Diabetes Mellitus Impact on Body's Mineral Profile, Relative Imbalance of ROS level Regulating Enzymes

By

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In The Name Of Allah,

The Most Beneficient, The Most

Merciful

CERTIFICATE

This thesis, submitted by **Ms. Nuzhat Shaheen Abbasi** to the Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan, is accepted in its present form as satisfying the thesis requirement for the Degree of Master of Philosophy in Biochemistry/Molecular Biology.

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Declaration

I hereby declare that the work presented in the following thesis is my own effort and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

NUZHAT SHAHEEN ABBASI

Dedicated

To my loving father, my sweet mother

To my brother and sisters

Whose love and prayers are source of light

In darkness.

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List of Abbreviations

%	Percentage
<	Less than
>	Greater than
°	Degree
°C	Celsius
µC	Micro coulomb
β	Beta
ACE inhibitor	Angiotensin-Converting Enzyme Inhibitor
AGE	Advanced Glycation End Product
ANOVA	Analysis of variance
ASK1	Apoptosis Signal-regulating Kinase 1
BMI	Body Mass Index
Ca	Calcium
CCCP	Carbonyl cyanide m-chlorophenyl hydrazone
CHD	Coronary Heart Disease
COX2	Cyclooxygenase-2
Cr	Chromium
Cu	Copper

Cu/ZnSOD	Copper/Zinc SOD
CVD	Cardio Vascular Disease
DAG	Diacylglycerol
DEEPD	N,N-diethyl-para-phenyldiamine
DM	Diabetes mellitus
DNA	Deoxyribonucleic Acid
DOS	Disk operating system
EDX	Energy dispersive X-ray
$^{\cdot}NO$	Nitric Oxide
$^{\cdot}NO_2^-$	Nitrogen Dioxide
eNOS	Enothelial Nitric Oxide Synthase
Fe	Iron
$FeSO_4$	Ferrous Sulphate
FeSOD	Iron SOD
FFA	Free Fatty Acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDI	GDP-dissociation Inhibitor
GSH	Glutathione (reduced)
GTP	Guanine tri phosphate

H	Calibration factor
H_2O_2	Hydrogen peroxide
HBP	High Blood Pressure
HD	Hypertensive Diabetes Mellitus
HDL	High Density Level
He^+	Helium ions
HIF-1α	Hypoxia-inducible factor 1 alpha
HNO_2	Nitrous Oxide
HOCl	Hydrochlorous acid
HRO_2^-	Hydroperoxyl
IDDM	Insulin dependent diabetes mellitus
IKK-β	IKappaB Kinase – beta subunit
IR	Insulin receptor
IRS	Insulin Resistance Syndrome
IRS	Insulin receptor substrate
JAK/STAT	Janus tyrosine Kinase / Signal Transducer and Activator of Transcription
JNK	c-Jun NH_2-terminal kinase
JNK/SAPK	c-Jun NH2-terminal kinase / Stress-activated protein kinase
K	Potassium

kDa	Kilo Dalton
KeV	Kilo electron volt
Kg	Kilogram
LPO	Lipid Peroxidation Product
MAPK	Mitogen-activated protein kinase
MeV	Mega electron volt
Mg	Magnesium
Mg	Milli gram
MIDD	Maternally Inherited Diabetes Mellitus and Deafness
Min	Minute
Mn	Manganese
Mn SOD	Manganese Superoxide Dismutase
MS	Metabolic Syndrome
mt DNA	Mitochondrial DNA
Na	Sodium
nA	Nano Ampere
Na	Sodium
NADPH	Nicotinamide adenine dinucleotide Phosphate (reduced)
NCP	National Centre of Physics

NF-KB	Nuclear factor – kappa light chain enhancer of activated B cell
NHD	Non Hypertensive Diabetic
Ni	Nickel
NIDDM	Non- insulin dependent Diabetes Mellitus
NOX	Nitric Oxidase Product
$^{\cdot}O_2$-	Superoxide
O_2	Molecular oxygen
$^{\cdot}OH$	Hydroxyl
$ONOO^-$	Peroxynitrite
P value	Significant value
P13k	Phosphotidylinositol 3-kinase
PGE2	Prostaglandin E2
pH	Negative logarithm of hydrogen ion
Phox	Phagocyte oxidase
PIXE	Proton induces X-Rays emission
PKC	Protein kinase C
Ppm	Parts per million
pS/T	Serine or Theronine sites
pY	Phsophorylation

RBS	Rutherford backscattering spectroscopy
RNS	Reactive nitrogen species
˙RO2	Peroxyl
RONOO	Alkyl peroxynitrates
ROS	Reactive oxygen species
Rpm	Revolution per minute
RRS	Relative risk factor
S.D	Standard deviation
SDS	Sodium dodecyl sulphate
SE	Standard error
Ser/Thr	Serine/Theronine
Serum i – Mg	Serum ionized magnesium
SH3	Src homology 3
Si Li	Lithium drifted silicon
SNICSII	Source of negative ions by cesium sputtering II
SOD	Superoxidase dismutase
T2DM	Type 2 diabetes mellitus
TNF-α	Tumor necrosis factor-alpha
UCP-1	Uncoupling protein 1

UV-Visible	Ulta violet- Visible
VEGF	Vascular endothelial growth factor
Vs	Verses
VSMC	Vascular smooth muscle cell
Z	Atomic number

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Nuzhat Shaheen Abbasi

Abstract

Diabetes Mellitus is one of the most common metabolic disorders. Diabetes Mellitus Type 2 is much more prevalent form (approx 90 to 95 %) as compared to other type of diabetes. The leading complications of diabetes are cardiovascular disease, diabetes retinopathy, diabetes nephropathy and many others. The level of ROS and concentrations of micronutrients in the serum of patients was evaluated by using the DEPPD method and PIXE technique respectively. For the measurement of ROS calibration curve constructed by using hydrogen peroxidase as a standard. The level of ROS in diabetic patients under the some risk factors like physical activity, hypertension, age, family history and cardiovascular disease was evaluated. We found the level of ROS was higher in diabetic patient having risk factor as compared to those with no risk factor for this disease. The endogenous serum level of different essential elements or micronutrients was also altered in diabetic patients. Levels of Cu, Mn, Fe and Se were higher in diabetic patients as compared to normal individual (control). Contrary, levels of Cr, Zn and Mg were lower in Diabetic patients as compared to control. Out of these elements some act as cofactors of different enzymes involved in the antioxidant mechanism. The level of these elements was either increased or decreased in the serum of patients. Due to the low level of these cofactors in the serum of patient the activity of enzymes was down regulated. The level of ROS/RNS up regulated when the level of enzymes down regulated in diabetic patients and the level of several antioxidant such as vitamin B, E and C down regulated. Due to the higher levels of ROS in patient several stress sensitive pathways were activated such as NF-KB, P38-MAPK and JNK/SAPK. Present study concluded that the progression and complication of disease occurred due to higher level of ROS in diabetic patients in concern with altered level of regulating enzymes co-factors.

INTRODUCTION

1. Introduction

Diabetes is one of the most common metabolic disorders. According to Malecki and Klupa. (2005) diabetes is a group of metabolic diseases that are characterized by hyperglycemia (elevated glucose level). Diabetes is classified into type 1 diabetes (insulin-dependent diabetes mellitus or IDDM) and type 2 diabetes (non-insulin dependent diabetes mellitus NIDDM). Type 2 diabetes is the much more prevalent form that is responsible for 90% of the disease prevalence (Zimmet *et al.*, 2001).

Type 2 diabetes (which is previously known as non insulin-dependent diabetes mellitus or adult – onset diabetes and here after referred as DM or diabetes) account for approximately 90 to 95 % of totally diagnosed cases of diabetes. Type 2 diabetes is a major cause of disability, morbidity and mortality (Schoenberg *et al.*, 2005).

The prevalence shows no difference between males and females. There is a significant increase in the prevalence of diabetes with increasing age; impaired glucose tolerance was diagnosed in 3.9%, while impaired fasting glucose was diagnosed in 1.9%. (Melidonis *et al.*, 2006).

Non-insulin dependent diabetes mellitus (NIDDM) is characterized by the presence of two basic abnormalities: impairment of insulin secretion and other is decrease in insulin sensitivity (Kahn, 1994). Type 2 diabetes mellitus (T2DM) resulting from autoimmune destruction of β cells of pancreas and genomic deoxyribonucleic acid (DNA) mutations in the gene linked to insulin, insulin receptor, enzyme adenosine deaminase, and glucokinase gene (Wahid *et al.*, 2006). Impairment of insulin secretion may be defects in mitochondrial function or mitochondrial DNA (mt DNA). In pancreatic beta cells, mitochondria are responsible for glucose induced insulin secretion (Abdul *et al.*, 2009).

They introduces that the mitochondrial DNA has 10 times the rate of spontaneous mutation when compared with nuclear genome. The mitochondrial DNA has neither protective histones nor an effective DNA repair system, as present in the nucleus, due to which mitochondria is less efficient in repairing DNA damage (Zeviani and Carelli, 2003).

In ''maternally inherited diabetes mellitus and deafness (MIDD)" the most common heteroplasmic point mutation associated with diabetes was found to be mitochondrial DNA tRNA gene (i.e., A3243G) mutation. This affects the transcription and translation of mt DNA and has been found to be one of the causes of type 2 diabetes mellitus with sen-sorineural hearing loss. Other homoplasmic mutations i.e., G1888A, T4216G, A4917G and T14709C were also detected and found to play important role in pathogenesis of type 2 diabetes mellitus (Brunmair *et al.*, 2004).

1.1 Risk factor for the diabetes mellitus for other disease

In a multivariate analysis glutamic pyruvic transaminase, blood glucose, body mass index, bilirubin, systolic blood pressure, uric acid and a family history of diabetes were all significantly associated with the development of diabetes (Ohlson *et al.*, 1988).

The glucose uptake in other tissues is restrained by two hormonally controlled mechanisms: 1) depression of insulin secretion and 2) increase in the concentration of free fatty acids in plasma (which by direct means depresses glucose uptake). The body can meet stress when these hormonal interactions provide large amounts of metabolic fuel and distribute them in an effective manner. In diabetes, an increased secretion of stress hormones in connection with trauma is observed (Efendic *et al.*, 1974).

1.1.1 Obesity

The associations between obesity and type 2 diabetes have confirmed many cross sectional and prospective studies. Most people with type 2 diabetes are overweight or obese in southeast Scotland in 2005 more than 85% of people with type 2 diabetes had a body mass index (weight in kilograms divided by height in meters squared) of over 25. It is reported that high waist circumference may be an even better indicator than body mass index (BMI) of increased risk of type 2 diabetes. In United States, earlier onset of type 2 diabetes is associated with a higher BMI, and increasing prevalence of overweight and obesity is the most important factor in the increasing number of younger people diagnosed with type 2 diabetes (Wild and Byrne, 2006).

Obesity is the principle and more powerful risk factor for both type 2 diabetes and of coronary heart disease (CHD). The most widely used epidemiological and clinical

definition of obesity has BMI > 30 kg/m^2. Women of BMI > 35 have > 90 fold increased risk factor of developing disease. The prevalence of obesity has increased rapidly in most population in whole world, as a result the prevalence of type 2 diabetes and CHD is rising. The loss of weight has shown clearly to improve glycemic control in overweight patient with type 2 diabetes. Previous investigations showed that sustained weight loss of just 3-4 Kg in overweight individuals with impaired glucose tolerance resulted in 55% risk reduction for diabetes at four year (Jonathan, 2001).

This data suggest that, at even average weight, women can develop risk of clinical non-insulin-dependent diabetes and that the relation between body mass index and risk of diabetes is unceasing (Colditz *et al.*, 1990).

1.1.2 Sleep

Short sleep duration could be a significant risk factor for diabetes. The association between long sleep duration and diabetes incidence is more likely to be due to some un-measured confounder such as poor sleep quality (Gangwisch *et al.*, 2007).

Independent of confounding factors, short and long sleep durations increase the risk of developing diabetes. Sleep duration may be a novel risk factor for diabetes. They have reported that 7 h of sleep per night served as the reference group. In men short sleep duration (≤ 5 and 6 h of sleep per night) were twice as likely to develop diabetes, and men reporting long sleep duration (> 8 h of sleep per night) were more than three times as likely to develop diabetes over the period of follow-up. Relative risk (RRs) were altered considerably for the two extreme sleep groups when adjusted for testosterone (1.51 [0.71–3.19] for ≤ 5 h and 2.81 [1.34–5.90] for > 8 h), suggesting that the effects of sleep on diabetes could be mediated via changes in endogenous testosterone levels (Yaggi *et al.*, 2006).

According to Karine Spiegel, chronic sleep loss, behavioral or sleep disorder may represent a novel risk factor for weight gain, insulin resistance, and Type 2 diabetes (Spiegel *et al.*, 2005).

1.1.3 Life Style

The results of ecological and migration studies indicate that a western lifestyle is associated with a higher prevalence of type 2 diabetes. Physical activity and diet can affect the development of type 2 diabetes through changes of body fatness, but also through other pathways. Due to higher consumption of whole grain products and exchanging unsaturated fat for saturated fat may lower risk for type 2 diabetes (Van, 2003).

Consumption of alcohol from light to moderate may also reduce risk for type 2 diabetes, whereas high alcohol consumption and cigarette smoking may increase risk for type 2 diabetes. The prevention of weight gain by balancing energy intake and expenditure is of dominant importance to limit current increases in the prevalence of type 2 diabetes. In addition, other effects of lifestyle may play an important role in reducing risk for type 2 diabetes (Van, 2003).

The effect of alcohol consumption on complications of diabetes mellitus suggests that moderate alcohol consumption is associated with a decreased risk for diabetes, whereas heavy alcohol consumption may be associated with an increased risk. The ingestion of alcohol along with use of a sulfonylurea or thiazolidinedione does not increase the risk for an adverse drug event (Andrea A. Howard, 2004).

The association between excessive use of alcohol and increased risk for diabetes in some studies may have been mediated by an increase in obesity, and particularly in truncal adiposity, which is a strong risk factor for type 2 diabetes (Ohlson, 1985).

1.1.4 Inheritance

The prevalence of diabetes was lower among patients with diabetic father (21.2%) compared to patients with diabetic mother (25.6%) and there was early onset of type 2 diabetes among patients having both parents with diabetes when compared to other patients. A strong family history for diabetes would signal an early age, onset of diabetes possibly with its complications (Jali *et al.*, 2009).

Those with a positive family history of diabetes had 2.4-fold higher risk for developing diabetes than those without such a history. Disturbed liver function and increased levels

of lactate are early risk factors for diabetes presumably indicators of the presence of impaired glucose tolerance and/or hyper-insulinaemia (Ohlson *et al.*, 1988).

1.1.5 Hypertension

The association between diabetes mellitus and hypertension has been described in 60 to 65% of diabetics. In hypertension we find insulin resistance mainly in skeletal muscle involving the conversion of glucose to glycogen independently of blood flow. States of hyperinsulinaemia and insulin-resistance have been postulated as causes and/or consequences of hypertension. Regardless of the type of diabetes, hypertension is two to three times more common among diabetics compared with non-diabetics (Contreras *et al.*, 2000).

Type 2 diabetes mellitus was nearly 2.5 times as likely to produce in subjects with hypertension as in subjects with normal blood pressure. Gress *et al.* (2000) reported that the high risk of diabetes in subjects with hypertension, they found that those who took a thiazide diuretic, ACE inhibitor, or calcium-channel antagonist were not at high risk of diabetes. They found that the risk of diabetes mellitus among those subjects who taking a thiazide diuretic was not greater than among those taking no medication.

1.2 Diabetes Mellitus as a risk factor

The interrelations between risk factors for non-insulin dependent diabetes and coronary heart disease and the potential value of an integrated approach to the prevention of these conditions based on the prevention of obesity and the promotion of physical activity (Ivan *et al.*, 1995). One of the leading complications of diabetes is cardiovascular disease. The frequency of cardiovascular disease in people with diabetes mellitus is three to four times of that in non-diabetic individuals. Additionally, established risk factors such as dyslipidemia, hypertension, and smoking cannot explain this increased occurrence of macro vascular disease in diabetes (Rahimi *et al.*, 2005).

The pathophysiology of cardiovascular disease in subjects with diabetes is multifactorial, and in addition to the classic risk factors (such as hypertension, dyslipidaemia and obesity) in type-II diabetes, increased urinary albumin excretion, endothelial dysfunction, and chronic inflammation are inter-related processes that develop in parallel, progress

with time, and are strongly and independently associated with risk of death (Varughese and Lip, 2005).

1.3 Ionic profile of Diabetes mellitus

In diabetes mellitus, the metabolism of several essential elements is altered and that these nutrients might have specific roles in the pathogenesis and progress of this disease. Previously study was carried out to compare the level of essential elements, potassium (K), calcium (Ca), magnesium (Mg), and sodium (Na), in biological samples (whole blood, urine, and scalp hair) of patients who have hypertensive diabetes mellitus type 2 (n = 254) and non hypertensive diabetes mellitus type 2 (n = 228) with those of non diabetic as control subjects (n = 182; age range of both genders 45-75). In this study they find that the mean value of K, Mg, and Ca were significantly reduced, while Na level were higher in blood and scalp hair samples of hypertensive diabetic (HD) patients and non hypertensive diabetic (NHD) patients as compared to control subjects of both genders ($p < 0.05$), but level of K in the biological samples of non hypertensive diabetic patient was found to be higher in both HD and NHD patients than healthy controls (Afridi, *et al.*, 2008).

Kazi *et al.* (2008) compared the level of essential trace elements, chromium (Cr), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), and zinc (Zn) in biological samples (whole blood, urine, and scalp hair) of patients who have diabetes mellitus type 2 (n = 257), with those of non diabetic control subjects (n = 16), age ranged (45-75) of both genders. In the result of this study, the mean values of Zn, Mn, and Cr were significantly reduced in blood and scalp-hair samples of diabetic patients as compared to control subjects of both genders ($p < 0.001$). The levels of these elements in urine were found to be higher in the diabetic patients than in the age-matched healthy controls. In comparison, high mean values of Cu and Fe were detected in scalp hair and blood from patients versus the non diabetic subjects, but the differences found in blood samples was not significant ($p < 0.05$).

Diabetes mellitus (DM) is associated with the modification in the metabolism of copper (Cu), zinc (Zn), and magnesium (Mg). The association between glycated hemoglobin and

levels of metals was also calculated. In Plasma the concentrations of Cu, Zn, and Mg were measured by atomic absorption spectrometry. Atomic absorption spectrophotometer showed higher levels of Cu ($P < .001$) and Cu/Zn ratio ($P < .0001$) and decreased levels of Zn ($P < .01$) and Mg ($P < .0001$) in patients with DM when compared with controls. Study reported that the patients with DM had altered metabolism of Cu, Zn, and Mg and this may be related to increased values of glycated hemoglobin. The impaired metabolism of these elements may contribute to the progression of DM and diabetic complications (Viktorinovaa *et al.*, 2009).

In Diabetes mellitus, the Zn deficiency may play a role in the development of some form of glucose intolerance. The effects of zinc on insulin secretion are biphasic with higher concentration impairing insulin release. Another micronutrient is chromium whose deficiency can lead to glucose intolerance. Chromium rarely plays a role in the pathogenesis of diabetes mellitus and most diabetic patients are Cr deficient. The majority of diabetic patients do not have micronutrient deficiencies. Zinc, chromium and magnesium deficiencies have been identified in a subgroup of patients. Serum concentration of certain elements such as copper, manganese, iron and selenium can be higher in diabetic patients than in non diabetic controls. Serum of diabetic patient contains ascorbic acid, group B vitamins and possibly 1, 25-dihydroxycholecalciferol but their concentration may be low in diabetic patients. The levels of vitamin A and E were normal or increased (Mooradian *et al.*, 1987).

Alzaid *et al.* (1995) reported that the Insulin influences both glucose metabolism and magnesium homeostasis in humans. They conclude that insulin resistance in subjects with NIDDM impairs the ability of insulin to stimulate magnesium as well as glucose uptake.

1.3.1 Magnesium

Magnesium is the fourth most abundant cation in the body and second most abundant intracellular cation. Magnesium is a cofactor of various enzymes in carbohydrate oxidation and plays an important role in glucose transporting mechanism of the cell membrane. It's also involved in insulin secretion, binding, and activity. Magnesium deficiency and hypomagnesemia can result from a wide variety of causes, including deficient magnesium intake, gastrointestinal, and renal losses. Chronic magnesium

deficiency has been associated with the development of insulin resistance (Chaudary *et al.*, 2009).

Magnesium is an important and major component of many unprocessed foods, such as whole grains, nuts, and green leafy vegetables, and it is largely lost during the processing of some foods (Saris *et al.*, 2000). Hypomagnesemia (low level of magnesium) is a common and important feature in patients with type 2 diabetes (Folsom, *et al.*, 1995). The inverse association with magnesium intake was consistent across different subgroups defined by the main predictors of type 2 diabetes, such as physical activity, BMI, and family history of diabetes. Higher intake of magnesium is likely more beneficial among individuals with some degree of magnesium deficiency. However, there is no generally accepted test for magnesium status in individuals (Lopez-Ridaura *et al.*, 2004).

Magnesium is the second most abundant intracellular cation (Lopez *et al.*, 1997). The main and important dietary sources for magnesium are whole grains, leafy green vegetables, legumes, and nuts. Hypomagnesemia, commonly due to insufficient magnesium intake or increased magnesium loss (Lefebvre *et al.*, 1994) is strongly related to metabolic syndrome (Guerrero-Romero and Rodriguez-Moran, 2002) and has been correlated with the development of type 2 diabetes (Kao *et al.*, 1999).

In the serum low levels of magnesium are related to diabetes mellitus (DM) and high blood pressure (HBP). They studied that there was a strong independent relationship between low serum magnesium levels and metabolic syndrome. Among the components of MS, dyslipidemia and HBP were strongly related to low serum magnesium levels. Reported that a strong relationship between decreased serum magnesium and MS (Guerrero-Romero and Rodriguez-Moran, 2002).

The depletion of cellular and extracellular Mg is characterized by type 2 diabetes mellitus. Insulin and glucose are important regulators of Mg metabolism. Intracellular Mg plays a key role in regulating insulin action, insulin-mediated-glucose uptake and vascular tone. The reduced intracellular Mg concentrations results in a defective tyrosine-kinase activity, post-receptorial impairment in insulin action, and worsening of insulin resistance in diabetic patients. Mg deficit has been proposed as a possible underlying common mechanism of the "insulin resistance" of different metabolic conditions. Low

intake of Mg in diet is also related to the development of type 2 diabetes (Barbagallo and Dominguez, 2007).

The serum ionized magnesium (i-Mg) levels were significantly reduced in patients with low HDL cholesterol, high triglycerides values, high waist circumference, high blood pressure, microalbuminuria and clinical proteinuria according to univariate analysis. Hypomagnesemia was highly prevalent in studied population (Corica *et al.*, 2006).

1.3.2 Zinc

Zinc is one of the most important functional metals in the human body with over 300 zinc-containing enzymes. These enzymes play important and critical roles in structural stabilization and as cofactors in catalysis, whereas a large number of proteins involved in transcription factors contain Zinc fingers and similar structural motifs. The concentration of Zinc ion in the human body is tightly regulated. Significant disturbances of zinc homeostasis have been associated with diverse ill effects including reproductive abnormalities, growth retardation, hypogonadism, impaired wound healing, skin lesions and anemia, diarrhea, anorexia, cognitive impairment, immune dysfunction, diabetes mellitus, impaired visual function, osteoporosis, cirrhosis of the liver, bowel disease, and even tumors (Nriagu, 2011).

The treatment of diabetes mellitus includes oral anti-diabetic agents, insulin, and dietary regimens. There is strong evidence that there is an abnormal metabolism of several micronutrients in diabetic individuals. Zinc is one of the essential and important micronutrients and its metabolism is altered in diabetic patient. Previously study reported a close relation among zinc, glucose metabolism, and insulin physiology (Salgueiro *et al.*, 2001).

1.3.3 Chromium

Chromium has been established to be an essential trace element in mammals for the maintenance of normal carbohydrate metabolism. The deficiency of chromium in human may improve glucose levels. It is reported that a clinical response to chromium (i.e. decreased glucose and improved insulin sensitivity) may be more likely in insulin-

resistant individuals with type 2 diabetes who have higher level of fasting glucose and hemoglobin A(1c) levels (Wang and Cefalu, 2010).

The supplementation of chromium is seems to improve glycaemic control in type 2 diabetic patients, which appears to be due to an increase in insulin action rather than stimulation of insulin secretion (Ghosha *et al.*, 2002).

Chromium is mainly present in many foods, especially in liver, Brewer's yeast, American cheese, wheat gram, vegetables such as carrots, potatoes, broccoli, and spinach, and is also present in alfalfa, brown sugar, molasses, dried beans, nuts, seeds, and mushrooms (Guerrero-Romero and Rodriguez-Moran, 2005). Generally, it is recognized that a chromium inta ke of 30–40 μg/day is sufficient for achieving the daily requirements (Anderson, 1998). Tyrosine kinase, the enzyme required for phosphorylation, is chromium dependent, and phosphotyrosine phosphates, an enzyme that deactivates the insulin receptor, is inhibited by chromium (Anderson, 1998). In addition to the increase in the number of insulin receptors (Anderson, 1998). Chromium improves the action of insulin by improving activity of tyrosine kinase on the insulin receptor. It has been reported that Cr also exerts a powerful cellular antioxidant action (Anderson *et al.*, 2001) and decreases the hepatic extraction of plasma insulin (Guan *et al.*, 2000). Deficiency of chromium may result in similar clinical demonstration to those observed in insulin resistance and type 2 diabetes, and supplementation with chromium remained to improve insulin sensitivity, leading to a more efficient peripheral glucose uptake (Guerrero-Romero and Martha Rodriguez, 2004).

In large cohort study of middle-aged women, found that both vitamin D and calcium intakes were inversely associated with progression of type 2 diabetes, and the benefits of the two nutrients appear to be additive. For both vitamin D and calcium, intakes from supplements rather than from diet were appreciably associated with a lower risk of type 2 diabetes (Pittas *et al.*, 2006).

The chromium lost and excreted from human body increases with aging and is related to the diabetics. Thus, it is counseled to supplement a certain amount of chromium to the elderly diabetics according to their nutritional level (Ding *et al.*, 1998).

1.3.4 Copper

Their studies showed that copper concentrations were significantly higher in NIDDM patients than non-diabetic subjects ($p < 0.01$). In a study by Schlienger et al on the effect of diabetes on trace elements, elevated levels of serum copper were found in patients with IDDM and NIDDM; glycaemic control did not affect copper levels (Zargar *et al.*, 1998). The mean values of the copper are high in the diabetics compared to the controls (the results have statistical significance $p < 0.01$). The concentration tends to rise as the illness progress. It is known that some diseases evolve with high levels of copper serum concentration. This fact was also proved in the groups we studied. Thus, for patients with diabetes with vascular lesions the Cu level was around 142.10 μg/dl, whereas in those with diabetes and artheriopathy it was 150.20 μg/dl and in tho se with diabetes and nephropathy or retinopathy it was 160.71 μg/dl (Rusu *et al.*, 2005).

1.4 Reactive oxygen species in diabetes mellitus

Oxidative stress is defined as surplus formation and/*or* deficient removal of highly reactive molecules such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Turko *et al.*, 2001; Maritim *et al.*, 2003). Oxidative stress is enormous production of reactive oxygen species (ROS) in the presence of diminished antioxidant substances (Opara, 2002). ROS comprise free radicals such as superoxide ($^{\bullet}O_2^-$), hydroxyl ($^{\bullet}OH$), peroxyl ($^{\bullet}RO_2$), hydroperoxyl ($^{\bullet}HRO_2^-$) as well as non-radical species such as hydrogen peroxide (H_2O_2) and hydrochlorous acid (HOCl) (Turko *et al.*, 2001; Evans *et al.*, 2002). RNS comprise free radicals like nitric oxide ($^{\bullet}NO$) and nitrogen dioxide ($^{\bullet}NO_2^-$), as well as non-radicals such as peroxynitrite ($ONOO^-$), nitrous oxide (HNO_2) and alkyl peroxynitrates (RONOO) (Turko *et al.*, 2001; Evans *et al.*, 2002). These reactive molecules, $^{\bullet}O_2^-$, $^{\bullet}NO$ and $ONOO^-$ are the most extensively stud ied species and play significant roles in the diabetic cardiovascular complications (Johansen *et al.*, 2005).

ROS are those species of oxygen which are in a more reactive state as compared to molecular oxygen. For example, a primary ROS is super- oxide, which is produced by the one-electron reduction of molecular oxygen (equation 1). This is the reaction catalyzed by NADPH oxidase (equation 2, and see below), with electrons provided by NADPH (Hancock *et al.*, 2001).

$$O_2 + e^- \rightarrow O_2^{\cdot -} \text{ (superoxide)} \qquad \text{equation.1}$$

$$2O_2 + NADPH \rightarrow 2O_2^{\cdot -} + NADP^+ + H^- \qquad \text{equation.2}$$

Additional reduction of oxygen produces hydrogen peroxide. This can proceed from the dis-mutation of superoxide (equation 3), which can occur spontaneously, specifically at low pH. However, this reaction can also be accelerated, by a family of enzymes known as superoxide dismutase (SOD) (Hancock *et al.*, 2001).

$$2O_2^{\cdot -} + 2H+ \rightarrow H_2O_2 + O_2 \qquad \text{equation.3}$$

Data suggested that reactive oxygen species, such as superoxide anions and hydrogen peroxide, behave as intracellular second messengers (Finkel, 1998). ROS, in particular hydrogen peroxide, are now known as important signaling molecules in both the animal and plant kingdoms (Hancock *et al.*, 2001).

Hydroxyl radicals are dreadfully reactive, with a short half-life, and they credibly react with the first molecule they encounter. In neutrophils, myeloperoxidase catalyses the formation of hypochlorous acid (HOCl), while superoxide may also react with nitric oxide (NO) to form another relatively reactive molecule, peroxynitrite (equation 4) (Hancock *et al.*, 2001).

$$NO\cdot + O_2^{\cdot -} \rightarrow OONO^- \text{ (peroxynitrite)} \qquad \text{equation.4}$$

ROS contribute to the development of various human diseases, including type 2 diabetes mellitus (T2DM) (Tiganis, 2 011). Oxidative stress has an adverse effect on glucose metabolism (Opara, 2002). ROS can repress the insulin response and contribute to the progression of insulin resistance, a key pathological feature of T2DM. Growth factors, cytokines and hormones such as insulin promote the propagation of ROS for the coordinated inactivation of protein tyrosine phosphatases and the elevation of tyrosine phosphorylation-dependent signaling (Tiganis, 2011).

ROS have "purposeful" roles as "regulators" of cell function or as "signaling molecules". ROS has acquired important recognition over the past many years from studies done in laboratories worldwide. The data supporting this concept is based largely on the following criteria: *1*) growth factors and cytokines are able of generating ROS in a

different number of cell types, *2*) anti-oxidants and inhibitors of ROS- initiating enzymatic systems prevent specific growth factor- and/or cytokine-activated signaling events or physiological effects, and *3*) exogenous addition of oxidants activates the same cytokine and/or growth factor-mediated signaling pathway or generates the same physiological effects (Thannickal and Fanburg, 2000).

In endothelial cells, high-glucose treatment enhances the mitochondrial ROS and normalization of the ROS production by inhibitors of mitochondrial metabolism. Over expression of UCP-1 or Mn-SOD, prevents glucose-induced activation of PKC, formation of AGE, and aggregation of sorbitol. All above are believed to be the main molecular mechanisms of diabetic complications (Nishikawa and Araki, 2007).

Elevated glucose and possibly free fatty acids (FFA) levels contribute to the patho-physiology of diabetes via the generation of ROS and subsequent activation of numerous stress-sensitive pathways (Evans *et al.*, 2003).

ROS (and RNS), by imposing macromolecular damage, may play a key role in the pathological process of diabetes. ROS also play role as signaling molecules (analogous to second messengers) to initiate several stress-sensitive pathways (indirect role). In addition, in type 2 diabetes, there is growing evidence that activation of stress-sensitive pathways, such as NF-kB, p38 MAPK, JNK/SAPK, and hexosamine, by increase in glucose and possibly FFA levels leads to both insulin resistance and impaired insulin secretion. Thus ROS and oxidative stress, induced by elevations in glucose and possibly FFA levels, may play a major role in causing insulin resistance and β-cell dysfunction by their ability to activate stress-sensitive signaling pathways. The proposed sequence of events may also include other stress pathways, such as the increased formations of AGE, sorbitol, cytokines, and prostanoids along with PKC activation, (DAG) Diacylglycerol. (Evans *et al.*, 2003).

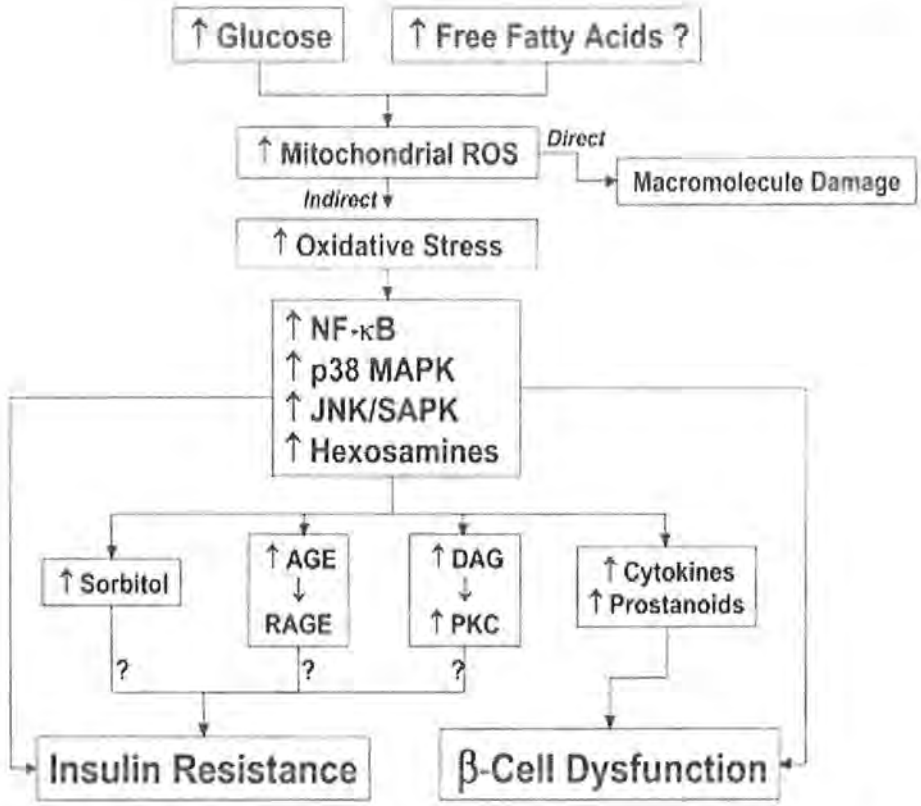

Fig 1.1: Causative link among hyperglycemia, mitochondrial ROS generation, oxidative stress, and the development of diabetic complications (Evans et al., 2003).

$^{\bullet}O_2^-$ can activate a number of damaging pathways in diabetes comprises of accelerated formation of advanced glycation end products (AGE), polyol pathway, hexosamine pathway and PKC, all of which have been demonstrated to be involved in micro- and macro vascular complications. $^{\bullet}O_2^-$ and H_2O_2 stimulate stress-related signaling mechanisms such as NF-κB, p38-MAPK and STAT-JAK resulting in vascular smooth muscle cell (VSMC) migration and proliferation. In endothelial cells, H_2O_2 facilitate apoptosis and pathological angiogenesis (Taniyama and Griendling, 2003).

When endothelial cells are exposed to hyperglycemia at the levels related to clinical diabetes, there is increased production of ROS and especially $^{\bullet}O_2^-$, which introduce the activation of four major pathways involved in the progression of diabetic complications. Nishikawa and colleagues elegantly evidenced, that production of surplus pyruvate via enhanced glycolysis under hyperglycemic conditions floods the mitochondria and causes

$^{\bullet}O_2^-$ propagation at the level of Complex II in the respiratory chain. The obstruction of $^{\bullet}O_2^-$ radicals by three different approaches using either a small molecule un-coupler of mitochondrial oxidative phosphorylation (CCCP), over expression of uncoupling protein-1 (UCP1) or over expression of Mn-SOD, avoid the changes in NF-κB as well as polyol pathway, AGE formation and PKC activity. According to this information, it has been suggested by several groups that mitochondrial $^{\bullet}O_2^-$ is the initiating snowball that turns oxidative stress increase in diabetes by motivating more ROS and RNS production via downstream activation of NF-κB-mediated cytokine formation, PKC and NADPH oxidase (Fig 1.2). Thus, inhibition of intracellular free radical accumulation would provide a causal therapy access in the prevention of oxidative stress and associated vascular complications in diabetes (Johansen *et al.*, 2005).

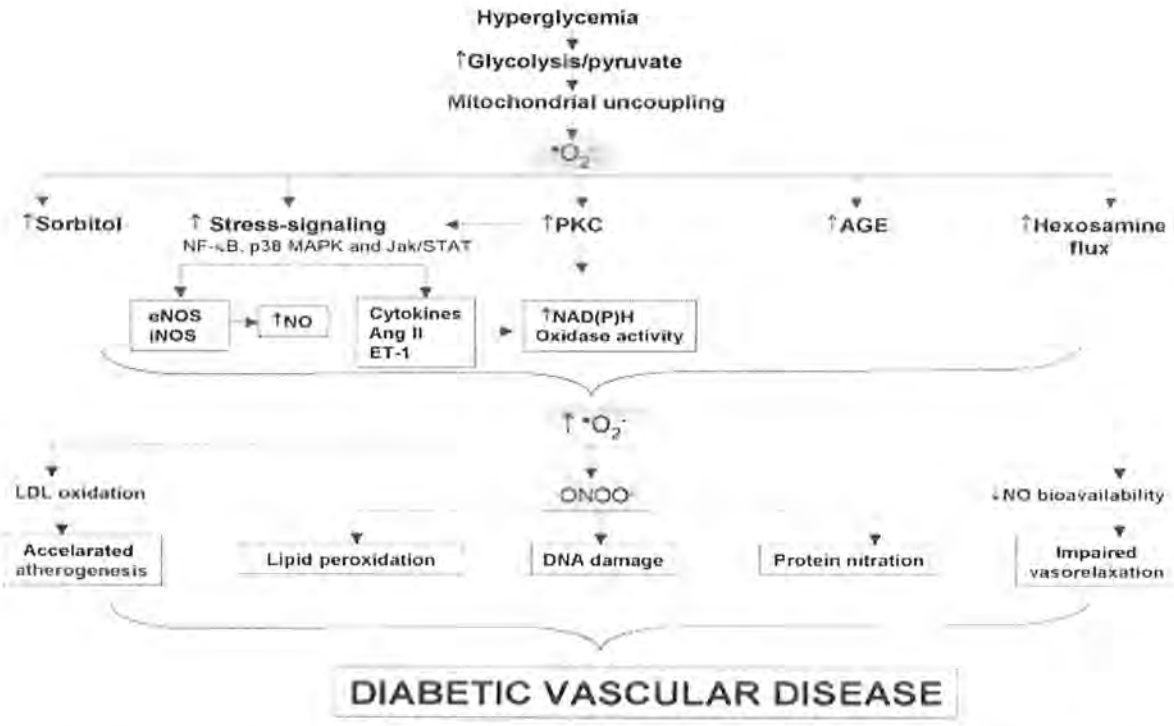

Fig 1.2: Current working model for the generation of reactive species and downstream targets in diabetes. Overflow generation of mitochondrial ROS due to hyperglycemia introduce a vicious circle by: accelerating stress-sensitive pathways such as NF-κB, p38 MAPK and Jak/STAT, polyol (sorbitol) and hexosamine pathways, PKC

and AGEs. Elevated production of AGEs, sorbitol and pro-inflammatory cytokines maintains a positive feedback on ROS and RNS synthesis and potentiates PKC-mediated vascular dysfunction by changing gene expression as well as vascular function and structure (Johansen *et al.*, 2005)

Insulin resistance syndrome (IRS) or metabolic syndrome X (Reaven, 1995; Chisholm, 1997) is characterized by a group of metabolic and haemostatic abnormalities, most of which represent independent risk factors for the progression of type II diabetes (see Fig. 1.3). These include impaired glucose tolerance, hyperinsulinemia, hypertension, dyslipidemia, a pro-thrombotic/hypo-fibrinolytic state, oxidant stress and endothelial dysfunction (Reaven, 1995; Chisholm, 1997).

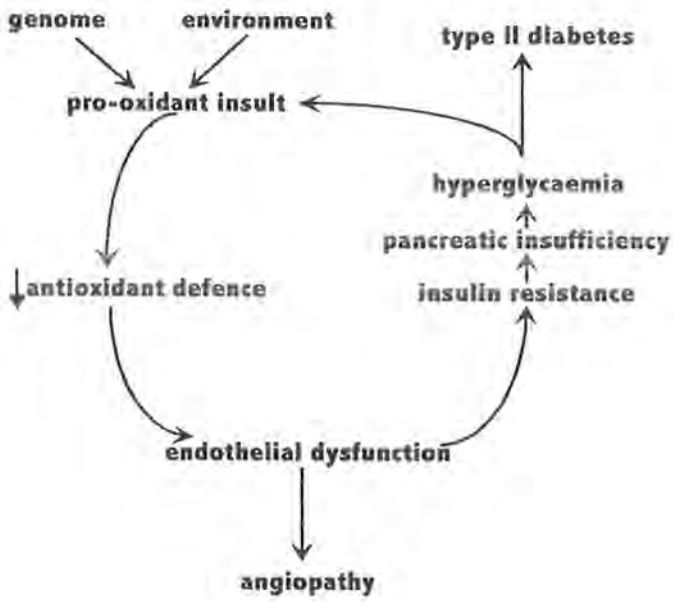

Fig 1.3: Hypothetical scheme linking oxidant stress, endothelial dysfunction and insulin resistance in the setting of type II diabetes (Laight *et al.*, 2000).

It is reported that an increased risk of macroangiopathy in both diabetes and the prediabetic state represented by IRS, resulting especially from atherosclerotic and thrombotic pathologies (Stamler *et al.*, 1993). Such macroangiopathy is often present at the diagnosis of type II diabetes (Took *et al.*, 1996) and the correlated coronary artery,

cerebrovascular and peripheral vascular disease are principal causes of diabetic morbidity and mortality (Laight *et al.,* 2000).

In liver cells, as in hyperglycemia, TNF-α increases mitochondrial ROS, which in turn initiates apoptosis signal-regulating kinase 1 (ASK1) and c-jun NH_2-terminal kinases (JNK), increases serine phosphorylation of IRS-1, and decreases insulin-stimulated tyrosine phosphorylation of IRS-1, dominating to insulin resistance (Nishikawa and Araki, 2007).

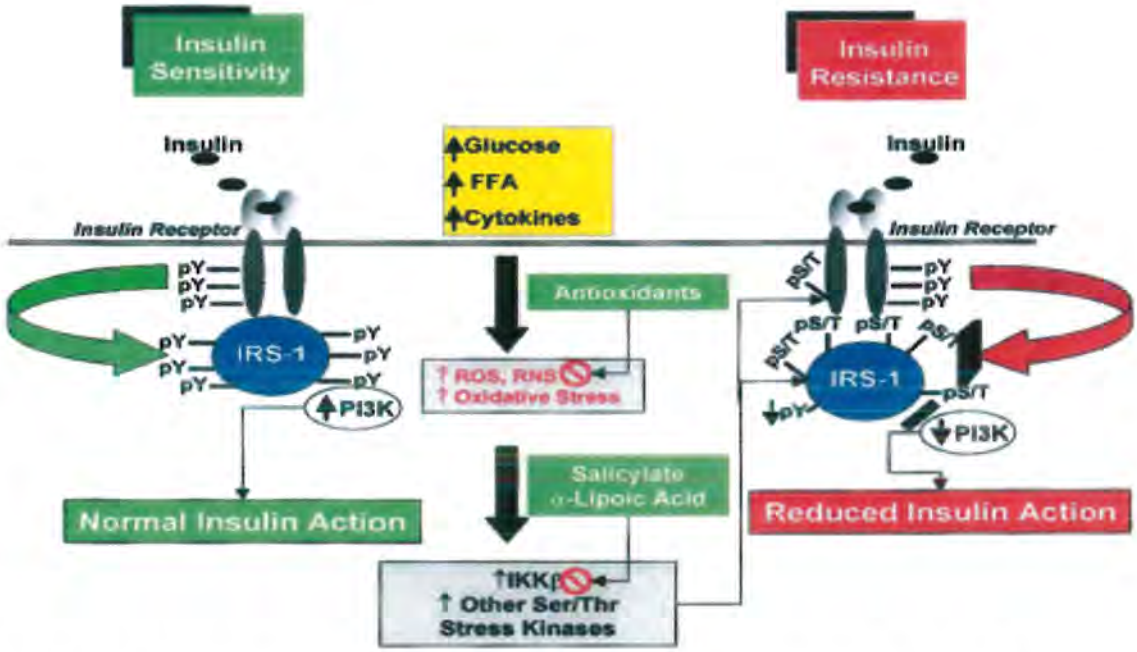

Fig 1.4: The role of serine kinase activation in oxidative stress induced insulin resistance. A range of stimuli, including hyperglycemia, raised, FFA levels, cytokines and others increase ROS (and RNS) formation and oxidative stress. In the result the initiation of several stress-sensitive serine/threonine (Ser/Thr) kinase signaling avalanche such as IKK-β and others. Once activated, these kinases are able to phosphorylate many targets, such as the IR and IRS proteins (including IRS-1 and IRS-2). Increased phosphorylation of IR or IRS proteins on distinct serine or threonine sites (pS/T) decreases the effect of insulin-stimulated tyrosine phosphorylation (pY) (Birnbaum, 2001; Evans *et al.,* 2003).

Accordingly the association and/or activities of downstream signaling molecules (e.g., phosphatidylinositol 3-kinase [PI3K]) are decreased, resulting in reduced insulin action (insulin resistance). The possessive effects of antioxidants (e.g., LA) on oxidative stress induced insulin resistance could relate to their ability to protect the intracellular redox balance (neutralizing ROS) or, corresponding to pharmacological agents (e.g., salicylates, p38 MAPK inhibitors), to prevent the activation of stress-sensitive kinases (Maddux *et al.*, 2001).

Glomerular hyper filtration is the important characteristics of early diabetic nephropathy, could be caused by mitochondrial ROS through activation of COX-2 gene transcription and continued by PGE_2 overproduction. In pancreatic β cells, mitochondrial ROS also increases by hyperglycemia which block out the first phase of glucose-induced insulin secretion, at least in part, through the inhibition of GAPDH activity (Nishikawa and Araki, 2007).

The oxidative stress is involved in the development of diabetes and its complications. Use of antioxidants decreased oxidative stress and diminished diabetic complications. Diabetes is differentiated by absolute or relative deficiencies in insulin secretion and/or insulin action correlated with prolonged hyperglycemia and disturbances of carbohydrate, lipid, and protein metabolism (Rahimi *et al.*, 2005).

1.4.1 Antioxidant

Antioxidants counter the action of free radicals by different mechanisms. These mechanisms comprise: (1) enzymes that impair free radicals, (2) proteins such as transferrin that can bind metals which excite the formation of free radicals, and (3) antioxidants such as vitamins E and C that act as free radical scavengers (Penckofer *et al.*, 2002).

The body's defense against oxidative stress is achieved by interconnecting systems of enzymes and antioxidant micronutrients (vitamins and minerals). Specifically, it has been reported that high doses of single micronutrient antioxidant supplements, such as vitamin E, may be effective to patients suffering from this disease (Opara, 2002).

However, micronutrient antioxidants correlate with each other in a biochemical chain of defense against free radicals, and the use of high doses of a single antioxidant poses potential risks because it could disturb the antioxidant-pro oxidant balance (Opara, 2002).

Previously it is reported that a high vitamin C intake from supplements is correlated with an increased risk of cardiovascular disease mortality in postmenopausal women with diabetes (Lee *et al.*, 2004).

New supplement reported to be formulated as a mixture of anti-diabetic trace elements and antioxidant vitamins, adequately balanced with other vitamins and minerals that intensify the metabolic processes (Opara, 2002). In comparison to vitamin A, the vitamin C and E combination can also be safely used in high doses to prevent diabetes and cardiovascular disease (Rahimi *et al.*, 2005).

1.4.2 Platelets, oxidative stress, and Diabetes Mellitus

In addition to cardiovascular syndromes, other diseases have been coupled with redox imbalance, reactive oxygen species production, and altered platelet function. Intracellular Ca^{2+} homeostasis in platelets of patients with non-insulin-dependent diabetes mellitus (NIDDM) has been reported to be distorted, leading to an augmented adhesiveness and spontaneous aggregation. Treatment of platelets from NIDDM patients with reactive oxygen species inhibitors can change these processes (Jardin *et al.*, 2006). It has also been revealed that hydrogen peroxide and peroxyl radicals are likely involved in the improved Ca^{2+} mobilization. It is observed in platelets from patients with type 2 diabetes, prospectively leading to platelet hyperactivity and hyperaggregability (Redondo *et al.*, 2005). In patients with hypercholesterolemia, platelet-associated NADPH oxidase creates a thrombogenic phenotype and mediates the arteriolar dysfunction (Stokes *et al.*, 2007).

Platelet superoxide production in patients with hypertension alone and in patients with coexistent diabetes mellitus has been observed. It was exposed that eNOS (endothelial nitric oxide synthase) can reside in the uncoupled state in patients with hypertension and, to a great level, in patients with coexisting hypertension and diabetes, and that this contributes significantly to increased superoxide production in these disease states (Dixon *et al.*, 2005).

1.5 Role of NADPH oxidase enzyme and it cofactor

NADPH oxidase is an enzyme that catalyzes the production of superoxide from NADPH and oxygen. It is a complex enzyme comprises of two membrane-bound components and three components in the cytosol, plus rac 1 or rac 2. The phosphorylation of one of the cytosolic components is essential for the activation of the oxidase. Latest crystallography data specify that the tail of this cytosolic component lies in a groove between two Src homology 3 domains and, when phosphorylated, the tail leaves the groove and is replaced by the tail of one of the membrane-bound components (Babior, 2004).

1.5.1 Composition of the NADPH oxidase

1.5.1.1 Multiprotein enzyme complex

The NADPH oxidase is a vastly regulated membrane bound enzyme complex that is composed of a number of cytosolic and membrane-bound proteins (Henderson and Chappel, 1996). In resting cells, the NADPH oxidase is inactive and its protein components are isolated into cytoplasmic and plasma membrane compartments. Because the active enzyme complex is located at the plasma membrane, the cytosolic proteins must translocate from the cytosol to the membrane through the assembly of the functional enzyme complex (Clark *et al.*, 1990).

1.5.1.2 Flavocytochrome b

The vital component of the NADPH oxidase is a heterodimeric transmembrane protein known as flavocytochrome b (Rotrosen *et al.*, 1992), Flavocytochrome b is situated in the specific granule and plasma membranes (90 and 10%, respectively) of resting neutrophils (Jesaitis *et al.*, 1990) and is consisted of a glycosylated 91-kDa subunit, gp9l-phox (phox refers to phagocyte oxidase), and a non glycosylated 22-kDa subunit, p22-phox (Parkos *et al.*, 1988). Subsequently neutrophils activation, flavocytochrome b in the precise granule membranes is transported to the plasma or phagosomal membrane (Quinn *et al.*, 1992) possibly chaperoned by the low-molecular-weight GTP-binding protein, RaplA, which is coupled with flavocytochrome b in neutrophils (Quinn *et al.*, 1992).

1.5.1.3 RaplA

RaplA is a low-molecular-weight GTP-binding protein of the Ras super family of GTP-binding proteins and has been expose to be coupled with flavocytochrome b (1:1 complex) (Quinn *et al.*, 1992). The localization of RaplA in human neutrophils mirrors that of flavocytochrome b in the plasma membrane and precise granules; on the other hand, in resting neutrophils there is three to four times more RaplA at the plasma membrane than flavocytochrome b, and this ratio becomes equimolar only after full degranulation and flavocytochrome b translocation (Quinn *et al.*, 1992).

1.5.1.4 P47-phox

P47-phox travels as a 47-kDa protein on sodium dodecyl sulfate (SDS) polyacrylamide gels (Volpp *et al.*, 1989) and is plentiful in the cytosol of myeloid cells (Nauseef, 1993). In resting neutrophils cytosol, p47-phox exists in a free form, as well as in a 240-kDa complex, comprising of equimolar amounts of p47-phox, p67-phox, and p40-phox (Iyer *et al.*, 1994). Subsequent neutrophils activation, the entire complex apparently translocates to and associates with the plasma membrane-bound components. In addition, free p47-phox is also transferred to the membrane (Iyer *et al.*, 1994). In any case, p47-phox seems to be the first cytosolic component to interact with flavocytochrome b during the gathering process (Heyworth *et al.*, 1991).

1.5.1.5 P67-phox

P67-phox migrates as a 65- to 68-kDa protein on SDS-polyacrylamide gels and consists of two Src homology 3 (SH3) domains surrounding residues 245-295 and 458-517 (Leto *et al.*, 1991). Like p47- phox, p67-phox has been accounted to exist in a 240-kDa cytosolic complex in resting neutrophils (Iyer *et al.*, 1994).

1.5.1.6 P40-phox

P40-phox was recognized in fractionated resting neutrophils cytosol via its binding to immune precipitated p67-phox (Wientjes *et al.*, 1993). It travels as a 40-kDa protein on SDS polyacrylamide gels, contains a single SH3 domain (residues 175-226), and considerable homology with the amino terminus of p47-phox. As with p47-p/wx and

p67-phox, p40-phox emerges to reside within a 240-kDa complex in neutrophils cytosol (Wientjes *et al.*, 1993).

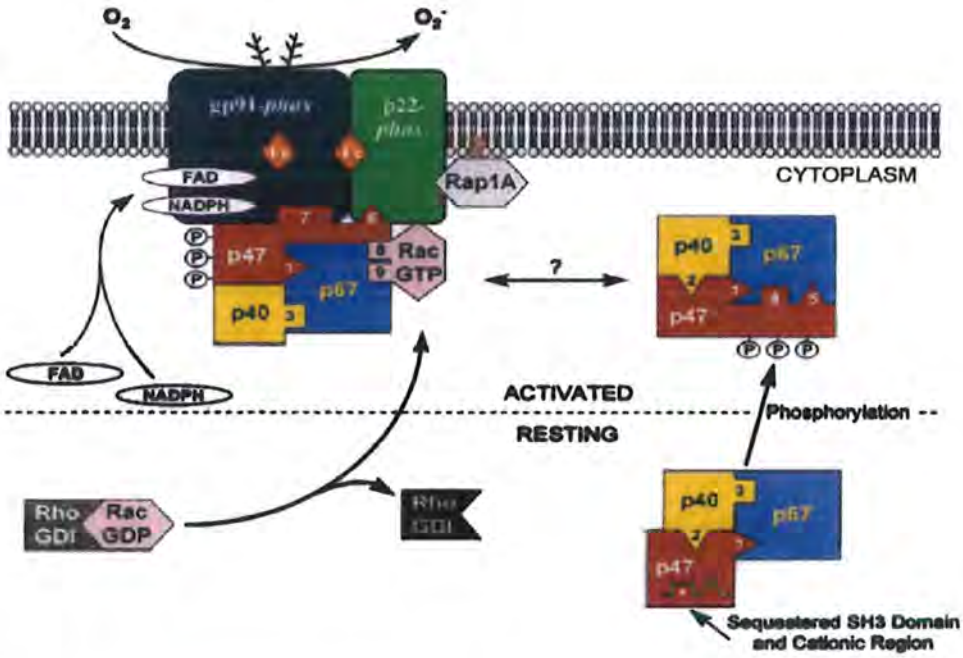

Fig 1.5: Model illustrating the role of protein. Protein binding relations in assembly and activation of the human neutrophil NADPH oxidase. This hypothetical model of the NADPH oxidase characterize a comprehensive review of the activation and assembly process. Residency of the oxidase protein components is based on compiled information obtained from presently reported interactions. In this model, SH_3-mediated binding interactions are revealed as a rectangular shapes and non-SH_3 interactions are revealed as rectangular shapes.

1.4.1.7 Rac

Rac is an 22 kDa low-molecular-mass GTP-binding protein (Didsbury *et al.*, 1989) that was known as an NADPH oxidase cofactor concurrently by two groups who showed that it was vital for optimal O_2^- generation in neutrophils Rac1 was identified in guinea pig neutrophils (Abo *et al.*, 1991) whereas Rac2, 92% homologous with Rac1, was simultaneously identified in human neutrophils (Knaus *et al.*, 1991).

In resting neutrophils cytosol, Rac is multifaceted with a guanine nucleotide exchange factor known as Rho GDP-dissociation inhibitor (GDI) (Abo *et al.*, 1991), and it has been suggested that GDI functions in part to keep Rac in a soluble, GDP-bound form in the resting cell cytosol (Quinn, 1995). Following neutrophils activation, Rac disconnects from Rho GDI, and Rac subsequently translocate to the plasma membrane (Abo *et al.*, 1994).

1.5.2 Role of NADPH oxidase in Diabetes Mellitus

Diabetic patients have raised risk of developing micro vascular complications and cardiovascular disease (CVD), with development of the disease leading to blindness, end-stage renal failure, and atherosclerosis (Mazzone *et al.*, 2008). Reactive oxygen species (ROS) produced hyperglycemia are concerned in the development and progression of diabetic vascular complications and often correlated with endothelial dysfunction (Srinivasan *et al.*, 2004). In diabetes, Vascular NADPH oxidase, mitochondrial dysfunction, and uncoupled endothelial nitric oxide synthase (eNOS) all contribute to oxidative stress (Gao and Mann, 2009).

NADPH oxidase has been implicated as the most important source of ROS generation in the vasculature in response to high glucose and advanced glycation end-products. Sustained activation of NADPH oxidase in diabetes may reduce intracellular levels of NADPH, a critical cofactor for endothelial NO synthase (eNOS) and several antioxidant systems (Gao and Mann, 2009). Latest data recommends that basal ROS production via

NADPH oxidase may up regulate antioxidant enzyme defenses via redox signaling. Thus, NADPH oxidase might be serve as a double-edged sword, with transient activation provided that a feedback defense against excessive ROS generation through the activation of receptor tyrosine kinases and the redox-sensitive Nrf2-Keap1 signaling pathway. Overproduction of ROS leads to eNOS uncoupling, mitochondrial dysfunction and weakened antioxidant defenses owing to reduction of intracellular NADPH (Gao and Mann, 2009).

The first known example of regulated production of reactive oxygen species (ROS) in mammalian cells was in the course of the respiratory burst of phagocytic cells by

nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. This enzyme complex uses electrons derived from intracellular NADPH to create superoxide anion, which is further processed to form hydrogen peroxide and other ROS-providing host defense against bacterial and fungal pathogens (Quinn and Gauss, 2004).

1.5.3 Homologues of NADPH oxidase

Homology searches in human genome databases consequence in the discovery of six novel NADPH oxidase enzymes: Nox1, Nox3, Nox4, Nox5, Duox1 and Duox2 (Sumimoto *et al.*, 2005). They all have at least moderately similar structure and generate ROS in response to various stimuli. Nox1, 3 and 4 are related in size and domain structure to $gp91^{phox}$ (Cheng *et al.*, 2001). Among them, Nox1 and Nox3 illustrate the closest homology to Nox2, while Nox4 is more distantly associated to Nox2. Nox5 is larger than Nox2 because it has four additional cytosolic EF hands (motifs from the parvalbumin E & F helices) on its *N*-terminus (Banfi *et al.*, 2001). These motifs are conscientious for the regulation of proteins by Ca^{2+}. Duox1 and 2 are the leading members of the Nox/Duox family (Donko *et al.*, 2005). They are called Dual oxidases because they acquire an *N*-terminal peroxidase-homology domain in addition to their C-terminal NADPH oxidase domain. Between the NADPH oxidase portion and the peroxidase-like domain there is an additional trans-membrane domain and two EF-hand motifs, representing ends of regulation by calcium ions (Orient *et al.*, 2007).

1.5.4 Activation of NADPH oxidase in Diabetic Nephropathy

In Diabetes Mellitus the excretion of hydrogen peroxide (H_2O_2), lipid peroxidation products (LPO), nitric oxide products (NOx), and protein has been increased. The kidneys of rats with DM had increased expression of p47phox and gp91phox and endothelial nitric oxide synthase (eNOS), and improved mesangial matrix with expression of fibronectin and collagen I. Apocynin averted the increase in excretion of H_2O_2, LPO, and protein in diabetic rats, increased renal NOx generation, and prevented the raised renal expression of gp91phox and the membrane fraction of p47phox, and reverted the mesangial matrix expansion (Kensuke *et al.*, 2004).

ROS from a Nox be the cause of the progression of diabetic nephropathy. Diabetes

mellitus increased excretion of H_2O_2, lipid peroxidation, and protein (Asaba *et al.*, 2005; Ohshiro *et al.*, 2006). Kidneys of rats with diabetes mellitus had increased expression of Nox2, $p47^{phox}$, and Nox4 (Ohshiro *et al.*, 2006), increased membrane translocation of $p47^{phox}$ (reflecting Nox2 activation) (Asaba *et al.*, 2005) and better mesangial matrix.

Apocynin prohibited the increased H_2O_2, lipid peroxidation, and protein in diabetic rats, prevented the increased renal expression of Nox2 and membrane translocation of $p47^{phox}$, and blocked the mesangial matrix expansion (Asaba *et al.*, 2005; Lambeth 2007)

1.6 Technique used for Elemental analysis

1.6.1 Proton Induced X-ray Emission (PIXE) Technique

1.6.1.1 History of PIXE

The foundation of proton induced x-ray emission (PIXE) was laid in 1914 by Mosley in his pioneering study of the energy of characteristics X-ray lines of different elements of the periodic table. In 1922 the Swedish Geologist Hadding at the University of Lund reported on the analysis of various minerals. He detected 10 to 12 elements in sample but the main problem in his work was the heating of the sample.

During 1960, nuclear detector technology was improved. The Lithium drifted Silicon (Si Li) detector became available for X-ray Spectroscopy at the energy resolution of about 150Kev which makes it possible for K X-rays. In 1970 Johansson at the Lund Institute of Technology showed a combination of excitation with 2 MeV protons with Si Li detector constituted a very powerful method for elemental analysis. This new analytical technique is known as Particle Induced X-ray Emission based on a Nuclear Physics technique.

1.6.1.2 Introduction of PIXE

PIXE is a powerful and relatively simple analytical technique that can be used to identify and quantify trace elements typically ranging from atomic number 11 to 92. PIXE is an elemental analysis technique in which the energy of emitted characteristics X-rays, when a sample is bombarded with a beam of energetic protons is used to identify the elements present in the sample. PIXE technique depends on the X-rays production cross-section, the fluorescence yield and the background radiation for a particular energy of the proton

beam. Sample irradiation is usually performed by means of 2-3 MeV protons produced by an accelerator. X-ray detection is usually done by energy dispersive semiconductor detectors such as Si (Li) detectors. With the use of proton beam, two major advantages follow. There is a higher rate of data accumulation. Secondly, although it does still contain a range for best sensitivity ($20 < Z < 40$), the overall sensitivity in lower Z elements is increased.

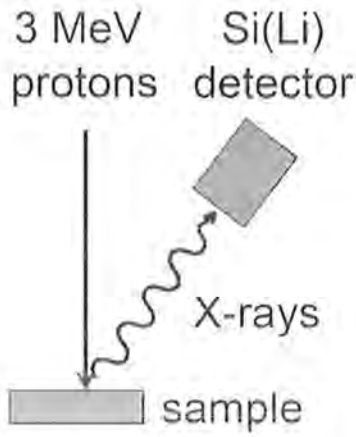

Fig 1.6 Schematic of PIXE

(http://www.ams.ethz.ch/research/material/iba/pixe/pixe.png?hires)

1.6.1.3 Specification of PIXE

i. The sample damage is very low, PIXE is considered to be non-destructive technique.

ii. Precision for PIXE analysis is 10%.

iii. It has sensitivity up to 1ppm for detection with probing depth of tens of μm.

iv. PIXE is 100 times more sensitive than EDX.

v. PIXE is a multi elemental technique allowing the detection of elements in the periodic table with atomic number from 11 to 92.

vi. PIXE is rapid and fast technique.

vii. Quantifying ratios of elements that cannot be resolved by RBS.

viii. For most of the elements and samples the limit of detection is of order of 1 ppm.

ix. Quantitative analysis is possible for extremely small amount of samples (less than 1 mg).

1.6.2 Applications of PIXE

1.6.2.1 Biological Study

Considerable efforts extended in the past ten years in the development of PIXE have been investigating biological and medical samples. This arises not only because of the versatility and ease with which PIXE can be used to obtain quantitative and multi-elemental information on small samples but also because of the nondestructive nature of the technique with minimal beam induced effects on the specimen itself, as compared with more classical physical and chemical analyzing methods. Trace elements have an important function in biological systems and the various concentrations play an important part in diagnosis of diseases. For example, many clinical and pathological disorders arise in animals and men as a consequence of trace element deficiencies or excesses. Cadmium concentrations in kidney found a correlation with age and the state of the disease under study. The trace element present varied with age, sex and dietary habits of the patients from a particular region. PIXE has been extensively used for the analysis of hair, blood serum and saliva samples. Other applications of PIXE are in aerosol study, archaeological study, geological study and plants (Johansson and Campbell, 1988).

MATERIALS AND METHODS

2. Materials and Methods

2.1 Blood sampling

Blood sampling was carried out, after taking permission from Executive Director of Pakistan Institute of Medical Sciences and head of medical department. Samples were obtained from 60 patients of diabetes mellitus. Blood was taken from patients after their consent under the International rules and regulations. In addition 30 blood samples of healthy control individuals were taken. 3 ml blood collected in BD-Vacutainer through BD-syringes. BD-Vacutainer tubes were placed in ice box for purpose of quenching of ROS (Reactive Oxygen Species).

2.2 Serum extraction

After half an hour of blood sampling, these tubes were centrifuged at 4000 rpm for 10 minutes for serum extraction. Serum was then collected in autoclaved eppendorf tubes with the help of micropipette and stored at -20°C in refrigerator.

2.3 Reagents

N,N-diethyl-para-phenylendiamine (DEPPD) sulphate and ferrous sulfate ($FeSO_4$) were purchased from SIGMA chemicals. H_2O_2 was obtained from E-Merck. All the chemicals were commercially available.

2.3.1 Reagent preparation

DEPPD was dissolved in 0.1 M sodium acetate buffer (pH 4.8) to attain final concentration of 100 µg/ml (R_1 solution as a chromagen) and ferrous sulphate was dissolve in 0.1 M sodium acetate buffer (pH 4.8) to attain a final concentration of 4.37 µM (R_2 solution as a transition metal ion). H_2O_2 solution attenuated to specified concentration was used as standard solution for generating a calibration curve.

2.4 Total ROS assay system by DEPPD method

For this process Pyrex cuvette was used and following steps were involved :

i. 1000 µl of mixture solution which was prepared from R_1 and R_2 at a ratio of 1:25 in falcon tube.

ii. 1400 µl of sodium acetate buffer (pH 4.8) was added in it and placed in the dark room for 30 minutes.

iii. 60 µl of serum added to above mixture of sodium acetate buffer and R_1 and R_2 in cuvette and gently mixed.

iv. Agilent 8453 UV-Visible Spectrophotometer was used for study purpose. Absorbance at 505 nm was measured for a fixed time (between 60 second) at 15 second interval.

v. A calibration curve was automatically constructed from the slopes, which were calculated based on varying absorbance at 505 nm each time (min) corresponding to the concentration of hydrogen peroxide.

2.5 Sample loading for mineral analysis through PIXE

100 µl of serum sample loaded on the accelerator graphite discs, and dried overnight with the help of sticky graphite discs were sticked on the aluminum holder and placed in the 15° end station of 5UDH-2 Pelletron accelerator for the sample analysis.

2.6 5UDH-2 Pelletron accelerator at National Centre for Physics

2.6.1 Introduction

We utilized the accelerator at NCP. The accelerator at NCP is U-series accelerator. The model 5-UDH pelletron accelerator is a dual acceleration Tandem electrostatic accelerator. This accelerator is capable of delivering energies of 0.8 to 30 MeV. The 5 MV rated terminal voltage can be reached within a few minutes after accelerator start up. The 5 MV Tandem (Pelletron) accelerator equipped with two types of ion sources: radio frequency ion source (Alphatross) which supplies beams of alpha particle and protons while the source of negative Ions by cesium sputtering (SNICSII) supplies ions of almost all elements of the periodic table. Currently ion beams of hydrogen, carbon, silicon, nickel, copper and gold are available. Two end stations are available at 15° and 30° beam lines. The 15° end station is meant for the materials characterization using all IBA techniques while the 30° end station is used for the nuclear physics experiments.

Table 2.1 5UDH-2 Pelletron accelerator technical specifications

Model	5UDH-2
Manufacturer	NFC, USA
Terminal voltage	5 MV(maximum)
Charging	Pelletron charging system
Ion source	SNICII and RF Source
Ion beams	H, He, C, Si, Ni, Cu, & Au
Beam lines	Two (15° and 30°)
Beam energy	Up to 30 MeV
Beam current	200 nA
Vaccum requirement	10^{-7} to 10^{-9} Torr
Number of targets	6 samples at a time can be placed in the target chamber
Control system	LINUX based control system

2.6.2 5UDH-2 Pelletron accelerator operation

There are two ion sources SNICSII and RF. Negative ion beam produces from sources. Extractor extracts the beam and accelerates the emerging beam from the source to a maximum of 75 KeV. The beam current is measured by Faraday cup from the extractor. The beam reaches to the injector magnet. The beam is injected by the magnet to the accelerator tube. The beam is also focused by steerer in a horizontal as well as in vertical direction. Now beam enters in to the tank through the Enzel lens. The acceleration tube is enclosed by a tank which is filled by with insulating gas i.e., SF6. In the centre of the tank is the high potential terminal. The charging of high voltage terminal to 5 MV is done by using the pelletron charging chains. In the terminal negative ions are stripped off few

electrons using carbon foil or some gases as a stripper. The beam energy gain by the ion in the accelerating tube at the end of the tube is determine by

$$E = (n + 1) V$$

V is the terminal voltage and n the positive charge after stripping. The beam will be stripped and become positive beam and then accelerated towards the high energy side. The accelerated beam reaches at the End station through switching magnet.

2.6.3 Component of 5UDH-2 Pelletron accelerator

The building blocks of 5UDH 2 Pelletron are the following

i. Two ion sources
ii. 5.0 MV Tandem ion electrostatic accelerator
iii. Injecting and analyzing magnets
iv. Beam lines
v. End stations for materials modification and analysis

The various components are described below:

2.6.3.1 Ion sources

An ion source is an electro magnetic device that is used to create charged particles. These are used primarily with in mass spectrophotometers, particle accelerators, ion implanters and ion engines. In particle accelerators an ion sources creates a particle beam at the beginning of the machine, the source. Protons are generated with a plasma based device, like a duoplasmatron or a magnetron.

The purpose of the ion source is to produce either positive or negative ions from neutral atoms. Different types of sources may be used depending on the mass and charge of the desired ion. These ions beam with few KeV are injected to pre accelerator tubes which are accelerated up to 70 KeV. Then the ion beams are injected to the accelerator for gaining higher energy.

There are two ion sources available at NCP 5 MV pelletron namely; Alpha tross exchange ion source and the SNICS II ion source. And the current study analysis was carried out by SNICS II.

2.6.3.1.1 SNICS-II ion source

The source of negative ions by cesium sputtering (SNICS) produces a negative ion beam. Cesium vapour comes from the cesium oven into an enclosed area between the cooled cathode and the heated ionizing surface. Some of the cesium condenses on the front of cathode and some of the cesium is ionized by the hot surface. The ionized cesium accelerates towards the cathode, sputtering particles from the cathode which pass through the condensed cesium layer. Some materials will preferentially sputter neutral or positive particles which pick up electrons as they pass through the condensed cesium layer, producing negative ions. SNICS II components are:

i. Cathode
ii. Cs oven
iii. Ionizer
iv. Extractor
v. Focus
vi. Cs supply line heater
vii. Coolant pump

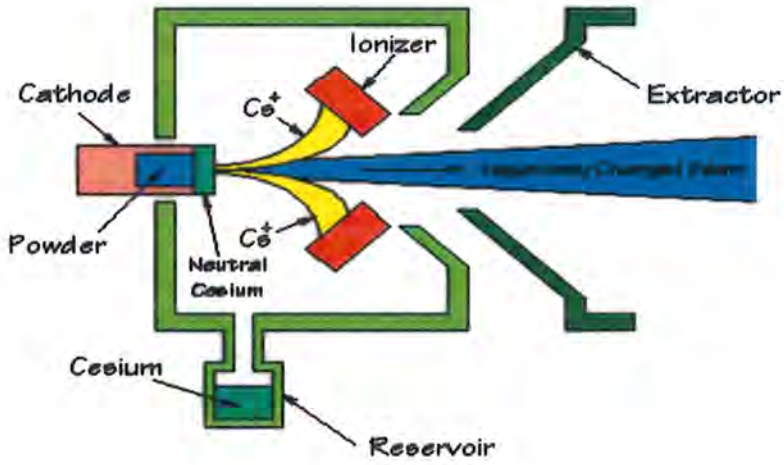

Fig 2.1: SNICS II source

(http://nd.edu/~nsl/html/research_SNICS.html)

The cathode contains element(s) for beams. Current of the order of hundreds of microamperes emerges from the source depending on the cathode material.

5UDH 2 Pelletron consists of Injected magnet, Einzel lens, Electrostatic beam steerers, Beam profile monitors, Faraday cups, Switching magnet, Tank, Vacuum system, Electrostatic quadrupole lenses, Slit system (remote or manual).

2.6.3.2 Beam Line Extension

There are two extended beam lines available at the NCP 5MV Pelletron.

i. 15 degree beam line for the material analysis through ion beam techniques.
ii. 30 degree beam line for ion scattering /nuclear physics reaction studies.

The 15^0 beam line is dedicated to carrying out materials analysis work and current study analysis was carried out by 15 degree beam line.

2.6.3.3 End stations

There are two end stations at the 5 MV NCP Pelletron.

i. NEC RC43 Analytical End station.
ii. NEC RS61 Scattering End station.

Currently we utilized NEC RC43 Analytical End station and Silicon Lithium (SiLi) Detector was used for analysis of trace elements.

2.6.4 Data analysis

The analysis of PIXE spectrum presents little difficulties when a sample contains only a few elements. Each of these elements may give rise to one or more X-ray peaks due to the K, L and M shell transitions, subdivided to give peaks corresponding to K_α, K_β, L_α, L_β, L_γ etc. Some of which are capable of being resolved. Thus a large number of both small and large peaks can be expected in samples. There is a high probability that a number of the X-ray peaks will be overlapping and interfering with each other. This can make data reduction, and interpretation complicated. Even when the number of samples to be analyzed is small, the use of a computer program to unravel overlapping peaks is often useful. When routine analyses are to be performed on a variety of samples of

differing compositions, a spectral analysis program is essential. Software, like AXIL, DATTPIXE, GEOPIXE, GUPIX (GUPIXWIN), PIXAN etc can be used for this purpose.

2.6.4.1 Data Analysis Software

Data Analysis Software at NEC RC43 end stations are.

i. RMP for RBS/ERDDDDA spectrum analysis.
ii. SIMNRA for nuclear reaction analysis.
iii. GUPIX for X-ray spectrum analysis.

Currently we utilized GUPIX for X-ray spectrum analysis.

2.6.4.1.1 Gupixwin software

Maxwell *et al.* (1989) GUP IXWIN is a versatile software package for fitting PIXE spectra from thin, thick, intermediate and layered specimens. It extracts peak intensities and converts these to concentrations via the H-value standardization method. X-ray excitation may be via protons, deuterons, helium ions. The original GUPIX package contains the FORTRAN programs GUPIX, GUCSA and GUYLS. GUPIX is an extensive program for least-square fitting PIXE spectra and converting peak areas into elemental concentrations. GUSCA provides ionization cross section, stopping powers and other aspects of the GUPIX database. GUYLS computes the X-rays yields from thin and thick layered target. GUPIX has been developing since the late 1980s and it now includes many helpful and sophisticated features that enable the user to get highly accurate results from PIXE analysis. But the final responsibility for accuracy and precision rests fully with the user, because issues of sample preparation, beam optics charge integration are as important as the PIXE software and also experience with PIXE is required in order to use the GUPIX package competently. GUPIX runs under DOS on a Pentium based computer. GUPIXWIN runs under windows XP PRO.

GUPIX capabilities include:

i. Calculating X-ray excitation by different sources such as protons, deuterons and helium ions.
ii. Evaluation of a spectra of up to 60 elements from Z=6 to Z=92.
iii. Evaluation of data from thin, intermediate, thick and multilayered targets.

When using GUPIX to calculate the elemental concentration in a specimen these input information are required such as:

i. The bombarding particle type and the incident and exit energies.
ii. A calibration factor, H, which is used to convert peak areas to concentration values. H is calculated after analysis of a standard.
iii. The target geometry, which is specified by the incident angle of the beam to the target.
iv. Information on materials between the target and the detector (e.g. filters).
v. Matrix composition as well as identifying the elements present in the sample.

After the calculations are completed, GUPIX produces information for the elemental concentrations of the elements present, detector efficiency, statistical error, fit error and limits of detection.

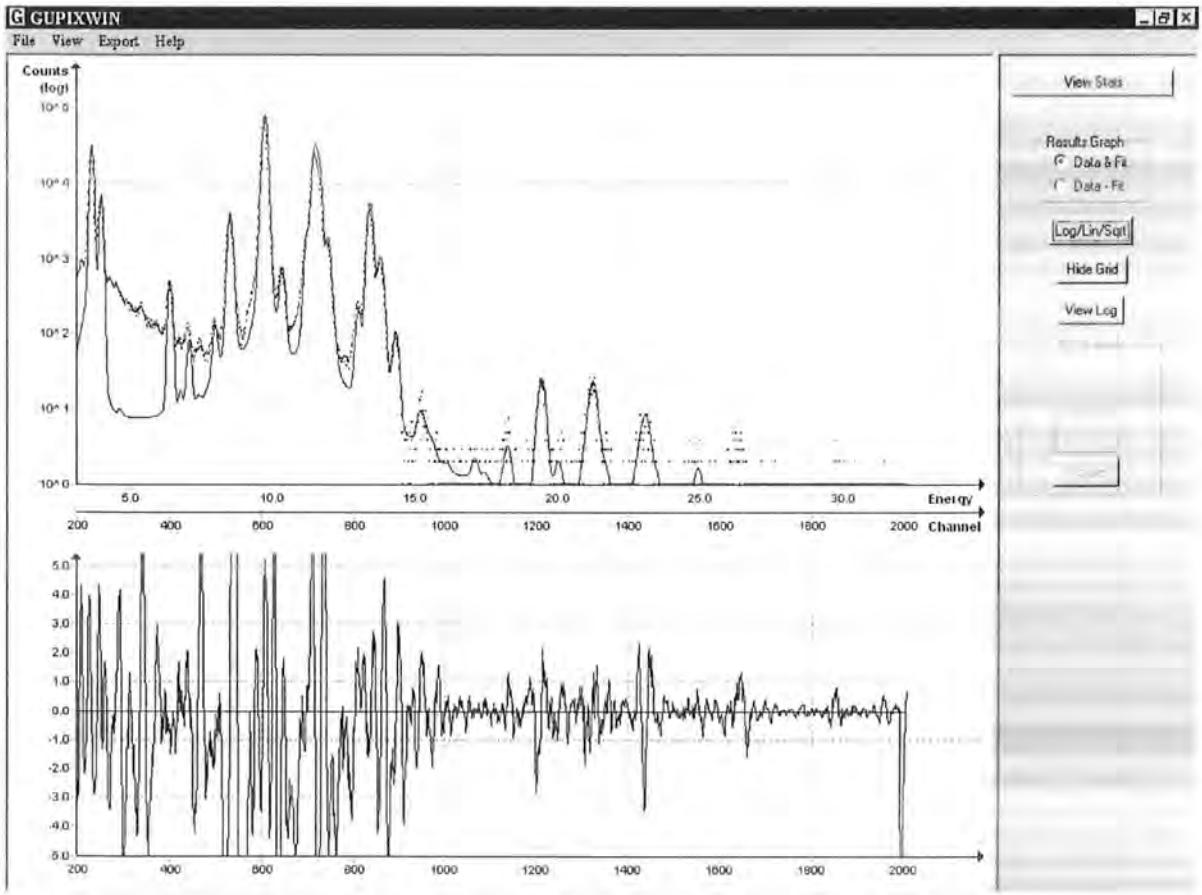

Fig 2.2: Schematic diagram of GUPIXWIN window (J. A. Maxwell *et al.*,. 1989)

2.7 Statistical analysis

SPSS for windows program (version 9.0) was used for statistical analysis. GUPIX WIN software was used for elemental analysis. Association between serum ROS levels and parameters such as physical activity, family, age, hypertension, cardiovascular disease in control and patient samples was examined by HSD Tukey test using one way ANOVA. Comparison between different parameters was constructed by graphed prism software (version 5.04). Comparison of trace elements counts in patient and control samples was performed by one way analysis of variance ANOVA with the Bonferroni multiple comparison test.

RESULTS

3. Results

Oxidative stress is enormous production of reactive oxygen species in the presence of diminished antioxidant substances. Oxidative stress contributes a significant role in diabetes mellitus type 2. Current study emphasized on evaluation and measurement of reactive oxygen species in diabetes mellitus type 2, their relation and effects on different parameters and antioxidants. Diabetes Mellitus type 2 is most common metabolic disorder in adults. Total 60 patients out of these 16 male patients and 44 female patients were studied. In addition 30 samples of healthy control individuals were studied. There was no age limits for diabetes mellitus patients. In current study, check the level of ROS in patients under different parameter like physical activity, hypertension, age, family history and cardiovascular disease. To measure or evaluates the level of ROS in patient serum by DEPPD method. For this purpose we construct a calibration curve and use as a standard.

3.1. Calibration curve

To measure and correlates the reactive oxygen species in the serum of the diabetes mellitus patients and control samples a calibration curve of hydrogen peroxide (H_2O_2) was constructed. With ten (10) different concentration of hydrogen peroxide standard solution (5000, 10,000, 15,000, 20,000, 25,000, 30,000, 35,000, 40,000, 45,000, 50,000, 55,000, 60,000, 65,000 and 70,000 mg) and 1 blank solution absorbance at 505 nm was measured. As shown in Fig 3.1 a calibration curve for the standard solution was developed by calculating the slopes. Formula to calculate slopes was absorbance at 505 nm/min ×1000. The calibration curve for hydrogen per oxide was linear up to (14) units.

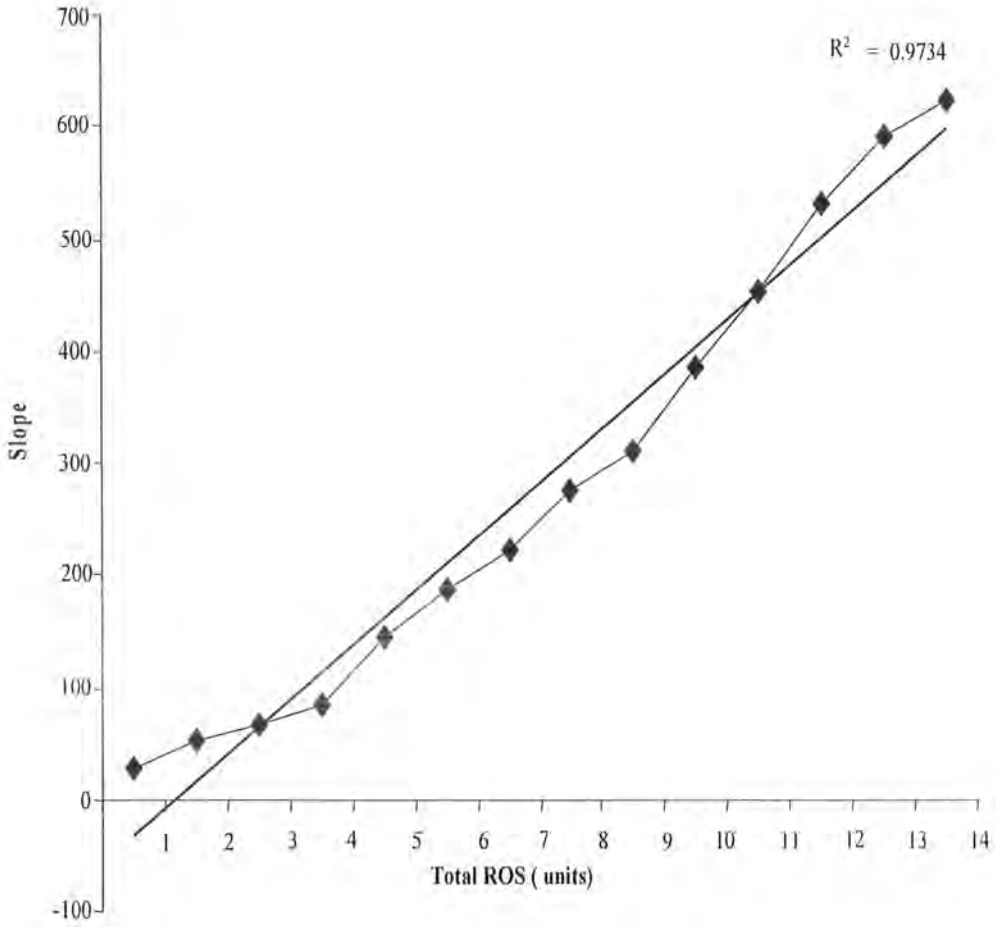

Fig 3.1: Calibration curve by hydrogen peroxide

3.2 Physical activity as a risk factor

Evaluation of ROS by DEPPD method to check the oxidative stress between control and patients with physical activity and no physical activity. The result showed a remarkable significant difference between control and physical activity, control and no physical activity. And also observed a remarkable significant difference between physical activity and no physical activity.

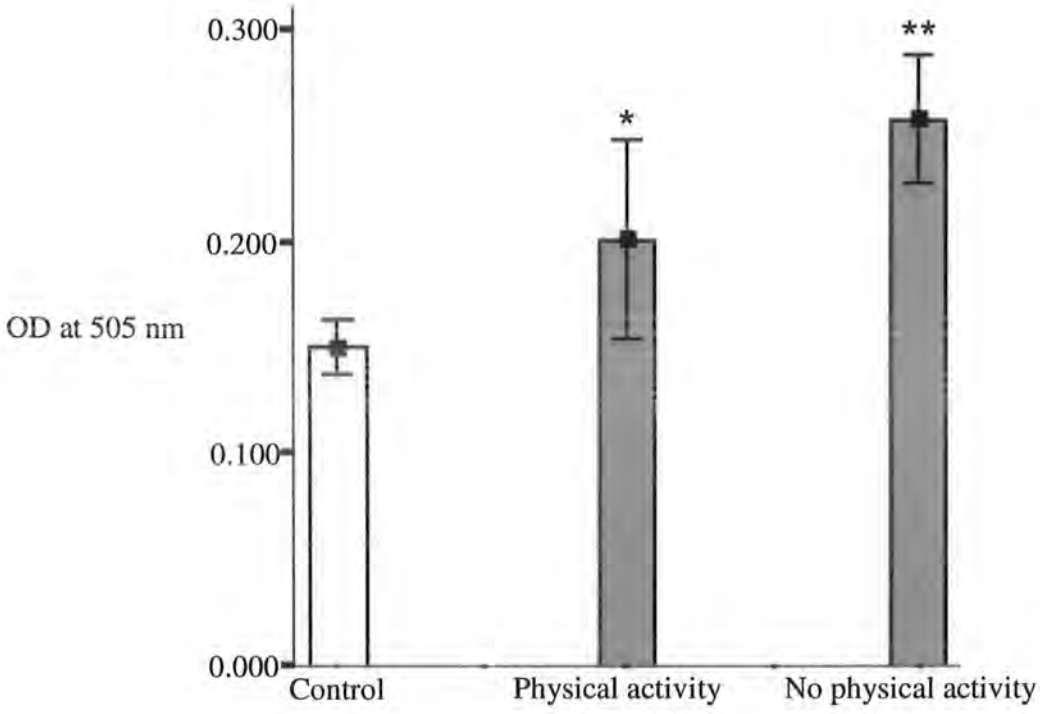

Fig 3.2: A comparison between control, physical activity and no physical activity patients at significant value $p < 0.05$. * Represents a remarkable significant difference between control and physical activity. ** Represents a remarkable significant difference between control and no physical activity, physical activity and no physical activity.

Table 3.1: Group comparison between control, physical activity and no physical activity Patients.

Tukey HSD

Group Comparison		Mean Difference	Std. Error	Sig.
1.00	2.00	-5.100 E-02*	1.09E-02	.008
	3.00	-0.10800*	1.09E-02	.000
2.00	1.00	5.1000E-02*	1.09E-02	.008
	3.00	-5.700E-02*	1.09E-02	.005
3.00	1.00	0.10800*	1.09E-02	.000
	2.00	-5.7000E-2*	1.09E-02	.005

In this table 1.00 = the average value of control and 2.00 = the average value of physical activity patients and 3.00 = the average value of no physical activity patients. * shows the mean difference is significant at the 0.05 level.

3.3 Hypertension as a risk factor

We have evaluated the level of ROS by DEPPD method to measure the oxidative stress between different groups like control and no hypertension, control and hypertension, no hypertension and hypertension. Result showed a remarkable significant difference between control and hypertension. There was no remarkable significant difference observed between hypertension and no hypertension, control and no hypertension.

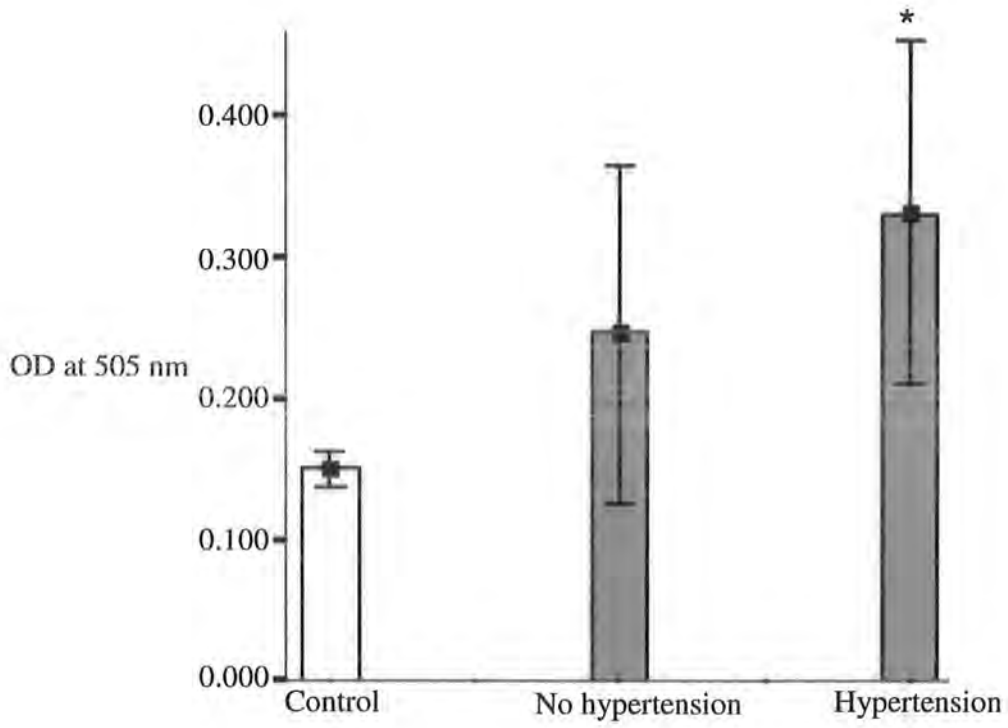

Figure 3.3: A comparison between control, no hypertension and hypertension Patient at Significant value is $p < 0.05$. In this figure * Represents a remarkable significant difference between control and hypertension patients. There is no remarkable significant difference among control, hypertensive and non hypertensive patients.

Table 3.2: Group comparison between control, hypertension and no hypertension Patients.

Tukey HSD

Group Comparison		Mean Difference	Std. Error	Sig.
1.00	2.00	-9.533E-02	3.19E-2	.055
	3.00	0.18133*	3.19E-2	.003
2.00	1.00	-9.5333E-02	3.19E-2	.055
	3.00	-8.600E-02	3.19E-2	.080
3.00	1.00	0.18133*	3.19E-2	.003
	2.00	8.6000E-2	3.19E-2	.080

In this table 1.00 = the average value of control and 2.00 = the average value of non hypertension patients and 3.00 = the average value of hypertension patients. * Represents the mean difference is significant at the 0.05 level.

3.4 Age as a risk factor

We have checked the ROS by DEPPD method to evaluate the oxidative stress between control and age < 50 years, control and age > 50 years, age < 50 years and age > 50 years these three group and our result showed a remarkable significant difference between all these three groups.

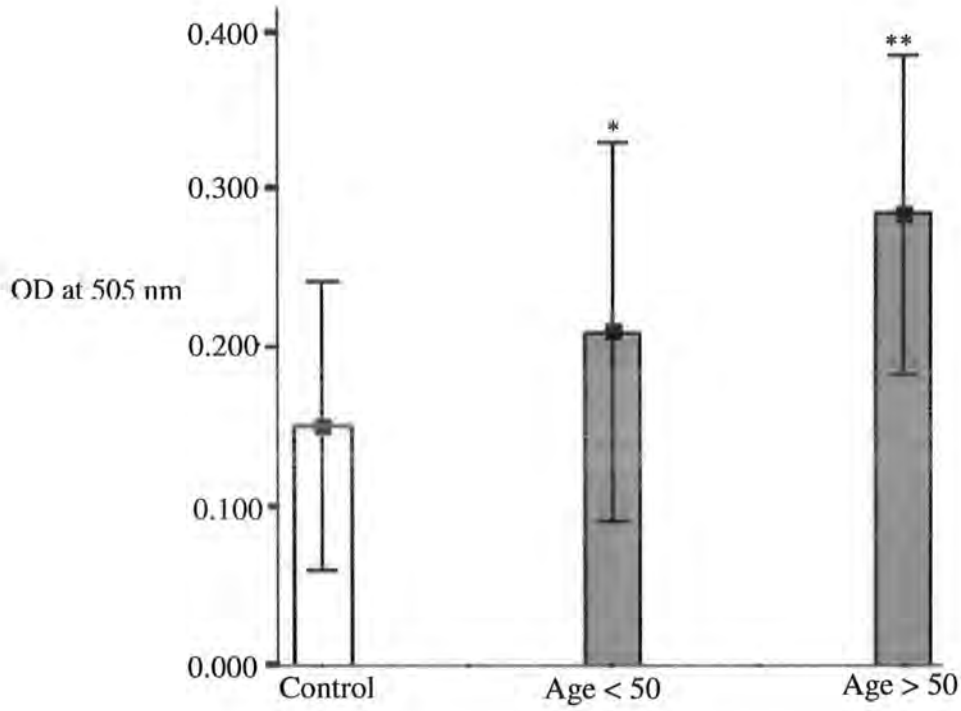

Fig 3.4: A comparison between control, age < 50 and Age > 50 at significant value $p < 0.05$. * Represents a remarkable significant difference between control and those patients whose age is less than 50. ** Represents a remarkable significant difference between control and those patients whose age is more than 50.

Table 3.3: Group comparison between control, age < 50 and age > 50 Patients.

Tukey HSD

Group Comparison		Mean Difference	Std. Error	Sig.
1.00	2.00	6.033 E-02*	4.66E-03	.000
	3.00	-0.13433*	4.66E-03	.000
2.00	1.00	6.0333E-02*	4.66E-03	.000
	3.00	-7.400E-02*	4.66E-03	.000
3.00	1.00	0.13433*	4.66E-03	.000
	2.00	7.4 000E-2*	4.66E-03	.000

In this table 1.00 = the average value of control and 2.00 = the average value of those patients whose age is less than 50 and 3.00 = the average value of those patients whose age is more than 50. * shows the mean difference is significant at the 0.05 level.

3.5 Family history as a risk factor

To evaluate the ROS by DEPPD method and to check the oxidative stress between different groups like control and no family history, control and family history, no family history and family history. Result showed a remarkable significant difference between control and family history. And did not observed a remarkable significant difference between no family history and family history, control and no family history.

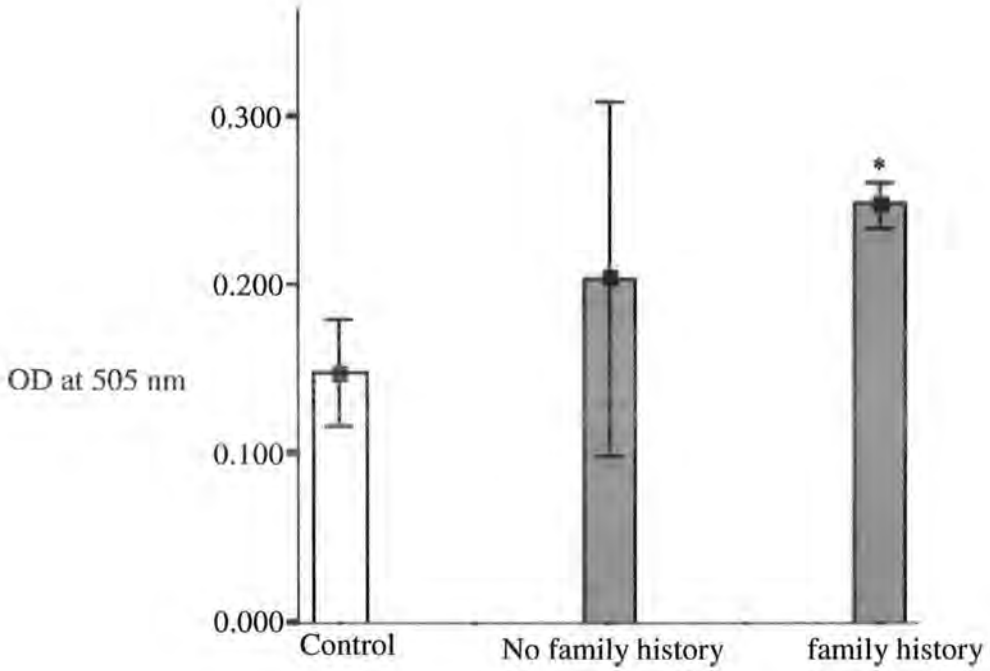

Fig 3.5: A comparison between control, no family history and family history patients at significant value $p< 0.05$. * Represents a remarkable significant difference between control and family history patients.

Table 3.4 Group comparison between control, no family history and family history patients.

Tukey HSD

Group Comparison		Mean Difference	Std. Error	Sig.
1.00	2.00	-5.617E-02	2.29E-02	.109
	3.00	-9.950E-02*	2.18E-02	.009
2.00	1.00	5.6167E-02	2.29E-02	.109
	3.00	-4.333E-02	1.92E-02	.139
3.00	1.00	9.9500E-02*	2.18E-02	.009
	2.00	4.3333E-02	1.92E-02	.139

In this table 1.00 = the average value of control and 2.00 = the average value of those patients has no family history and 3.00 = the average value of those patients has family history. * shows the mean difference is significant at the 0.05 level.

3.6 Diabetes Mellitus as a risk factor for cardiovascular disease

To evaluate the ROS by DEPPD method between control and no cardiovascular disease, control and cardiovascular disease, no cardiovascular disease and cardiovascular disease these three group and our result showed a remarkable significant difference between all these three groups.

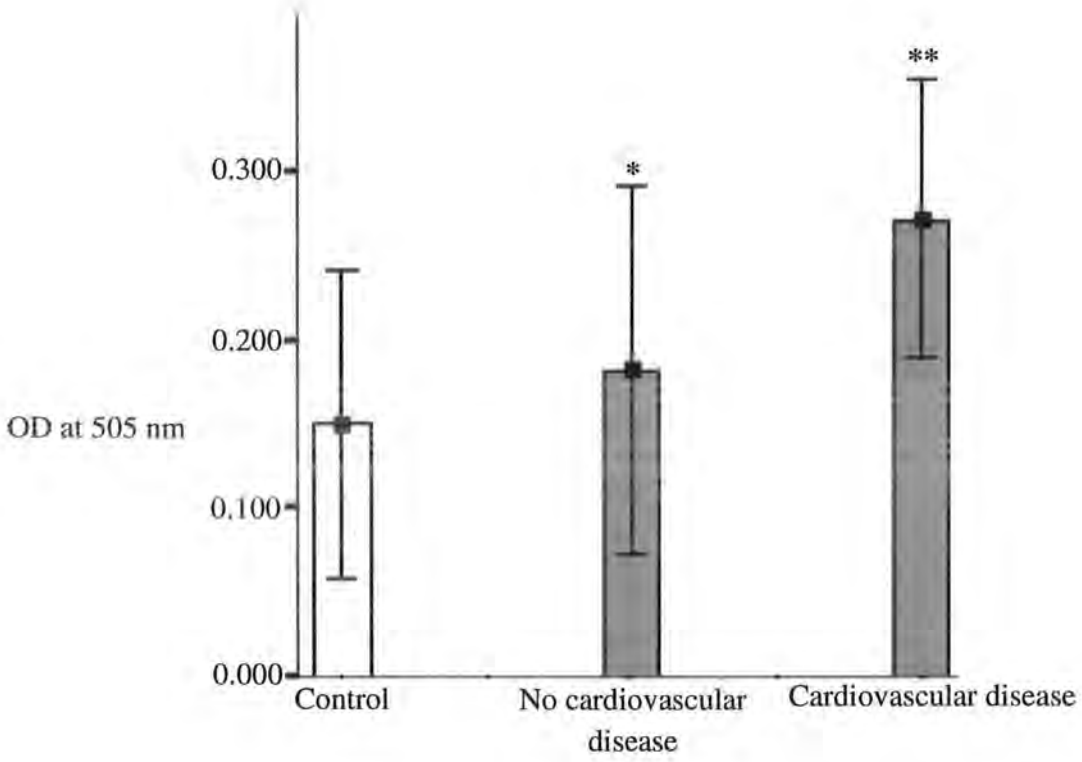

Fig 3.6: A comparison between control, cardiovascular disease and no cardiovascular disease at significant value $p < 0.05$. * Represents a remarkable significance difference between control and no cardiovascular disease. ** Represents a remarkable significance difference between control and cardiovascular disease.

Table 3.5 Group comparison between control, no cardiovascular disease and cardiovascular disease.

Tukey HSD

Group Comparison		Mean Difference	Std. Error	Sig.
1.00	2.00	-3.200E-02*	4.25E-03	.001
	3.00	-0.12133*	4.25E-03	.000
2.00	1.00	3.2000E-02*	4.25E-03	.001
	3.00	-8.933E-02*	4.25E-03	.000
3.00	1.00	0.12133*	4.25E-03	.000
	2.00	8.9333E-02*	4.25E-03	.000

In this table 1.00 = the average value of control and 2.00 = the average value of those patients has no cardio vascular disease and 3.00 = the average value of those patients has cardiovascular disease. * shows the mean difference is significant at the 0.05 level.

Table 3.6: Comparison between different parameters of diabetes mellitus patients and control individuals.

Parameter	Patients (average ± S.D) SE	Control (average ± S.D) SE	P value
Hypertension	(0.331 ± 0.04801) 0.0277 SE	(0.150 ± 0.005) 0.00289 SE	0.004*
Non hypertension	(0.24533 ± 0.04750) 0.0274 SE	(0.150 ± 0.005) 0.00289 SE	0.004*
Physical activity	(0.201 ± 0.01900) 0.0110 SE	(0.150 ± 0.005) 0.00289 SE	0.000*
No physical activity	(0.25800 ± 0.01200) 0.00693 SE	(0.150 ± 0.005) 0.00289 SE	0.000*
No family history	(0.20367 ± 0.0421) 0.0244 SE	(0.150 ± 0.005) 0.00289 SE	0.011*
Family history	(0.24700 ± 0.008406) 0.00420 SE	(0.150 ± 0.005) 0.00289 SE	0.011*
Cardiovascular disease	(0.27133 ± 4.509E-03) 2.60E-03 SE	(0.150 ± 0.005) 0.00289 SE	0.000*
No cardiovascular disease	(0.18200 ± 6.000E-02) 3.46E-03 SE	(0.150 ± 0.005) 0.00289 SE	0.000*
Male **Female**	16 (26.88 %) 44 (73.33 %)	17(56.66%) 13(43.33%)	0.0107*

* Represents the mean difference is significant at the 0.05 level. P value was calculated by one way ANOVA

3.7 Analysis of trace elements in the serum of diabetes mellitus by PIXE

To evaluate and measure the counts of trace elements in the serum of diabetes mellitus, proton induced x-rays emission (PIXE) analysis has been performed using 5-UDH tandem accelerator at National Centre for Physics Islamabad. We studied the difference in the number of counts of trace elements between 30 samples of controls and 60 patients of diabetes mellitus.

We selected few trace elements whose number of counts is altered in diabetes mellitus patients. Due to the up regulation and down regulation of these elements the progression of the disease occurs. The trace elements magnesium (Mg) (fig 3.7, 3.8), manganese (Mn) (fig 3.9, 3.10), zinc (Zn) (fig 3.11, 3.12), iron (Fe 3.13, 3.14), copper (Cu) (fig 3.15, 3.16), chromium (Cr) (fig 3.17, 3.18) and selenium (Se) (fig 3.19, 3.20) were identified in control and patients. Their counts were calculated by Gupixwin software. We performed one way analysis of variance (ANOVA) with Bonferroni multiple comparison test to find the difference in concentration of trace elements between control and diabetes mellitus patients.

The average number of counts of magnesium (Mg) in the control samples was 21 ± 7.4498 (table 3.7) and in the diabetes mellitus was 16 ± 6.2048 (table 3.7). The mean difference between control and diabetes mellitus was 4.000 which were insignificantly (0.683) decreased in DM patients (table 3.8). The average concentration of manganese (Mn) in the control samples and in the patients were 13 ± 4.7434 and19 ± 3.8079 (table 3.7), respectively. The mean difference of Mn between control and patient was 6.000 which were insignificantly (0.227) increase in patients (table 3.8). Similarly we observed the average number of counts of zinc (Zn) in the control samples was 38 ± 5.7009 and in the DM patient was 5 ± 1.5811 (table 3.7).The mean difference of Zn between control and patients was 34.000 which were significantly (0.000) decreased in patients as compared to control (table 3.8).

The average number of counts of Iron (Fe) in the serum of control sample was 14 ± 5.0990 (table 3.7) and in the serum of patients was 29 ± 5.6125 (table 3.7). The mean difference of Fe was 15.000 which were significantly (0.001) higher in patient as

compared to patient (table 3.8). Likely, the average counts of copper (Cu) was 242 ± 34.1467 (table 3.7) and 07 ± 2.2361 (table 3.7) in the control samples and in the patients, respectively. The mean difference of copper between control samples and patients was 2.000 that were insignificantly (0.519) increased in patients (table 3.8).

The chromium (Cr) was contributing with average number of counts of 112 ± 8.9443 in the serum of control individuals and 11 ± 3.1623 in the serum of patient (table 3.7). The counts of Cr was significantly (p = 0.000) low in control as compared to patients at the mean difference of Cr between control individuals and patients was 101.00 (table 3.8).

The average number of counts selenium (Se) in the serum of control individual was 5 ± 1.5811 and in the serum of patients was 4 ± 1.5811 (table 3.7). The mean difference of Se between control and patient was 2.000 that were insignificantly increased in the patients of diabetes mellitus as compared to control individuals (table 3.8).

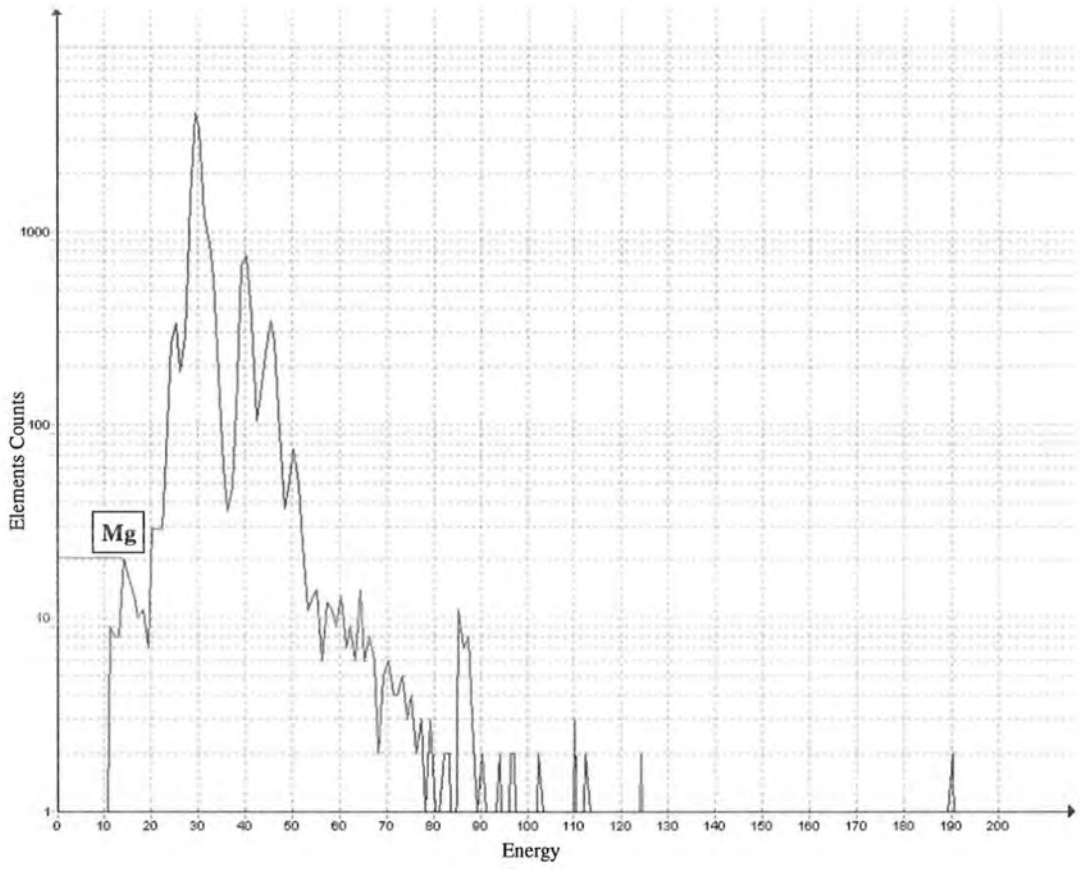

Fig 3.7: The level of magnesium in serum of control samples at energy **(KeV).** The Parameters are Beam He^{+} Energy 0.812MeV Charge 40μC Current 000.00nA.

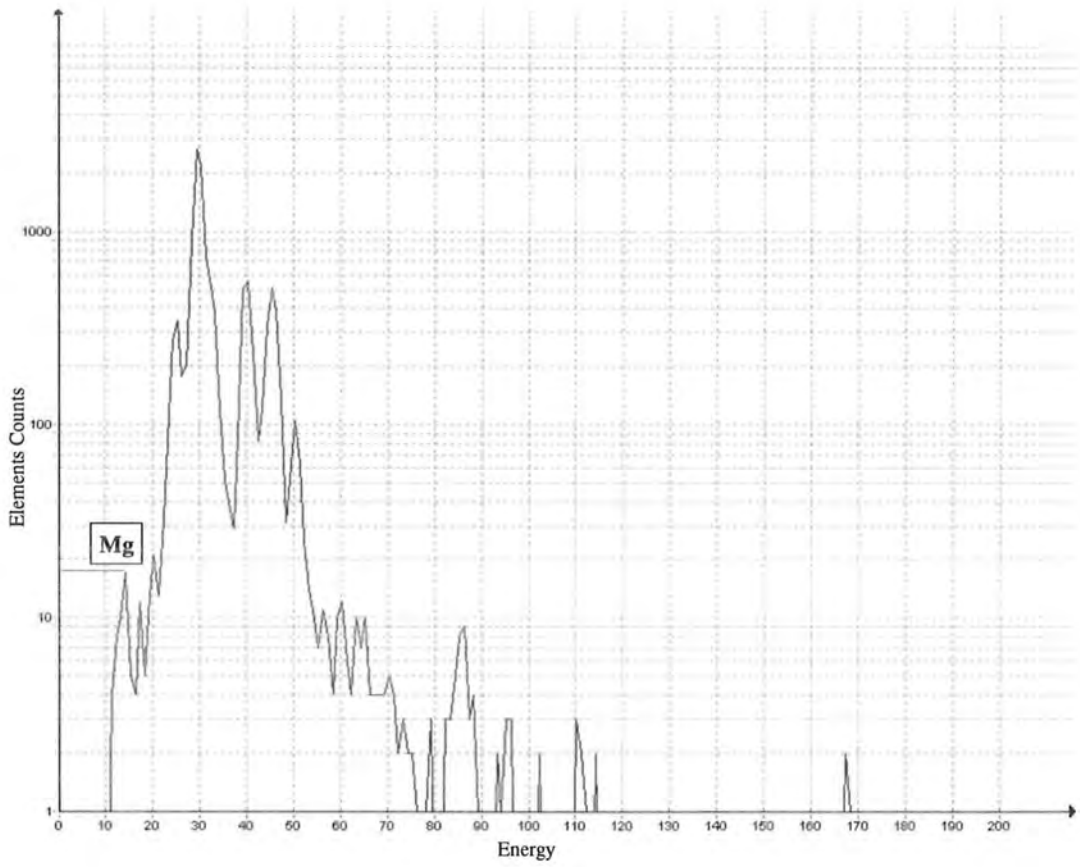

Fig 3.8: The level of magnesium in serum of Patient samples at energy **(KeV).** The Parameters are Beam He^{+} Energy 0.812MeV Charge 40μC Current 000.00nA.

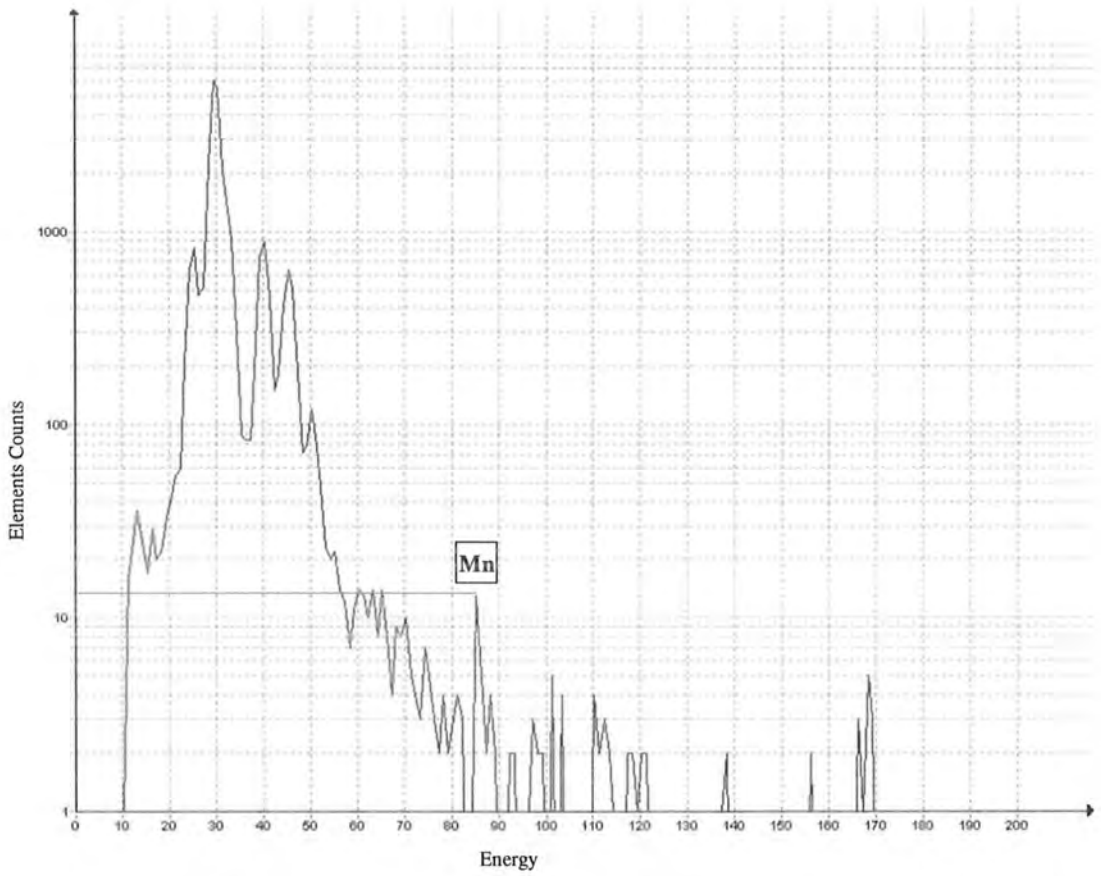

Fig 3.9: The level of manganese in serum of control samples at energy **(KeV).** The Parameters are Beam He^{+} Energy 0.812MeV Charge 40μC Current 000.00nA.

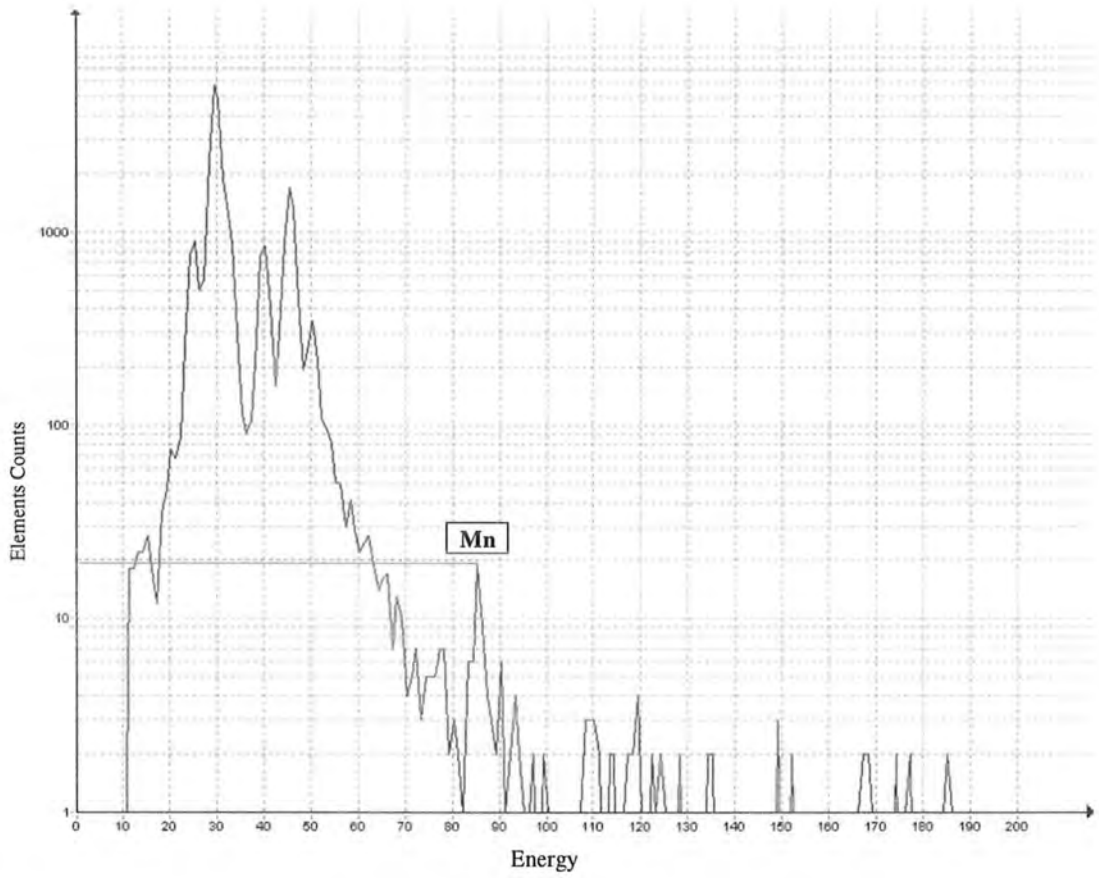

Fig 3.10: The level of manganese in serum of patient samples at energy **(KeV).** The Parameters are Beam He^{+} Energy 0.812MeV Charge 40μC Current 000.00nA.

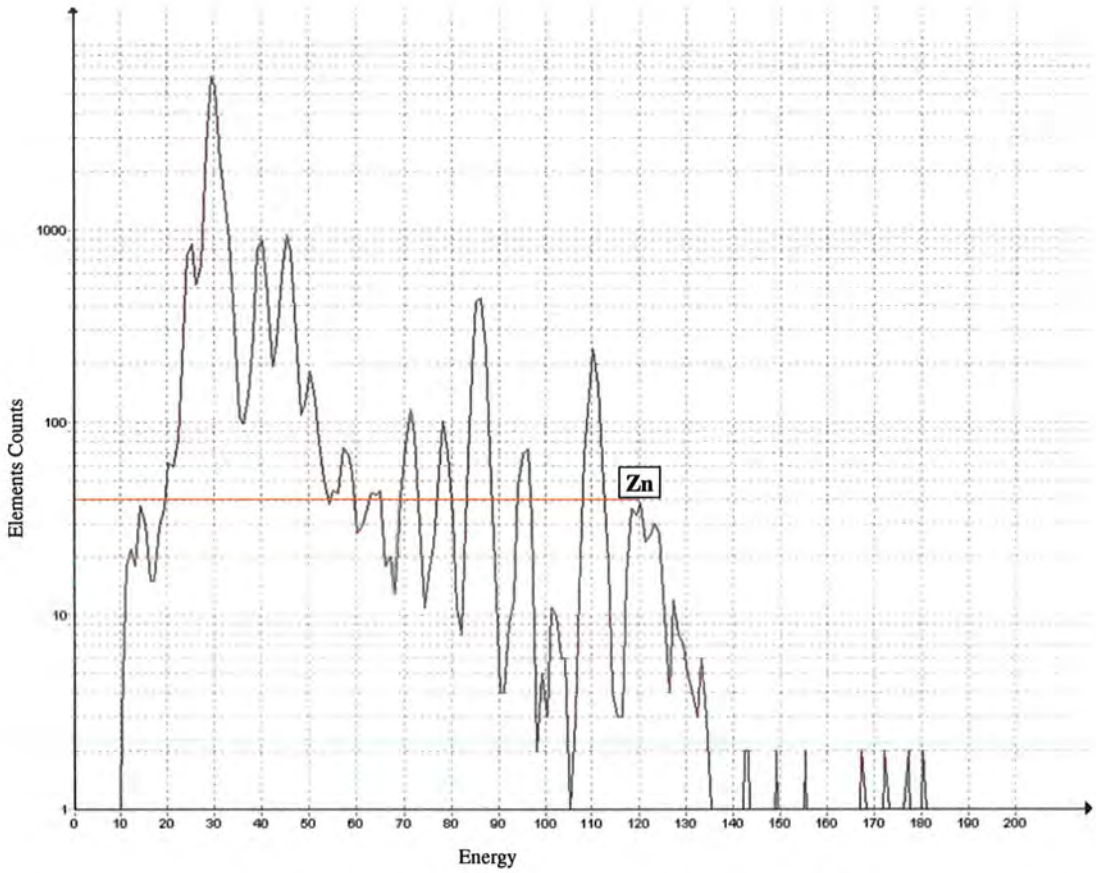

Fig 3.11: The level of zinc in serum of control samples at energy **(KeV).** The Parameters are Beam He^{+} Energy 0.812MeV Charge 40μC Current 000.00nA.

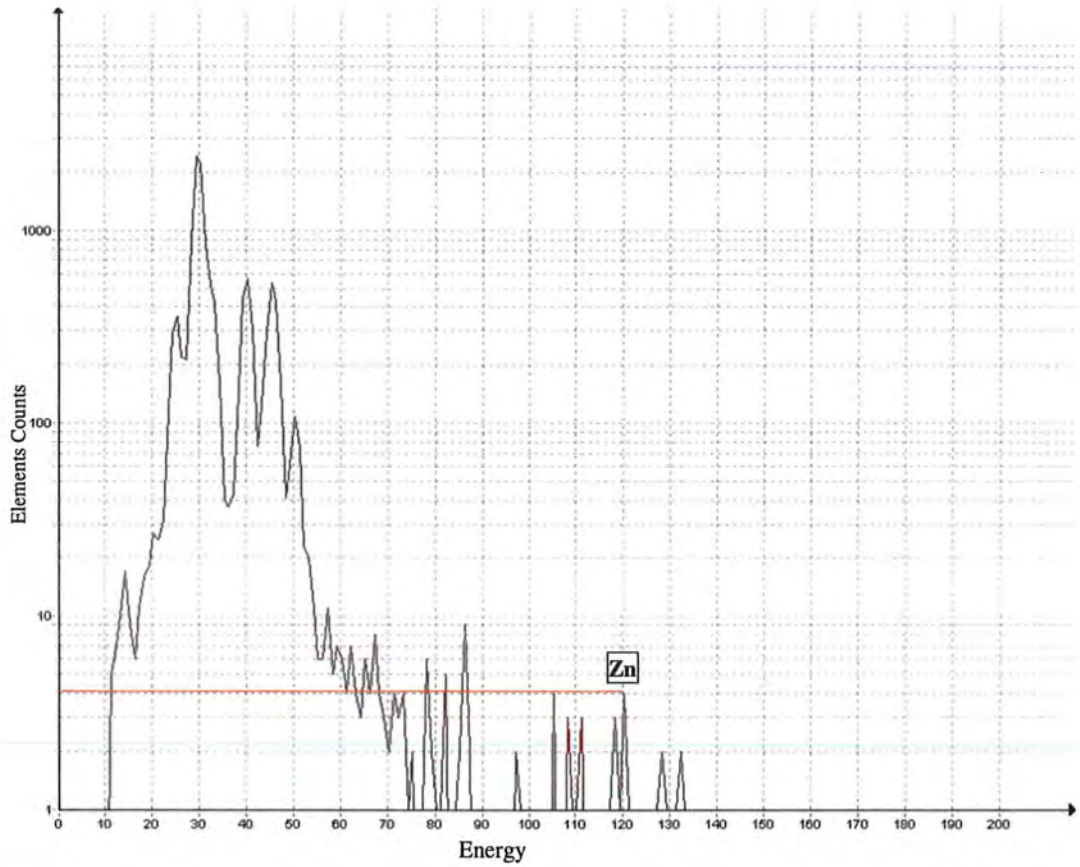

Fig 3.12: The level of zinc in serum of patient samples at energy **(KeV).** The Parameters are Beam He^{+} Energy 0.812MeV Charge 40μC Current 000.00nA.

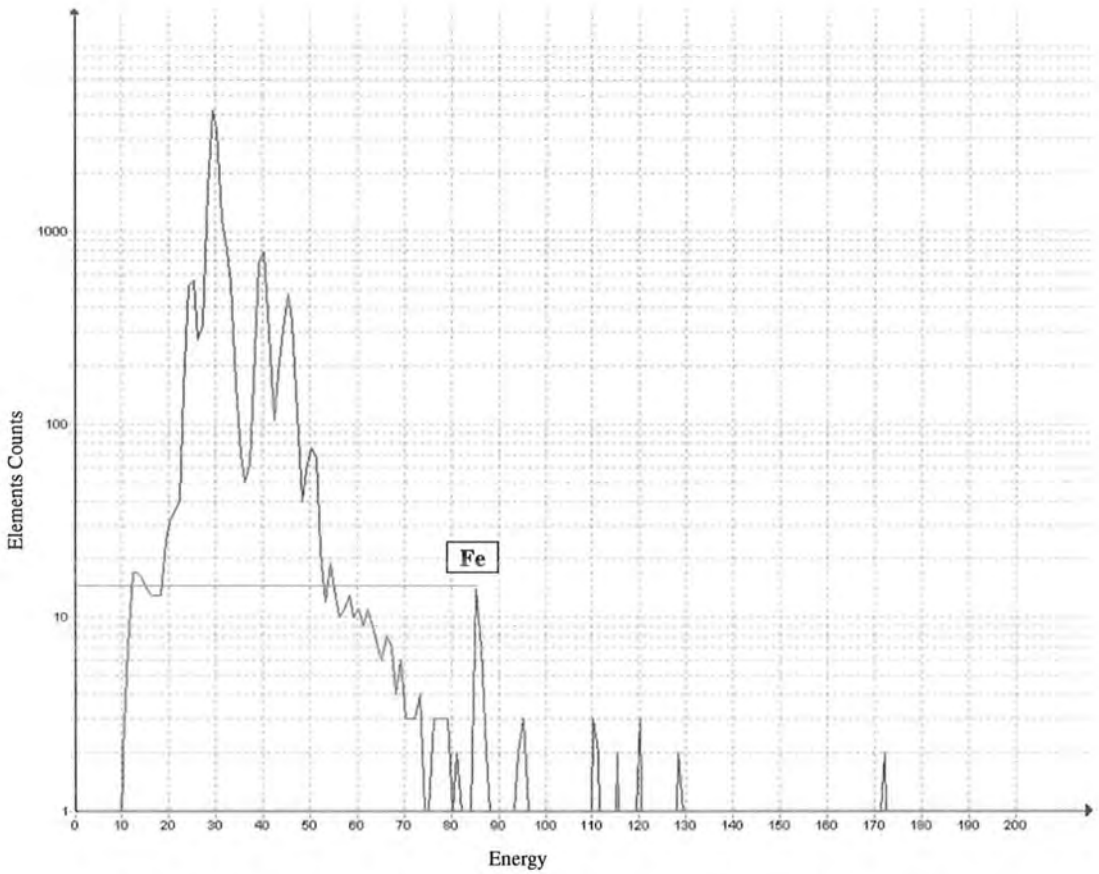

Fig 3.13: The level of iron in serum of control samples at energy **(KeV).** The Parameters are Beam He^{+} Energy 0.812MeV Charge 40μC Current 000.00nA.

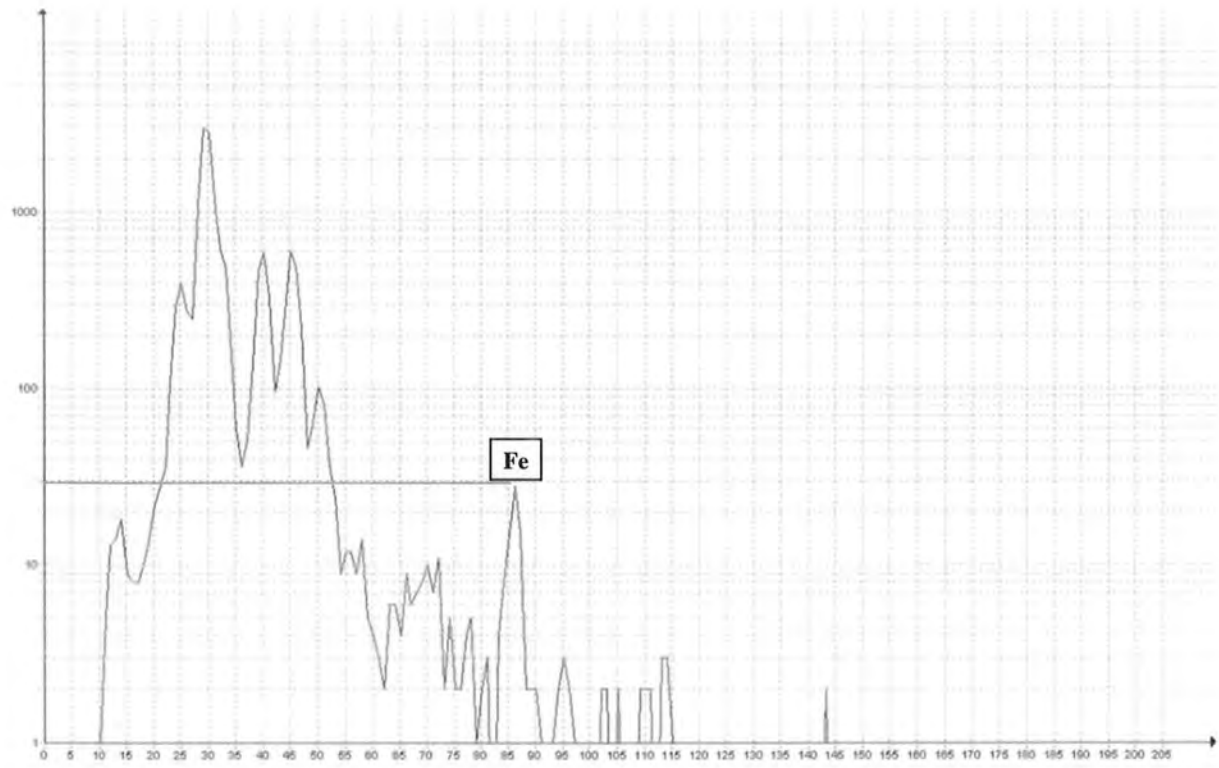

Fig 3.14: The level of iron in serum of patient sample at energy **(KeV).** The Parameters are Beam He^{+} Energy 0.812MeV Charge 40μC Current 000.00nA.

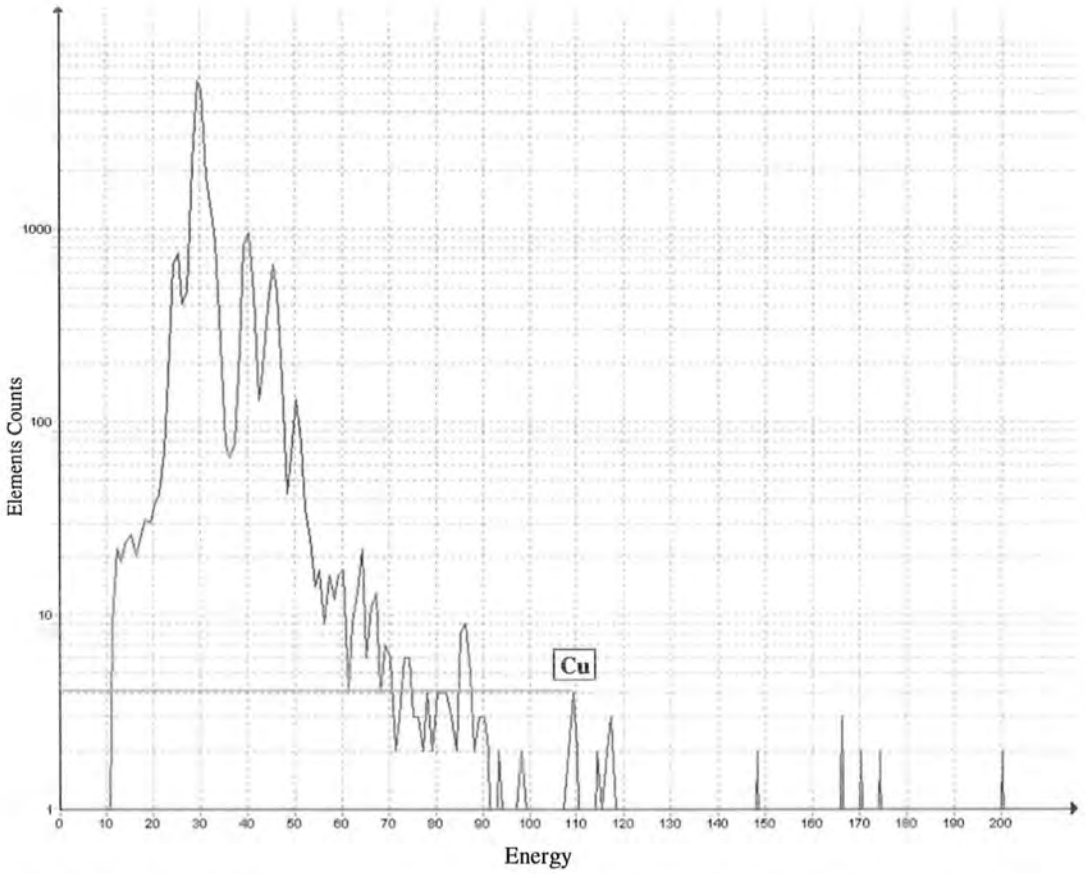

Fig 3.15: The level of copper in serum of control samples at energy **(KeV).** The Parameters are Beam He^{+} Energy 0.812MeV Charge 40μC Current 000.00nA.

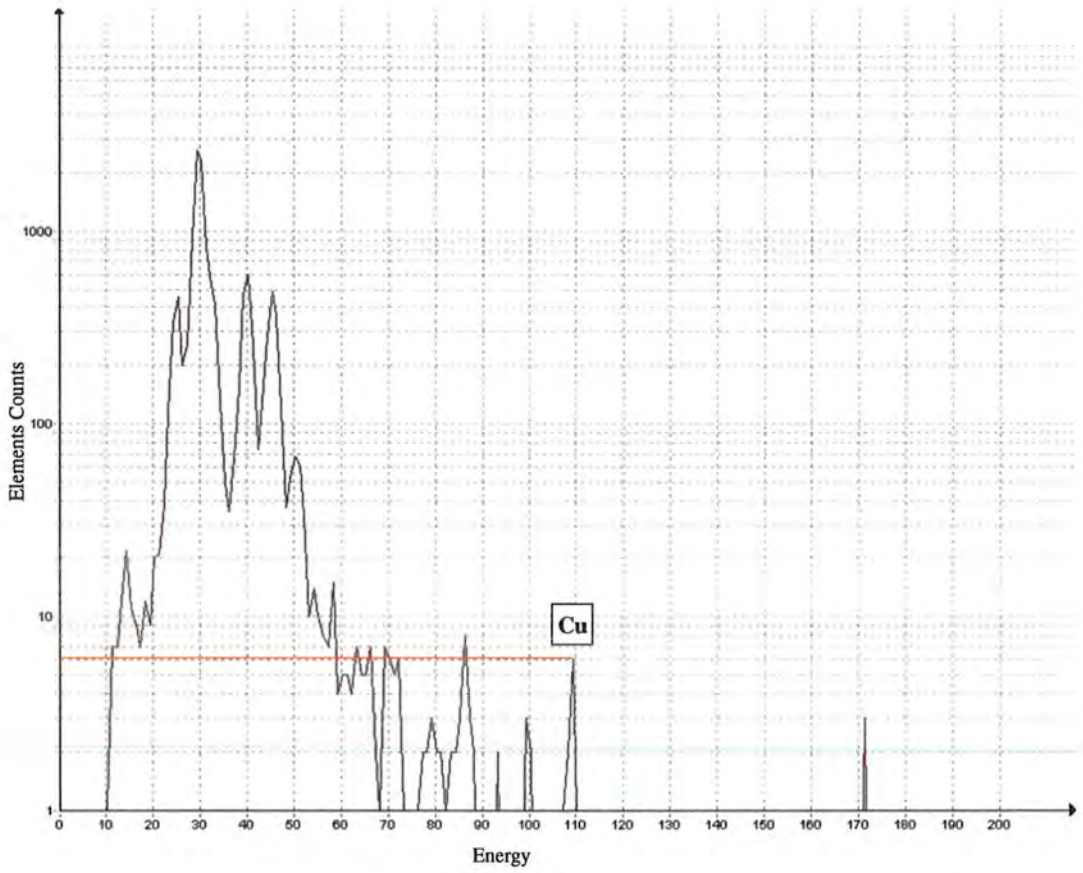

Fig 3.16: The level of copper in serum of patient samples at energy **(KeV).** The Parameters are Beam He^{+} Energy 0.812MeV Charge 40μC Current 000.00nA.

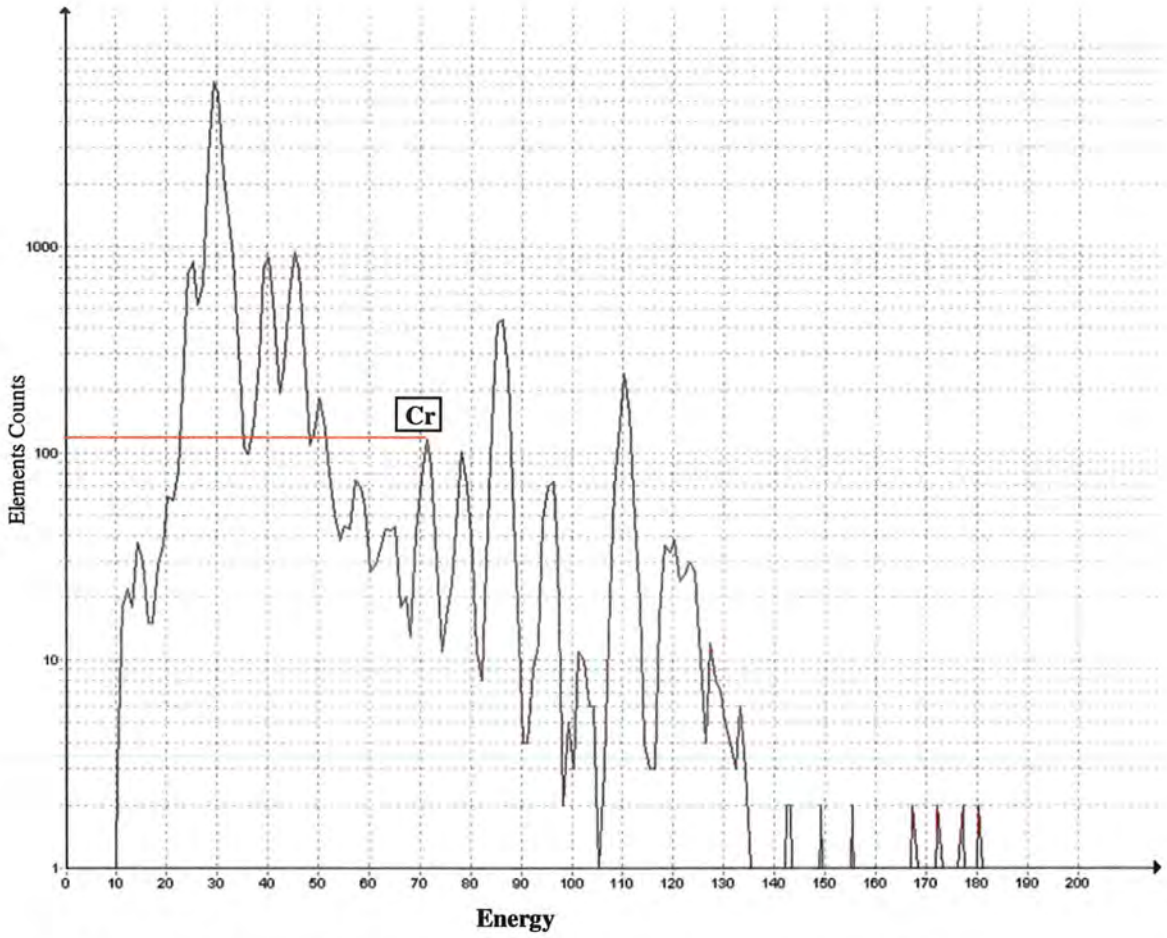

Fig 3.17: The level of chromium in serum of control sample at energy **(KeV).** The Parameters are Beam He^{+} Energy 0.812MeV Charge 40μC Current 000.00nA.

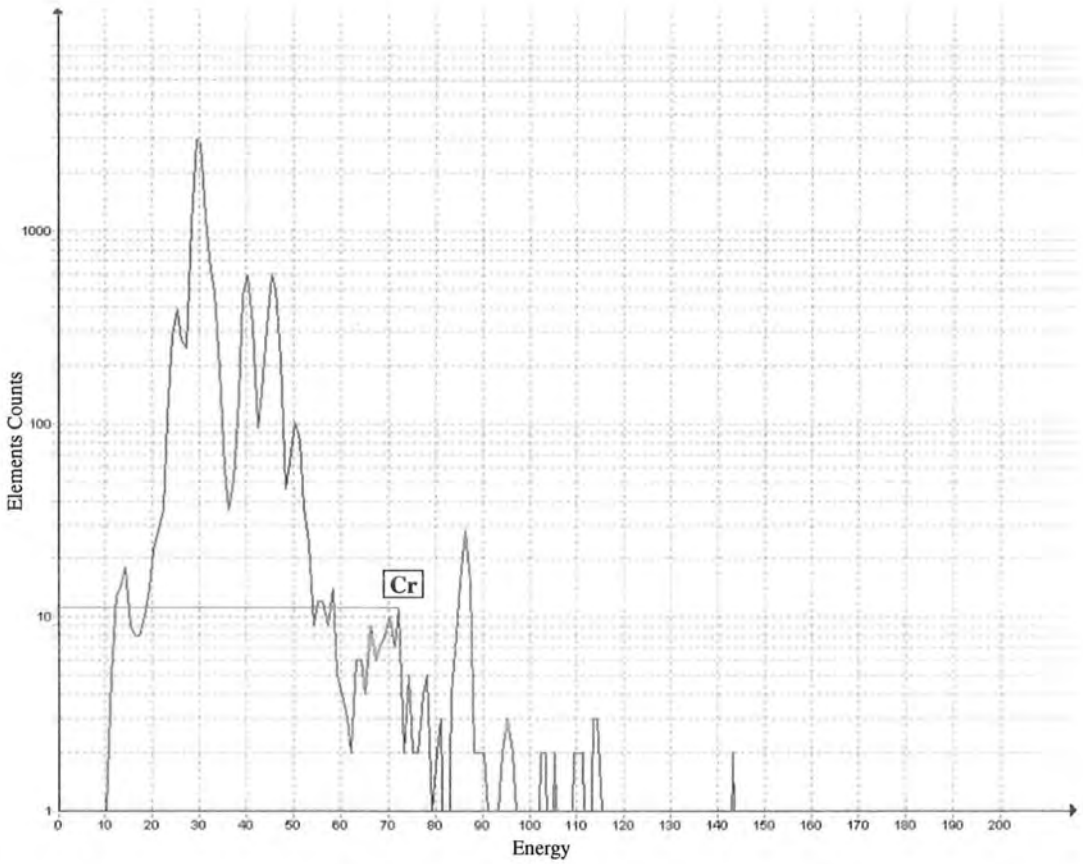

Fig 3.18: The level of chromium in serum of patient samples at energy **(KeV).** The Parameters are Beam He^{+} Energy 0.812MeV Charge 40μC Current 000.00nA.

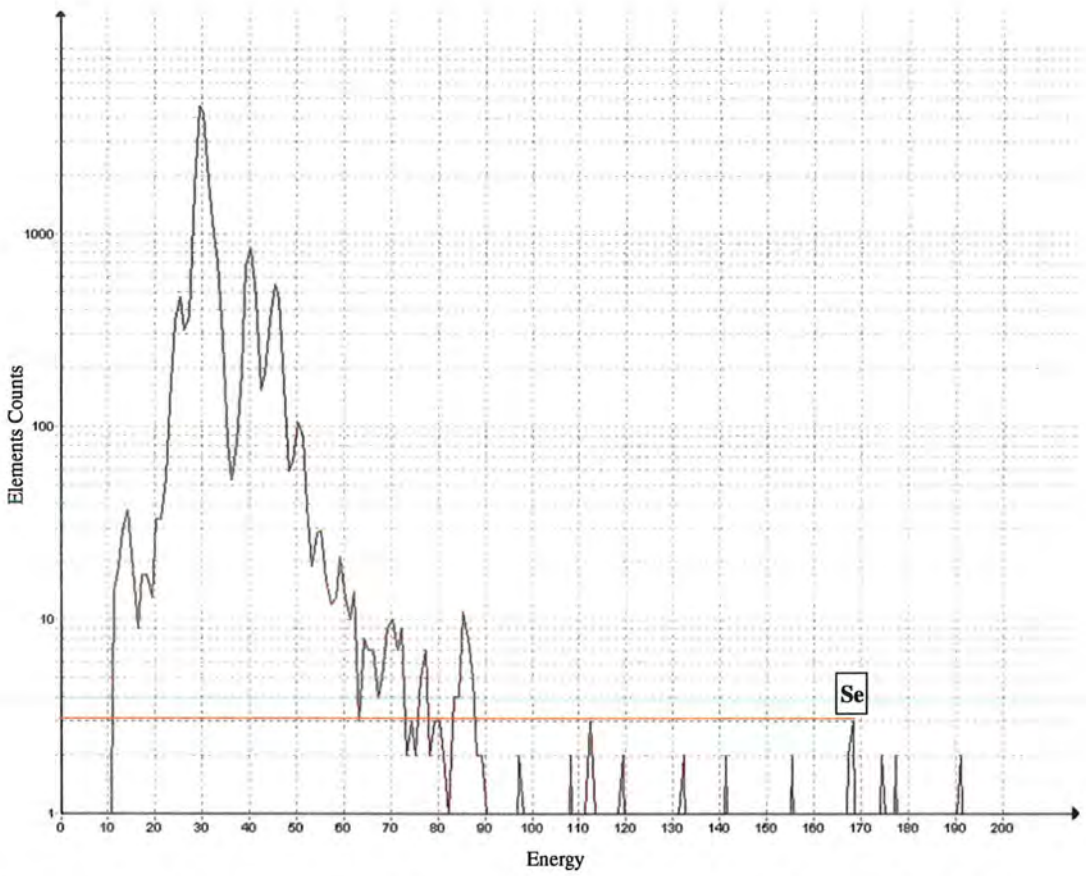

Fig 3.19: The level of selenium in serum of control samples at energy **(KeV).** The Parameters are Beam He^{+} Energy 0.812MeV Charge 40μC Current 000.00nA.

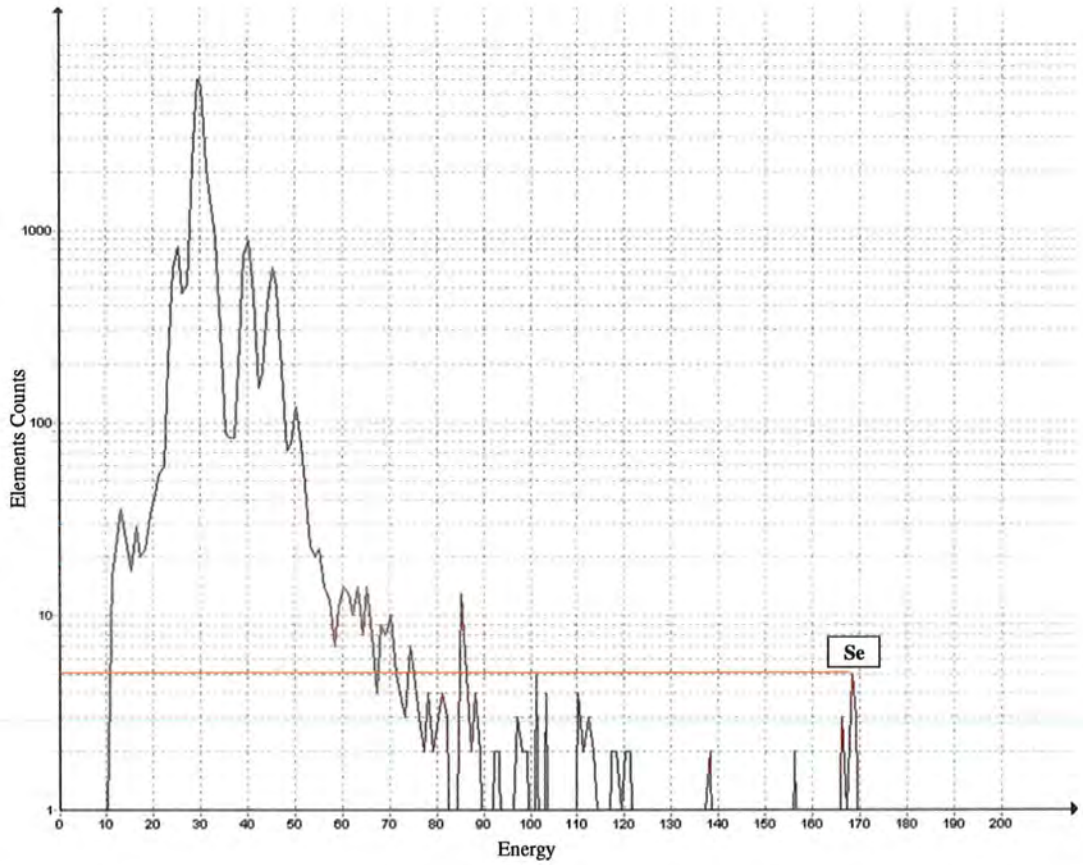

Fig 3.20: The level of selenium in serum of Patient samples at energy **(KeV).** The Parameters are Beam He^{+} Energy 0.812MeV Charge 40μC Current 000.00nA.

Table 3.7: Comparison of trace elements between Diabetes Mellitus and controls individuals.

Elements	**Controls (n=30)** **Average ± SD**	**Patients (n=60)** **Average ± SD**
Chromium (Cr)	112 ± 8.9443	11 ± 3.1623
Manganese (Mn)	13 ± 4.7434	19 ± 3.8079
Iron (Fe)	14 ± 5.0990	29± 5.6125
Copper (Cu)	242 ± 34.1467	07 ± 2.2361
Zinc (Zn)	38 ± 5.7009	5 ± 1.5811
Magnesium (Mg)	21 ± 7.4498	16 ± 6.2048
Selenium (Se)	5 ± 1.5811	4 ± 1.5811

Table 3.8: Difference in trace elements counts between control and patients

Comparisons	Means Difference	p Value
Chromium (Cr)		
Control Vs DM	101.00*	0.000
Iron (Fe)		
Control Vs DM	15.000*	0.001
Copper (Cu)		
Control Vs DM	2.000	0.519
Zinc (Zn)		
Control Vs DM	34.000*	0.000
Magnesium(Mg)		
Control Vs DM	4.000	0.683
Selenium (Se)		
Control Vs DM	2.000	0.473
Manganese (Mn)		
Control Vs DM	6.000	0.227

DM = Diabetes mellitus

Bonferroni multiple comparison tests by oneway ANOVA test was performed to show the difference in trace elements counts between control and patients. * Represents the mean difference is significant at the 0.05 level.

DISCUSSION

4. Discussion

Diabetes is a chronic metabolic disorder that continues to present a major worldwide health problem. It is characterized by absolute or relative deficiencies in insulin secretion and/or insulin action associated with chronic hyperglycemia and disturbances of carbohydrate, lipid, and protein metabolism. As a consequence of the metabolic instability in diabetes, various complications develop including both macro- and micro-vascular dysfunctions (Rahimi *et al.*, 2005). Type 2 diabetes mellitus is characterized by resistance of peripheral tissues to insulin and a relative deficiency of insulin secretion (Martin *et al.*, 1992). Type 2 diabetes is the most prevalent and serious metabolic disease all over the world and its hallmarks are pancreatic β-cell dysfunction and insulin resistance. Under diabetic conditions, chronic hyperglycemia and subsequent augmentation of reactive oxygen species (ROS) deteriorate β-cell function and increase insulin resistance which leads to the aggravation of type 2 diabetes (Kaneto *et al.*, 2010).

There is an impact of mitochondrial reactive oxygen species (ROS) on diabetes and its complications. ROS play a significant role as a signaling molecule to initiate several stress regulating pathways. In type 2 diabetes, activation of several stress regulating pathways, like NF-kB, p38 MAPK, JNK/SAPK, and hexosamine by increase in glucose and possibly FFA levels leads to both insulin resistance and impaired insulin secretion. (Evans *et al.*, 2003)

Several potential risk factors for diabetes mellitus include family history of diabetes, hypertension, obesity, alcohol consumption, sleep duration (short sleep duration and long sleep duration) and physical activity. In this study some risk factor were studied.

In present study, there was a significant difference of ROS level between control and patient with physical activity ($p = 0.008$) (table 3.1) and patient with no physical activity ($p = 0.000$) (table 3.1). Zinman *et al.* (2003) suggests that the importance of promoting physical activity as a vital component of the prevention as well as management of type 2 diabetes must be viewed as a high priority. It must also be recognized that the benefit of physical activity in improving the metabolic abnormalities of type 2 diabetes is probably greatest when it is used early in its progression from insulin resistance to impaired

glucose tolerance to overt hyperglycemia requiring treatment with oral glucose-lowering agents and finally to insulin. This study shows that the ROS level in diabetic patient with physical activity was decreased as comparison to those patients with no physical activity (fig 3.2).

Data shows that there is an insignificant (0.055) (table 3.2) difference between control and non hypertension patients and a remarkable significant (0.003) (table 3.2) difference between control and hypertension patients. Sowers *et al.* (2001) supported our result that hypertension is approximately twice as frequent in patients with diabetes compared with patients without the disease. Conversely, recent data suggest that hypertensive persons are more predisposed to the development of diabetes than are normotensive persons. This study showed that the level of ROS is higher in those diabetic patients has a hypertension as compared to those with no hypertension (fig 3.3).

This data shows that there is remarkable significant (0.000) (table 3.3) difference between control and age < 50 and remarkable significant (0.000) (table 3.3) difference between control and those patients whose age > 50. As study reported that the prevalence of diabetes was much higher in people aged 40–64 years than in people aged 20–39 years (Zhao *et al.*, 2006). In our results the level of ROS is higher in those patients whose age is more than 50 years old as comparison to those has less than 50 years old (fig 3.4).

In our study, the relation between control and no family history is insignificant (0.109) (table 3.4) as compared to control and family history is significant (0.009) (table 3.4). As earlier data suggest that a strong positive family history of diabetes had 2.4 fold higher risk as compared to those patients without such history (Ohlson *et al.*, 1988). A result showed that the level of ROS was higher in diabetic patient with family history as compared to those patients has no family history with diabetes (fig3.5).

In this study, there is a significant (0.001) (table 3.5) difference between control and no cardiovascular disease and also a significant (0.001) (table 3.5) difference between control and cardiovascular disease. Type 2 diabetes is a common disorder and an important risk factor for cardiovascular disease (CVD). Diabetes is associated with increased morbidity and mortality, specifically due to complications, including diabetic retinopathy, neuropathy, nephropathy, and cardiovascular disease. CVD accounts for the

primary cause of death of all patients with diabetes (Fox, 2010). A result showed that the level of ROS is higher in those diabetic patients has cardiovascular disease as compared to those has no cardiovascular disease (fig 3.6).

In present study, we used PIXE technique to identify and quantify trace elements. PIXE is an elemental analysis technique in which the energy of emitted characteristics X-rays, when a sample is bombarded with a beam of energetic protons is used to identify the elements present in the sample. The energies of these X-rays are characteristics of the element and therefore can be used to identify elemental composition. In present study, we analyzed the serum of 60 diabetes mellitus patients and 30 control individuals. In our study, we compared the counts of some elements in both control and patients. In patients, we observed the up regulation or down regulation of some elements like magnesium (Mg), manganese (Mn), zinc (Zn), iron (Fe), copper (Cu), chromium (Cr) and selenium (Se) as compared to control. Due to the up regulation and down regulation of these elements progression and complication of diabetes mellitus occur. These elements also act as cofactor for various enzymes and these enzymes involved in the production of reactive oxygen species (ROS) or reactive nitrogen species (RNS).

In diabetic patients, the down regulation of magnesium (Mg) (fig 3.7) has been observed as compared to control (fig 3.8). Earlier data suggests that magnesium deficiency, defined on the basis of intracellular free magnesium levels, and or serum ionized magnesium is a common feature of both diabetic and hypertensive states as well as various other cardiovascular and metabolic processes and aging that magnesium supplementation is indicated in conditions associated with magnesium deficit although well-designed therapeutic trials of magnesium is essential in hypertension and type 2 diabetes mellitus (Barbagallo *et al.*, 2007).

A result showed that the up regulation of manganese (Mn) has been observed (fig 3.9) in the serum patients as compared to control (fig 3.10). As earlier data suggests that, Mn status in human diabetics is controversial. One studies reported that Mn blood levels to be approximately one-half of the normal values In contrast, another studies found elevated Mn levels in diabetic patients aged 61-70 yr. In a study of serum Mn levels in patients with various diseases, 62% of diabetic patients had elevated Mn levels Although serum

Mn levels reported in the older literature are high according to modern standards, inclusion of control subjects in each study allows a valid comparison between serum Mn concentrations of diabetic patients and that of control subjects (Mooradian *et al.*, 1987). In one study reported that, Superoxide dismutase (SOD) is a metabolic enzyme that specifically catalyzes the conversion of the superoxide radical (O_2-) to H_2O_2 and O_2. The SOD metalloenzymes can be separated into three classes based on the metalcofactors at their active sites: copper/zinc SOD (Cu/ZnSOD), manganese SOD (MnSOD), and iron SOD (FeSOD) (Areekit *et al.*, 2011). This study showed that, manganese as a cofactor of SOD and the enzyme SOD involved in antioxidant mechanism. In patients the level of Mn decreased due to this down regulation of enzyme.

In contrast, the number of counts of zinc in the serum of diabetic patients (fig 3.11) was low as comparison to control individuals (fig 3.12). Prasad, 2009 reported that zinc as an antioxidant, reduces formation of free radicals by several ways. Zinc acts as an inhibitor of NADPH oxidase, inducer of metallothionein (effective scavenger of radicals) and is an integral metal of Cu, Zn-SOD. ROS are known to activate NF-kappaB which in turn activates growth factors, antiapoptotic molecules resulting in cell proliferation (cancer), inflammatory cytokines and adhesion molecules. Thus zinc functions not only as an antioxidant but also as an anti-inflammatory agent. Salgueiro *et al.* (2001) reported that, zinc is one of the essential micronutrients of which status and metabolism is altered in diabetes mellitus. Zinc deficiency may play a role in abnormal immune function in type II diabetes mellitus (Niewoehner *et al.*, 1986). Zinc deficiency has been associated with increased levels of oxidative damage including increased lipid, protein and DNA oxidation. Zinc as an antioxidant, reduces formation of free radicals by several ways (Prasad, 2009). Thus zinc functions not only as an antioxidant but also as an anti-inflammatory agent. So, this study also suggests that Zn which acts as a cofactor is decreased in serum of patient showing enzyme activity is down regulated.

The up regulation of iron was observed in patients (fig 3.13) as compared to control individuals (fig 3.14). Many studies documented that mutations in superoxide dismutase enzymes (Deng *et al.*, 1993) and iron-uptake regulator (Iolascon *et al.*, 2009) may lead to excess levels of superoxide anion radicals and iron overload. Such a condition leads to

the possibility of redox active iron to participate in organic and inorganic oxygen radical reactions, such as stimulating lipid peroxidation and catalyzing the formation of damaging hydroxyl radicals with subsequent tissue damage. Iron metabolism has been found to be significantly disturbed in type 2 diabetes and interferes with glucose metabolism (Lee *et al.*, 2006). Lowering iron pools generally improves insulin sensitivity in others words, higher level of iron improves insulin resistance. In addition, iron has been strongly implicated in nonalcoholic steatohepatitis, considered an early marker of insulin resistance (Machado and Cortez-Pinto, 2006). So, this study also suggests that Fe as a cofactor of superoxide dismutase is increased in sample of patient enzyme activity is downregulated, due to either mutation in enzyme or chelation of iron is not occur with enzyme and it is present as a free state.

The data and result showed that the number of counts of copper was higher in the serum of patients (fig 3.15) as compare to the control subject (fig 3.16). Speisky *et al.* (2009) elevated levels of copper significantly d ecreases glutathione levels. Glutathione is a substrate for several enzymes that removes ROS and is also a powerful cellular antioxidant present in the cells in millimolar concentration. It has multiple functions in intracellular copper metabolism and detoxification. Glutathione can suppress copper toxicity by directly chelating the metal (Mattie and Freedman, 2004) and maintaining it in a reduced state making it unavailable for redox cycling. O'Dell, (1976) reported that disruption of copper homeostasis resulting in elevated pools of copper may contribute to a shift in redox balance towards more oxidizing environment by depleting glutathione levels. The depletion of glutathione may enhance the cytotoxic effect of ROS and allow the metal to be more catalytically active, thus producing higher levels of ROS. The large increase in copper toxicity following GSH depletion clearly demonstrates that GSH is important cellular antioxidant acting against copper toxicity (Steinebach and Wolterbeek, 1994). Animal models have been adopted to reveal the association between abnormal copper metabolism and diabetes (Eaton and Qian, 2002). It has been recently characterized that hyperglycemic complications contributing to cardiovascular disease are linked with disturbed copper homeostasis. Chelatable copper level was found to be increased in the diabetic hearts and elevated extracellular copper might be implicated in the mechanism of cardiovascular damage in diabetes (Cooper *et al.*, 2004). This study

also suggests that Cu level is increased in serum of patients due to this the activity of enzymes is downregulated.

The level of chromium was down regulated in the serum of patients (fig 3.17) as compared to the control subject (fig 3.18). Jomova and Valko, (2011) repo rted that chromium is known to activate the MAP kinase signal transduction pathway. NF-kB, ATF-2 and p53 participate in regulation of critical cellular processes, including apoptosis. Cr (VI)-induced oxidative stress triggers the hypoxia signaling pathways, leading to increase in HIF-1α and VEGF protein levels. Chromium (III) deficiency in humans has been associated with cardiovascular disease, metabolic disease (e.g. diabetes) and infertility. As earlier reported that the Chromium (III) is an essential mineral which has a beneficial role in the regulation of insulin, metabolic syndrome and cardiovascular disease. Chromium potentiates insulin and therefore plays a role in the normal glucose metabolism. Decreased levels of chromium in human tissues have been found which correlated with the incidence of type 2 diabetes. Deficiency of chromium has been associated with disturbed glucose tolerance, fasting hyperglycemia, glucosuria, increased body fat, dyslipidemia and impaired fertility (Flora *et al.*, 1995). There is growing evidence that chromium may facilitate insulin signaling and chromium supplementation therefore may improve systemic insulin sensitivity (Hummel *et al.*, 2007). This study shows, the downregulation of chromium has been observed in diabetic patients. Chromium is invovled in activation of several stress signaling pathways.

This study suggested that the number of counts of selenium was higher in patients (fig 3.19) as compared to control subjects (fig 3.20). The high serum selenium levels were positively associated with the prevalence of diabetes. Selenium intake including selenium supplementation should not be recommended for primary or secondary diabetes for prevention in populations with adequate selenium status such as the U.S. population (Bleys *et al.*, 2007). Selenium acts as a cofactor of glutathione peroxidase which is involved in antioxidant defense mechanism.

Present study concluded that the progression and complication of disease occurred due to higher level of ROS in diabetic patients in concern with altered level of regulating enzymes co-factors. Future prospective of this study is that the antioxidant enzyme

activity can be enhanced in diabetic patient by providing supplements of deficient co-factor. ROS regulating enzyme activity can be enhanced by therapeutic interventions.

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(http://www.ams.ethz.ch/research/material/iba/pixe/pixe.png?hires)

(http://nd.edu/~nsl/html/research_SNICS.html)

Patient Consent Form

Research title:

Diabetes Mellitus impact on Body's Mineral Profile, Relative Imbalance of ROS level Regulating Enzymes

Introduction

My name is Nuzhat Shaheen Abbasi, student of M.Phil at Quaid-e-Azam University Islamabad Department of Biochemistry. I am collecting data and blood samples for my research project on (Diabetes Mellitus) and I would like to ask you for your help by answering a few questions regarding to my project related to your disease. Your participation in this would take some minutes.

This research has been reviewed and approved by Quaid-e-Azam University. If you have any concerns about this research project, you can contact University. These data will be strictly confidential. The results of the treatment may be published for scientific purposes.

Purpose of Study

The purpose of this study is to find better way to detect and treat diabetic patients and help to improve current understanding of this disease.

Consent Statement:

I have read the above comments and agree to investigator. I have no objection to give my blood sample and data under the terms outlined above. I understand that if I have any question or concerns regarding this project I can contact the investigator at the above location.

_____

Name of Patient

_____

Signature of Patient

PATIENT HISTORY PERFORMA

DATE: _____

Code _____
Gender _____
Name _____
Place _____

Age _____ Wt/Ht _____ Status _____

Pulse rate _____ BP _____ Temp _____

Physical examination_____
_____
_____

Patient History _____
_____

Family history_____
_____

Time duration _____ Smoking _____

Any kind of depression_____

Hypertension _____ Diabetic _____

Cardiovascular disease _____

Blood picture

BSR/BSF _____