COMPARATIVE STUDY OF BIOCHEMICAL PARAMETERS OF CARDIAC PATIENTS FROM DIFFERENT REGIONS OF BANGLADESH.

A THESIS SUBMITTED TO THE UNIVERSITY OF DHAKA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSOPHY IN BIOCHEMISTRY AND MOLECULAR BIOLOGY

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MRS. SABINA YASMIN

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BANGLADESH

DEDICATION

DEDICATED TO MY BELOVED PARENTS AND TEACHERS

TO WHOM IT MAY CONCERN

This is to certify that thesis entitled **"COMPARATIVE STUDY OF BIOCHEMICAL PARAMETERS OF CARDIAC PATIENTS FROM DIFFERENT REGIONS OF BANGLADESH."** submitted by Mrs. Sabina Yasmin. The author carried out her research under my supervision and she performed the relevant investigations from Bangladesh Medical College Hospital, Dhanmondi, Dhaka. No part of this thesis has been submitted to any other university or institution for any degree or any other award. I therefore, recommend the submission of the thesis for the degree of Master of Philosophy.

(PROF: DR. MD. ENAMUL HAQUE)

Supervisor
Professor
Department of Biochemistry and Molecular Biology
Dhaka University
Dhaka-1000
Bangladesh

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ABSTRACT

Globally, cardiovascular diseases are the number one cause of death and are projected to remain so. An estimated 17.5 million people died from cardiovascular disease in 2005, representing 30 % of all global deaths. Of these deaths, 7.6 million were due to heart attacks and 5.7 million due to stroke. About 80% of these deaths occurred in low- and middle-income countries. If current trends are allowed to continue, by the year 2015, an estimated 20 million people will die from cardiovascular disease (mainly from heart attacks and strokes). Cardiovascular disease (CVD) is an abnormal function of the heart or blood vessels. It can cause an increase in risk for heart attack, heart failure, sudden death, stroke and cardiac rhythm problems, thus resulting in decreased quality of life and decreased life expectancy. The causes of cardiovascular disease range from structural defects, to infection, inflammation, environmental and genetical. Cardiovascular diseases include coronary heart disease (heart attacks), cerebrovascular disease, raised blood pressure (hypertension), peripheral artery disease, rheumatic heart disease, congenital heart disease and heart failure. We studied total 290 cases whose serum were collected from different areas of Bangladesh and these are coastal area, urban area and hilly area and each group has same age level controls. Values in controls vs. patients were found to be: Age (year, Mean±SD) 52.94 ± 8.93 vs. 58.12 ± 9.31, Sex (F/M) 42/48 vs. 66/134, respectively. It is reported, that CVD risk varies with geographical inhabitants, ethnic lifestyle and cultural history and typical food habits. In this study we examined various risk factors and cardiovascular markers among the urban, hilly and coastal populations. From our study we found the following average cholesterol level in urban, hilly and coastal area's populations respectively: (mean ± sd) 170.62 ± 41.92 mg/dL, 158.14 ± 37.75 mg/dL and 168.96 ± 40.5 mg/dL, where the hilly population showed a significant lower cholesterol level then the other two groups. The average HDL level of hilly, urban and coastal populations were as follows respectively: (mean ± sd) 36.69 ± 5.08 mg/dL, 37.85 ± 8.56 mg/dL and 42.37 ± 8.53 mg/dL and interestingly HDL known as good cholesterol was clearly higher in coastal groups populations than the other two groups. Hilly populations had average LDL level (mean ± sd) 117.02 ± 23.08 mg/dL, urban patients had 123.19 ± 22.18 mg/dL and coastal area's people had average LDL concentration is 120.58 ± 25.97 mg/dL. Serum electrolytes,

and AST and CKMB variation in cardiac patients in these geographic regions is also reported.

Additionally, a positive correlation between age and total cholesterol and LDL and a negative correlation between age and serum Cl was shown. This study is a preliminary investigation to get a relationship between heart diseases and geographical stratifications in Bangladesh.

CHAPTER 1

INTRODUCTION

1.1.1 : Cardiovascular disease

Cardiovascular disease (CVD) is an abnormal function of the heart or blood vessels. It can cause an increase in risk for heart attack, heart failure, sudden death, stroke and cardiac rhythm problems, thus resulting in decreased quality of life and decreased life expectancy. The causes of cardiovascular disease range from structural defects, to infection, inflammation, environmental and genetical. Cardiovascular diseases include coronary heart disease (heart attacks), cerebrovascular disease, raised blood pressure (hypertension), peripheral artery disease, rheumatic heart disease, congenital heart disease and heart failure. The major causes of cardiovascular disease are use of tobacco, physical inactivity, and an unhealthy diet. To help prevention of cardiovascular disease one must adopt a healthy lifestyle and avoid smoking, fattening foods and stress [1].

Globally, cardiovascular diseases are the number one cause of death and are projected to remain so. An estimated 17.5 million people died from cardiovascular disease in 2005, representing 30% of all global deaths. Of these deaths, 7.6 million were due to heart attacks and 5.7 million due to stroke. About 80% of these deaths occurred in low- and middle-income countries. If current trends are allowed to continue, by the year 2015, an estimated 20 million people will die from cardiovascular disease (mainly from heart attacks and strokes) [2].

1.1.2 : Rationale of this study

CVD is a global disease and includes patients of different socioeconomic status as well as different geographical regions. CVD risk factors and disease progression are often related with socioeconomic status and life styles. In a large-scale clinical study in caNADa from 1986 to 1992 including 29,855 samples, inverse correlation between socioeconomic status (education and income) and prevalence of CVD risk factors was reported.[3] interestingly, high CVD mortality in males compared to females was also observed historically. [4] along with socioeconomic status and race/ethnicity, CVD death rates strikingly differ by geography in usa. [5] higher death rates by premature coronary heart disease (CHD) were observed in the rural south of the us compared to urban areas.[6] this is may be an artifact of treatment availability, education, communications etc. These types of disparities were also reported in

Europe and Australia.[7,8]

High CVD mortality in south asian compared to other ethnic regions was reported, but there are very few such type of study in this sub-continent. [9] higher CVD death rates was also observed in south asian migrants in different countries. Major risk factors identified in south Asian populations are; impaired glucose tolerance (IGT), central obesity, high TG (triglycerides), and low HDL (high density lipoprotein) [9]. Prevalence of diabetes in urban India is at least 3 times higher than the rural which may contribute to more urban CVD deaths in this region [9].

Consistent high blood pressure was observed among urban gambian populations compared to Gambian rurales. [10] national blood pressure survey in China reported increased hypertension caused by the modernizations. Body mass index (BMI), blood pressure and total serum cholesterol were significantly higher in urban China compared to rural China.[11] in urban north India, diabetes, hypertension and coronary artery disease (CAD) was significantly higher compare to rural north India. [12] an elevated level of proinflammatory cytokines and leptin was reported in the blood of urban Indians compared to rural Indians which may contribute in increased risk of diabetes and CHD. [13]

Lower CVD mortality was observed in mountains compared to coastal regions in north Carolina. [14] among coastal south Indians, fish consumers had lower blood pressure compared to non-fish consumers. [15]. Bangladesh is a small but geographically and ethnically diverse country with high density population and high CVD mortality rates. Rheumatic fever and rheumatic heart disease were reported in rural Bangladeshi children, age between 5 and 15 with a density of 1.2 and 1.3 per thousand, respectively.[16] arsenic was identified as a risk factor for diabetes in Bangladesh which can be geographically stratified. [17] along with other physiological problems arsenic exposure was also reported as hypertension inducer in rural Bangladeshi adults.[18] prevalence of tobacco consumption, hypertension, and central obesity in females was reported in general clinic-based rural population of Bangladesh.[19] diabetes was found prevalent in urban but paradoxically IGT in rural Bangladesh. [20] in a cross-sectional survey, a higher prevalence of diabetes was reported in urban compared with rural Bangladeshi populations. [21]

The urbanizations and modernizations increase the urban population but still most of the people in Bangladesh live in rural areas. Here, we have one of the largest coasts in the world and many

people live in coastal regions as well. In the coastal regions of Bangladesh, there are ~9.2 million people living and in hilly areas ~1.4 million (Bagerhat, Khulna, Satkhira, Barguna, Patuakhali and Pirojpur are considered as coast and Bandarban, Khagrachari and Rangmati as mountains) (https://www.citypopulation.de/Bangladesh-mun.html). But there is limited study includes CVD risks with geographical stratifications. Study that includes biochemical markers such as lipid profiles and related enzymes with demographic considerations is also insufficient. This study will identify CVD risk parameters in different regions of Bangladesh which will benefits us to create strategies of CVD management and prevention. The rationale to study lipid profile is that, these biochemical markers can confirm CVD risk and its progression. Lipid profiles are also important to identify therapeutic outcomes. Along with lipid profiles, two important enzymes namely creatine kinase (ck) and aspartate aminotransferase (AST) will also be investigated as CVD markers. Serum electrolyte imbalance is associated with multiple disorders including renal, cardiovascular and hematological complications. [22] important electrolytes, such as sodium (Na), potassium (K), chlorine (Cl) and carbon-di-oxide (Co2) will be measured in subject's serum to assess the disease conditions.

1.1.3 Thesis aim and objectives

The aim of this study is to compare biochemical parameters of cardiac patients of urban, hilly and coastal populations of Bangladesh. This aim will be achieved through three specific objectives. These are:

1. Measuring and comparing serum CKMB and AST/SGOT status of cardiac patients among the above-mentioned populations.
2. Measuring and comparing serum lipid profile (Total cholesterol, HDL, LDL and TG) of cardiac patients among the above-mentioned populations.
3. Measuring and comparing serum electrolyte (K, Na, Cl and Co2) status of cardiac patients among the above-mentioned populations.

1.1.4 Thesis structure

This thesis is divided into four chapters namely, introduction, methods, results and discussion. In the rest of the introduction, cardiovascular disease, related terms, and different risk factors will be discussed along with different parameters. In the method section, each analytical procedure will be briefly discussed with details in the supplementary sections followed by description of samples. In the results and discussion, the statistical tests of the data points of different parameters will be illustrated with density plots, boxplots, correlation plot and linear regression along with explanations before drawing a general conclusion.

1.1.5 Awareness

Atherosclerosis is a process that develops over decades and is often silent until an acute event (heart attack) develops in later life. Population based studies in the youth show that the precursors of heart disease start in adolescence. The process of atherosclerosis evolves over decades, and begins as early as childhood. The path biological determinants of atherosclerosis in youth study demonstrated that intimal lesions appear in all the aortas and more than half of the right coronary arteries of youths aged 15–19 years. However, most adolescents are more concerned about other risks such as hiv, accidents, and cancer than cardiovascular disease[3].this is extremely important considering that 1 in 3 people will die from complications attributable to atherosclerosis. In order to stem the tide of cardiovascular disease, primary prevention is needed. Primary prevention starts with education and awareness that cardiovascular disease poses the greatest threat and measures to prevent or reverse this disease must be taken [23].

1.1.6 : Stroke and heart attack

An ischemic stroke (the most common type) happens when a blood vessel that feeds the brain gets blocked, usually from a blood clot. When the blood supply to a part of the brain is shut off, brain cells will die. The result will be the inability to carry out some of the previous functions as before like walking or talking. A hemorrhagic stroke occurs when a blood vessel within the brain bursts. The most probable cause is uncontrolled hypertension.

Some effects of stroke are permanent if too many brain cells die after a stroke due to lack of blood and oxygen to the brain. The good news is that some brain cells don't die — they're only temporarily out of order. Injured cells can repair themselves. Over time, as the repair takes place, somebody functioning improves. Also, other brain cells may take control of those areas that were injured. In this way, strength may improve, speech may get better and memory may improve. This recovery process is what rehabilitation is all about.

A heart attack occurs when the blood flow to a part of the heart is blocked by a blood clot. If this clot cuts off the blood flow completely, the part of the heart muscle supplied food and nutrition by that artery begins to die. Most people survive their first heart attack and return to

their normal lives to enjoy many more years of productive activity. But having a heart attack does mean you have to make some changes. The doctor will advise you of medications and lifestyle changes according to how badly the heart was damaged and what degree of heart disease caused the heart attack [24].

1.2 : Myocardial infarction

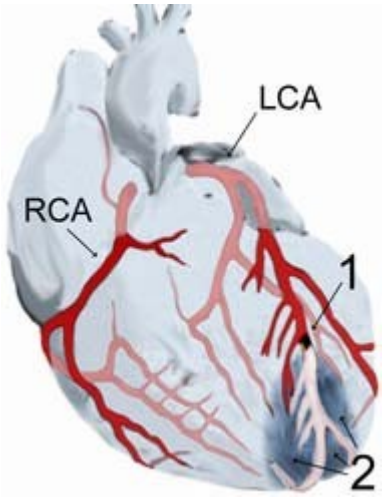


Figure-1.1: Myocardial infarction

Diagram of a myocardial infarction (2) of the tip of the anterior wall of the heart (an *apical infarct*) after occlusion (1) of a branch of the left coronary artery (lca, right coronary artery = rca).

Myocardial infarction (mi) or acute myocardial infarction (ami), commonly known as a heart attack is the interruption of blood supply to part of the heart, causing some heart cells to die. This is most commonly due to occlusion (blockage) of a coronary artery following the rupture of a vulnerable atherosclerotic plaque, which is an unstable collection of lipids (like cholesterol) and white blood cells (especially macrophages) in the wall of an artery. The resulting ischemia (restriction in blood supply) and oxygen shortage, if left untreated for a sufficient period of time, can cause damage or death (*infarction*) of heart muscle tissue (*myocardium*).

Classical symptoms of acute myocardial infarction include sudden chest pain (typically radiating to the left arm or left side of the neck), shortness of breath, nausea, vomiting, palpitations, sweating, and anxiety (often described as a sense of impending doom). Women may experience fewer typical symptoms than men, most commonly shortness of breath, weakness, a feeling of indigestion, and fatigue.[25] approximately one quarter of all myocardial

infarctions are silent, without chest pain or other symptoms. A heart attack is a medical emergency, and people experiencing chest pain are advised to alert their emergency medical services because prompt protection with an external defibrillator can save your life from primary ventricular fibrillation which occurs unexpectedly in 10% of all myocardial infarctions especially during the first hours of symptoms. Contemporary treatment of many myocardial infarctions can result in survival and even good outcomes. While it is true that certain less amenable cases are very massive and rapIDLy fatal "widowmakers", it is also true that in small attacks with limited damage and optimal treatment the heart muscle can be salvaged.

Heart attacks are the leading cause of death for both men and women all over the world.[26] important risk factors are previous cardiovascular disease (such as angina, a previous heart attack or stroke), older age (especially men over 40 and women over 50), tobacco smoking, high blood levels of certain lipids (triglycerides, low-density lipoprotein or "bad cholesterol") and low levels of high density lipoprotein (HDL, "good cholesterol"), diabetes, high blood pressure, obesity, chronic kidney disease, heart failure, excessive alcohol consumption, the abuse of certain drugs (such as cocaine and methamphetamine), and chronic high stress levels.[27-28]

Immediate treatment for suspected acute myocardial infarction includes oxygen, aspirin, and sublingual glyceryl trinitrate (colloquially referred to as nitroglycerin and abbreviated as nTG or gtn). Pain relief is also often given, classically morphine sulfate.[29] however, a 2009 review about the use of high flow oxygen for treating myocardial infarction found its administration increased mortality and infarct size, calling into question the recommendation for its routine use.[30]

The patient will receive a number of diagnostic tests, such as an electrocardiogram (ecg, ekg), a chest x-ray and blood tests to detect elevations in cardiac markers (blood tests to detect heart muscle damage). The most often used markers are the creatine kinase-mb (ck-mb) fraction and the troponin i (tni) or troponin t (tnt) levels. On the basis of the ecg, a distinction is made between st elevation mi (stemi) or non-st elevation mi (nstemi). Most cases of stemi are treated

with thrombolysis or if possible with percutaneous coronary intervention (pci, angioplasty and stent insertion), provided the hospital has facilities for coronary angiography. Nstemi is managed with medication, although pci is often performed during hospital admission. In patients who have multiple blockages and who are relatively stable, or in a few extraordinary emergency cases, bypass surgery of the blocked coronary artery is an option.

The phrase "heart attack" is sometimes used incorrectly to describe sudden cardiac death, which may or may not be the result of acute myocardial infarction. A heart attack is different from, but can be the cause of cardiac arrest, which is the stopping of the heartbeat, and cardiac arrhythmia, an abnormal heartbeat. It is also distinct from heart failure, in which the pumping action of the heart is impaired; severe myocardial infarction may lead to heart failure, but not necessarily.

Acute myocardial infarction refers to two subtypes of acute coronary syndrome, namely non-st- elevated myocardial infarction and st-elevated myocardial infarction, which are most frequently (but not always) a manifestation of coronary artery disease. The most common triggering event is the disruption of an atherosclerotic plaque in an epicardial coronary artery, which leads to a clotting cascade, sometimes resulting in total occlusion of the artery. Atherosclerosis is the gradual buildup of cholesterol and fibrous tissue in plaques in the wall of arteries (in this case, the coronary arteries), typically over decades. Blood stream column irregularities visible on angiography reflect artery lumen narrowing because of decades of advancing atherosclerosis. Plaques can become unstable, rupture, and additionally promote a thrombus (blood clot) that occludes the artery; this can occur in minutes. When a severe enough plaque rupture occurs in the coronary vasculature, it leads to myocardial infarction (necrosis of downstream myocardium).

If impaired blood flow to the heart lasts long enough, it triggers a process called the ischemic cascade; the heart cells in the territory of the occluded coronary artery die (chiefly through necrosis) and do not grow back. A collagen scar forms in its place. Recent studies indicate that another form of cell death called apoptosis also plays a role in the process of tissue damage subsequent to myocardial infarction.[31] as a result, the patient's heart will be permanently damaged. This myocardial scarring also puts the patient at risk for potentially life-threatening

arrhythmias and may result in the formation of a ventricular aneurysm that can rupture with catastrophic consequences.

Injured heart tissue conducts electrical impulses more slowly than normal heart tissue. The difference in conduction velocity between injured and uninjured tissue can trigger re-entry or a feedback loop that is believed to be the cause of many lethal arrhythmias. The most serious of these arrhythmias is ventricular fibrillation (*v-fib*/vf), an extremely fast and chaotic heart rhythm that is the leading cause of sudden cardiac death. Another life threatening arrhythmia is ventricular tachycardia (*v-tach*/vt), which may or may not cause sudden cardiac death. However, ventricular tachycardia usually results in rapid heart rates that prevent the heart from pumping blood effectively. Cardiac output and blood pressure may fall to dangerous levels, which can lead to further coronary ischemia and extension of the infarct.

The cardiac defibrillator is a device that was specifically designed to terminate these potentially fatal arrhythmias. The device works by delivering an electrical shock to the patient in order to depolarize a critical mass of the heart muscle, in effect "rebooting" the heart. This therapy is time dependent, and the odds of successful defibrillation decline rapIDLy after the onset of cardiopulmonary arrest.

1.3: Other types of cardiovascular disease:

Heart failure: this doesn't mean that the heart stops beating. Heart failure, sometimes called congestive heart failure, means the heart isn't pumping blood as well as it should. The heart keeps working, but the body's need for blood and oxygen isn't being met. Heart failure can get worse if it's not treated. If your loved one has heart failure, it's very important to follow the doctor's advices.

Arrhythmia: this is an abnormal rhythm of the heart. There are various types of arrhythmias. The heart can beat too slowly, too fast or irregularly. Bradycardia is when the heart rate is less than 60 beats per minute. Tachycardia is when the heart rate is more than 100 beats per minute. An arrhythmia can affect how well the heart works. The heart may not be able to pump enough blood to meet the body's needs.

Heart valve problems: when heart valves don't open enough to allow the blood to flow through as it should, it's called stenosis. When the heart valves don't close properly and allow blood to leak through, it's called regurgitation. When the valve leaflets bulge or prolepses back

into the upper chamber, it's a condition called mitral valve prolapse. When this happens, they may not close properly. This allows blood to flow backward through them. [32]

1.4: Risk factors:

Despite public apprehension over the risks of developing cancer, in most countries the public has more to fear from cardiovascular disease (CVD). Coronary heart disease (CHD) and stroke, the principal manifestations of CVD, are the first and second most common causes of death worldwide.

A risk factor is an attribute which is positively associated with the development of a disease but is not enough to cause the disease. [33]

1.4.1 : Severity of major risk factors

Framingham scoring takes into account gradations in risk factors when estimating absolute risk. The scoring does not adequately account for severe abnormalities of risk factors, eg, severe hypertension, severe hypercholesterolemia, or heavy cigarette smoking. In such cases, framingham scores can underestimate absolute risk. This underestimation is particularly evident when only 1 severe risk factor is present. Thus, heavy smoking or severe hypercholesterolemia can lead to premature CHD even when the summed score for absolute risk is not high. Likewise, the many dangers of prolonged, uncontrolled hypertension are well known. These dangers underscore the need to control severe risk factors regardless of absolute short-term risk estimates. (The American heart association, inc. And the American college of cardiology)

1.4.2 : Diabetes

That diabetes mellitus is a major risk factor for CVD is well established. Both type 1 diabetes and type 2 diabetes confer a heightened risk for CVD. Type 2 diabetes is of particular concern because it is so common and usually occurs in persons of advancing age, when multiple other risk factors coexist. There is a growing consensus that most patients with diabetes mellitus, especially those with type-2 diabetes, belong in a category of high short-term risk. When the risk factors of diabetic patients are summed, their risk often approaches that of patients with established CHD. The absolute risk of patients with type 2 diabetes usually exceeds the framingham score for hyperglycemia because other risk factors almost always coexist. Another reason to elevate the patient with diabetes to a higher risk category than suggested by framingham scoring is the poor prognosis of these patients once they develop CHD.these factors point to the need to intensify the management of coexisting risk factors in patients with diabetes.

These considerations about the very high risk of patients with diabetes apply to ethnic groups that have a relatively high population risk for CHD. The inclusion of patients with type 2 diabetes in the very-high-risk category may not be appropriate when they belong to ethnic groups with a low population risk. (the american heart association, inc. And the american college of cardiology).

1.4.3 : Age

One of the more prominent features of the framingham risk scoring is the progressive increase in absolute risk with advancing age. This increase undoubtedly reflects the cumulative nature of atherogenesis. With advancing age, people typically accumulate increasing amounts of coronary atherosclerosis. This increased plaque burden itself becomes a risk factor for future coronary events [34]. Framingham scoring for age reflects this impact of plaque burden on risk. Still, average scores mask the extent of variability in plaque burden in the general population. To apply average risk scores for age to individual patients may lead to miscalculation of true risk, particularly because framingham applies so much weight to age as a risk factor. Miscalculation of risk could lead to inappropriate selection of patients for aggressive risk-reduction therapies. This fact points to the need for flexibility in adapting treatment guidelines to older persons. The tempering of treatment recommendations with clinical judgment becomes increasingly important with advancing age, particularly after the age of 65. In the future, measures of subclinical atherosclerosis may improve the accuracy of global risk assessment in older patients. When risk scoring is used to adjust the intensity of risk factor management in elderly patients, relative risk estimates may be more useful than absolute risk estimates. Relative risk estimates essentially eliminate the age factor and are based entirely on the major risk factors. These estimates allow the physician to stratify and compare patients of the same age, and patients at highest relative risk could be selected for the most aggressive risk management. (the american heart association, inc. And the american college of cardiology).

1.4.4 : Male sex (gender)

Men have a greater risk of heart attack than women do, and they have attacks earlier in life. Even after menopause, when women's death rate from heart disease increases, it's not as great as men's.

1.4.5 : Heredity (including race)

Children of parents with heart disease are more likely to develop it themselves. African americans have more severe high blood pressure than caucasians and a higher risk of heart disease. Heart disease risk is also higher among mexican americans, american Indians, native hawaiians and some asian americans. This is partly due to higher rates of obesity and diabetes. Most people with a strong family history of heart disease have one or more other risk factors. Just as you can't control your age, sex and race, you can't control your family history. Therefore, it's even more important to treat and control any other risk factors you have.

1.4.6 : Major risk factors can be controlled by changing lifestyle or taking medicine

Tobacco smoke — smokers' risk of developing coronary heart disease is 2–4 times that of nonsmokers. Cigarette smoking is a powerful independent risk factor for sudden cardiac death in patients with coronary heart disease; smokers have about twice the risk of nonsmokers. Cigarette smoking also acts with other risk factors to greatly increase the risk for coronary heart disease. People who smoke cigars or pipes seem to have a higher risk of death from coronary heart disease (and possibly stroke) but their risk isn't as great as cigarette smokers'. Exposure to other people's smoke increases the risk of heart disease even for nonsmokers.

High blood cholesterol — as blood cholesterol rises, so does risk of coronary heart disease. When other risk factors (such as high blood pressure and tobacco smoke) are present, this risk increases even more. A person's cholesterol level is also affected by age, sex, heredity and diet.

High blood pressure — high blood pressure increases the heart's workload, causing the heart to thicken and become stiffer. It also increases your risk of stroke, heart attack, kidney failure and congestive heart failure. When high blood pressure exists with obesity, smoking, high blood cholesterol levels or diabetes, the risk of heart attack or stroke increases several times.

Physical inactivity — an inactive lifestyle is a risk factor for coronary heart disease. Regular, moderate-to-vigorous physical activity helps prevent heart and blood vessel disease. The more vigorous the activity, the greater your benefits. However, even moderate-intensity activities help if done regularly and long term. Physical activity can help control blood cholesterol level, diabetes and obesity, as well as help to lower blood pressure in some people.

Obesity and overweight — people who have excess body fat — especially if a lot of it is at the waist — are more likely to develop heart disease and stroke even if they have no other risk factors. Excess weight increases the heart's work. It also raises blood pressure and blood cholesterol and triglyceride levels, and lowers HDL ("good") cholesterol levels. It can also make diabetes more likely to develop. Many obese and overweight people may have difficulty losing weight. But by losing even as few as 10 pounds, you can lower your heart disease risk.

1.4.7 : Other factors contribute to heart disease risk

Individual response to stress may be a contributing factor. Some scientists have noted a relationship between coronary heart disease risk and stress in a person's life, their health behaviors and socioeconomic condition. These factors may affect established risk factors. For example, people under stress may overeat, start smoking or smoke more than they otherwise would.

Drinking too much alcohol can raise blood pressure, cause heart failure and lead to stroke. It can contribute to high triglycerides, cancer and other diseases, and produce irregular heartbeats. It contributes to obesity, alcoholism, suicide and accidents.

The risk of heart disease in people who drink moderate amounts of alcohol (an average of one drink for women or two drinks for men per day) is lower than in nondrinkers. One drink is defined as 1-1/2 fluid ounces (fl oz) of 80-proof spirits (such as bourbon, scotch, vodka, gin, etc.), 1 fl oz of 100-proof spirits, 4 fl oz of wine or 12 fl oz of beer. It's not recommended that nondrinkers start using alcohol or that drinker’s increase the amount they drink. (american heart association, inc. - 2009)

1.5 : Fat

Fats consist of a wide group of compounds that are generally soluble in organic solvents and largely insoluble in water. Chemically, fats are generally triesters of glycerol and fatty acids. Fats may be either solid or liquid at normal room temperature, depending on their structure and composition. Although the words "oils", "fats", and "lipids" are all used to refer to fats, "oils" is usually used to refer to fats that are liquids at normal room temperature, while "fats" is usually used to refer to fats that are solids at normal room temperature. "lipids" is used to refer to both liquid and solid fats, along with other related substances. The word "oil" is used for any substance that does not mix with water and has a greasy feel, such as petroleum (or crude oil) and heating oil, regardless of its chemical structure [35]. Fats form a category of lipid, distinguished from other lipids by their chemical structure and physical properties. This category of molecules is important for many forms of life, serving both structural and metabolic functions. They are an important part of the diet of most heterotrophs (including humans). Fats or lipids are broken down in the body by enzymes called lipases produced in the pancreas.

Examples of edible animal fats are lard (pig fat), fish oil, and butter or ghee. They are obtained from fats in the milk, meat and under the skin of the animal. Examples of edible plant fats are peanut, soya bean, sunflower, sesame, coconut, olive, and vegetable oils. Margarine and vegetable shortening, which can be derived from the above oils, are used mainly for baking. These examples of fats can be categorized into saturated fats and unsaturated fats.

1.6 : Lipid

Lipids are a broad group of naturally-occurring molecules which includes fats, waxes, sterols, fat-soluble vitamins (such as vitamins a, d, e and k), monoglycerides, diglycerides, phospholipids, and others. The main biological functions of lipids include energy storage, as structural components of cell membranes, and as important signaling molecules.

Lipids may be broadly defined as hydrophobic or amphiphilic small molecules; the amphiphilic nature of some lipids allows them to form structures such as vesicles, liposomes, or membranes in an aqueous environment. Biological lipids originate entirely or in part from two distinct types of biochemical subunits or "building blocks": ketoacyl and isoprene groups [35].

1.6.1 : Phospholipid

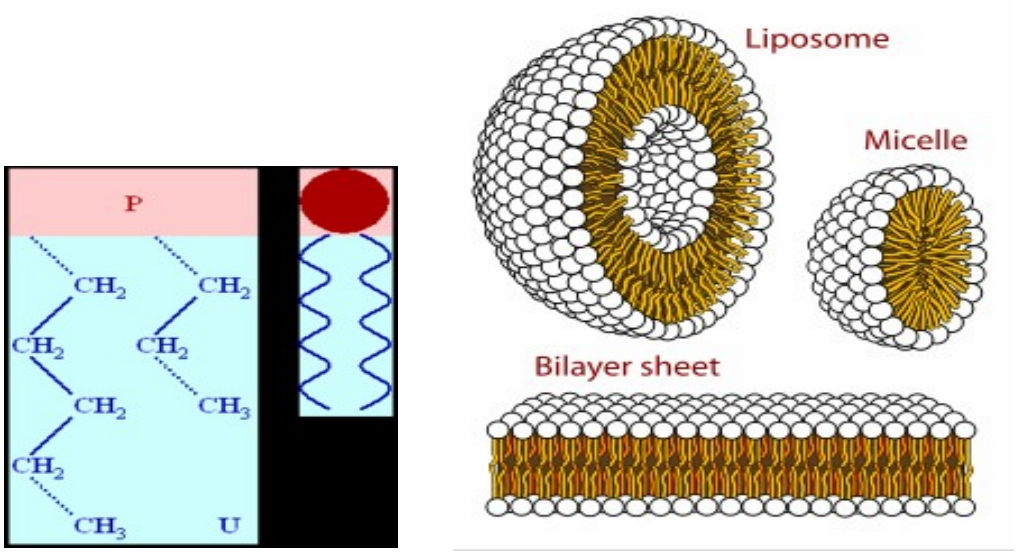


Figure-1.2: Structure and organization of self-organization of phospholipids: a spherical liposome, a micelle and a lipid bilayer.

Phospholipids are a class of lipids and are a major component of all cell membranes. Most phospholipids contain a diglyceride, a phosphate group, and a simple organic molecule such as choline; one exception to this rule is sphingomyelin, which is derived from sphingosine instead of glycerol. They are a type of molecule. They form a lipid bilayer within a cell membrane. Phospholipid synthesis occurs in the cytosol adjacent to er membrane that is studded with proteins that act in synthesis (gpat and lpaat acyl transferases, phosphatase and choline phosphotransferase) and allocation (flippase and floppase). Eventually a vesicle will bud off from the er containing phospholipids destined for the cytoplasmic cellular membrane on its exterior leaflet and phospholipids destined for the exoplasmic cellular membrane on its inner leaflet.[36]

1.6.2 : Calcification and lipids

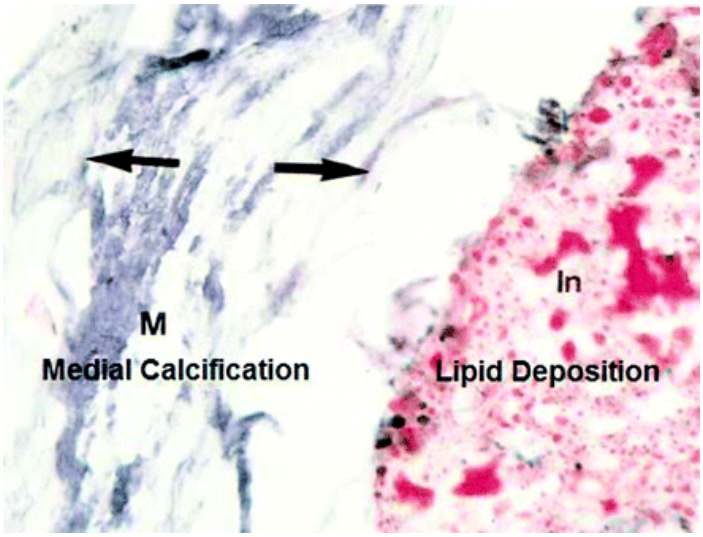


Figure: 1.3 Histologic section demonstrating purported mönckeberg's sclerosis with medial calcification; however, inspection of the intimal space shows a large lipid deposit characteristic of atherosclerosis. The correct interpretation is that this is medial calcification in the setting of atherosclerosis, not mönckeberg's sclerosis. [37]

Intracellular micro calcifications form within vascular smooth muscle cells of the surrounding muscular layer, specifically in the muscle cells adjacent to the atheromas. In time, as cells die, this leads to extracellular calcium deposits between the muscular wall and outer portion of the atheromatous plaques. A similar form of an intramural calcification, presenting the picture of an early phase of arteriosclerosis, appears to be induced by a number of drugs that have an antiproliferative mechanism of action (rainer liedtke 2008).

Cholesterol is delivered into the vessel wall by cholesterol-containing low-density lipoprotein (LDL) particles. To attract and stimulate macrophages, the cholesterol must be released from the LDL particles and oxidized, a key step in the ongoing inflammatory process. The process is worsened if there is insufficient high-density lipoprotein (HDL), the lipoprotein particle that removes cholesterol from tissues and carries it back to the liver.

The foam cells and platelets encourage the migration and proliferation of smooth muscle cells, which in turn ingest lipids, become replaced by collagen and transform into foam cells themselves. A protective fibrous cap normally forms between the fatty deposits and the artery

lining (the intima).

These capped fatty deposits (now called 'atheromas') produce enzymes that cause the artery to enlarge over time. As long as the artery enlarges sufficiently to compensate for the extra thickness of the atheroma, then no narrowing ("stenosis") of the opening ("lumen") occurs. The artery becomes expanded with an egg-shaped cross-section, still with a circular opening. If the enlargement is beyond proportion to the atheroma thickness, then it is created. [38]

1.6.3 : Relation of high TG–low HDL cholesterol and LDL cholesterol to the incidence of ischemic heart disease (IHD)

Lipid abnormalities are frequent in the elderly, and are associated with the presence of CVD. Low HDL-c and/or abnormal TG levels, when added to abnormal LDL-c, are associated with a higher prevalence of CVD, suggesting the advisability of a comprehensive lipid evaluation and treatment earlier in life.

An increased plasma TG and decreased HDL-c concentration is the characteristic dyslipidemia seen in insulin-resistant subjects. It has been suggested that insulin resistance is a basic metabolic abnormality that plays an important role in the etiology of IHD. A high level of LDL-c is a well- established major risk factor of IHD. This lipid abnormality is not associated with insulin resistance per se. So far, it is not well defined which of these two metabolically different dyslipidemic syndromes is the more powerful predictor of IHD in a free-living, healthy population.

Using high TG–low HDL-c as a marker of insulin resistance, the aim of this prospective cardiovascular study was to examine the relative contribution to IHD risk of this dyslipidemic condition compared with that of high LDL-c in a cohort of middle-aged and elderly white men. High triglyceride (TG) and low HDL cholesterol (HDL-c) is the characteristic dyslipidemia seen in insulin-resistant subjects. We examined the role of this dyslipidemia as a risk factor of ischemic heart disease (IHD) compared with that of high LDL cholesterol (LDL-c) in the copenhagen male study. In total 2910 white men, aged 53 to 74 years, free of cardiovascular disease at baseline, were subdivided into four groups on the basis of fasting concentrations of serum TG, HDL-c, and LDL-c. "high TG–low HDL-c" was defined as belonging to both the

highest third of TG and the lowest third of HDL-c; this group encompassed one fifth of the population. "high LDL-c" was defined as belonging to the highest fifth of LDL-c. A control group was defined as not belonging to either of these two groups. "combined dyslipidemia" was defined as belonging to both dyslipidemic groups. Age-adjusted incidence of IHD during 8 years of follow-up was 11.4% in high TG–low HDL-c, 8.2% in high LDL-c, 6.6% in the control group, and 17.5% in combined dyslipidemia. Compared with the control group, relative risks of IHD (95% confidence interval), adjusted for potentially confounding factors or covariates (age, body mass index, alcohol consumption, physical activity, non–insulin-dependent diabetes, hypertension, smoking, and social class), were 1.5 (1.0-2.1), $p<.05$; 1.3 (0.9-2.0), $p=.16$; and 2.4 (1.5-4.0), $p<.01$, in the three dyslipidemic groups, respectively. In conclusion, the present results showed that high TG–low HDL-c, the characteristic dyslipidemia seen in insulin-resistant subjects, was at least as powerful a predictor of IHD as isolated high LDL-c. The results suggest that efforts to prevent IHD should include intervention against high TG–low HDL-c, and not just against hypercholesterolemia. [39]

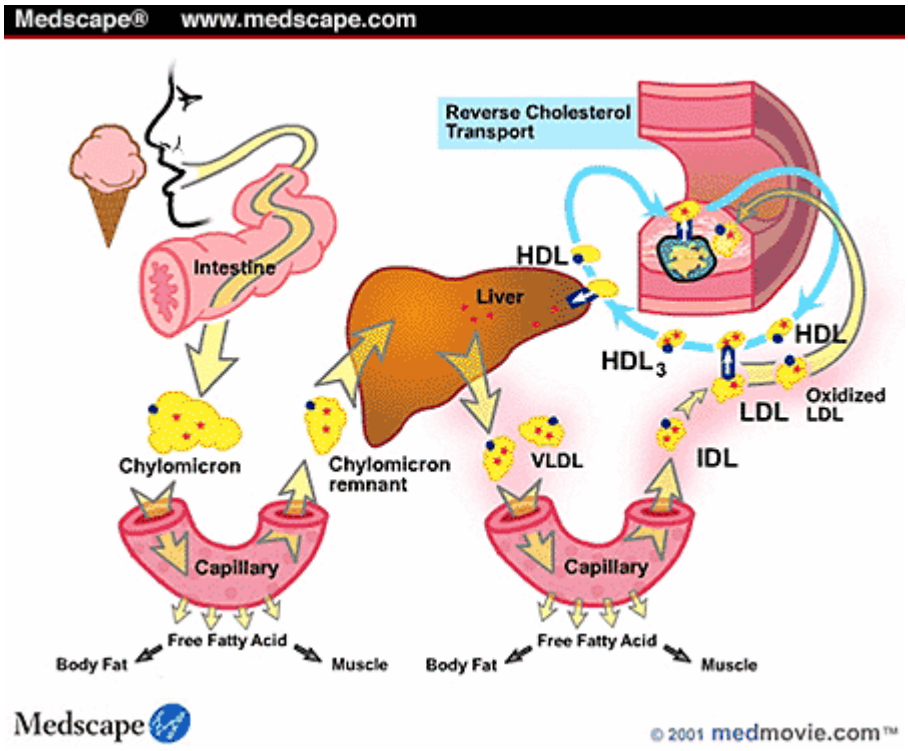


Figure: 1.4 Build-up of plaque in arterial walls (role of cholesterol, HDL & LDL)

1.7: Cholesterol

cholesterol is a type of fat that is part of all animal cells and cell membrane. It is essential for many of the body metabolic processes, including hormone and bile production, and to help the body produce vitamin d. Cholesterol is more abundant in tissues which either synthesize more or have more abundant densely-packed membranes, for example, the liver, spinal cord and brain.

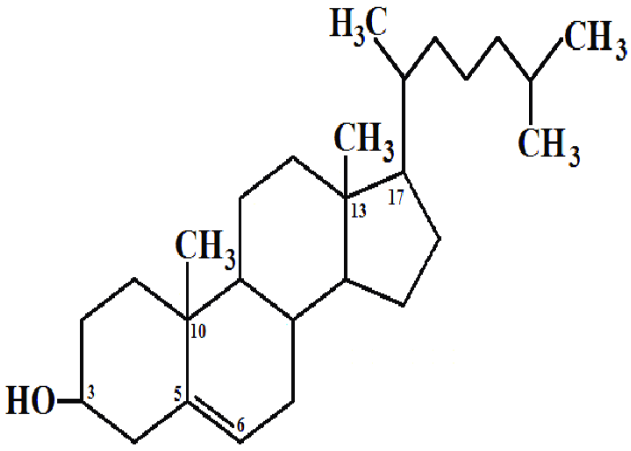


Figure 1.5 Molecular structure of cholesterol

according to the lipid hypothesis, abnormally high cholesterol levels (hypercholesterolemia), or, more correctly, higher concentrations of LDL and lower concentrations of functional HDL are strongly associated with cardiovascular disease because these promote atheroma development in arteries (atherosclerosis). This disease process leads to myocardial infarction (heart attack), stroke and peripheral vascular disease. Since higher blood LDL, especially higher LDL particle concentrations and smaller LDL particle size, contribute to this process more than the cholesterol content of the LDL particles, LDL particles are often termed "bad cholesterol" because they have been linked to atheroma formation. On the other hand, high concentrations of functional HDL, which can remove cholesterol from cells and atheroma, offer protection and are sometimes referred to colloquially as "good cholesterol". These balances are mostly genetically determined but can be changed by body build, medications, choice of foods and other factors [40].

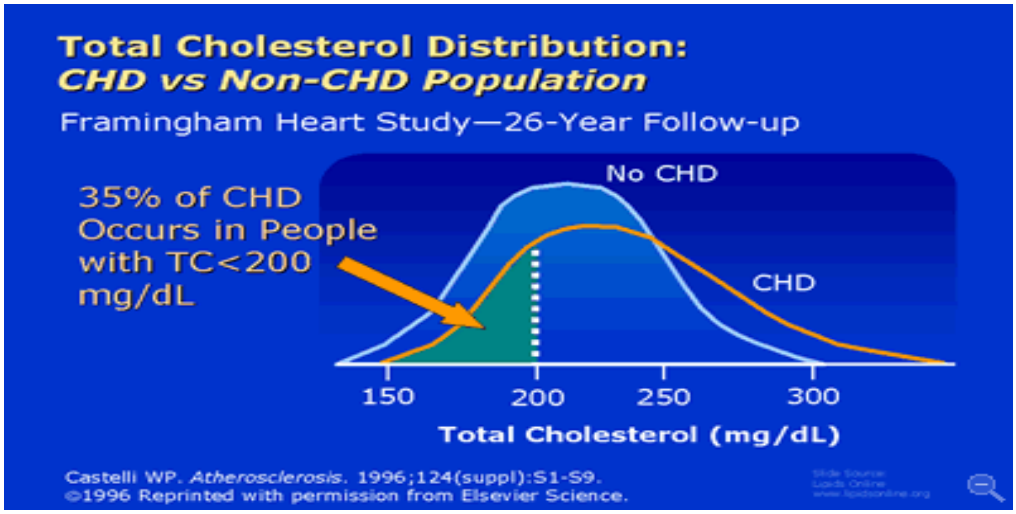


Figure : 1.6 Total cholestérol population distribution CHD vs non-CHD population.

1.8: High-density lipoprotein

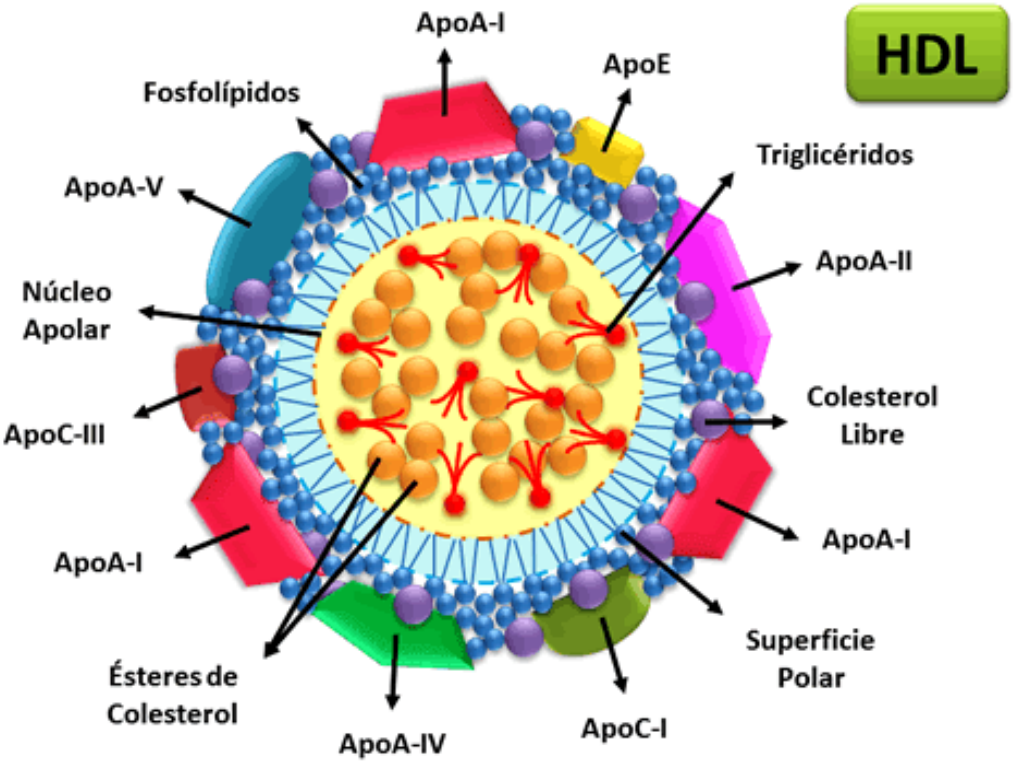


Figure: 1.7 Structure of high density lipoprotein.

High-density lipoproteins (**HDL**) is one of the 5 major groups of lipoproteins (chylomicrons, VLDL, IDL, LDL, HDL) which enable lipids like cholesterol and triglycerides to be transported within the water based blood stream. In healthy individuals, about thirty percent of blood cholesterol is carried by HDL.it is hypothesized that HDL can remove cholesterol from atheroma within arteries and transport it back to the liver for excretion or re-utilization—which is the main reason why HDL-bound cholesterol is sometimes called **"good cholesterol"**, or HDL-c. A high level of HDL-c seems to protect against cardiovascular diseases, and low HDL cholesterol levels (less than 40 mg/dl) increase the risk for heart disease. When measuring cholesterol, any contained in HDL particles is considered as protection to the body's cardiovascular health, in contrast to "bad" LDL cholesterol. [41]

1.8.1 : Aapolipoprotein a-i (lpa-i), apolipoprotein a-iı (lpa-i:a-ii) HDL and CHD-risk

Investigators tested the hypothesis that concentrations of lpa-i and/or lpa-i:a-ii HDL subclasses are significantly associated with CHD prevalence and recurrent cardiovascular events. They observed slightly but significantly higher lpa-i levels in CHD cases compared to all or to HDL-c-matched controls and slightly but significantly higher lpa-i:a-ii levels in CHD cases compared to HDL-c-matched controls it the fos. Neither lpa-i nor lpa-i:a-ii levels were significantly different between groups with and without recurrent cardiovascular events in the va-hit. No significant differences were observed in lpa-i and lpa-i:a-ii levels in low HDL- c (< or = 40 mg/dl) subjects with CHD (va-hit, n = 711) and without CHD (fos, n = 373). Plasma lpa-i concentration had a positive correlation with the large lpa-i HDL particle (alpha-1) but no correlation with the small lpa-i HDL particle (prebeta-1). Lpa-i:a-ii concentration had a positive correlation with the large (alpha-2) and an inverse correlation with the small (alpha-3) lpa-i:a-ii HDL particles.[42]

1.9 : low-density lipoprotein

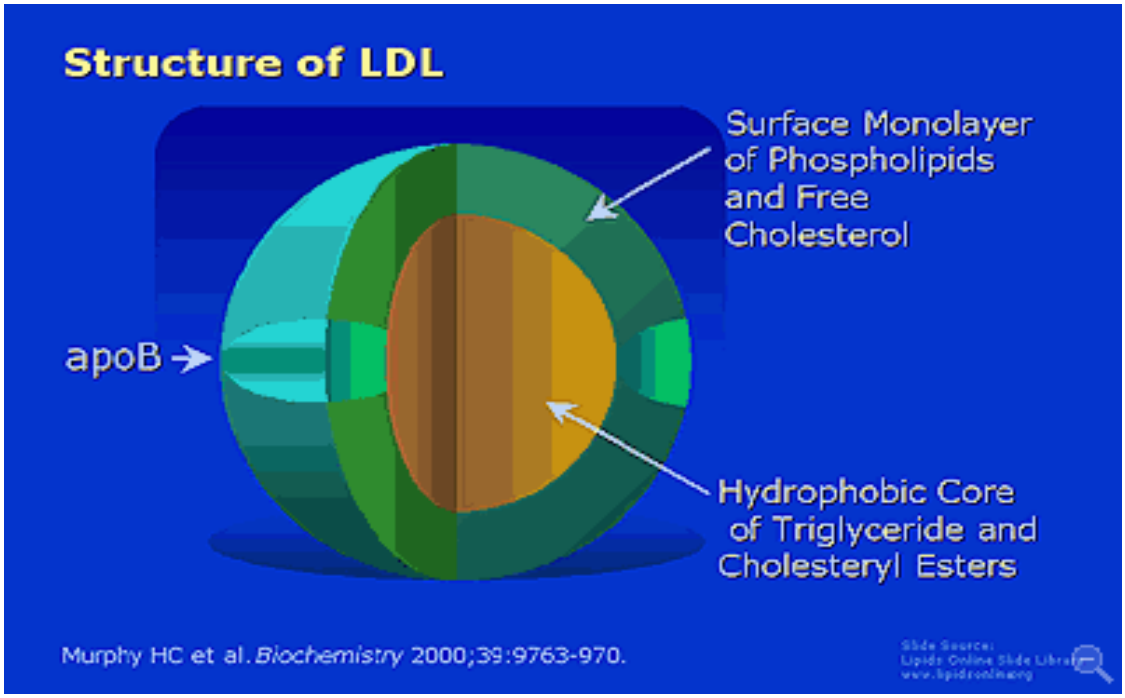


Figure: 1.8 Structure of low density lipoprotein.

Low-density lipoprotein (LDL) is a type of lipoprotein that transports cholesterol and

triglycerides from the liver to peripheral tissues. LDL is one of the five major groups of lipoproteins; these groups include chylomicrons, very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein, and high-density lipoprotein (HDL). Like all lipoproteins, LDL enables fats and cholesterol to move within the water based solution of the blood stream. LDL also regulates cholesterol synthesis at these sites. It commonly appears in the medical setting as part of a cholesterol blood test, and since high levels of LDL cholesterol can signal medical problems like cardiovascular disease, it is sometimes called "bad cholesterol," (as opposed to HDL, which is frequently referred to as "good cholesterol" or "healthy cholesterol).[43]

1.10 : Very-low-density lipoprotein (VLDL)

Very-low-density lipoprotein (**VLDL**) is a type of lipoprotein made by the liver. VLDL is one of the five major groups of lipoproteins (chylomicrons, VLDL, intermediate-density lipoprotein, low-density lipoprotein, high-density lipoprotein) that enable fats and cholesterol to move within the water-based solution of the bloodstream. VLDL is assembled in the liver from cholesterol and apolipoproteins. VLDL is converted in the bloodstream to low-density lipoprotein (LDL). VLDL particles have a diameter of 30-80 nm. VLDL transports endogenous products, whereas chylomicrons transport exogenous (dietary) products. VLDL transports endogenous triglycerides, phospholipids, cholesterol, and cholesteryl esters. It functions as the body's internal transport mechanism for lipids.

1.11 : Intermediate-density lipoproteins

Intermediate-density lipoproteins belong to the lipoprotein particle family and are formed from the degradation of very low-density lipoproteins. IDL is one of the five major groups of lipoproteins (chylomicrons, VLDL, IDL, LDL, HDL) that enable fats and cholesterol to move within the water-based solution of the bloodstream. Each native IDL particle consists of protein that encircles various fatty acids, enabling, as a water-soluble particle, these fatty acids to travel in the aqueous blood environment as part of the fat transport system within the body. Their size is, in general, 25 to 35 nm in diameter, and they contain primarily a range of triacylglycerols and cholesterol esters. They are cleared from the plasma into the liver by receptor-mediated endocytosis, or further degraded to form LDL particles.

In general, IDL, somewhat similar to low-density lipoprotein (LDL), transports a variety of triglyceride fats and cholesterol and, like LDL, can also promote the growth of atheroma.[44]

1.12 : Free fatty acids

Fatty acids can be bound or attached to other molecules, such as in triglycerides or phospholipids. When they are not attached to other molecules, they are known as "free" fatty acids. The uncombined fatty acids or free fatty acids may come from the breakdown of a triglyceride into its components (fatty acids and glycerol). However as fats are insoluble in water they must be bound to appropriate regions in the plasma protein albumin for transport around the body. The levels of "free fatty acid" in the blood are limited by the number of albumin binding sites available.free fatty acids are an important source of fuel for many tissues since they can yield relatively large quantities of atp. Many cell types can use either glucose or fatty acids for this purpose. In particular, heart and skeletal muscle prefer fatty acids. The brain cannot use fatty acids as a source of fuel; it relies on glucose, or on ketone bodies. Ketone bodies are produced in the liver by fatty acid metabolism during starvation, or during periods of low carbohydrate intake. [45]

1.13 : Chylomicron

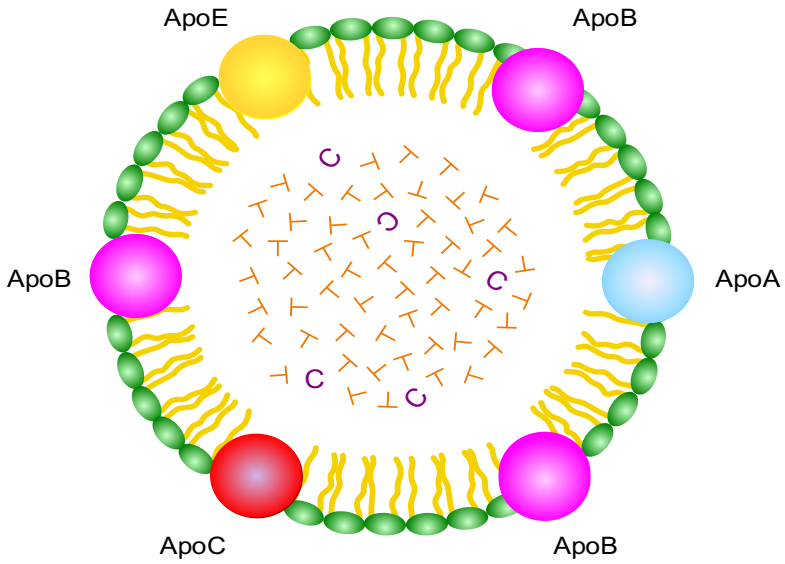


Figure-1.9: Chylomicron structure. ApoA, ApoB, ApoC, ApoE (apolipoproteins); T (triacylglycerol); C (cholesterol); green (phospholipids)

Chylomicrons are large lipoprotein particles that transport dietary lipids from the intestines to other locations in the body. Chylomicrons are one of the five major groups of lipoproteins (chylomicrons, VLDL, IDL, LDL, HDL) that enable fats and cholesterol to move within the water-based solution of the bloodstream. Chylomicrons transport exogenous lipids to liver, adipose, cardiac, and skeletal muscle tissue, where their triglyceride components are unloaded by the activity of lipoprotein lipase. As a consequence, chylomicron remnants are left over and are taken up by the liver. [46]

1.14 : Tri-glyceride

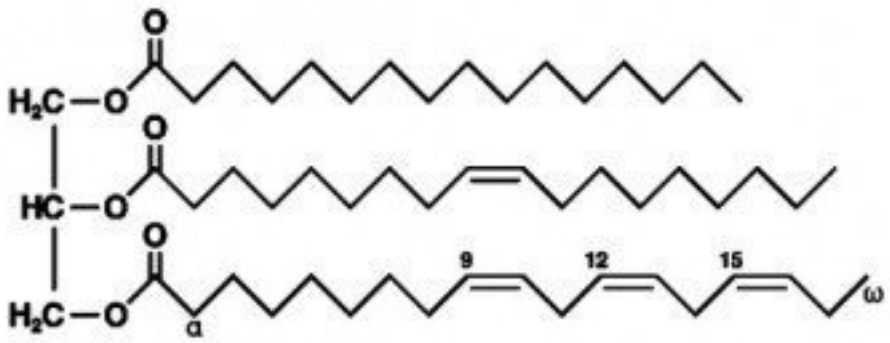


Figure: 1.10 Structure of tri-glyceride

Triglycerides are formed from a single molecule of glycerol, combined with three fatty acids on each of the oh groups, and make up most of fats digested by humans. Ester bonds form between each fatty acid and the glycerol molecule. This is where the enzyme pancreatic lipase acts, hydrolyzing the bond and 'releasing' the fatty acid. In triglyceride form, lipids cannot be absorbed by the duodenum. Fatty acids, monoglycerides (one glycerol, one fatty acid) and some diglycerides are absorbed by the duodenum through villi, once the triglycerides have been broken down.in the human body, high levels of triglycerides in the bloodstream have been linked to atherosclerosis, and, by extension, the risk of heart disease and stroke. However, the relative negative impact of raised levels of triglycerides compared to that of LDL: HDL ratios is as yet unknown. The risk can be partly accounted for by a strong inverse relationship between triglyceride level and HDL-cholesterol level. Another disease caused by high triglycerides is pancreatitis. [47]

1.14.1 : The role of triglycerides as a CHD risk factor

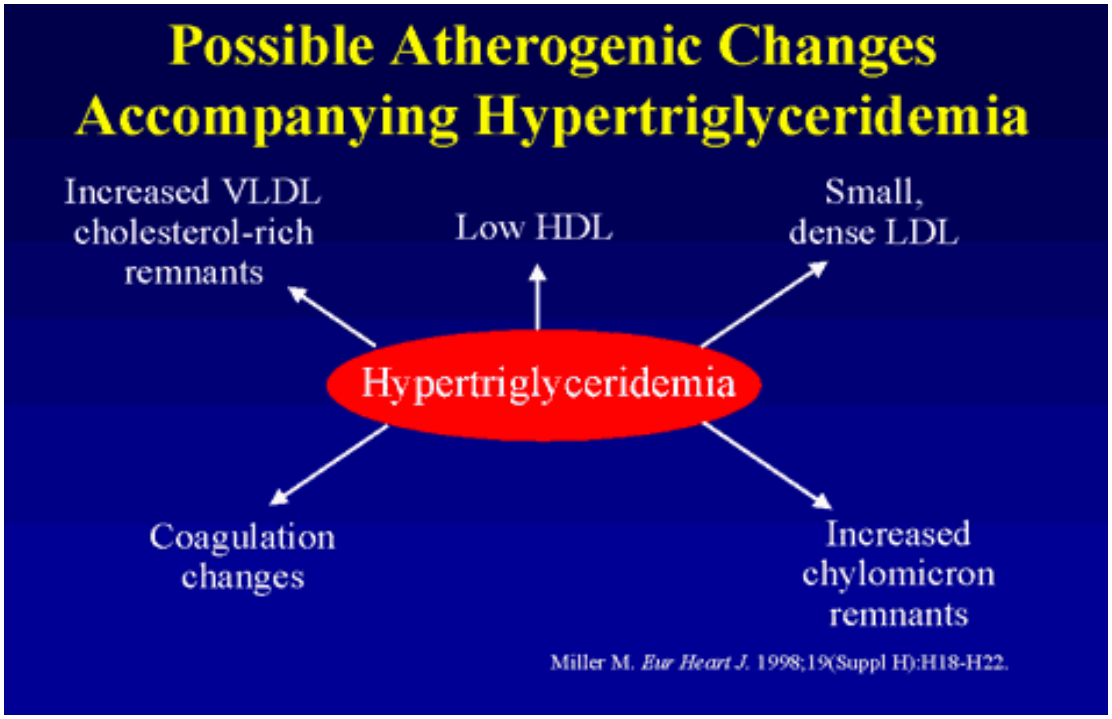


Figure: 1.11: Mechanism of action of triglyceride as risk factor for cardiovascular disease

1.15 : Role of bile acid in lipid metabolism

Bile acids are steroid acids found predominantly in the bile of mammals. Bile salts are formed by the bile acids conjugated to glycine or taurine. In humans, taurocholic acid and glycocholic acid (derivatives of cholic acid) represent approximately eighty percent of all bile salts. The two major bile acids are cholic acid, and chenodeoxycholic acid. Bile acids, glycine and taurine conjugates, and 7-alpha-dehydroxylated derivatives (deoxycholic acid and lithocholic acid) are all found in human intestinal bile. An increase in bile flow is exhibited with an increased secretion of bile acids. The main function of bile acid is to facilitate the formation of micelles, which promotes processing of dietary fat. Bile acids are made in the liver by the cytochrome p450-mediated oxidation of cholesterol. They are conjugated with taurine or the amino acid glycine, or with a sulfate or a glucuronide, and are then stored in the gallbladder. In humans, the rate limiting step is the addition of a hydroxyl group on position 7 of the steroid nucleus by the enzyme cholesterol 7 alpha-hydroxylase. Upon eating a meal, the contents of the gallbladder are secreted into the intestine, where bile salts serve the purpose of emulsifying dietary fats. Bile acids serve other functions, including eliminating cholesterol from the body,

driving the flow of bile to eliminate catabolites from the liver, emulsifying lipids and fat soluble vitamins in the intestine, and aiding in the reduction of the bacteria flora found in the small intestine and biliary tract.

Bile acid refers to the protonated (-cooh) form. *Bile salt* refers to the deprotonated or ionized (- coo^-) form. Conjugated bile acids are more efficient at emulsifying fats because at intestinal ph, they are more ionized than unconjugated bile acids.[48]

Synthesis of bile acids is a major route of cholesterol metabolism in most species other than humans. The human body produces about 800 mg of cholesterol per day and about half of that is used for bile acid synthesis. In total about 20-30 grams of bile acids are secreted into the intestine daily. About 90% of excreted bile acids are reabsorbed by active transport in the ileum and recycled in what is referred to as the enterohepatic circulation. Bile is also used to break down fat globules into tiny droplets. Bile from slaughtered animals can be used in the preparation of soap.

As surfactants or detergents, bile acids are potentially toxic to cells, and their concentrations are tightly regulated. They function as a signaling molecule in the liver and the intestines by activating a nuclear hormone receptor, fxr, also known by its gene name *nr1h4*. Such activation inhibits synthesis of bile acid in the liver when bile acid levels are too high. Emerging evidence associates fxr activation with alterations in triglyceride metabolism, glucose metabolism, and liver growth.

Since bile acids are made from endogenous cholesterol, the enterohepatic circulation of bile acids may be disrupted to lower cholesterol. Bile acid sequestrants bind bile acids in the gut, preventing reabsorption. In so doing, more endogenous cholesterol is shunted into the production of bile acids, thereby lowering cholesterol levels. The sequestered bile acids are then excreted in the feces.

Tests for bile acids are useful in both human and veterinary medicine, as they help to diagnose a number of conditions, including cholestasis, portosystemic shunt, and hepatic microvascular dysplasia.

1.16 : Atherosclerosis and coronary artery disease

Recent research has shown that inflammation plays a key role in coronary artery disease (CAD) and other manifestations of atherosclerosis. Immune cells dominate early atherosclerotic lesions, their effector molecules accelerate progression of the lesions, and activation of inflammation can elicit acute coronary syndromes. This review highlights the role of inflammation in the pathogenesis of atherosclerotic cad. It will recount the evidence that atherosclerosis, the main cause of cad, is an inflammatory disease in which immune mechanisms interact with metabolic risk factors to initiate, propagate, and activate lesions in the arterial tree.

A decade ago, the treatment of hypercholesterolemia and hypertension was expected to eliminate CAD by the end of the 20th century. Lately, however, that optimistic prediction has needed revision. Cardiovascular diseases are expected to be the main cause of death globally within the next 15 years owing to a rapIDLy increasing prevalence in developing countries and eastern europe and the rising incidence of obesity and diabetes in the western world [31]. Cardiovascular diseases cause 38 percent of all deaths in north america and are the most common cause of death in european men under 65 years of age and the second most common cause in women. These facts force us to revisit cardiovascular disease and consider new strategies for prediction, prevention, and treatment.

1.16.1 : Key features of atherosclerotic lesions

Atherosclerotic lesions (atheromata) are asymmetric focal thickenings of the innermost layer of the artery, the intima (figure-1.12). They consist of cells, connective-tissue elements, lipids, and debris. [49] blood-borne inflammatory and immune cells constitute an important part of an Atheroma, the remainder being vascular endothelial and smooth-muscle cells. The atheroma is preceded by a fatty streak, an accumulation of lipid-laden cells beneath the endothelium.[33] most of these cells in the fatty streak are macrophages, together with some t cells. Fatty streaks are prevalent in young people, never cause symptoms, and may progress to atheromata or eventually disappear.

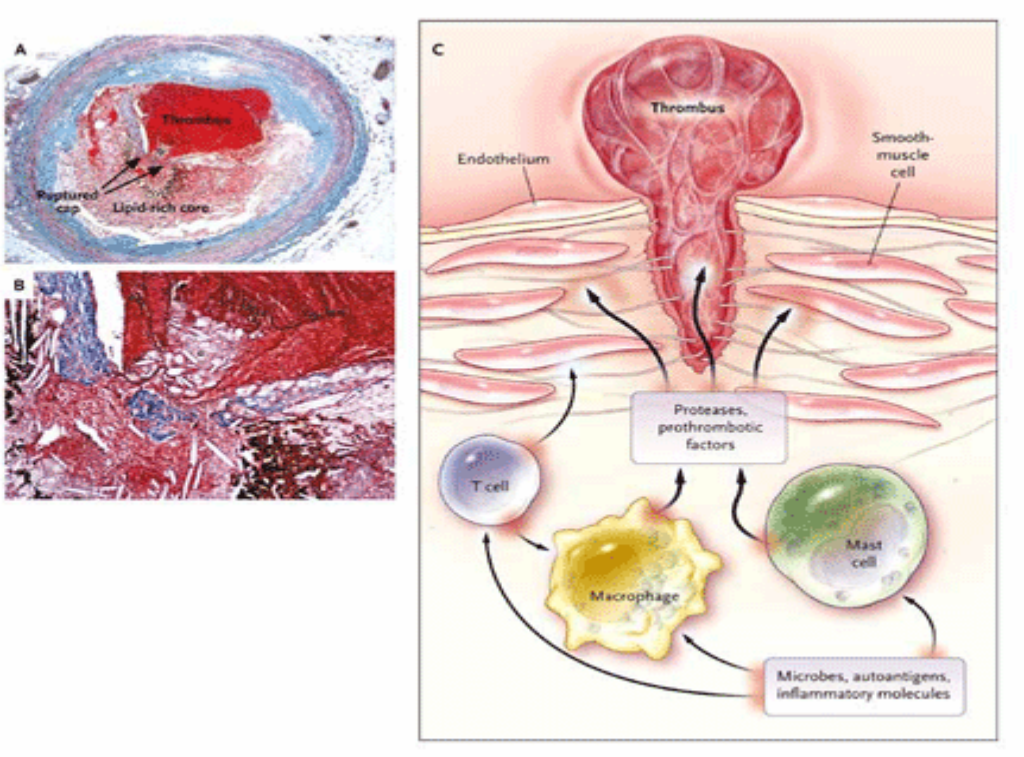


Figure- 1.12: Atherosclerotic lesion in a human artery.

Panel a shows a cross-sectioned coronary artery from a patient who died of a massive myocardial infarction. It contains an occlusive thrombus superimposed on a lipid-rich atherosclerotic plaque. The fibrous cap covering the lipid-rich core has ruptured (area between the arrows), exposing the thrombogenic core to the blood. Trichrome stain was used, rendering luminal thrombus and intraplaque hemorrhage red and collagen blue. Panel b is a high-power micrograph of the area in panel a indicated by the asterisk and shows that the contents of the atheromatous plaque have seeped through the gap in the cap into the lumen, suggesting that plaque rupture preceded thrombosis (the asterisk indicates cholesterol crystals). (panels a and b courtesy of dr. Erling falk, university of aarhus, aarhus, denmark.) Panel c illustrates the consequences of the activation of immune cells in a coronary plaque. Microbes, autoantigens, and various inflammatory molecules can activate t cells, macrophages, and mast cells, leading to the secretion of inflammatory cytokines (e.g., interferon- and tumor necrosis factor) that reduce the stability of plaque. The activation of macrophages and mast cells also causes the release of metalloproteinases and cysteine proteases, which directly attack collagen and other components of the tissue matrix. These cells may also produce prothrombotic and procoagulant

factors that directly precipitate the formation of thrombus at the site of plaque rupture.

In the center of an atheroma, foam cells and extracellular lipid droplets form a core region, which is surrounded by a cap of smooth-muscle cells and a collagen-rich matrix. T cells, macrophages, and mast cells infiltrate the lesion and are particularly abundant in the shoulder region where the atheroma grows. [50-52] many of the immune cells exhibit signs of activation and produce inflammatory cytokines. [53-56] myocardial infarction occurs when the atheromatous process prevents blood flow through the coronary artery. It was previously thought that progressive luminal narrowing from continued growth of smooth-muscle cells in the plaque was the main cause of infarction. Angiographic studies have, however, identified culprit lesions that do not cause marked stenosis, [57] and it is now evident that the activation of plaque rather than stenosis precipitates ischemia and infarction (figure 1.12). Coronary spasm may be involved to some extent, but most cases of infarction are due to the formation of an occluding thrombus on the surface of the plaque. [58]there are two major causes of coronary thrombosis: plaque rupture and endothelial erosion. Plaque rupture, which is detectable in 60 to 70 percent of cases, [59] is dangerous because it exposes prothrombotic material from the core of the plaque — phospholipids, tissue factor, and platelet-adhesive matrix molecules — to the blood (figure 1.12). Ruptures preferentially occur where the fibrous cap is thin and partly destroyed. At these sites, activated immune cells are abundant.[44] they produce numerous inflammatory molecules and proteolytic enzymes that can weaken the cap and activate cells in the core, transforming the stable plaque into a vulnerable, unstable structure that can rupture, induce a thrombus, and elicit an acute coronary syndrome (figure 1.12). To understand how this can happen, we need to identify the key steps leading from a normal artery wall to a rupture-prone atherosclerotic plaque.

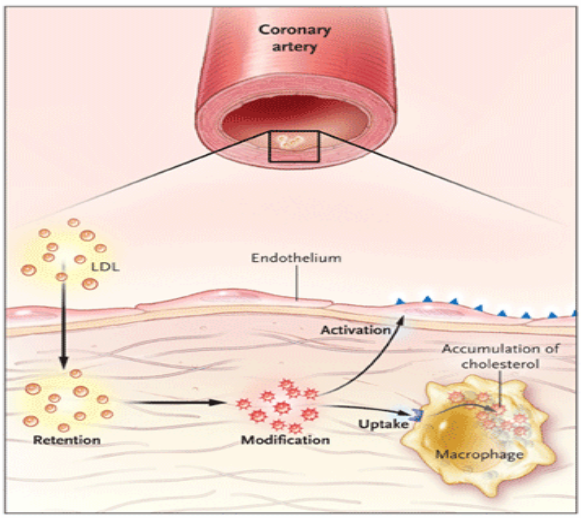


Figure 1.13: Activating effect of LDL infiltration on inflammation in the artery.

In patients with hypercholesterolemia, excess LDL infiltrates the artery and is retained in the intima, particularly at sites of hemodynamic strain. Oxidative and enzymatic modifications lead to the release of inflammatory lipids that induce endothelial cells to express leukocyte adhesion molecules. The modified LDL particles are taken up by scavenger receptors of macrophages, which evolve into foam cells. [60-63]

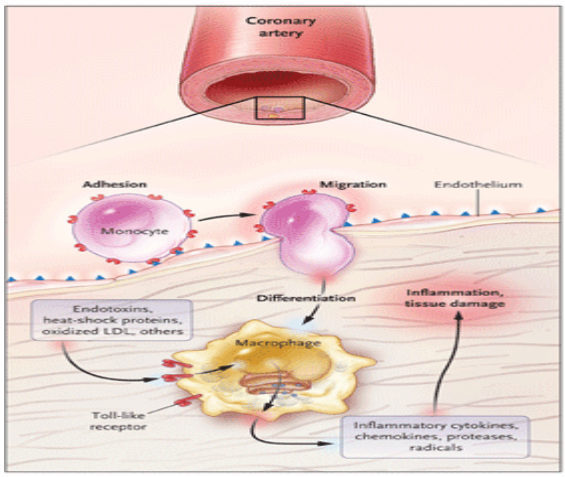


Figure 1.14: Role of macrophage inflammation of the artery.

Monocytes recruited through the activated endothelium differentiate into macrophages. Several endogenous and microbial molecules can ligate pattern-recognition receptors (toll-like receptors) on these cells, inducing activation and leading to the release of inflammatory cytokines, chemokines, oxygen and nitrogen radicals, and other inflammatory molecules and, ultimately, to inflammation and tissue damage.

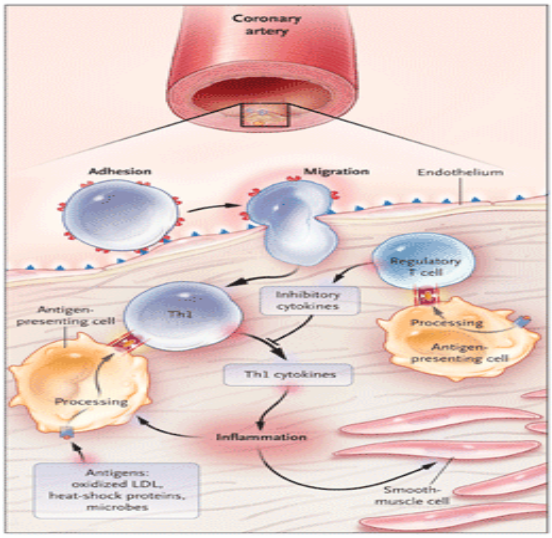


Figure 1.15: Effects of t-cell activation on plaque inflammation.

antigens presented by macrophages and dendritic cells (antigen-presenting cells) trigger the activation of antigen-specific t cells in the artery. Most of the activated t cells produce th1 cytokines (e.g., interferon-), which activate macrophages and vascular cells, leading to inflammation. Regulatory t cells modulate the process by secreting anti-inflammatory cytokines (such as interleukin-10 and transforming growth factor). [64-66]

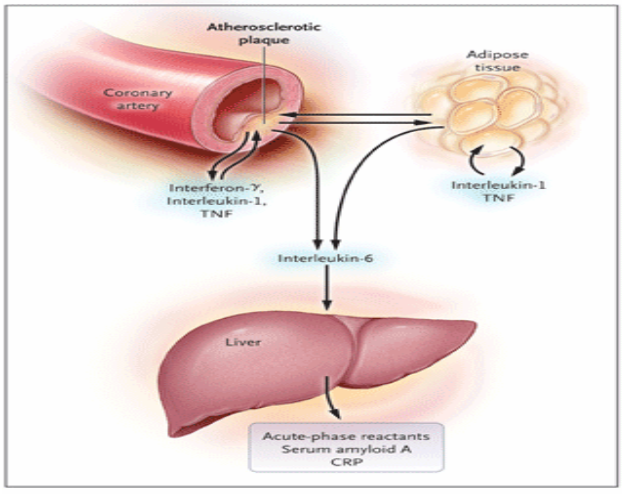


Figure 1.16: The cytokine cascade.

activated immune cells in the plaque produce inflammatory cytokines (interferon-γ, interleukin-1, and tumor necrosis factor [tnf]), which induce the production of substantial amounts of interleukin-6. These cytokines are also produced in various tissues in response to infection and in the adipose tissue of patients with the metabolic syndrome. Interleukin-6, in turn, stimulates the production of large amounts of acute-phase reactants, including c-reactive protein (crp), serum amyloid a, and fibrinogen, especially in the liver. Although cytokines at all steps have important biologic effects, their amplification at each step of the cascade makes the measurement of downstream mediators such as crp particularly useful for clinical diagnosis. [67-69]

1.17 : Acute coronary syndromes

Mechanisms of plaque rupture

What causes a silent atherosclerotic lesion to rupture? Activated macrophages, t cells, and mast cells at sites of plaque rupture [70-72] produce several types of molecules — inflammatory cytokines, proteases, coagulation factors, radicals, and vasoactive molecules — that can destabilize lesions (figure 1.12). They inhibit the formation of stable fibrous caps, attack collagen in the cap, and initiate thrombus formation. [73-76] all these reactions can conceivably induce the activation and rupture of plaque, thrombosis, and ischemia.

Two types of proteases have been implicated as key players in plaque activation: matrix metalloproteinases (mmps) and cysteine proteases. [77-78] several members of these families of enzymes occur in the plaque and may degrade its matrix. Mmp activity is controlled at several levels: inflammatory cytokines induce the expression of mmp genes, plasmin activates proforms of these enzymes, and inhibitor proteins (tissue inhibitor of metalloproteinase) suppress their action. Similarly, cysteine proteases are induced by certain cytokines and checked by inhibitors termed "cystatins." [79] several of these molecules play decisive roles in the formation of aneurysms, as shown by experiments in gene-targeted mice. However, mechanistic studies in models of atherosclerosis have yielded complex results, with certain mmps reducing rather than increasing the size of the lesions. At the same time, these enzymes clearly affect the composition of plaque. Therefore, they may represent future therapeutic

targets. Study of plaque rupture in animal models should be helpful in determining the role of these proteases in the activation of plaque and myocardial infarction.

1.18 : Pathophysiology of coronary heart disease

Coronary heart disease (CHD) remains a persistent public health burden in the united states, and it is the cause of one of every five deaths each year. The link between lipids and CHD has been firmly established, first by epidemiologic studies and, more recently, by long-term outcomes trials that demonstrated that lowering low-density lipoprotein cholesterol (LDL-c) levels significantly reduced the risk of major coronary events. Based on this evidence, the national cholesterol education program recommends lowering the LDL-c level to reduce CHD risk, particularly for patients at highest risk. Recently, evidence has emerged that suggests that c- reactive protein may be a mediator of atherosclerosis and its presence may be indicative of increased risk of CHD. Although these data are intriguing, their relevance has yet to be established in prospective outcomes trials. Until then, lipid lowering through lifestyle modification and the use of safe and effective modes of therapy should be the emphasis of CHD risk reduction strategies. Emerging evidence strongly suggests that coronary heart disease (CHD), once considered the result of vessel-occluding deposition of lipids, is a manifestation of a chronic inflammatory response to injury or infection. Elevated plasma cholesterol levels have long been established as risk factors for CHD, and lowering cholesterol levels, particularly low- density lipoprotein cholesterol (LDL-c), has been the focus of the prevention of CHD and its sequelae for almost 25 years. However, the complex mechanisms by which these molecules act are only beginning to be appreciated. Evidence suggests that lipid-lowering modes of therapy also reduce inflammation, which may reduce the risk of cardiovascular events, even for individuals with LDL-c levels in the normal range (<130 mg/dl) based on the national cholesterol education program (ncep) adult treatment panel iii (atp iii) guidelines. [80] this article reviews the role of lipids in plaque development, the data supporting atherosclerosis as an inflammatory disease, and the impact of these data on clinical practice.

Although some of these criteria can be visualized with noninvasive procedures such as

magnetic resonance imaging or computer-enhanced tomography, none is easily used for routine screening purposes. Thus, the atp iii risk factor assessment based on a 10-year cardiovascular risk remains the best tool to identify patients at high risk. [81]

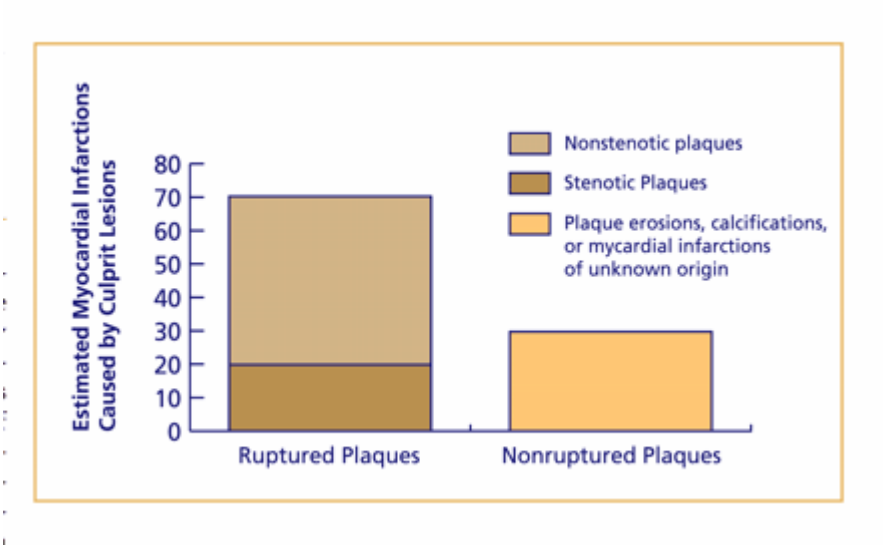


Figure 1.17: Underlying pathologic processes of coronary lesions determined to be responsible for coronary occlusion and death. (source: naghavi m, et al. Circulation. 2003; 108:1664-1672.)

1.18.1 : Role of lipids in plaque development

Injury or infection can disrupt normal endothelial function and initiate formation of atherosclerotic lesions known as *fatty streaks*. Fatty streaks typically consist of macrophages and t cells embedded in a thin layer of lipids on the arterial wall. [66-69] macrophages engulf lipids, becoming activated foam cells that release an array of chemoattractant molecules, cytokines, and growth factors. More lymphocytes are attracted to the lesion and, in turn, add to the pool of effector molecules that expand and perpetuate the inflammatory response. As this cycle is repeated, the plaque develops a fatty core covered by a fibrous matrix that stabilizes the structure.
[82]

The frequent presence of fatty streaks in young children is consistent with the chronic nature of atherosclerotic progression. Although the possible events that can initiate fatty streak formation remain controversial, LDL-c, modified by oxidation, glycation, and association with proteoglycans and immune complexes, can become trapped in the arterial wall, injuring the endothelium and vascular smooth muscle.[83-87] once trapped, LDL-c particles become progressively more oxidized, form lipid peroxides, and facilitate accumulation of cholesterol esters.[88] also, modified LDL-c is chemotactic for circulating monocytes and stimulates the proliferation of macrophages already in the lesion.[89] inflammatory mediators increase the binding of LDL-c to endothelial cells and vascular smooth muscle cells that have migrated into the lesion.[90] as the plaque becomes thicker, the arterial wall responds by "remodeling," that is, gradually dilating to maintain the diameter of the vessel lumen. Eventually, macrophages may be stimulated to release metalloproteinases that degrade the fibrous cap and render the plaque vulnerable to rupture. [91-92]

Although several types of plaque can result in serious coronary events, retrospective analyses have demonstrated that 70% of all fatal acute myocardial infarctions and sudden coronary deaths are attributable to plaque rupture [93] or plaque erosion [94] (figure 1.12). This observation is not surprising because plaque destabilization is often accompanied by release of prothrombotic factors. [95] however, a recently developed consensus document emphasizes that all types of atherosclerotic plaques can result in coronary events and sudden death. [96]

vulnerable plaques are defined as thrombosis-prone or at risk of rapid progression and exhibit some combination of the following: active inflammation, thinning cap with a large lipid core, endothelial denudation with superficial platelet aggregation, fissures, or greater than 90% stenosis. The authors further conclude that the thrombotic status of the blood and the electrical instability of the myocardium are important to the ultimate outcome for the patient. [97]

1.18.2 : Inflammation and atherosclerosis

An accumulating body of evidence suggests that atherosclerotic progression results from microinflammation mediated by proinflammatory cytokines. The observation that monocytes and t lymphocytes are present at all stages of plaque development is consistent with active inflammation. Chronic low-level inflammation increases atherosclerotic plaque deposition in animal models. In addition, heightened levels of the acute-phase reactant c-reactive protein (crp) is believed to be a marker of inflammatory processes and may also be of value in the prediction of coronary events.[98-99] a recently published report suggests that crp is more than a marker and may be a mediator of atherosclerosis. [100]

The association between elevated levels of crp and cardiovascular risk has been the object of extensive research and is the topic of much current debate. Some evidence suggests that crp is an independent predictor of risk of cardiovascular events. In a study that followed nearly 28,000 apparently healthy women for 8 years, ridker and colleagues [84] found that the crp level was a stronger predictor than the LDL-c level for myocardial infarction, ischemic stroke, coronary revasculature, or death due to cardiovascular causes. However, because crp and LDL-c levels appeared to identify somewhat different risk groups, the combined risk assessment was superior to that of either marker alone. [101]

In this same study, multivariate analysis indicated that increasing crp levels were associated with increased risk of cardiovascular events at all levels of estimated risk based on the framingham risk score and ncep atp iii risk categories. [102] although the women in this study who had high crp and low LDL-c levels were at higher absolute risk than those with low crp and high LDL-c levels, only the latter group would be considered eligible for aggressive therapy.[103]

Danesh et al [104] recently reported data from a study of circulating inflammatory markers that evaluated the relevance of crp to the prediction of CHD. These investigators prospectively observed 18,569 individuals enrolled in the reyk-javik heart study and measured inflammatory markers in blood samples obtained at baseline from up to 2459 patients who had a nonfatal myocardial infarction or died of CHD during the study and from up to 3969 control subjects without CHD. Results of the study suggested that crp was not as strong a predictor for CHD as more traditional risk factors such as total cholesterol level or cigarette smoking. Therefore, the authors concluded that recommendations regarding the use of the crp level in predicting the likelihood of CHD may need to be reviewed. [105]

Newer information reveals that crp is a modulator of inflammation and may have both proinflammatory and antiinflammatory actions, which may directly contribute to endothelial dysfunction by inducing cytokine release and surface expression of adhesion molecules. Through a conformational rearrangement in crp from a pentameric to a monomeric structure, the atherogenic effects of crp are noted on human endothelial cells. C-reactive protein now appears to be more than a marker of cardiovascular events. [106]

1.18.3 : The emerging concept of the vulnerable patient

Vulnerable plaques are not the only culprit factors for the development of acute coronary syndromes, myocardial infarction, and sudden cardiac death. Vulnerable blood (prone to thrombosis) and vulnerable myocardium (prone to fatal arrhythmia) play an important role in the outcome. Therefore, the term *vulnerable patient* may be more appropriate and is proposed now for the identification of subjects with high likelihood of having cardiac events develop in the near future (figure 1.18). A quantitative method for cumulative risk assessment of vulnerable patients needs to be developed that may include variables based on plaque, blood, and myocardial vulnerability.

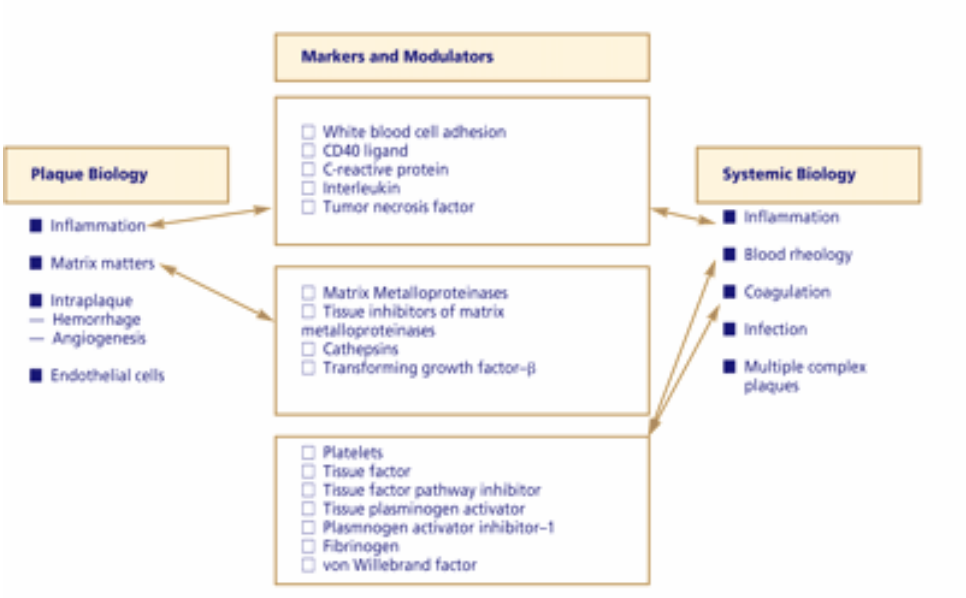


Figure 1.18: Factors contributing to the "vulnerable patient."

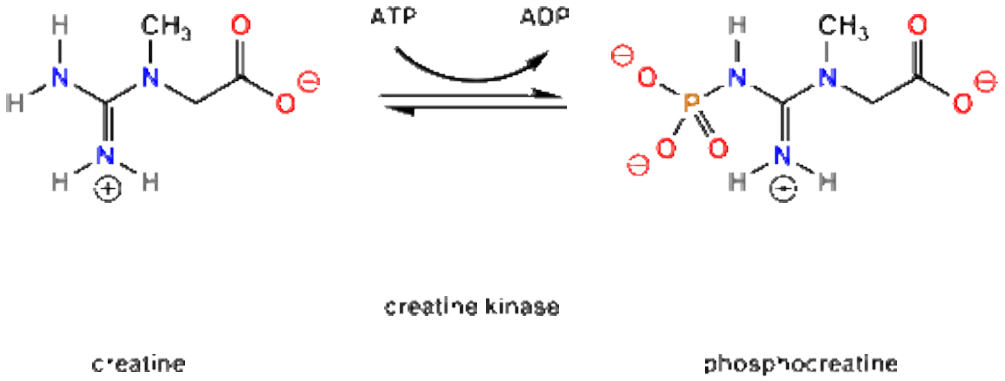
According to naghavi et al, [106] markers suggesting the presence of vulnerable blood include increased levels of crp and circulating interleukin-6 levels, which are elevated in patients with acute coronary syndromes. Investigators have also shown that high plasma concentrations of soluble cd40 ligand may indicate an increased vascular risk in apparently healthy women. [107] likewise, hwang et al [108] reported that circulating levels of soluble intracellular adhesion molecule were predictive of future acute coronary events. Markers of systemic inflammation, such as soluble adhesion molecules, circulating bacterial endotoxin, soluble human heat-shock protein 60, and antibodies to mycobacterial heat-shock protein 65, may predict an increased risk of atherosclerosis.[109] pregnancy-associated plasma protein a is present in unstable plaques, and its circulating levels are elevated in patients with acute coronary syndromes. [110] increased serum levels of pregnancy-associated plasma protein a may reflect instability of atherosclerotic plaques. [111]

Lipids, particularly cholesterol, play a fundamental role in the development of CHD. A substantial volume of evidence indicates that reducing cholesterol levels reduces the risk of CHD in both primary and secondary prevention populations. Despite the emergence of new markers of CHD (such as crp), LDL-c currently remains the primary target for reduction of

risk of CHD. However, emerging evidence suggests the intriguing notion that the anti-inflammatory activities of lipid-lowering modes of therapy such as statins may have a more significant role in reducing risk of CHD than previously believed. However, until the relevance of these nontraditional risk factors is established in well-designed prospective outcomes trials, physicians and patients should strive to decrease CHD risk by reducing lipids through the initiation of therapeutic lifestyle changes and use of appropriate modes of lipid therapy.

1.19 : Creatine kinase

creatine kinase (ck), also known as **creatine phosphokinase** (cpk) or **phospho-creatine kinase** or sometimes wrongfully also **creatinine kinase**, is an enzyme (ec 2.7.3.2) expressed by various tissues and cell types. Ck catalyses the conversion of creatine and consumes adenosine triphosphate (atp) to create phosphocreatine and adenosine diphosphate (adp). This ck enzyme reaction is reversible, such that also atp can be generated from pcr and adp.[112]



in tissues and cells that consume atp rapidly, especially skeletal muscle, but also brain, photoreceptor cells of the retina, hair cells of the inner ear, spermatozoa and smooth muscle, phosphocreatine serves as an energy reservoir for the rapid buffering and regeneration of atp *in situ*, as well as for intracellular energy transport by the phosphocreatine shuttle or circuit.[112] thus creatine kinase is an important enzyme in such tissues.[113]

Clinically, creatine kinase is assayed in blood tests as a marker of myocardial infarction (heart attack), rhabdomyolysis (severe muscle breakdown), muscular dystrophy, and in acute renal failure. Ck-mb is a more sensitive marker of myocardial injury than total ck activity, because

it has a lower basal level and a much narrower normal range. Medical literature commonly states that ck-mb levels become elevated in 4 to 6 hours, peak at 10 to 24 hours, and return to normal within 3 to 4 days after an acute myocardial infarction. However, these enzyme kinetics were determined using an insensitive electrophoretic method. In 1991, most laboratories began measuring ck-mb mass instead of enzyme activity. Ck-mb mass assays are able to measure small, but significant changes during the early hours following onset of chest pain.

1.20 : Serum electrolytes and coronary heart disease

Twenty-four hour urinary excretion of sodium (Na), potassium (K), na/k ratio and creatinine (cr), serum cr, na, and k, and plasma renin activity (pra) were evaluated in 623 untreated hypertensive men. Blacks (n = 407) and whites (n = 216) were similar in weight, heart rate, systolic blood pressure (sbp), diastolic blood pressure (dbp), 24-hour urine cr excretion, and cr clearance. Twenty-four hour urine Na excretion was the same in blacks and whites, but whites excreted 62% more K than blacks: 73 +/- 41 (mean +/- sd) vs 45 +/- 40 meq/24h (p less than 0.001). Urinary na/k ratio was 4.51 +/- 2.18 in blacks and 2.85 +/- 1.40 in whites (p less than 0.001). Serum K and pra were also lower in blacks. Serum and urine na/k ratios, serum Na and age were positively associated with sbp; serum K and pra were negatively associated with sbp. Serum na/k ratio, heart rate and weight were positively associated with dbp; serum K was weakly negatively associated with dbp. The racial difference in urinary K excretion and serum K is believed to reflect a difference in dietary K intake between blacks and whites. This difference may be an important factor in the greater prevalence of hypertension among blacks. The role of stress in the precipitation of hypertension is often described in clinical studies, although the underlying mechanism remains unknown. The present study concerns the role of electrolytes in stress induced hypertension in rats. Acute immobilization stress of one hour elevated systolic blood pressure (sbp) in rats. Restraint induced blood pressure elevation was associated with increased sodium concentration in the red cells, heart and kidney, and decreased potassium in the red cells. Magnesium concentration increased and calcium concentration decreased in the serum. Increases of calcium and decreases of magnesium were also observed in the heart and kidney tissues. The results may help toward an understanding of the relationship between hypertension and electrolyte homeostasis. A possible role of na^+-k^+-atpase activity leading to observed changes of electrolytes or vice versa is discussed. [114]

1.21 : Troponin: the biomarker of choice for the detection of cardiac injury

The 3-unit troponin complex (troponin i, t and c) along with tropomyosin is located on the actin filament and is essential for the calcium-mediated regulation of skeletal and cardiac muscle contraction. There are tissue-specific isoforms of troponin i, t and c. Because the cardiac isoform of troponin c is shared by slow-twitch skeletal muscles, troponin c does not have cardiac specificity and thus is not used in assays for the diagnosis of cardiac injury.

There is one cardiac troponin i (ctni) isoform in myocardial tissue. This isoform has a post-translational tail of 32 amino acids on the n-terminus. This sequence and the 42% and 45% dissimilarity with sequences of the other isoforms have made possible the generation of highly specific monoclonal antibodies without cross-reactivity with other noncardiac forms.

Three genes control cardiac troponin t (ctnt). These genes and alternative mrna splicing produce a series of isoforms with variable sequences close to the regions of the n-terminus and c-terminus. Human cardiac muscle contains 4 troponin t isoforms, but only one is characteristic of the normal adult heart.highly specific antibodies have been made to the n-terminus–specific sequence of this ctnt isoform.

The skeletal isoforms present in the fetal heart are replaced by ctni and ctnt late during fetal development. Ctni is not expressed in skeletal muscle or other tissues during development or in response to degenerative or regenerative muscle disease processes.[115] thus, it is unlikely to be re- expressed in damaged tissues. The situation is more complex for ctnt. Re-expression of fetal forms occurs in cardiac tissue and in diseased skeletal muscle. With the first-generation ctnt assay, this problem was compounded by a nonspecific tag antibody that cross-reacted with troponin t in skeletal muscle.once this antibody was replaced by one with high specificity, false- positive elevations from skeletal muscle were eliminated.studies using immunohistochemistry and polymerase chain reaction have confirmed that these fetal isoforms are not detected by the assay used today. Thus, the assay used to measure ctnt levels has cardiac specificity equivalent to that of assays for ctni.[116-125]

CHAPTER 2

METHODS AND METARIALS

2.1 Subjects

In the present study two hundred patients suffering from cardiovascular disease of age (37-79) were examined against ninety healthy normal control subjects of age (37-71). Cardiovascular diseases were diagnosed according to american heart association criteria. Subjects were considered dyslipidemic to the following cutoff points: LDL-c (150mg/dl), TG (190mg/dl), total cholesterol (120-200mg/dl), HDL-c (35mg/dl), CKMB (0 - 24 iu/l (37°c). 0 - 14 iu/l (30°c)), AST (10-40 iu/l at 37°c.), Na (135 - 155 meq/l), K (1.0 to 15.0 meq/l), Cl (serum –98-106 meq/l), and Co2 (23 - 34 mmol/l).

2.2 Instruments and materials:

1. Estimations were carried out by semi-automatic biochemical analyzer. Made in france by sfri sarl, bsa 3000, 0101055, ref no. Rsl30/08
2. Turbox. Made in finland,2002
3. Centrifuge.
4. Freezer.
5. Micropipette.

Procedure for the estimation of following parameters and reference values are provided by the literature supplied with above reagent cartridge

2.3 Sample collection:

Blood samples (10ml) were collected from cardiovascular patients and respective controls by using sterile syringe and stored in test tube having heparin as antiquagulant. Then blood was left at room temperature for 30 minutes and the serum was separated from the cells within 3 hours of venipuncture. Samples were refrigerated at (2-8 °c) for up to 3 days, if not tested within 24 hours. For longer storage, sample may be frozen at (-20°c) for up to one month or at (-70°c) for up to 2 years. Serum and heparinized plasma yield identical results. Lipemic or icteric specimens do not interfere with the validity of the results.

2.4 Estimation of serum total cholesterol:

Reference value: serum total cholesterol; 120-200 mg/dl

Summary: the **chol** method is based on the principle first describe (stadman 1957) and later adapted by other workers (flegg, 1973. Roschlau, 1974), including (rautela and liedke, 1978).

Principle of the procedure: cholesterol esterase (ce) catalyzes the hydrolysis of cholesterol ester to produce free cholesterol which, along with preexisting free cholesterol, is oxidized in a reaction by cholesterol oxidase (cd) to form choles-4-ene-3one and hydrogen peroxide. In the presence of horse radish peroxidase (hpo), the hydrogen peroxide thus formed is used to oxidize n, n-diethyl aniline-hcl/4-aminiantipyrine (dea-hcl/aap) to produce a chromophore that absorb at 540nm. The absorbance due to oxidized dea- hcl/aap is directly proportional to the total cholesterol concentration and is measured using a polychromatic (452,540,700 nm) endpoint technique.

cholesterol ester	Ce →	Cholesterol + fatty acids
Cholesterol	Co →	Choles-4-ene-3one + 2h2o2
2h2o2+dea hcl/aap	Hpo →	4h2o + oxidized dea-hcl/aap

Details methods including reagent preparation and calculations are in supp. S2.1

2.5 Estimation of serum triglyceride

Reference value: serum triglyceride (fasting); 35-160 mg/dl

Summary: the triglyceride method was based on an enzymatic procedure in which a combination of enzymes were employed for kinetic bichromatic (340,383 nm) measurement of serum triglycerides (hagen, 1962. Rautela,1974). Fluctuation in relative concentration of the tri, di, monoglycerides and free glycerol which occur in patient sample upon storage (tiffany, 1974.rautela, 1973) are automatically accommodated since this method measures the sum of this metabolites. The contribution of di and monoglycerides is negligible.

Principle of the procedure: the sample was pre-incubated with lipase enzyme reagent which converts triglycerides into free glycerol and fatty acids. The liberated glycerol was determined enzymatically using glycerol dehydrogenase (GDH) and NAD. The change in absorbance at 340 nm due to the formation of NADH is proportional to the total amount of glycerol and it's precursors in the sample and is measured using a bichromatic (340,383 nm)

rate technique.

triglyceride	Lipase →	Glycerol + fatty acids
glycerol + NAD^+	GDH →	Dihydroxyacetone + NADH+H+GDH

Details methods including reagent preparation and calculations are in supp. S2.2

2.6 Estimation of serum HDL-cholesterol:

Reference value: serum HDL-cholesterol; for men >35 mg/dl, and for women >45 mg/dl

Summary: the reference method for the quantification of HDL combines ultracentrifugation and chemical precipitation to separate HDL from other lipoproteins, followed by cholesterol measurement using abell-kendall assay. The method is too time consuming and labour intensive for use routine analysis. Therefore most laboratories utilize one of LDL and VLDL, followed by enzymatic measurement of the HDL in the supernatant fraction. Since these methods require off-line pretreatment and separation steps in the assay procedure can not be fully automated. As a result, routine determination of HDL has suffered from long handling times and poor reproducibility.

Principle of the procedure: the HDL cholesterol method is homogenous method for direct measuring HDL without the need for off-line pretreatment or centrifugation step. The method is in a two reagent format and depends on the properties of unique detergent, which stabilizes only the HDL lipoprotein particles, thus releasing the HDL cholesterol to react with cholesterol esterase to produce color. In addition to selectively disrupt the HDL lipoprotein particles, this detergent also inhibits the reaction of the cholesterol enzyme with LDL, VLDL and chylomicron lipoproteins by adsorbing to their surface. A polyanion is contained in the first reagent to assist with complexing LDL, VLDL and chylomicron lipoproteins, further enhancing the selectivity of the detergent and enzymes for HDL cholesterol.

LDL, VLDL and chylomicron	Detergent →	Stable complexes polyanion
HDL	Detergent →	HDL disrupted
HDL disrupted	Cholesterol esterase →	Δ' cholestenone + h2o2
h2o2 + dsmt + 4aa	Peroxidase →	Color development

Details methods including reagent preparation and calculations are in supp. S2.3

2.7 Estimation of serum LDL-cholesterol:

Reference value: serum LDL-cholesterol;<150mf/dl

Summary: methods for LDL-c measurement assume that total cholesterol is a composed primarily of cholesterol in VLDL, IDL, LDL, HDL and lp. Friedewald equation developed in 1972 the most frequently used indirect method for estimating LDL-c concentration. Using this equation, LDL-c concentration is calculated as follow:

[LDL-c]= [total cholesterol]-[HDL-c]-[triglyceride]/5

All concentration were in mg/dl. The factor [triglyceride]/5 are an estimate of VLDL-c concentration and are based on the average ratio of triglyceride to cholesterol in VLDL. In practice, the friedewald calculation works reasonably well. However it should not be used with samples that have triglyceride concentration above 400 mg/dl, when chylomicrons are present or in patients with dysbetalipoproteinemia. At high triglyceride concentration, LDL-c concentrations are estimated. Until the only means of consistently measuring LDL-c concentration accurately was to perform beta-quantification; an expensive, time consuming and labour intensive approach that most clinical laboratories are unable to perform. The automated low density lipoprotein (aLDL) is a direct assay not dependent on the friedewald calculation and is referenced to the beta-quantification determination of LDL-c concentration. Details methods including principle, reagent preparation and calculations are in supp. S2.4

2.8 Estimation of serum CKMB:

Ck-mb kinetic reagent set code: rcc0033

Format: 1 x 60 tests

For in vitro diagnostics use only

Intended use

Ck-mb is intended to measure the activity of isoenzyme CKMB in human serum.

Introduction

Creatine kinases are dimeric molecules composed of m and b subunits and exist as the isoenzymes mm, mb, and bb.the subunits m and b are immunologically distinct; ck-mm and ck-mb are distributed primarily in the skeletal muscle and heart muscle, respectively. While

ck-bb is present mainly in the brain and in tissues composed of smooth muscle. Following acute myocardial infarction, ck-mb activity increases significantly and this elevation is highly specific for the laboratory diagnosis of myocardial infarction. Although total ck activity usually increases following myocardial infarction, in some patients only the ck-mb activity increases, while the total ck remains in the normal range.

In conventional methods, ck isoenzymes are quantitated after first separating the three species by electrophoresis, column anion exchange, or batch anion exchange chromatography. However, these methods are time consuming. In recent times, wurzburg et al. Has introduced an immunoinhibition method8. This methodology forms the basis of our ck-mb reagent. Wagner, et al,, *circulation*: 47:263 (1973).

Principle

The sample is incubated in the ck-mb reagent which includes the anti-ck-m antibody. The activity of the noninhibited ckb is then determined using the following series of reactions:

adp + creatine phosphate	$\xrightarrow{Ck}$	Creatine + atp
atp + glucose	$\xrightarrow{Hk}$	Adp + glucose-6-phosphate
g-6-p + NAD+	$\xrightarrow{G6p\text{-}dh}$	6-phosphogluconate + NADH + h+

Ck-b catalyzes the reversible phosphorylation of adp, in the presence of creatine phosphate, to form atp and creatine. The auxiliary enzyme hexokinase (hk) catalyzes the phosphorylation of glucose by the atp format, to produce adp and glucose-6-phosphate (g-6-p) and later which is oxidized to 6phosphogluconate with the concomitant production of NADH. The rate of NADH formation, measured at 340 nm, is directly proportional to serum ck-b activity.

Details methods including reagent preparation, precautions and calculations are in supp. S2.5

2.9 Estimation of serum potassium (K) in refrigerated serum:

Summary of test principle and clinical relevance

The dxc800 system uses indirect (or diluted) i.s.e. (ion selective electrode) methodology to measure potassium in biological fluids. The system determines potassium ion concentration by measuring electrolyte activity in solution. The potassium electrode consists of valinomycin membrane. The voltage (potential) change that takes place within the membrane follows the nernst equation and allows the calculation of potassium concentration in solution.

Potassium measurements are used in the diagnosis and treatment of hypokalemia, hyperkalemia, renal failure and diseases involving electrolyte imbalance.

Specimen collection, storage, and handling procedures; criteria for specimen rejection

a. Interferences:

1) No interference from ≤30.0 mg/dl bilirubin

2) Do not use hemolyzed specimens.

3) No interference from <4+ lipemia

4) On samples with >3+ lipemia, perform potassium on lipoclear-treated serum.

b. Separated serum or plasma should not remain at +15°c to +30°c longer than 8 hours. If assays are not completed within 8 hours, serum or plasma should be stored at +2°c to +8°c. If assays are not completed within 48 hours, or the separated sample is to be stored beyond 48 hours, samples should be frozen at -15°c to -20°c.

c. Fasting is not required.

d. A minimum of 0.6 ml serum is needed for the multi-analyte panel.

e. Sample volume for individual test is 40 µl added to 1.27 ml buffer reagent, and 40 µl added to 3.23 ml reference reagent.

f. Sample is run singly as part of multi-analyte biochemistry panel.

Reference ranges (normal values)

Potassium

Serum or plasma **Age group**	**Reference range** **Meq/l**
0-1 month	3.9-6.9
1 mo. - 1 y	3.6-6.8
1-5 y	3.2-5.7
5-10 y	3.4-5.4
>10 y	3.5-5.0

2.10 Estimation of serum sodium (Na) in refrigerated serum

Intended use

For the colorimetric determination of sodium in human serum and plasma.

Introduction

Sodium is the major cation of extracellular fluid. It plays a central role in the maintenance of the normal distribution of water and the osmotic pressure in the various fluid compartments. The main source of body sodium is sodium chloride contained in ingested foods. Only about one-third of the total body's sodium is contained in the skeleton since most of it is contained in the extracellular body fluids. 1,2 hyponatremia (low serum sodium level) is found in a variety of conditions including the following: severe polyuria, metabolic acidosis, addison's disease, diarrhea, and renal tubular disease. Hypernatremia (increased serum sodium level) is found in the following conditions: hyperadrenalism, severe dehydration, diabetic coma after therapy with insulin, excess treatment with sodium salts.

Principle

The present method is based on modifications of those first described by maruna3 and trinder4 in which sodium is precipitated as the triple salt, sodium magnesium uranyl acetate,with the excess uranium then being reacted with ferrocyanide, producing a chromophore whose absorbance varies inversely as the concentration of sodium in the test specimen.

Details methods including reagent preparation, precautions and calculations are in supp. S2.6

2.11 Estimation of serum chloride (Cl) in refrigerated serum

(by chloridometry)

Principle (coulometric method)

The labconco digital chloridometer is an instrument for measuring chloride levels in fluids. The instrument automatically titrates chloride ions by generating silver ions at a constant rate. These silver ions combine with the chloride ions in the sample and they precipitate as silver chloride. The end point occurs when all the chloride ions have combined with the silver ions in the precipitation of silver chloride. At this time, free silver ions appear in the sample which changes the conductivity of the solution. This change is sensed by the detector electrodes and the digital readout is stopped, displaying the results directly in milliequivalents of chloride per

liter. The digital display is held until automatically reset by the start of another measurement.

Clinical significance measurement of chloride in serum is a vital part of the determination of a patient's electrolyte status and useful in managing the patient's electrolyte status and useful in managing the patient's intravenous therapy or other forms of fluid therapy as well as indicating other disease processes.

Hyperchloremia is found in patients with decreased renal blood flow such as congestive heart failure. It may occur in dehydration or when inappropriate excess administration of saline occurs.

Hypochloremia occurs with severe vomiting or excess removal of gastric contents by gastric suction. It is also seen in metabolic acidosis due to the excessive production and diminished excretion of acids. It may also be seen in disease states involving decreased production of anti-diuretic hormone (adh) or when inappropriate excessive administration of intravenous glucose has occurred. Low cl– values are found in salt-losing nephritis, in addison's disease crisis and in metabolic alkalosis when the hco– 3 ion is increased the cl– falls reciprocally. Chloride concentration in normal cerebrospinal fluid (csf) is higher than serum levels. A decrease in csf chloride concentration occurs when spinal fluid protein increases as in tubercular and bacterial meningitis.

Normal values

Serum – 98-106 meq/l csf – 118-132 meq/l

Urine – 110-250 meq/24 hours

Details methods including reagent preparation, precautions and calculations are in supp. S2.7

2.12 Estimation of serum carbon di oxide (Co2) in refrigerated serum

Intended use

For manual or automated quantitative determination of carbon dioxide content in human plasma or serum at 340 nm.

Introduction

Carbon dioxide (Co2) in serum or plasma exists primarily as dissolved Co2 and bicarbonate anion (hco_3-). The plasma Co2 content is decreased in metabolic acidosis and respiratory alkalosis, whereas the level is increased in metabolic alkalosis and respiratory acidosis. In

pathologic conditions such as in diabetes mellitus, glomerulonephritis, pyloric obstruction, diarrhea, etc., acidosis or alkalosis could be anticipated. Therefore, determination of plasma Co2 content as part of an electrolyte profile can help establish to a degree, the anticipated change in the patient. Plasma Co2 content can be measured manometrically or using an ion selective electrode or by spectrophotometric procedures. This Co2 reagent measures Co2 content enzymatically and the procedure is a modification of the method of forrester et al.

Principles of the procedure

Phosphoenol pyruvate + hco_3- pepc > oxalate + $h2po_4$ oxalate + NADH mdh > malate + NAD

Phosphoenol pyruvate carboxylase (pepc) catalyzes the reaction between phosphoenol pyruvate and carbon dioxide (bicarbonate) to form oxalacetate and phosphate ion. Oxalacetate is reduced to malate with simultaneous oxidation of an equimolar amount of reduced nicotinamide adenine dinucleotide (NADH) to NAD; the reaction is catalyzed by malate dehydrogenase (mdh). This results in a decrease in absorbance at 340 nm that is directly proportional to Co2 concentration in the sample.

Details methods including reagent preparation, precautions and calculations are in supp. S2.8

2.13 Estimation of serum serum aspartate aminotransferase (AST) in refrigerated serum

Ast (SGOT) reagent (colorimetric endpoint procedure) for the quantitative colorimetric determination of ast(SGOT) in serum.

Introduction

Serum aspartate aminotransferase (AST) also known as serum glutamic oxalacetic transaminase (SGOT) is a tissue enzyme that catalyzes the exchange of amino and ketogroups between alpha amino acids and alpha-keto acids. AST is widely distributed in tissue principally cardiac hepatic muscle and kidney. Injury to these tissues results in the release of the AST (SGOT) enzyme to general circulation. Following a myocardial infarction serum level of AST (SGOT) are elevated and reach a peak 48 to 60 hours after onset. Hepatobiliary diseases such as cirrhosis metastatic carcinoma and viral hepatitis also will increase serum AST levels.

Methods for the determination of serum AST (SGOT) include ultraviolet kinetic analysis and colorimetric methods. Earlier colorimetric methods were based on the reaction of oxalacetate

with dinitrophenylhydrazine. However this reaction is time consuming and non-specific reactions. The present method is based on a modification of the colorimetric method by doumas and briggs which offers increased specificity and shortened incubation time.

Principle

Ast catalyzes the following reaction. Ast

L-aspartate + 2-oxoglutarate ----- oxalacetate + l-glutamate

In the present method a diazonium salt is used which selectively reacts with the oxalacetate to produce a color complex that is measured photometrically.

Details methods including reagent preparation, precautions and calculations are in supp. S2.9

2.14: History of the patients

the history of all cardiac patients compared with controls at the onset of the study is listed in tabular form below. Age, sex, lipid profile, enzymes (ck-mb, SGOT) and electrolytes status are used as markers to differentiate between CVD and no-CVD subjects among urban, hilly and coastal areas. Table 3.1 shows the history patients age, sex and percentage of menopause in women. There were 290 subjects included in the present study, 200 of which were as patients group and 90 were as control groups. The total 290 study population's serum were collected from different areas and these are coastal area, urban area and hilly area and each group has same age level controls. There are total 108 females and 182 males included in this study who are divided into patient-control and coastal-hill-urban groups (table 2.1). The mean age of the study population (290) is 56.51 with a standard deviation of 9.49 ranges from 37 to 79 (table 3.1). Age is normally distributed across separate groups (figure 2.1). The mean and standard deviation of age does not change too much in different experimental group (figure 2.2).

Region	Condition	F	M
Coastal	Control	10	15
	Patient	15	35
Hill	Control	13	12
	Patient	22	28
Urban	Control	19	21
	Patient	29	71

table 2.1 Sample counts in different experimental groups

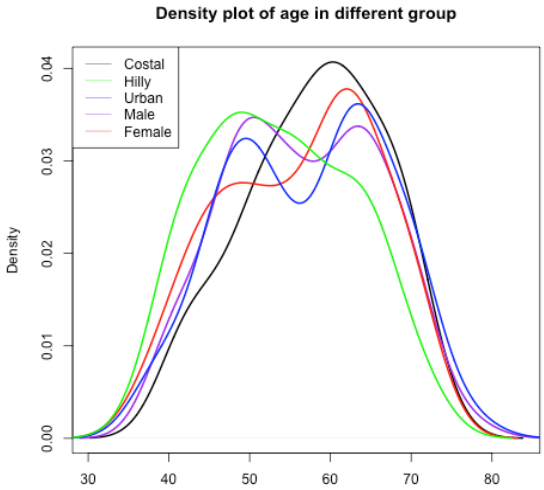


Figure 2.1 Density plot of age in experimental populations

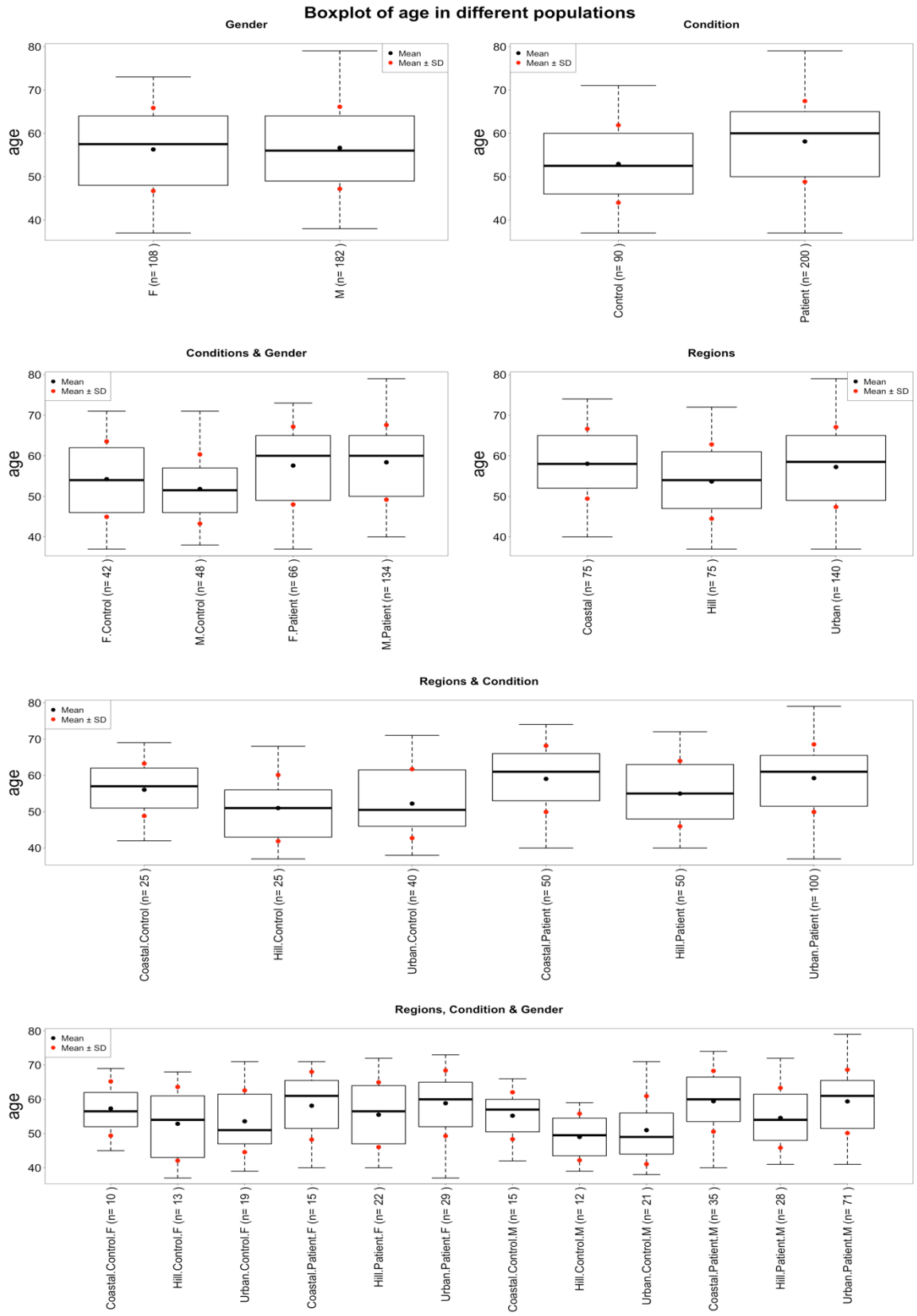


Figure 2.2 Boxplot of age in experimental populations

2.15: Sample collection period, locations, and exclusion criteria

Samples were collected in between march 2009-march 2010 from:

Urban:

1. Birdem general hospital
2. Ibn sina hospital
3. Mitford hodpital
4. Dhaka medical college and hospital
5. Bangladesh medical college and hospital

Hilly:

1. Christian hospital chondroghona
2. Imperial hospital chittagong
3. Chittagong general hospital

Coastal:

1. Fouad- al- khateeb hospital
2. Central hospital coxs bazar
3. Khulna medical college and hospital
4. Shaheed sheikh abu naser specialised hospital

Subject that are already diagnosed with heart diseases and under regular follow up was considered as patients. Patients from any other city except the above mentioned nine, were considered as urban. Age below 30 and missing any variable were excluded. Controls were chosen for similar age and respective region's healthy individuals. Smoking, drinking, diabetes, kidney and other conditions were not included in this study.

CHAPTER 3

RESULTS

3: Results:

In this section all the above-mentioned parameter's statistics will be described, their distributions and comparisons among different experimental groups will be illustrated.

	N	Mean	Sd	Median	Trimmed	Mad	Min	Max	Range	Skew	Kurtosis	Se
Age	290	56.51	9.49	57	56.64	11.86	37	79	42	-0.08	-0.96	0.56
Cholesterol	290	166.96	40.72	155.43	163.81	41.54	99.78	279.45	179.67	0.61	-0.47	2.39
TG	290	141.13	53.11	136.22	138.64	49.65	50.55	278.34	227.79	0.4	-0.3	3.12
HDL	290	38.46	7.44	37.62	37.93	7.71	25.01	74.63	49.62	0.85	1.29	0.44
LDL	290	120.92	23.5	120.13	120.26	26.69	80.08	191.57	111.49	0.24	-0.67	1.38
CKMB	290	45.94	16.22	44.31	45.34	19.43	20	79.12	59.12	0.24	-1.04	0.95
K	290	4.09	1.28	3.92	3.93	0.9	2.06	9.71	7.65	1.54	3.43	0.08
Na	290	139.93	6.16	140.21	140.05	4.81	117.09	165.19	48.1	-0.15	1.89	0.36
Cl	290	102.72	5.02	103.28	102.96	4.51	81.61	122.79	41.18	-0.44	1.62	0.29
Co2	290	26.26	2.47	25.91	26.14	2.09	19.62	36.78	17.16	0.6	1.15	0.15
Ast	290	52.69	19.59	51.22	51.76	22.44	20.04	96.75	76.71	0.31	-0.85	1.15

Table 3.1 Descriptive statistics of all the parameters used in this study

3.1: Cholesterol status among different populations

mean and sd (standard deviation) of total cholesterol value observed in all the samples is 163.23 and 59.91 and ranging from 99.78 to 279.45 mg/dl. This variable (cholesterol) is normally distributed in different populations with a variable mean (figure 3.1). Cholesterol level does not significantly change between male and female (t = -0.61313, df = 214.83, p-value = 0.5404) but in control and patient (t = -11.341, df = 285.99, p-value < 2.2e-16, welch two sample t-test (all t and df indicates this test); mean in group control=138.7152 and mean in group patient=179.6773). Both male and female in patient group has higher cholesterol level

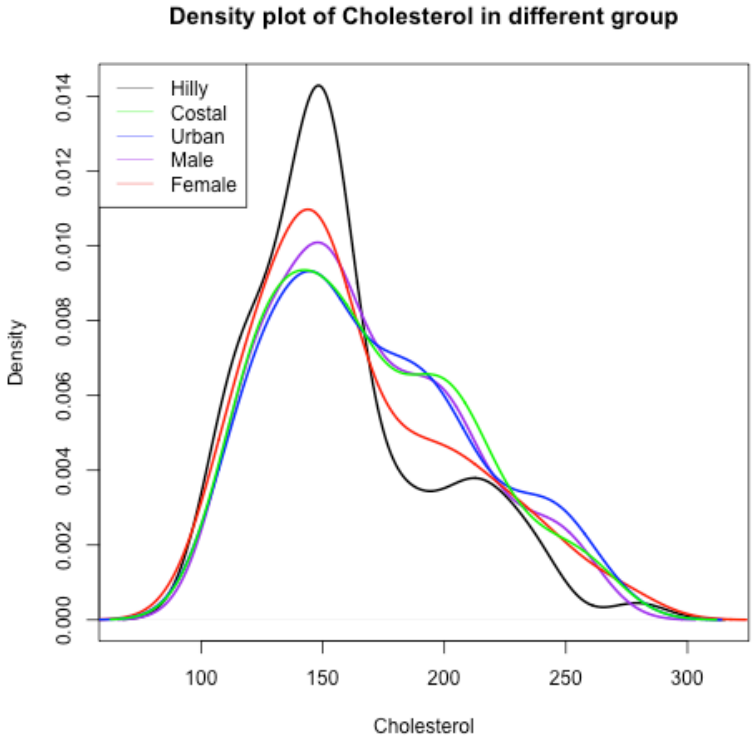


Figure 3.1.1 Density plot of serum total cholesterol in distinct groups

(figure 3.1.2). Hilly people have lower cholesterol compared to urban and coastal population. Patient groups in these regions have higher serum cholesterol level than controls and no notable difference between male and female across regions (figure 3.1.2).

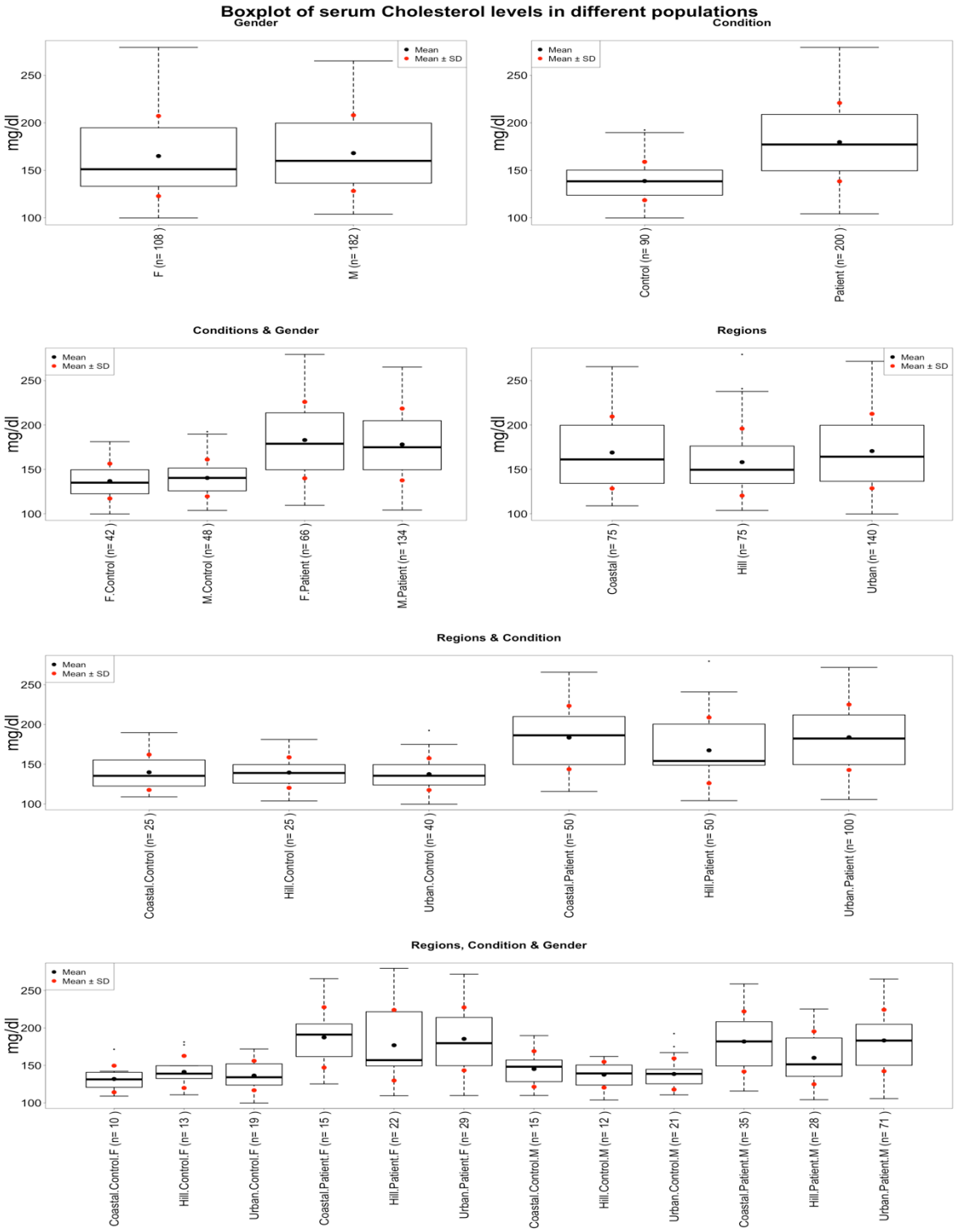


Figure 3.1.2 Boxplot of serum cholesterol level in experimental groups

3.2: Serum TG status among different populations

serum TG ranged from 50.55 to 278.34 mg/dl with a mean and sd of 141.13 and 53.11 respectively (table 3.1). From the distribution hilly population have a significantly lower TG compared to other two populations (figure 3.2.1). There is now significant different between male and female (t = -0.21044, df = 212.68, p-value = 0.8335, mean in group f=140.2573 mean in group m=141.6412). A significant difference was observed between control and patient (t = -3.9268, df = 285.2, p-value = 0.000108, mean in group control=126.8852 mean in group patient=147.5341). Both male and female patients have higher TG than their control counterpart (figure 3.2.2). Interestingly, urban population has higher serum TG level compared to coastal and hilly populations (even urban control has higher values than hilly patients). No visible difference was found between urban, patient female group and urban patient male group (figure 3.2.2).

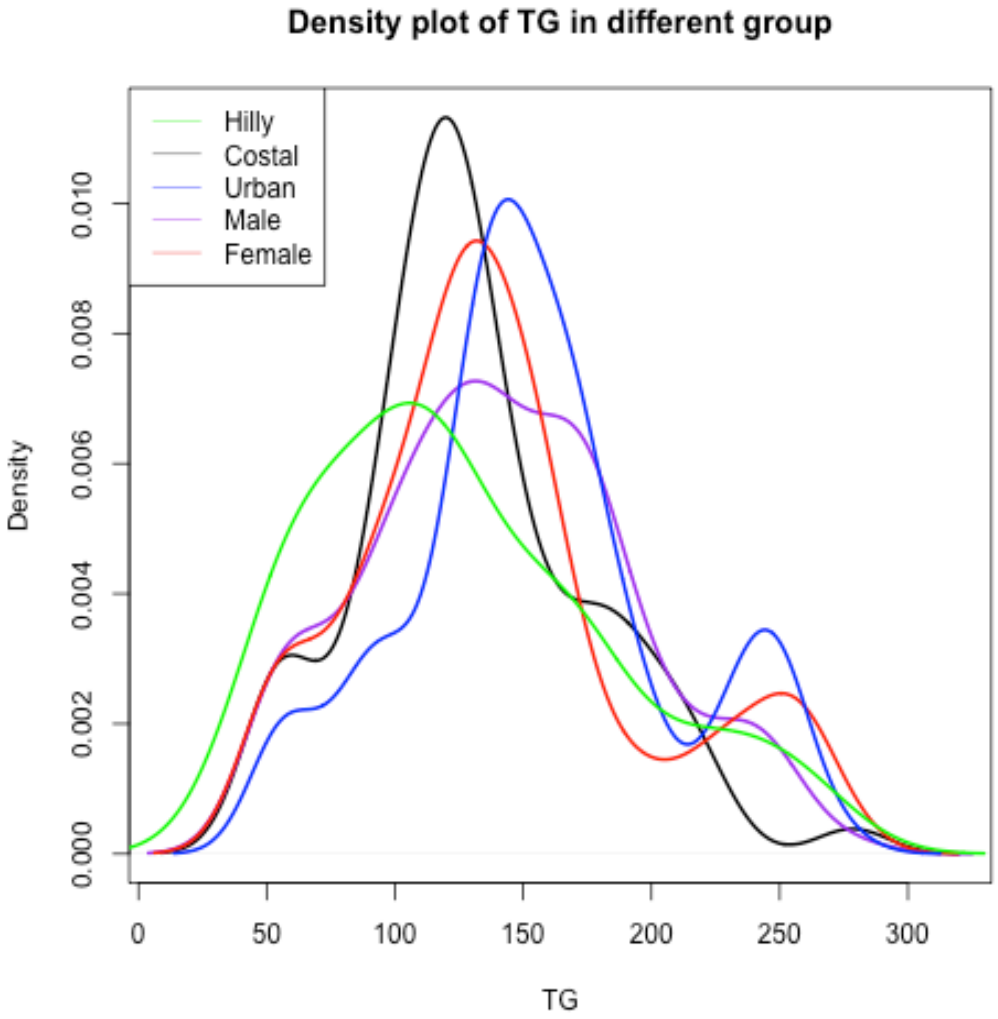


Figure 3.2.1 Density plot of serum TG level in different populations

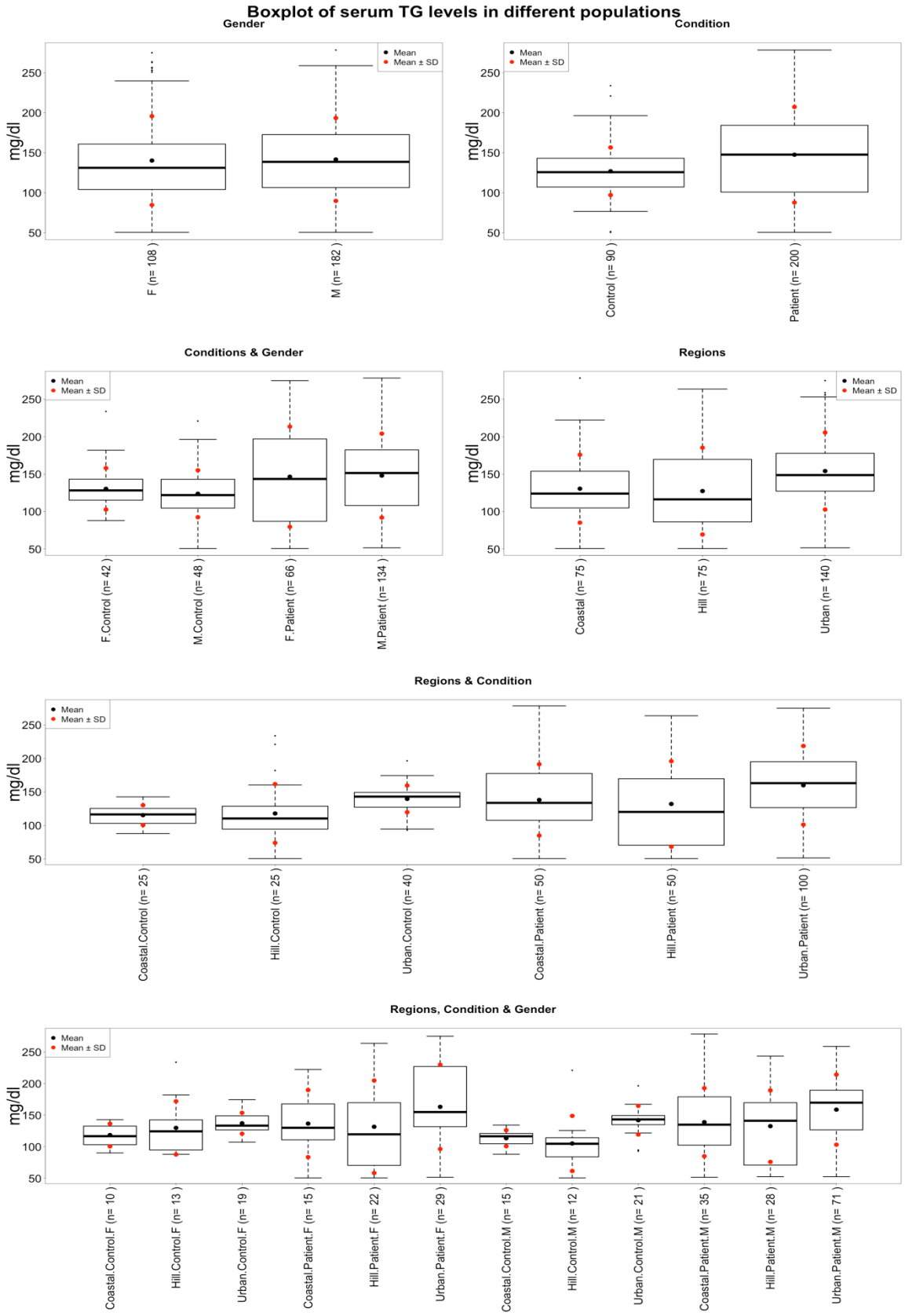


Figure 3.2.2 Boxplot with mean and sd of TG in experimental groups

3.3: Serum HDL level among different populations

mean and sd of serum HDL is 38.46 and 7.44 respectively with range from 25.01 to 74.63 mg/dl. There is no significant change between male and female serum HDL level (t = 0.22406, df = 212.02, p-value = 0.8229, mean in group f=38.58861 mean in group m=38.38198). But there is significant difference between controls and patients. Patients have lower HDL level than controls (t = 2.4332, df = 121.4, p-value = 0.01642, mean in group control=40.3110 mean in group patient=37.6255). Both control male and female have higher HDL than patient male and female, respectively. Urban people have lower HDL than other two groups and urban male patients have the lower mean values. Overall, coastal population has higher HDL and coastal male controls have the larger mean than any other groups.

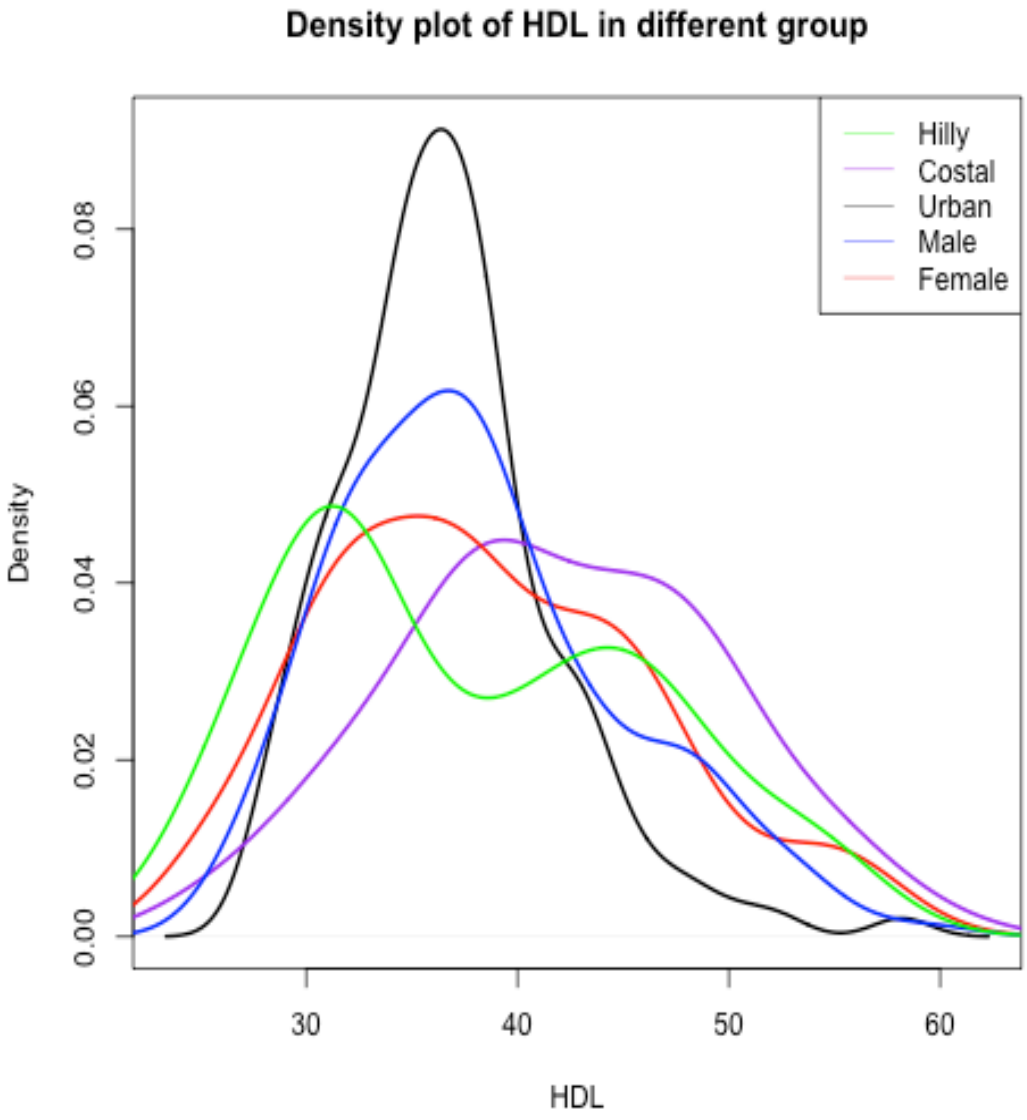


Figure 3.3.1 Density plot of serum HDL level in experimental groups

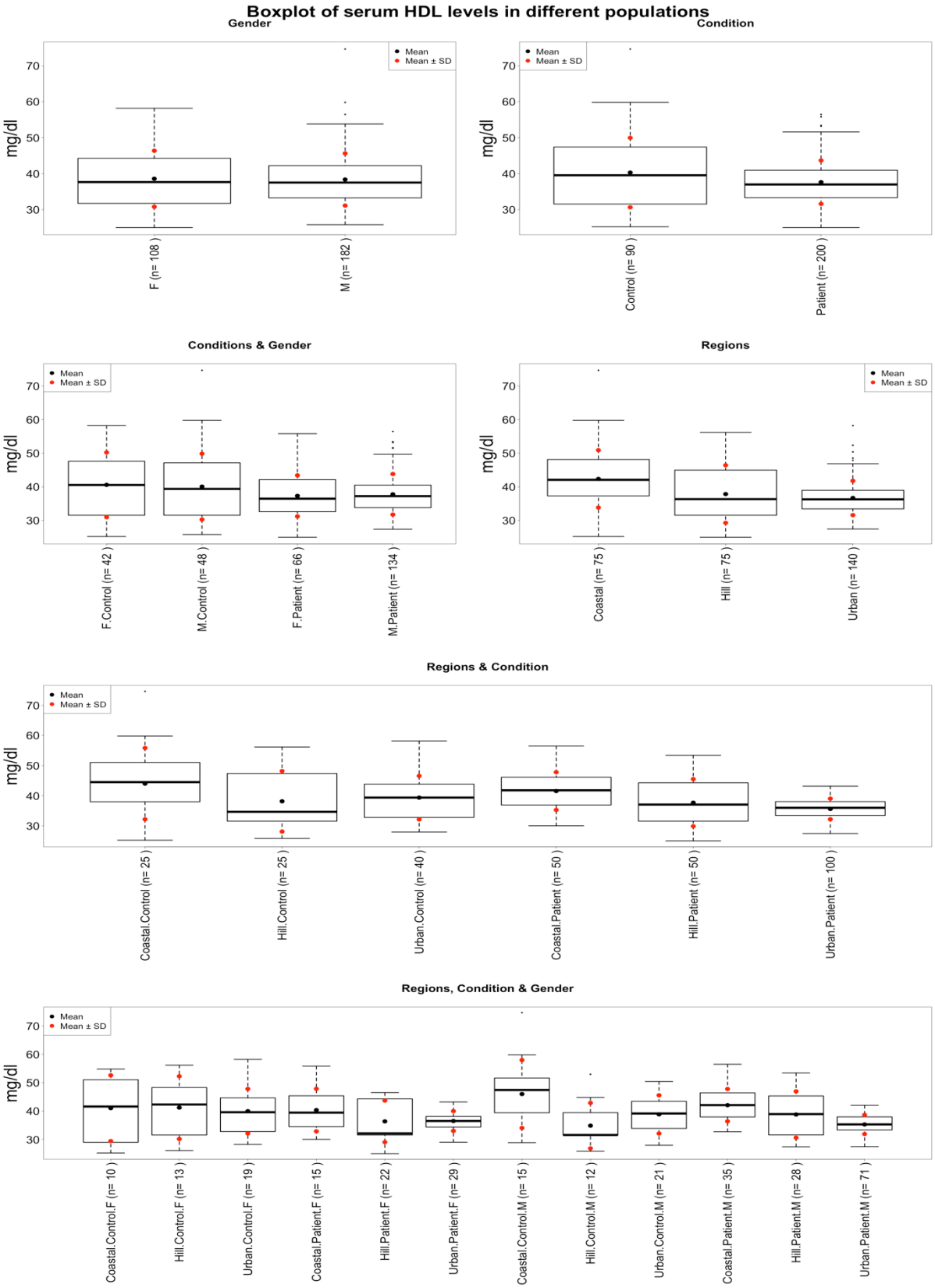


Figure 3.3.2 Boxplot of serum HDL level in experimental populations in mg/dl.

3.4: Serum LDL level among different populations

serum LDL was ranged from 80.08 to 191.57 mg/dl with a mean of 120.92 and sd 23.5 (table 3.1). This variable also normally distributed across different populations (figure 3.4.1). LDL does not significantly vary between male and female (t = -0.81593, df = 227.04, p-value = 0.4154, mean in group f=119.4616 mean in group m=121.7843), but in patients it is significantly higher than controls (t = -4.6296, df = 228.97, p-value = 6.14e-06, mean in group control=112.6610 mean in group patient=124.6356). Both male and females in patients have higher serum LDL level than their control counterparts. There is no pattern observed for different locations but both male and female of hilly control group have the smallest mean values than any other groups (figure 3.4.2).

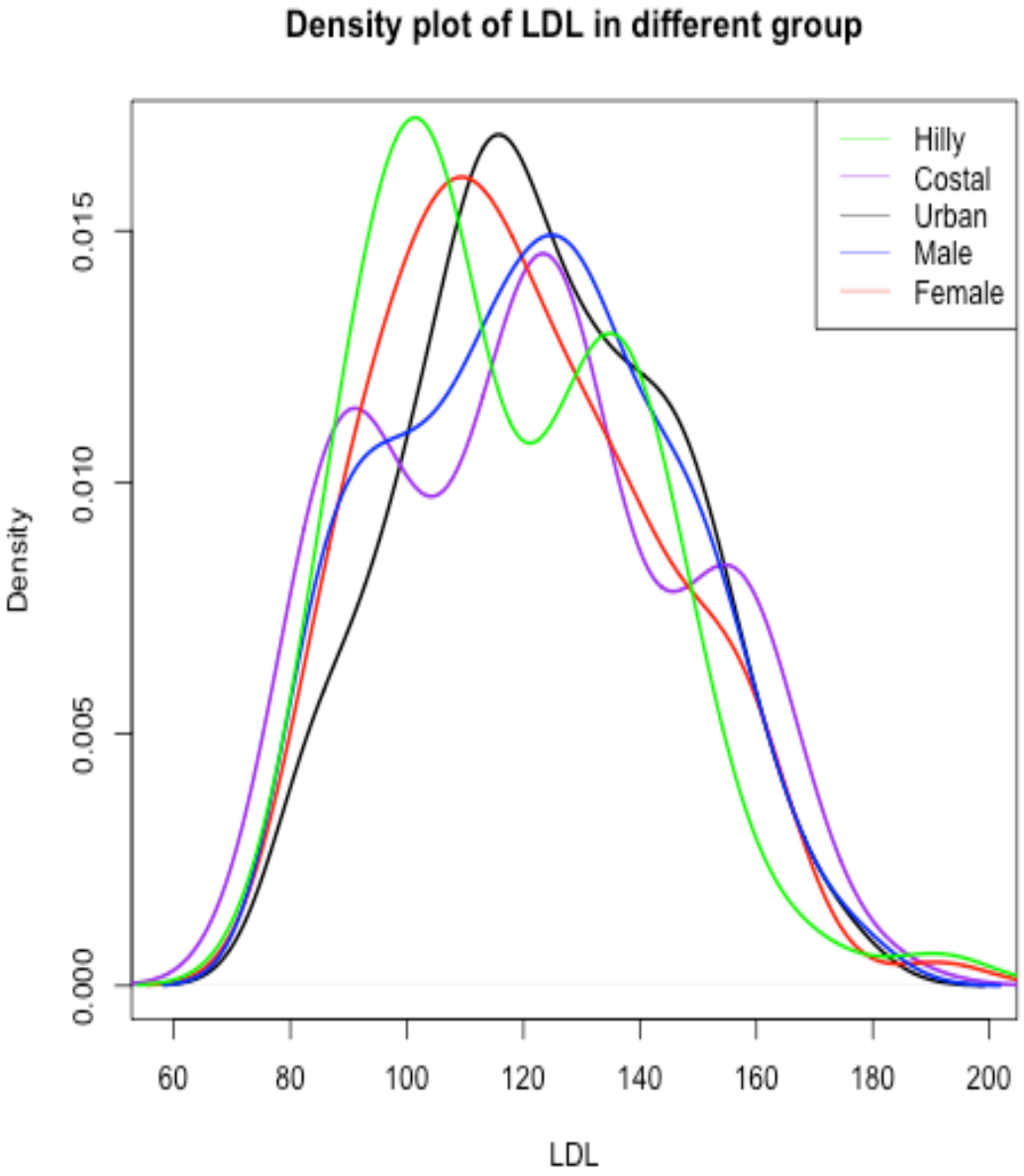


Figure 3.4.1 Density plot of serum LDL level in experimental samples

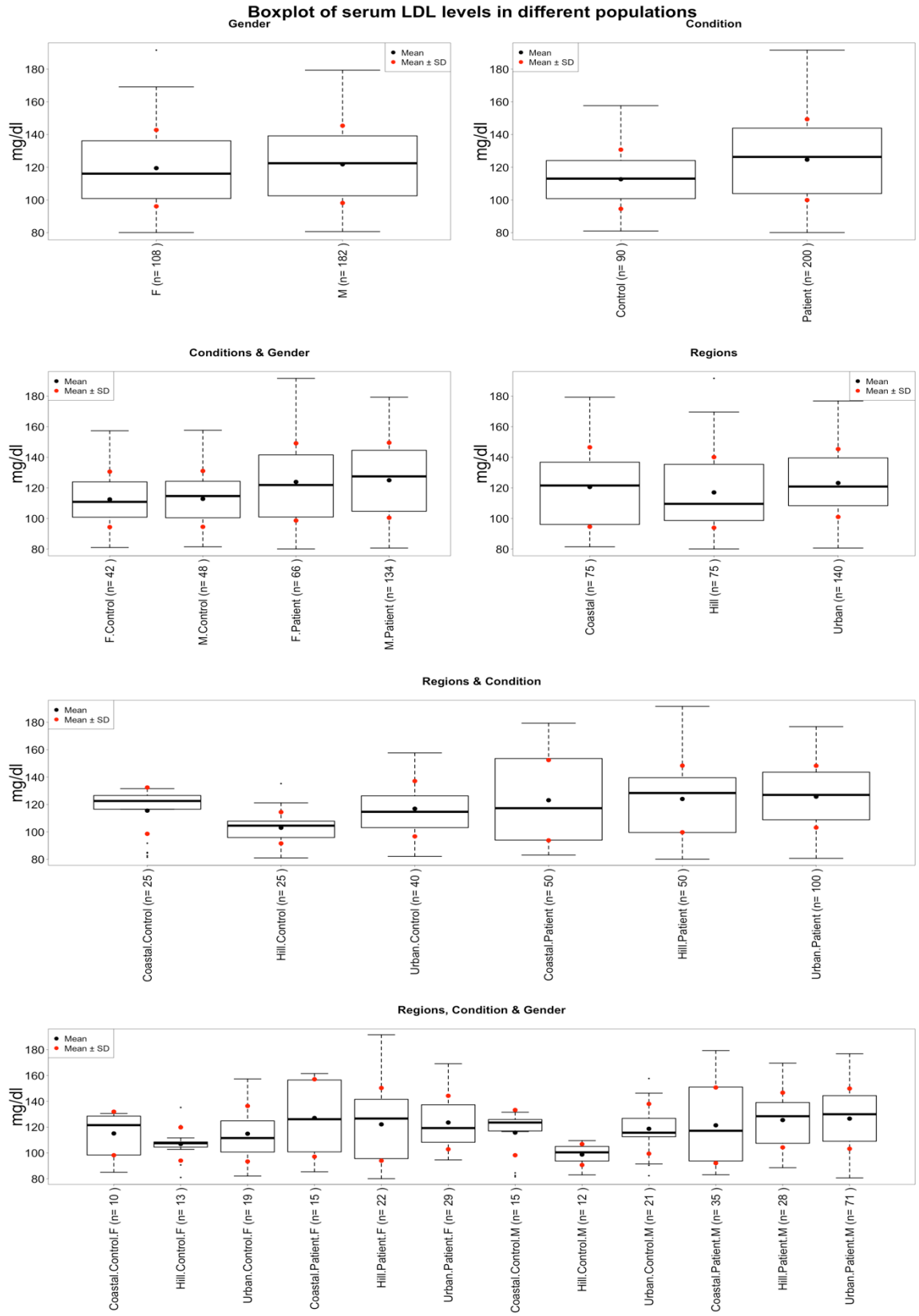


Figure 3.4.2 Boxplot of serum LDL level in different experimental groups

3.5: Serum CKMB level among different populations

Serum CKMB was ranged from 20 to 79.12 iu/l with a mean and sd respectively 45.94 and 16.22 (table 3.1). It is normally distributed across population though hilly data showed a strange bimodal distribution (figure 3.5.1). Males have little higher CKMB compared females but not significantly (t = -1.3215, df = 243.01, p-value = 0.1876, mean in group f=44.34407 mean in group m=46.87934). In patients it is significantly higher than controls (t = -11.348, df = 262.5, p-value < 2.2e-16, mean in group control=34.12233 mean in group patient=51.25095). Both male and female inpatients have higher mean values than control male and female respectively (figure 3.5.2). Urban population has higher CKMB and urban patient male and female have higher values than other experimental groups (figure 3.5.2).

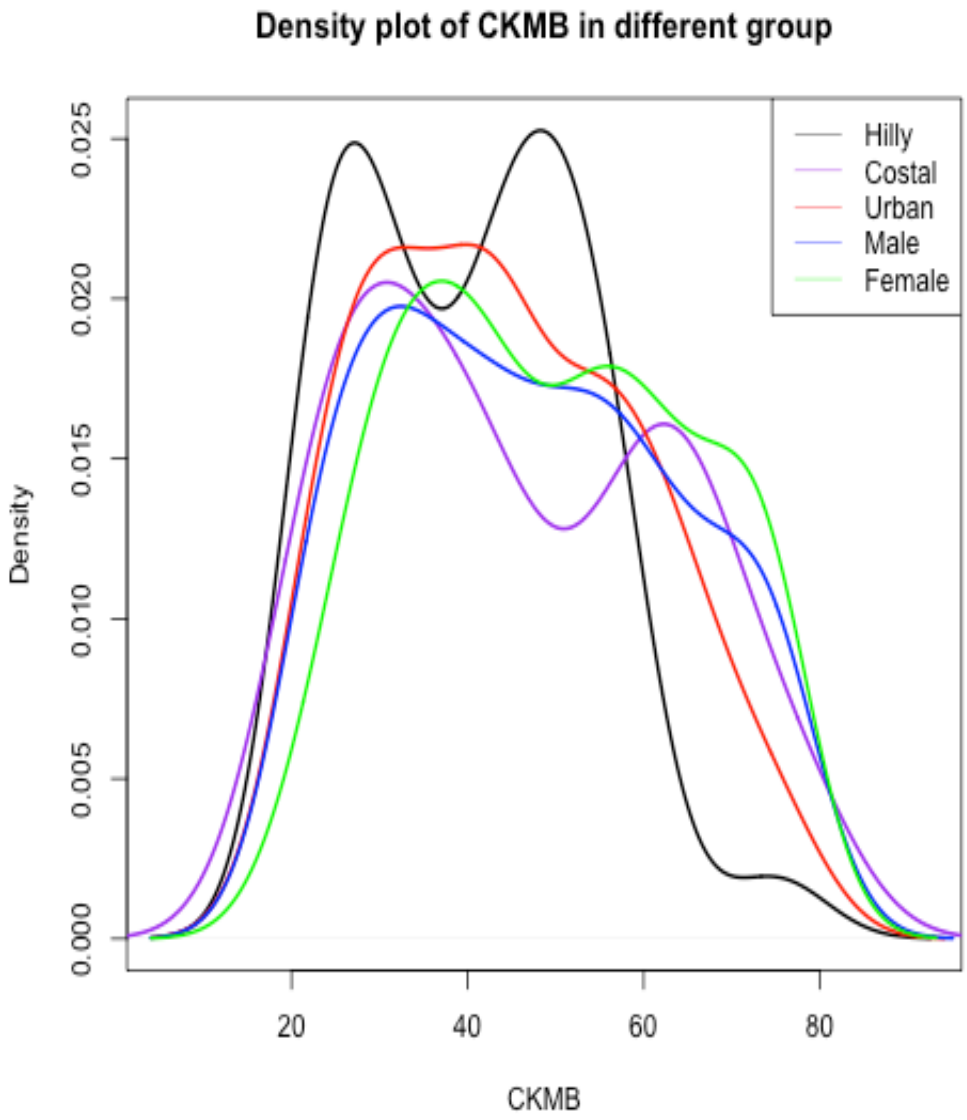


Figure 3.5.1 Density plot of serum CKMB level in different experimental populations

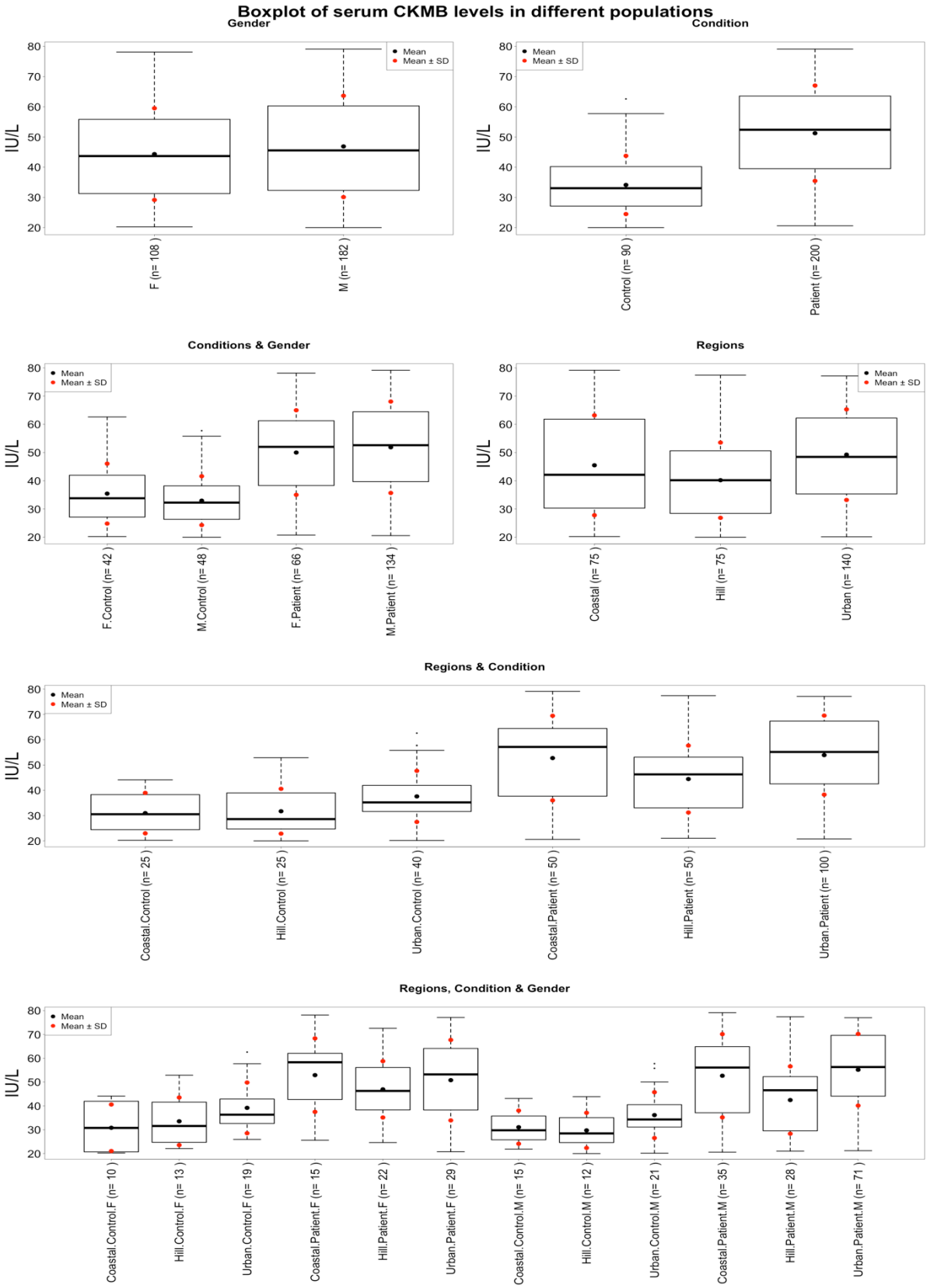


Figure 3.5.2 Boxplot of serum CKMB levels in different experimental groups

3.6: Serum AST level among different populations

mean serum AST level in all sample is 52.69 iu/l with a sd of 19.59 and ranged from 20.04 to 96.75 iu/l (table 3.1). It is also normally distributed in all groups and hilly populations have the most steep density plot (figure 3.6.1). Males have higher serum AST compared to females (t = -1.7938, df = 226.62, p-value = 0.07418, mean in group f=50.02426 mean in group m =54.26549) and patients have significantly higher values than controls (t = -8.6474, df = 194.33, p-value = 1.958e-15, mean in group control=39.97700 mean in group patient=58.40505). Both males and females in patients have higher serum AST that their control counterparts and no significant difference between hilly, urban, and coastal areas (figure 3.6.2). Urban controls and urban controls male and females have the lowest mean among all experimental populations (figure 3.6.2).

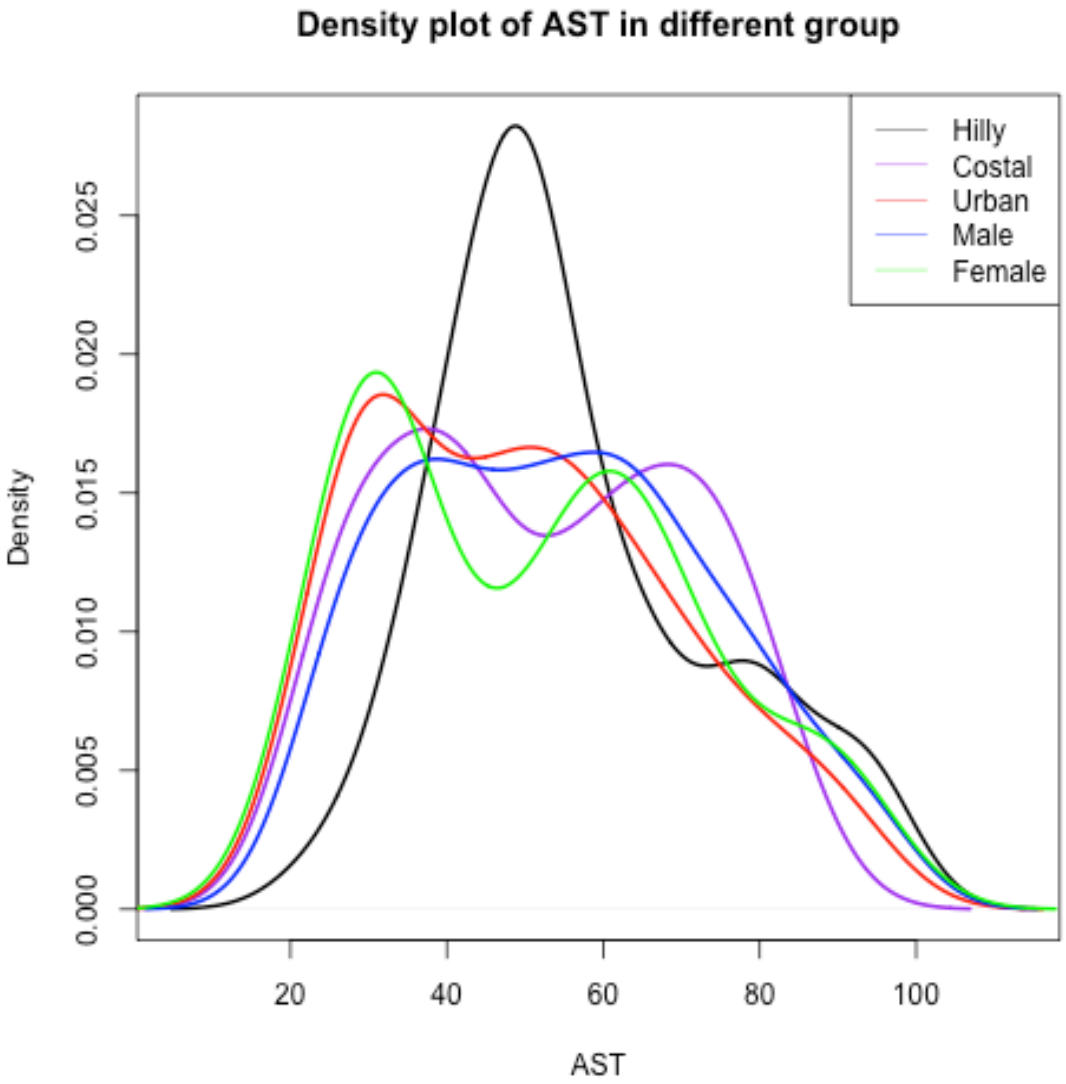


Figure 3.6.1 Density plot of serum AST level in experimental subjects

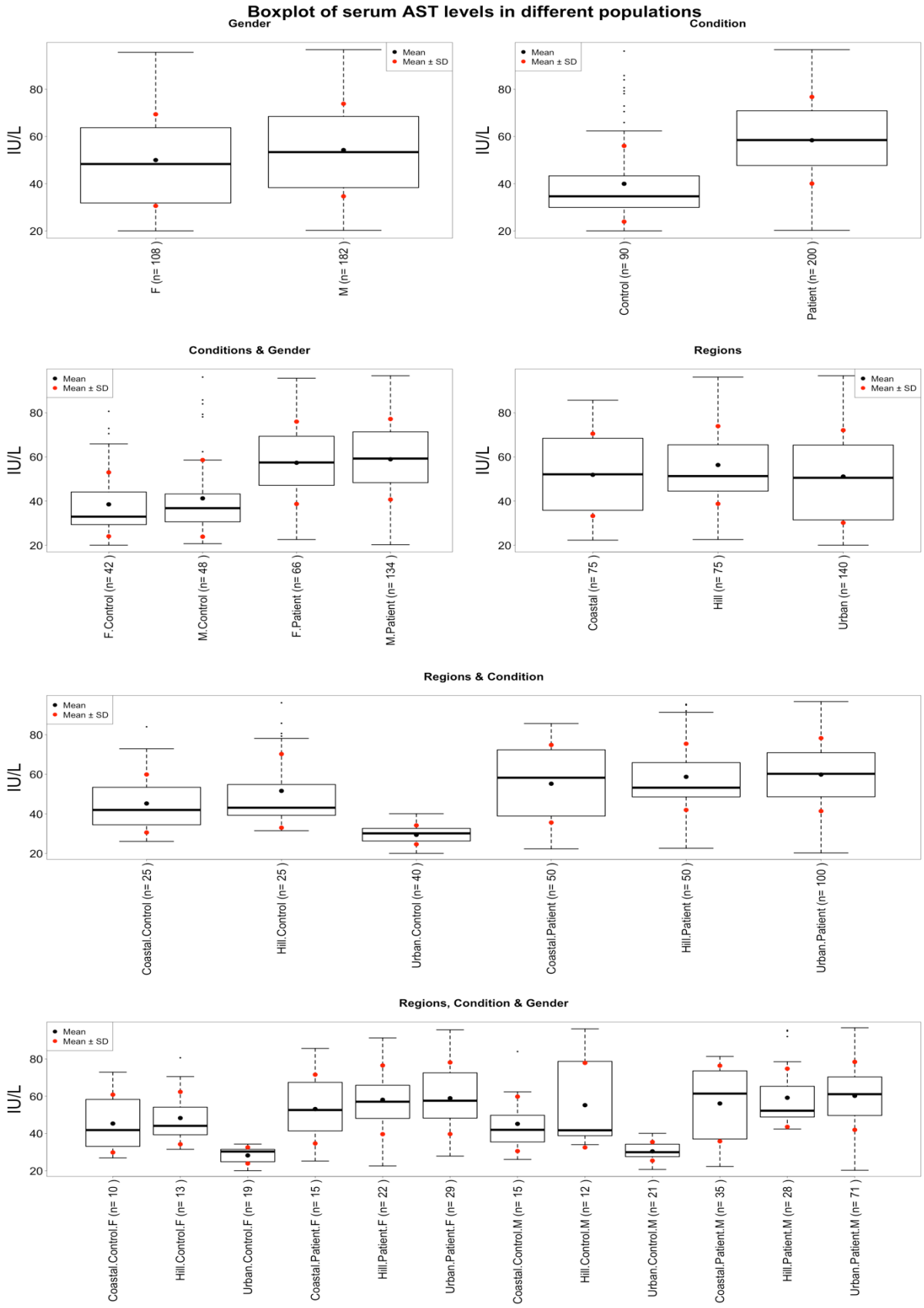


Figure 3.6.2 Boxplot of serum AST level in experimental subjects

3.7: Serum K level among different populations

serum potassium (K) is not as variable as previous seven parameters (figure 3.7.1). Mean value of K in all samples is 4.09 with an sd of 1.28 and ranged from 2.06 to 9.71 meq/l (table 3.1). The hilly population has the most steep density curve which means they are less variable (figure 3.7.1). There is no statistical significant difference between values in male and female (t = 1.1159, df = 211.51, p-value = 0.2657, mean in group f=4.203981 mean in group m=4.027198). In patients K is significantly lower compared to controls (t = 3.9737, df = 114.11, p-value = 0.0001244, mean in group control=4.619667 mean in group patient=3.856050). There is no visible differences in hilly, coastal and urban populations, but urban controls males has the

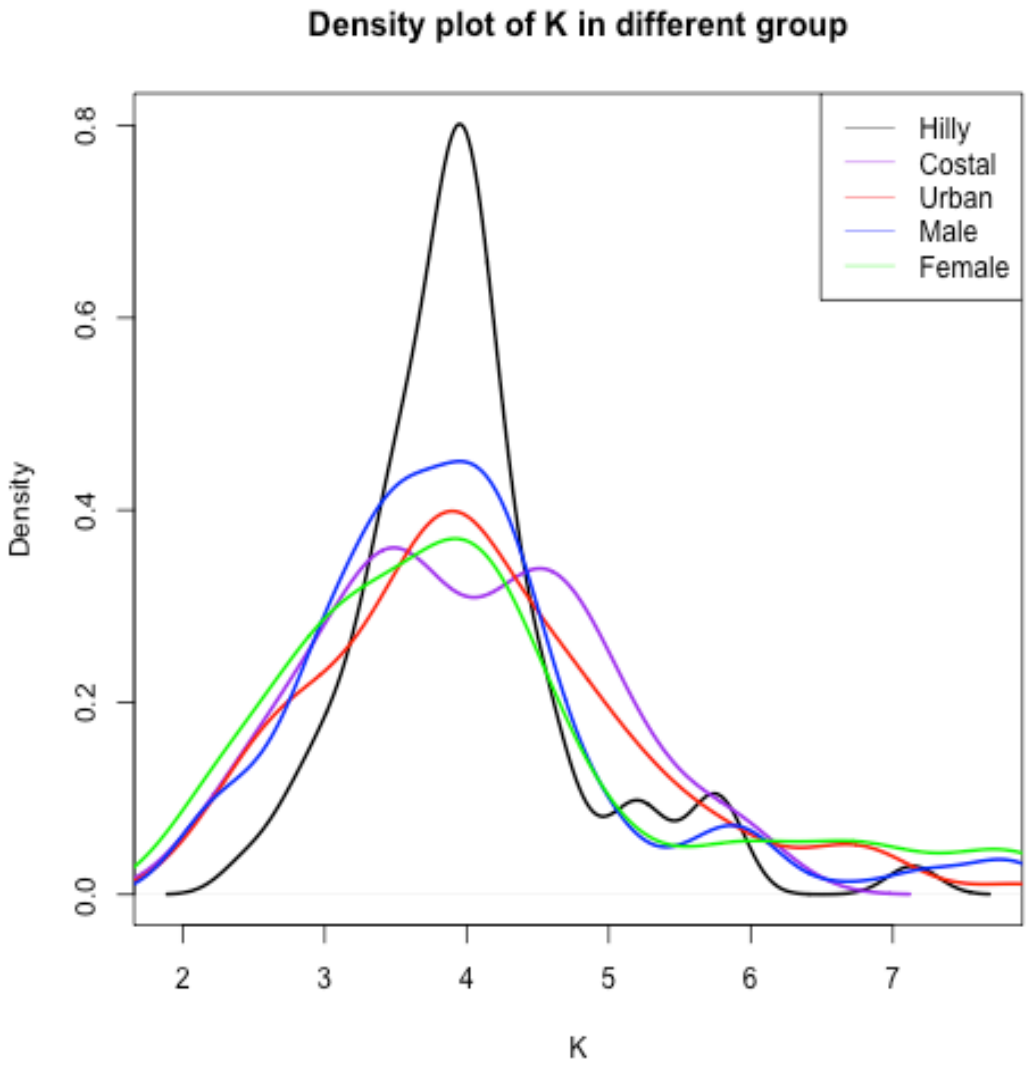


Figure 3.7.1 Density plot of K in different populations

largest mean among all groups (figure 3.7.2).

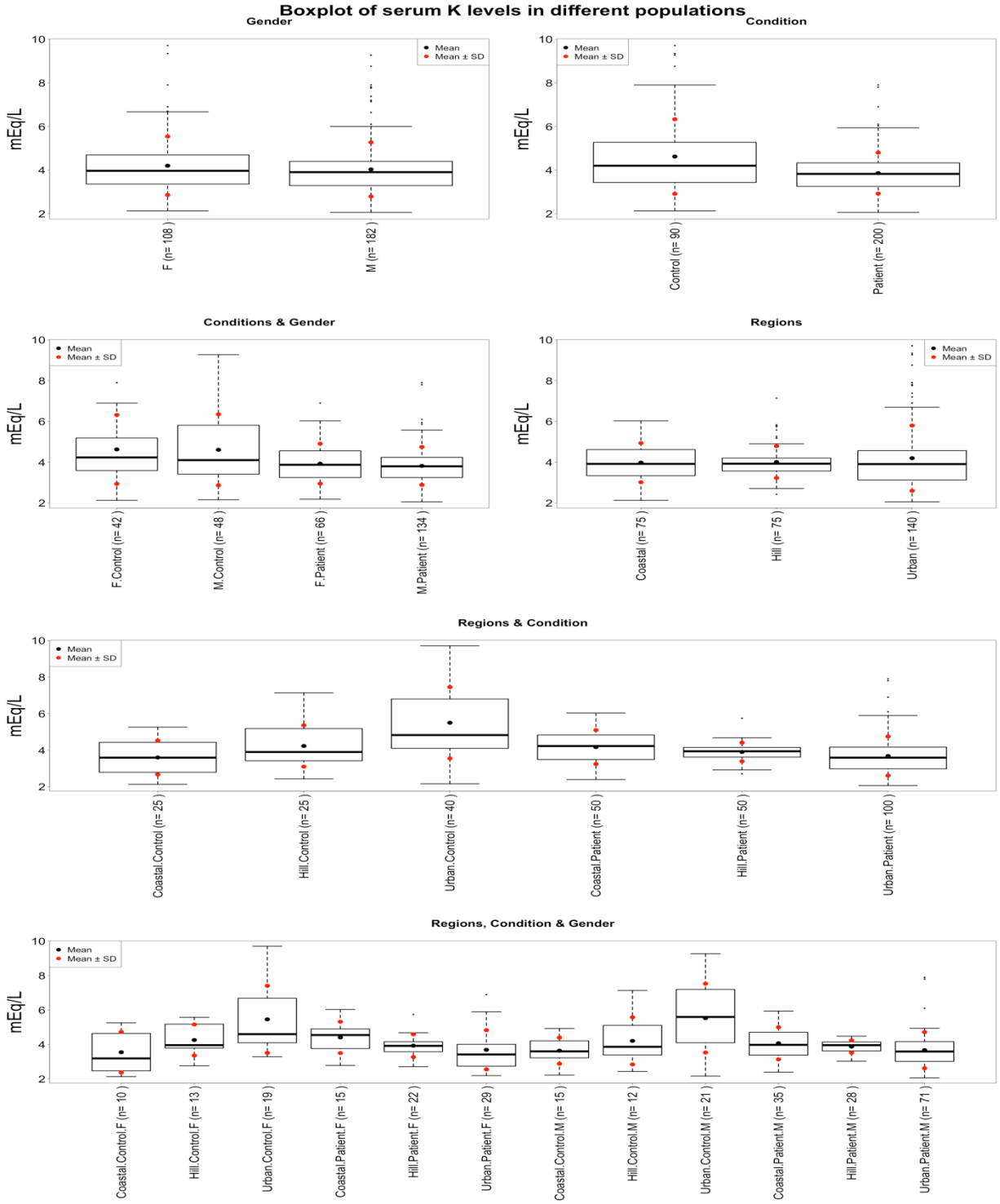


Figure 3.7.2 Serum K level in different subjects

3.8: Serum Na level among different populations

mean serum level of Na in all samples is 139.93 meq/l with a sd of 6.16 and ranges from 117.09 to 165.19 meq/l (table 3.1). Overall it has a normal distribution in different populations with steeper curve than lipids (figure 3.8.1). Na is neither significantly different between male and female (t = 1.8204, df = 213.02, p-value = 0.0701, mean in group f=140.7948 mean in group m=139.4147) nor between controls and patients (t = 1.6866, df = 138.86, p-value = 0.09392, mean in group control=140.9259 mean in group patient=139.4799). There is no significant different between populations also and coastal control male and females have higher variability than other groups (figure 3.8.2).

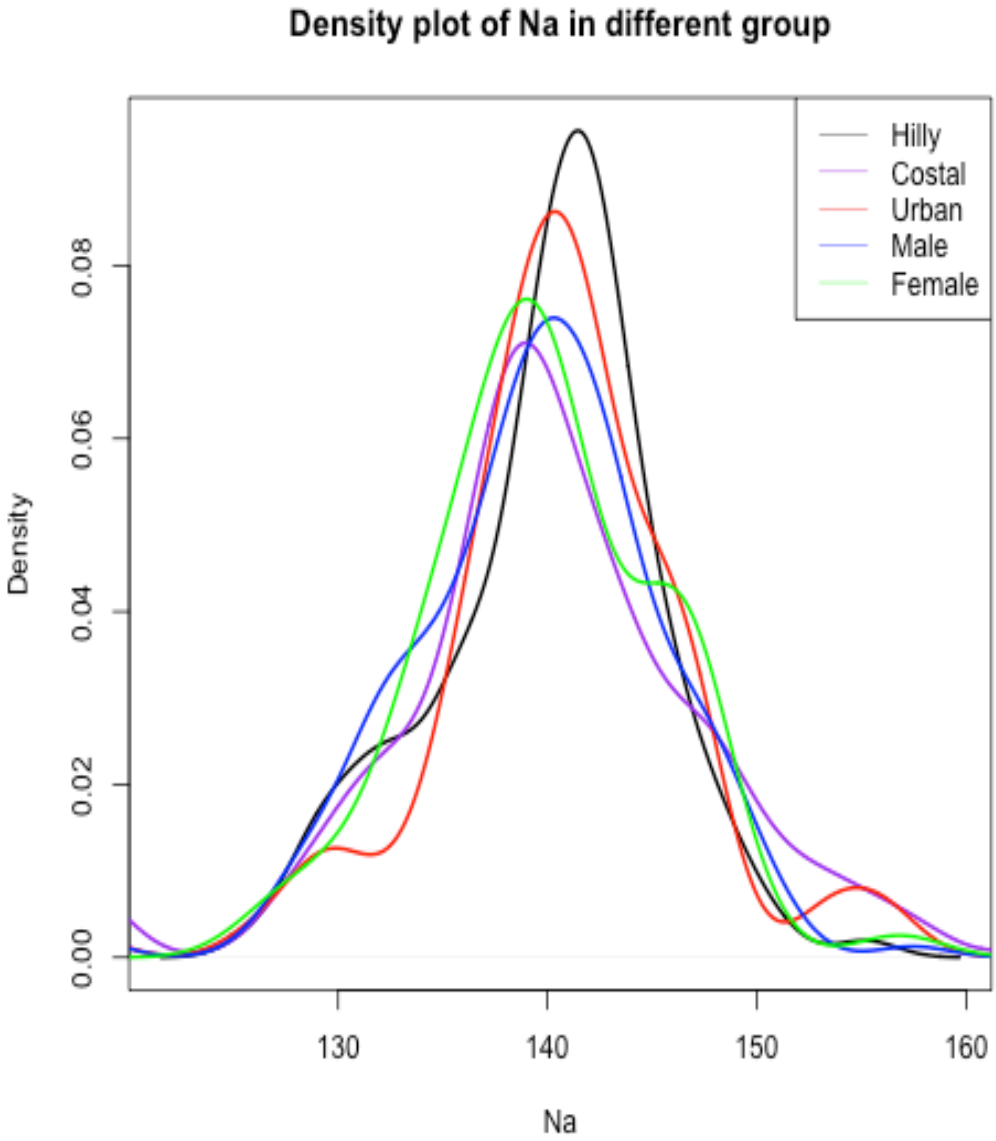


Figure 3.8.1 Density plot of serum Na in experimental populations

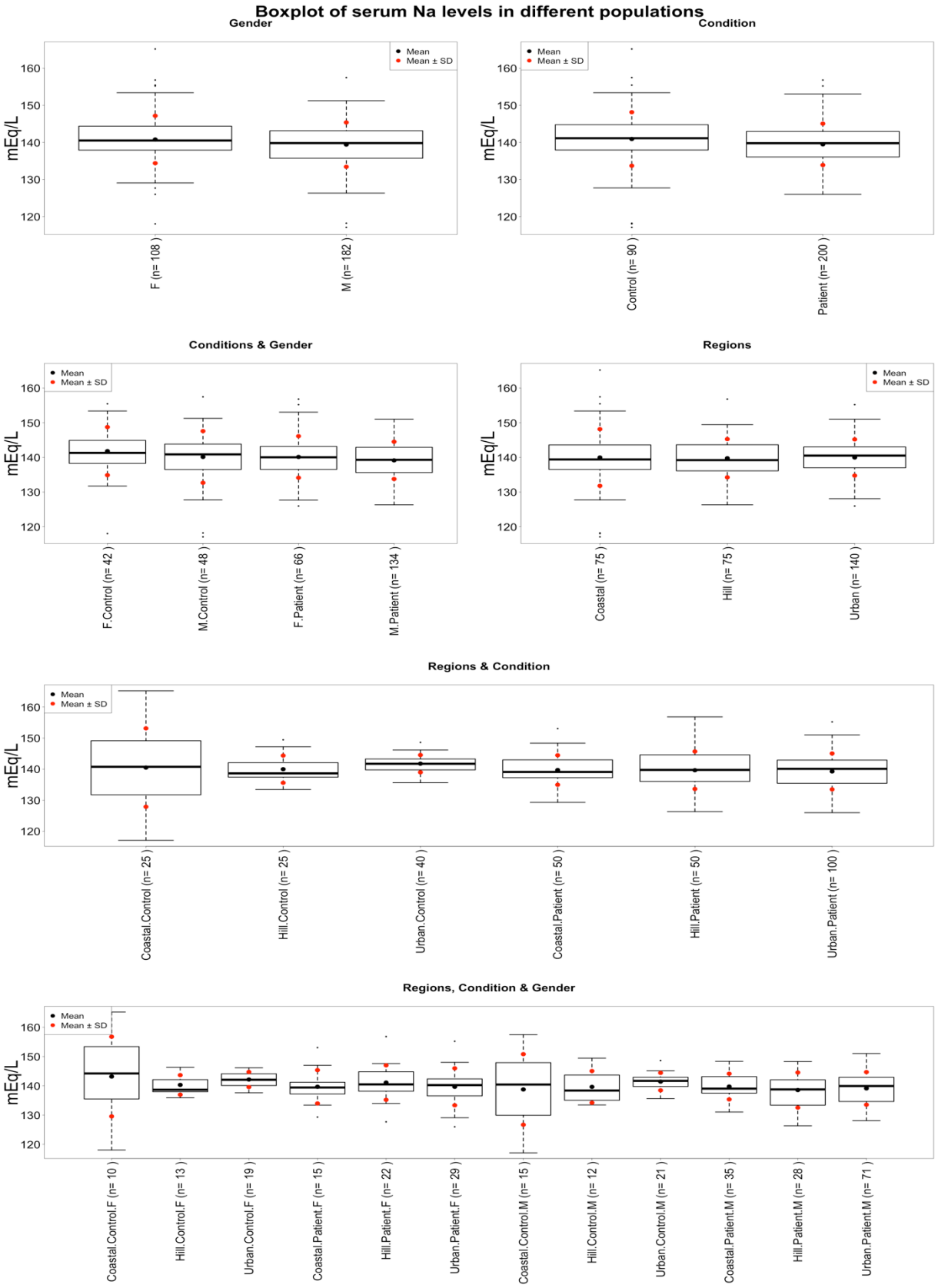


Figure 3.8.2 Boxplot of serum Na level in all populations

3.9: Serum Cl level among different populations

mean serum Cl in all samples is 102.72 with a sd of 5.02 ranging from 81.61 to 122.79 meq/l (table 3.1). It is also normally distributed with different area in different populations (figure 3.9.1). Cl is not also significantly different in any comparing groups, neither between male and female (t = 1.0981, df = 234.62, p-value = 0.2733, mean in group f=103.1385 mean in group m=102.4781) nor between control and patients (t = 0.83141, df = 134.44, p-value = 0.4072, mean in group control=103.1331 mean in group patient=102.54). Among all the experimental groups costal control males and females have highly variable Cl level than any other populations in the experimental groups (figure 3.9.2).

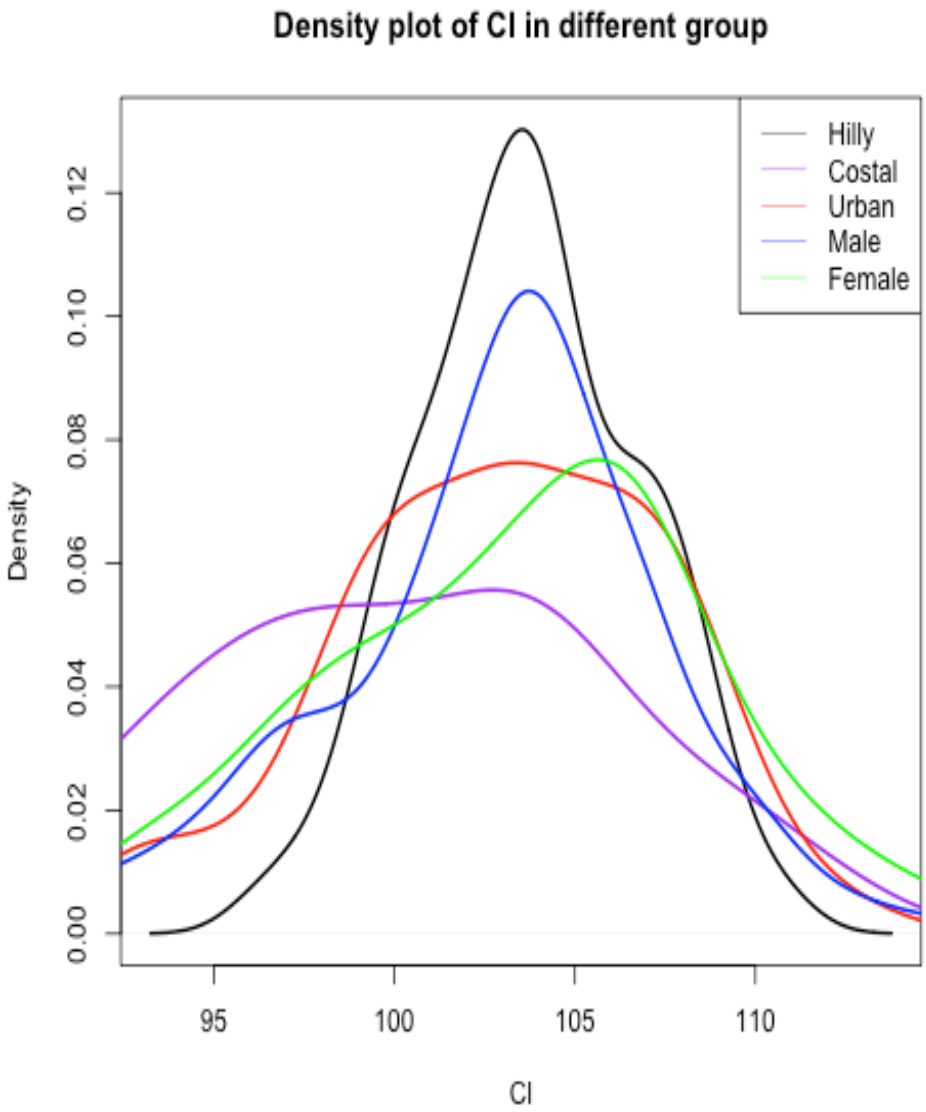


Figure 3.9.1 Density plot of serum Cl values in experimental populations

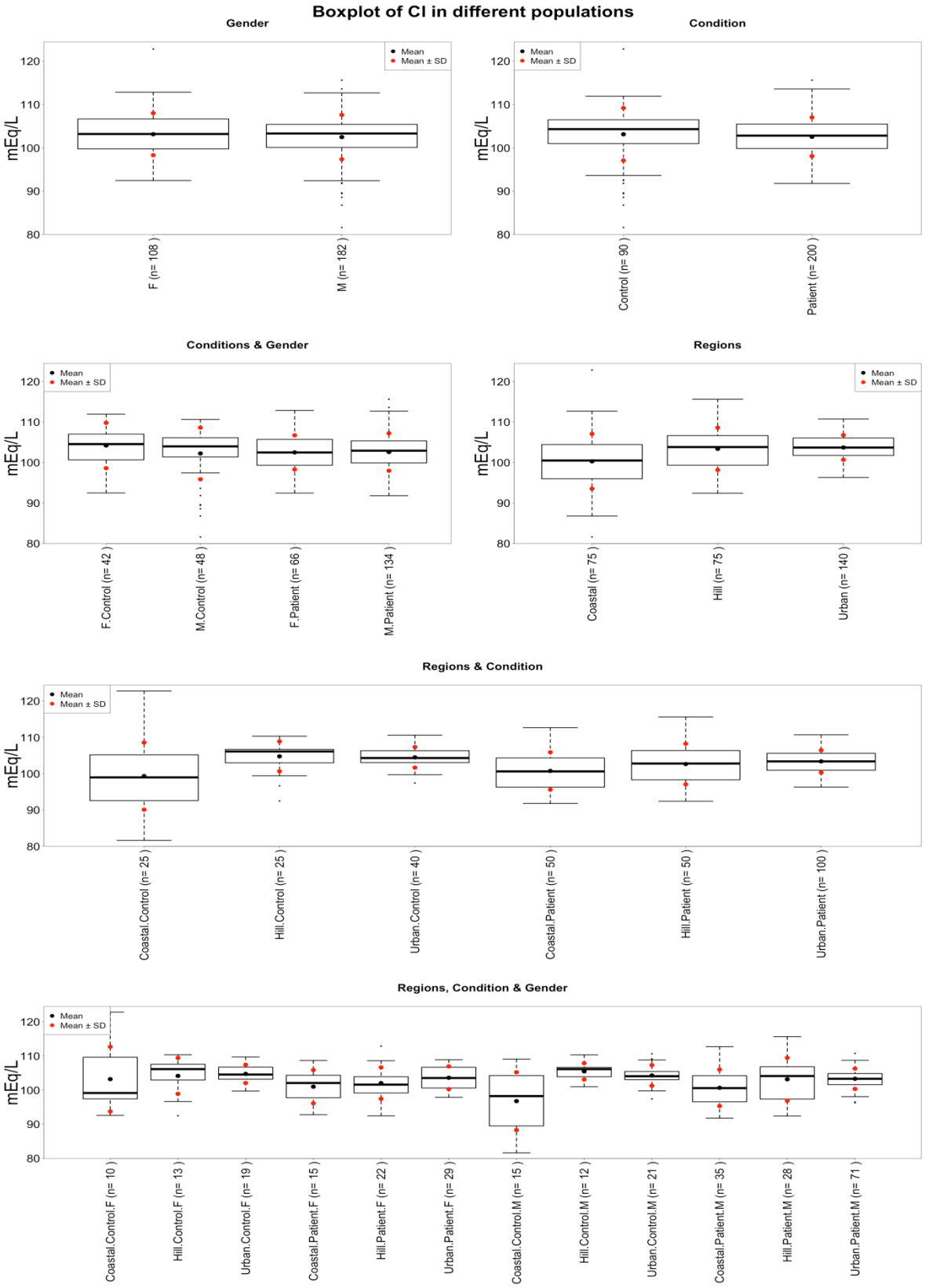


Figure 3.9.2 Boxplot of serum Cl level in all experimental groups

3.10: Serum Co2 level among different populations

mean serum Co2 level in all samples is 26.26 with a sd of 2.47 and ranges from 19.62 to 36.78 mmol/l (table 3.1). Among all the parameters it has the most uniform normal distribution in all groups (figure 3.10.1). There is no significant difference between male and females (t = -0.95963, df = 225.16, p-value = 0.3383, mean in group f=26.07593 mean in group m=26.36407). But, between controls and patients serum Co2 level is significantly different (t = -4.974, df = 287.17, p-value = 1.131e-06, mean in group control=25.4544 mean in group patient=26.6178). From the boxplot, patients have higher serum Co2 than controls, hilly and coastal populations also have higher values than urbans (figure 3.10.2).

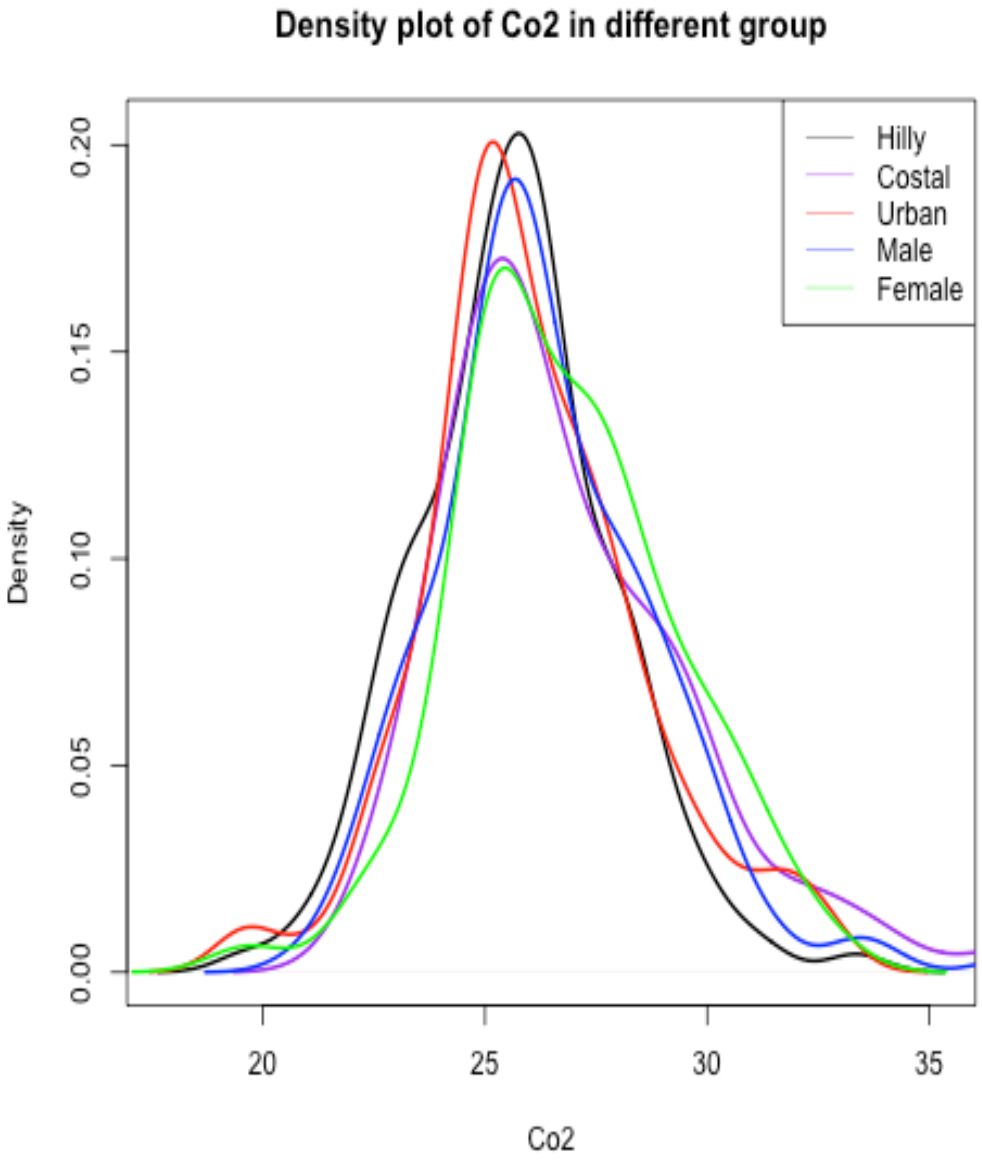


Figure 3.10.1 Density plot of serum Co2 in experimental groups

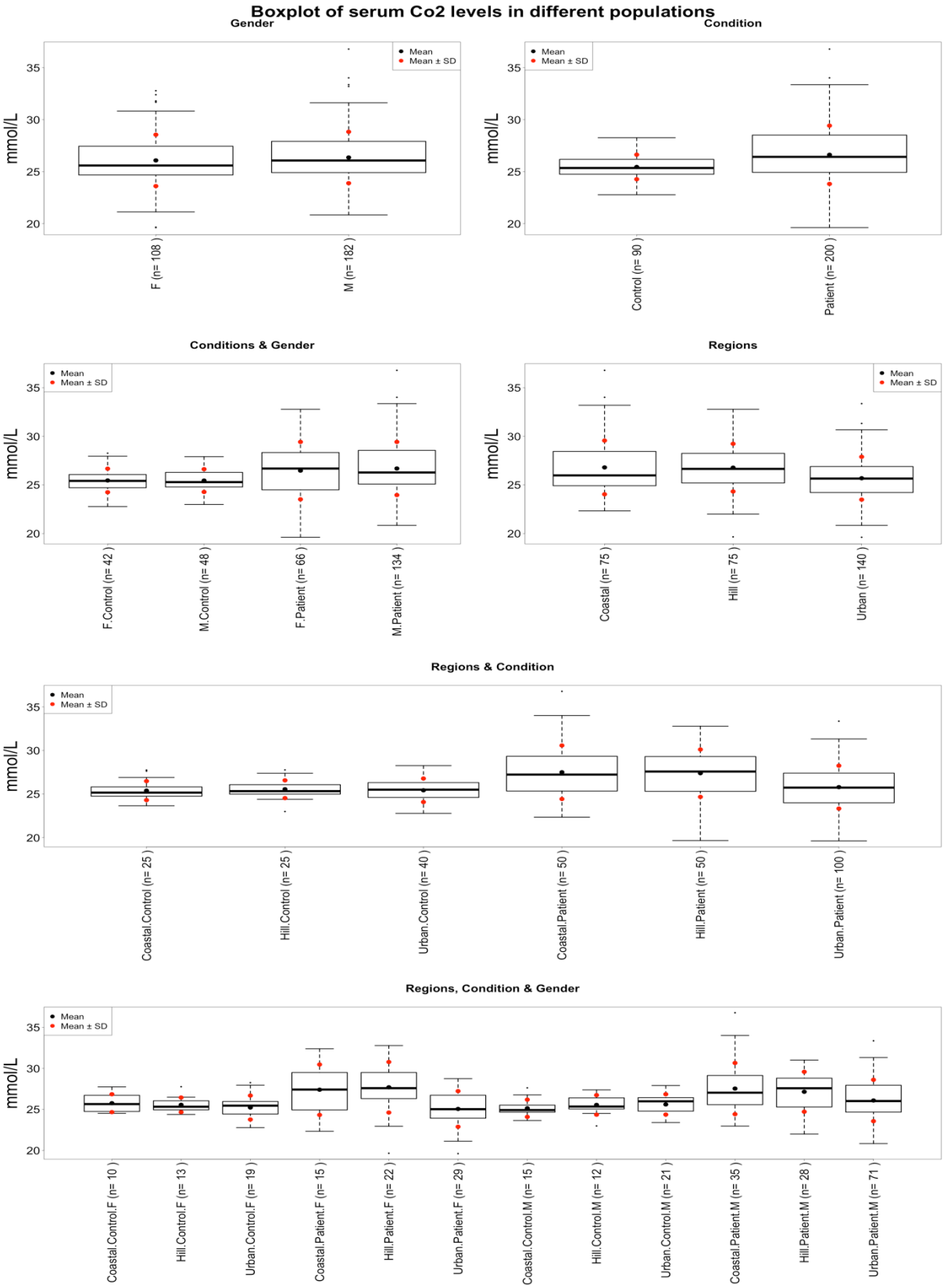


Figure 3.10.2 Boxplot of serum Co2 level in all observed populations

3.11: Correlation tests

there are total 11 numerical variables including age in this data set. Serum cholesterol and LDL are positively, and Cl is negatively correlated with age (table 3.2 and figure 3.11). Pearson's product-moment correlation for cholesterol~age, t = 7.0512, df = 288, p-value = 1.318 e-^{11}, cor=0.3836938; for LDL~age, t = 2.5251, df = 288, p-value = 0.0121, cor=0.1471755 and for Cl ~ age, t = -2.1689, df = 288, p-value = 0.03091, cor = -0.1267698.

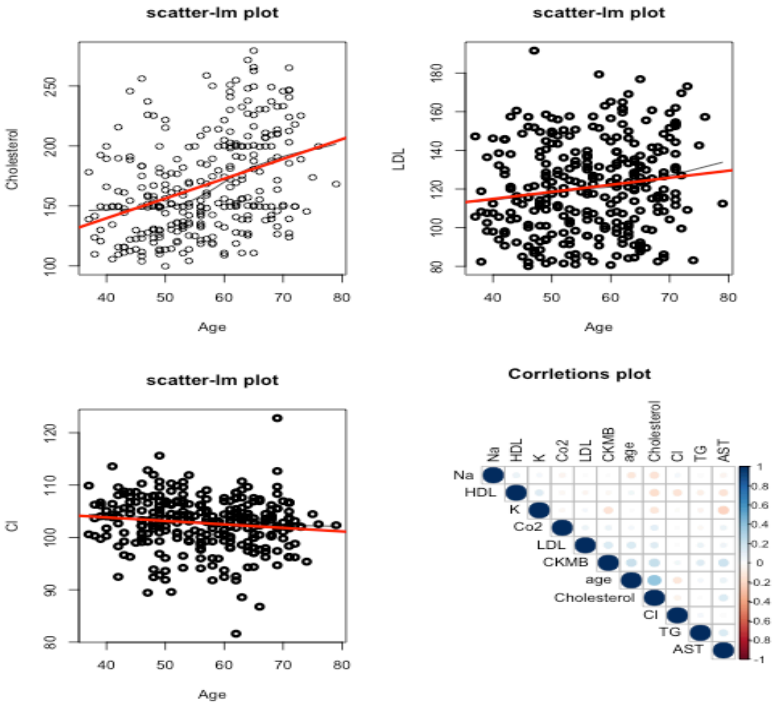


Figure 3.11 Pearson correlation and linear regression plot. The correlation plot describe pearson correlations between all variables. The scatter plots shows the data points of respective variable with a black smooth line and a red linear regression (Details models with stats are in Supplementary: S3.2)

	Age	Cholesterol	TG	HDL	LDL	CKMB	K	Na	Cl	Co2	Ast
Age	1	0.38	0.04	0.05	0.15	0.21	-0.01	-0.12	-0.13	0.04	0.08
Cholesterol	0.38	1	0.01	-0.17	0.12	0.22	-0.13	-0.15	-0.07	0.1	0.14
TG	0.04	0.01	1	-0.08	0.07	0.12	-0.05	0	0.04	-0.04	0.14
HDL	0.05	-0.17	-0.08	1	-0.05	-0.04	0.13	0.08	-0.14	-0.04	-0.14
LDL	0.15	0.12	0.07	-0.05	1	0.15	0.03	0.01	0.01	0.07	0.06
CKMB	0.21	0.22	0.12	-0.04	0.15	1	-0.15	-0.01	0.05	0.07	0.2
K	-0.01	-0.13	-0.05	0.13	0.03	-0.15	1	0.04	-0.02	-0.04	-0.22
Na	-0.12	-0.15	0	0.08	0.01	-0.01	0.04	1	0.04	-0.06	-0.06
Cl	-0.13	-0.07	0.04	-0.14	0.01	0.05	-0.02	0.04	1	-0.01	0
Co2	0.04	0.1	-0.04	-0.04	0.07	0.07	-0.04	-0.06	-0.01	1	0.07
Ast	0.08	0.14	0.14	-0.14	0.06	0.2	-0.22	-0.06	0	0.07	1

Table 3.2 Pearson correlation table of all numerical variables

CHAPTER 4

DISCUSSION AND CONCLUSION

4.1 Discussion:

Cardiovascular disease (CVD) is a major cause of disability and mortality in the developed countries. CVD represents a cluster of disorders, associated with complex interactions between multiple risk factors. The risk factors, or co-morbidities, that lead to enhanced risk of developing CVD has been recognized for many years.[116] the main CVD risk factors include smoking,[117] hypertension[118] and dyslipidemia[119]. Further major influences relate to familial risk (premature CVD in men before 55 years old and women before 65 years old) and diabetes, which results in a gross acceleration of the pathological progresses involved in CVD. Other important predisposing factors include diet, physical activity, obesity and genetic influences. Although more than 200 risk factors for CHD have now been found, the single most powerful predictor of CHD risk is abnormal lipid values. All these risk factors are multiplicative, acting to exaggerate the damage caused by each risk factor alone.

In this study, samples were divided based on geographical areas. Age, sex, lipid profile, cardiac enzymes (ck-mb and SGOT) and serum electrolyte level were explored to investigate the correlation of these risk factors with CVD. For this purpose, two hundred cardiac patients and ninety control subjects from urban, hilly and coastal area were examined. When age (year) was compared among cardiac patients vs. Control, it has been found that age was significantly higher in patients (figure 2.2). The bimodal distribution of age in separate groups of subjects confirms that there are two different local means for controls and patients (figure 2.1).

One of the more prominent features of the framingham risk scoring is the progressive increase in absolute risk with advancing age. This increase undoubtedly reflects the cumulative nature of atherogenesis. With advancing age, people typically accumulate increasing amounts of coronary atherosclerosis. This increased plaque burden itself becomes a risk factor for future coronary events [126-128]. Framingham scoring for age reflects this impact of plaque burden on risk. Still, average scores mask the extent of variability in plaque burden in the general population.

It has been observed that men are more prone to CVD than women. This is because female hormone estrogen acts as a barrier for CVD till menopause. In this study, there are 108 females

were included versus 182 males (table 2.1). In Bangladeshi socioeconomic structures, women are less prone to go to the doctor than males, males are exposed in more stress, introvertive nature about disease, sample collection methods etc. May also contribute to this male/female sample number discrepancy in this study.

The number of controls seems much less than the number of patients (90 vs 200). However, all cardiac biochemical markers have a lower variability in general healthy individuals compared to patients (supplementary figure s3.1). In that sense, this number of controls can be used for statistical correlations and other tests. None the less, equal or similar number of controls and patients are always better than varying numbers.

Lipid abnormalities are frequent in the elderly and are associated with the presence of CVD. Low HDL-c and/or abnormal TG levels, when added to abnormal LDL-c, are associated with a higher prevalence of CVD, suggesting the advisability of a comprehensive lipid evaluation and treatment earlier in life. Lipid profile of patient state the concentration of four parameters of lipids such as total cholesterol, high density lipoprotein, low density lipoprotein and triglycerides in serum. Figure 3.1.2, 3.2.2, 3.3.2 and 3.4.2 shows the lipid profiles among different study groups. The ncep (national cholesterol education program) recommends aggressive treatment with lower goals for serum LDL-c in diabetic patients (ncep.1993). The usual lipidic parameters recommended for the evaluation of lipid-related cardiovascular risk are tc, TG, LDL-c, and HDL-c. However, the above parameters which were usually recommended for cardiovascular risk do not reflect the total of atherogenic particles in these patients. Therefore, additional parameters that do so, are needed.

Total cholesterol — an elevated total cholesterol level is associated with an increased risk of CHD (figure 3.1.2). A desirable total cholesterol level is usually less than 200 mg/dl (5.17 mmol/l). A total cholesterol level of 200 to 239 mg/dl (5.17 to 6.18 mmol/l) is borderline high, while a value greater than or equal to 240 mg/dl (6.21 mmol/l) is high. However, most decisions about treatment are made based upon the level of LDL or HDL cholesterol, rather than the level of total cholesterol.

The total cholesterol can be measured any time of day. It is not necessary to fast (avoid eating

for 12 hours) before testing. In our study all groups of populations (urban, hilly, and coastal) showed a significant higher value of cholesterol in patients than the respective controls (fig. 3.1.2).

LDL cholesterol — the low density lipoprotein (LDL) cholesterol (sometimes called bad cholesterol) is a more accurate predictor of CHD than total cholesterol. Higher LDL cholesterol (figure 3.4.2) concentrations are associated with an increased incidence of CHD in many studies.

Most healthcare providers prefer to measure LDL cholesterol after the person has fasted (not eaten) for 12 to 14 hours. A test to measure LDL in people who have not fasted is also available, although the results may differ slightly from the fasting result.

People with hyperlipidemia should know their own LDL cholesterol level, as well as their goal LDL. This goal depends upon several factors, including the person's history of CHD or CHD risk equivalents and their 10-year risk score of developing CHD. In this study, all groups of populations (urban, hilly, and coastal areas) showed a significant higher value of LDL cholesterol in patients than the respective controls (fig. 3.4.2).

Triglycerides — high triglyceride levels are also associated with an increased risk of CHD (figure 3.2.2). Triglyceride levels are divided as follows:

- Normal - less than 150 mg/dl (1.69 mmol/l)
- Borderline high - 150 to 199 mg/dl (1.69 to 2.25 mmol/l)
- High - 200 to 499 mg/dl (2.25 to 5.63 mmmol/l)
- Very high - greater than 500 mg/dl (5.65 mmol/l)

Triglycerides should be measured after fasting for 12 to 14 hours. In this study urban populations showed a significant higher value of LDL cholesterol in patients than the respective controls and hilly or coastal populations (fig. 3.2.2).

HDL cholesterol — not all cholesterol is bad. Elevated levels of HDL cholesterol lower the risk of heart disease. In fact, a remarkably high HDL (greater than or equal to 60 mg/dl or 1.55 mmol/l) is considered a negative risk factor for CHD (removes one risk factor). On the other hand, treatment is sometimes recommended for people with low levels of HDL cholesterol

(<40 mg/dl or 1.03 mmol/l), particularly if they already have heart disease. In this study, urban patients have the lowest HDL level and coastal people have the largest serum HDL level (figure 3.3.). Similar to total cholesterol, the HDL-cholesterol can be measured on any blood specimen. It is not necessary to be fasting [129-130].

Creatine kinase — plasma concentrations of total ck are elevated in approximately 30 to 70 percent of dialysis patients [131-132]. The underlying reasons for this increase are probably multifactorial; these include a skeletal myopathy, intramuscular injections, deficiencies of vitamin d or carnitine, muscle trauma, and reduced clearance [133-136]. Among patients with esrd, elevations of total ck are usually less than three times the upper limit of normal. Ck isoenzymes consist of dimers of m and b chains; as a result, three isoenzymes are found: ck-mm, ck-mb, and ck-bb [137]. (see "troponins and creatine kinase as biomarkers of cardiac injury".) Ck-mb fraction — in the absence of esrd and acute myocardial damage, less than 5 percent of total ck is present as the mb fraction. With myocardial injury, the fraction of ck- mb among patients without renal failure rises beyond this level in a well-described temporal pattern. However, approximately 30 to 50 percent of dialysis patients without evidence of myocardial injury exhibit an elevation in the mb fraction (defined as greater than 5 percent) [132-134]. The percentage is even higher among those with elevations of total ck. As an example, one longitudinal study of 18 stable hemodialysis patients found that 72 percent had at least one elevated level of total ck. More than 80 percent of these individuals also had at least one increased fraction of ck-mb. In the current study, serum CKMB was found significantly higher in cardiac patients compared to the controls and hilly populations have lower serum CKMB compared to the urban and coastal populations (figure 3.5.2).

Although usually elevated, the ck-mb fraction is usually less than 8 percent among dialysis patients without evidence of myocardial injury. Thus, interpretation of an elevation of ck- mb alone in dialysis patients with atypical chest pain is not reliable.

Other ck isoenzymes — increased serum levels of ck-mm and ck-bb are not used in diagnosing myocardial damage in patients with or without renal failure; however, elevations in these isoenzymes may suggest other conditions, such as a myopathy with increased values for ck-

mm:

Ck-mm — the levels of ck-mm are increased in a significant percentage of dialysis patients. Possible etiologies include myopathy, hepatitis, and deficiencies of vitamin d and/or carnitine .

Ck-bb — in a review of patients with chronic renal failure, ck-bb was universally present in the serum of 13 of 14 peritoneal dialysis patients, all 60 hemodialysis patients, and nearly all of those with a serum creatinine concentration 4.5 mg/dl (398 μmol/l) [138]. By comparison, serum ck-bb was absent in nine patients with acute renal failure (eight of whom needed dialysis) and in 10 renal transplant patients

This test measures the amount of an enzyme called glutamic-oxaloacetic transaminase (got) in patient's blood. This enzyme is found in the liver, muscles (including the heart), and red blood cells. It is released into the blood when cells that contain it are damaged. Other names for this enzyme are aspartate aminotranskinase, aspartate transaminase, and ast.

The SGOT level is measured to check the function of our liver, kidney, heart, pancreas, muscles, and red blood cells. It is also measured to monitor medical treatments that may lead to liver inflammation.

The normal AST/SGOT range for adults is 0 to 35 units per liter (u/l). The normal range may vary slightly from lab to lab. Blood level of SGOT may be higher than normal because: kidneys, heart, or liver are injured, have heart failure or have had a heart attack or recent heart catheterization.[136] in the this study, figure 3.6.2 showed a higher value of SGOT in cardiac patient's serum than the controls in each group of populations (urban, hilly and coastal) and urban female controls has the lowest serum AST level.

Hypercholesterolemia (literally: high blood cholesterol) is the presence of high levels of cholesterol in the blood.[139] it is not a disease but a metabolic rearrangement that can be secondary to many diseases and can contribute to many forms of disease, most notably

cardiovascular disease. It is closely related to the terms "hyperlipidemia" (elevated levels of lipids) and "hyperlipoproteinemia" (elevated levels of lipoproteins).[140]

Elevated cholesterol in the blood is due to abnormalities in the levels of lipoproteins, the particles that carry cholesterol in the bloodstream. This may be related to diet, genetic factors (such as LDL receptor mutations in familial hypercholesterolemia) and the presence of other diseases such as diabetes and an underactive thyroid. The type of hypercholesterolemia depends on which type of particle (such as low density lipoprotein) is present in excess.[138] the hilly populations showed a significantly lower cholesterol level than other two groups which may reflect their diet habit and industrious life style. The urban patients have a relatively higher cholesterol level which explained their unhealthy diet habit especially rich food, fast food and junk food consuming and their relatively lower industrious and exercise. It also explained the relatively healthy food consuming and industrious life style of hilly populations.

Since 1950, researchers have recognized the cardioprotective effect of high-density lipoprotein cholesterol (HDL-c). Subsequent epidemiologic and clinical data demonstrate the important role of HDL-c in coronary heart disease (CHD) risk. Notably, elevated HDL-c reduces the risk of CHD, while low HDL-c is a strong independent risk factor for CHD. Despite this convincing evidence, however, there is still controversy as to whether treatment of low HDL-c reduces the risk of CHD. In this study, coastal area's populations have a higher HDL level in their serum among the three groups of populations. It occurred may be for the ethnicity and availability of sea fishes as well as fish oil. Omega-3 essential fatty acids from fish oil can be helpful against arthritis. Its many other benefits include the reduction of abnormal clotting inside blood vessels, high triglyceride levels, and many forms of chronic inflammation. The free fatty acids form of omega-3 rich fish oil allows this product optimal absorption of epa and dha. Epa (eicosapentaenoic acid) is anti-inflammatory, lowers triglycerides and raises HDL cholesterol, and reduces the tendency of the blood to thrombosis. Dha (docosahexaenoic acid) is a highly unsaturated fatty acid found abundance in the membranes of mitochondria and neurons where it may aid their function by increasing their fluidity.

Gla (gammalinolenic acid) and dha have repeatedly shown a remarkable effect on the reduction of cholesterol and triglyceride levels in both animal and human studies.

In a clinical trial involving 12 hyperlipidemic men epa supplementation of 240 mg/day was given for four months. The results demonstrated a significant average reduction of triglyceride levels (48%), most of which was achieved as early as four weeks after the start. Total cholesterol and LDL-cholesterol levels were significantly decreased, whereas the good HDL cholesterol was significantly increased (22%).[141]

While most studies of omega-3 supplementation have been done on men, an interesting study on the effects of omega-3 fatty acids on serum lipids in post-menopausal women was recently published [140]. In this placebo-controlled, double-blind trial, 36 women received either omega-3 fatty acids (2.4 g/d epa and 1.6 g/d dha) or placebo oil. After 28 days of supplementation there was a marked reduction in serum triglycerides (26%) and a 28% lower ratio of triglycerides to HDL-cholesterol. Women with and without hormone replacement had the same results.

The long-term prevention of atherosclerosis does not, as we now know, depend entirely on lowering cholesterol and triglyceride levels, but on increasing the good HDL- cholesterol. Reduced incidence of cardiovascular disease has been observed in the presence of high HDL levels. Specific subfractions of HDL appear to be involved in this process.
A study on 350 men and women with normal blood pressure demonstrated an increase of HDL2, a particularly beneficial subgroup of HDL-cholesterol, particularly in women, when given omega-3 fatty acids for six months[142].

The effects of gla on subfractions of HDL were studied in rabbits due to their similarity of plasma lipoprotein to humans [143]. After four weeks of gla supplementation there were no changes in the total cholesterol and triglyceride levels, but large changes in the distribution of HDL subfractions. A relative increase in the proportion of HDL2b and HDL3c was observed, an alteration that returned to basal levels 12 weeks after gla withdrawal. This is especially noteworthy since the HDL2b subfraction is increased in centenarians as compared to both ‘middle-aged’ and ‘elderly’ subjects according to a study on long-lived individuals conducted by barbagallo et al. (1998). In the same study HDL2b was also found to be inversely correlated with coronary heart disease and therefore likely to favor healthy aging. In this study, a lower

serum TG in coastal population was observed compared to urban populations.

Potassium (k^+) is the only electrolyte among the four electrolytes (sodium, potassium, chloride and carbon di oxide) which is directly related with heart disease. Figure 3.7.2, 3.8.2, 3.9.2 and 3.10.2 showed the electrolyte status of cardiac patients among the three groups of populations (urban, hilly, and coastal) which presented that urban controls has higher K and lower Co2 compared to other groups.

Resting transmembrane potential difference depends on intracellular and extracellular potassium concentrations. Hypokalemia causes cellular hyperpolarity, increases resting potential, hastens depolarization, and increases automaticity and excitability [142-143]. Because cardiac repolarization relies on potassium influx, hypokalemia lengthens the action potential and increases qt dispersion (reflecting electrical inhomogeneity). Hypokalemic ventricular ectopy is suppressed by potassium replacement [144-145]. Thus, hypokalemia increases risk of ventricular arrhythmia and sudden cardiac death (scd) [146].

Hypokalemia predisposes to digitoxicity by reducing renal clearance and promoting myocardial binding of the drug [147-148]. This produces increased automaticity and ventricular arrhythmias [149]. Hyperkalemia depolarizes myocytes and exacerbates digoxin's atrioventricular nodal blocking [150]. Hypomagnesemia reduces intracellular potassium by reducing the membrane concentration of the sodium-potassium-atpase pump and, thus, predisposes to digitoxicity. Hypokalemia and hypomagnesemia should be avoided in patients taking digitalis. Potassium depletion produces diastolic dysfunction in animal and human models [151]. Experimentally, high potassium protects against hypertensive and sodium-induced endothelial dysfunction independent of blood pressure (bp) [152-156]. In humans, intravenous potassium ameliorates hypertensive endothelial dysfunction [157]. This effect is blunted by the competitive nitric oxide (no) synthase inhibitor, n-monomethyl-l-arginine, implicating the no pathway. Potassium partly mediates vasodilation via strong inwardly rectifying potassium channels and the sodium-potassium-atpase pump of vascular smooth muscle cells (vsmcs) [158]. This may be important when no bioavailability is low. Potassium also blunts angiotensin-ii–induced vasoconstriction [159-160]. It is increasingly apparent that endothelial dysfunction is associated

with a worse prognosis in cardiovascular disease [161-162].

In vitro, high extracellular potassium concentration impairs platelet aggregation. In animal models, increasing plasma potassium reduces the rate of thrombosis on endothelial lesions. These effects occur with physiologically relevant increases. Increasing dietary potassium reduces neointimal formation after angioplasty and reduces atherosclerotic load. Potassium ameliorates oxidative stress by reducing free-radical formation, impairing vsmc proliferation, and reducing monocyte adherence to vessel walls [163]. Thus, potassium retards the progression of atherosclerosis. Ischemic myocardium extrudes potassium, causing hypopolarization and reducing the arrhythmic threshold [164]. Ventricular arrhythmia aggravates the hypopolarization and further lowers the arrhythmic threshold[165].

Adrenaline stimulates the sodium-potassium-atpase pump via $beta_2$-receptors and shifts potassium intracellularly [166]. The catecholamine surge that accompanies acute myocardial infarction (ami) causes redistributional hypokalemia and hyperpolarizes non-ischemic myocardium, producing electrical inhomogeneity and ventricular arrhythmias. Potassium repletion abolishes these effects[167].

Clinical observations suggest that these mechanisms are important. Serum adrenaline levels are inversely correlated with serum potassium in ami and are higher in scd victims. Beta-blockers lessen hypokalemia in ami, and this may partly explain their benefit. Hypokalemia is associated with ventricular fibrillation (vf) in ami independent of diuretic usage. Hulting et al. Found an inverse relationship between serum potassium and vf incidence. None occurred when serum potassium was over 4.6 mmol/l [168-178.] Hypokalemia is a strong independent predictor of mortality in heart failure (hf). Heart failure activates the raas and sympathetic nervous system and induces hypokalemia. Diuretics aggravate hypokalemia and heighten neurohormonal activation. Plasma and muscle magnesium and potassium concentrations are reduced in hf. Most hf patients have increased ventricular ectopy, and 50% exhibit non-sustained ventricular tachycardia. A total of 50% of hf deaths are sudden, presumably due to malignant arrhythmias. In scd victims, myocardial potassium is significantly lower than in controls, and survivors are often hypokalemic. In hf, all-cause and cardiac mortality rates are higher in individuals taking non–potassium-sparing diuretics [179-185]. Serum potassium is negatively correlated with plasma

renin activity and plasma noradrenaline, and patients who respond to treatment show increases in intracellular potassium concentrations. Thus, neurohormonal activation contributes significantly to potassium depletion in hf.

Hypokalemia due to prior non-potassium-sparing diuretic use results in more pronounced hypokalemia during ami[184]. Therefore, it seems sensible to avoid unopposed non– potassium-sparing diuretics in patients at risk for ami. The effect of angiotensin-converting enzyme (ace) inhibitors on mortality when started soon after ami further support the evidence that normokalemia is beneficial in ami[185-189].

In this experiment, there was no significant different found in serum electrolytes among different groups of subjects. Electrolytes are also less variable than all other parameters and Cl is found to be negatively correlated with age.

Conclusion

Cardiovascular disease (CVD) is a global problem and cause huge mortality each year in every part of the world. The risk factors, or co-morbidities, that lead to enhanced risk of developing CVD has been recognized for many years. Despite limited scope and samples, this study reports several biochemical markers related with cardiovascular disease in different geographical area and showed significant variation of this parameters in different area's patient's serum. So, form this study it can be demonstrated that cardiovascular disease risk varies in urban, coastal and hilly areas which may arise for their typical lifestyles and health disparity exists in this small country among different gender and regions.

Overall, urban population appeared as more dyslipidemic compared to costal or hilly populations. Unhealthy life style and food habit, pollutions etc. Might contribute this phenomenon. Some sub-population of hilly and coastal area showed a better parameter status compared to each other which can be attributed as geographical advantages/disadvantages. Lipid profiles and enzymes (CKMB and ast) are more variable compared to electrolytes (mean ± sd of sd of lipid profile and enzymes (6 parameters) and electrolytes (4 parameters) are 26.76 ± 16.94 and 3.73 ± 2.25, respectively) which indicate a stringent electrolyte maintenance by human body. Despite this stringent regulation of body, electrolyte imbalance is observed in cardiac patients and a negative correlation between age and Cl is reported. A positive correlation between age and total cholesterol and LDL conforms age as a risk factor of CVD in this area.

Further study including more subjects in all the experimental groups, including other parameters like smoking, drinking, education, income, diabetes etc. Will help us to reach a general theory about regional health disparity in CVD patients in this area.

CHAPTER 5

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Supplementary s2.1: estimation of serum total cholesterol

Reagents:

Wells	Form	Ingredient	Concentration	Source
1	Tablet	Ce	0.7u/ml	Fungal
		Co	0.1u/ml	
		Hpo	2.4mmol/l	
1-3	Tablet	Aap buffers	4.5mmol/l	Microbial
4-6	Liquid	Dea surfactant	5.8umol	Plant

Reagent preparation: mixing and diluting are automatically performed by the instrument.

Storage: concentration: store at 2-8°c.

Calculation: the instrument automatically calculates and prints the concentration of cholesterol in mg/dl using calculation scheme illustrated in dimension system manual.

Supplementary s2.2: estimation of serum total triglyceride

Reagents:

Wells	Form	Ingredients	Concentration	Source
1-4	Tablet	Lipase	1590u	Yeast Bacterial
		GDH	1.06u	
		Buffer	0.78mmol/l	
		NAD+	5.27mm	
		K2co3	0.17m	

Reagent preparation: mixing and diluting are automatically performed by the instrument.

Storage: concentration: store at 2-8°c.

Calculation: the instrument automatically calculates and prints the concentration of TG in mg/dl using calculation scheme illustrated in dimension system manual.

Supplementary s2.3: estimation of serum total HDL cholesterol

Reagents:

Wells	Form	Ingredient	Concentration	Source
1,2,3	Liquid	Polyanion 4-aminopyrine Mes-buffer (ph-6.5)		Fungal
4,5,6	Liquid	Cholesterol oxidase, esterase hpo, dsmt, mes, naoh	0.53m	Bacterial

Reagent preparation: mixing and diluting are automatically performed by the instrument.

Storage: concentration: store at 2-8°c.

Calculation: the instrument automatically calculates and prints the concentration of HDL cholesterol in mg/dl using calculation scheme illustrated in dimension system manual.

Supplementary s2.4: estimation of serum total LDL cholesterol

Principle of the procedure: the LDL cholesterol assay is homogenous method for direct measuring LDL-c levels in human serum or plasma without the need for off-line pretreatment or centrifugation step. The method is in a two reagent format and depends on the properties of detergent 1 which solubilizes only non L D L particles. The cholesterol released is consumed by cholesterol esterase and cholesterol oxidase in a non-color forming reaction. Detergent 2 solubilizes the remaining LDL particles. The soluble LDL-c is then oxidized by the action of cholesterol esterase and cholesterol oxidase forming cholestenone and h_2o_2. The enzymatic action of peroxidase on h_2o_2 produce color in the presence of n, n-bis (4-sulfobutyl)-m-toluidine, disodium salt (dsbmt) and 4-aminantipyrine (4-aa) that is measured in bichromatic (540,700 nm) endpoint technique. The color produce directly proportional to the amount of LDL-c present in the sample.

Non soluble LDL-c, VLDL-c, HDL-c, chylomicrons	Detergent 1	Soluble non- LDL-c
Soluble non- LDL-c	Cholesterol esterase	Non color forming
Non soluble LDL-c	Detergent 2	Soluble non- LDL-c

Soluble LDL-c + o_2	Cholesterol oxidase	Cholestenone + h_2o_2
H2o2 + dsbmt + 4-aa	Peroxidase	Color development

Reagents:

Wells	Form	Ingredient	Ph	Source
1,2,3	Liquid	Mes buffer	6.5	Cellulomonas Pseudomonas Horseradish Curcubita sp.
		Dtergent 1		
		Cholesterol esterase		
		Cholesterol oxidase		
		Peroxidase		
		Ascorbic acid Preservatives		
4,5,6	Liquid	Mes buffer	6.5	
		Detergent 2		
		Dsbmt, preservatives		

Reagent preparation: mixing and diluting are automatically performed by the instrument.

Storage: concentration: store at 2-8°c.

Calculation: the instrument automatically calculates and prints the concentration of LDL cholesterol in mg/dl using calculation scheme illustrated in dimension system manual.

Supplementary s2.5: estimation of serum CKMB

Materials provided

10 r1 ck-mb reagent 10 r2 ck-mb diluent

Reagents composition

1.

Ck-mb reagent:

Creatine phosphate 30 mm; adenosine-5'-phosphate 2mm; nicotinamide adenine dinucleotide (NAD) 2mm; hexokinase (yeast) = 3000 u/l; glucose-6-phosphate dehydrogenase (bacterial) = 2000 u/l;

2.

Ck-mb diluent:
Buffer 100 mm, anti-human ck-m antibody (goat)sufficient amount to inhibit up to 1500 u/l of ck-mm at 37°c.

Warnings and precautions

1. For in vitro diagnostic use.
2. Exercise the normal precautions required for the handling of all laboratory reagents. Pipetting by mouth is not recommended for any laboratory reagent.

Reagent preparation

Each vial of ck-mb reagents was reconstituted with the 6 ml CKMB diluent. Swirl to dissolve.

Reagent storage and stability

The unreconstituted reagent and diluent should be stored at 2 - 8°c. They are stable until the expiration date. The reconstituted reagent is stable for at least 7 days in refrigerator (2 - 8^{o}c) and 24 hours at room temperature (15 - 30^{o}c).

Specimen collection

The serum sample used for this assay. Exposure of samples to strong light prevented. The samples were stored in refrigerator (2-8^{o}c), but no longer than one week. Freezing of samples (-20^{o}c) results in minimal loss of activity.

Interfering substances

Extremely hemolyzed samples are not suitable for the test since they may contain high levels of adenylate kinase, atp, and glucose-6-phosphate, which interfere with the assay to yield false results. Drugs and other substances, which may interfere with the determination of creatine kinase activity, have been listed by young et al: clin chem 21:10 (1975). The described procedure may overestimate ck-mb values if ck-bb activity in the serum is very high. However, ck-bb activity is usually absent in sera from normal individuals and patients with myocardial infarction. Some investigators have observed a macro form of bb (immunoglobulin complexed), which may be measured as b in this assay. The presence of macro bb in the specimen should be suspected if the ck-b activity measured by this procedure represents more than 20% of the total ck activity.

Materials required

Sample and reagent pipettes, test tubes or cuvettes, timer, thermoregulated flowcell, spectrophotometer, control serum.

Calculations

1. Total ck activity:

Total ck activity in serum was determined according to the directions provided in the package insert for ck reagent.

2. Ck-b activity:

Iu/l = .abs./min. X tv x 1000 = abs./min. X 1.050 x 1000 d ×.× sv 1× 6.22 ×0.050 = .abs./min. X 3376 where: .abs./min. = average absorbance change per minute tv = total reaction volume (1.050) 1000 = conversion of iu/ml to iu/l d = light path in cm (1.0) = millimolar absorptivity of NADH (6.22), sv = sample volume in ml (0.050)

3. Ck-mb activity: ck-mb activity is calculated from ck-b activity as follows: ck-mb activity (u/l) = ck-b activity u/l x 2*

*ck-mb molecule is a dimer consisting of a b subunit and an m subunit. Antibody complexing with the m subunit results in loss of half the catalytic activity of the ck-mb molecule. Therefore, ck-mb activity in the sample is equal to twice the ck-b activity.

Total ck activity

For example, if the total ck activity is 1007 iu/l, the ck- b activity is 67.5 iu/l, and the ck-mb activity is 135 iu/l then % ck-mb activity = 135 x 100% = 13.5% 1007

Supplementary s2.6: estimation of serum Na

Materials provided

1 r1 acid reagent

1 r2 sodium color reagent 1 r3 filtrate reagent

1 r4 sodium standard

Reagent composition

1. Filtrate reagent: uranyl acetate 2.1 mm and magnesium acetate 20 mm in ethyl alcohol.
2. Acid reagent: a diluted acetic acid.
3. Sodium color reagent: potassium ferrocyanide, nonreactive stabilizers, and fillers.
4. Sodium standard: sodium chloride solution: 150 meq/l of sodium

Procedure

Filtrate preparation:

1. Label test tubes: blank, standard, control, patient, etc.
2. Pipette 1.0 ml of filtrate reagent to all tubes.
3. Add 50 µl of sample to all tubes and distilled water to the blank.
4. Shake all tubes vigorously and mix continuosly for 3 minutes.

5. Tubes were centrifuged at high speed (1,500g) for 10 minutes and test the supernatant fluids as described below, taking care not to disturb the protein precipitate.

Color development

1. Test tubes were leveled corresponding to the above filtrate tubes.
2. 1.0 ml acid reagent to all tubes were added,
3. 50 µl of supernatant to respective tubes were added and mixed.
4. 50 µl of color reagent to all tubes were added and mixed.
5. Zero spectrophotometer with distilled water at 550 nm.
6. Read and record absorbance of all tubes.

Note: the chemistry reaction of this procedure involves a reduction in absorbance, as opposed to the usual absorbance increase. The absorbance of blank should be higher than the test samples.

Calculations

Abs. = absorbance s = sample

Std = standard

(abs. Of blank - abs. Of s) x conc. Of std = conc. Of s (abs. Of blank - abs. Of std) (meq/l) (meq/l) rcc0060/e3/tc/ce 2

Example:

Assume the standard with a sodium value of 150 meq/l, gave an absorbance of 0.803 while the sample and the blank had absorbances of 0.880 and 1.406 respectively. The sodium concentration of the sample may then be calculated as follows: (1.406 - 0.880) x 150 = 0.526 x 150 = 131 meq/l (1.406 - 0.803) 0.603

Reference values

135 - 155 meq/l

It is strongly recommended that each laboratory establish its range of expected values, since differences exist between instruments, laboratories, and local populations.

Supplementary s2.7: estimation of serum Cl

Patient samples should be within normal value range. Abnormal values should be brought to the attention of an instructor.

Specimens

Serum and heparinized plasma yield identical results. Lipemic or icteric specimens do not interfere with the validity of the results. Urine specimens must be from a well-mixed 24-hour timed collection. No special additivesare required for collection. (see results for urine calculations.)

Reagents

Acid buffer – provided as a stock item in a 16 oz. Bottle. Contains acetic and nitric acids.

Stored at room temperature.

Standard solution – 100 meq/l of chloride. Store at room temperature. Avoid contamination.

Materials required
Direct reading digital chloride meter oxford pipet (100 :l size) and clean tips

Beaker – large enough to hold chloride electrodes

Procedure
Electrode preparation

1. For best results, clean the electrodes and anode before use each day using a soft cloth and small amount of silver polish.
2. When clean, thoroughly rinse the electrodes in distilled water.
3. Remove the electrodes from the distilled water. Be certain that no residue remains between the indicator electrodes and their common mounting post. Avoid getting skin oils on the electrodes. When not in use, the electrodes should be lowered into a beaker of distilled water to prevent oxidation.

Supplementary s2.8: estimation of serum c02

Materials provided
10 r1 carbon dioxide reagent . 1 r2 carbon dioxide standard.

Reagent preparation
Reconstitute Co2 reagent with the 6 ml of co_2-free water . Mix by inversion 5-6 times. Do not shake. Co2 standard is ready to use. Note: avoid contamination of reagents with co_2. Do not blow into pipette, since breath contains a high content of co_2. Do not let bottles open unnecessarily, since Co2 from air can contaminate reagent. Keep container tightly stoppered

Reagent composition

1. When reconstituted according to the direction, Co2 reagent contains: pep 1.8 mm, magnesium sulfate 10 mm, NADH 0.40 mm, mdh (porcine) 1,250 u/l, pepc (microbial) 200 u/l, sodium oxamate 2.5 mm, buffer, (ph 7.0), non-reactive fillers and stabilizers with sodium azide 0.1% as preservative.

2. Co2 standard contains 30 mmol/l of sodium bicarbonate in an aqueous solution.

Reagent storage and stability

1. Store the unopened vial of Co2 reagent and Co2 standard refrigerated (2 - 8°c). Reagent and standard are stable until the expiration date shown on the labels.
2. Reconstituted reagents stored in the closed tightly stoppered bottle are stable for 1 day at room temperature (18 - 26° c) and 7 days refrigerated (2 - 8° c).

Procedure

1. Co2 reagent was prepared according to reagent preparation.
2. Tubes were leveled as "blank", "standard", "controls", "patients", etc.
3. 1.0 ml carbon dioxide reagent was added into each tube.
4. All tubes were incubated for 3 minutes at 37°c.
5. Spectrophotometer wavelength at 340 nm, temperature to 37°c and the absorbance reading to zero were set up with water as reference.
6. 5 µl (0.005 ml) of water, standard, and sample were added to the cuvette labeled "blank", "standard" and "patients", respectively.
7. Gently mixed by inversion and incubated for 5 minutes.
8. Read and recorded absorbance (abs.) Of all cuvettes at 340 nm.

Calculations

Determine Co2 content of sample as follows:

Co2 content of sample (mmol/l) = abs. Blank-abs. Sample x concentration of standard

abs. Blank-abs. Standard

Example:

Abs. Sample = 0.95 abs. Standard = 0.82 abs. Blank = 1.66

Concentration of standard = 30 mmol/l

Co2 (mmol/l) = 1.66 - 0.95 x 30 1.66 - 0.82= 25.4 mmol/l

Reference values

23 - 34 mmol/l.

It is strongly recommended that each laboratory establish its own range of expected values, since differences exist between instruments, laboratories, and local populations.

Supplementary s2.9: estimation of serum ast

Reagent composition

Ast (SGOT) substrate: 33 mm aspartic acid 5 mm ketoglutaric acid phosphate buffer ph 7.4.

Ast (SGOT) color reagent: 0.25% w/v diazonium salt preserved with formalin. AST (SGOT) calibrator: a lyophilized serum with ast(SGOT) value provided in each lot

reconstitute with distilled water let stand until dissolved and swirl to mix. Stable for five (5) days at 2 - 8°c after reconstitution. Aliquot into small portions and keep frozen.

Storage and stability
Store AST (SGOT) substrate AST (SGOT) color reagent and AST (SGOT) calibrator in refrigerator (2 - 8°c).

Materials required

1. Pipetting devices.
2. Test tubes/rack.
3. Timing device.
4. Spectrophotometer.
5. Heating block or bath (37°c).
6. 0.1 n hydrochloric acid.

Manual endpoint procedure

1. Place 0.5 ml of AST (SGOT) substrate into test tubes labeled "blank" "calibrator" "control" and "unknowns". Warm vials in 37°c heating bath for at least four (4) minutes.
2. At timed intervals add 0.1 ml (100µl) of samples into their respective tubes gently mix and return to 37°c heating bath for exactly ten (10) minutes. (use distilled water for sample blank)
3. After ten (10) minutes and in the same timed sequence add 0.5 ml of AST (SGOT) color reagent mix gently and immediately return to 37°c heating bath for another ten (10) minutes.
4. After ten (10) minutes add 2.0 ml of 0.1 n hydrochloric acid and mix by inversion.
5. Set the wavelength of the spectrophotometer at 530 nm and zero the instrument with the blank. Read and record the absorbance of all tubes. (wavlength range: 500 - 550) note: the final color developed in the reaction must be read within sixty (60) minutes.

Calculation of results
Use the absorbance reading of the calibrator and unknown(s) to
Calculate AST (SGOT). Abs. Of unknown x conc of calibrator (iu/l) = AST (SGOT)

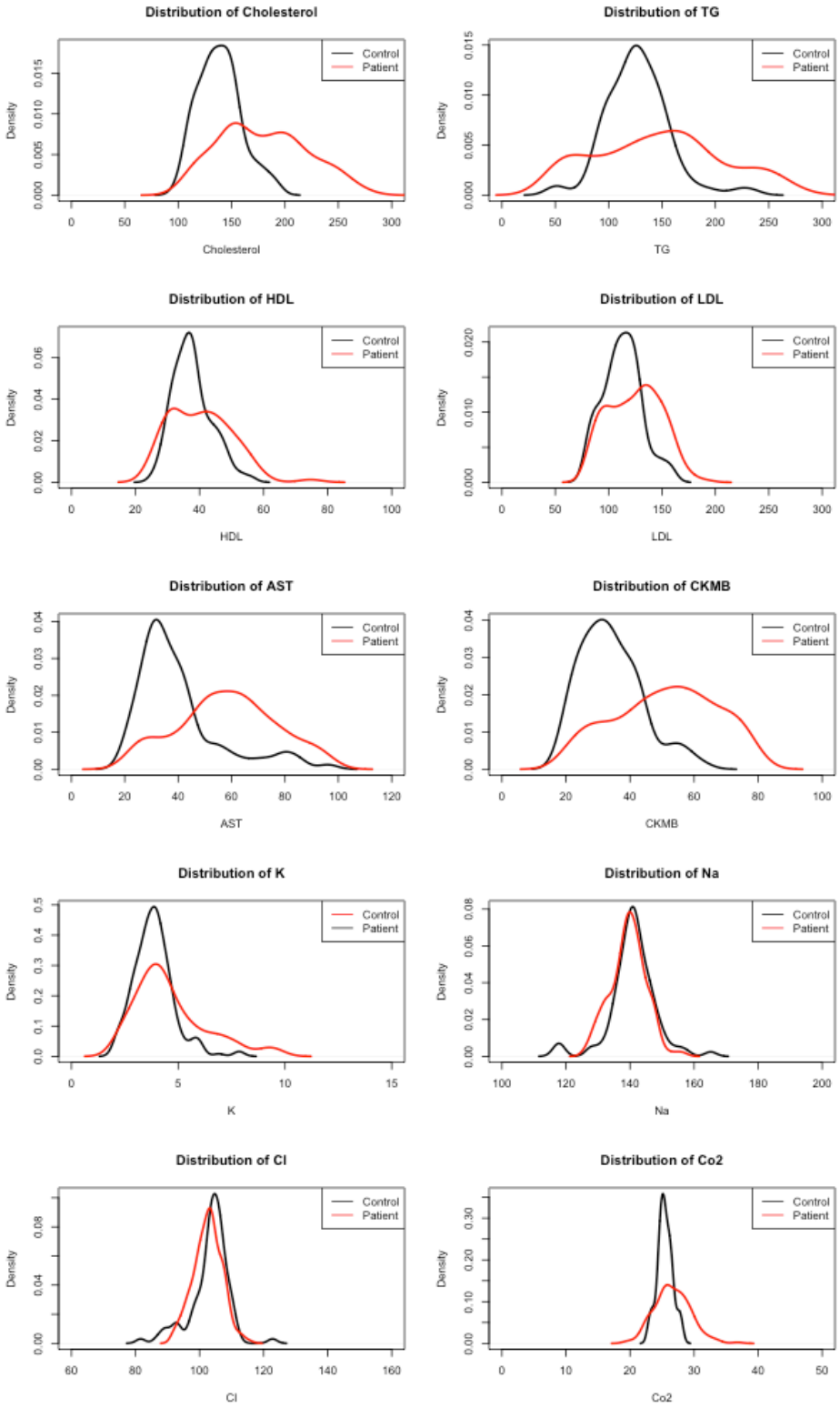
values

Abs. Of calibrator of unknown (iu/l)

References value

10-40 iu/l at 37°c.

It is strongly recommended that each laboratory establish its own normal range.



Supplementary figure S3.1: Distribution of all the variables in controls vs patients

Supplementary S3.2: linear regression of all variables versus age

```
> m1=lm(cholesterol~age,data = sb1)
> summary(m1)

Call:
Lm(formula = cholesterol ~ age, data = sb1)

Residuals:
   min      1q  median      3q     max
-70.141 -28.730  -3.706  23.600 106.520

Coefficients:
            estimate std. Error t value pr(>|t|)
(intercept)  73.8929    13.3835   5.521 7.52e-08 ***
Age           1.6469     0.2336   7.051 1.32e-11 ***
---
Signif. Codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 37.67 on 288 degrees of freedom
Multiple r-squared:  0.1472,   adjusted r-squared:  0.1443
F-statistic: 49.72 on 1 and 288 df,  p-value: 1.318e-11

> m2=lm(HDL~age,data = sb1)
> summary(m2)

Call:
Lm(formula = HDL ~ age, data = sb1)

Residuals:
   min      1q  median      3q     max
-13.371  -5.773  -1.035   4.743  35.908

Coefficients:
            estimate std. Error t value pr(>|t|)
(intercept) 36.16382    2.64494   13.67   <2e-16 ***
Age          0.04061    0.04616    0.88     0.38
---
Signif. Codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 7.444 on 288 degrees of freedom
Multiple r-squared:  0.002681,          adjusted r-squared:  -0.0007822
F-statistic: 0.7741 on 1 and 288 df,  p-value: 0.3797

> m3=lm(LDL~age,data = sb1)
> summary(m3)

Call:
```

```
Lm(formula = LDL ~ age, data = sb1)

Residuals:
   min     1q  median     3q    max
-44.184 -17.399  -0.809  16.680  74.119

Coefficients:
            estimate std. Error t value pr(>|t|)
(intercept) 100.3182     8.2721  12.127   <2e-16 ***
Age           0.3645     0.1444   2.525   0.0121 *
---
Signif. Codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 23.28 on 288 degrees of freedom
Multiple r-squared:  0.02166, adjusted r-squared:  0.01826
F-statistic: 6.376 on 1 and 288 df,  p-value: 0.0121

> m4=lm(TG~age,data = sb1)
> summary(m4)

Call:
Lm(formula = TG ~ age, data = sb1)

Residuals:
   min     1q  median     3q    max
-93.692 -35.819  -5.322  30.564 140.338

Coefficients:
            estimate std. Error t value pr(>|t|)
(intercept) 127.0162    18.8843   6.726 9.38e-11 ***
Age           0.2497     0.3296   0.758    0.449
---
Signif. Codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 53.15 on 288 degrees of freedom
Multiple r-squared:  0.001989,          adjusted r-squared:  -0.001476
F-statistic: 0.5739 on 1 and 288 df,  p-value: 0.4493

> m5=lm(ast~age,data = sb1)
> summary(m5)

Call:
Lm(formula = AST ~ age, data = sb1)

Residuals:
   min     1q  median     3q    max
-34.377 -17.518  -0.958  13.281  44.561
```

```
Coefficients:
            estimate std. Error t value pr(>|t|)
(intercept)  43.0848     6.9488   6.200 1.95e-09 ***
Age           0.1699     0.1213   1.401    0.162
---
Signif. Codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 19.56 on 288 degrees of freedom
Multiple r-squared:  0.006769,          adjusted r-squared:  0.00332
F-statistic: 1.963 on 1 and 288 df,  p-value: 0.1623

> m6=lm(CKMB~age,data = sb1)
> summary(m6)

Call:
Lm(formula = CKMB ~ age, data = sb1)

Residuals:
   min      1q  median      3q     max
-27.844 -12.428  -1.163  11.788  34.828

Coefficients:
            estimate std. Error t value pr(>|t|)
(intercept) 25.36703    5.64259   4.496 1.01e-05 ***
Age          0.36395    0.09847   3.696 0.000262 ***
---
Signif. Codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 15.88 on 288 degrees of freedom
Multiple r-squared:  0.04528, adjusted r-squared:  0.04197
F-statistic: 13.66 on 1 and 288 df,  p-value: 0.0002621

> m7=lm(k~age,data = sb1)
> summary(m7)

Call:
Lm(formula = K ~ age, data = sb1)

Residuals:
   min      1q  median      3q     max
-2.0460 -0.7722 -0.1658  0.4365  5.6027

Coefficients:
             estimate std. Error t value pr(>|t|)
(intercept)  4.169901   0.455597   9.153   <2e-16 ***
Age         -0.001360   0.007951  -0.171    0.864
```

```
---
Signif. Codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 1.282 on 288 degrees of freedom
Multiple r-squared:  0.0001016,         adjusted r-squared:  -0.00337
F-statistic: 0.02926 on 1 and 288 df,  p-value: 0.8643

> m8=lm(na~age,data = sb1)
> summary(m8)

Call:
Lm(formula = Na ~ age, data = sb1)

Residuals:
    min      1q   median      3q     max
-22.3536  -3.1952   0.1405   3.5406  25.0734

Coefficients:
            estimate std. Error t value pr(>|t|)
(intercept) 144.15501    2.17867  66.167   <2e-16 ***
Age          -0.07478    0.03802  -1.967   0.0502 .
---
Signif. Codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 6.131 on 288 degrees of freedom
Multiple r-squared:  0.01326, adjusted r-squared:  0.009829
F-statistic: 3.869 on 1 and 288 df,  p-value: 0.05015

> m9=lm(cl~age,data = sb1)
> summary(m9)

Call:
Lm(formula = Cl ~ age, data = sb1)

Residuals:
    min      1q   median      3q     max
-20.7459  -2.8610   0.5058   3.2747  20.9038

Coefficients:
            estimate std. Error t value pr(>|t|)
(intercept) 106.51646    1.77295  60.079   <2e-16 ***
Age          -0.06711    0.03094  -2.169   0.0309 *
---
Signif. Codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 4.99 on 288 degrees of freedom
Multiple r-squared:  0.01607, adjusted r-squared:  0.01265
```

F-statistic: 4.704 on 1 and 288 df, p-value: 0.03091

```
> m10=lm(co2~age,data = sb1)
> summary(m10)

Call:
Lm(formula = Co2 ~ age, data = sb1)

Residuals:
   min      1q  median      3q     max
-6.6624 -1.4845 -0.3127  1.4530 10.4460

Coefficients:
            estimate std. Error t value pr(>|t|)
(intercept) 25.67350    0.87946  29.192   <2e-16 ***
Age          0.01032    0.01535   0.672    0.502
---
Signif. Codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 2.475 on 288 degrees of freedom
Multiple r-squared:  0.001568,           adjusted r-squared:  -0.001899
F-statistic: 0.4522 on 1 and 288 df,  p-value: 0.5018
```