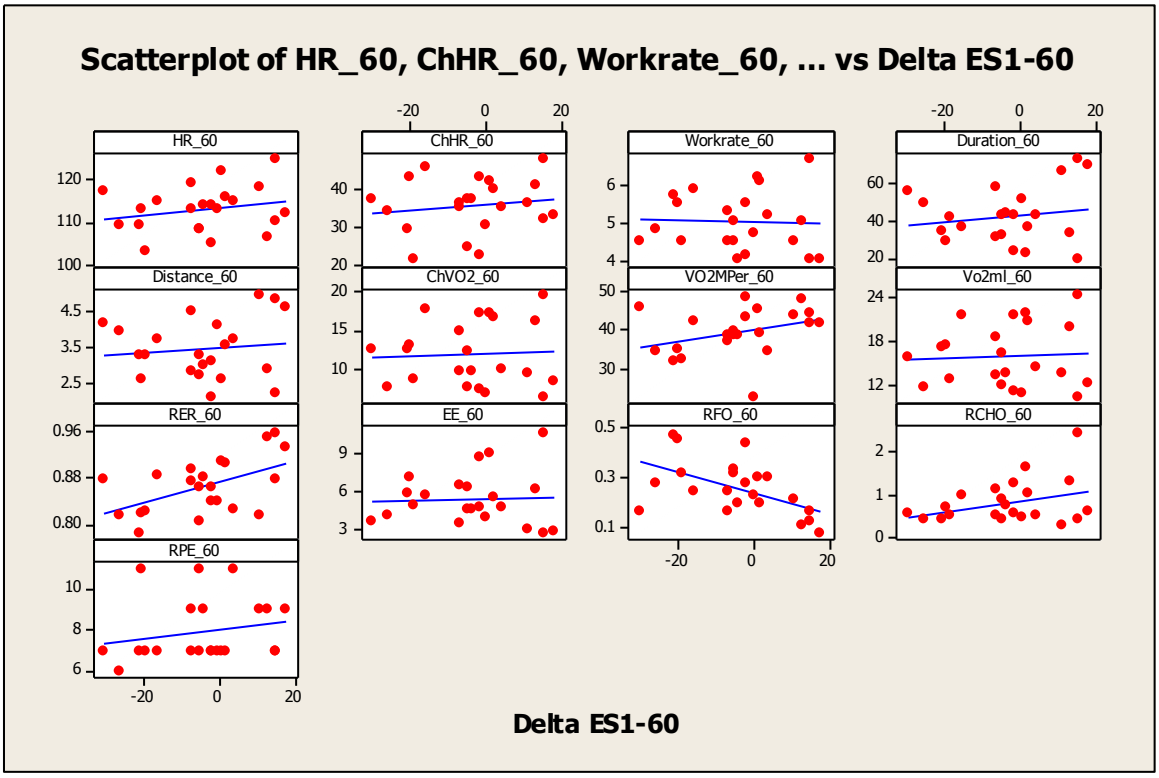
Variable	Baseline	ΔES10	ΔES 30	ΔES 60	ΔES 120	ΔES 240	ΔES 24
Age (years)	0.077	-0.080	-0.340	-0.099	0.347	**0.751**	**0.727**
	0.857	0.851	0.410	0.816	0.400	**0.032**	**0.041**
Height (cm)	-0.366	-0.385	-0.253	-0.179	0.042	0.148	-0.230
	0.372	0.346	0.545	0.672	0.920	0.726	0.585
Weight (kg)	0.196	-0.454	-0.499	-0.591	0.003	0.683	0.272
	0.642	0.258	0.208	0.122	0.994	0.062	0.515
Body Fat (Kg)	0.332	-0.091	-0.329	-0.584	-0.144	0.617	0.488
	0.422	0.831	0.426	0.128	0.735	0.103	0.220
Body Fat (%)	0.440	-0.116	-0.424	-0.253	-0.163	0.491	0.525
	0.275	0.785	0.295	0.545	0.700	0.217	0.182
Lean weight (kg)	-0.045	-0.570	-0.396	-0.284	0.114	0.358	-0.119
	0.915	0.140	0.332	0.495	0.787	0.384	0.779
Body density	-0.372	-0.148	0.057	0.453	0.195	-0.367	-0.328
	0.364	0.726	0.894	0.260	0.643	0.371	0.427
body volume	0.051	-0.468	-0.497	-0.638	0.073	0.697	0.314
	0.913	0.290	0.256	0.123	0.876	0.082	0.493
BMI (kg/m^2)	0.442	-0.089	-0.231	-0.394	-0.031	0.456	0.409
	0.273	0.834	0.581	0.334	0.943	0.256	0.315
SBP (mmHg)	0.633	-0.009	-0.282	0.595	0.072	0.502	0.570
	0.092	0.983	0.499	0.120	0.865	0.205	0.141
DBP (mmHg)	-0.314	-0.198	-0.493	-0.083	-0.110	0.272	0.308
	0.449	0.638	0.214	0.845	0.795	0.515	0.458
WC (cm)	0.530	-0.386	-0.489	-0.044	-0.784	-0.283	-0.412
	0.280	0.450	0.325	0.934	0.065	0.587	0.417
HC (cm)	0.174	-0.755	-0.671	-0.038	-0.376	-0.287	-0.468
	0.741	0.083	0.145	0.943	0.462	0.581	0.349
W/H ratio	0.215	**0.815**	0.585	0.047	-0.119	0.215	0.377
	0.682	**0.048**	0.223	0.930	0.822	0.682	0.461
FBG (mmol)	0.450	**-0.765**	**-0.796**	-0.136	-0.130	0.428	0.045
	0.263	**0.027**	**0.018**	0.748	0.760	0.291	0.916
TG (mmol)	0.342	0.446	0.117	0.312	-0.015	0.161	0.554
	0.407	0.269	0.783	0.452	0.972	0.704	0.154
Cholesterol (mmol)	0.360	0.226	-0.149	-0.022	-0.218	0.243	0.525
	0.381	0.591	0.724	0.959	0.603	0.562	0.181
HDL (mmol)	-0.318	-0.364	-0.336	**-0.694**	-0.417	-0.004	-0.326
	0.442	0.376	0.416	**0.056**	0.304	0.993	0.430
Insulin Resistance	0.629	-0.026	-0.352	0.350	-0.235	0.191	0.414
	0.095	0.952	0.392	0.396	0.575	0.650	0.307
SDLDL (mg/dl)	**0.750**	0.012	-0.181	0.724	-0.093	0.187	0.307
	0.032	0.978	0.669	0.042	0.827	0.657	0.459
NEFA (mg/dl)	0.480	0.109	0.389	0.278	-0.095	-0.407	-0.510
	0.229	0.796	0.341	0.505	0.823	0.317	0.196

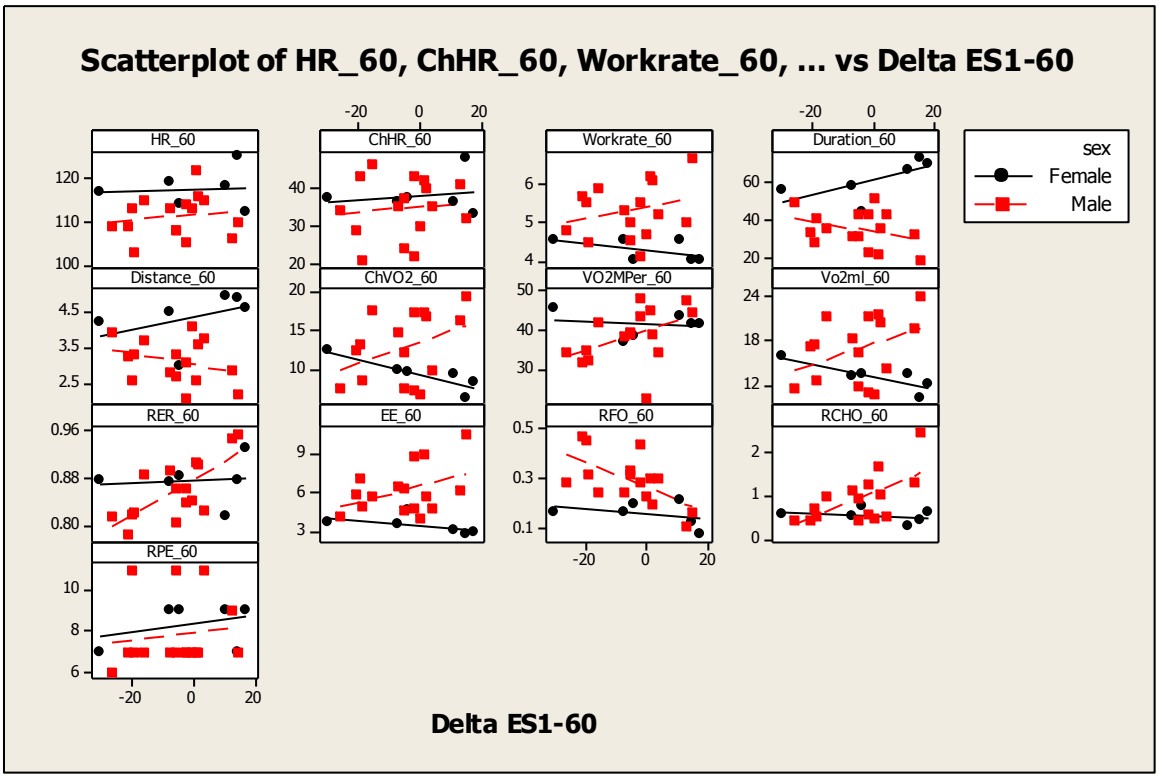
4-Change in endostatin with different exercise parameters of the participants

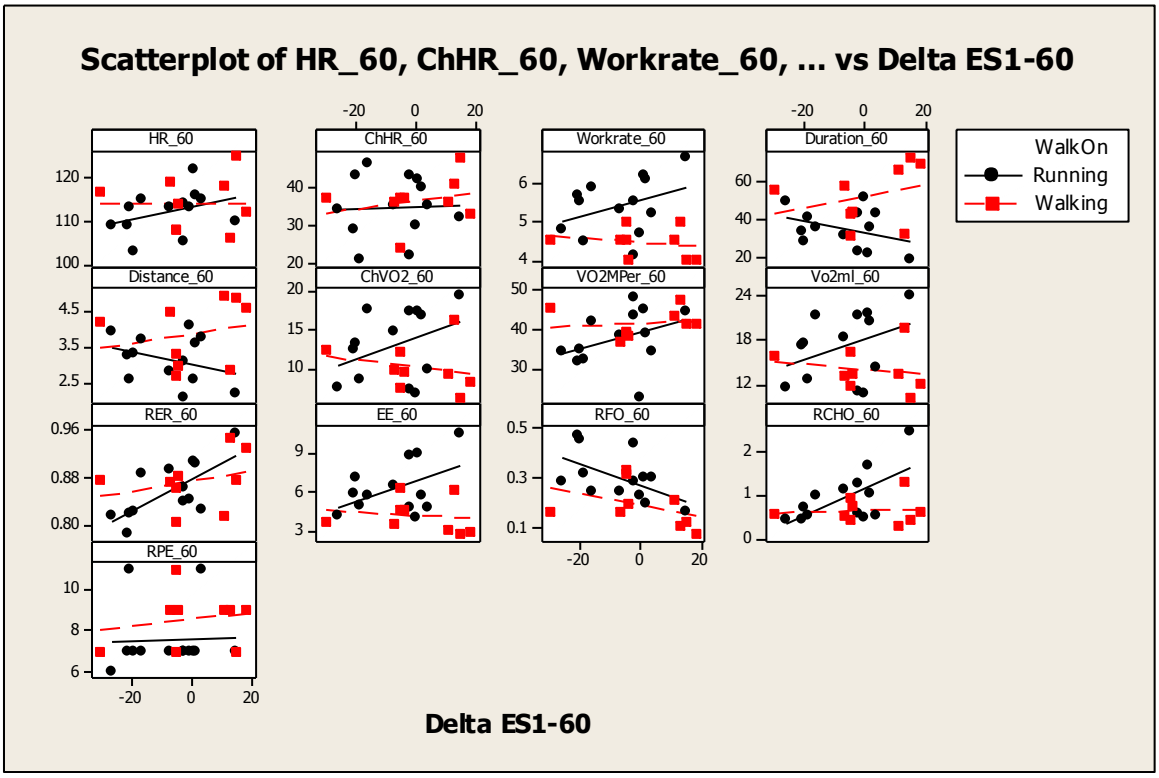
Variable	ΔES10	ΔES 30	ΔES 60	ΔES 120	ΔES 240	ΔES 24
Basal HR	-0.376	-0.165	**0.703**	0.179	-0.384	-0.509
	0.359	0.696	**0.052**	0.672	0.348	0.198
Max HR	0.116	0.014	0.165	**-0.717**	-0.395	-0.269
	0.785	0.973	0.696	**0.045**	0.333	0.519
ΔHR	0.337	0.116	-0.292	**-0.747**	-0.111	0.078
	0.415	0.785	0.482	**0.033**	0.794	0.854
Change HR %	0.478	0.609	0.330	0.098	-0.222	-0.170
	0.230	0.109	0.425	0.817	0.598	0.687
Duration	0.475	0.612	0.320	0.106	-0.201	-0.164
	0.234	0.107	0.440	0.802	0.634	0.697
distance	0.189	0.325	0.259	-0.078	-0.223	-0.301
	0.654	0.433	0.536	0.855	0.596	0.469
Max Gradient	0.404	0.475	**0.750**	0.039	-0.263	-0.050
	0.321	0.234	**0.032**	0.927	0.528	0.906
Basal VO_2	0.378	0.552	0.407	0.532	0.176	0.141
	0.356	0.156	0.317	0.174	0.677	0.739
VO_2-Max	0.350	0.529	0.331	0.571	0.229	0.160
	0.395	0.178	0.424	0.139	0.585	0.705
ΔVO_2 max	0.148	0.140	0.358	-0.233	0.093	0.010
	0.726	0.741	0.385	0.578	0.826	0.981
ΔVO_2 %	0.090	0.085	-0.361	-0.097	0.391	0.214
	0.832	0.841	0.379	0.820	0.338	0.611
Basal-VCO_2	0.082	0.077	-0.409	-0.082	0.402	0.223
	0.847	0.856	0.315	0.848	0.323	0.595
VCO_2-max	0.460	0.411	-0.005	-0.579	-0.214	-0.284
	0.251	0.312	0.992	0.133	0.610	0.496
ΔVCO_2	0.367	0.196	-0.214	-0.680	-0.218	-0.003
	0.371	0.642	0.611	0.064	0.605	0.994
ΔVCO_2%	0.169	-0.028	-0.288	-0.510	-0.142	0.199
	0.689	0.947	0.489	0.197	0.737	0.636
Basal RER	0.275	0.091	-0.347	0.103	0.612	0.669
	0.510	0.830	0.400	0.808	0.107	0.070
RER-max	-0.326	-0.332	-0.425	-0.551	-0.444	-0.325
	0.430	0.422	0.294	0.157	0.271	0.432
ΔRER	-0.413	-0.344	-0.253	-0.552	-0.658	-0.572
	0.309	0.404	0.545	0.156	0.076	0.139

Appendix- I; Figures for correlations in chapter 4

For pooled/ gender and walking/running based groups



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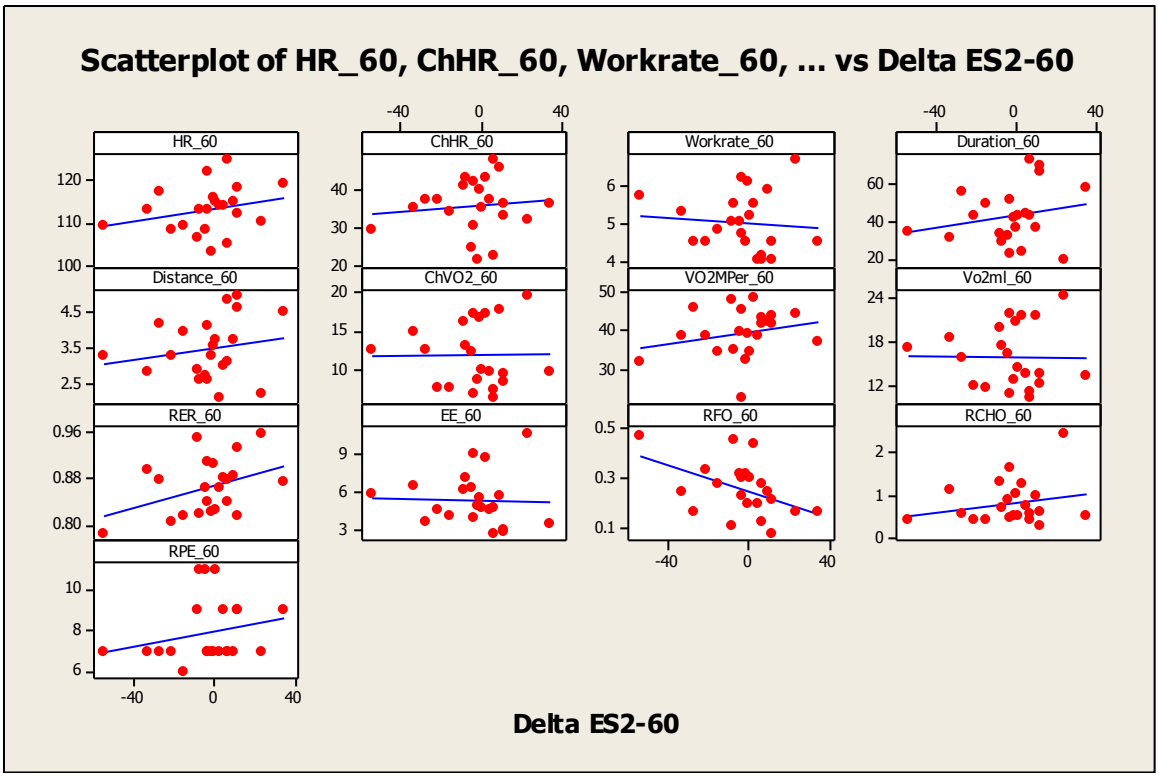
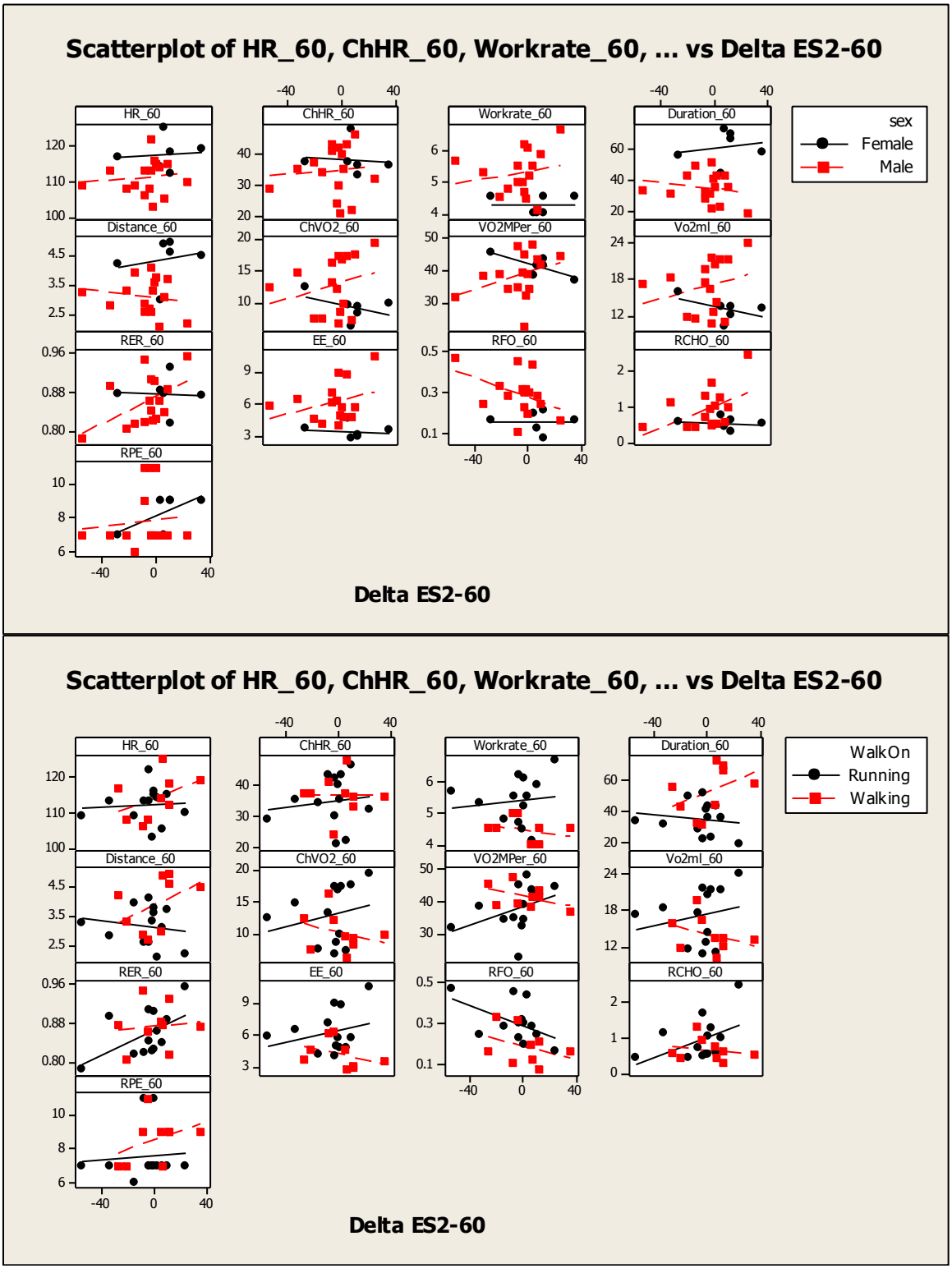
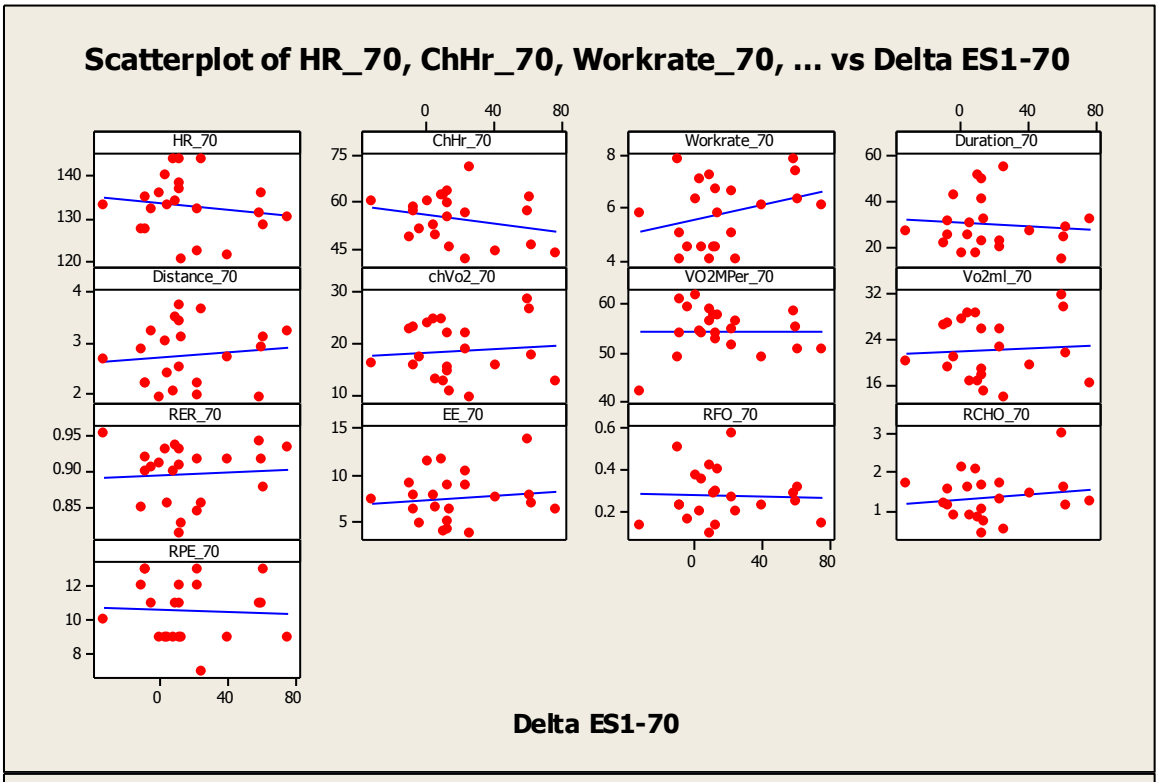
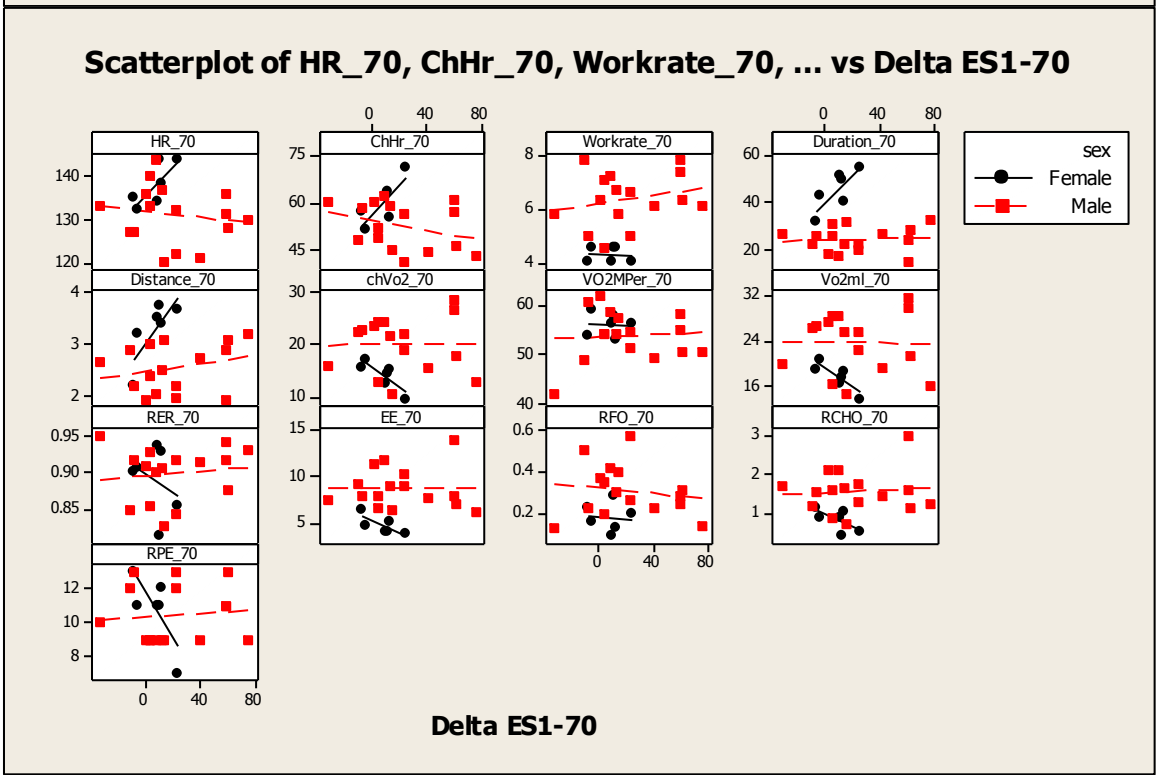
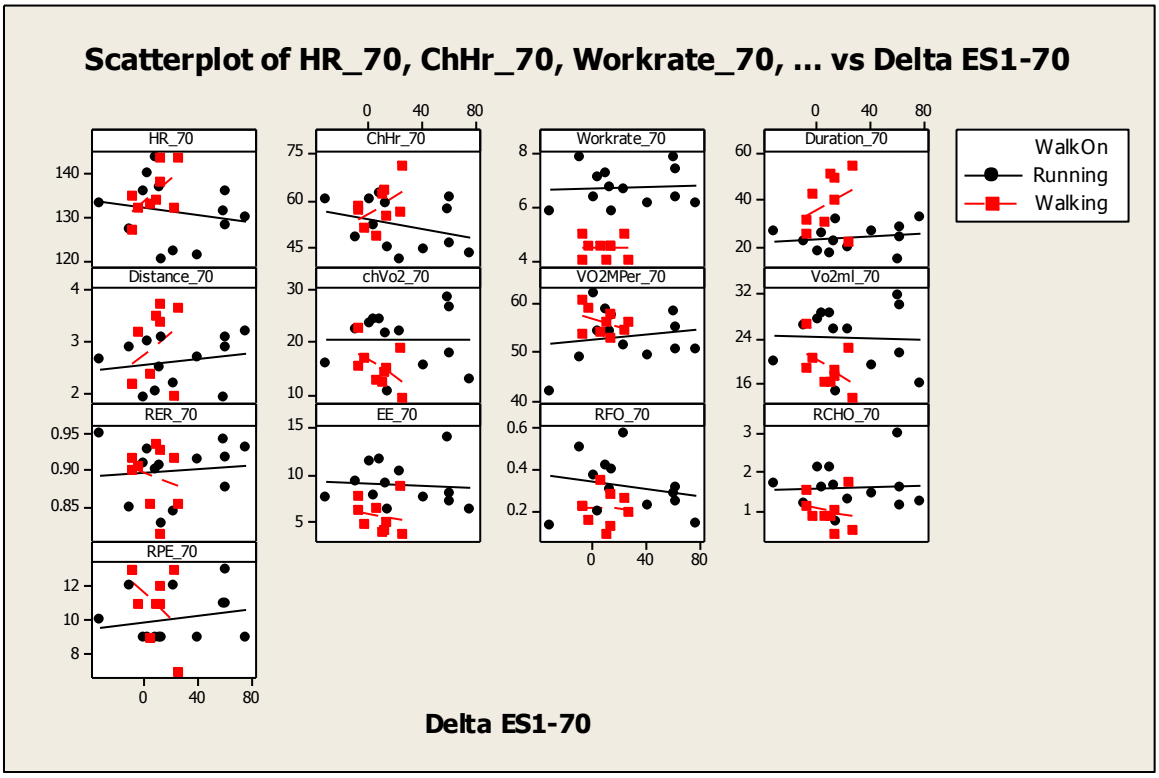
HR_60
ChHR_60
Workrate_60
Duration_60
Distance_60
ChVO2_60
VO2MPer_60
Vo2ml_60
RER_60
EE_60
RFO_60
RCHO_60
RPE_60
WalkOn
Running
Walking
Delta ES1-60

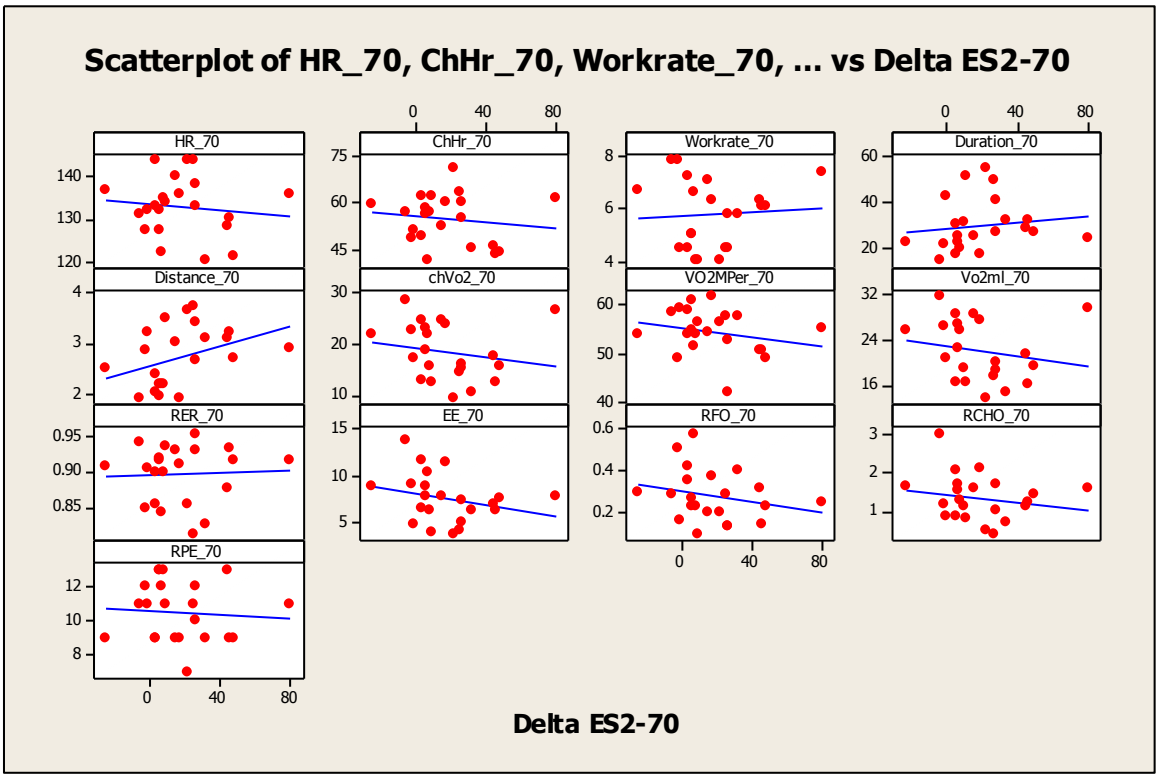

Scatterplot of HR_60, ChHR_60, Workrate_60, ... vs Delta ES2-60
HR_60
ChHR_60
Workrate_60
Duration_60
Distance_60
ChVO2_60
VO2MPer_60
Vo2ml_60
RER_60
EE_60
RFO_60
RCHO_60
RPE_60
Delta ES2-60

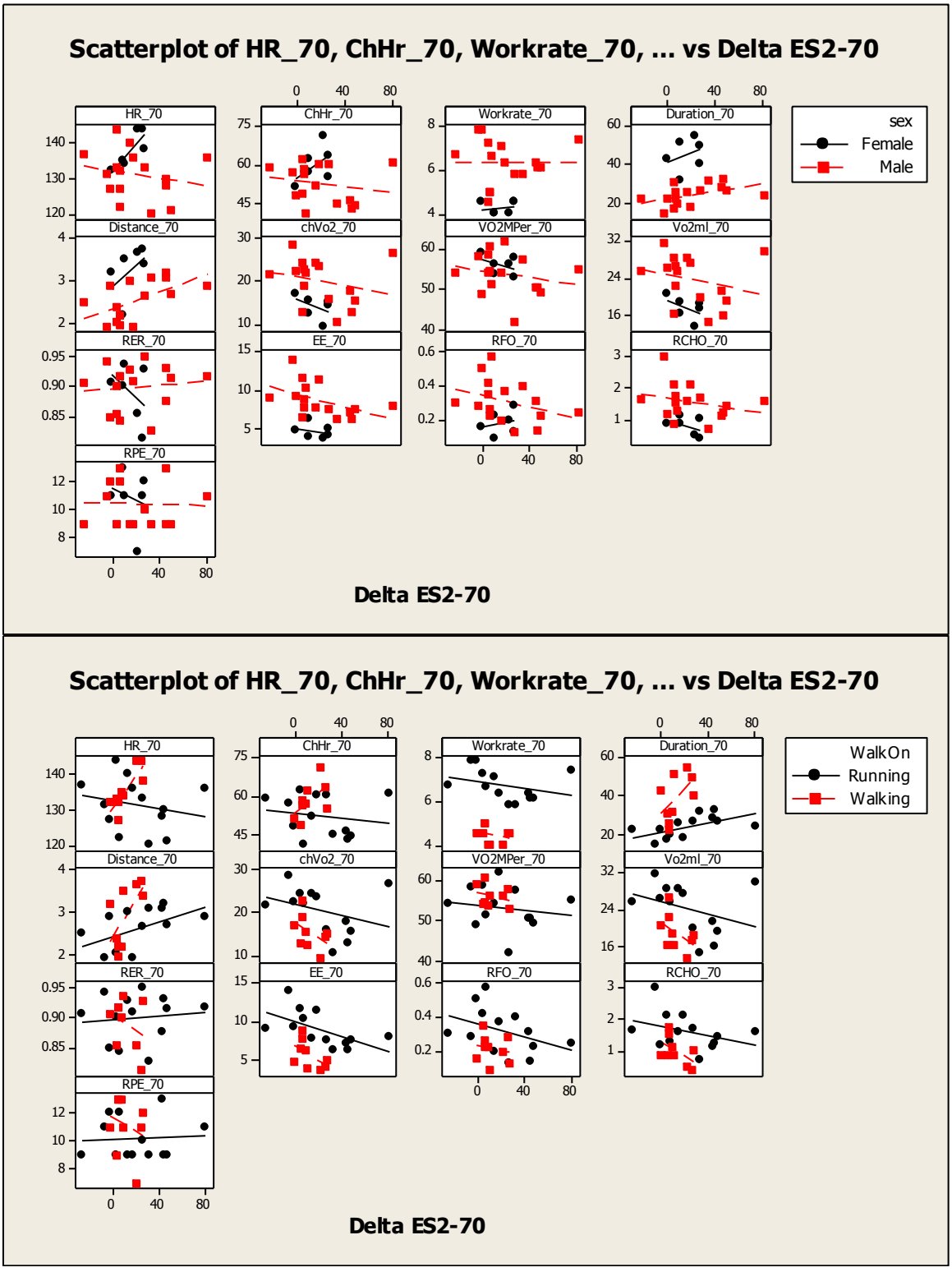

Scatterplot of HR_60, ChHR_60, Workrate_60, ... vs Delta ES2-60
HR_60
ChHR_60
Workrate_60
Duration_60
Distance_60
ChVO2_60
VO2MPer_60
Vo2ml_60
RER_60
EE_60
RFO_60
RCHO_60
RPE_60
sex
Female
Male
Delta ES2-60
Scatterplot of HR_60, ChHR_60, Workrate_60, ... vs Delta ES2-60
HR_60
ChHR_60
Workrate_60
Duration_60
Distance_60
ChVO2_60
VO2MPer_60
Vo2ml_60
RER_60
EE_60
RFO_60
RCHO_60
RPE_60
WalkOn
Running
Walking
Delta ES2-60

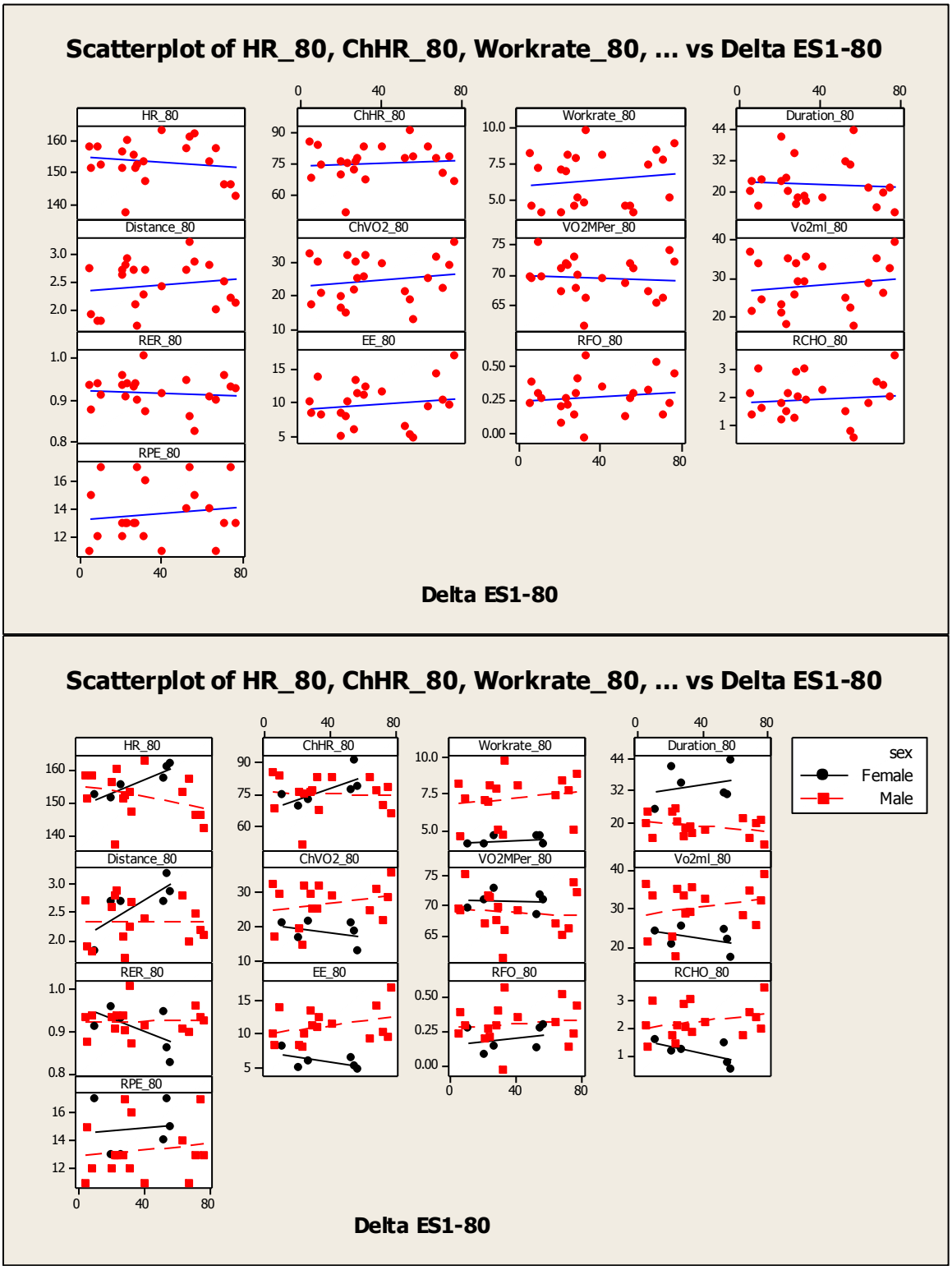

Scatterplot of HR_70, ChHr_70, Workrate_70, ... vs Delta ES1-70
HR_70
ChHr_70
Workrate_70
Duration_70
Distance_70
chVo2_70
VO2MPer_70
Vo2ml_70
RER_70
EE_70
RFO_70
RCHO_70
RPE_70
Delta ES1-70

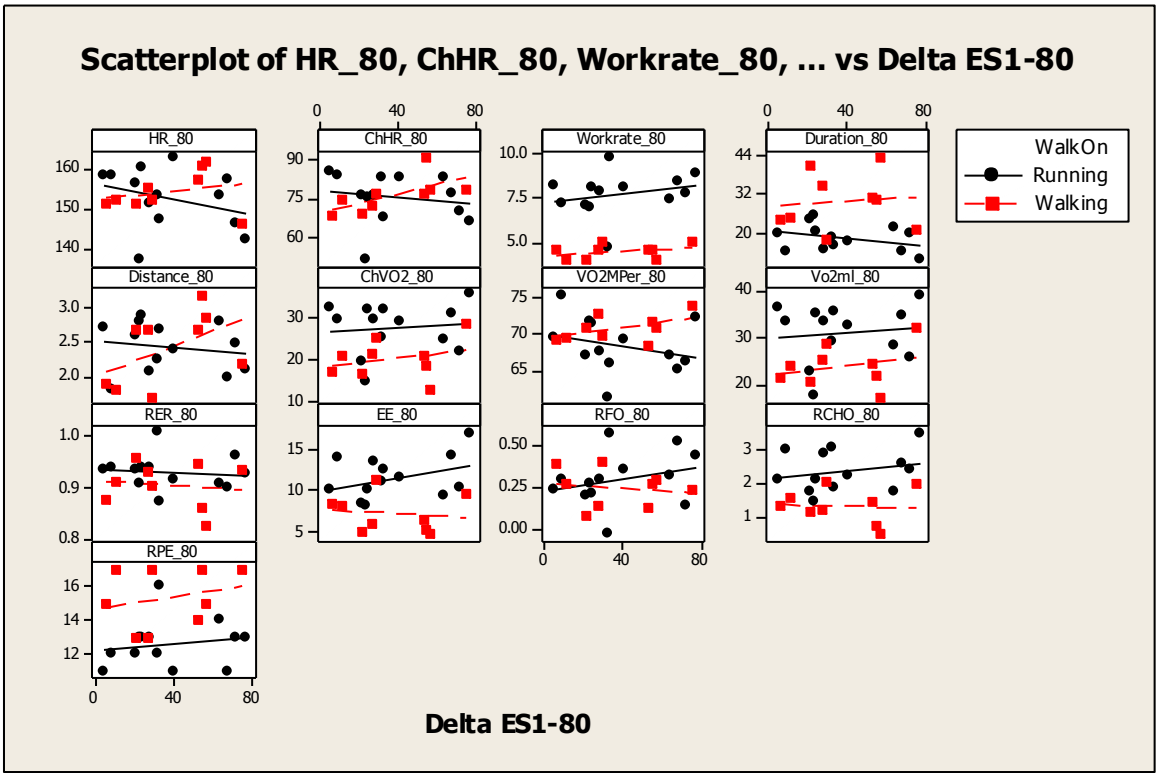

Scatterplot of HR_70, ChHr_70, Workrate_70, ... vs Delta ES1-70
HR_70
ChHr_70
Workrate_70
Duration_70
Distance_70
chVo2_70
VO2MPer_70
Vo2ml_70
RER_70
EE_70
RFO_70
RCHO_70
RPE_70
sex
Female
Male
Delta ES1-70

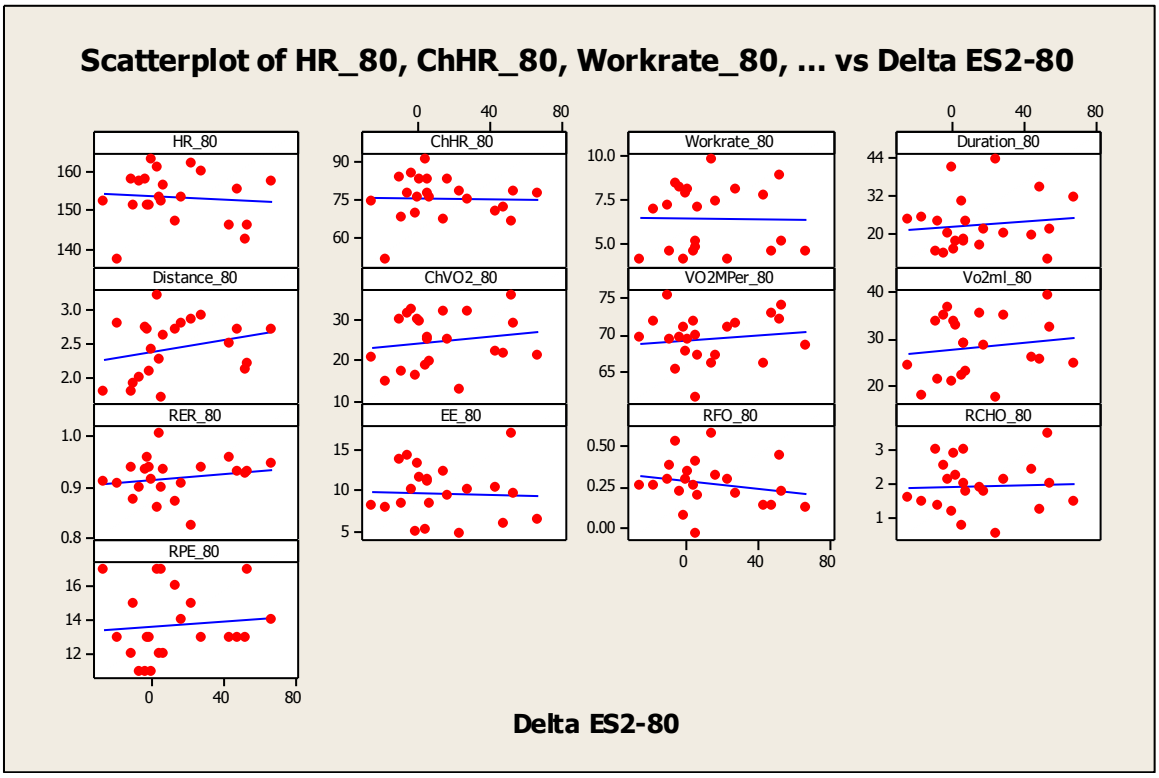

Scatterplot of HR_70, ChHr_70, Workrate_70, ... vs Delta ES1-70
HR_70
ChHr_70
Workrate_70
Duration_70
Distance_70
chVo2_70
VO2MPer_70
Vo2ml_70
RER_70
EE_70
RFO_70
RCHO_70
RPE_70
WalkOn
Running
Walking
Delta ES1-70

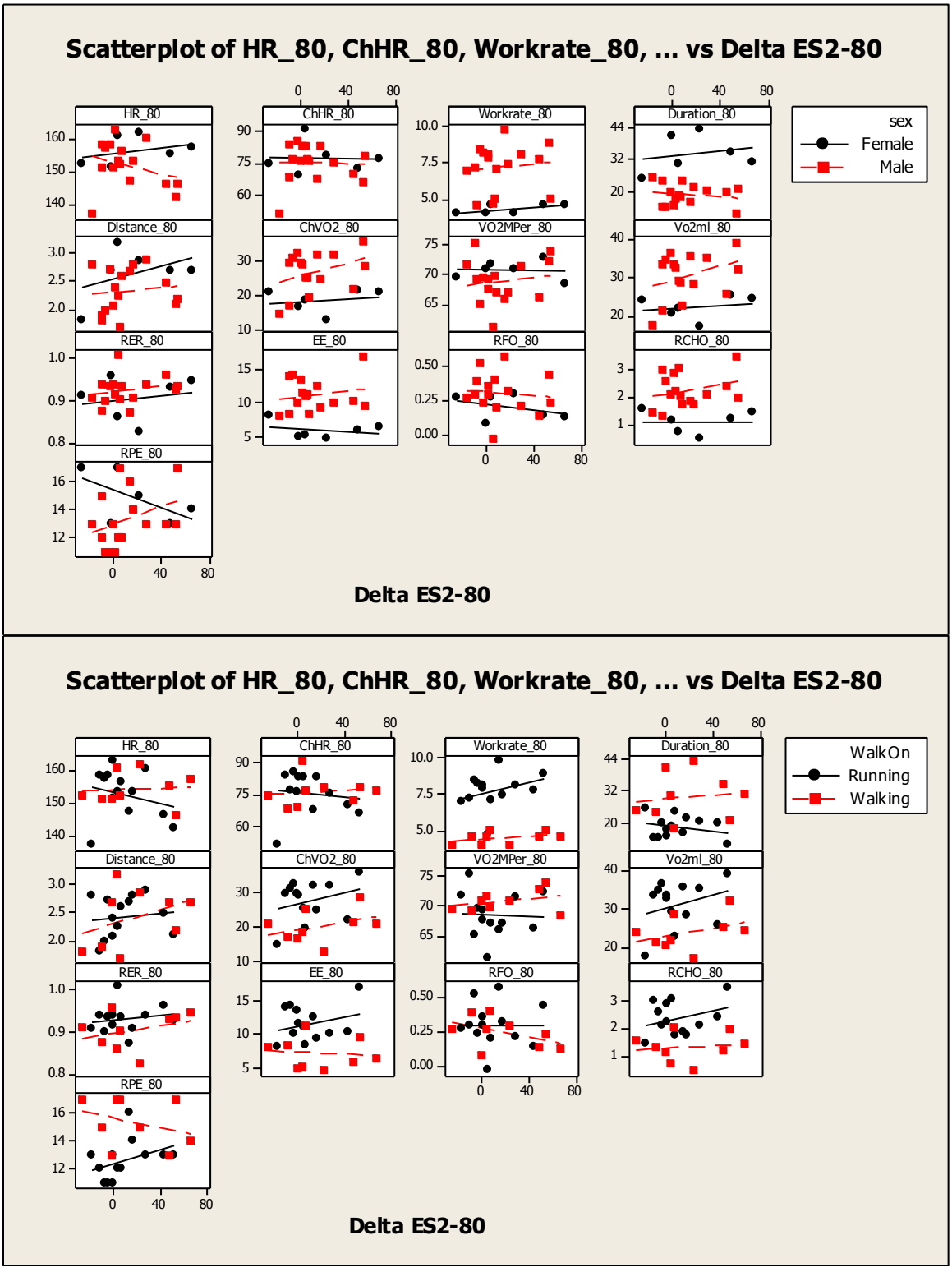

Scatterplot of HR_70, ChHr_70, Workrate_70, ... vs Delta ES2-70
HR_70
ChHr_70
Workrate_70
Duration_70
Distance_70
chVo2_70
VO2MPer_70
Vo2ml_70
RER_70
EE_70
RFO_70
RCHO_70
RPE_70
Delta ES2-70


Scatterplot of HR_70, ChHr_70, Workrate_70, ... vs Delta ES2-70
HR_70
ChHr_70
Workrate_70
Duration_70
Distance_70
chVo2_70
VO2MPer_70
Vo2ml_70
RER_70
EE_70
RFO_70
RCHO_70
RPE_70
sex
Female
Male
Delta ES2-70
Scatterplot of HR_70, ChHr_70, Workrate_70, ... vs Delta ES2-70
HR_70
ChHr_70
Workrate_70
Duration_70
Distance_70
chVo2_70
VO2MPer_70
Vo2ml_70
RER_70
EE_70
RFO_70
RCHO_70
RPE_70
WalkOn
Running
Walking
Delta ES2-70


Scatterplot of HR_80, ChHR_80, Workrate_80, ... vs Delta ES1-80
HR_80
ChHR_80
Workrate_80
Duration_80
Distance_80
ChVO2_80
VO2MPer_80
Vo2ml_80
RER_80
EE_80
RFO_80
RCHO_80
RPE_80
Delta ES1-80
Scatterplot of HR_80, ChHR_80, Workrate_80, ... vs Delta ES1-80
HR_80
ChHR_80
Workrate_80
Duration_80
Distance_80
ChVO2_80
VO2MPer_80
Vo2ml_80
RER_80
EE_80
RFO_80
RCHO_80
RPE_80
sex
Female
Male
Delta ES1-80


Scatterplot of HR_80, ChHR_80, Workrate_80, ... vs Delta ES1-80
HR_80
ChHR_80
Workrate_80
Duration_80
Distance_80
ChVO2_80
VO2MPer_80
Vo2ml_80
RER_80
EE_80
RFO_80
RCHO_80
RPE_80
WalkOn
Running
Walking
Delta ES1-80


Scatterplot of HR_80, ChHR_80, Workrate_80, ... vs Delta ES2-80
HR_80
ChHR_80
Workrate_80
Duration_80
Distance_80
ChVO2_80
VO2MPer_80
Vo2ml_80
RER_80
EE_80
RFO_80
RCHO_80
RPE_80
Delta ES2-80


Scatterplot of HR_80, ChHR_80, Workrate_80, ... vs Delta ES2-80
HR_80
ChHR_80
Workrate_80
Duration_80
Distance_80
ChVO2_80
VO2MPer_80
Vo2ml_80
RER_80
EE_80
RFO_80
RCHO_80
RPE_80
sex
Female
Male
Delta ES2-80
Scatterplot of HR_80, ChHR_80, Workrate_80, ... vs Delta ES2-80
HR_80
ChHR_80
Workrate_80
Duration_80
Distance_80
ChVO2_80
VO2MPer_80
Vo2ml_80
RER_80
EE_80
RFO_80
RCHO_80
RPE_80
WalkOn
Running
Walking
Delta ES2-80