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Assessment of Osteoprotegerin, Sclerostin and Procollagen I N-Propeptide in Patients with Chronic Kidney Disease on Hemodialysis

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بسم الله الرحمن الرحيم

(ن وَالْقَلَمِ وَمَا يَسْطُرُونَ مَا أَنتَ بِنِعْمَةِ رَبِّكَ بِمَجْنُونٍ

وَإِنَّ لَكَ لأَجْرًا غَيْرَ مَمْنُونٍ وَإِنَّكَ لَعَلَى خُلُقٍ عَظِيمٍ

فَسَتُبْصِرُ وَيُبْصِرُونَ)

سورة القلم

Supervisor Certification

We certify that this thesis entitled " **Assessment of Osteoprotegerin, Sclerostin and Procollagen I N-Propeptide in Patients with Chronic Kidney Disease on Hemodialysis**."

Was carried under our supervision at the College of Medicine, University of Babylon, as partial fulfilment for the requirement of the degree of Master of Science in Clinical Biochemistry

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DEDICATION

To My supervisor (Dr. Thana M.Joda and Dr.Ali Alsultanie). My parents For their endless supportand they were always a source of strength duringmoments of despair and discouragement,to Abu meirza (Hussein K.)Who taught me that even the largest task can be accomplished if I do it one step at a time, to all my family, my friends, I dedicate this

research.

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Summary

Chronic kidney disease (CKD) is a chronic impairment in the structure or function of the kidney in which glomerular filtration rate [GFR] <60 mL/min/1.73 m^2 or albuminuria ≥30 mg per 24 hours. Diabetes, hypertension, chronic glomerulonephritis, chronic pyelonephritis, chronic use of anti-inflammatory medicine, autoimmune illnesses, polycystic kidney disease, Alport disease, congenital anomalies, and prolonged acute renal disease are the most common causes of Chronic kidney disease. Diagnosis is often made as a result of randomly screening test results, or if symptoms worsen. Chronic kidney disease –mineral and bone disorder (CKD-MBD) is term for describe the systemic disorder of mineral and bone metabolism due to CKD. Renal osteodystrophy is a change in bone morphology in CKD patients, and it is one marker of the skeletal component of the systemic condition of CKD-MBD. Bone turnover is consists of two processes the removing of old bone (resorption) and the production of new bone (formation). The essential actors in chronic kidney disease-mineral and bone disorders (CKD-MBD) include parathyroid hormone (PTH), Fibroblast growth factor 23 (FGF23) calcium, phosphate, and the vitamin D hormonal system.

The study's objective is to investigate the relationship between serum levels of bone turnover markers Osteoprotegerin (OPG), Sclerostin (SOST), procollagen type I N-propeptide (PINP), with traditional biomarker PTH, and ALP in patients with CKD and healthy controls. This study was designed as a case-control study. Forty-five hemodialysis patients were involved in this study (27females and 18 males) in addition to forty-five healthy controls (27females and 18 males) well-matched with the patients in age and gender.

Age was (20 – 75) years mean age 50.51 ± 2.07years, BMI with (24-27) Kg/m^2 Patients were subjected to the dialysis unit at Almahmodia and Alyarmouk Hospitals.

The levels of OPG, PTH, SOST &PINP were measured by ELISA Technique whereas the levels of other parameters (Ca, PO4, ALP& albumin) were measured by colorimetric method according to the manufacturer manual. As the results of the tests that were conducted showed that the levels of Calcium and albumin in the people with CKD were significantly lower than healthy people, as the value of $P < 0.05$ the levels of OPG, Parathyroid hormone, Phosphorus, SOST, ALP and PINP in patients were significantly higher than healthy people, as the value of $P < 0.05$. Relationship showed no significant correlation between OPG and (ALP,Ca,Alb) in CKD patients($P > 0.05$) otherwise there was a significant positive correlation between OPG and PINP ($P \leq 0.01$). and OPG with SOST&PTH where ($P \leq 0.01$).also correlation between SOST and Ca,Alb was significant negative ($p < 0.001$) within CKD patients. Otherwise there were a significant positive correlation between SOST and other bone turnover biomarker (PINP, OPG, ALP, Phos, PTH) ($p < 0.001$). The receiver operating characteristic curve test showed a good discriminative value of OPG, SOST, and PINP between CKD Patients and Controls. However, the ROC curve test showed a good discriminative value of OPG&SOST between CKD Patients and Controls. The current study that we conducted leads us to the conclusion that the level of the osteoportogrin ,seclerostin,PINP ,parathyroid hormone & phosphors is higher in patient CKD than those healthy control ,The OPG&SOST could be served as a prospective marker in CKD patients to predict the possibility to develop renal ostodystrophy (ROD) disease at a different stages of CKD The current study showed that the BMI is not significant in patient compared with healthy control ,There was a decrease in albumin and calcium levels in patients compared to healthy people , The

presence of a positive correlation between the bone turnover biomarker (OPG ,SOST, PINP) with parathyroid hormone & ALP, The presence of a positive correlation between the ALP, parathyroid hormone and phosphorus that were performed and negative correlation between Calcium, albumin & other studied parameters

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Lists of abbreviations

Symbols	Full term
AKD	Acute kidney disease
AKI	Acute Kidney Injury
ALP	alkaline phosphatase
AUC	area under curve
bALP	Bone alkaline phosphatase
BMI	Body mass index
BMPs	bone morphogenetic proteins
BTMs	Bone turnover marker
CaSR	Calcium-sensing receptors
CKD	Chronic Kidney Disease
CKD-MBD	chronic kidney disease-mineral and bone disorder
CTX	C-terminal telopeptides of type I collagen
Da	Dalton
DTPA	renal DTPA scan
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assays
ESKD	End-Stage Renal Disease
FGF23	Fibroblast growth factors23
GFR	Glomerular Filtration Rate
HD	Heamodialysis
HRP	Horseradish Peroxidase
IFCC	International Federation of Clinical Chemistry
IL-1	Interleukin-1
IL-6	Interleukin-6
IOF	International Osteoporosis Foundation
Ipth	Intact parathyroid hormone
KDIGO	Kidney Disease Improving Global Outcomes
Kg	Kilogram
Ml	Milliliter
NF-κB	nuclear factor kappa B
NKD	No kidney. Disease
NPT	sodium-dependent phosphate transport protein
NTX	N-terminal telopeptides of type I collagen
OD	Optical density
OPG	Osteoprotegerin
PICP	procollagen type I C- propeptides
PINP	procollagen type I N-propeptides

PTH	Parathyroid hormone
P-val	p value
RANKL	Receptor activator of NF-κB ligand
ROC	Receiver operating characteristic
ROD	Renal osteodystrophy
SCr	Serum creatinine
SD	Standard deviation
SE	Standard error
SHP	secondary hyperparathyroidism
SN	Sensitivity
SOST	Sclerostin
SP	Specificity
THP	tertiary hyperparathyroidism
TMB	Tetramethylbenzidine
TMV	turnover, mineralization, volume system
TNF-α	tumor necrosis factor-Alpha
US	United stat
Wnt	The name Wnt is a portmanteau created from the names Wingless and Int-1

1.1 The kidneys

The kidneys are specialized organs that play a critical role in the maintenance of normal vital processes. Human survival is strongly dependant on the critical activities and processes accomplished by the kidneys. Because it maintains bodily fluid balance and maintains other organ systems working effectively, the renal system has an influence on all sections of the body(1).

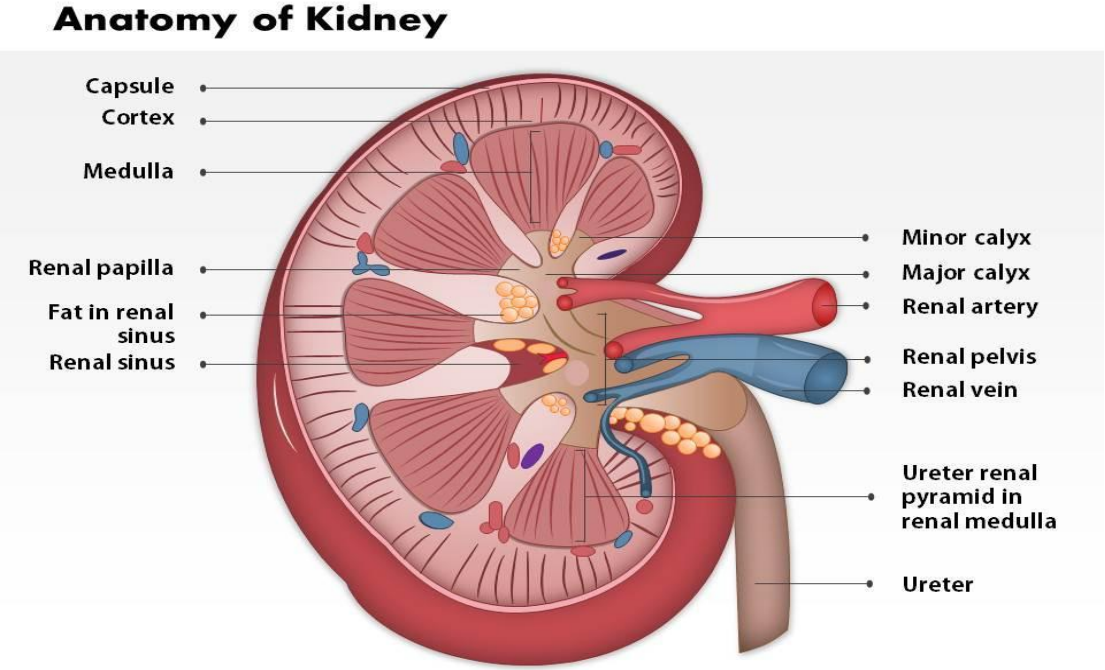


Figure (1-1) kidney anatomy(2).

1.2.1 Chronic kidney disease (CKD)

Is a clinical condition caused by a permanent alteration in kidney function and/or Structure. It is characterised by its irreversibility as well as its gradual and steady progression(3).Is defined as a chronic impairment in the structure or function of the kidney. (eg, glomerular filtration rate [GFR] <60 mL/min/1.73 m^2 or albuminuria ≥30 mg per 24 hours) for more than 3 months, Around the world, CKD affects between 8% and 16% of the population. CKD is the world's

16th largest cause of lost years of life. Proper screening, diagnosis, and treatment by primary care physicians are required to prevent adverse CKD-related outcomes, involving cardiovascular disease, end-stage renal disease, and death Those who are at high risk of developing CKD (eg, estimated GFR <30 mL/min/1.73 m^2, albuminuria ≥300 mg per 24 hours, or a significant decrease in estimated GFR) must be referred to an urologist as earliest as possible(4, 5). Some evidence of kidney injury are Albuminuria, alterations in renal imaging, hematuria/leukocyturia, recurrent hydroelectrolytic problems, histological changes in renal biopsy, and recent kidney transplantation(6).

1.1.2.2 The causes of CKD

Diabetes, hypertension,chronic glomerulonephritis, chronic pyelonephritis, chronic Use of anti-inflammatory medicine, autoimmune illnesses, polycystic kidney disease, Alport disease, congenital anomalies, and prolonged acute renal disease are the most common causes of CKD(7, 8).

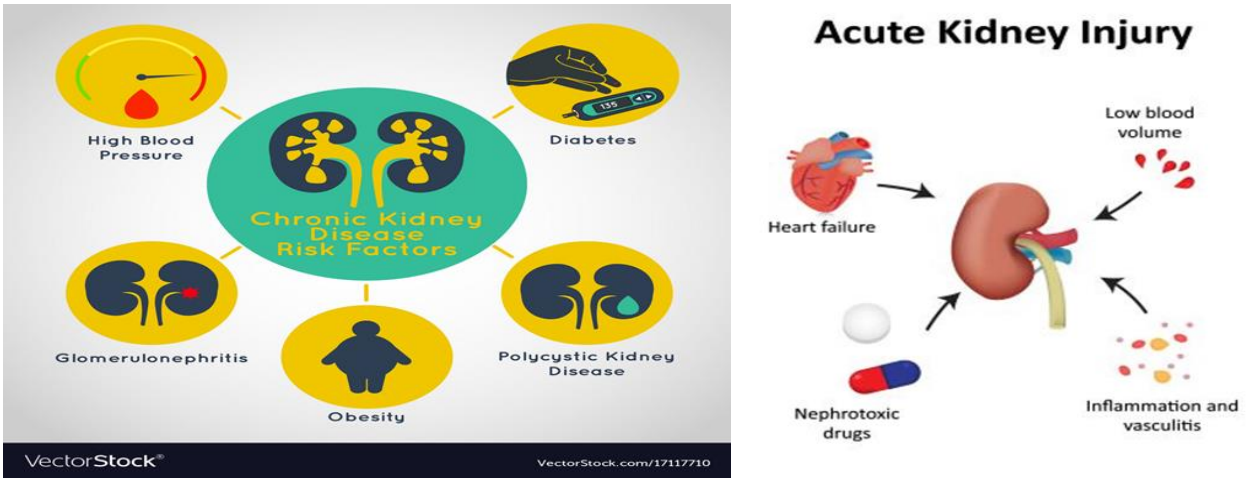


Figure (1-2) risk factors of chronic & acute kidney injury (9).

1.2.3 Clinical and laboratory findings in CKD

As CKD progresses over time, the following complications become more likely:

• Anemia, often known as a low erythrocyte count characterized by fatigue and weakness.

• Blood calcium levels drop, while phosphorus rates are elevated lead to bone problems.

• Elevated potassium content in the blood may result in an irregular or unstable heartbeat

• Nausea or loss of appetite

• Excessive bodily fluid can result in elevated blood pressure, leg edema, or breathlessness

• Infections or a compromised immune system.

• Depression(10-12).

1.2.4 Kidney disease classification

Both CKD & AKI (acute kidney injury) are categorized into stages based on the degree of abnormalities in these parameters, and clinical guidelines for both conditions include a stage-based approach to diagnosis and therapy.

The identification of the etiology of renal disease isn't included in descriptions of CKD or AKI. This gap was deliberately, enable kidney impairment to be detected in epidemiology which in many clinical situations, where the cause of renal disease is unclear or undocumented. Defining CKD and AKI only based on laboratory information risks "overdiagnosis." However, the work groups opinion that the danger of "underdiagnosis" was possibly higher (12-14).

Table (1- 1) Criteria for Definitions of Kidney Diseases and Disorders

	Functional Criteria	Structural Criteria
AKI	Increase in SCr by 50% within 7 day, *or* increase in SCr by 0.3 mg/dL within 2 day, *or* oliguria	No criteria
CKD	GFR <60 mL/min for >3 month	Kidney damage for >3 month
AKD	AKI, *or* GFR <60 mL/min/1.73 m^2 for <3 month, *or* decrease in GFR by ≥35% or increase in SCr by >50% for <3 month	Kidney damage for <3 month
NKD	GFR ≥60 mL/min/1.73 m^2, stable SCr	No damage

(14, 15).

1.2.5 Diagnosis of CKD

Is often made as a result of randomly screening test results (urinalysis or blood tests), or if symptoms worsen. A first assessment of CKD is commonly produced in general care by general practitioners using serum creatinine (SCr) values. GFR is the greatest available indication of total renal function, This can be assessed by exogenous markers (e.g., DTPA, iohexol) or calculated using equations Proteinuria is linked to an increased risk of CKD progression and mortality. Renal histology specimens can provide definite evidence of CKD by common alterations such tubular atrophy, glomerular sclerosis, and interstitial fibrosis(16-18).

1.2.5.1 Renal Function Tests

The most common clinical tests to measure renal function are to determine the glomerular filtration rate (GFR) and to look for proteinuria (albuminuria).

1. Glomerular Filtration Rate GFR: is the rate at which molecules in circulation are filtered through glomerulus in milliliters per minute.

Creatinine is an endogenous marker used to measure GFR. The estimated creatinine clearance is used as an indication of GFR.

- The following equation is used to determine creatinine clearance:
- C = (V x U) / P

C = clearance, V = urinary flow rate (volume / time i.e. ml / min), U = urinary concentration, & P = plasma concentration

- Stages of chronic kidney disease (CKD) identified by Kidney Disease Improving Global Outcomes (KDIGO):
- Stage1 GFR > 90 ml/min/1.73 m²
- Stage2 GFR(60 -89) ml/min/1.73 m²
- Stage3a GFR 45 to 59 ml/min/1.73 m²
- Stage3b GFR (30 – 44) ml/min/1.73 m²
- Stage4 GFR of (15 - 29) ml/min/1.73 m²
- Stage5 GFR < 15 ml/min/1.73 m² (end stage renal disease)

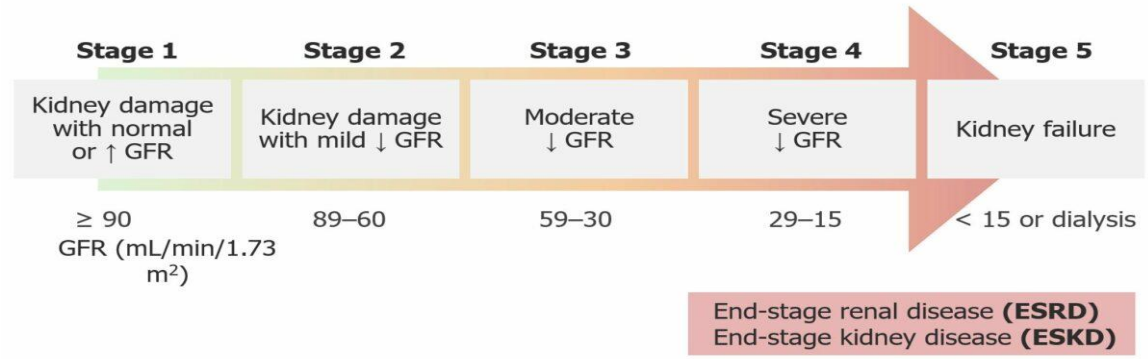


Figure (1-3) stages of CKD(19)

2. Albuminuria and Proteinuria

Albuminuria is the abnormal appearance of albumin in the urine. Because there is no such biological molecule as microalbumin, the phrase is now simply referred to as urine albumin.

3. Urinalysis: Urine analysis is the evaluation of urine characteristics to assist in illness diagnosis. Physical examination, chemical analysis, and microscopic examination are all part of it(15, 20).

1.2.6 Complications of CKD

Complications of CKD Include:

1.2.6.1Anemia

Related to decreased erythropoietin synthesis by the kidney, decreased red blood cell survival, and iron deficient. The pathophysiology of anemia in chronic renal disease is complicated, but a prominent aspect is a relative lack of erythropoietin. The hypoxia-sensing system plays a vital role in modulating erythropoietin production and release, according to new research. Iron deficiency is a secondary important factor in CKD anemia(19, 21). Patients with CKD have both absolute & functional iron inadequacy. Absolute iron insufficiency is defined by iron reserves that are severely depleted or absent, whereas functional iron insufficiency is characterised by adequate iron storage but inadequate iron availability for absorption into erythroid precursors. This is related to elevated hepcidin levels(22).

1.2.6.2. Mineral Bone Disease

caused by a disruption in the metabolism of vitamin D, calcium, and phosphate(23).

1.2.6.3 Hypertension

Hypertension is both a cause as well as a result of CKD, and it is common in people with both CKD and ESKD(24).

1.2.6.4 Hyperkalemia and Hypokalemia in CKD

Serum potassium abnormalities are prevalent in CKD patients despite the fact that hyperkalemia is a well-known CKD consequence, the prevalence rates of hyperkalemia (14% to 20 %) and hypokalemia (12 % to18%) are comparable. Serum potassium content in CKD is heavily influenced by the severity of the disease, the use of drugs such as renin-angiotensin-aldosterone system inhibitors and diuretics, and dietary potassium consumption(25, 26).

1.2.6.5 Secondary and Tertiary Hyperparathyroidism

Secondary hyperparathyroidism (SHP) is a common complication of renal illness. Renal SHP contains a number of pathogenetic features. Furthermore when chronic kidney disease (CKD) progresses, SHP can sometimes convert into a hypercalcemic syndrome approaching the autonomous type of hyperparathyroidism tertiary hyperparathyroidism (THP) (27, 28).

1.1.3 Epidemiology

Chronic kidney disease has been identified as a major public health issue across the world. The estimated worldwide prevalence of CKD is 13.4% (11.7-15.1%), and the number of people with end-stage kidney disease (ESKD) who require renal replacement treatment is predicted to range between 4.902 and 7.083 million. CKD has a direct impact on the global burden of morbidity and mortality

due to its effect on cardiovascular risk and ESKD. The global rise in this condition is being driven mostly by an increase in the prevalence of diabetes, hypertension, obesity, and aging. However, in some areas, additional reasons such like infection, chemical and environmental poisons are still prominent. The high number of mortality in developing world due to a lack of access to kidney transplant therapy, Furthermore, a huge increase in the number of patients with ESKD in the future would impose a significant financial burden on even the richest countries. The cost-effectiveness of disease-prevention strategies should be assessed in relation to local economic growth and resource availability. Strategies for lowering heart disease risk in CKD need to be tested in major studies, particularly in individuals with severe renal impairment or end-stage kidney disease(29-31).

1.4.1 Renal Osteodystrophy

Renal osteodystrophy is a change in bone morphology in CKD patients, and it is one marker of the skeletal component of the systemic condition of CKD-MBD that can be quantified using histomorphometry on bone biopsies (32). Osteoporosis is the most serious clinical condition associated with bone remodeling disorders and raises the risk of bone fracture(33). Osteoporosis is described as a skeletal condition characterized by decreased bone strength, which predisposes people affected to an increased risk of fracture. According to bone histology, osteoporosis is merely one component of a skeletal problems spectrum that also includes osteomalacia numerous types of renal osteodystrophy of chronic kidney disease–mineral and bone disorder (CKD-MBD)(34, 35).Furthermore, the term "kidney-induced osteoporosis" has been proposed, despite the fact that the alterations generated by CKD do not qualify as osteoporosis by histological definition. A new label, "CKD-MBD/osteoporosis," may be a more acceptable name because it incorporates osteoporosis within the official title of CKD-MBD. Neither laboratory

and neither noninvasive diagnostic procedures can distinguish osteoporosis from the various types of renal osteodystrophy. Transiliac crest bone biopsy can rule out other kidney-associated bone illnesses and help diagnose osteoporosis, although it is not widely available. Currently, a categorization of metabolic bone disorders from low to high bone turnover, as well as mineralization and bone mass, has been presented (36-38).

1.4.2 Pathogenesis

The pathophysiology of renal osteodystrophy (ROD) is complicated. The major focus on bone health has been to manage parathyroid hormone (PTH) with calcitriol or other vit D analogs, and more recently with calcimimetics(39). Secondary and tertiary hyperparathyroidism clearly have a significant role in developing bone remodeling in CKD. Despite an extensive focus on therapies, PTH and phosphate reducing therapy for renal osteodystrophy research have shown that the incidence of age-adjusted hip fracture has actually grown over the last few decades (40). Thus, aberrant bone in CKD patients is caused by more than only PTH(41).

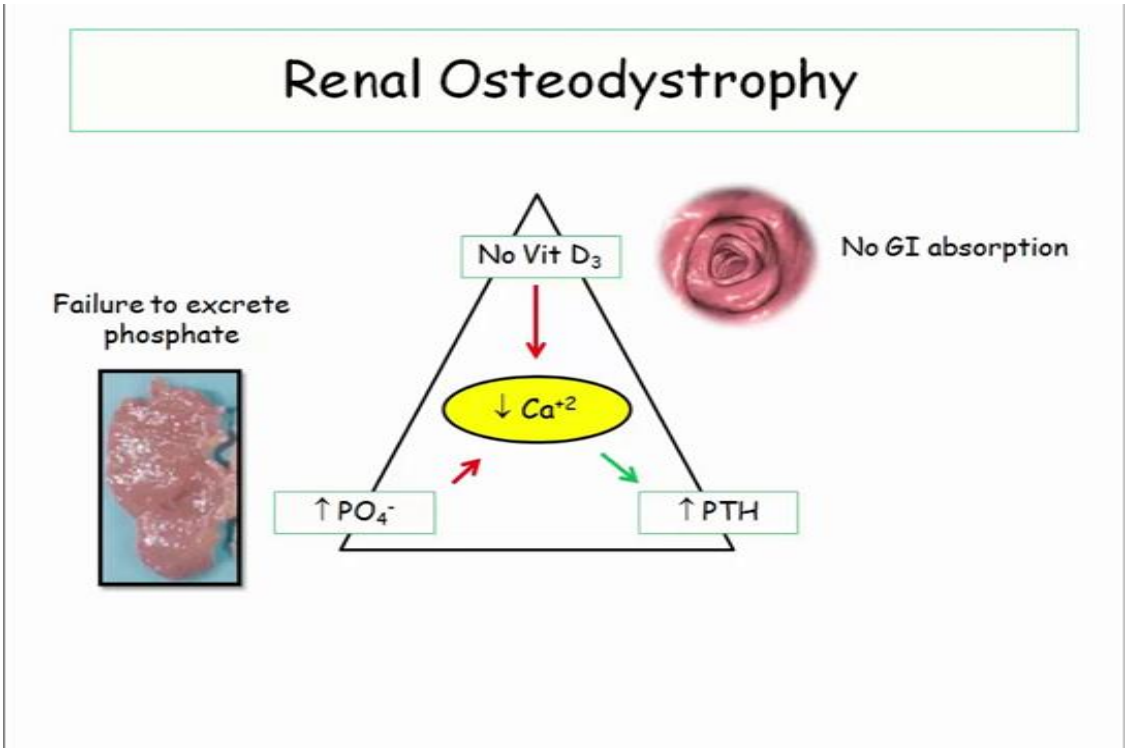


Figure (1-4) factor stimulate SHPT & causing ROD(40)

1.4.3 Treatment

The US Food and Drug Administration have not authorized any antifracture therapies for people with kidney-associated bone disease. Hyperparathyroid bone disease is treated with agents that inhibit parathyroid hormone (vitamin D analogues and calcimimetics). Antiresorptive and osteoanabolic medicines licensed treatment osteoporosis are used off-label for treating CKD stages 3b-5 in high-risk individuals. Intermittent use of PTH as early as CKD stage 2 has now been proposed as an effective treatment option(36, 42).

1.4.4 Characteristics of CKD-MBD:

(i) Inappropriate parathyroid hormone (PTH), phosphorus, calcium , vitamin D metabolism.

(ii) Defects in bone metabolism, mineralization, volume linear growth, or strength

(iii) soft-tissue calcifications (vascular calcifications). The most prevalent consequences associated with CKD-MBD are uremic vascular calcification and osteoporosis (43, 44).

1.1.5 Bone Turnover

Bone is constantly being replaced and renewed, with osteoclasts removing a certain amount of mineralized bone tissue, leaving a resorptive space that is filled by the migration and development of osteoblast precursors into mature osteoblasts that generate a mineralized extracellular matrix (45). In additional to its structural role, the skeleton functions as an endocrine organ, regulating mineral metabolism and energy balance. Three main cell types in bone – osteoblasts, osteoclasts, and osteocytes – actively form and maintain bone while secreting systemic substances(46). Bone turnover is consists of two processes: the removing of old bone (resorption) and the production of new bone (formation) (47). Osteocytes, Cells characterize terminal stage of the osteoblast progenitor implanted in the bone matrix play a crucial role in the control of both the remodeling and mineralization processes(48).

1.5.1 Kidney Role in Mineral Metabolism

The essential actors in chronic kidney disease-mineral and bone disorders (CKD-MBD) include PTH, FGF23, calcium, phosphate, and the vitamin D hormonal system. The increasing loss of renal function has a significant impact on the closely linked mechanisms that regulate these parameters. As a result, significant alterations occur in the bone & mineral hormonal axis, resulting in changes in bone turnover having clinically meaningful outcomes(49). Such as decreased bone mass, increased bone fragility and fractures, and increased vascular calcification, all of which have a significant influence on cardiovascular outcomes.

So far, increasing understanding of mineral and bone problems in CKD, as well as a wider range of effective medicines, should lead to better prevention and management of CKD-MBD(50, 51).

1.5.2 Effect of CKD on bone turnover and mineralization

Chronic renal disease is frequently accompanied with skeletal deformities that might last for years after a successful kidney transplant. Patients suffer from fractures as well as calcification of soft tissues and blood vessels, which is referred to as chronic kidney disease - mineral bone disorder (CKD-MBD) (52-54).

Table (1-2) Factors affecting bone strength in chronic kidney disease-mineral and bone disorder (CKD-MBD).

Factor	Main Effect	Category
↓ Kloth	↑ FGF-23 level	Humoral
↑ FGF-23 [1]	↑ phosphate excretion ,↓ calcitriol synthesis	Humoral
↑ Sclerostin	↓ bone formation , ↑ osteoclastogenesis	Humoral
↑ dickkopf1	↓ bone formation	Humoral
↑ phosphate	↑ SPTH↓ calcitriol synthesis	Mineral
↑ uremic toxins [2]	↓ PTH receptor, ↑ skeletal resistance to PTH	Uremia
↓ $1,25(OH)_2$ D	↑ PTH secretion , ↓ calcium	Humoral
↓ calcium	↑ SPTH ↑ abnormal bone remodeling	Mineral
↑Skeletal resistance to PTH	↑ SPTH	Humoral

FGF-23: fibroblast growth factor-23; Uremic toxins refer to indoxyl sulfate and p-cresyl sulfate; SPTH: secondary hyperparathyroidism; ↓: decrease, ↑: increase(54).

1.5.3 Characteristic of Mineral & Hormonal Disturbance in CKD

As GFR decreases, unbound serum calcium levels drop and serum phosphorus levels rise. Because of GFR loss compensatory synthesis of fibroblast growth factor 23 (FGF-23), reduces sodium-dependent phosphate transport protein (NPT2a) and (NPT2c) levels in the kidney As a result, phosphate excretion in the urine is increased(55).

In response, the parathyroid glands produce more PTH, which reduces the quantity of NPT2a and NPT2c in proximal tubule, resulting in greater urine Pi excretion and lower serum Pi levels(56). FGF23 also suppresses the synthesis of 1,25(OH)2D, which reduces intestinal Pi absorption and, as a result, serum Pi levels(57). Reduced 1,25(OH)2D causes hypocalcaemia, and increased PTH production continues, resulting in secondary hyperparathyroidism (SHPT). However, when GFR continues declines, these compensatory mechanisms fail, resulting in hyperphosphatemia, hyperparathyroidism, and increased serum FGF-23 levels. SHPT and uremic toxin buildup expedites bone turnover by stimulating osteoclastogenesis and increasing calcium and phosphate release from bone. FGF-23 reduces PTH from the gland under physiological conditions. By way of progressive GFR loss, PTH was resistant to FGF-23 inhibition, and successive nodular parathyroid hyperplasia developed (58, 59). In considerations of vascular health, hyperphosphatemia, SHPT, and hypovitaminosis have all been linked to increased cardiovascular morbidity and mortality, as well as an increased risk of calciphylaxis(60). Thus, SHPT therapy focuses on (i) phosphate limit, (ii) calcimimetics and (iii) supplementation with vitamin D analogue (61).

1.5.4 Diagnosis CKD BMD

Non-invasive imaging has proven to be effective in assessing the microstructure/microarchitecture of cancellous and cortical bone in patients. On the other hand, In vivo imaging methods cannot offer information on mineralization abnormalities in CKD-MBD patients. Therefore A bone biopsy sample might be useful in the diagnosis of bone disorders(62). It was formerly known as renal osteodystrophy (ROD). and are commonly described by the TMV (turnover, mineralization, volume) –system In CKD-MBD, histomorphometrically defined bone turnover ranges from adynamic bone disease to osteitis fibrosa with substantially accelerated bone turnover. Also, the biopsy sample contains information about bone quantity and mineralization problems (63, 64).

1.1.6 Bone Turnover Biomarker

Bone turnover markers (BTMs) are a group of protein or protein derivatives biomarkers generated by osteoblasts or osteoclasts during bone remodeling. BTMs can provide predictive information on fracture risk that supports radiographic measurements of bone mass, but BTM testing must account for the huge number of preanalytic variables and concomitant clinical disorders that influence BTM levels(65). BTMs respond quickly to changes in bone physiology, making them useful in evaluating patient response to and conformity with osteoporosis therapy(66). Biomarkers like bALP & iPTH may aid in determining bone turnover. Before starting an antiresorptive or anabolic drug to treat osteoporosis in CKD patients, lifestyle changes such as exercise, calcium and vitamin D intake, smoking cessation, and avoiding excessive alcohol consumption are critical. It is also critical to manage hyperphosphatemia and SHPT(54).

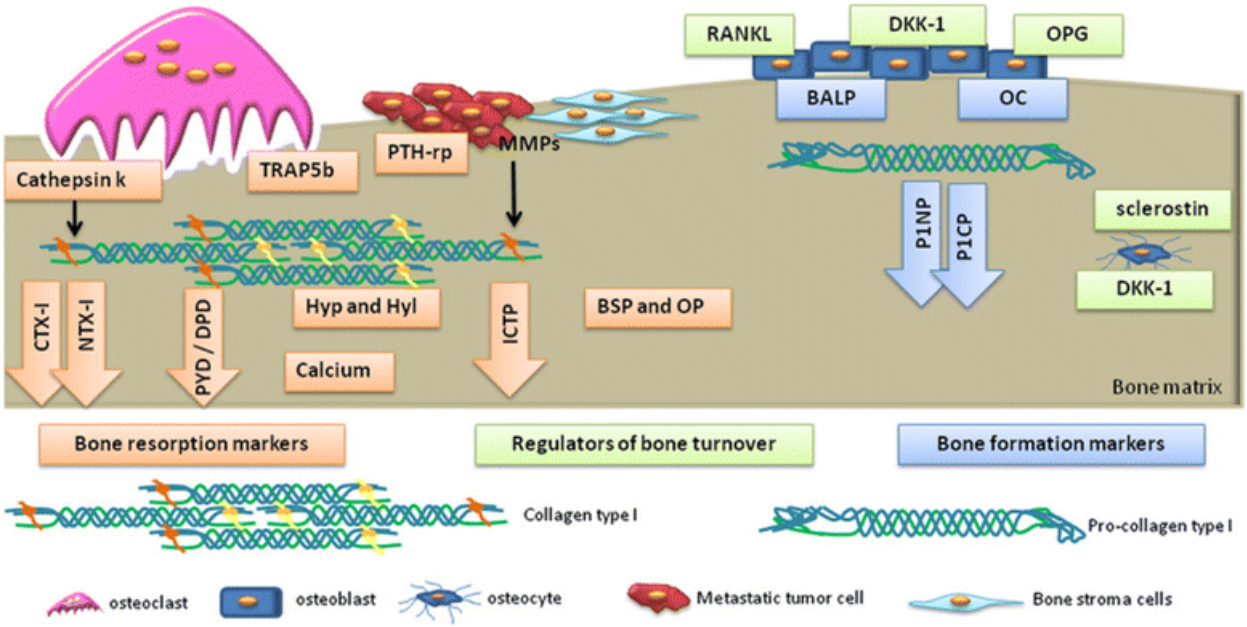


Figure (1-5) Biochemical biomarkers of bone turnover.

Blue boxes/arrows represent bone formation markers: bone-specific alkaline phosphatase (BALP); osteocalcin (OC); propeptides of type I procollagen (P1NP and P1CP). Orange boxes/arrows represent bone resorption markers: pyridinoline (PYD); deoxypyridoline (DPD); carboxy-terminal crosslinked telopeptide of type 1 collagen (CTX-1); amino-terminal crosslinked telopeptide of type 1 collagen (NTX-1); hydroxyproline (HYP); hydroxylysine (HYL); bone sialoprotein (BSP); osteopontin (OP); tartrate-resistant acid phosphatase 5b (TRAP 5b); cathepsin K (CTSK). Green boxes represent regulators of bone turnover: receptor activator of NF-κB ligand (RANKL), osteoprotegerin (OPG), dickkopf-1 (DDK-1) and sclerostin(67).

1.6.1 The most commonly used bone turnover markers (BTMs)

1. Type I Collagen C- and N-Terminal Telopeptides The most prevalent protein component of bone is type I collagen, while C- & N-terminal telopeptides of type I collagen (CTX and NTX) are both portions of type I collagen from the telopeptide portion, a nontriple-helical area at the ends of mature collagen.

2. Type 1 Collagen N- & C-Terminal Propeptides (PINP and PICP) Type 1 collagen is secreted by osteoblasts as a complete molecule comprising the N- and C-terminal propeptides, that are then cleaved in the extracellular environment.

3. Alkaline Phosphatase Specified for Bone

4.Osteocalcin (66).

1.7 Bone Formation Biomarkers

1.7.1 Total alkaline Phosphatase (ALP)

Alkaline phosphatase is a blood enzyme. ALP remove phosphate (dephosphorylation) from several metabolic pathways (from various molecules such as proteins, nucleotides, pyrophosphate). ALPs in various forms can degrade proteins in the human body. The majority of ALPs are created in the liver, although some are also produced in the bones, intestines, and kidneysThe total ALP level is determined by measuring the quantity of alkaline phosphatase enzyme with in circulation(68). Total ALP measurement requires merely a simple blood sample and is frequently performed as part of regular blood testing. ALP levels in persons vary according on their age, blood type, gender, and pregnancy(69). ALP levels in the blood that are very high usually signal a problem with the liver, gallbladder, or bones. A recent research also found that serum total ALP activity more than 129 U/L is utilized as an indication of osteoporosis in men (70, 71).So, APs are necessary for bone mineralization, but they may also be harmful to other activities, like vascular calcification as well as the increasingly recognized cross-talk with bone and arteries. A balance of beneficial and destructive activities is made more difficult in the situation of chronic kidney disease (CKD)(72). Elevated total blood alkaline phosphatase concentrations have been linked to an increased risk of death in the general

public, CKD patients, and dialysis patients(73). Bone alkaline phosphatase (BALP) is essential for biomineralization. Recent findings demonstrate a crucial role for BALP in the pathogenesis of vascular calcification and identified it as a promising predictor of mortality in CKD. In conjunction with parathyroid hormone (PTH), serum BALP has been suggested as a biomarker of bone turnover in CKD-MBD. In contrast to PTH, serum BALP demonstrates a lower variability and may thus be better suited for the diagnosis and longitudinal follow-up of bone turnover. The linear association with mortality, compared to the U-shaped curve for PTH, is an additional advantage, making BALP more suitable than PTH as a treatment target in CKD (74, 75).

1.7.2.1 procollagen type I N-propeptide

The osteogenesis marker procollagen type I N-propeptide (PINP) can be found in the organic bone matrix (> 90%), that is developed in bone from procollagen type 1 PINP is a trimeric peptide with such a molecular mass of about 35,000 kDa, consisting of two types procollagen-1 chains and a procollagen-2 chain that are non-covalently bonded(76).

1.7.2.2 Synthesis of PINP

Procollagen I molecule is synthesised by fibroblasts and osteoblasts, and it has pro-peptide extensions at the amino-N-terminal extensions and carboxy-terminals C-terminal extensions (PINP and PICP respectively) of the procollagen molecule are cleaved off and released into circulation when collagen molecule is laid down to form the osteoid matrix during bone formation(77). Specific proteases remove procollagen type 1 during the transformation of procollagen to collagen. P1CP and P1NP from procollagen type 1 are then bonded onto the bone matrix (78).

1.7.2.3 Diagnostic role of PINP

Procollagen type I N-propeptide is released into the intracellular compartment during the synthesis of type 1 collagen, and it finally ends up in the bloodstream. P1NP is often produced in a trimeric structure (formed from a trimeric collagen structure) and then rapidly degrades to a monomeric state due to heat degradation processes. P1NP antibodies are used in enzyme-linked immunosorbent assays (ELISA) or radioimmunoassays to identify the trimeric structure of P1NP(79). Serum PINP (sPINP) has been shown to reflect histomorphometric markers of bone formation in practice(80). It has been selected as the reference biomarker for bone formation during osteoporosis by an International Osteoporosis Foundation (IOF) as well as the International Federation of Clinical Chemistry and Laboratory Medicine as the most promising marker of bone formation (IFCC) (81).

The monomeric pieces are most likely eliminated by the kidney and aggregate abnormally as renal function is reduced. As a result, when PINP is tested using 'total PINP' assays, which recognize the monomer in additional to the complete PINP molecule, significant amounts are observed in dialysis patients (77).

1.8 Regulators of Bone Turnover

1.8.1 Receptor activator of NF-κB ligand (RANKL)

RANKL and OPG are produced by osteoblasts throughout the bone remodeling process to govern the differentiation and maturation of osteoclasts. Dextromethorphan has recently been shown to decrease RANKL-induced osteoclastogenesis and bone resorption in vitro via inhibiting NF-B signaling activation human serum RANKL levels have been measured to assess the states in metabolic bone disorders(70, 82, 83)

1.8.2 Osteoprotegerin (OPG)

Osteoprotegerin is generally considered to be a secreted soluble receptor and is produced by many different tissues and cell types including osteoblasts.it has key roles in bone biology and the immune system(84). The role of OPG is used as a decoy receptor for RANKL and inhibitor of osteoclastogenesis.

1.8.2.1 Structure

Osteoprotegerin (OPG) is a glycoprotein that belongs to the tumor necrosis factor receptor superfamily(85).

1.8.2.2 Diagnostic role of OPG

In individuals with renal illness, OPG might be a biomarker. Circulating OPG levels are higher in CKD patients on predialysis, dialysis, and transplant, and they may indicate vascular calcification development and patient survival. Circulating OPG, on the other hand, is reduced in nephrotic syndrome(86).

1.8.2.3 Biological Effect of OPG

Despite the link between high OPG levels and illness, experimental functional data shows that OPG may be beneficial in renal disease and vascular damage in the context of uremia. Thus, tissue injury causes an increase in OPG, whereas OPG may protect against tissue harm(87). Osteoblasts mediate osteoclastogenesis by expressing the membrane-associated cytokine receptor activator of nuclear factor-kappa B ligand (RANKL). Osteoprotegerin (OPG) is a soluble RANKL decoy receptor that is primarily generated by osteoblasts and inhibits the RANKL-RANKL receptor interaction, hence preventing osteoclast development and osteoclastic bone resorption(82, 88).

1.8.3 Seclerostin

Sclerostin, a 22-kDa protein produced by the SOST gene, is a well-known suppressor of bone growth. Although SOST mRNA is commonly thought to be an osteocyte-specific protein, it is also found in the renal, liver, marrow, lung, heart, and pancreas(89, 90). Sclerostin is primarily generated by osteocytes and is well recognized as a paracrine regulator of WNT signaling and osteoblast and osteoclast activity on bone surfaces (46). Sclerostin (SOST) is a glycoprotein that is primarily released by osteocytes and is involved in the control of bone metabolism. It inhibits the Wnt/-catenin metabolic pathway in bone cells and influences the activity of bone morphogenetic proteins (BMPs). In response to mechanical stimuli acting on bone, osteoocytes limit the production of sclerostin, promoting the activation of the osteogenic pathway Wnt/-catenin in osteoblasts. This signaling system is essential for osteogenesis and bone turnover. (91) is one of the biomarkers that serves as a connection between vascular and bone disease. Sclerostin may have an effect on bone metabolism in chronic kidney disease and end-stage renal disease patients on maintenance dialysis. Sclerostin buildup has been demonstrated to limit PTH release in individuals with chronic renal illness, which may potentially delay an excessive bone turnover caused by secondary hyperparathyroidism.(92)

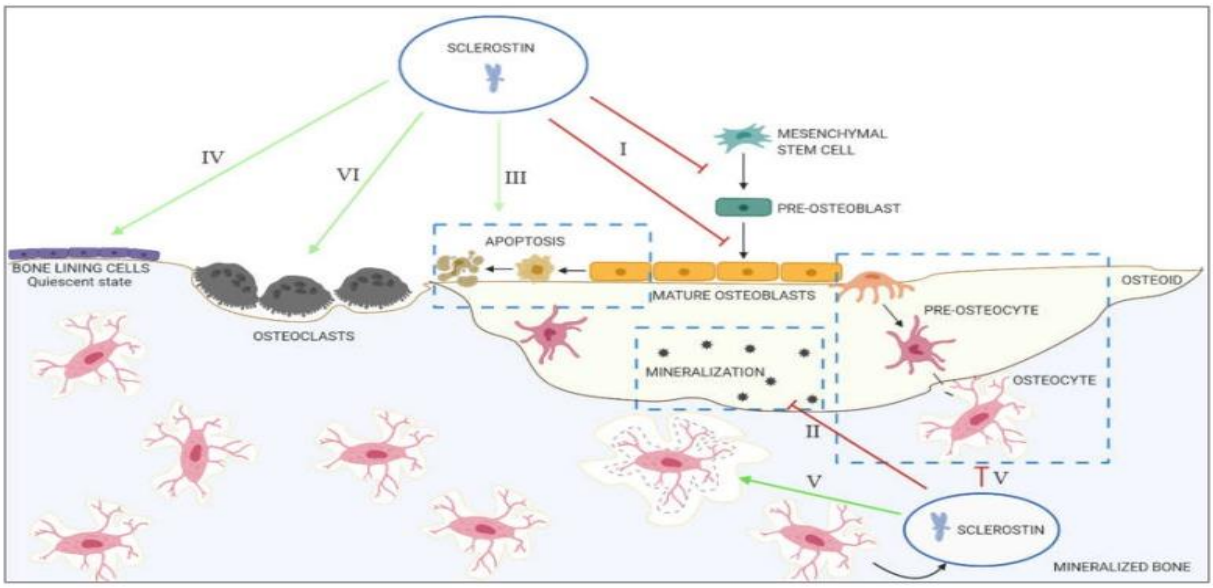


Figure (1-6) Overview of the actions of sclerostin in the bone.

I: Inhibition of proliferation and differentiation of osteoprogenitor/pre-osteoblastic cells, as well as decreased activation of mature osteoblasts; II: decreased mineralization; III: increased apoptosis of the osteogenic cells; IV: maintenance of bone lining cells in their quiescent state; V: regulation of osteocyte maturation and osteocytic osteolysis; VI: stimulation of bone resorption(89).

1.9 PTH, Calcium and Phosphorus interaction

The equilibrium of calcium and phosphorus is carefully maintained by the coordinated management of circulating hormones such as PTH, FGF-23, and vitamin D. The PTH-Vitamin D axis modulates blood calcium concentrations by combining the effects of increased 1,25(OH)2D to enhance gastrointestinal calcium absorbing and PTH effect on bone to promote calcium release and on the kidney to decrease calcium elimination in the urine. Calcitonin, which is produced by C-cells in the thyroid, also inhibits bone resorption and calcium efflux and protects against hypercalcemia(33, 89). Fibroblast growth factor 23 (FGF23) and parathyroid hormone (PTH) are hormones that regulate renal phosphate excretion

and vitamin D metabolism. As renal function diminishes in chronic kidney disease (CKD), circulating FGF23 and PTH concentrations rise. (93)

Secondary hyperparathyroidism is a component of the chronic kidney disease-related mineral and bone disorders (CKD-MBD) complex, and it is associated with rapid bone turnover, ectopic calcification, and higher cardiovascular mortality. (94) In the course of CKD, both PTH production and secretion are constantly increased, resulting in secondary hyperparathyroidism . Downregulation of vitamin D receptor, calcium-sensing receptor, and Klotho expression in parathyroid tissue increases PTH overproduction even more. Prolonged stimulation of parathyroid secreted activity is distinguished not only by a persistent increase in serum PTH, but by parathyroid gland hypertrophy. (95) The fundamental determinant of parathyroid hormone (PTH) secretion is serum calcium content.

1.10 Albumin

Serum albumin is a plasma protein found in large amounts in mammalian blood. This protein's significance stems from its activities in both bioregulation and transporter phenomena. Serum albumin binds different metal ions and aids in the storage and transport of fatty acids, bilirubin, steroids, amino acids, and a variety of other ligands, typically with hydrophobic surface areas. Although serum albumin's primary purpose is to transport multiple ligands, its diverse binding capabilities and high concentration allow it to perform a variety of other activities (96). Serum albumin is still commonly utilized in research and as an indicator of nutritional status in the therapeutic context. Overhydration and proteins losses into urine and dialysate diminish serum albumin concentrations in CKD patients(97).

Aims of study

1. investigate the possible relationship between serum sclerostin levels and bone markers in CKD.

2.Assessment of osteoprotegerin as a mediated mineral bone metabolism marker in CKD is important in clinical decision-making to select and initiate treatment.

3.Assessment of circulating procollagen I N-Propeptide in CKD.

4.Evaluation of the association between serum level of sclerostin, procollagen I N-Propeptide and osteoprotegerin in CKD

2. Materials and Methods

2.1. Materials

2.1.1. Chemicals and Kits

This investigation's chemicals and kits were used just the same as they arrived from the store, without any further purification.

Table 2-1 lists the kits and compounds used in this study.

No.	CHEMICALS	COMPANY AND COUNTRY
1	Albumin	RANDOX (UK)
2	Alkalin phosphatase (ALP)	RANDOX (UK)
3	Calcium	RANDOX (UK)
4	Human Osteoprotegerin (OPG) ELISA Kit	mybiosource (USA)
5	Human P Ⅰ NP(Procollagen Ⅰ N-Terminal Propeptide) ELISA Kit	mybiosource (USA)
6	Human Sclerostin (SOST) ELISA Kit	mybiosource (USA)
7	Phosphorus	Mybiosource (USA)
8	PTH ELISA Kit	CUSABIO. (USA)

Table 2-2 contains a list of the instruments and equipment utilized in this study.

No	Instruments and Equipments	Company and Country
1	0.1 ml pipette tips	China
2	1 ml pipette tips	China
3	Centrifuge EBA 20	Hettich/Germany
4	Deep Freezer	Samsung/Korea
5	Disposable cuvette	China
6	Disposable syringe (5 ml)	China
7	Elisa washer, reader and printer	Biotek /USA
8	Eppindorf tube (0.5 ml)	China
9	Kan tube	China
10	Micropipettes (5-50µl),(100-1000µl)	Slamed / Germany
11	Multichannel micropipette (0-250 µl)	Slamed / Germany
12	Refrigerator	agur/Turkish
13	Spectrophotometer	Jenway/Italian
14	Tank for multichannel micropipette	China
15	Test tube with Separating gel	AFCO , Jordan
16	Water Bath	HH-2 Chain

2.2. Subjects

2.2.1. The Place and Date of Study

This study was carried out in private laboratories. The samples were collected from dialysis units at Almahmmodia general hospital and Al Yarmouk teaching hospital between September 1st and December 1st, 2021, and were patients diagnosed by the aforementioned clinics nephrologist depending on measuring the renal diagnostic parameters such as blood urea, serum creatinine, proteinuria , e GFR ,renal ultrasound show diminished in kidney size.

2.2.2. Study Designs

The study was designed as a case-control study.

2.2.3. Patients Group

The sample size for the patient group was determined using Fisher's exact test for sample size formulas equation. Consist of 45 patients (18 male and 27female). All patients were diagnosed by a specialist physician when they attended to heamodialysis Unit at alyarmouk Teaching Hospital.and almahmmodia generl hospital

Fisher's exact test for sample size: $N = P Z^2 (1-P) / D^2$, where

N = sample size ,Z = statistic for a level of confidence interval 95%(98).

which = 1.96 ,P = expected prevalence or proportion of CKD which is 3.0% in KSA(99). ,D = precision (in proportion of one , if 5 % , D = 0.05)

n= (1.96)2 *0.03*0.97 / 0,0025 , n= 45

2.2.4 The Diagnosis of Patients Group

In this study, the diagnosis of CKD patients was confirmed by a nephrologist, depending on measuring the renal diagnostic parameters such as blood urea, serum creatinine, proteinuria , e GFR ,renal ultrasound show diminished in kidney size. The entire history was provided, and a detailed questionnaire was filled out, that contains (age, gender, weight, hight, habitation, pregnant, occupational status, plus if the patient suffered from any chronic or acute diseases such as diabetes mellitus DM and hypertension).

2.2.5 Control Groups

A control group of 45 apparently healthy individuals (18 men and 27 females) was used, were matched with patients in terms of gender and age to improve the accuracy of the results. They were obtained from our families and friends and health workers. They were had a normal renal functions test.

2.2.6. Exclusion Criteria

The persons have inherited metabolic abnormality, dawn's syndrome and any other chromosomal abnormalities, Parathyroid disease, thyroid & Parathyroid ectomy, pregnant, and breastfeeding women.

2.2.7 Inclusion Criteria

Persons were proven with CKD and sample taken at predialysis time, within the age from (20 – 75) years mean age (50.51 ± 2.07) were in the current study as a patient group, in addition to apparently healthy individuals as control group within the age from (21-71) years mean age (50.57 ± 1.81).

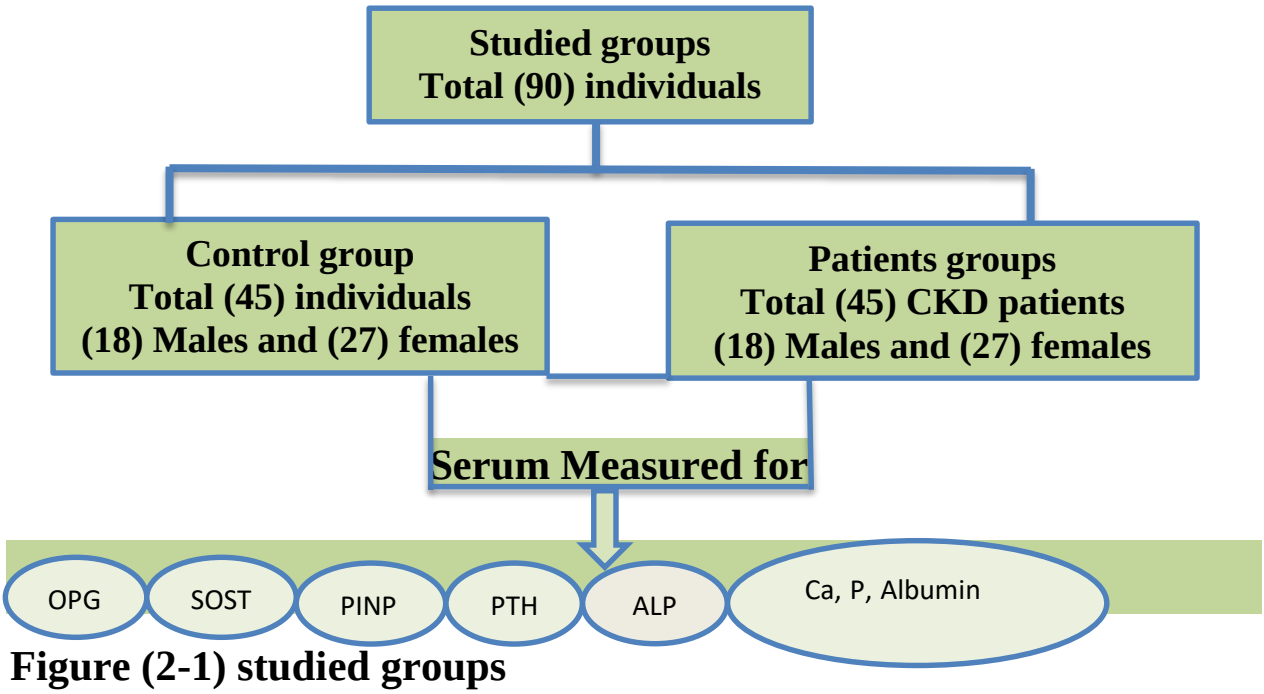


Figure (2-1) studied groups

2.2.8. Ethical issues

Depends on the following:

1- Approval by a scientific committee of Babylon Medical College (University of Babylon, Iraq) and the Biochemistry Department in the same college.

2- The aims and procedure of this study were clarified to all participants in the present study to gain their verbal acceptance.

3- Ethical and scientific committee in hospital.

2.3. Methods

2.3.1. Collection of Blood Sample

A blood sample was collected from the vein of each participant, the patients sample was taken at prehemodialysis time, using a needle puncture approximately

(5ml) was placed in disposable plan tubes with separating gel. The blood sample in the plan tubes was kept at room temperature for five minutes to coagulate. and later it centrifuged for (10 minutes) at 3600 rpm subsequently, then Pipetting the transparent serum into three clear dry Eppendorf tubes and stored at (-20^{o}C) until used for the several examinations. The serum was thawed at (20-25^{o}C) temperature for 2 hours then submitted to the centrifuge for 5 minute at 3600 rpm.

2.3.2. Body Mass Index (BMI)

Body mass index was measured in all individuals based on a weight-to-height ratio derived by achieving a mathematical equation that divided the weight in kilograms by the square height in meters, and the results were considered as follows:

1. Underweight ≤ 18.5
2. Normal weight (18.5 - 24.9)
3. Overweight 25-29.9
4. Obese ≥ 30

BMI (kg/m^2) = weight (kg) / height (m^2) (100)

2.3.3. Measurement Human Osteoprotegerin (OPG) by ELISA method.

A. Principle

The "Double Antibody Sandwich" method was used in this investigation with an ELISA kit. The Double Antibody Sandwich concept is based on the properties of a target analyte that has more than two potential epitopes that can be detected by both the pre-coated capture antibody and the detection antibody at the same time.

The process is:

1. Pre-coat the plate with anti-Human OPG monoclonal antibody, and then wash away any antibodies or contaminants that did not attach to the plate.

2. When an OPG-containing sample is added, the OPG is immobilized by the OPG-specific capture antibodies, resulting in the formation of an antigen-antibody complex. The wells are then washed.

3. The wells are subsequently treated with a biotin-labeled antibody that is also specific for the OPG, resulting in an antibody-antigen-antibody combination. The plate is again washed.

4. In the wells, horseradish peroxidase + avidin binds to the biotin-labeled antibodies. The wells are then washed.

5 Substrates for the HRP reaction are added, the reaction is stopped using an acidic stop solution, and absorbance at 450 nm is measured.

The sample concentrations may then be determined based on the color changes. The amount of reporter enzyme is now proportional to the amount of OPG in the sample.

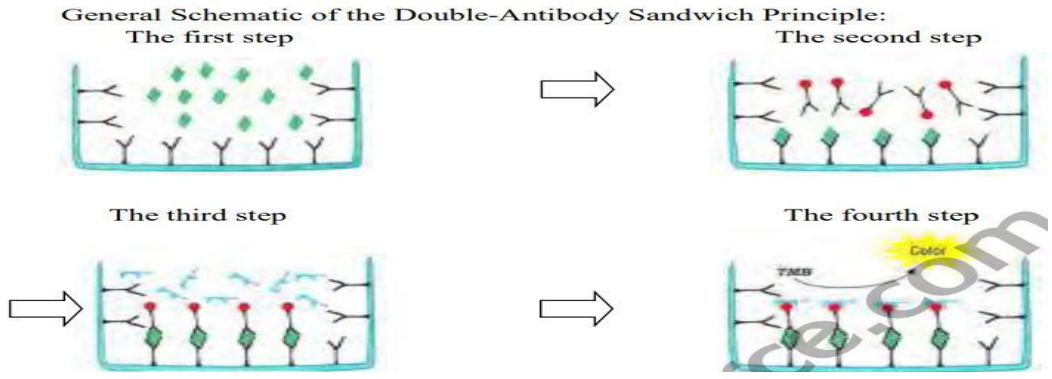


Figure (2-1) principle of sandwich ELISA

Detection range：10 ng /mL-0.156 ng/mL

Sensitivity：the minimum detectable Human OPG up to 0.05 ng/mL

B.Reagents& Preparation of reagents Procedure:

Pre-coated plate 12×8

Human OPG Standards 2 vial

Biotinylated antibody (1:100) 1vial

Enzyme conjugate (1:100) 1vial

Enzyme diluent 1vial 1

Antibody diluent 1vial

Standard diluent 1vial

Sample diluent 1vial

Washing buffer (1:25) 1vial

Color Reagent A 1vial

Color Reagent B 1vial

Color Reagent C 1vial and Manual 1 set

1.The Elisa Kit was taken out of the refrigerator 20 minutes before the test and tested after it had adjusted to room temperature.

2. Distilled water has been used to dilute the concentrated washing solution (1:25).

3. Human OPG standard sample: 1.0ml of diluent was added to the human OPG lyophilized standard sample After the sample has been thoroughly dissolved, gently mixed, and labeled on the tube①, it should be diluted as needed. the following concentration values was utilized for the standard curve: 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156ng/mL.

4. Standard sample dilution technique legend: 7 clean tubes has been taken and labeled them with②,③,④,⑤,⑥,⑦,⑧ respectively. A 300μl standard sample diluent was added to each tube.

The Pipette has been used out 300µl diluent from tube ① to tube ② and mixed well. Further Pipette has been used out 300µl diluent from tube ② to tube ③, and mixed well. Steps above was Repeated up to tube ⑦. Standard sample dilution in tube ⑧ is Negative control.

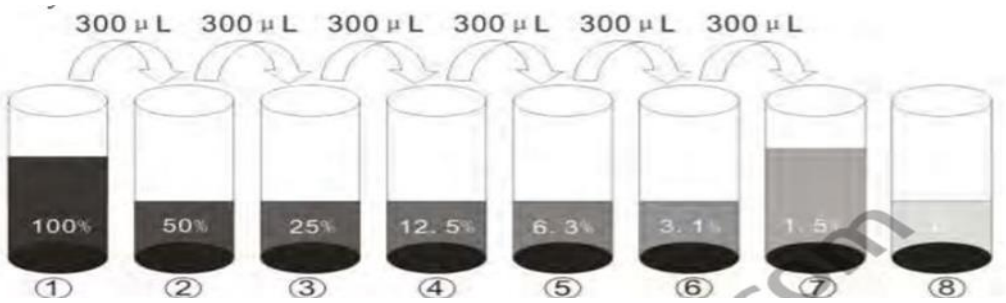


Figure (2-2) serial dilution of standard solution

5. Biotinylated human OPG antibody liquid: it has been employed diluent to dilute the concentrated biotinylated antibody (1:100) to form biotinylated antibody liquid.

6. Enzyme-conjugate liquid: the concentrated enzyme-conjugate has been diluted by enzyme-conjugate diluent (1:100) to form enzyme-conjugate liquid.

7. Color Reagent liquid: Color Reagent liquid has been prepared 30 min in advance with Color Reagent A and Color Reagent B by the proportion of 9:1.

C. Procedure

1.All reagents, standard solutions, and samples were prepared as directed. Before use, all reagents were brought to room temperature. The experiment is done at room temperature.

2. In the respective wells, samples or varying concentrations of human OPG standard samples have been introduced. (100µl for each well), 0pg/ml

3. The Elisa plate had already been rinsed twice.

4. Each well had a biotinylated human OPG antibody liquid added to it. (100µl for each). Reaction wells were sealed with adhesive tapes before hatching in an incubator at 37ºC for 60 minutes. then washing 3 times

5. Except for the blank wells, enzyme-conjugate liquid had been added to each well. (100µl for each). the reaction wells had been Sealed with adhesive tapes, hatching in incubator at 37ºC for 30 min. Then the Elisa had been Washed 5 times again.

11. 100µl Color Reagent liquid had been applied to different well (also into blank well), kept in dark incubator at 37ºC. When color for high concentration of standard curve develop darker and color gradient appears, the incubation had been stopped. The chromogenic reaction must be controlled within 30 min.

12. 100µl Color Reagent C had been Added to separate well (also into blank well. OD Readed（450nm） within 10 min.

D. Result determination

1. OD value of each sample and specimen should minus that of blank well (if not, the standard curve of zero well should intersect at Y axis)

2. Standard curve was drawn manually. The concentration value of samples took as abscissa and OD readings as vertical coordinate. Smooth line Used to connect each coordinate point of standard sample. The concentration of samples can be found by checking sample OD reading. It is recommended to employ the professional curve software (e.g. curve expert 1.3) to analyze and compute the result.

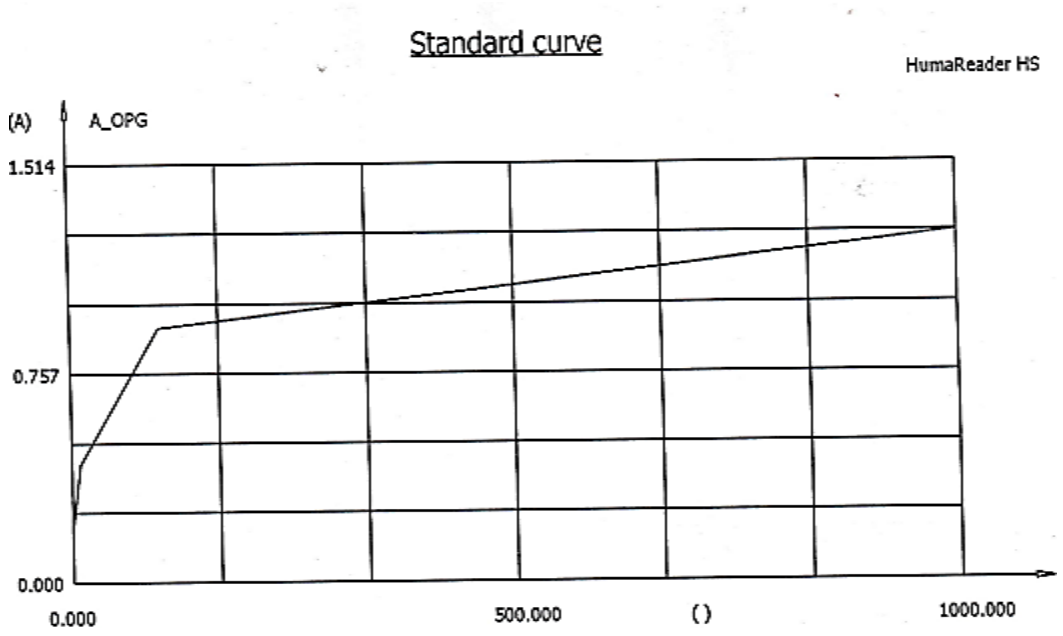


Figure (2-3) standard curve OPG test

2.3.4. Measurement Human P Ⅰ NP (Procollagen Ⅰ N-Terminal Propeptide) By ELISA method.

A. Principle

This study employs the Sandwich-ELISA principle. The kit's micro ELISA plate has been pre-coated with an antibody specific to Human PINP. Standards or samples are placed in the micro ELISA plate wells and mixed with the specific antibody. Then, in each microplate well, a biotinylated detection antibody specific for Human PINP and an Avidin Horseradish Peroxidase (HRP) conjugate are added and incubated. Free components are swept away. Each well is filled with the substrate solution. Only the wells containing Human PI NP, biotinylated detection antibody, & Avidin-HRP conjugate will be blue in color The enzyme-substrate reaction is stopped by the administration of stop solution and the color becomes yellow. At 450 nm ± 2 nm, the optical density (OD) is determined spectrophotometrically. Human PINP concentration is proportional to the OD

value.Finally concentration of Human PINP calculated the in the samples through matching the OD of the samples to the standard curve.

Detection range: 15.63-1000pg/mL

Sensitivity: 9.38pg/mL.

B. Reagents& Preparation of reagents Procedure:

Micro ELISA Plate 96T: 12 strips× 8 wells
2 vials of Reference Standard (96T)
1 vial, 120 μL of Concentrated Biotinylated Detection Ab (100×) 96T:
1 vial, 120 μL of Concentrated HRP Conjugate (100×) 96T
1 vial, 20 mL of Reference Standard & Sample Diluent
1 vial, 14 mL of Biotinylated Detection Ab Diluent
1 vial, 14 mL of HRP Conjugate Diluent
Concentrated Wash Buffer (25×) 1 vial, 30 mL
Substrate Reagent (1 vial, 10 mL)
Stop Solution (1 vial, 10 mL)
Plate Sealer 5 pieces

C.Reagent preparation

1. all reagents brought to room temperature (18~25^{o}C) before use.

2. Wash Buffer: 30 mL of Concentrated Wash Buffer was Diluted with 720 mL of deionized or distilled water to prepare 750 mL of Wash Buffer.

3.Standard working solution: after Centrifuged the standard at 10,000×g for 1 min. Added 1.0 mL of Reference Standard &Sample Diluent, stand for 10 min .

1000pg/mL of working solution was produced by test reconstitution. Then serial dilutions make as needed. The dilution gradient is as follows:

1000、500、250、125、62.5、31.25、15.63、0 pg/mL.

Dilution method: 7 ependrof tubes has been Taken 500µL added of Reference Standard & Sample Diluent to each tube. 500µL has been pipetted of the 1000pg/mL working solution to the first tube and mixed up to yield a 500pg/mL working solution. Then 500µL of the solution had been pipetted from the former tube into the latter one according to this step.

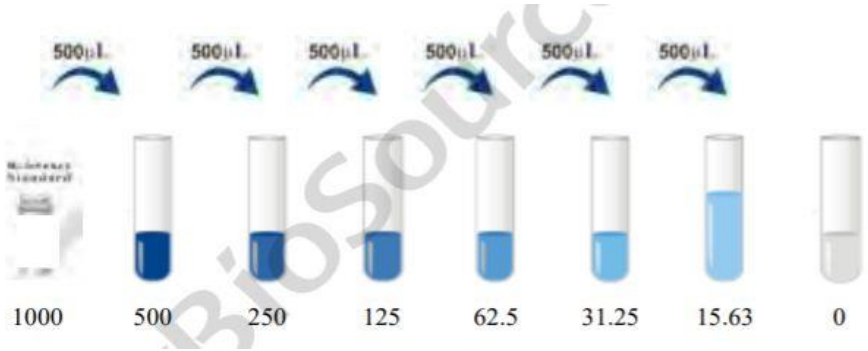


Figure (2-4) serial dilution of standard solution of PINP

4. Biotinylated Detection Ab working solution: required amount (100µL/well). the Concentrated Biotinylated Detection Ab has been Centrifuged, then it diluted with Biotinylated Detection Ab Diluent the 100× Concentrated Biotinylated Detection Ab to 1× working solution (Concentrated Biotinylated Detection Ab: Biotinylated Detection Ab Diluent= 1: 99).

5. HRP Conjugate working solution: the required amount (100µL/well). the 100 Concentrated HRP Conjugate diluted to 1× working solution with HRP Conjugate Diluent(Concentrated HRP Conjugate: HRP Conjugate Diluent= 1: 99).

D. Assay procedure

1. 100 µL standard or sample had been Added to each well. Then it had been incubated for 90 min at 37°C.

2. The liquid was removed. 100 µL Biotinylated Detection Ab was added. Then Incubated it for 1 hour at 37ºC.

3. Aspirated & washed 3 times.

4 100 µL HRP Conjugate had been Added then Incubated at 37ºC for 30 min

5. Washed 5 times again

6. Substrate Reagent (90 µL) was Added. Incubated at 37ºC for 15 min

7. Stop Solution (50 µL) was Added. Immediately OD was Read at 450 nm.

8. OD value had been Determined of each well at once with a micro-plate reader set to 450 nm.

E. Calculation of results

Average the duplicate readings for each standard and samples, then subtract the average zero standard optical density. Plot a four-parameter logistic curve on log-log graph paper, with standard concentration on the x-axis and OD values on the y-axis. If the OD of the sample surpasses the upper limit of the standard curve, it should re-test it with an appropriate dilution. The actual concentration is the calculated concentration multiplied by the dilution factor.

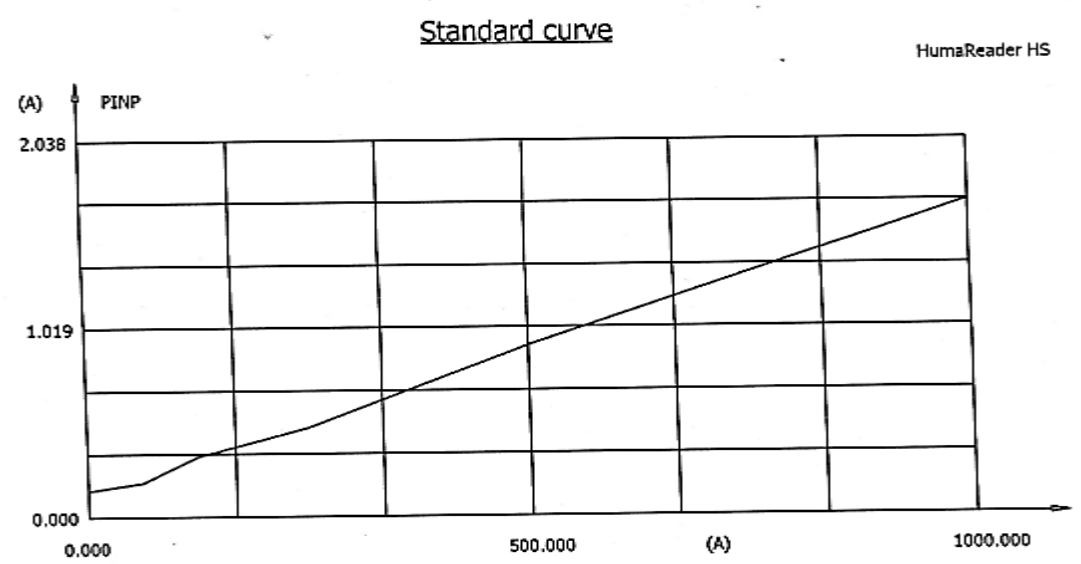


Figure (2-5) standard curve PINP test

2.3.5 Human parathyroid hormone (PTH) ELISA

A. Principle

The quantitative sandwich enzyme immunoassay method is used in this assay. A microplate has been pre-coated with a PTH specific antibody. Pipette standards and samples into the wells, and any PTH present is bound by the immobilized antibody. After eliminating any unattached compounds, a biotin-conjugated antibody specific for PTH is applied to the wells. After washing, avidin conjugated Horseradish Peroxidase (HRP) is applied to the wells. A substrate solution is added to the wells after a wash to remove any unbound avidin-enzyme reagent, and color develops in proportion to the quantity of PTH bound in the initial step. The color development is terminated, and the color intensity is measured.

Detection range 62.5 pg/ml-4000 pg/ml.

Sensitivity: less than 15.6 pg/ml.

Specificity : No significant cross-reactivity or interference between human PTH and analogues was observed.

B. Material

Reagents Quantity

Assay plate 1(96 coated Microwells)

Standard (Freeze dried) 2vial

Biotin antibody (100 x concentrate) 1vial x 120 µl

HRP avidin (100 x concentrate) 1vial x 120 µl

Biotin antibody Diluent 1vial x 15 ml

HRP avidin Diluent 1vial x 15 ml

Sample Diluent 1vial x 50 ml

Wash Buffer (25 x concentrate) 1vial x 20 ml

TMB Substrate 1vial x 10 ml

Stop Solution 1vial x 10 ml

Adhesive Strip (For 96 wells) 4

C.Reagent prepration

All of the reagents have been used. Before usage, allow 30 minutes at room temperature (18-25ºC).

1.Biotin-antibody had been centrifuged before the vial is opened.then A 100x dilution was prepared. As the following: 990 µl of Biotin-antibody Diluent. +10 µl of Biotin-antibody

2.HRP-avidin before the vial was opened it had been centrifuged then A 100-fold dilution was prepared. As the following: 990 µl of HRP-avidin Diluent+10 µl of HRP-avidin.

3. Wash Buffer To avoid crystals forming in the concentrate, it is warmed to room temperature and mixed gently until the crystals have completely dissolved

20 ml of Wash Buffer Concentrate (25 x) was diluted into deionized or distilled

water to prepare 500 ml of Wash Buffer (1 x)

Standard

1. standard vial centrifuged for 30 seconds at 6000–10,000 rpm.

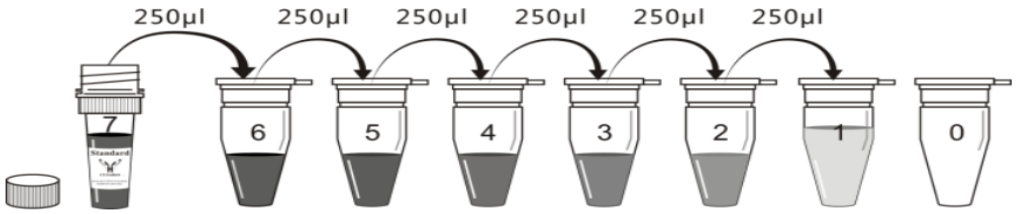
solution of 4000 pg/ml was prepared by reconstituting the standard with 1.0 ml of sample diluent.

250 µl of sample diluent was pipetted into each tube (S0-S6).

The stock solution is used to produce a 2-fold dilution series (below).

by Mix each tube thoroughly before the next transfer.

The undiluted Standard serves as the high standard (4000 pg/ml). Sample Diluent serves as the zero standard (0 pg/ml



Tube	S7	S6	S5	S4	S3	S2	S1	S0
pg/ml	4000	2000	1000	500	250	125	62.5	0

Figure (2-6) serial dilution of standard solution of PTH

C. steps of PTH measurement

1. Prepare reagents, samples and standards as instructed.
2. Add 100µl standard or sample to each well. Incubate 2 hours at 37°C.
3. Remove the liquid of each well, don't wash.
4. Add 100µl Biotin-antibody(1x) to each well. Incubate 1 hour at 37°C.
5. Aspirate and wash 3 times.
6. Add 100µl HRP-avidin (1x) to each well. Incubate 1 hour at 37°C.
7. Aspirate and wash 5 times.
8. Add 90µl TMB Substrate to each well. Incubate 15-30 minutes at 37°C. **Protect from light.**
9. Add 50µl Stop Solution to each well. Read at 450nm within 5 minutes.

D. Calculation of results

by Using the professional soft "Curve Expert" to make a standard curve .

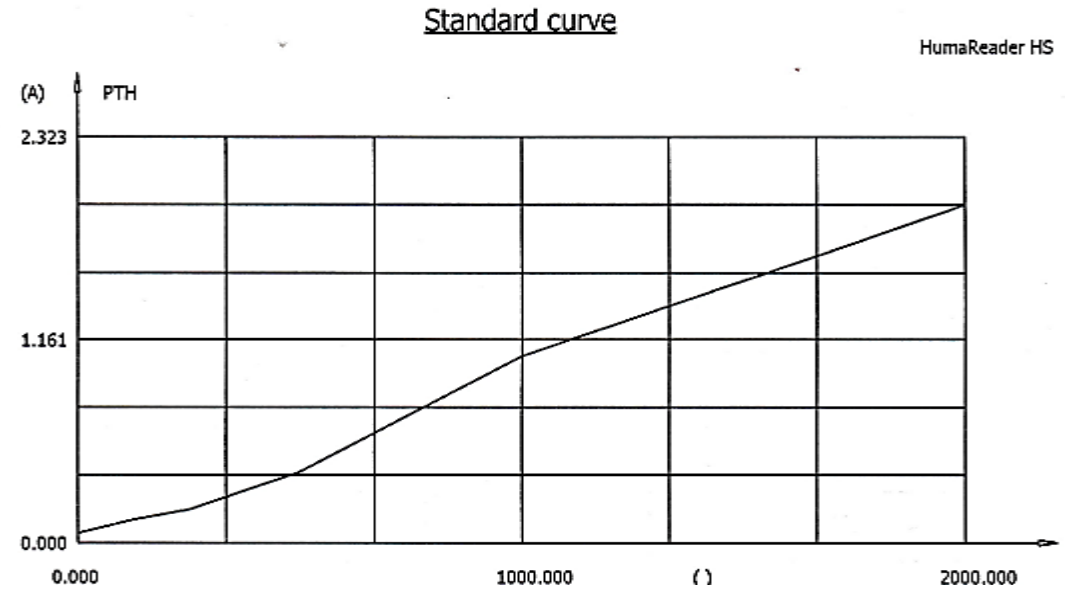


Figure (2-7) standard curve PTH test

2.3.5 Assesment of Human Sclerostin (SOST) by Elisa

A. Principle

The competitive enzyme immunoassay technique is used in the SOST ELISA kit which includes a monoclonal anti-SOST antibody and an SOST-HRP conjugate.

In a pre-coated plate, the test sample and buffer are incubated for one hour with the SOST-HRP conjugate. The wells are decanted & washed 5 times after incubation. The wells then are incubated with an HRP enzyme substrate. The result of the enzyme-substrate reaction is a blue complex. Lastly, a stop solution is added to terminate the reaction, which causes the solution to become yellow. Color intensity is measured spectrophotometrically in a microplate reader at 450nm. Because SOST from samples & SOST-HRP conjugate compete for the anti-SOST Ab

binding site, the color intensity is inversely proportional to SOST concentration. Because the number of sites is limited, since more SOST from the sample occupy sites, less sites are left to bind SOST-HRP conjugate. A standard curve is drawn that relates the color intensity (OD) to the concentration of standards. This standard curve is used to interpolate the SOST concentration in each sample.

Sensitivity: is 0.1 ng/mL.

Specificity: No significant cross-reactivity or interference between SOST and analogues was observed

B. Material

1. microtiter plate stripwell,96 wells
2. enzym conjugate 1 vial, 6.0 mL
3. standard A 1 vial l,0 ng/mL
4 .standard B 1 vial,1.0 ng/mL
5 .standard C 1 vial, 2.5 ng/mL
6 .standard D 1 vial, 5.0 ng/mL
7 .standard E 10 ng/mL 1 vial
8 .standard F 1 vial25 ng/mL,
9 .substrate A 1 vial,6 mL
10 .substrate B 1 vial,6 mL
11 .stop solution 1 vial,6 mL
12 .wash solution (100 x) 1 vial, 10 mL
13 .balance substrate 1 vial, 3 mL

C. reagent prepration

Before use, all kit components and samples were brought to room temperature (20–25^{o}C).Wash Solution - Dilute 10 mL of Wash Solution concentrate (100×)

with 990 mL of deionized or distilled water to prepare 1000 mL of

Wash Solution (1×). If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved.

D.Assay procedure

1. The coated wells in the holder were secured with the desired numbers and then 100 µL of Standards or Samples be added to the appropriate well in the antibody pre-coated Microtiter Plate. Add 100 µL of PBS (pH7.0-7.2) in the blank control well.

2. Each well receives 50 µL of conjugate, which is then mixed well.a plate is covered and incubated for 1 hour at 37°C.

Calculate the concentration of samples corresponding to the mean absorbance from the standard curve.

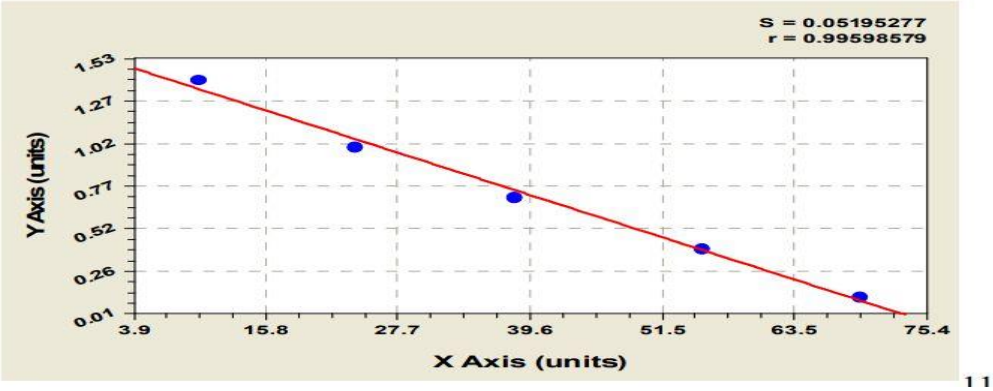


Figure (2-8) standard curve SOST test

2.3.6 Determination of serum Calcium

A-Principle

Calcium ions form a violet complex with O-Cresolphthalein complexone in an alkaline medium. that absorbs at 570nm .

Calcium + O-Cresolphthalein Complexone $\xrightarrow[\text{medium}]{\text{Alkaline}}$ Calcium-Cresolphthalein Complexone Complex(purple color)

B. Assay procedure:

	Blank	Standard	Sample
Standard		20μl	
Sample			20μl
Working Reagent (R1+R2)	1ml	1ml	1ml

equal volumes of color & buffer reagent was Combined, mixed and stand for twenty minutes at room temperature then used and incubated for 1minute at room temperature. The absorbance (A) of standard and sample was read against the blank at 570 nm.

C .Calculations

Calcium (mg/ dl) = $\frac{A\ Sample}{A\ Standard}$ x Standard conc (10)

* According to this procedure, the reference values for serum calcium concentration were 8.5-10.5mg/dl.

2.3.7 Determination of serum albumin

A. Principle

Serum albumin is measured by its quantitative binding to the indicator 3,3',5,5'-tetrabromo-m cresol sulphonephthalein (bromocresol green, BCG). The albumin-BCG-complex absorbs maximally at 578 nm , The absorbance is directly proportional to the albumin concentration in the sample.

B. procedure

Pipette into test tubes:			
	Reagent	Standard	Sample
Distilled H2O	0.01 ml	----	----
Standard (CAL)	----	0.01 ml	----
Serum or Plasma	----	---	0.01ml
BCG reagent (R1)	3.00 ml	3.00 ml	3.00ml

Mix and incubate for 5 minutes at +20 to +25C. Measure the absorbance of the sample (Asample) and of the standard (Astandard) against the reagent blank.

Manual calculation

The following formula may be used to calculate the albumin concentration in a sample:

Albumin Concentration (g/l or g/dl)
= Asample/Astandard x Concentration of standard
Normal values

Adults 38 - 44 g/l (3.8 - 4.4 g/dl) , Neonates 38 - 42 g/l (3.8 - 4.2 g/dl)

2.3.8 Determination of serum Phosphorous

A. princeple

The Phosphorus Microplate Assay Kit is a sensitive colorimetric method for measuring phosphorus levels in a variety of samples. Phosphorus concentration is

determined via the reaction of phosphorus with ammonium molybdate, which produces a blue product. The color intensity at 620 nm is proportional to the concentration of phosphorus in the sample.

B. Sample Preparation

For serum and other biological fluids sample Add 100 µl sample and 900 µl Assay buffer into the microcentrifuge tube, mix, centrifuged at 8,000g 25 °C for 10 minutes, take the supernatant into a new centrifuge tube for detection.

C. Assay Procedure

Add following reagents into the microplate:

Reagent	Blank	Standard	Sample
Reaction Buffer	50 µl	50 µl	50 µl
Dye Reagent	50 µl	50 µl	50 µl
Distilled water	100 µl	-	-
Standard	-	100 µl	-
Sample	-	-	
Mix, wait for 10 minutes, measured at 620 nm and record the absorbanc			

D .Calculations

According to the serum sample Phosphorus(mmol/L) = C Standard × (ODSample - ODBlank) / (ODStandard - ODBlank) × 10 = 4 × (OD Sample - ODBlank) / (ODStandard - ODBlank)

C Standard: the concentration of Standard, 0.4 mmol/L.

2.3.9 Determination of serum alkaline phosphatase ALP

A. princeple

p-nitrophenylphosphate + H2O ALP → phosphate + p-nitrophenol

Pipette into cuvette:	Macro	Semi-Micro	Micro
Sample	0.05 ml	0.02 ml	0.01
Reagent (25 º C, 30 º C, 37 º C)	3.00 ml	1.00 ml	0.50

Mix, read initial absorbance and start timer simultaneously.Read again after 1, 2and 3 min.

C. manual caliculation

To calculate the ALP activity use the following formulae:

U/l = 3300 x ΔA 405 nm/min MACRO

U/l = 2760 x ΔA 405 nm/min SEMI-MICRO

U/l = 2760 x ΔA 405 nm/min MICRO

D.Normal value in serum

	25 º C	30 º C	37 º C
Men/women	60-170 U/l	73-207U/l	98-279 U/l

2.4 Statistical Analysis

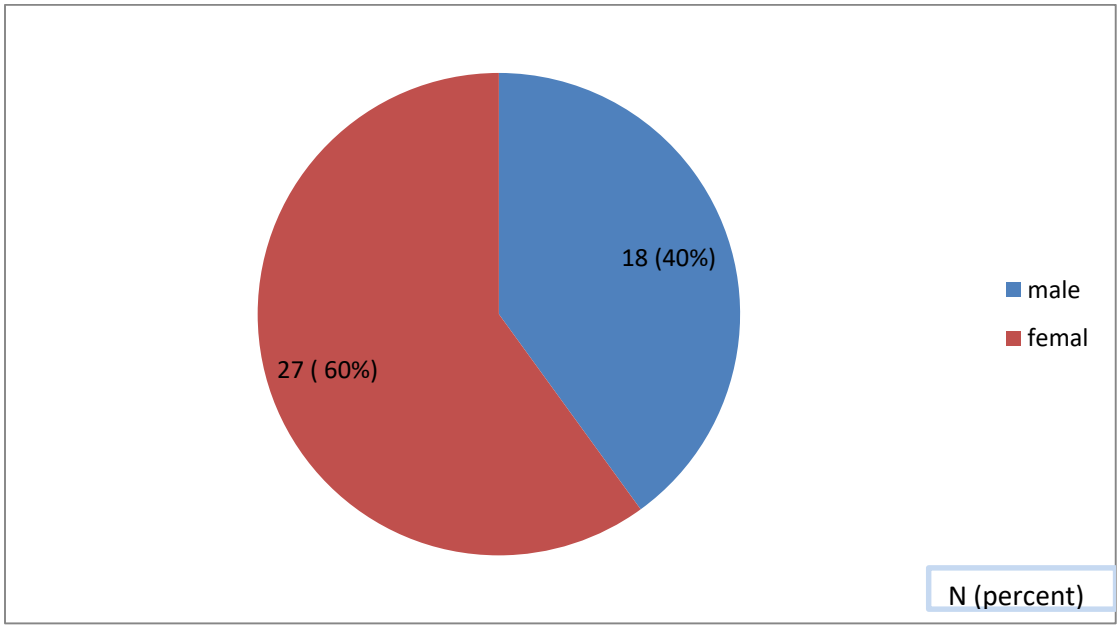
This study was a case -control research design. Statistical analyses were performed using SPSS statistical package for Social Sciences (version 26.0 for Windows, SPSS, Chicago, IL, USA). Data normality distribution was examined using the Shapiro-Wilk test.OPG, SOST, PINP, and PTH distribution were considered normal. Student's t and ANOVA tests were used to test differences in studied parameters within different groups. Quantitative data are represented as mean, standard deviation. Qualitative data are represented as count and percentage. Scatterplots was used for visual demonstrating of relationships between independent variables. Pearson correlation conducted for measurement of correlation coefficients and their significance. ROC survival test was used for evaluating the ability of study markers to discriminate disease from nondisease subjects. A p-value of <0.05 was considered statistically significant(101).

3. The Results and Discussion

3.1. Demographic and Clinical Characteristics of the Study Group

3.1.1 Distribution of study groups According to gender

The gender distribution of the studied groups was 45 patients with CKD on hemodialysis, 18 (40.00%) male and 27 (60.00%) female, matching with controls and the results represented in figure (3-1).

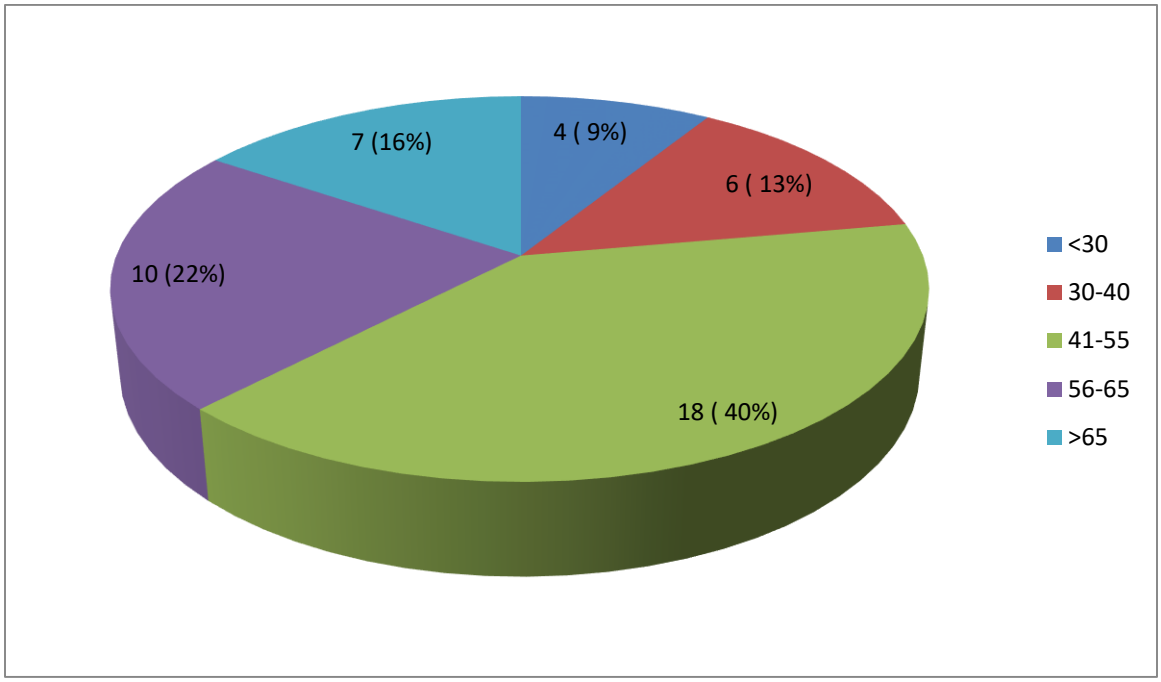


(Figure 3-1) Gender distribution of patients & controls

3.1.2. Age

In this study, 90 participants were divided into 45 patients and other healthy people, and their ages ranged between 20 and 75 years. A comparison between study groups regarding age was performed using a t-test there was not statistically significant difference p value (0.980). The dispersion of age the rate malady is appeared in table

(3-1) &Figure (3-2), the mean of CKD (50.51 ± 2.07) compared with healthy control (50.57 ± 1.81) P value (0.980).



(Figure 3-2) Age of patients with CKD

Acording to the results of this study were found more cases of CKD in (41-65)age that agree with previous study of Ranasinghe, Kumara *et al* ,The most susceptible age group was between the ages of 40 and 60(102)

Table (3-1) comparison between patient & control according age

variable	Patients (Mean ± SE)	Control(Mean ± SE)	P-value
Age (years)	50.51 ± 2.07	50.57 ± 1.81	0.980 NS
NS: Non-Significant.			

3.1.3 Comparison Body Mass Index (BMI) between patients and healthy groups

showed that there were BMI was significantly higher in Control ($p \leq 0.05$) the mean BMI and SE was (28.99 ± 0.58) compared to Patients of CKD the mean BMI and SE was (26.96 ± 0.82) , as illustrated in table 3.2

Table (3-2): Comparison between patients and control in BMI

Variable	Patients,Mean ± SE	Control ,Mean ± SE	P-value
BMI (kg/m^2)	26.96 ± 0.82	28.99 ± 0.58	0.0494*
* (P≤0.05)			

Patients with chronic kidney disease (CKD) undergoing hemodialysis (HD) are at high risk for malnutrition.

(103)

3.2 Calcium and Phosphate in Patient and Control Groups.

The current study show that were been a greater significant difference in calcium and phosphate between CKD prehemodialysis patients from healthy control (P≤0.01), as shown in Table (3-3).

Table (3-3): Comparison between patients and control in Calcium and Phosphate

Variable	**Patients, Mean ± SE**	**Control ,Mean ± SE**	**P-value**
Ca (mg /dl)	**7.34 ± 0.16**	**8.98 ± 0.08**	**0.0001****
P (mg/dl)	**6.46 ± 0.36**	**2.68 ± 0.03**	**0.0001****
** **(P≤0.01).**			

this study showed that the Mean and SE of the calcium and phosphate for patient was (7.34 ± 0.16, 6.46 ± 0.36) and for healthy control was (8.98 ± 0.08, 2.68 ± 0.03) respectively , phosphate was significantly higher in CKD vs. Control while calcium was significant lower CKD vs. Control. The most common complication occurring in chronic kidney disease (CKD) is mineral bone disease. Ca and P homeostasis are hormonally controlled by a four-tissue axis comprising the gut, bone, kidney, and parathyroid gland. that closely regulate serum P & ionized Ca levels within a restricted range. The primary two hormones involved in calcium balance are parathyroid hormone (PTH) & 1,25 vitamin D(1,25D). fibroblast growth factor 23 (FGF-23) (49). Hypocalcemia stimulates PTH secretion and hypercalcemia suppresses it. Calcium-sensing receptors (CaSR) on the parathyroid gland detect low levels of ionized calcium in the blood, which stimulates PTH synthesis and secretion. , hyperphosphatemia, Hypocalcemia, secondary hyperparathyroidism (SHPT) occur (104). The biochemical changes of CKD-MBD include raised fibroblast growth factor-23 (FGF23) and parathyroid hormone (PTH), declined (1,25D), raised serum P, and decreased serum Ca. **(105)** the results of the current study are agreed Keung and Perwad (106), DiMeglio and Imel (107), Barreto *et al* (108)that found significantly lowe plasma Ca levels and high phosphate level in patients with CKD compared with healthy controls.

3.3 Serum Albumin

Estimation of serum albumin in patients and control groups was represented in figure (3.7). It shows that the mean and SE of CKD patients and healthy controls were (3.72 ± 0.07, 4.47 ± 0.05) respectively. Albumin was significantly ($p \leq 0.01$) lower in CKD compared to control, however both within normal range

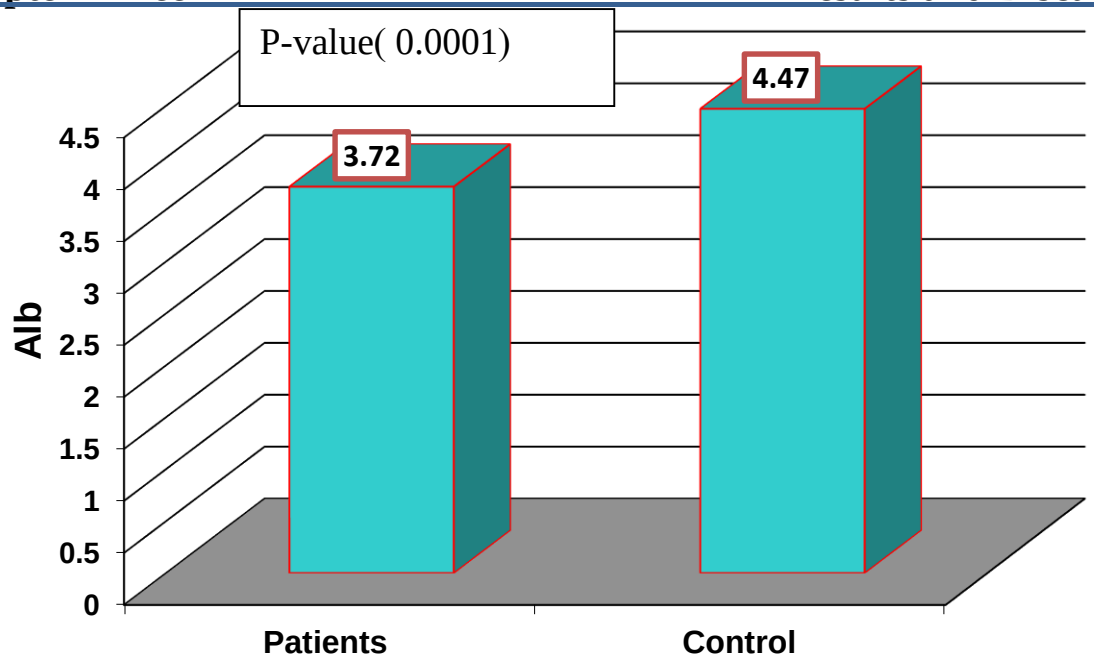


Figure (3-3) showed Albumin in patient & control group.

Albumin is commonly considered as a nutritional status-assessing biological marker. Hypoalbuminemia can be caused by a variety of factors, including malnourishment(109), hepatic damage, diminished hepatic production of albumin due to tumor necrosis factor (TNF)-α and interleukin (IL)-1, IL-6(110), and protein lossing via gastrointestinal tract or the kidney (111) the results of the current study are agreed with Lang, Katz et al that demonstrate Lower blood albumin levels are strongly and independently related with declines in renal function in the elderly. (112)also agree with Alves, Sun et al that has been reported Hypoalbuminemia is a common feature of chronic renal disease(113)

3.4 Serum Alkaline Phosphatase ALP

The current study's ALP results revealed highly significant differences in CKD patients versus healthy controls ($P \leq 0.01$). Where the mean and SE were (381.71 ± 46.62, 86.37 ± 4.46) for the patient and control recipectivly .

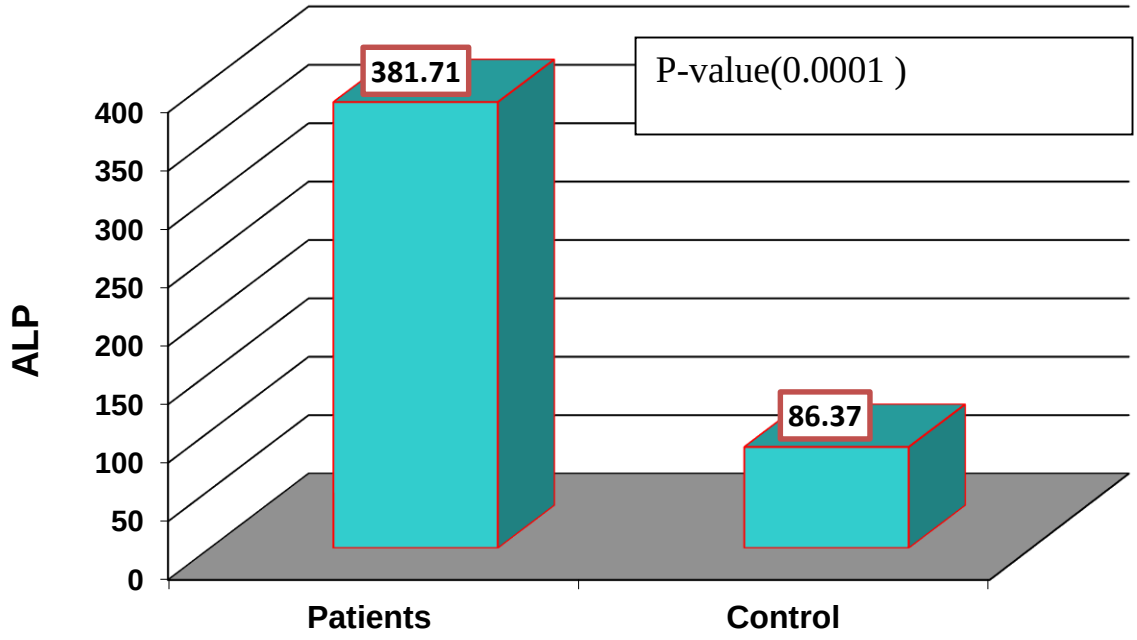


Figure (3-4) demonstrate ALP in patient and control

renal osteodystrophy in a CKD patient, could result in a considerable rise in the ALP bone isoenzyme (b-ALP) This contributed to elevated total ALP levels in the blood. Actually, Higher ALP levels have been linked to an increased risk of mortality. in pre-dialysis CKD in addition to patients undergoing constant haemodialysis (114)Disorders in bone and mineral metabolism are virtually always noticed in patients with End-Stage Renal Disease (ESRD) on dialysis and have been linked to an increased risk of negative clinical outcomes high level of serum alkaline phosphatase (ALP), a well-known characteristic of Mineral and bone abnormalities in ESRD patients (115) Combination of elevated alkaline phosphatase with high PTH indicated a effective diagnostic predictive value of high-turnover bone disease (116). This study agrees with Wally that presented raised ($P<0.001$) in activity level of (ALP) in serum of CKD patients before and after dialysis in matched with control group for male and female.(117).also agree

with Latiwesh, Younis et al that show When CKD patients were compared to healthy controls, their ALP levels were considerably higher.(114)

3.5 Bone Turnover Biomarker:

Metabolism of Bone is characterized by the continuous coordination of bone cells. Comprising osteoblasts, osteoclasts, and osteocytes in in order to keep bone tissue quantity & the integrity of bone organization. The creation of blood and urine biochemical marker tests that reflect either the enzymatic activity of osteoblasts and osteoclasts or the breakdown products of bone tissue., has proved extremely beneficial in studying the intricate routes of bone metabolism and their changes in bone disorders. (118)

Table (3-4) represents comparisons between patients and controls in OPG, PINP, SOST, and PTH. Biomarkers used in this study.

Table (3-4): Comparison between patients and control in OPG, PINP, SOST and PTH.

Variable	Patients,Mean ± SE	Control ,Mean ± SE	P-value
OPG (ng/ml)	219.54 ±56.31	4.88 ±0.61	0.0003
PINP (pg/ml)	500.82 ±30.76	252.00 ±9.14	0.0001
SOST (ng/ml)	3.55 ±024	1.61 ±0.07	0.0001
PTH (pg/ml)	858.03 ±42.62	613.84 ±23.12	0.0001

3.5.1 Osteoprotegerin

Measurement of serum OPG in patients and control groups representing in the figure (3.5) revealed that significantly ($P \leq 0.01$).higher in CKD compared to Control .

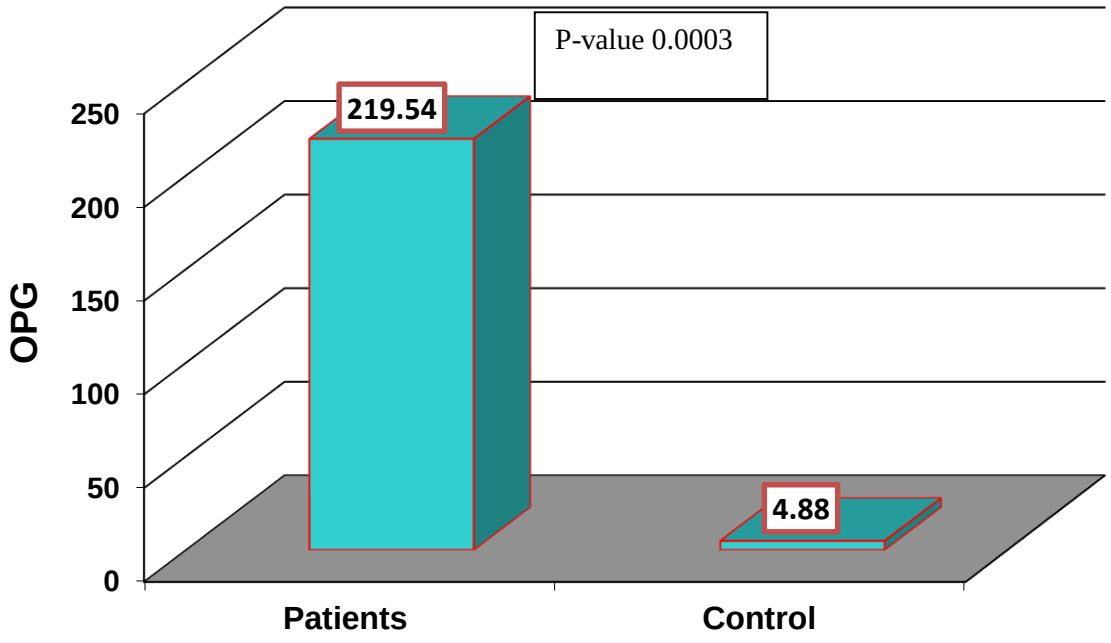


Figure (3-5) represent OPG levels in patients and control

Osteoprotegerin (OPG) is a member of the tumor necrosis factor superfamily and has been linked to an increase in endothelial dysfunction and vascular calcification. (119) It prevents the cytokine receptor activator of nuclear factor kappa-B ligand (RANKL) and TNF-related apoptosis-inducing ligand (TRAIL) from binding to cell membrane signaling receptors. Inhibiting the RANK/TRAIL pathway reduces osteoclast differentiation as well as activation and survival of mature osteoclasts. OPG is also implicated in metabolic bone disease and may have a role in CKD prognosis. (120)

OPG levels are higher in patients with chronic renal disease.(121). this result agree with Ameen and Ali study in which serum OPG levels are raised in individuals with chronic renal disease, with the goal of predicting kidney function decline.

(122) our study also was agreed with Bernardi, Toffoli et al demonstrating the Circulating OPG levels were considerably greater in CKD patients with hypertension, and there was a strong negative relationship between OPG and renal function. (123)

3.5.2 Procollagen type I N-Propeptide

In this investigation, individuals with CKD had considerably higher blood concentrations of PINP than the control group, as seen in the figure (3-6)

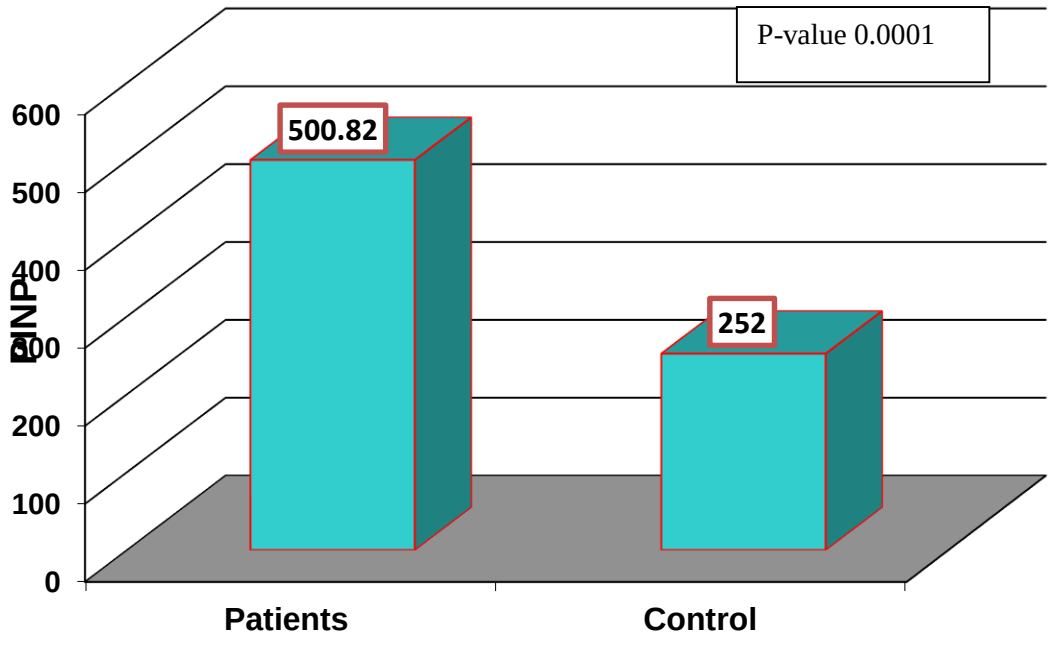


Figure (3-6) differences between patients and control in PINP

The most collagen in mineralized bone is procollagen type I propeptides (PINP), which are generated by active osteoblasts in the form of procollagen. As a result, PINP is regarded as a dependable biomarker of bone formation.(124) The serum procollagen type I N-propeptide (PINP) has been selected as the reference marker of bone formation in osteoporosis. The C-terminal telopeptide of type I collagen (CTX) is used as a resorption marker PINP can also be used to diagnose Paget's

disease of the bone, as well as to evaluate response to therapy and detect recurrence. Although Bone turnover marker(BTM) other than bone alkaline phosphatase are not currently indicated for usage in metabolic bone disease or chronic renal disease, Hung, Chang et al and Cai, Hu et al. show (77)Serum P, iPTH, BALP, PINP, and -CTX levels were substantially higher in the CKD stage 3-5 group (P<0.05), but eGFR and serum Ca levels were lower (P<0.05). Changes in their levels were more significant (P<0.05) as CKD staging increased (125, 126) that agreed with our findings.

3.5.3 Seclerostin

In the current study, plasma seclerostin levels in CKD patients were significantly higher than in matched healthy controls, as shown in figure (3-7).

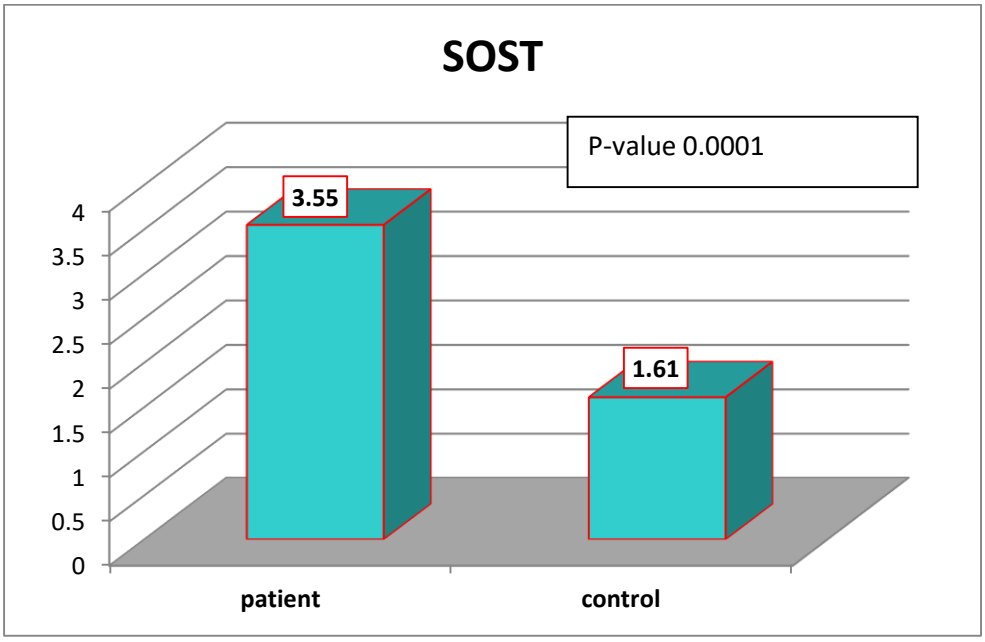


Figure (3-7) reveald SOST in patients & control

In heamodialysis (HD) patients, however, sclerostin release by osteocytes appears to be promoted by inflammation.(127) Sclerostin, a canonical Wnt/-catenin signaling inhibitor(126)Behets *et al* They found that serum SOST levels were greater in CKD patients compared to controls.

(128) Rija, Almahdawi et al showed When HD CKD patients were compared to the control group, the level of SOST raised with extremely significant differences (P≤0.01) .(129) that agreed with our outcome

3.6 Parathyroid hormon PTH

Estimation of PTH in patients and control groups were representing in figure (3.8) They show that the mean and SE for CKD and control were (858.03 ±42.62), (613.84 ±23.12). respectively so that PTH was significantly (P≤0.01)) higher in CKD patients compared Control.

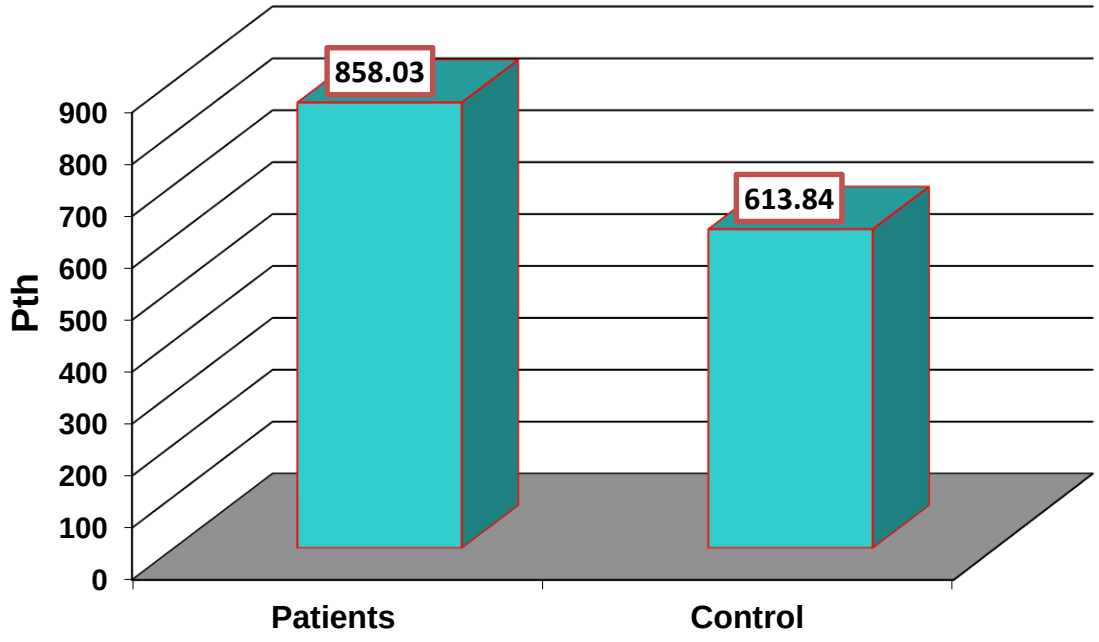


Figure (3-8) comprasion between pateint and control in PTH

Chronic kidney disease (CKD) causes hypocalcemia and hyperphosphatemia by impairing vitamin D activation in the kidneys, leading in a compensatory increase in parathyroid gland cellularity and parathyroid hormone synthesis and secondary hyperparathyroidism (SHP).(130) Mnazzal and Abdullah indicate that

PTH levels were significantly higher in the patient group (235.032±3.841) pg/ml compared with healthy control group (49.96±0.308) pg/mL(131) Isakova, Cai et al show In CKD, PTH and phosphate levels are elevated somewhat, but calcium levels decreased slightly(132) that agrees with the current study results.

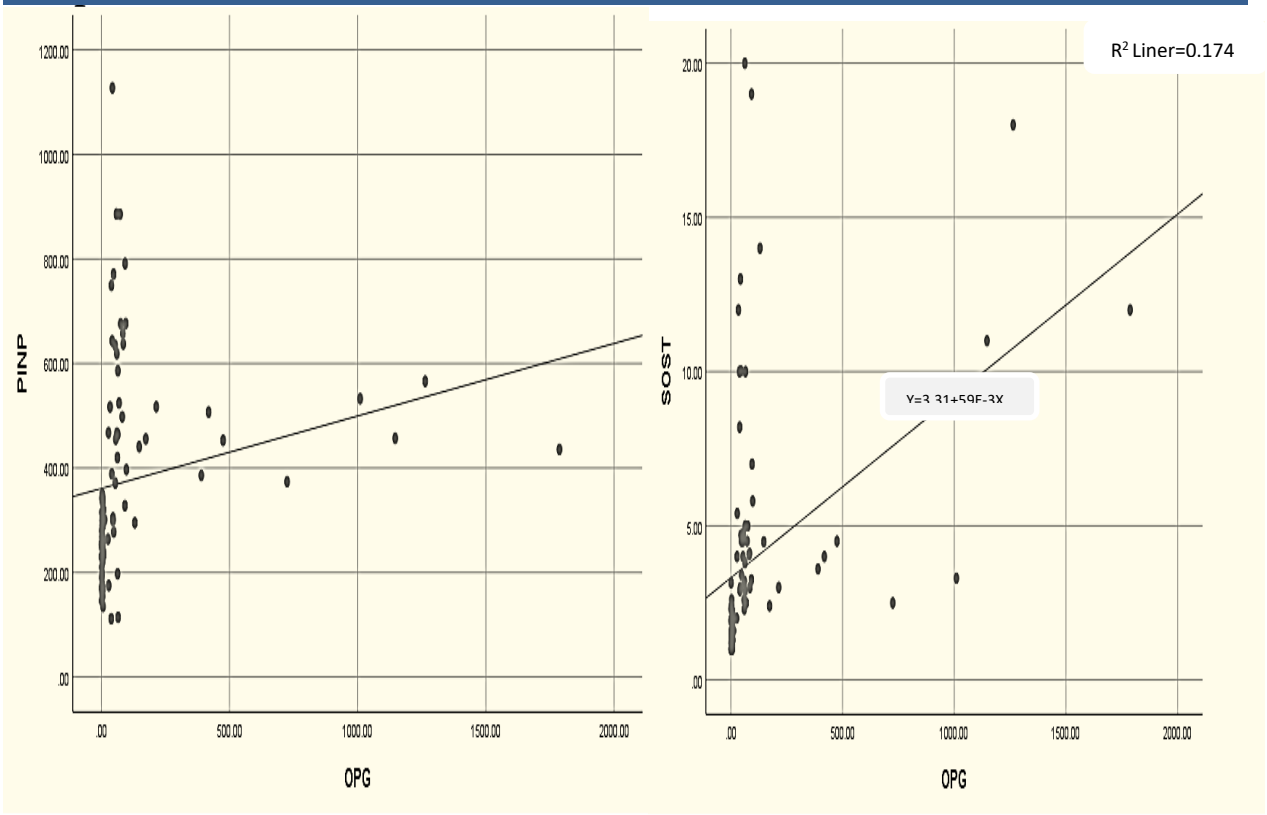
3.7 Correlation between OPG and other parameter

Table (3-5) correlation between OPG and another parameter in CKD patients.

	Parameter	Person correlation (r)	P-vale
OPG	**PINP**	**0.21 ***	**0.050**
	SOST	0.31 **	**0.011**
	ALP	**0.16 NS**	**0.131**
	PTH	**0.36 ****	**0.0006**
	Ca	**-0.18 NS**	**0.0929**
	Pi	**0.23 ***	**0.0298**
	Albumin	**-0.18 NS**	**0.0741**

Correlation is significant at the 0.05 level (p-vale): * (P≤0.05), ** (P≤0.01).

The present study showed no significant correlation between OPG and (ALP,Ca,Alb , PINP) in CKD patients(P>0.05) .otherwise in our study, there was a significant positive correlation between OPG and SOST,PTH where (P≤0.01).The information exposed in table (3-5) and figure (3-9)



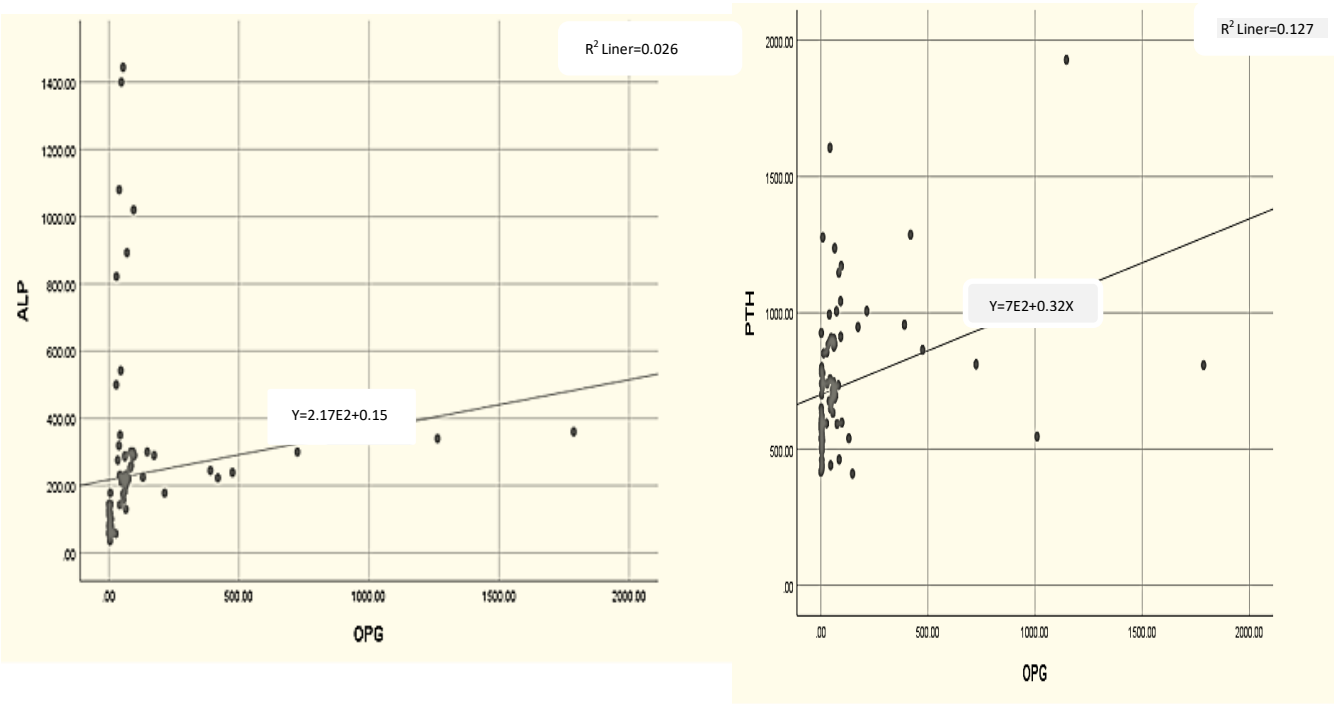


Figure (3-9) the association between OPG and other parameters (PINP,SOST,PTH) among CKD patients

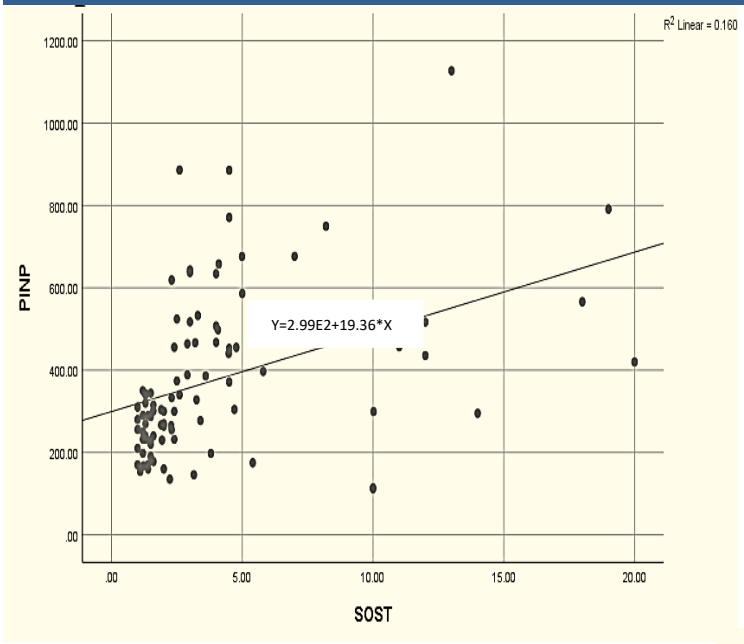
3.8 Correlation between SOST and other parameter

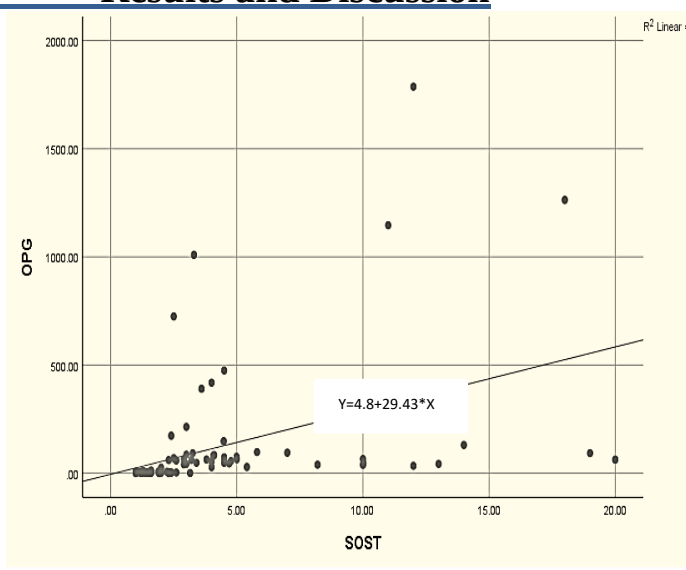
Table (3-6) Relationship between SOST and other parameters among CKD patients

	Parameter	Person correlation (r)	P-vale
SOST	**PINP**	**0.65 ****	**0.0001**
	OPG	**0.31 ****	**0.011**
	ALP	**0.63 ****	**0.0001**
	PTH	**0.49 ****	**0.0001**
	Ca	**-0.56 ****	**0.0001**
	Pi	**0.71 ****	**0.0001**
	Albumin	**-0.56 ****	**0.0001**

* ($P \leq 0.05$), ** ($P \leq 0.01$).

The current study showed significant negative correlation between SOST and Ca,Alb ($p<0.001$) within CKD patients. Otherwise in our study showed a significant positive correlation between SOST and other bone turnover biomarker (PINP, OPG,ALP,Phos,PTH) ($p<0.001$) Details are exposed in table (3-6) and figure (3-10).





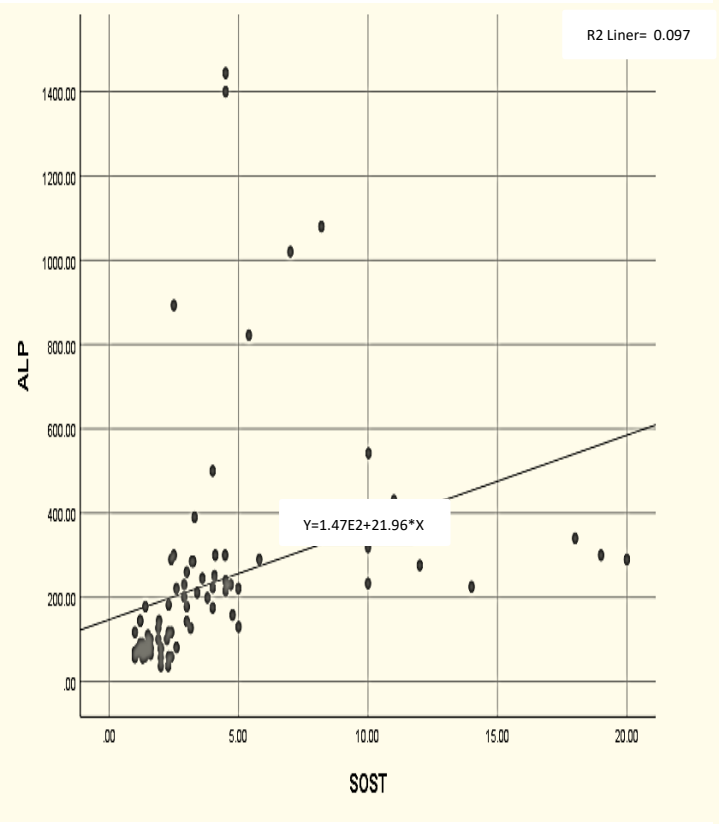


Figure (3-10) the association between SOST and other parameters (PINP,SOST,PTH) among CKD patients

3.9 Correlation between PINP and other parameter

Table (3-7) Correlation between PINP and other parameters among CKD patients

	Parameter	Person correlation (r)	P-vale
PINP	**OPG**	**0.21 ***	**0.050**
	SOST	**0.65 ****	**0.0001**
	ALP	**0.41 ****	**0.0001**
	PTH	**0.38 ****	**0.0002**
	Ca	**-0.32 ****	**0.0017**
	Pi	**0.37 ****	0.0004
	Albumin	**-0.45 ****	**0.0001**

*** (P≤0.05), ** (P≤0.01).**

the recent study showed significant negative correlation between PINP and Ca,Alb ($p<0.001$) within CKD patients, as shown in table (3-7). moreover, in our study, there was a significant positive correlation between SOST and other bone turnover biomarker(PINP, ALP,Pi,PTH) ($p<0.001$) as publicized in figure (3-11).

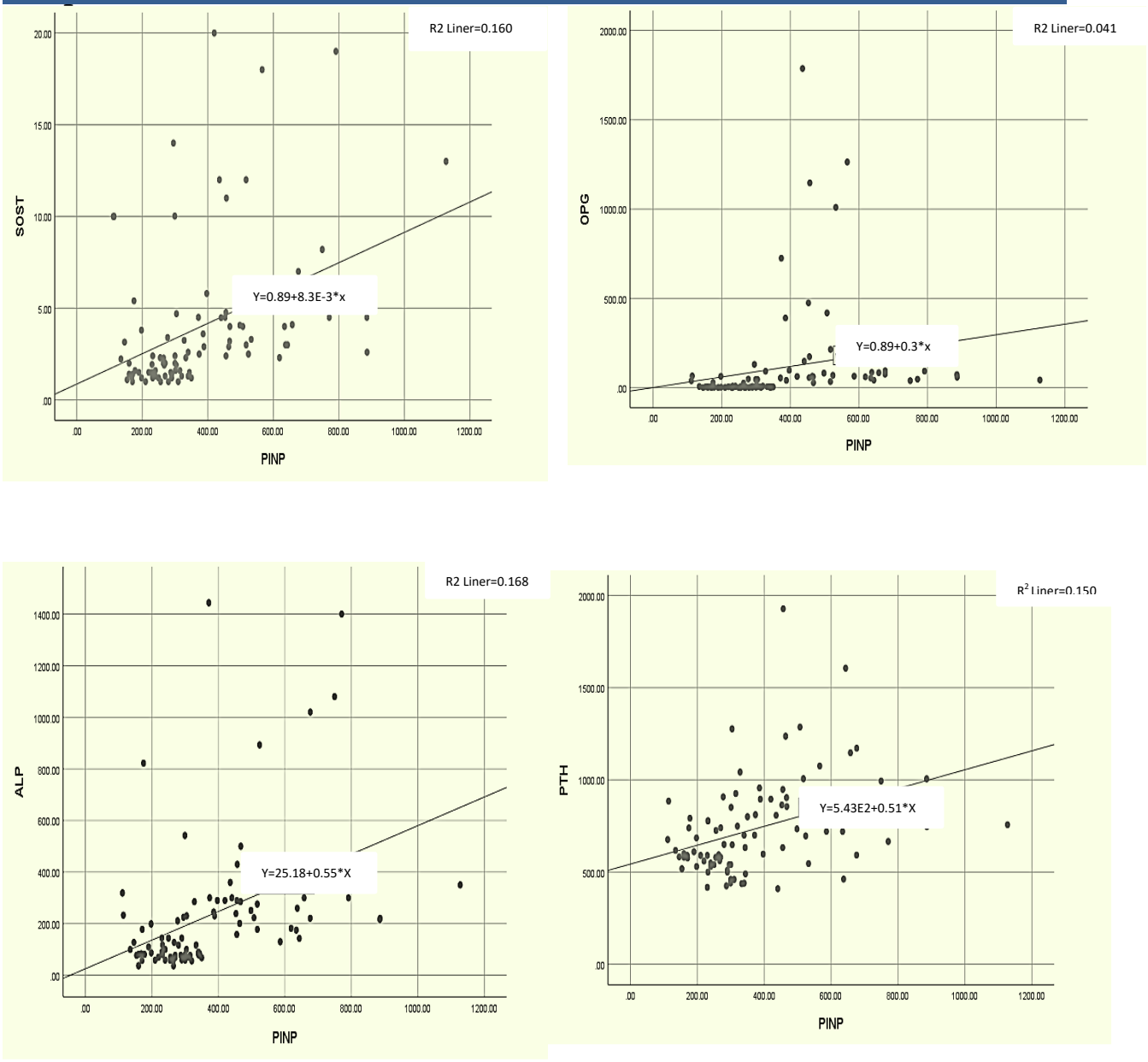


Figure (3-11) the association between PINP and other parameters (PINP,SOST,PTH) among CKD patients

3.10 Correlation between ALP and other parameter

Table (3-8) Correlation between ALP and other parameters in pateint group.

	Parameter	Person correlation (r)	P-vale
ALP	**PINP**	**0.41 ****	**0.0001**
	SOST	**0.63 ****	**0.0001**
	OPG	**0.16 NS**	**0.131**
	PTH	**0.22 ***	**0.0411**
	Ca	**-0.40 ****	**0.0001**
	Pi	**0.24 ***	**0.0214**
	Albumin	**-0.37 ****	**0.0003**

* ($P \leq 0.05$), ** ($P \leq 0.01$).

The current study showed no significant correlation between Plasma ALP and OPG ($p>0.05$) in CKD patients, as revealed in a table (3-8). additionally, in the present study, there was a significant negative correlation between plasma Ca,Alb and ALP($p<0.01$) forther more significant posetive correlation between ALP and PINP,SOST ($p<0.01$)and ALP with PTH ,Pi ($p<0.05$) as publicized in figure (3-11)

3.11 Correlation between PTH and other parameter

Table (3-9) Correlation between PTH and other parameters.

	Parameter	Person correlation (r)	P-vale
PTH	**PINP**	**0.38 ****	**0.0002**
	SOST	0.49 **	**0.0001**
	ALP	**0.22 ***	**0.0411**
	OPG	**0.36 ****	**0.0006**
	Ca	**-0.29 ****	**0.0061**
	Pi	**0.37 ****	**0.0003**
	Albumin	**-0.37 ****	**0.0003**

The result of the current study showed significant correlation between traditional biomarker PTH, ALP ,Pi, ($p<0.05$), ($p<0.01$)recpctivaly for diagnosis CKD-MBD and PTH with new biomarker SOST,PINP($p<0.01$ showed in a table (3-9).

3.12 The ability of biomarkers to interpret CKD-MD from Control.

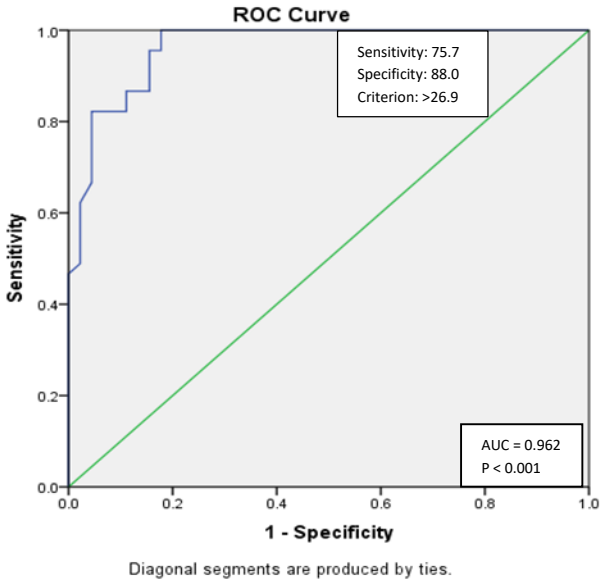
The receiver operating characteristic curve (ROC curve) was used to evaluate the diagnostic values of Biomarkers in discrimination between **CKD-MD** patients and controls.

Table (3-10) ROC analysis of ELISA , PINP ,OPG and SOST markers as bone turnover marker of CKD-MD from Control

Marker	SE	Cut-off	SN%	SP%	AUC	P vale
PINP	**0.04**	**≥ 292.6**	**88.9 %**	**71.1 %**	**0.891**	**0.0001**
OPG	**0.18**	**≥ 26.9**	**88.4%**	**75.6%**	**0.962**	**0.0003**
SOST	**0.07**	**≥ 2.45**	**91.0%**	**88.6 %**	**0.97**	**0.0001**
SN: sensitivity, SP: specificity, AUC: area under curve, P-val.: p value						

3.12.1. Predictor of OPG CKD-MD patients form controls Groups

Figure (3-12) shows the ROC curve between well CKD-MD patients and controls. The test revealed that the area under the curve (AUC) was **0.962** (standard error) **0.18**, 95 % CI = 0.92 – 0.99, p=0.0003. The sensitivity and specificity of the test at the cut-off value of **≥ 26.9** ng/mL were **88.4**% and **75.6**%, respectively, indicating a fair discriminative value.



(Figure 3-12) Criterion values and coordinates of the ROC curve analysis for OPG as differentiating patients from control subject

3.12.2. Interpreter of plasma PINP CKD-MD patients from healthy Groups

ROC test revealed that the AUC= 0. 891 (SE) 0.04, 95%CI= 0. 813-0. 970, p=0.0001. The sensitivity and specificity of the test at a cut-off value of ≥ **292.6** ng/ml were 88.9% and 71.1%, respectively, indicating a fair discriminative value (Figure 3-13).

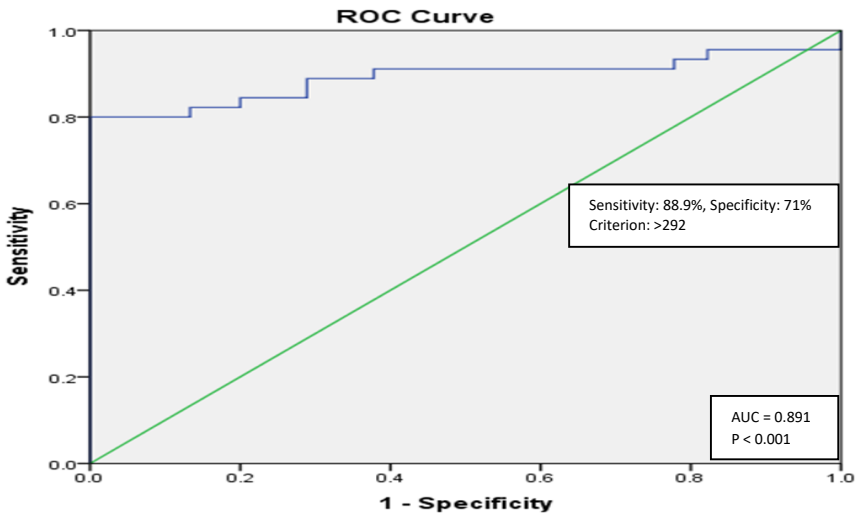


Figure (3-13) Receiver operating characteristic curve for PINP in the context of discrimination of CKD patients from healthy controls.

3.12.3. expect of plasma SOST CKD-MD patients from healthy Group

According to Figure (3-14), the AUC was **0.97** (SE) **0.07**, **95 % CI = 0.94–0.98**, p=0.001. Sensitivity and specificity of the test at cut off value of **2.45** ng/ml were **91.0 %a**nd **: 88.6 %** in that order, indicating a good characteristic value

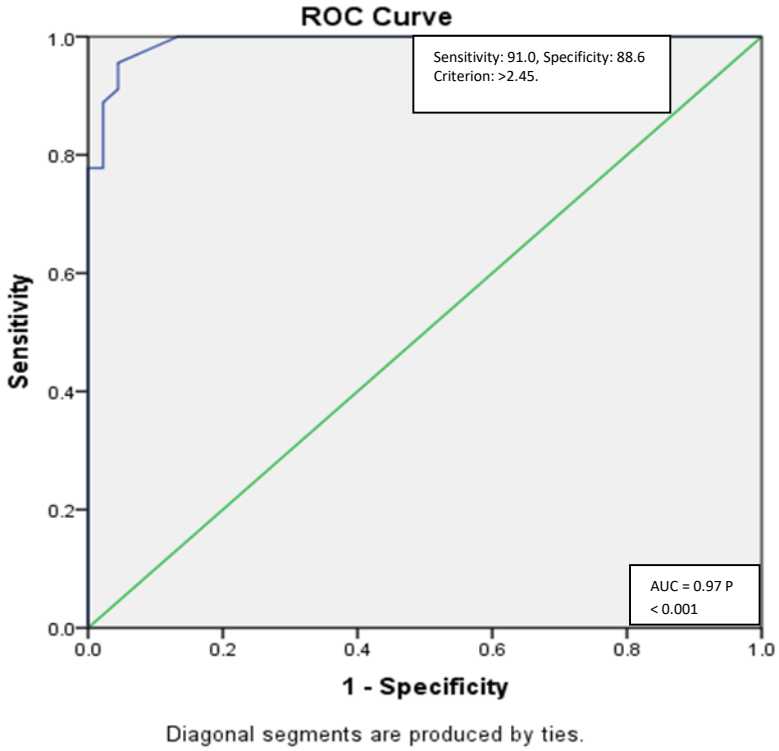


Figure (3-14) ROC curve of the SOST that expected CKD from healthy

Conclusions

Conclusions

This study concluded that:

1- The OPG, SOST, PINP, ALP and parathyroid hormone have a Beneficial association to indicates bone mineralization status.

2- OPG, SOST, PINP could be used as a predictive marker in individuals with chronic kidney disease to predict the development of bone mineral disease

3.Could be guide the markers in diagnosis and follow up

Recommendations

Recommendations:

1- Future studies performed with a larger size

2- The follow-up study on the same patients yields a more accurate picture of the variability in the levels of OPG, SOST, and PINP.

3. Estimate OPG, SOST, and PINP as bone turnover parameter in CKD patients before and after treatment for bone minral disease.

4. uses OPG, SOST, and PINP with other biomarkers related to bone trunover, such as fibroblast growth factore-23, CTX, and osteocalcin in futuer studies to be more knowledgeable about bone mineral status and to have a better understanding of the pathophisylogy of CKD-BMD

5. Assess bone turnover biomarkers in correlation with CKD stagings.

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APPENDIX A

Questionnaire

Case No. Date:

Hospital name:

Patient name:-

Age: -

Sex:-

Weight: - Kg

BMI:

Height: - cm

Telephone number:-

Profession:-

Residence:-

Duration of disease:-

Final diagnosis of the disease:-

Other disease

الخلاصة

امراض الكلية الزمنة تتميز بضعف مزمن في بنية أو وظيفة الكلى حيث معدل الترشيح الكبيبي (GFR) < 60 مل / دقيقة / 1.73 م^2 أو وجود الألبومين في البول بتركيز 30 ملغ لكل 24 ساعة.
السكري ، ارتفاع ضغط الدم ، التهاب كبيبات الكلى المزمن ، التهاب الحويضة والكلية المزمن ، استخدام الأدوية المضادة للالتهابات بشكل دائم ، أمراض المناعة الذاتية ، مرض الكلى متعدد الكيسات ، مرض ألبورت ، التشوهات الخلقية ،والامراض الحادة للكلية لفترات طويلة جميعها من الأسباب الأكثر شيوعًا لامراض الكلية المزمنة. التشخيص غالبا يكون عرضياً او بالصدفة نتيجة الفحص الروتيني لعينة الادرار، أوعند تفاقم الاعراض لدى المريض ومراجعة الطبيب.
اضطراب المعادن والعظام المرتبط بمرض الكلية المزمن CKD-MBD هو مصطلح لوصف الاضطراب الجهازي في العملية الايضية للمعادن في العظام. الحثل(الضمور) العظمي الكلوي هو تغيير في تشكيل العظام لدى مرضى الكلى المزمن ، وهو احد المؤشرات التي تتعلق بالحاله الهيكلية لمكون الهيكل العظمي ضمن الاضطراب الجهازي لـ اضطراب المعادن والعظام المرتبط بمرض الكلية المزمن CKD-MBD.
يتكون ايض العظام من عمليتين هما إزالة العظام القديمة (ارتشاف) وإنتاج عظم جديد (تكوين). تشمل العناصر الفاعلة الأساسية في أمراض الكلى المزمنة والاضطرابات المعدنية والعظام CKD-MBD: النظام الهرموني(لفيتامين د. ، هرمونات جار الدرقية وعامل النمو الليفي 23) ،الكالسيوم ،الفوسفات.

الهدف من الدراسة هو تقييم العلاقة بين مستويات المصل لمؤشرات ايض العظام أوستيوبروتيجرين) (OPG)، سكليروستين(SOST) ، طليعة الكولاجينPINP1 ، مع المؤشرات الحيوية التقليدية هرمون الغدة جار الدرقيةPTH ، وانزيم الفوسفوتيز القلوي ALP في المرضى الذين يعانون من امراض مزمنة في الكلى والاشخاص الاصحاء. تم تصميم هذه الدراسة كدراسة حالة وضبط. شارك في هذه الدراسة خمسة وأربعون مريضًا من مرضى غسيل الكلى (27 إناثًا و 18 ذكرًا) بالإضافة إلى خمسة وأربعين شخصًا سليمًا (27 إناثًا و 18 ذكرًا) متطابقين جيدًا مع المرضى في العمر والجنس. كان العمر (20 - 75) سنة متوسط العمر 50.51 ± 2.07 سنة ، مؤشر كتلة الجسم (24-27) كغم / م^2 المرضى من وحدات الغسيل الكلى في مستشفى المحمودية ومستشفى اليرموك.

تم قياس مستويات OPG و PTH و SOST و PINP بواسطة تقنية ELISA بينما تم قياس مستويات المعلمات الأخرى (الكالسيوم ، الفوسفات ، انزيم الفوسفوتيز ، الألبومين) بطريقة القياس اللوني وفقًا لدليل

الشركة المصنعة للفحص. كما أظهرت نتائج الاختبارات التي أجريت أن مستويات الكالسيوم والألبومين لدى الأشخاص المصابين بمرض الكلى المزمن أقل بكثير من الأشخاص الأصحاء ، حيث أن قيمةP < 0,05بينما مستويات OPG وهرمون الغدة الجار درقية والفوسفور و SOST و انزيم الفوسفوتيز القلوي و PINP في المرضى أعلى بكثير من الأشخاص الأصحاء ، حيث كانت قيمة P< 0,05

نستنتج من الدراسة الحالية التي أجريناها أن مستوى وجود اوستيوبروتجرين و سكليروستين و طليعة الكولاجين1 وهرمون الغدة جار الدرقية والفوسفور أعلى في مرضى الكلى المزمن مقارنة بالاصحاء ، ويمكن استخدام OPGوSOST كعلامة تنبؤية في مرضى الكلى المزمن للتنبؤ بإمكانية الإصابة بمرض الحثل(الضمور العظمي_ الكلوي) (ROD) في مراحل مختلفة من مرض الكلى المزمن ، وأظهرت الدراسة الحالية أن مؤشر كتلة الجسم اقل في المرضى مقارنة بالاصحاء قيمة P< 0,05 ، وكان هناك انخفاض في مستويات الألبومين والكالسيوم لدى المرضى مقارنة بالأشخاص الأصحاء ، مع وجود علاقة إيجابية بين المؤشرات الحيوية لايض العظام (OPG، SOST، PINP) مع هرمون الغدة جار الدرقية و انزيم الفوسفوتيز القلوي ، وجود علاقة إيجابية بين انزيم الفوسفوتيز القلوي وهرمون الغدة الجار درقية والفوسفور التي تم إجراؤها وهناك ارتباط متضاد بين الكالسيوم والألبومين و المعلمات المدروسة الأخرى.

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قسم الكيمياء والكيمياء الحياتية السريرية

تقييم مستويات أوستيوبروتيجرين, سكليروستين, طليعة الكولاجين1 لدى مرضى امراض الكلى المزمن الذين يجرون الغسل الكلوي

رسالة

مقدمة إلى عمادة كلية الطب في جامعة بابل وهي جزء من متطلبات نيل
درجة الماجستير في علوم الكيمياء / الحياتية السريرية

من قبل

براء خالد خلف ابراهيم
بكالوريوس تقنيات التحليلات المرضية

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