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Genetic, biochemical and nutritional markers of cardiovascular events in Chronic Kidney Disease patients

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A Emanuele,
l'Amore
che auguro a chiunque di poter incontrare sul proprio cammino,
il regalo più bello di questo Dottorato.

SOMMARIO

Introduzione. *I pazienti con insufficienza renale cronica (IRC) sono ad alto rischio di mortalità cardiovascolare, superiore rispetto alla popolazione generale della stessa età. Infatti, la malattia cardiovascolare è la prima causa di morte nei pazienti affetti da IRC, a causa della presenza dei classici fattori di rischio insieme ai rischi specifici della patologia renale, come i disturbi del metabolismo minerale osseo. Le complicanze a lungo termine includono iperparatiroidismo e iperfosfatemia, legati ad un aumento del livello sierico di FGF23 (Fibroblast Growth Factor 23). FGF23 è un fattore di crescita prodotto dal tessuto osseo che agisce sulla riduzione della fosfatemia. La letteratura riporta che una prolungata esposizione all'assunzione di fosfato aumenta la produzione di FGF23. I dati mostrano un legame tra FGF23 e iperfosfatemia, con il conseguente aumento del rischio cardiovascolare. Una restrizione dietetica del fosfato può essere una strategia contro la morbilità cardiovascolare e la formazione di calcificazioni vascolari. Un altro interessante marker del metabolismo osseo è la sclerostina, una proteina prodotta dagli osteociti ad attività inibitoria sull'osteogenesi e la sua produzione è aumentata nell'IRC. L'attuale studio si propone di indagare la mortalità e la morbilità cardiovascolare identificando marcatori genetici, biochimici e funzionali. Il progetto è uno studio osservazionale longitudinale e i pazienti reclutati sono stati seguiti per un follow-up di 5 anni. Sono stati studiati la predisposizione genetica al rischio di insorgenza di eventi cardiovascolari nell'IRC e i fattori nutrizionali e biochimici che potrebbero influenzare lo sviluppo e la progressione degli eventi cardiovascolari in questi pazienti.*

Popolazione e Metodi. *L'attuale studio ha reclutato 332 pazienti affetti da IRC, sia in pre-dialisi che in emodialisi (HD) e ha raccolto i recall dietetici delle 24 ore, storia medica, parametri biochimici e strumentali e campioni di sangue per le analisi genetiche. La genotipizzazione degli SNPs è stata eseguita su 208 pazienti in HD utilizzando Illumina® MegaEX Array (SNP 2M) confrontando i pazienti con precedenti eventi cardiovascolari (CV1, 135 soggetti) con pazienti senza eventi cardiovascolari (CV0, 73 soggetti). Per ciascun marker è stato calcolato l'indice di fissazione (Fst) utilizzando CV1 e CV0 come sottopopolazioni al fine di identificare gli SNPs più differenziati tra i due sottogruppi. Utilizzando il cut-off del 99° percentile della distribuzione Fst, sono stati identificati 3218 SNPs come differenziati tra CV1 e CV0. Sono stati interrogati due strumenti di pathway funzionale (Panther and Ingenuity Pathway Analysis - IPA) per definire qualsiasi possibile ruolo biologico/patologico degli SNPs associati.*

Risultati. *È stato osservato che i pazienti in HD hanno peso corporeo, calcemia e vitamina D inferiori e fosfatemia, PTH e FGF23 più elevati rispetto ai pazienti pre-dialisi con IRC allo stadio 3-5. Inoltre, la regressione lineare multipla mostra una relazione positiva tra fosfatemia, FGF23 e PTH in entrambi i pazienti HD e pre-dialisi, mentre è stata trovata una relazione negativa tra l'assunzione di fosfato e la fosfatemia. Probabilmente questo è dovuto all'effetto di FGF23. Il campione di soggetti è stato poi diviso in tre gruppi in base all'assunzione di fosfato. Per i pazienti in HD il livello sierico di FGF23 aumenta all'aumentare dell'assunzione di fosfato, mentre nei pazienti in pre-dialisi questa relazione non viene osservata. Inoltre, sono stati valutati i fattori di rischio legati alle malattie cardiovascolari. Un'analisi di*

regressione (Cox) sul campione ha dimostrato che un'assunzione elevata di fosfato nella dieta (>1200 mg/die) aumenta il rischio di eventi cardiovascolari. La sclerostina sierica, invece, è aumentata in 87 pazienti con IRC e il suo aumento era proporzionale al declino del tasso di filtrazione glomerulare (GFR). Considerando i terzili di sclerostina sierica, i pazienti nel terzile più alto hanno mostrato valori di GFR e di vitamina D inferiori e valori più elevati di FGF23 rispetto ai pazienti nel terzile più basso. Lo z-score osseo lombare (BMD) era maggiore nei pazienti nei terzili più alti e nel terzile medio, considerati insieme, rispetto ai pazienti nel terzile più basso. I pazienti che non assumevano calcitriolo come terapia cronica (n=60) avevano valori di GFR più alti e sclerostina sierica inferiore rispetto ai pazienti trattati cronicamente con calcitriolo. La regressione multipla ha mostrato che la sclerostina sierica era negativamente associata alla vitamina D e positivamente a FGF23 nell'intero campione e nei pazienti che non assumevano calcitriolo. Anche questa analisi ha mostrato che solo la sclerostina sierica era positivamente associata allo z-score della BMD in tutti i partecipanti e nei pazienti che non assumevano calcitriolo. Lo score di calcificazione dell'aorta e gli eventi cardiovascolari non erano associati alla sclerostina sierica. Per l'analisi genetica, sono stati uniti i risultati più significativi dei due strumenti di analisi e identificati due geni coinvolti in entrambi i pathway di malattia cardiovascolare (da IPA) e nello sviluppo del cuore (Panther): il recettore del peptide-1 simile al glucagone (GLP1R) e il delta Sarcoglicano (SGCD). Usando la tree regression analysis è stato scoperto che l'allele minore GLP1R rs10305445 è associato a percentuali più alte di CV1 (p=0.03) e, inversamente, l'allele minore SGCD rs145292439 è associato a percentuali più basse di CV1 (p=0.038) e livelli più elevati di HDL (p=0.015). La valutazione nutrizionale è stata condotta su 148 soggetti (98 in HD e 50 in pre-dialisi). Le analisi hanno mostrato una correlazione positiva tra lo stadio della malattia e l'assunzione di vitamina E, mentre aveva una correlazione negativa con l'assunzione di beta-carotene. Tuttavia, non sono state osservate correlazioni significative tra l'analisi dei dati nutrizionali raccolti e le analisi genetiche condotte.

Conclusioni. *I livelli sierici di FGF23 aumentano con un'elevata assunzione di fosfato nella dieta (> 1200 mg/die) nei pazienti in HD. I pazienti in HD hanno un più alto apporto di fosfato rispetto ai pazienti affetti da IRC allo stadio 3-5. I valori di FGF23 sono principalmente influenzati dall'assunzione di fosfato con il cibo nei pazienti in HD. In particolare, un'assunzione elevata di fosfati nella dieta (>1200 mg/die) aumenta il rischio di eventi cardiovascolari durante tutti gli stadi della IRC. Inoltre, i nostri risultati suggeriscono che la sclerostina sierica possa essere un determinante della sintesi di vitamina D e possa predire la massa ossea nei pazienti affetti da IRC. Da questa analisi preliminare, è possibile concludere che i fattori genetici possono contribuire più della dieta nello sviluppo di malattie cardiovascolari nei pazienti con IRC ed ipotizzare GLP1R come marcatore genetico di rischio e SGCD come marcatore protettivo per IRC. Rimangono da investigare i meccanismi che si nascondono sotto queste varianti alleliche e che influenzano le vie metaboliche specifiche associate alla malattia cardiovascolare nella IRC.*

ABSTRACT

Introduction. *Chronic Kidney Disease (CKD) patients at different stages suffer of high risk of cardiovascular mortality, which is higher than the age-matched general population. In fact, cardiovascular disease (CVD) is the first cause of death in CKD patients because of the simultaneous presence of the classical risk factors and the ones specific of the renal disease, among which there are metabolic mineral disorders. The long-term complications include hyperparathyroidism and hyperphosphatemia that are linked to an increase of FGF23 (Fibroblast Growth Factor 23) serum level. FGF23 is a growth factor produced by the bone tissue that acts to reduce the level of serum phosphate. Literature reports that a prolonged exposure to phosphate intake increases FGF23 production. Data show a link between FGF23 and hyperphosphatemia with an increased risk of CVD. A dietary restriction of phosphate can be useful to fight against cardiovascular morbidity and vascular calcifications. Another interesting bone metabolism marker is sclerostin, a protein produced by osteocytes having an inhibitory activity on bone formation and its production is increased in CKD patients.*
The current study is called "The Cardiovascular Mortality project: study of functional markers" (CREMA) and aims to investigate cardiovascular mortality by identifying the genetic, biochemical and functional markers of arterial damage in relation to the diet. The project is a longitudinal observational study. Recruited patients were followed for a 5-year follow-up period. The main purpose of the study was to identify the genetic factors predisposing the risk of occurrence of cardiovascular events in CKD and, together with these, the nutritional and biochemical factors that could influence the development and progression of cardiovascular events.

Population and Methods. *The current study recruited 332 CKD patients, both in pre-dialysis and in hemodialysis (HD) and collected dietary 24hrs recalls, medical history, biochemical and instrumental parameters and blood samples for genetic analysis. SNPs genotyping was performed in 208 HD patients using Illumina® MegaEX Array (2M SNPs) comparing patients with previous cardiovascular events (CV1; 135 subjects) to patients without cardiovascular events (CV0; 73 subjects). For each marker, we calculated the Fixation Index (Fst) using the CV1 and CV0 as subpopulations in order to identify the most differentiated SNPs between the two subgroups. Using the cut-off of the 99th percentile of the Fst distribution we identified 3218 SNPs as differentiated between CV1 and CV0. We interrogated two functional pathway tools (Panther and Ingenuity Pathway Analysis - IPA) to define any possible biological/pathological role of the associated SNPs.*

Results. *We observed that HD patients had lower body weight, lower calcium and lower vitamin D serum and higher phosphatemia, PTH and FGF23 than pre-dialysis CKD patients at stage 3-5. Multiple linear regression showed positive correlation between serum phosphate, FGF23 and PTH in both HD and pre-dialysis CKD patients, while a negative correlation between phosphorous intake and serum phosphate was found. Probably this is due to the effect of FGF23. The sample was split in three sets based on phosphate intake. Serum FGF23 was evaluated for the three sets. For HD patients, FGF23 serum level increases with an increase of*

phosphate, whereas in pre-dialysis CKD patients this relation is not observed. Moreover, risk factors linked to CVD were evaluated for our sample. A Cox regression analysis on the sample showed that a high dietary phosphate intake (> 1200 mg/die) increases the risk of cardiovascular events.
Serum sclerostin was increased in 87 patients with CKD and its increase was proportional to estimated glomerular filtration rate (eGFR) decline. Considering tertiles of serum sclerostin, patients in the highest tertile showed lower GFR and 1,25(OH)$_2$D serum values and higher FGF23 serum values than in patients in the lowest tertile. Lumbar-spine bone mineral density (BMD) z-score was greater in patients in the highest and the middle tertiles, taken together, than in patients in the lowest tertile. Patients not-taking calcitriol as chronic therapy (n=60) had higher eGFR and lower serum sclerostin than patients chronically treated with calcitriol.
Multiple stepwise regression showed that serum sclerostin was negatively associated with serum 1,25(OH)$_2$D and positively with serum FGF23 in the whole patient sample and in patients not-taking calcitriol. Multiple stepwise regression also showed that only serum sclerostin was positively associated with BMD z-score in all participants and patients not-taking calcitriol. Aorta calcification score and cardiovascular events were not associated with serum sclerostin.
For the genetic analysis, we merged the most significant results from the two tools and we identified two genes involved in both cardiovascular disease pathways (from IPA) and hearth development (Panther): Glucagon-like peptide-1 receptor (GLP1R) and Sarcoglycan delta (SGCD). Using regression tree analysis, we found that GLP1R rs10305445 minor allele A is associated with higher percentages of CV1 (p=0.03) and, inversely, SGCD rs145292439 minor allele A is associated with lower percentages of CV1 (p=0.038) and higher levels of HDL (p=0.015).
Nutrition evaluation was conducted on 148 subjects (98 in hemodialysis and 50 in pre-dialysis). The analyses showed a positive correlation between the stage of the disease and the intake of vitamin E, while it had a negative correlation with beta-carotene intake. However, no significant correlations have been observed between the analysis of the collected nutritional data and the conducted genetic analyses.

Conclusions. *Serum FGF23 levels increase with high dietary phosphate intake (>1200 mg/die) in HD patients. HD patients have a higher intake of phosphate than pre-dialysis CKD patients at stage 3-5. FGF23 is mainly influenced by phosphate dietary intake in hemodialysis patients. Moreover, a high dietary phosphate intake (>1200 mg/die) increases the risk of cardiovascular events during all stages of CKD. Our findings suggest that serum sclerostin may be a determinant of 1,25(OH)$_2$D synthesis and may predict bone mass in CKD patients.*
From this preliminary analysis, we can conclude that the genetic factors may contribute more than diet to cardiovascular disease in CKD patients and we can speculate a role of GLP1R as genetic risk marker and SGCD as protective marker. The mechanisms hiding under these allelic variants influence the specific metabolic pathways associated with cardiovascular disease in CKD remain to be investigated. Further studies are needed to evaluate the impact of vitamin E and beta-carotene on CKD.

ABBREVIATIONS

1,25 (OH)2 D 1,25 dihydroxy Vitamin D
1,25 (OH)2 D 3 Calcitriol
25 (OH) D 25-hydroxy Vitamin D
AKT Protein Kinase B
ALP Alkaline Phosphatase
BMD Bone Mass Density
BMI Body Mass Index
CAPD Continuous Ambulatory Peritoneal Dialysis
CCPD Continuous Cyclic Peritoneal Dialysis
CKD Chronic Kidney Disease
CKD-EPI Chronic Kidney Disease EPIdemiology collaboration equation
CRP C-Reactive Protein
CVD Cardiovascular Disease
DCM Dilated Cardiomyopathy
DGC Dystrophic-Glycoprotein Complex
DPP-4 dipeptidyl peptidase 4
DXA Dual wavelength X-ray Absorptiometry
eGFR estimated Glomerular Filtration Rate
ESRD End-Stage Rena Disease
FFA Free Fatty Acids
FGF-23 Fibroblast Growth Factor-23
FGFR Fibroblast Growth Factor Receptor
GFR Glomerular Filtration Rate
GLP-1 Glucagon Like Peptide-1
GLP-1R Glucagon Like Peptide-1 Receptor
GPCR G-protein-coupled receptor
GPR G-protein Receptor
HD Hemodialysis
HDL High Density Lipoprotein
IPA Ingenuity Pathway Analysis
IRC Insufficienza Renale Cronica
IRS-1 Insulin Receptor Substrate-1

KAU severity Kauppila calcification score
LDL Low Density Lipoprotein
LVH Left Ventricular Hypertrophy
MAF Minor Allele Frequency
MDRD Modification of Diet in Renal Disease
NaPi2a, b, c Sodium/Phosphate Cotransporters
PANTHER Protein Analysis Through Evolutionary Relationships
PEW Protein-Energy Wasting
PI3K Phosphatidyl Inositol 3-Kinase
PiT-1, -2 Sodium-dependent Phosphate Transporter
PTH Parathyroid Hormone or Parathormone
PTHR-1 Parathyroid Hormone Receptor-1
SGCD Sarcoglycan Delta
SLC Solute Carriers
SNP Single Nucleotide Polymorphism
SOST Sclerostin Gene
T2DM Type 2 Diabetes Mellitus
TGF-β Transforming Growth Factor-β
TNF- Tumor Necrosis Factor-α
VC Vascular Calcification
VEP Variant Effect Predictor

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INTRODUCTION

Chronic Kidney Disease (CKD)

Chronic Kidney Disease (CKD) is a condition due to the irreversible loss of renal function, both depurative and homeostatic. The evidence of the chronicity of the syndrome is provided by repeated evaluations of the glomerular filtrate through the creatinine clearance, measured directly or calculated, which document a gradual reduction over a period of at least three months. According to data from the National Health and Nutrition Examination Survey III, CKD prevalence reaches over 10% of the population. CKD is now recognized as a real health emergency, because of its epidemic dimensions[29].

CKD Definition and Staging

In 2004, the National Kidney Foundation (Kidney Disease Outcome Quality Initiative (NKF-KDOQI)[136] proposed the definition and staging of chronic kidney disease. CKD is defined as the condition in which it has been present for at least three months:

- a reduction in Glomerular Filtration Rate (GFR) that is below 60 ml / min / 1.73 m^2 estimated with the MDRD formula, or
- renal damage documented by biopsy, transplantation history or presence of blood markers or urinary renal damage such as proteinuria. The most correct method for measuring glomerular filtrate is the creatinine clearance. However, to avoid frequent errors related to urine collection and to make the evaluation of renal function easier, the estimation of GFR (estimated GFR or eGRF) obtained by formulas that disregard the urinary data was introduced. The formula used is MDRD, which requires 4 variables: creatinine, age, sex and race. The MDRD formula can be found directly at the following site: http://nephron.org/mdrd_gfr_si.

CKD staging involves 6 progressive stages, evaluated through the GFR (Table 1).

Stage III-b is the most monitored and intensively treated because it represents a group of patients at high risk of complications, with consequent evolution towards the terminal stages[109].

However, this type of staging does not take into account the progressive reduction of filtration velocity secondary to the physiological process of aging and this has led to a problem of incorrect classification.

Stage	Renal Damage	GFR (ml/min/1.73m^2)
I	normal or increased function	> 90
II	mildly decreased function	89-60
III-a	mildly to moderately decreased function	59-45
III-b	moderately to severely decreased function	44-30
IV	severely decreased function	29-15
V	kidney failure	< 15 (Dialysis)

Table 1: CKD staging[87].

Epidemiology

The increased prevalence in the general population of clinical conditions characterized by a high risk of developing kidney damage, the general aging of the population, the physiological aging of the kidney, the greater attention paid to the diagnosis and the increase of life expectancy thanks to innovative therapies, have led to an epidemic increase in chronic kidney disease. In recent years, many studies have been conducted in many countries to identify the real prevalence of CKD (Table 2). All data collected in the North American and Northern European population cannot, however, be applicable to the Italian reality, because, for the cardiovascular risk profile, the countries

of the Mediterranean area represent a specificity not comparable to other areas.

In Italy, the most recent and relevant study was conducted by the Italian Society of Nephrology in collaboration with the Istituto Superiore di Sanità (ISS) and the Association of Hospital Cardiologists. This is the STUDIO CARHES[36,139] which showed a prevalence of 8.1% in men and 7.8% in women, with a higher prevalence in stages I and II. In Italy, CKD is a major problem but is characterized by a lower prevalence than other Western countries.

Study	Date	Country	Sample(n)	Prevalence (%)
NANHES III[30]	1988-1994	USA	15488	11
CARHES[139]	2010	Italy	9020	7.9
PREVEND[38]	1997	Holland	8459	11.6
HUNT[72]	1995-1997	Norway	65181	10.2
NANHES IV[29]	1999-2004	USA	13233	13.1
NHI[105]	2003	Taiwan	176365	9.8
EPRICE[141]	2004-2008	Spain	2756	9.2

Table 2: CKD prevalence - international data.

Etiology

Renal damage is secondary to numerous and heterogeneous pathological events. Many of them specifically target the kidney, others affect the kidney as a vascularized organ and belong to the category of risk factors for systemic vascular damage. Therefore, renal disorders are distinguished in:

- typically renal diseases;
- diabetic nephropathy and nephroangiosclerosis;
- ischemic nephropathy due to renal artery stenosis[192].

Typically, renal diseases include congenital or hereditary diseases like polycystic kidney or Alport syndrome, glomerulonephritis due to defects of the immune response, diseases of infectious origin, calculi (and its consequences) and tubulo-interstitial diseases from drugs. *Glomerulonephritis* is the most common cause of CKD. These are diseases in which the renal glomerulus is damaged and progressively loses its filtration capacity. This damage occurs at different ages as a consequence of renal injury more often caused by an impaired function of the immune system (primitive glomerulonephritis) or diseases affecting other organs (secondary glomerulonephritis). *Interstitial diseases* are the second cause of chronic renal failure and are known as pyelonephritis, because the most frequent cause is a chronic infection of the urinary tract that is favored by an obstacle to flow during the urinary tract. This obstruction can be determined by diseases present since birth, such as the bladder-ureteral reflux, or others that occur in the course of life, such as calculosis. These diseases can also be the consequence of metabolic disorders and/or drug abuse. Nephroangiosclerosis and diabetic nephropathy, however, are disorders linked to risk factors that affect the kidney as a vascularized organ: hypertension, dyslipidemia and metabolic syndrome. In these cases we must also consider a special sensitivity to insults from the vascular system of the kidney; in fact, only a part of diabetics or hypertensive patients shows renal insufficiency[165].

Replacement Therapy

The term "replacement therapy" refers to a method that can replace the function of the damaged kidney and allow the survival of the patient affected by CKD. All dialysis systems currently available are a renal replacement therapy (removal of toxic substances, electrolyte and acid-base re-balancing, removal of liquids), but not for the production of hormones for

which it is often necessary to provide drugs. The types of dialysis available today are differentiated according to the type of membrane that is used to perform the purifying function of blood and in the case of hemodialysis this membrane is artificial, while in peritoneal dialysis it is natural. In hemodialysis, it is necessary to create a monitored extra-corporeal circulation, where blood with heparin and a solution called "dialysis bath" are pumped through the dialysis filter and then re-enter the organism through a vascular access, called fistula. Hemodialysis is the most common type of dialysis and it is applied in hospitals or dialysis centers. Peritoneal dialysis, on the other hand, is based on the spread vascularization of the peritoneal membrane. The simplest method, called CAPD (Continuous Ambulatory Peritoneal Dialysis), consists in introducing a dialysate solution bag (2 liters) into the peritoneal cavity through a catheter. After 4-5 hours, the liquid is conveyed into a special drain bag together with the toxic substances and the cycle is resumed with a new bag. Another method is the CCPD (Continuous Cyclic Peritoneal Dialysis), a continuous treatment in which specific devices are used to manage the exchange of dialysis solution and is performed using a device at night and a manual method during the day[201].

CKD and Cardiovascular Disease (CVD)

CKD is a risk condition for two reasons: it may be a prerequisite for the uremic syndrome and the development of End Stage Renal Disease (ESRD), and may increase the risk of cardiovascular complications. There are several epidemiological and clinical evidence showing that patients with chronic renal failure present a much higher cardiovascular risk than those found in the population of the same age[58,123]. It is also evident that this risk increases directly in proportion to the progressive decay of renal function. A study published by Go et al. shows that patients with GFR between 45 and 59 ml/min/1.73 m^2 have a risk of developing cardiovascular events increased

by about 40% compared to patients with normal or slightly reduced renal function. Furthermore, the same risk is increased by 240% in patients with GFR below 15 ml/min/1.73 m^2 (Table 3)[66].

GFR (ml/min/1.73 m^2)	Relative Risk	Risk Increase
> 60	1	-
45-59	1.4	40
30-44	2	100
15-29	2.8	180
< 15	3.4	240

Table 3: Risk of cardiovascular events and GFR values.

In most patients, nephropathy is a consequence of damage from diabetes mellitus and/or arterial hypertension, which are important cardiovascular risk factors. Furthermore, the worsening of arterial hypertension is conditioned by nephropathy and is associated with dyslipidemia, both of which contribute to increase the risk. The risk profile is justified by the simultaneous presence of traditional cardiovascular risk factors (hypertension, hypercholesterolemia, metabolic syndrome and diabetes, obesity, familiarity) and other special factors related to nephropathy, among which there are the mineral metabolism disorders, such as hyperphosphatemia, secondary hyperparathyroidism, vitamin D deficiency and increase of the calcium-phosphate product, which contribute to the progressive vascular calcification (VC) of vessels and cardiac valves[80].

Other emerging risk factors are hyperhomocysteinemia[132] and chronic inflammation associated with an increase in oxidative stress, with the consequent accumulation of products that play a fundamental role in inducing endothelial dysfunction[131]. All these factors together largely legitimize the increased cardiovascular risk in the CKD population[32]. The

possible pathogenetic interactions that associate the renal pathology with the cardiovascular risk are not yet completely clear, being linked both to the sharing of pathogenetic mechanisms, and to the ability of the renal and cardiovascular factors to enhance each other. CVD is the leading cause of death and morbidity in patients with CKD. In addition to the increased number of cardiovascular events, a worse prognosis is reported in these patients than in the general population. All CKD patients are considered at high risk for CVD, including atherosclerotic coronary artery disease, cerebrovascular disease and heart failure; therefore, careful monitoring and the adoption of preventive measures must be carried out. In the presence of coronary heart disease, it accelerates the reduction of glomerular filtration that, in turn, increases the risk of coronary events. With the progression of renal disease to more advanced stages, other risk factors predominate over traditional ones, such as changes in bone and lipid metabolism, oxidative stress and anemia.

Nutrition in pre-dialysis CKD

In CKD patients the main objective is to preserve the residual renal function to achieve adequate biochemical and clinical parameters in order to maintain an acceptable quality of life. The approach is to recommend an hypo-protein diet, proposed for the first time in 1939, with the aim of relieving uremic symptoms through the control of urea and acidosis, to reduce nephropathy progression.

The hypo-protein diet can help to delay the start of dialysis treatment and improve the quality of life; in fact, it allows to slow down the progression of renal disease, to have a better control of calcium/phosphate metabolism and a better control of metabolic acidosis. However, this type of diet requires constant monitoring of the macronutrients contributions to prevent the appearance of protein-caloric malnutrition. There are several factors that can

interfere with adherence to a low-protein diet plan, such as the patient's ability to self-control in nutrition and the interference of the diet with the relationship life. Generally, patients have to undergo a major change from their habitual behaviour and to follow a straight diet. Patients with GFR higher than 70 ml/min/1.73 m^2 (stage II) usually do not have to follow a protein restriction, unless renal function is rapidly decreasing; in the latter case, patients are advised not to exceed a protein intake of 0.8-1 g/kg of body weight/day. In case the GFR is between 25 and 70 ml/min/1.73m^2 (stage III), a hypo-protein and hypo-phosphate diet is recommended, which may delay the dialysis treatment. The indicated amount of protein is 0.6 g/kg of body weight/day, of which 35g/kg/day should be high biological value proteins, to ensure an adequate intake of essential amino acids. At the IV stage of the CKD, with a GFR value less than 25 ml/min/1.73 m^2 (not yet in dialysis), a hypo-protein and hypo-phosphate diet may no longer be equally valid. Toxic nitrogen metabolism products accumulate in large quantities. Since there is no evidence on how to behave from the nutritional point of view at this stage of the disease, it is advisable to follow a diet equal to that followed in stage III of the disease. Following a hypo-protein diet plan means avoiding purine rich foods, mainly meat. Other nutritional indications are to reduce the salt intake added in the preparation of food, but also the hidden one, found in sausages, aged cheeses, canned foods, stock cubes and spices/salt preparations. It would also be advisable to avoid the consumption of alcohol, drink at least 1.5 liters of water per day (preferably natural mineral content) and avoid prolonged fasting. Today there are several commercially available aprotein products available free of charge for patients at an advanced CKD stage; these products allow patients not to completely change their usual diet by replacing "normal" products with those protein-free.

Dialysis Treatment

Although the all-cause mortality rate for dialysis patients remains higher than in the general population, since the introduction of dialysis in the 1960s, life expectancy of patients with ESRD has gradually increased. According to a 2013 report, in the United States, the 60-months survival probabilities measured from the first day of therapy for all patients were 30% in 1998 compared to 36% in 2006[1]. In Europe and Japan, substantially higher survival rates have been reported, even after adjustment of cases by age, sex and renal disease. Japanese patients have far fewer comorbid-associated risk factors than those in the United States[83,26]. One of the highest survival rates was reported by Tassin, in France, where patients were in dialysis treatment 24 hours a week, a longer period than almost all the other centers (usual dialysis duration is 12 hours)[25]. Dialysis has improved the quality and life conditions of patients with CKD. ESRD patients are subjected to dialysis, a purifying treatment that replaces the kidney in its four main functions: the removal of waste and toxic substances from the body; maintaining the balance of electrolytes; maintaining the pH balance; removal of excess fluids. Dialysis, therefore, removes from the body all the toxic substances that the kidneys are no longer able to expel. During the dialytic process, the blood that is rich in substances to be eliminated, is put in contact with the "dialysis bath", a solution with an established composition, which contains electrolytes and other molecules aimed at mimicking the blood plasma. The dialysis solution and the blood are divided by a semi-permeable membrane through which the molecules pass at low/medium molecular weight by simple diffusion, according to concentration gradient, so that the toxic products contained in the blood pass into the dialysate solution, whereas the other electrolytes more concentrated in the solution tend to pass to the blood.

The substances useful to the body are not however reabsorbed, as happens in the functioning kidney.

There are two methods of dialysis[87]:

- Hemodialysis: the patient's blood is drawn from a fistula, constructed through the anastomosis of an artery and a vein in the upper limb, and passed through a disposable filter containing a semi-permeable membrane that allows, through the diffusion, to correct the blood imbalances of the solutes.
- Peritoneal dialysis: allows the purification of the blood with the use of a dialysis solution placed inside the peritoneal cavity, thus exploiting the natural semi-permeability of the peritoneum. The peritoneum is a serous mesothelial membrane with a wide, thin and almost transparent surface, which is the lining of the abdominal cavity and part of the pelvic one (parietal peritoneum) and also covers most of the viscera contained within it (visceral peritoneum), at the same time fixing them to the walls of the cavity (viscera ligaments). The choice of the type of dialysis depends on several factors, such as the patient's age and the presence of associated diseases, the ability to perform the procedure and the patient's preferences. Peritoneal dialysis is preferred for younger patients for their better dexterity. Furthermore, young patients prefer the independence and flexibility of the peritoneum-home dialysis method. For the execution of the hemodialysis treatment it is necessary to have an artificial kidney and an adequate blood flow, allowed thanks to the creation of a vascular access. The artificial kidney consists of a filter, a dialysis bath and a dialysis monitor. The most important component of the filter is represented by the dialysis membrane which must be semi-permeable, in order to allow the selective passage for some substances. The dialysis monitor is used to prepare the dialysis solution, regulate the blood flow and the dialysate and monitor the main parameters of the hemodialysis treatment.

The dialysis bath is obtained by diluting a concentrated saline solution with deionized water. The composition varies according to clinical needs, the standard solution consists of[215]:

- sodium 142 mmol/l;
- potassium 2 mmol/l;
- calcium 1,5 mmol/l;
- magnesium 0.5 mmol/l;
- chlorine 109 mmol/l;
- acetate 4 mmol/l;
- bicarbonate 35 mmol/l;
- glucose 5.55 mmol/l.

Various complications related to hemodialysis can occur. The most common complications are hypotension due to numerous factors, including excessive ultra-filtration with inadequate intra-vascular volume balance, altered autonomic vasoactive response, osmolar variations, food ingestion, altered cardiac reserve, use of anti-hypertensive drugs and vasodilatation due to the use of hot dialysate. Another common complication of this procedure is muscle cramps, probably caused by changes in muscle perfusion due to excessively rapid volume removal[192]. During the hemodialysis session, the rapid correction of plasma potassium and metabolic acidosis can cause changes in the intra-/extra-cellular potassium concentration, with possible occurrence of changes in heart rhythm. Dreadful complications may be bacterial contamination of the dialysate, gaseous embolism and hemorrhage.

Malnutrition and Dialysis Treatment

Although the dialytic treatment is able to regress most of the symptoms associated with uremia, not all the consequences of renal failure are effectively contained. In the first place, dyslipidemia and cardiovascular

complications (accelerated atherosclerosis) may continue to worsen, as well as some neurological symptoms. Secondly, the release of chemical mediators such as interleukin-1, -6 and TNF-α and complement system activation due to blood contact with dialysis membranes may, together with the loss of amino acids and peptides in the dialysis fluid, contribute to the instauration of a specific framework of protein-caloric malnutrition called Protein Energy Wasting (PEW).

Protein-caloric malnutrition is a very common factor among patients with terminal uremia or in dialytic treatment, with a prevalence ranging from 18 to 70%, and is associated with an increased risk of cardiovascular mortality[16]. In CKD patients malnutrition can occur relatively early and generally worsens with the disease progression. At the beginning of dialysis therapy, different degrees of protein-caloric malnutrition are usually detected with the use of specific parameters (anthropometric, biochemical and food intake). These parameters are monitored systematically over time. The diagnosis of PEW is confirmed when there are at least 3 alterations of the 4 parameters listed below:

1. Alteration of biochemical markers. Reduction of albumin, pre-albumin, cholesterol.
2. Reduced body mass. BMI <23 kg/m^2, unintentional weight loss greater than 5% in 3 months or 10% in 6 months, body fat percentage <10%.
3. Reduced muscle mass. Muscle mass reduction of 5% in 3 months or 10% in 6 months, reduction of muscle area of the arm by 10% compared to the 50th percentile of the reference population.
4. Inadequate dietary intake. Calorie intake less than 25 kcal/kg/day and protein less than 0.8 g/kg of body weight, for a period of at least 2 months.

The pathogenesis of malnutrition is multifactorial and is mainly caused by[65,28]:

- <u>inadequate food intake.</u> The guidelines of the Dialysis Outcome Quality Initiative (DOQI) indicate a daily protein and caloric intake of 1.2 g/kg of

protein and 30-35 kcal/kg of ideal weight per day. Furthermore, 50% of the protein intake should derive from high biological value proteins, such as meat and fish, rich in all the essential amino acids. However, even in this case compliance with the diet is often inadequate for several reasons. First of all, the need to restrain the amount of phosphate can lead to a reduction of the protein intake, could be insufficient and therefore unable to support the negative nitrogen balance. Secondly, in at least 1/3 of hemodialysis patients anorexia is present, as a consequence of complex interactions between metabolic signals, accumulation of uremic toxins, altered regulation of homeostatic mechanisms of the digestive system, alteration of the circulating levels of factors that regulate appetite (gastric mediators, adipokines and cytokines) and altered hypothalamic signalling. Finally, other factors such as the alteration of taste and smell, comorbid diseases such as gastric diseases, impaired mobility and intestinal infections, as well as social factors such as a low economic level, alcohol abuse, the impossibility of obtaining, preparing or ingesting inadequate foods and depression contribute to the reduction of dietetic intake.

- catabolizing disorders;
- loss of nutrients during dialysis procedures, both macro- and micronutrients (during a hemodialysis session, 4-9 grams of amino acids are lost if the patient is fasting and about 8-12 grams in the postprandial session);
- blood loss due to gastrointestinal bleeding, repeated laboratory and/or diagnostic tests and blood seizure in the dialyzer;
- endocrine-metabolic alterations typical of uremia, in particular insulin resistance, reduction of circulating anabolic factors and increase of catabolic cytokines;
- accumulation of uremic toxins (acidosis stimulates muscle proteolysis due to the increase in decarboxylation of valine, leucine and isoleucine);
- reduced renal metabolic capacity;
- drugs that inhibit appetite.

During CKD progression, the loss of renal function leads to a series of alterations including inflammation, acidosis, and anorexia that are able to trigger protein catabolism generating malnutrition[16]. The alterations in protein metabolism caused by uremia are reflected in the nutritional status, which, in turn, promotes an increase in cardiovascular risk and infections. The classic cardiovascular risk factors (advanced age, lifestyle, smoking, hypertension, dyslipidemia, diabetes) are expressed more in patients with renal insufficiency, partly because of the clinical features of CKD patients (elderly, most of whom with cardiovascular problems and/or diabetes)[51]. The nutritional status of patients tends to worsen gradually, because nutritional needs in dialysis increase and often cannot be reached for the aforementioned reasons. Inadequate nutrient intake is one of the most important causes of malnutrition and, together with other comorbidities, induces anorexia, a very high risk factor for mortality. During dialysis treatment the protein and energy needs increase. According to a study conducted by Rao et al.[162] most patients taking at least 1.1 g/kg/day showed a neutral or positive nitrogen balance, but not in all patients. For this reason we tend to consider a protein intake of 1.2 g/kg ideal weight/day as a safety value of the EBPG Guideline on Nutrition[61] to guarantee the nitrogen balance, and 25-35 kcal/kg ideal weight/day respectively, depending on the patient's dry weight. If the patient has a normal weight, 30 kcal/kg/day are enough; if the patient is overweight or malnourished the kcal will be less than 30 or higher than 30, respectively. The spontaneous protein intake of uremic patients seems to be lower than the recommended needs and range from 0.94 to 1 g/kg/day[114,92], and the energy intake is around 23-28 kcal/kg/die. Such insufficient intake, due to decreased appetite and other pathological and psychological conditions, is linked to a progressive development of caloric-protein malnutrition[101].

Several studies have shown that a protein intake of at least 1.2 g/kg/day is required to ensure a positive or at least neutral nitrogen balance[28], thus

avoiding a catabolic state. Three retrospective studies of uremic patients have shown that a lower protein intake of less than 1.2 g/kg/day corresponds to lower albumin values and a higher mortality risk[2,189,14]. In fact, a low level of albumin (<4 g/dl) is an important independent risk factor for cardiovascular mortality in these patients[118]. Patients are unlikely to achieve the recommended minimum intake: many dialysis patients consume less than 80% of their recommended needs[46]. Prescribing a hypo-protein diet requires specific skills and every patient must be monitored carefully in terms of compliance. Through a careful nutritional counselling carried out on the patient by specialized personnel, such as a dietician or a nutritionist expert in kidney disease, can try to increase the caloric and protein intake through strategies aimed at motivating the patient. If these goals are not fully achieved, then oral or enteral nutritional supplementation may be considered by assessing its effect on nutritional inputs related to biochemical values. In addition to quantitative factors, the qualitative factor plays a fundamental role in the determination of nutritional status. Patients not only tend to spontaneously reduce both caloric and protein intake due to lack of appetite, but encounter numerous difficulties in maintaining adequate energy intake by having to comply with a controlled and low potassium diet. Therefore, they tend to take large amounts of food containing atherogen fat. In addition, such patients may undergo micronutrient deficiencies, which may increase the risk of cardiovascular mortality related to caloric-protein malnutrition[101]. A study carried out on a group of patients in hemodialysis, subjected to food investigations, showed that they consume significantly less amounts of some cardio-protective carotenoids[92]. Substantial nutrient losses occur during dialysis, and this is the reason why nutritional needs in this type of treatment increase. At the same time there is also a loss of micronutrients (vitamins and minerals), both during the dialytic procedure and for other causes, such as deficient diets, reduced intestinal absorption or impaired renal metabolism. The vitamin status varies from patient to patient depending on

age, sex, vitamin intake with diet, any supplementation, loss during dialysis session and the residual renal function. For this reason, a possible integration of vitamins must be personalized according to the individual.

Cardiovascular Diseases (CVD)

Cardiovascular diseases are one of the most important public health problem in the Western world: they are the main causes of mortality, morbidity and disability. Those who survive an acute form become a chronic patient with repercussions on the quality of life and an increase in social costs.

Definition and Epidemiology

The term CVD is intended to describe a series of diseases that affect the heart and blood vessels; 43% of deaths in men and 54% of deaths in women in Europe are attributable to cardiovascular disease[209]. In Italy, this pathology remains the leading cause of death, being responsible for 35.4% of all deaths[108]. According to the statistical classification of diseases and related health problems (ICD-10)[129], CVD include:

- Ischemic heart disease: angina pectoris, acute myocardial infarction,
- Hypertensive diseases;
- Cerebrovascular diseases: cerebral infarction (stroke);
- Acute articular rheumatism;
- Chronic rheumatic cardiopathies;
- Other forms of cardiopathies;
- Diseases of the arterial systemic circulation: atherosclerosis; aneurysm; dissection of the aorta;
- Diseases of the venous systemic circulation, lymphatic vessels and lymph nodes;
- Pulmonary heart and diseases of the pulmonary circle.

Among the diseases that are of great importance from the epidemiology point of view are: angina pectoris, acute myocardial infarction, cerebral infarction, atherosclerosis and aneurysm. Ischemic heart disease is a disease caused by a reduced supply of oxygen to the heart muscle due to the reduction of blood flow through the coronary arteries. Angina pectoris is a pain or discomfort due to a temporary disparity between the oxygen demand and the ability of the coronaries to carry blood. Myocardial infarction is necrosis of the heart tissue due to insufficient blood flow. Cerebral stroke or stroke is the necrosis of brain cells due to a hemorrhage (rupture of a blood vessel) or an obstruction of the flow through a vessel downstream of the site that went into necrosis. The term atherosclerosis indicates a group of three pathological conditions that have in common the thickening and loss of elasticity of the arterial walls: real atherosclerosis, medial calcific sclerosis, atherosclerosis. The aneurysm is a circumscribed dilatation of the aorta caliber, often asymptomatic, which above 5 cm can lead to rupture of the endothelium with fatal consequences. In particular, coronary heart disease accounts for 21% of deaths in men and 22% of deaths in women, stroke causes more death in women (17% compared to 11% in men), as well as other CVD. The incidence, mortality and prevalence data were obtained from longitudinal studies involved in the CUORE project, coordinated by the Istituto Superiore di Sanità, which enrolled 21,000 men and women aged 35-74 years since the 1980s with a follow-up of 13 years. The data obtained show a higher incidence of coronary events (6.1 ‰ per year in men and 1.6 ‰ per year in women) compared to cerebrovascular events (2.7 ‰ per year in men and 1.2 ‰ per year in women)[43].

Risk Factors for CVD

Cardiovascular diseases have multifactorial etiology, therefore more risk factors can act in synergy leading to an increased probability of onset.

Traditionally, risk factors can be classified into factors that are not modifiable and factors that can be modified with the lifestyle. Non-modifiable factors include age, gender and familiarity. The advancing age increases progressively the risk, being atherosclerotic disease a chronic degenerative pathology, however one death in four occurs in people under 65 years of age. Gender is a risk factor, as women have a lower incidence of coronary atherosclerosis before menopause, while it increases significantly after menopause and the risk between the two sexes becomes similar. A history of premature heart disease in siblings or in parents suggests a greater susceptibility that can be influenced by a genetic factor. The main modifiable factors are: obesity, hypertension, dyslipidemia, diabetes mellitus, diet, sedentary lifestyle, cigarette smoking. Alongside these traditional factors are emerging risk factors such as abdominal adiposity, inflammation, stress and depression. Blood pressure, blood glucose, and cholesterol threshold levels are derived from the American Heart Association guidelines (AHA 2000) and from the guidelines of the Italian Society for the Study of Atherosclerosis (SISA). Diet influences cardiovascular risk. Healthy dietary habits can help in reducing three of the major risk factors for atherosclerosis and CVD: dyslipidemia, hypertension and obesity. Dyslipidemia contributes directly to the development and progression of coronary heart disease. Epidemiological data indicate that both hypercholesterolemia, in particular the increase in LDL[86], and hypertriglyceridemia[77] (>150 mg/dl) are considered both powerful and independent risks of coronary heart disease. A meta-analysis of data collected from over one million adults between 40 and 70 years has shown that an increase of 20 mmHg of systolic pressure and 10 mmHg of diastolic pressure doubles the mortality from coronary heart disease[111]. Overweight and, above all, obesity[107] are also important risk factors. This association is particularly significant in young people and adults, whereas it declines in senile age. The circumference measurement and the Body Mass Index (BMI) can represent two clinical parameters that are easy to use.

The nutritional aims of the World Health Organization for the prevention of diseases with nutritional component and in particular CVD (WHO 2003) indicate 4 points for the preventive strategy: follow a balanced diet, control body weight, control hypercholesterolemia and hypertension. Consumption of a diet capable of supplying the necessary amount of energy and nutrients is essential for maintaining health. Several studies in the literature have shown that following a Mediterranean diet is a protective factor for the most common chronic diseases[76].

The Mediterranean diet includes: a high consumption of vegetables, legumes, fresh fruit and nuts, olive oil and whole grains; a moderate consumption of fish and dairy products and red wine during meals; low consumption of red meat, white flour, simple sugars and saturated fatty acids. It has emerged in numerous studies that the Mediterranean diet represents a protective factor against the causes of mortality linked to CVD[50]. It has not yet been fully clarified how this type of diet has a positive effect on the cardiovascular system. Experimental evidence is that this type of diet is associated with lower levels of cholesterol and apolipoprotein B, the two main components of atherogenic lipoproteins. In addition, there is a positive correlation between the Mediterranean diet and the plasma concentration of adiponectin, a cytokine secreted by adipose tissue with the function of improving insulin sensitivity. Moreover, it is associated with lower values of fasting glucose, triglycerides, LDL cholesterol. Finally, this diet provides a high intake of anthocyanins, polyphenols that have an antioxidant function[168].

Dialysis and CVD

CKD affects one adult in seven American adults and its prevalence is expected to increase even further as the age in the population tends to increase and augment the incidence of risk factors such as hypertension and diabetes[31]. CKD patients are more likely to die prematurely than the general

population[191] because CKD is strongly associated with the development of fatal and non-fatal cardiovascular diseases. Even in the absence of diabetes, the 10-year risk of incident cardiac events is equivalent to that observed in patients with diabetes but without CKD. This equivalence led to the belief that CKD by itself should be considered as an equivalent of cardiovascular risk[196]. Similarly, CVD remains the leading cause of death in patients with CKD[63], however trials that experiment pharmacological and non-drug treatment strategies for CVD systematically under-represent or exclude patients with advanced CKD. This leads to a paradox of "risk management": patients at greater risk are less likely to receive proven strategies or to be tested with new potential strategies.

Causes of cardiac death in CKD patients

Although it has been known that cardiovascular disease is the leading cause of death in patients with non-dialysis CKD, population information on the mechanisms of cardiovascular death is lacking. Recently, however, an analysis of data from about 81,000 people from the Alberta Kidney Disease Health Network has sought to shed light on this topic[196]. The study authors reported that when the baseline value of GFR was less than 60 ml/min/1.73m^2, CVD became the most common cause of death and the rate of deaths from cardiovascular disease increased inversely to the values of GFR. Ischemic heart disease accounted for just over 50% of cardiovascular deaths in patients with an GFR below 60 ml/ml/1.73m^2 and the percentage did not change in the lower GFRs. Likewise, the percentage of deaths attributed to heart failure and valvular heart disease increased with values progressively lower than baseline GFR values. The possible mechanisms underlying these observations include an increase in the degree of valvular calcification, an increased pressure overload due to arteriosclerosis, disturbed bone mineral metabolism and smooth muscle altered more

severely among people with low GFR. Furthermore, as renal function worsens, volume overload due to sodium retention and reduced free water excretion can become more problematic. Despite the limitations inherent to the analysis of retrospective data, in the same study, sub-classifying the causes of cardiac death within the non-dialysis CKD population, the authors highlighted the need for further research into strategies to reduce mortality from non-ischemic cardiac death.

The consequences of acute cardiovascular events in CKD

The risk of myocardial infarction is higher in CKD patients than in patients without CKD and its incidence increases at low levels of basic GFR. In addition, mortality following hospitalization for a non-fatal myocardial infarction is higher in CKD patients than in those without CKD[196], but little is known how these acute events alter the subsequent risk of ESDR and the concurrent risk of death before the ESRD. A recent study examined the cause-specific risks associated with non-fatal cardiac events in CKD patients. After a non-fatal myocardial infarction, both the risk of ESRD and death before ESRD were increased four-five times, compared to patients who did not have myocardial infarction during the study period.
These results suggest that a non-fatal myocardial infarction in a CKD patient may serve as a marker of a risk particularly high for both death and ESRD[191].
Among CKD patients, the risk of death, in particular due to CVD, is typically higher than the risk of a possible request for renal replacement therapy[57]. However, the risk depends on factors such as age. In fact, in many studies, elderly patients with less severe renal impairment and lower levels of proteinuria are more likely to die (usually due to the cardiovascular event) before needing dialysis therapy; while younger patients with proteinuria and kidney-related disease are more likely to reach dialysis[161,37].

Phosphate

Phosphate is a mineral that represents about 1% of the body weight of an adult. About 80% of the phosphate present in the body is found in the mineral component of the bone tissue in the form of crystalline hydroxyapatite, 9% in skeletal muscles, 10.9% in the visceral tissues and 0.1% in the environment extracellular.
In the intracellular environment it is present both in the form of free anions and in numerous organic compounds such as proteins, intermediate metabolites of carbohydrate and lipid metabolism, high energy molecules (ATP and phosphocreatine) and nucleic acids.
The mixture of free ions is referred to as "phosphates" and is present in plasma at concentrations between 0.75 and 1.45 mmol/l.

Physiological homeostasis of the phosphate

The absorption of phosphates occurs in the small intestine, particularly in the fast, through two mechanisms: a passive and an active one.
Paracellular and transcellular passive absorption is responsible for the absorption of about 60% of the phosphate load taken with the diet.
The sodium-dependent active mechanism can further increase up to 80% absorption of phosphate intake through a mechanism dependent on 1.25-$(OH)_2$D3 or calcitriol[13].
Intestinal absorption is very efficient, therefore the regulation of serum phosphate levels is mainly carried out at the renal proximal tubule[173].
Figure 1 shows schematically the phosphate homeostasis.

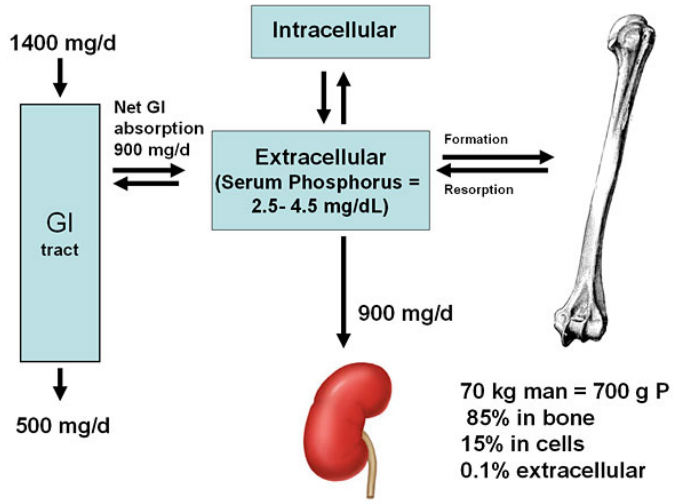


Figure 1: Phosphate homeostasis in human organism[154].

Active transport via the apical membrane of enterocytes and renal tubule cells is regulated by the expression and biological activity of three sodium-dependent membrane co-carriers, all members of SLC (solute carriers). NaPi2a and NaPi2c are expressed in the renal tubule, while NaPi2b in the intestine[115].

In both epithelia there is also a transporter on the basal membrane not yet identified. The expression of the carriers is finely regulated.

Phosphate homeostasis depends on the balance between active reabsorption at the renal level, both active and passive reabsorption at the level of the jejunum and bone tissue.

The regulation of homeostasis occurs through the interaction between parathormone (PTH) and calcitriol (hydroxylated Vitamin D), two hormones able to regulate renal and intestinal reabsorption[183].

At the intestinal level, in addition to NaPi2b there is a second type III sodium dependent carrier, PiT-1, which does not play a crucial role under physiological conditions. NaPi2b activity is regulated by the phosphate dike. Moreover, FGF23 (fibroblast growth factor 23) is a phosphatinine produced

by the bone tissue that regulates the absorption of phosphate, favoring the degradation of this carrier.

The kidney plays a fundamental role in homeostasis thanks to the reabsorption in the proximal tubule of 60% of the filtered phosphate and 20% in the distal tubule[78]. At the renal level, next to NaPi2a and NaPi2c, are located PiT-2 carriers on the brush band. The expression of these transporters and the ability to reabsorb phosphate are regulated by FGF23 and PTH[60].

Bone tissue is also a fundamental regulator of phosphate balance. PTH, calcitriol and acidosis can increase its release from the bone, while the PiT-1 carrier present in osteoblasts and chondrocytes can stimulate the synthesis of bone tissue. Finally, osteocytes and osteoblasts play a very important role in phosphate homeostasis as they are producers of FGF23[49].

In humans, changes in circulating phosphate levels lead to cell dysfunctions and systemic diseases. Mild hypophosphatemia is associated with bone pain, muscle weakness, rickets and/or osteomalacia; the severe one (<0.8 mg/dl) causes disturbances of energy metabolism with systemic symptoms such as lethargy, confusion, muscular paralysis, generalized paralysis, microcitemia, hemolysis, platelet dysfunction and leukocyte dysfunction. On the other hand, hyperphosphatemia, especially acute, is associated with widespread precipitation of calcium phosphate with secondary hypocalcemia. The clinical signs are tetany, convulsions and diffuse calcifications.

Phosphate homeostasis in CKD

Intestinal absorption of phosphate in patients with renal disease is decreased. At the initial stage, adaptive processes increase phosphate excretion and decrease its clearance to maintain serum phosphate levels[143]. The increase in PTH levels is one of the main phenomena of these adaptive

processes[19]. The importance of PTH is demonstrated by the increase in phosphatemia that is observed in patients not yet on dialysis treated with cinacalcet (a Calcium Sensing Receptor agonist) that inhibits the production of PTH[184].

When GFR values are less than 30 ml/min/1.73m^2, the adaptive mechanisms can no longer maintain normal phosphatemia values, due to the excretory inability of the kidney. The phosphoric effect of PTH and FGF23 is no longer sufficient to guarantee normal phosphatemia and the progressive growth of PTH levels together with increased acidosis can stimulate bone resorption with the paradoxical effect of releasing phosphate contributing to hyperphosphatemia.

Phosphate retention is responsible for the development of renal osteodystrophy in CKD by stimulating the production of PTH and FGF23, the attenuation of the calcemic response to PTH and the suppression of dihydroxylated vitamin D production. It also contributes to the development of vascular calcifications[54].

Phosphate and diet

The phosphate contained in foods is mainly present as a natural component of the food (organic phosphate and inorganic phosphate salts), but an often non-negligible portion can come from additives added as preservatives (inorganic phosphate).

The amount of phosphate naturally present in foods is proportional to the protein content, with relatively higher amounts in some foods (for example nuts, cheeses, egg yolk, meat, poultry, fish). D'Alessandro and colleagues have "built" a very useful pyramid to understand the content of phosphate in foods to help CKD patients in the conscious choice (Figure 2)[34].

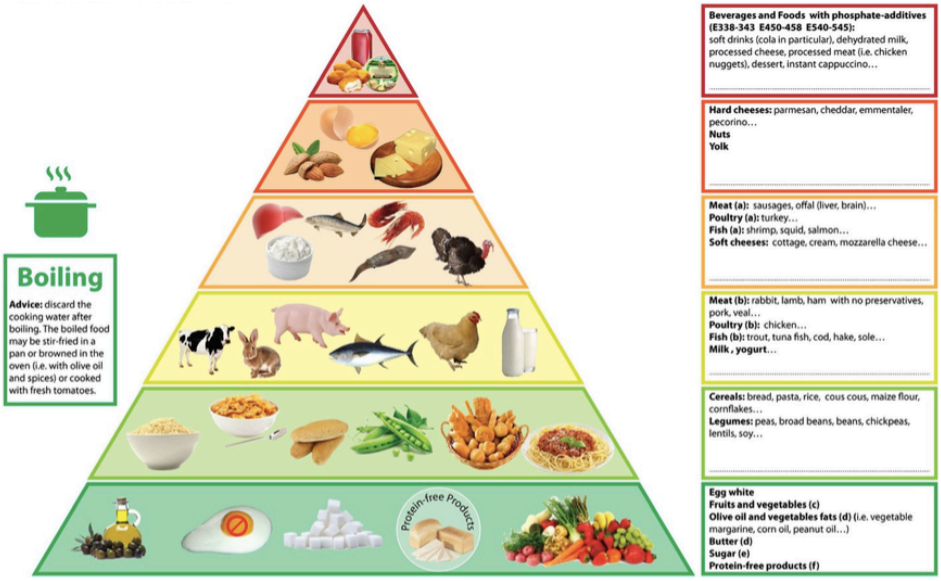


Figure 2: The phosphate pyramid[34]. Foods are distributed on six levels on the basis of their phosphate content, phosphate to protein ratio and phosphate bioavailability. Each level has a colored edge (from green to red, through yellow and orange) that corresponds to recommended consumption frequency, which is the highest at the base (unrestricted intake) and the lowest at the top (avoid as much as possible). a) foods with unfavorable phosphate to protein ratio (>12 mg/g); b) foods with favorable phosphate to protein ratio (<12 mg/g); c) fruits and vegetables must be used with caution in dialysis patients to avoid excessive potassium load; d) Fats must be limited in overweight/obese patients, to avoid excessive energy intake; e) sugar must be avoided in diabetic or obese patients; f) protein-free products are dedicated to patients not on dialysis therapy and who need protein restriction but a high energy intake.

Some industrial and food preparation methods can influence the content of phosphate in food[33].

The normal diet in industrialized societies guarantees an average intake of 1000-1500 mg/day, although the recommended dose is 700 mg/day.

In an omnivorous diet the phosphate content per gram of protein is about 12-14 mg/g. The reference to this relationship is particularly useful for identifying those foods that, with the same protein content, contain more or less phosphate. It is believed that a maximum reference value, not to be

exceeded, is equal to 12 mg/g. It is particularly important to consider the protein/phosphate ratio: for example, egg has an high protein content but a low phosphate content.

In addition to the quantitative intake, it is necessary to consider the qualitative one: while the phosphate contained in foods of animal origin is absorbed in a percentage equal to or greater than 60%, that contained in plants is absorbed at about 40%. In fact, the phosphate of foods of plant origin is largely represented by phytic or phytate which, due to the absence of the phytase enzyme in humans, is not metabolized and is not absorbed[15].

Finally, the average intake of phosphate with the diet can be increased by the presence of additives in preserved foods. The additives used to modify the duration, color and flavor of foods contain phosphate in the form of phosphoric acid, phosphates and/or polyphosphates. Being inorganic phosphates, the percentage of absorption is very high, close to 100%. Unfortunately, in Europe, these additives are indicated on labels not with their names but with an abbreviation ("E" series), which in fact prevents recognition. Even various drinks such as fruit juices, sports drinks and most carbonated drinks contain phosphate-based additives[93,134].

FGF23 - Fibroblast Growth Factor-23

Chronic kidney disease is characterized by a pathophysiology that includes changes in the mineral and bone tissue, changes in laboratory parameters and the formation of vascular calcifications.

All these factors are associated with clinical events such as fractures, mortality and vascular morbidity.

Although the alterations that characterize CKD appear in the early stages of renal failure, it is not clearly identified the initial mechanism of these alterations.

The long-term complications that occur in the course of the disease include an increase in serum Fibroblast Growth Factor (FGF) 23 values, associated with a reduction of Klotho, hyperparathyroidism, hyperphosphatemia and vitamin D deficiency.

FGF23 Physiology

FGF23 is a peptide hormone of 251 amino acids with a molecular weight of 30 kDa encoded by a gene on chromosome 12[208,206].
In 2000, a genetic study of patients with hypophosphatemic rickets led to the discovery of Fibroblast Growth Factor 23. From that moment, follow-up studies have elucidated the biological role of this hormone and have significantly improved our knowledge on the metabolism system of phosphates. FGF23 is a phosphaturic hormone that acts by degrading (down regulation) the co-carriers of the NaPi phosphate present in the brush-like band of renal epithelial cells. This leads to a reduction in the reabsorption of phosphate from the urine, and therefore to an increase in phosphate excretion[49].
In addition, FGF23 is also the only one of its family to have paracrine and endocrine activities.
In addition to blocking the co-transporters at the renal tubular epithelium, FGF23 regulates the renal expression of 1α-hydroxylase, decreasing the synthesis of calcitriol. The low level of silky calcitriol acts by decreasing the intestinal absorption of the phosphate introduced with the diet, with a consequent further decrease in the levels of circulating phosphate. Therefore, FGF23 plays a phosphaturic role both directly preventing its reabsorption in the renal tubule and indirectly, through calcitriol, preventing its absorption into the intestinal lumen.

Furthermore, FGF23 is synthesized from bone cells (osteocytes), whose synthesis is stimulated by high serum phosphate levels and $1,25(OH)_2D$, creating negative feedback between the kidney and the bone tissue.

Knock-out mice for FGF23 have a lower life expectancy, associated with growth retardation, skin atrophy, decreased bone density and numerous vascular calcifications. They also have hyperphosphatemia, hypercalcemia and high levels of dihydroxylated vitamin D. They show an increased sensitivity to insulin with the risk of protracting in hypoglycemic conditions. In these animals, a diet with a reduced phosphate intake can improve the clinical phenotype by correcting the levels of serum phosphate but not by modifying the levels of $1,25(OH)_2D3$ and calcium. Similarly, a diet low in vitamin D corrects the levels of $1,25(OH)_2D3$ and calcium without modifying phosphatemia[187]. In contrast, mice that over express FGF23, present a clinical phenotype of hypophosphataemic rickets, hyperthyroidism and elevated phosphaturia[120].

FGF23 biosynthesis occurs at the bone tissue level, while the main, but non-exclusive, site of action is the proximal part of the renal tubule. In the renal tubule, FGF23 controls both reabsorption of phosphates, regulating the cellular expression of the sodium phosphate co-transporter (NaPi2a and Napi2c) expressed in the brush-border membrane of renal cells, and the biosynthesis of $1,25(OH)_2D3$, regulating the activity of the enzyme 1α-hydroxylase[186].

It exerts its biological activities interacting with an FGFR membrane receptor of which 4 subtypes are described, accumulated by the presence of three similar extracellular immunoglobulin domains. FGF23 inhibits the synthesis and secretion of PTH by parathyroid glandular cells[102].The specific renal activity of FGF23 requires the interaction of the FGF23-FGFR complex with other protein molecules, one of which is Klotho[203]. Klotho is an enzyme formed from a single transmembrane protein with a wide extracellular domain composed of two KL1 and KL2 domains, homologous to the β-

glycosidase family and is expressed in different tissues, even if the main sites of expression are kidneys (renal tubules, distal and proximal), the choroid plexus and the parathyroid glands. Differently from the regulation of other minerals such as calcium, magnesium, potassium and sodium, the endocrine regulation of phosphate reabsorption in the kidney occurs in the proximal renal tubule. Studies by Shimada and collaborators on a transgenic mouse characterized by over expression of FGF23 have demonstrated the phosphaturic role of this hormone that suppresses the expression of NaPi2a and NaPi2c. However, the biological mechanism has remained misunderstood for many years. FGF23 can activate, with a mechanism that depends on Klotho, ERK1 and ERK2 (extracellular signal-regulated kinase), which in turn activates SKG1. The activation of ERK and SKG1 leads to the phosphorylation of NHERF1 and consequently to the down regulation of the expression of NaPi2a transporters. Furthermore, ERK1 and ERK2 suppress the transcription of 1α-hydroxylase in the renal tubule (Figure 3).

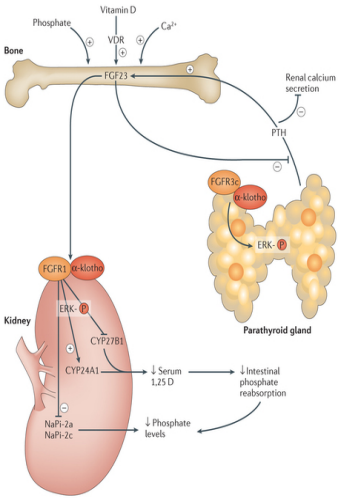


Figure 3: FGF-23 biological effects on phosphate metabolism[203].

FGF23 increase in CKD

In CKD there is a progressive increase in FGF23 levels associated with the reduction of renal function[42]. Several factors such as hyperphosphatemia, hypercalcemia, secondary hyperparathyroidism and Klotho deficiency can help to stimulate this phenomenon.

FGF23 increases progressively while the glomerular filtration rate decreases: this increase is observed already in the II and III stages of CKD. Many studies have shown clearly how the down-regulation of FGF23 metabolism occurs in the advanced stages of renal disease, while the mechanism of the increase from the early stages, before the serum phosphate or PTH increases, remains unclear.

With the decline of renal function there is a simultaneous reduction of the tissue levels of Klotho, which induce a resistance to FGF23[98]. The increase in FGF23 leads to the achievement of high concentrations of this hormone in dialysis patients (Figure 4).

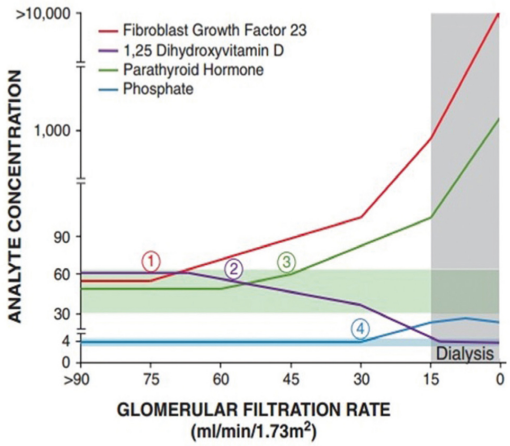


Figure 4: variation of FGF-23, 1,25$(OH)_2$D, PTH and phosphate on the basis of GFR values.

This increase can be explained by different factors:

– a diminished clearance of FGF23;

– a compensation mechanism to maintain the serum phosphate in the standard range;

– a response to treatment with activated vitamin D analogues;

– a compensatory mechanism to the loss of the Klotho protein (although it is not clarified if the biological effect on FGF23 would be of increase or reduction);

– an increase in the synthesis of FGF23 by bone cells[150].

Pereira et al. showed that in 32 patients with stage II-V CKD FGF23 expression in bone tissue was increased at all stages, with a positive association between gene expression in the osteocytes and circulating levels of FGF23. However, the trigger of this overproduction remains to be defined. During the development of the disease, the serum concentration of FGF23 is positively correlated with the serum phosphate and negatively with calcitriol and circulating PTH.

The role of FGF23 in the development of secondary hyperparathyroidism can be explained by various direct and indirect effects. First of all, since FGF23 has a counterproductive effect on vitamin D regulation, an increase in FGF23 during renal disease reduces vitamin D and consequently can facilitate the development of secondary hyperparathyroidism. Secondly, FGF23 can also stimulate the local expression of 1α-hydroxylase in the parathyroid and indirectly regulate PTH by increasing the local production of calcitriol. Finally, it has been demonstrated both in mice and in dialysis patients that there is a down-regulation of the FGF23 signalling pathway in the parathyroid glands with a reduced expression of FGFR1 and Klotho.

Another element that remains misunderstood is whether the accumulation of FGF23 corresponds to active or inactive fragments of this protein.

There are several studies in this regard that have however obtained discordant data[120,188].

As anticipated, in parallel to the increase of FGF23 a decrease in Klotho levels is observed. This was observed in several in vivo studies on murine models[216], but also in a cross-sectional study with 87 patients in CKD[149]. In this study, Klotho polymorphisms with different prognosis were associated with the same sample.

FGF23 and the Cardiovascular System

In the general population, Parker et al. described an increased risk of cardiovascular events and mortality in patients with coronary artery disease and higher levels of FGF23, while elevated serum levels of FGF23, even within a normal range, are associated with a left ventricular mass index correlated with an increased risk for the presence of ventricular hypertrophy[146]. Furthermore, recent studies have shown that FGF23 is a marker of the severity of coronary artery disease in the general population and predictive of serious vascular calcifications in the abdominal aorta[178].

The first clinical study that associated the presence of high levels of FGF23 with the risk of death in dialysis patients was conducted by Gutiérrez in 2008[71]. This was followed by numerous studies, including that of Jean et al.[89] who obtained similar results in a cohort of 219 patients on hemodialysis. Many evidences have associated FGF23 with risk of death, accelerated progression of CKD and other adverse events, particularly cardiovascular[82,56,211].

Several studies have shown that FGF23 has greater predictive power than traditional mineral metabolism markers.

The numerous results that associate FGF23 with the different clinical events has prompted scientific interest to clarify whether this growth factor is only a

marker that represents a range of metabolic alterations or if the high levels of FGF23 are dangerous themselves.

From these epidemiological data, a further step in understanding the relationship between cardiovascular events and CKD was done by describing FGF23 targets in cardiomyocytes.

Moreover, Faul et al.[53] demonstrated that FGF23 could regulate cardiac cell biology in an "independent Klotho" manner. This study showed that the left ventricular index increased in the quartiles with a high level of FGF23 in a prospective cohort of 3070 adults with CKD, and that the higher levels of FGF23 have been associated with an onset of left ventricular hypertrophy (LVH). After these results, the authors studied the direct effects of FGF23 on cell models (rat cardiomyocytes) and animal models (Wt mice and knock-out mice for Klotho). They showed that FGF23 is able to induce hypertrophy in cardiomyocytes in vitro by stimulating a pathway of pro-hypertrophic genes, which depend on the activation of FGFR1 and FGFR4.

Following in vitro evidence of the deleterious effects of FGF23, the authors infused this intravenous phosphatonin into wild-type mice for 5 days and directly into the heart muscle, demonstrating that LVH was induced in both conditions. Also in the murine knock-out model for Klotho mice developed LVH, highlighting independence from Klotho in the pathogenesis of these events.

All of these epidemiological and cellular data demonstrate an association between FGF23 and left cardiac hypertrophy.

In the pathophysiology of vascular calcifications (VC), the role of FGF23 is less clear. There are conflicting data in considering FGF23 as a protective value or not in the development of VC.

Scialla et al.[179] clearly showed that high quartiles of FGF23 in CKD patients were not associated with VC, while high quartiles of phosphate were significantly associated with VC.

Recent experimental studies support a role for FGF23 and its co-receptor Klotho in cardiovascular disease, but the underlying mechanisms remain unknown.

A deficiency of Klotho causes and increases VC in murine models. In a cohort of 114 CKD patients, a decrease in serum Klotho levels has been shown to be an independent biomarker associated with arterial stiffness[96].

Many conflicting data have been published on the role of FGF23 and Klotho in the heart and vessels, but FGF23 appears to be a deleterious actor in the heart, while Klotho appears to have beneficial effects in the vessels (Figure 5).

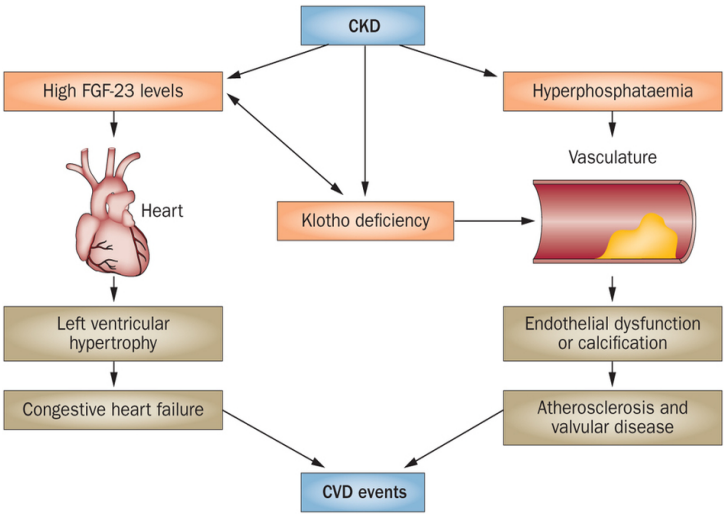


Figure 5: FGF23/Klotho effects on CVD events in CKD patients.

Clinical perspectives related to FGF23

FGF23 is an important regulator, and therefore the complete abolition of its effects can have more negative than positive aspects. The complete abolition of the effects of FGF23 with monoclonal antibodies in rats with CKD, led to hyperphosphatemia, aortic calcification and increased mortality, although the rats had resolution of secondary hyperparathyroidism and normalization of the bone mineral structure[5].

Other studies that used pharmacological inhibition of FGF23 did not demonstrate an increased mortality in animal models, suggesting that a down-regulation of FGF23 is important, but that the goal should be its modulation and not the complete abrogation of the effects.

Sclerostin

Sclerostin is a protein produced by osteocytes in the skeleton, that suppresses bone formation in bone remodelling and modelling. Sclerostin inhibits Wnt/β-catenin signalling pathway in osteoblasts and, thus, prevents β-catenin translocation into the nucleus and transcription of target genes addressing bone formation[133] (Figure 6).

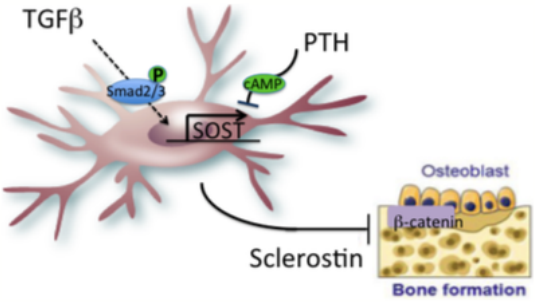


Figure 6: Actions of TGFβ and PTH on Sclerostin. TGFβ activates intracellular SMAD3 up-regulating sclerostin expression. Conversely, PTH acts through its receptor PTHR1 down-regulating sclerostin transcription[133].

Sclerostin production may be increased in CKD patients and its serum levels are increased since the early stages of the pathology[174], despite its renal clearance rises with the decline of renal function[23]. Therefore, sclerostin is a candidate to explain low turnover bone disease in CKD patients[45]. Bone histology showed that serum sclerostin was negatively related with bone formation rate and activation frequency in hemodialysis patients[22]. Consistent with these findings, serum sclerostin was negatively related with serum markers of bone formation and reabsorption and with serum PTH[22,84,213]. Moreover, fracture risk was associated with serum sclerostin levels in CKD patients and women with normal-kidney function[10]. Despite these findings, the relationship between serum sclerostin and bone mineral density (BMD) was positive in patients with normal or decreased renal function[84,142,163]; however, the decrease of BMD over time was more marked in hemodialysis patients with higher serum sclerostin levels[44]. Sclerostin also showed an *in vitro* inhibitory activity on the transformation of vascular muscle cells into osteoblast-like cells, but its relationship with vascular calcification remains controversial in hemodialysis patients, CKD patients and patients with normal renal function[17].

Sclerostin may also influence bone and mineral metabolism in CKD patients by altering the production of calciotropic hormones. It may inhibit renal synthesis of $1,25(OH)_2D$ and consequently the intestinal absorption of calcium and phosphate[172]. Complementary to this effect, $1,25(OH)_2D$ may stimulate osteocyte production of sclerostin[198]. Sclerostin may enhance FGF23 production by osteocytes and sustain its effect on renal phosphate excretion and directly inhibit calcium reabsorption in the renal distal tubule[172]. These effects contribute to the bone-kidney axis in calcium-phosphate metabolism. Finally, a negative relationship between serum sclerostin and PTH was observed in CKD patients[84,213], patients with primary hyperparathyroidism[6] and osteoporotic patients treated with teriparatide[155].

An inhibitory effect of PTH on sclerostin gene (*SOST*) transcription may sustain this relationship[9].

Despite the studies performed until now, the role of sclerostin remains unclear in calcium-phosphate metabolism of CKD patients and its relationship with the other components of the bone-kidney axis has been poorly investigated.

Genetic characteristics of CKD

In line with the most common multifactorial diseases, CKD has a complex etiology involving both a hereditary predisposition and exposure to environmental factors. Some studies have shown that there is a hereditary risk associated with the development of CKD, that seems to be linked to a different prevalence among ethnic groups. However, studies done to determine the hereditary risk factors for this pathology have rarely identified frequent genetic variants[197,135]. Thanks to the advancement and improvement of genotyping technologies and analytical methods, the international scientific community can now study and identify genetic sites more effectively. Moreover, improvements in laboratory techniques and associated bioinformatics tools have resulted in low costs and approaches with genotyping, including high-density microarrays, examining up to 5 million single-nucleotide polymorphisms (SNPs) and new-generation sequential approaches, that can interrogate the whole genome of an individual. More recent studies also seem to demonstrate that epigenetic modifications play a role both in hereditary predisposition to CKD and how the environment dynamically interacts with the genome to alter the risk of an individual's disease[24,176,177].

Molecules of interest related to the study

Some molecules and metabolic pathways have emerged as elements capable of determining or influencing the evolution of cardiovascular complications related to CKD: in particular, the diffuse intestinal endocrine system and, more specifically, the **Glucagon-like secretion peptide 1** and the **Sarcoglycan delta complex** related to the composition and function of muscle fiber.

Glucagon-like secretion peptide 1 (GLP-1)

The glucagon-like peptide 1 (GLP-1) is a hormone produced by the intestine that stimulates insulin secretion and inhibits glucagon secretion by the pancreas. Its release occurs after the meal following the raising of the glycemia due to the carbohydrate intake. GLP-1 slows gastric emptying, increasing the sense of satiety in response to food intake, and reduces appetite by acting directly on the central nervous system's centers of hunger regulation. Furthermore, it appears to have other potentially favorable actions, including a protection of pancreatic β-cells and an increase in cardiac output[190]. In humans, plasma insulin levels are higher after an oral glucose load, compared to those achieved after an intravenous loading that reproduces the same levels of glucose[138]. This phenomenon is known as the "incretinic effect" and is estimated to cause about 70% of insulin secretion after a meal[11]. Patients with type 2 diabetes mellitus (T2DM) have a reduced incretin effect[138]. The molecular mechanisms responsible for this alteration are not known, as we do not know if these are genetically transmitted alterations or secondary to acquired defects. The incretinic effect is mainly due to two hormones, GLP-1 and GIP, secreted in response to meal nutrients and able to enhance glucose-induced insulin secretion[104]. GLP-1 is a hormone secreted by intestinal L cells, the synthesis of which is under the

control of the proglucagon gene by the action of the proconvertase PC 1/3[202]. The mechanisms that regulate the secretion of GLP-1 are not fully known and are actively studied in these years. Data in the literature show that the loss of the incretinic effect in patients with diabetes could be secondary to glycemic changes or insulin resistance, thus presaging that this phenomenon could be reversible[97]. In recent years, Knop and collaborators have shown that in healthy subjects not predisposed to T2DM, an alteration of the incretinic effect could be detected by subjecting these patients to a short period of reduced glucose tolerance or even recreating a state of insulin resistance[74,73]. These evidences lay the foundations for the study of intestinal L cells as possible therapeutic targets and as important components in the pathogenesis of T2DM.

Intestinal L Cells

L cells are present along the walls of the intestinal epithelium with an increasing density towards the distal tract of the ileum and the colon. The placement in the intestine allows these cells to be in direct contact with the intestinal lumen and to be affected by the passage of nutrients along the gastrointestinal tract[47].

L cells are part of a larger entero-endocrine system that secrete many hormones, including cholecystokinin, serotonin and secretin. Although endocrine cells have been classified on the basis of the respective hormone product, it is now clear that many cells are capable of secreting more than one hormone.

In particular, L cells, in addition to GLP-1, produce GLP-2 and the YY peptide. In response to the meal, the course of GLP-1 secretion shows a biphasic profile: a first early stage that occurs immediately after food ingestion and which lasts for 15-30 minutes, followed by a persistent delayed phase for 1-2 hours[137]. The peak of early secretion seems to be mediated by

nerve stimuli and, in particular, by the action of the vagus nerve, while the late secretion is directly affected by direct stimulation of the L cells by nutrients[169,79,4]. Moreover, although the low density of L cells in the proximal portions of the intestine, had initially hypothesized that the early phase of secretion was only indirectly induced by nervous signals or hormonal factors; but it is now universally recognized that the small percentage of L cells present at the duodenum level is sufficient to cause early release of GLP-1 in response to food intake[195]. The half-life of GLP-1 active in the circulation is very short (about 2 minutes) and, in fact, once secreted, the hormone is rapidly inactivated by dipeptidyl peptidase 4 (DPP-4)[39], enzymes present on the surface of endothelial and epithelial cells, implementing the specific cleavage of dipeptides from the N-terminal portion of GLP-1[75]. As a result, it was estimated that only 10-15% of the secreted active form passes into the general circulatory system and is able to influence the other organs. The intestinal L cell is activated by the increase in intra-intestinal glucose concentrations and other nutrients present in a meal. Glucose is internalized by the cell, phosphorylated by glucokinase and metabolised in the mitochondrion for ATP production. This mechanism seems to be very similar to that shown in the pancreatic β-cell.

A series of receptors associated with G proteins (GPR) seems to be important, to which substances such as free fatty acids (FFA) or bile acids, both physiologic stimulators of GLP-1 secretion are bound[147,164]. Both the increase in intracellular glucose concentrations and levels of cyclic AMP (cAMP) lead to an increase in intracellular calcium concentrations and the secretion of GLP-1. In addition to nutrients such as glucose, amino acids and FFA, also insulin seems capable of stimulating the secretion of GLP-1 by intestinal L cells[112] through the activation of the chain phosphorylation system initiated at the IRS-1, PI3K and AKT levels.

The insulin signal in intestinal L cells seems to play an important role in modulating the signals that lead to the secretion of GLP-1. In fact, it was

observed that, in some experimental conditions, chronic exposure to high doses of insulin was able to induce specific insulin resistance in a murine cell line (GLUTag), in a human L cell line (NCI-H716) and in fetal rat intestinal cells. By this mechanism it was possible to alter the secretion of GLP-1[112]. Once released in response to the meal, especially if rich in carbohydrates, GLP-1 exercises several actions at the pancreatic level: stimulates insulin secretion in a manner closely dependent on glucose concentrations (thus, being unable to cause hypoglycemia), inhibits direct and indirect glucagon secretion and favors the processes of differentiation and proliferation of pancreatic β-cells (to date this phenomenon has been highlighted in animal models and there is no direct demonstration that a similar effect occurs in vivo, even in humans)[212].

The GLP-1 also carries out a series of activities at extra-pancreatic level: slowing of gastric emptying, regulation of appetite, reduction of body weight (mediated by a decrease in caloric intake and by direct and indirect effects on the apparatus gastrointestinal and central nervous system), endothelial-mediated vasodilation and improvement of cardiac contractile function in heart disease[124].

While the L cells of the proximal tract of the intestine appear to be more responsible for the incretinic effect, those of the distal tract seem to be related to the effects on appetite and intestinal motility. After its release, GLP-1 is rapidly degraded by a specific enzyme, DPP-4, therefore its use for therapeutic purposes is not practicable except by continuous infusion. To overcome the problem of rapid degradation of GLP-1, analogues have been developed, more correctly defined as "GLP-1 receptor agonists", with a structure similar to GLP-1, which resist the degradation action exerted by DPP-4 and that sometimes are bound to molecules that slow down their subcutaneous absorption. It is interesting that the first of these, exenatide, was developed starting from knowledge on a molecule isolated from a reptile that lives in the desert of Arizona (USA), the Gila Monster.

Glucagon Like Peptide - 1 Receptor (GLP-1R)

GLP-1R is a molecule of 463 residues similar to the secretin, parathormone and calcitonin receptor. With these and other molecules it forms a new class, called "B Family" or "secretin receptor like", belonging to the superfamily of G protein-coupled receptors (GPCR). These receptors possess a unique N-terminal extracellular domain of 100-150 residues that is connected to the intra-membrane domain (J domain) that is typical of all GPCRs. The receptors belonging to the B family of GPCRs bind to their ligands through a mechanism known as the "two-domain model" in which the N-terminal domain binds to the C-terminal region of the ligand thus allowing a second interaction between the N-terminal region of the ligand and the intramembrane domain of the receptor. Like all receptors in the B family, GLP-1R transmits the signal mainly through G-proteins, raising the levels of intracellular cyclic AMP (cAMP). Interactions have also been observed with other types of G-proteins. The receptor is coupled to the G-protein via its intracellular domains, and there is evidence that different regions and receptor residues mate with different G-proteins. In one study, Hallbrink et al. show, using synthetic peptides aimed at activating the G proteins, that the N-terminal residue is responsible for the coupling with G-proteins, while the C-terminal residue mediates the signal through the G-protein/hormones bond. However, it is the production of cAMP mediated by the activation of GLP-1R which is the main responsible for insulin secretion. Some studies have identified polymorphisms in the GLP-1R gene that may be related to diseases such as obesity and diabetes mellitus. GLP-1R is expressed predominantly in the pancreatic islets[181].

Delta-Sarcoglycan

Delta-sarcoglycan is a protein that in humans is encoded by the *SGCD* gene[148,140,200]. The protein encoded by this gene is one of the four components of the sarcoglycan complex, that is a sub-complex of the dystrophic-glycoprotein complex (DGC). In skeletal muscle, the associated glycoprotein complex is a dystrophin that forms a link between cytoskeletal actin and the extracellular matrix, that is critical for muscle integrity. Within this complex is the sarcoglycan sub-complex, which consists of four transmembrane glycoproteins (alpha, beta, gamma and delta-sarcoglycan). During the assembly, beta-sarcoglycan is closely associated with the delta-sarcoglycan to form a functional nucleus which then recruits gamma-alpha-sarcoglycan to form the sarcoglycan complex. Together with sarcospan, the sarcoglycan complex binds other components of the glycoprotein complex associated with dystrophin and integrates into the myofibre membrane. Once integrated, the sarcoglycan complex plays a fundamental role in the mechanical stabilization of the sarcolemma and of the glycoprotein complex associated with dystrophin. Furthermore, the sarcoglycan complex undergoes chemical modifications in response to muscle contractions, thus transferring mechanical information into a cellular signal. Mutations in sarcoglycans induce the muscular dystrophy of the limb girdle (set of hip and shoulder muscles) and different animal models have been established to study the molecular biology and function of the sarcoglycan complex[193]. Mutations in the genes encoding dystrophin and its associated proteins lead to muscular dystrophy and cardiomyopathy in vertebrates and invertebrates. Another study highlights the role of delta-sarcoglycan in dilated cardiomyopathy (DCM), a common cause of heart failure and the most common cardiomyopathy[167]. DCM can derive from myocardial infarction, myocarditis, mitochondrial dysfunction and genetic abnormalities, which represent the final result of different courses[94,185]. The complex nature of

DCM can be attributed in part to the progressive removal of tissues at cellular and molecular levels of the heart[152,52]. In the last decade progress has been made in understanding the molecular genetics of DCM, but remains unclear how molecular alterations that occur at the molecular level can mediate the development of the disease, that often occurs in cardiac-inflammatory autoimmune processes[122]. The identified DCM genes encode for cytoskeletal, sarcomeric, mitochondrial and membrane proteins[199]. The alterations of these proteins are thought to influence the generation and/or transmission of the mechanical force, the signal transduction, the energy metabolism and the stability of the sarcolemma[8]. In a hamster model with DCM, associated with the δ-sarcoglycan mutation, it has been observed that cardiac dysfunctions are evident and then a congestive heart failure resembles that observed in many patients[127,175]. Thus, a fragility of sarcolemma is shown, culminating in DCM in the presence of mutation[27]. Mutation of the human δ-sarcoglycan gene causes familial and idiopathic DCM associated with muscular dystrophy of the limb girdle[91]. Since the hamster DCM model shows successive and uniform phases of pathophysiological changes, it was then used to identify the early biochemical and molecular alterations involved in the initiation and progression of myocardiopathy. The loss of δ-sarcoglycan protein destabilises the dystrophy complex, thus the signalling link between the cytoskeletal network and the extracellular matrix should be altered in tissues lacking δ-sarcoglycan. It has been shown that δ-sarcoglycan physically interacts with the β1 integral signalling molecule and mediates cell adhesion[214].

AIMS OF THE STUDY

The Cardiovascular Mortality project: study of functional markers (CREMA) aims to investigate cardiovascular morbidity and mortality by identifying the genetic, biochemical and functional markers of arterial damage in relation to diet. The project is a longitudinal observational study. Recruited patients were followed for a 5-year follow-up period. The main purpose of the study was to identify the genetic factors predisposing the risk of occurrence of cardiovascular events in CKD and, together with these, the nutritional factors that could influence the development and progression of cardiovascular events.

POPULATION AND METHODS

Study Population

332 CKD consecutive patients, in hemodialysis (n=245) and in pre-dialysis treatment (n=87), were recruited at the hemodialysis Units of the Uboldo Hospital in Cernusco sul Naviglio (MI), San Raffaele Hospital and San Paolo Hospital in Milan. Patients were treated according to the clinical guidelines and patient needs.

In this group, 148 patients agreed to participate in the nutritional survey (n=98 HD patients; n=50 pre-dialysis patients). For genetic analyses, the DNA of 245 individuals on hemodialysis treatment was analysed; 84 of these subjects had no cardiovascular events, whereas 161 individuals had an history of cardiovascular events.

Inclusion criteria:

- subjects diagnosed with CKD at different stages, undergoing a replacement hemodialysis or conservative therapy (GFR < 60 ml/min/1.73m^2);
- age > 18 years old;
- availability to participate in the study;
- absence of acute autoimmune or inflammatory diseases;
- absence of neoplasia history in the five years prior to enrolment;
- absence of endocrinopathies not related to CKD.
- Caucasian race;

The evaluations performed on patients are:

- general objective examination at the beginning of the study and every six months;
- personal and family history;
- blood tests measured at the beginning of the study and every six months: renal function (creatinine, albumin and proteins), inflammatory indices (C reactive protein) and the variables involved in mineral metabolism (calcium, phosphate, parathormone, vitamin D, alkaline phosphatase and FGF23);
- nutritional survey;

- genetic studies.

Glomerular filtration (GFR) was estimated with the Chronic Kidney Disease Epidemiology Collaboration equation (CKD-EPI)[110]. The patient's clinical history was analysed through clinical documents, in order to define the acute cardiovascular events occurred in the 5 years of follow-up.

The cardiovascular events considered are as follows:

- sudden death (ICD-9 code 427.4);
- heart failure (ICD-9 code 428.23, 438.31, 428.33, 428.3, 428.43);
- myocardial infarction (ICD-9 code 410 and 411);
- stroke (ICD-9 code 431-434);
- transient cerebral ischemic attacks (ICD-9 code 435);
- acute peripheral coronary occlusion (ICD-9 code 445).

Study participants were enrolled after signing an informed consent and authorized the processing of personal data for research purposes. Data confidentiality was guaranteed by identification of each patient through a numerical code.

Biochemical and Instrumental Analysis

Blood was drawn for analyses after overnight fasting before the visit in stage 3-5 CKD patients and before the hemodialysis at the end of the longest interval among dialysis sessions in hemodialysis patients. A serum sample was stored at -80°C to measure PTH, FGF23, sclerostin, $1,25(OH)_2D$. Another serum sample was sent to the San Raffaele Hospital Central Laboratory to measure serum 25(OH)D, calcium, phosphate, creatinine, albumin, CRP and alkaline phosphatase with standard analytical systems. Intact serum PTH, sclerostin (reference range was 124-853 pg/ml) and $1,25(OH)_2D$ were determined by chemiluminescent CLIA assay (Diasorin, Stillwater, MN, USA). Serum 25(OH)D was measured by electrochemiluminescent ECLIA method assay (Roche Diagnostics,

Mannheim, Germany). Serum FGF23 was measured using a two-step ELISA method (Kainos Laboratories, Tokyo, Japan; reference range 10-50 pg/ml) determining the full-length molecule of FGF23. Serum concentrations of FGF23 were expressed as absolute values (pg/ml) and $\log_{10}$ of serum FGF23 to compact our data[130].

All stage 3-5 CKD and hemodialysis patients were available to undergo dual wavelength X-ray absorptiometry (DXA) and lateral abdominal plain radiography: lumbar spine bone mineral density (BMD) was measured by DXA (QDR4500W or 1000; Hologic, Bedford, MA) and expressed as z-score, which is the number of standard deviations from the mean in a control population with the same sex and age. Lumbar aorta calcification was quantified in patient lateral abdominal plain radiography by one single trained radiologist using the KAU severity score (range: 0-24; as already described by Kauppila et al.[95]) in a blinded fashion.

Nutritional Analysis

The nutritional survey of patients on conservative therapy was conducted during the outpatient visit, whereas patients on hemodialysis were interviewed during the dialysis session.

The dietary habits were assessed by:

- Food Frequency Questionnaire: a validated questionnaire (Annex 1) that investigates the weekly frequencies of eating habits, especially for calcium, phosphate and Vitamin D present in foods.
- 24-hour Dietary Recall: a diary on the food consumption of the day before the interview to better assess the real eating habits (Annex 2).

The quantity of portions was estimated with the help of the food photographic atlas Scotti-Bassani[59]. This information was used to verify the consistency with the weekly habits. The quantities and the quality of food consumed by the individual patients have been included in the database of a software

(Dietosystem, DS Medica, Milan) specifically prepared for the diet analysis, that includes the bromatological composition of over 1200 foods. The food database of this software has been implemented with the quantities of micronutrients, such as phosphate, calcium and vitamin D. Nutrient intake has been normalized to the body weight of patients.

Genetic Analysis

Genetic analyses were carried out to study the genetic polymorphisms associated with calcification and cardiovascular mortality and morbidity. Blood samples (in 15 ml EDTA) for DNA extraction were taken from patients enrolled in the study after obtaining informed consent. The samples, divided into different aliquots, were marked with an identification code and stored at -80 °C until DNA extraction.

DNA extraction

DNA was extracted from peripheral blood samples in EDTA tubes using the NucleoSpin Blood DNA Isolation kit, following the manufacturer's protocol (Macherey - Nagel GmbH & Co KG, Germany). Blood samples of 200 µl were lysed in the presence of proteinase K at 70° C. Ethanol was added to the lysate obtained to allow DNA binding to the silica membranes of the NucleoSpin Blood columns. The contaminations were removed by rinsing with two different buffers and then the NucleoSpin columns were centrifuged at 11,000 rpm for 1 minute to remove the residual ethanol. Finally, 30 µl of pre-heated elution buffer (TE) was added to the silica membrane and incubated for 1 minute at room temperature to allow the DNA to dissolve in the TE. Then, the Nucleospin columns were centrifuged at 11,000 rpm for 1 minute to elute the DNA in the 1.5 mL tube.

Genotyping

The DNA quality and quantity were checked using Nanodrop (Thermo Scientific, Waltham, Massachusetts, United States) and FlexStation3 (Molecular Devices, Sunnyvale, California, United States) and then processed for Illumina genotyping using Infinium Expanded Multi-Ethnic Genotyping Array (Mega-Ex, Illumina, San Diego, California, United States). For Infinium assays, 4 µl of genomic DNA (~ 200 ng) was used as input. The DNA was transferred onto a new plate, denatured, neutralized and incubated at 37° C for one night for isothermal amplification. After amplification, the DNA was enzymatically fragmented, precipitated and finally resuspended in a RA1 hybridization buffer. All subsequent steps were performed according to the standard Infinium protocol. The fragmented DNA was dispensed on the Multi-sample Mega Ex BeadChips and the hybridization was performed in an Illumina hybridization furnace for 20 hours. The beadChips were washed and treated by primer extension. The single base extension reaction was carried out on the Freedom Evo 150 automation system (Tecan, Männedorf, Switzerland).

Illumina® MegaEX Array

SNPs genotyping was performed using Illumina® MegaEX Array (2M SNPs). Illumina allows to test hundreds of thousands of gene variants simultaneously through the use of Chip-arrays. The association studies (Genome-Wide Association Study, GWAs) are based on the comparison of allele or genotype frequencies in a sample of patients, defined cases, compared to a sample of healthy individuals, defined controls, not related to each other. In this case, the two groups compared were CKD patients with or without vascular calcification. The test investigates statistical differences in the frequency of specific alleles in the two patient series. The hypothesis

underlying the association study is that the presence of genetic polymorphisms is related to the increase or decrease of the risk of developing complex pathologies.

Data Analysis

All quantitative variables were reported as mean ± standard deviation. Differences of means between groups were compared by the Student's t-test for paired data or one-way ANOVA with Tukey post-hoc or LSD post-hoc test for multiple comparisons.
Discrete variable distribution was compared between groups with chi-squared test and among groups with multinomial logistic regression. Relationships between variables were analysed with Pearson correlation.
Variables associated with serum FGF23 and sclerostin were evaluated by multiple stepwise regression: $\log_{10}$ of serum FGF23 concentrations were used as dependent variables and age, body weight, gender, dialysis vintage, serum concentrations of calcium, phosphate, PTH, phosphate and calcium intake and the number of phosphate binders taken by patients as independent variables; for serum sclerostin, was used a first model in which age, body weight, eGFR, serum concentrations of calcium, phosphate, $1,25(OH)_2D$, PTH and FGF23 (as $\log_{10}$) were independent variables. Variables associated with BMD z-score were also tested using body weight, eGFR, serum values of $1,25(OH)_2D$, PTH, sclerostin, FGF23 (as $\log_{10}$), phosphate and calcium as independent variables. Variables associated with Kauppila aorta calcification score were estimated with a regression model including age, body weight, eGFR, serum values of $1,25(OH)_2D$, PTH, sclerostin, FGF23 (as $\log_{10}$), phosphate and calcium as independent variables.

Conditional inference-based recursive partitioning (implemented in the R "party" package) was used to describe the genotype-phenotype relationship between genetic variants discovered, covariates and cardiovascular phenotype. The procedure utilizes the variable with the lowest p-value (after Bonferroni correction) as the first node of the decision tree, subsequently subgroups are created; for each subgroup the variable with lowest p-value (if there is one) is taken as the second or third node.

The association of genotype with cardiovascular events during a 5-year follow-up was assessed using Cox regression. An adjusted Cox regression model included age, body weight, serum phosphate, calcium and PTH and dialysis vintage as covariates. Odds ratio and 95% Confidence Interval were calculated to express the risk of cardiovascular events related to the patient genotype.

Statistical analysis was conducted at $\alpha=0.05$ level and performed using the SPSS statistical package (IBM, Armonk, NY, USA) and R environment.

The Pairwise identity by state between individuals was calculated using PLINK v 1.07[158] and all close relatives were removed (PI_HAT> 0.125). The initial dataset consisted of 245 individuals. After the removal of close relatives, we obtained a set of 208 CKD selected subjects were in dialysis therapy. Individuals on dialysis were grouped into two sub-populations: one with individuals without cardiovascular events (CV0: n=73) and one with individuals who had cardiovascular events (CV1: n=135). For analyses of markers with steep difference in allele frequency between CV0 and CV1 we applied the following filter in our dataset: we removed markers with minor allele frequency (MAF) <0.05, Hardy-Weinberg equilibrium exact test p-value <1E-7, marker in linkage disequilibrium (r2>0.4), genotyping rate<0.99.

Subsequently, the Fst (a measure of genetic differentiation for the same marker between two sub-populations)[207] was estimated for 32178 polymorphic variants, in order to identify the most differentiated SNPs between CV0 and CV1. Only variants that fell over the 99th percentile of the

Fst distribution have been considered. The variants were annotated using VEP (Variant Effect Predictor)[125]. Genetic ontology analyses were performed using the genetic PANTHER database[126]. Pathway analyses, on the other hand, were performed using Ingenuity Pathway Analysis (IPA)[103]. The gene variants present in the cardiovascular pathway enriched by IPA and the heart development process in PANTHER were subsequently analysed with the GTEx database[116]. In order to further confirm our results, we performed the analysis including the related subjects and compared the results.

Protein Analysis Through Evolutionary Relationships (Panther)

The Protein Classification System ANalysis THrough Evolutionary Relationships (PANTHER) is designed to classify proteins (and their genes) to facilitate high-percentage analysis.

Proteins have been classified according to:

- Family and subfamily: families are groups of related evolutionary proteins; subfamilies are related proteins that have the same function.
- Molecular function: the function of the protein itself or with proteins directly interacting at biochemical level.
- Biological process: the protein function in the context of a wider network of proteins that interact to achieve a process at cells or entire body levels.
- Pathway: similar to a biological process, but also specifies the relationships between the interacting molecules.

PANTHER Classifications are the result of human attention as well as sophisticated bioinformatics algorithms[144].

Ingenuity Pathway Analysis (IPA)

IPA is a web-based software for the analysis, integration and interpretation of data derived from homic experiments, such as RNAseq, small RNAseq, microarrays, including miRNA and SNPs, metabolomics, proteomics, and

other experiments that generate lists of genes. Powerful analysis and research tools discover the meaning of data and identify new candidate targets or biomarkers within the context of biological systems.

IPA goes beyond the pathways analysis:

• Identify key regulators and activities to explain expression patterns;

• Furnish downstream effects on biological processes and diseases;

• Provide targeted data on genes, proteins, chemicals and drugs;

• Construct interactive models of experimental systems.

RESULTS

Population

The total population of the study includes 332 subjects (male n=226, 68.1%; female n=106, 31.9%) aged between 19 and 90 years old, at different CKD stages, both in hemodialysis and in conservative therapy.

All personal, anthropometric, biochemical and instrumental data of the recruited patients are shown in Table 4, divided in groups on the basis of the CKD stage (from stage III to V - dialysis). Serum levels of the following biochemical parameters were evaluated: calcium, phosphate, creatinine, 25(OH) D, $1,25(OH)_2D$, parathormone (PTH), alkaline phosphatases (ALP), C Reactive Protein (CRP), FGF23 and sclerostin. Lumbar aorta calcification was evaluated through Kauppila score. Moreover, in Table 5 the same parameters are shown dividing CKD patients in two groups: in hemodialysis (n=245, 73.8%) and in conservative therapy (n=87, 26.2%).

	CKD Patients Stage III (n=50)	***CKD Patients Stage IV (n=24)***	***CKD Patients Stage V (n=13)***	***Hemodialysis Patients (n=245)***
Age (y)	69.4 ± 10.6	71.0 ± 7.2	71.0 ± 7.7	67.3 ± 14.0
Gender (M/F)	37/13	14/10	7/6	168/77
Weight (kg)	76.7 ± 15.2	72.9 ± 14.0	78.7 ± 9.9	66.3 ± 13.3
BMI (kg/m^2)	27.3 ± 4.9	26.7 ± 4.8	27.5 ± 4.6	23.9 ± 4.5
Calcium (mmol/l)	2.33 ± 0.11	2.34 ± 0.12	2.30 ± 0.14	2.21 ± 0.18
Phosphate (mmol/l)	1.06 ± 0.17	1.15 ± 0.22	1.32 ± 0.24	1.48 ± 0.54
25 (OH)D (ng/ml)	17.6 ± 8.9	21.1 ± 9.7	14.6 ± 7.3	13.1 ± 9.8
1-25 (OH)$_2$D (ng/ml)	31.1 ± 13.5	20.2 ± 6.6	16.6 ± 8.6	10.3 ± 10.1
Creatinine (mg/ml)	1.62 ± 0.25	2.50 ± 0.42	4.27 ± 1.00	8.34 ± 2.35
ALP (U/l)	101.2 ± 49.3	102.1 ± 59.1	92.3 ± 53.5	120.8 ± 73.8
CRP (mg/dl)	5.99 ± 8.76	3.54 ± 2.44	10.74±10.61	9.07 ± 16.09
PTH (pg/ml)	38.2 ± 23.7	36.9 ± 20.1	100.7±105.0	86.1 ± 94.3
FGF23 (pg/ml)	51.8 ± 26.3	204.0±349.0	351.5±694.6	2158 ± 3543
Log FGF23	1.64 ± 0.29	2.10 ± 0.36	2.27 ± 0.38	2.81 ± 0.76
Sclerostin (pg/ml)	991 ± 588	1096 ± 518	1223 ± 805	2806 ± 2312
KAU score (0-24)	8.39 ± 6.88	10.35 ± 6.18	13.08 ± 6.88	14.17 ± 6.88

Table 4: Personal, anthropometric, biochemical and instrumental characteristics of patients stratified by CKD stage.

	CKD Patients Stages III-V (n=87)	***Hemodialysis Patients (n=245)***	***p Value***
Age (y)	70.1 ± 9.3	67.3 ± 13.9	-
Gender (M/F)	59/29	168/77	-
Dialysis Vintage (mo)	-	61.8 ± 64.2	-
Weight (kg)	75.9 ± 14.2	66.3 ± 13.3	0.0001
BMI (kg/m^2)	27.1 ± 4.8	23.9 ± 4.5	0.0001
Calcium (mmol/l)	2.33 ± 0.12	2.22 ± 0.18	0.0001
Phosphate (mmol/l)	1.13 ± 0.21	1.48 ± 0.54	0.0001
25 (OH)D (ng/ml)	18.1 ± 9.1	13.1 ± 9.8	0.0001
1-25 (OH)2D (ng/ml)	26.1 ± 12.7	10.3 ± 10.1	0.0001
Creatinine (mg/ml)	2.3 ± 1.0	8.3 ± 2.4	0.0001
ALP (U/l)	100.1 ± 52.2	120.8 ± 73.8	0.027
CRP (mg/dl)	6.01 ± 8.01	9.07 ± 16.09	-
PTH (pg/ml)	46.0 ± 47.1	86.1 ± 94.3	0.0001
FGF23 (pg/ml)	138.6 ± 335.7	2158.0 ± 3543.2	0.0001
Log FGF23	1.86 ± 0.42	2.81 ± 0.76	0.0001
Sclerostin (pg/ml)	1055 ± 605	2806 ± 2312	0.0001
KAU score (0-24)	9.69 ± 6.84	14.17 ± 6.88	0.003

Table 5: Personal, anthropometric and biochemical characteristics of patients in conservative therapy vs patients in hemodialysis treatment.

HD patients and CV risk

Among 245 participants, 161 patients (65.7%) had suffered from cardiovascular events (CV1). Compared to 84 patients (34.3%) without a cardiovascular history (CV0), these patients were older, they spent higher time on dialysis and had higher frequency of hypertension. They also had higher CRP and lumbar aorta calcification score. Finally, they had more cardiovascular events during 5-years follow-up (Table 6).

Seventy-nine patients suffered from cardiovascular events during the 5-years follow-up. The effect of age, dialysis vintage, lumbar aorta calcification score and hypertension was confirmed and increased by the body weight and serum PTH, both lower in the CV1 patients than CV0 (Table 7).

	Hemodialysis Patients (HD)	***Patients with CV events (CV1)***	***Patients without CV events (CV0)***	***p Value***
N (M/F)	245 (168/77)	161(112/49)	84(56/28)	0.64
Age (y)	67 ± 14	70 ± 12	62 ± 15	0.0001
Weight (kg)	66 ± 14	66 ± 14	66 ± 13	0.87
Dialysis vintage (months)	62 ± 64	71 ± 67	45 ± 55	0.002
Protein (g/dl)	6.54 ± 0.67	6.55 ± 0.68	6.53 ± 0.69	0.81
Calcium (mmol/l)	2.22 ± 0.18	2.21 ± 0.18	2.24 ± 0.18	0.15
Phosphate (mmol/l)	1.48 ± 0.54	1.44 ± 0.55	1.55 ± 0.53	0.15
25 (OH)D (ng/ml)	13.1 ± 9.8	12.4 ± 10.5	14.3 ± 8.5	0.32
1-25 (OH)2D (ng/ml)	10.3 ± 10.1	9.5±6.2	11.7±14.5	0.23
Creatinine (mg/dl)	8.34 ± 2.36	8.25 ± 2.25	8.51 ± 2.54	0.41
PTH (pg/ml)	235 ± 197	228 ± 197	248 ± 197	0.44
FGF23 (pg/ml)	2158 ± 3543	1874 ± 3181	2654 ± 4078	0.13
CRP (mg/l)	9.1 ± 16.1	11.1 ± 18.8	5.1 ± 7.8	0.001
KAU score (0-24)	12.7 ± 7.1	14.8 ± 6.2	8.1 ± 6.9	0.0001
CV events in 5yr follow-up (%)	79 (32.2)	73 (45.3)	6 (7.1)	0.0001
CV death in 5yr follow-up (%)	34 (13.9)	34 (21.1)	0	0.0001
All-cause death in a 5yr follow-up (%)	62 (25.3)	49 (30.4)	13 (15.5)	0.011
T2DM (%)	76 (31)	54 (33.5)	22 (26.2)	0.24
Arterial hypertension (%)	235 (95.9)	159 (98.8)	76 (90.5)	0.002

Table 6: Patients' characteristics divided basing on cardiovascular history. P-value represents the statistical differences between CV0 and CV1.

	Patients CV1 5 yrs FU	***Patients CV0 5 yrs FU***	***p Value***
N (M/F)	79 (56/23)	166 (112/54)	0.59
Age (y)	70 ± 12	62 ± 15	0.031
Weight (kg)	63 ± 14	67 ± 13	0.038
Dialysis vintage (m)	70 ± 68	58 ± 62	0.002
Protein (g/dl)	6.59 ± 0.59	6.52 ± 0.71	0.81
Calcium (mmol/l)	2.20 ± 0.16	2.22 ± 0.19	0.39
Phosphate (mmol/l)	1.47 ± 0.48	1.48 ± 0.57	0.84
25 (OH)D (ng/ml)	13.4 ± 13.8	13.0 ± 7.4	0.85
1-25 (OH)2D (ng/ml)	9.2 ± 7.1	10.8 ± 11.2	0.31
Creatinine (mg/dl)	8.31 ± 2.31	8.36 ± 2.39	0.88
PTH (pg/ml)	197 ± 155	253 ± 211	0.039
FGF23 (pg/ml)	2092 ± 3482	2193 ± 3588	0.85
CRP (mg/l)	11.7 ± 18.7	7.8 ± 14.6	0.11
KAU score (0-24)	15.1 ± 6.8	11.4 ± 7.0	0.0001
T2DM (%)	23 (29.1%)	23 (29.1%)	0.66
A. hypertension (%)	78 (98.7%)	157 (94.6%)	0.038

Table 7: Clinical characteristics of patients who suffered from cardiovascular events during the 5-years follow-up.

Nutritional Assessment

For the nutritional study 148 patients were evaluated (99 males, 66.9%; 49 females, 33.1%), aged between 36 and 90 years, at different CKD stages; 98 (66 males; 32 females) patients under hemodialysis and 50 (33 males; 17 females) in conservative therapy (pre-dialysis). Out of the initial population of 332 subjects, only 148 patients gave the consent for the nutritional study. The nutritional survey on the dietary habits of recruited subjects was carried out using the 24-hours recall method prior to the visit. The correlation between foods and the CKD stage (or dialysis), mortality, body weight, gender, age, height, body max index (BMI), calcemia, creatinine, parathormone and phosphatemia was investigated (Figure 7). A positive correlation was found between CKD progression, dialysis and the number of years of dialysis treatment, blood creatinine values, phosphate, PTH and vitamin E intake. Whereas, a negative correlation with calcemia, beta-carotene, water intake and body weight was found.

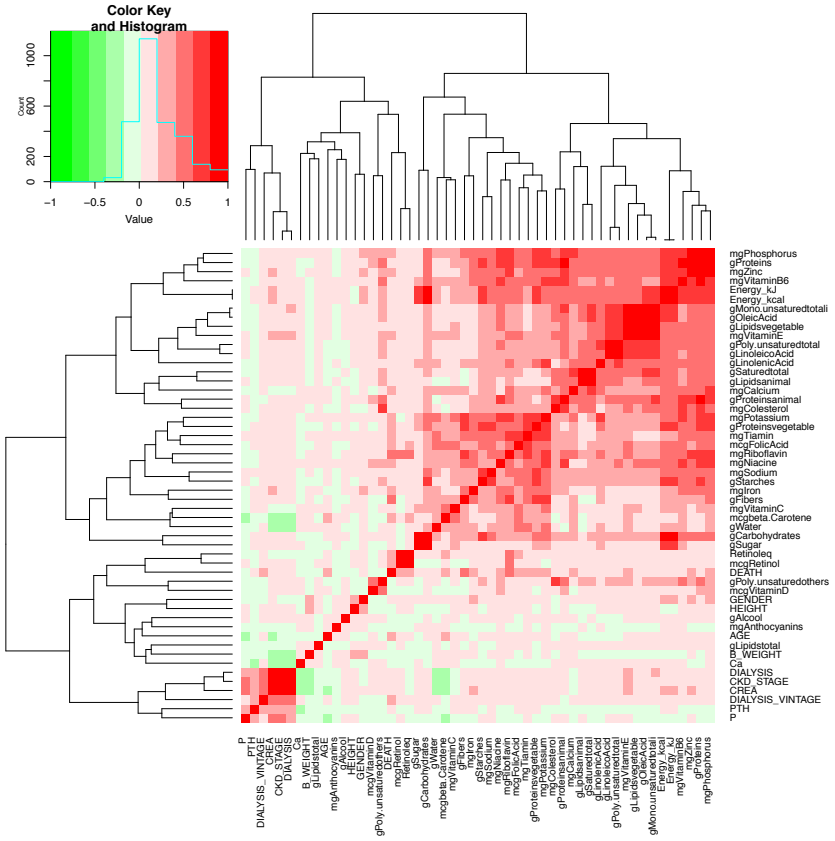


Figure 7: Heat map showing a Pearson's correlation of all the phenotypes measured in the population (HD and pre-dialysis patients).

In particular, vitamin E intake was investigated in the various CKD stages, including dialysis, and it was observed that the assumption was significantly higher in dialysis-treated patients than in patients in conservative therapy. In contrast, beta-carotene intake is lower in dialysis patients (Figure 8). Calcium intake is also reduced in hemodialysis patients compared to patients in conservative therapy (Figure 9). The regression analysis between serum calcium and proteins highlighted a reduction in serum calcium with the increase in the plant proteins intake (Figure 10).

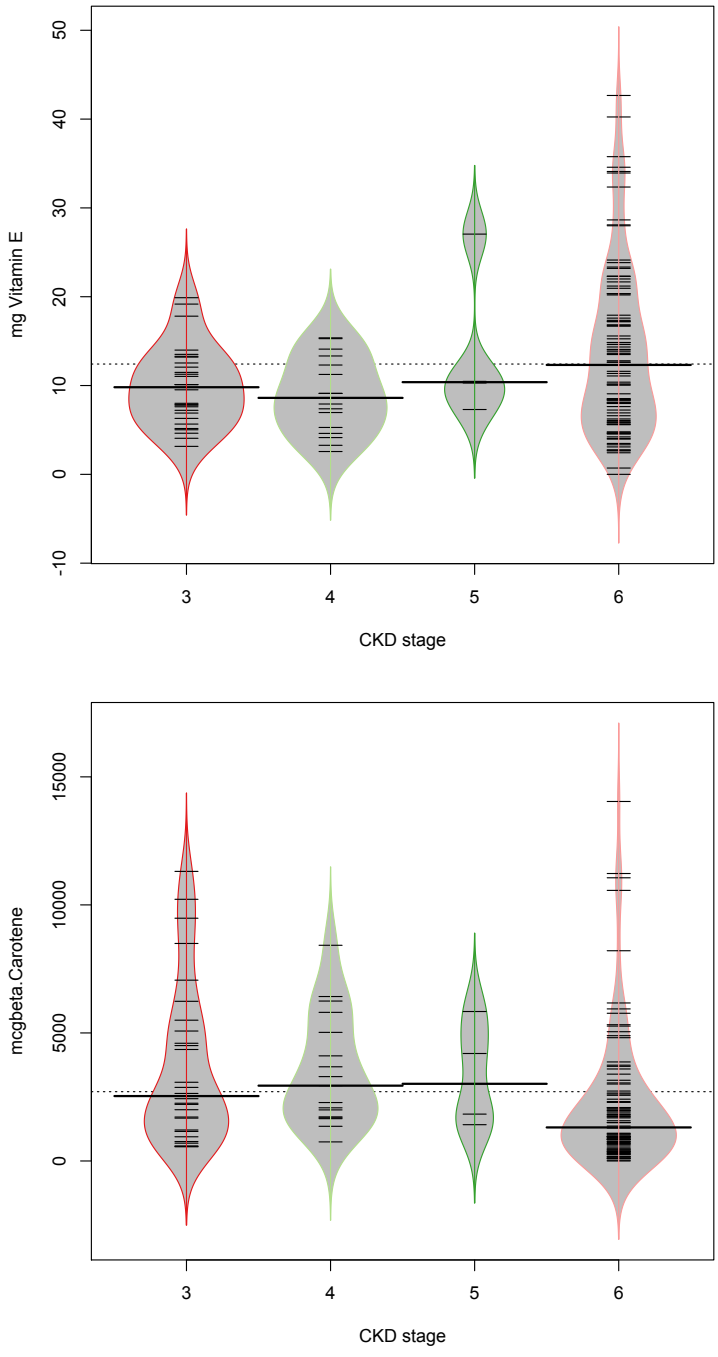


Figure 8: Association between CKD stages and (above) Vitamin E (mg) – (below) Beta-carotene (mcg) intake. Hemodialysis is considered as stage 6.

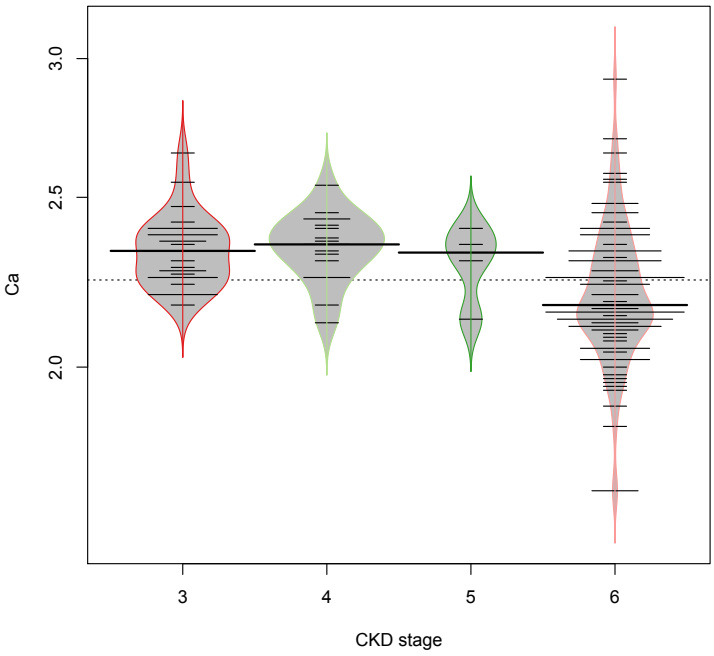


Figure 9: Association between CKD stages and calcium intake (mg). Hemodialysis is considered as stage 6.

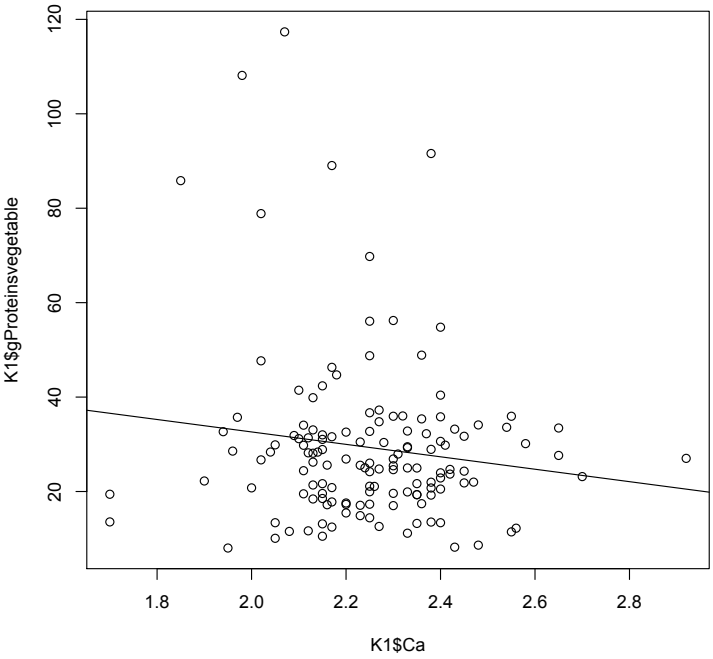


Figure 10: Regression analysis between plant proteins (g) and serum calcium (mmol/l).

One-hundred-fourteen individuals were selected for 68 variables that had less than 20% missing data. Figure 11 shows their correlation.

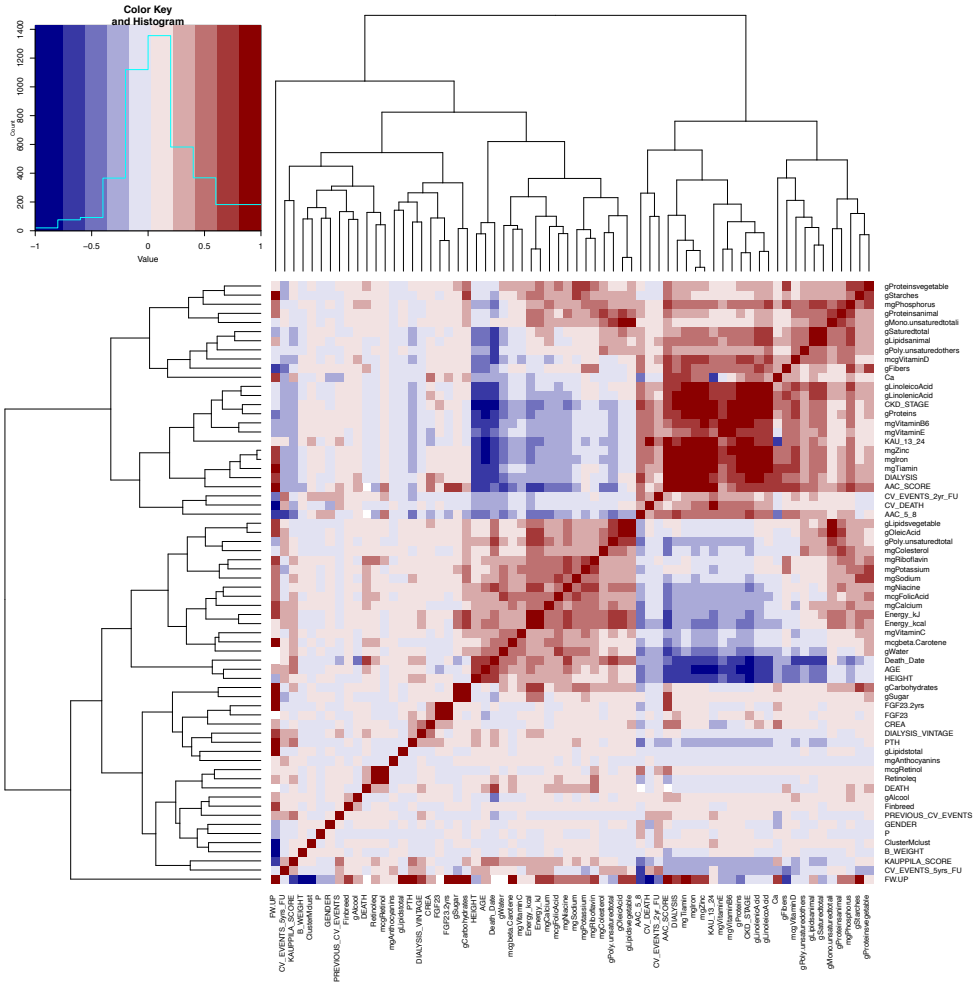


Figure 11: Pearson's correlation heat map of all phenotypes in the selected population (HD and pre-dialysis patients).

In Table 8 all significant results of Pearson's correlation are reported ($p \leq 0.05$).

Fixed Variable	*Association Variable*	*p Value*	*Pearson's r*
CV events 5yr Follow-up	Retinol (mcg)	1.69E-02	0.222
	Retinol (eq)	2.20E-02	0.213
	Previous CV events	3.09E-02	0.201
Previous CV events	Dialysis vintage	5.00E-03	0.256
	Age	6.00E-03	0.249
	CV events 2yr FU	2.20E-02	0.213
CKD Stage	Dialysis	0.00+E00	0.948
	Creatinine	0.00+E00	0.828
	Dialysis vintage	0.00+E00	0.542
	Phosphate	1.92E-06	0.422
	PTH	1.34E-05	0.392
	Ca	4.09E-04	-0.319
	FGF23	5.71E-04	0.316
	Vitamin E (mg)	6.30E-03	0.248
	Body Weight	9.16E-03	-0.237
	Vegetable Lipids (g)	1.62E-02	0.219
	Energy (kcal)	1.78E-02	0.216
	Total Mono-unsaturated Lipids	1.99E-02	0.212
	Oleic Acid (g)	2.08E-02	0.211
	Beta-carotene (mcg)	2.76E-02	-0.201
	Zinc (mg)	3.77E-02	0.190
	Proteins (g)	4.32E-02	0.185
FGF23	Creatinine	8.61E-06	0.402
	Age	1.00E-04	-0.355
	Phosphate	2.13E-04	0.344
	Dialysis	3.23E-04	0.330
	CKD Stage	5.71E-04	0.316
	PTH	8.72E-03	0.245
PTH	Dialysis	6.78E-06	0.404
	CKD Stage	1.34E-05	0.392
	Creatinine	3.06E-04	0.329
	Phosphate	1.18E-03	0.299
	FGF23	8.72E-03	0.245
	Ca	2.15E-02	-0.213
	Dialysis vintage	3.20E-02	0.199
	Age	4.12E-02	-0.190
Phosphate	Creatinine	1.78E-08	0.490
	CKD Stage	1.92E-06	0.422
	Age	6.43E-06	-0.402
	Dialysis	1.09E-05	0.393
	FGF23	2.13E-04	0.340
	PTH	1.18E-03	0.299
	Beta-carotene (mcg)	2.34E-03	-0.278
	Calcium (mg)	2.70E-02	-0.204
	Ca	4.57E-02	-0.184

Creatinine	CKD Stage	0.00+E00	0.828
	Dialysis	0.00+E00	0.834
	Dialysis vintage	7.21E-13	0.596
	Phosphate	1.78E-08	0.490
	FGF23	8.61E-06	0.402
	PTH	3.06E-04	0.329
	Age	1.94E-03	-0.280
	Beta-carotene (mcg)	7.54E-03	-0.243
	Iron (mg)	8.87E-03	0.238
	Energy (kcal)	1.07E-02	0.232
	Body Weight	1.33E-02	-0.226
	Vitamin E (mg)	1.64E-02	0.219
	Ca	2.32E-02	-0.208
	Total Mono-unsaturated Lipids	3.05E-02	0.198
	Zinc (mg)	3.17E-02	0.196
	Water (g)	3.17E-02	-0.196
	Vegetable Lipids (g)	3.21E-02	0.196
	Oleic Acid (g)	3.47E-02	0.193
	Carbohydrates (g)	3.55E-02	0.192
	Vegetable Proteins (g)	4.72E-02	0.182
Dialysis	CKD Stage	0.00+E00	0.948
	Creatinine	0.00+E00	0.834
	Dialysis vintage	0.00+E00	0.572
	PTH	6.78E-06	0.404
	Phosphorous	1.09E-05	0.393
	Ca	2.32E-04	-0.331
	FGF23	3.23E-04	0.330
	Vitamin E (mg)	5.91E-03	0.250
	Total Mono-unsaturated Lipids	6.69E-03	0.246
	Oleic Acid (g)	6.93E-03	0.245
	Vegetable Lipids (g)	7.70E-03	0.242
	Body Weight	9.32E-03	-0.236
	Energy (kcal)	1.01E-02	0.234
	Water (g)	1.86E-02	-0.215
	Beta-carotene (mcg)	2.27E-02	-0.208
	Proteins (g)	2.62E-02	0.203
	Zinc (mg)	2.78E-02	0.201
	Alcohol (g)	3.62E-02	0.191
	Cholesterol (mg)	4.94E-02	0.180
Age	Creatinine	1.94E-03	-0.280
	Phosphorous	6.43E-06	-0.402
	PTH	4.12E-02	-0.190
	Death	2.64E-03	0.272
	Beta-carotene (mcg)	1.60E-03	0.285
	Previous CV events	6.19E-03	0.249
	FGF23	1.00E-04	-0.355

Table 8: Statistically significant Pearson's correlation ($p \leq 0.05$) for all the population (HD and pre-dialysis patients).

FGF23, phosphate intake and cardiovascular risk

We analysed the relation between phosphate (P) dietary intake and FGF23 levels in HD and pre-dialysis patients. In both groups, the multiple linear regression showed a positive association between P intake and protein (r^2=0.824; p=0.0001) and calcium (r^2=0.132; p=0.0001) consumption.

After patients stratification by P intake, we obtained three groups for both HD and pre-dialysis patients: P intake ≤ 700 mg/day, P intake 700-1200 mg/day, P intake > 1200mg/day (Table 9). In Figure 12, FGF23 level variations are presented, expressed as logarithm value, in relation to different P dietary intake. HD patients with lower P intake (≤ 700 mg/day) showed lower FGF23 serum values (2.09 ± 0.9 pg/ml vs 2.84 ± 0.62 pg/ml; p=0.0008). In pre-dialysis patients, FGF23 values are not stratified in relation to P dietary intake. Moreover, in pre-dialysis patients phosphatemia was associated with 1-25 $(OH)_2D$ (r^2=0.210; p=0.001) and P intake (r^2=0.160; p=0.002). Whereas, phosphatemia in HD patients was associated with FGF23 (r^2=0.184; p=0.0001), age (r^2=0.084; p=0.005) and P intake (r^2=0.048; p=0.03).

We then investigated the link between P intake and CV mortality in all CKD patients during the 5 years follow-up. Cox regression model showed that CV risk was increased in CKD patients with P intake > 1200 mg/day (OR=3.8; p=0.008), diabetes mellitus (OR=2.8; p=0.027), previous CV events (OR=1.04; p=0.006) (Figure 13).

Phosphate intake (mg/day)	Pre-dialysis patients (n=50)	HD patients (n=98)
≤ 700	3 (6%)	14 (14.4%)
700-1200	29 (58%)	41 (42.3%)
> 1200	18 (36%)	42 (43.3%)

Table 9: Patients stratification on the basis of dietary phosphate intake.

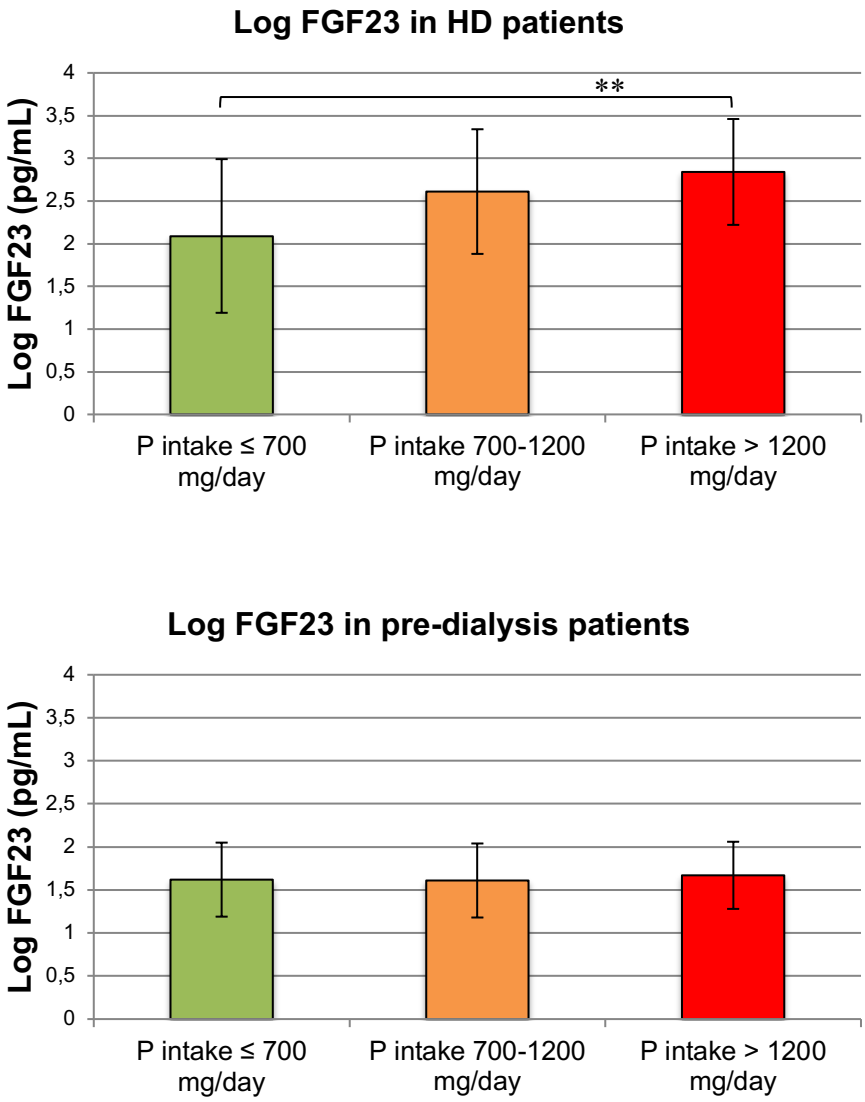


Figure 12: FGF23 level variations, expressed as log_{10}, in relation to different P dietary intake (≤ 700 mg/day, 700-1200 mg/day or > 1200 mg/day) in HD and pre-dialysis patients.

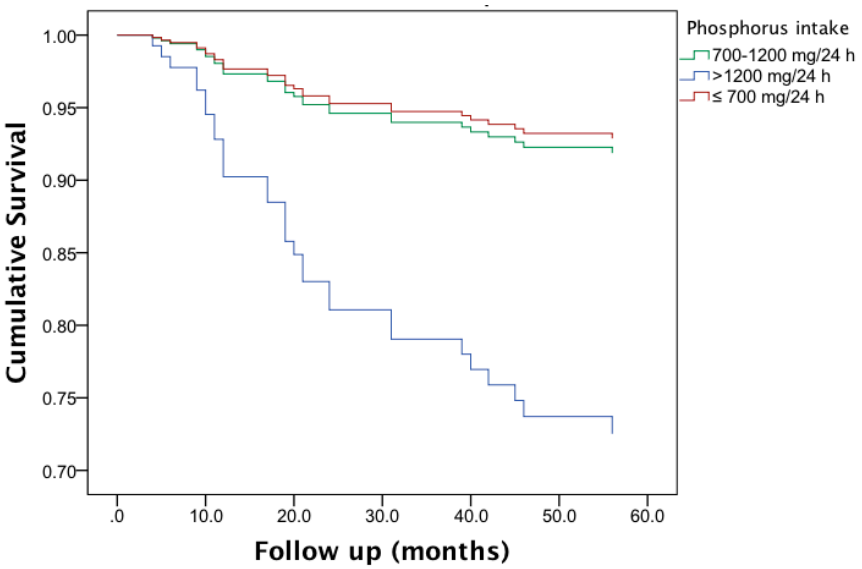


Figure 13: Survival analysis (Cox regression), link between P intake and CV mortality in all CKD patients during the 5 years follow-up.

FGF23 in HD patients

Serum FGF23 at Baseline

We analysed 97 consecutive HD patients (age range 36-90 yrs, M/F: 65/32) to study the role of FGF23 in end-stage renal disease and to understand the evolution of this parameter after 2 years of follow-up.
Serum FGF23 ranged from 20 to 23888 pg/ml in our HD patients cohort. Ten patients (9.7%) had normal serum concentrations of FGF23 (<50 pg/ml) at baseline control; these 10 patients had higher age (75 ± 6.3 vs 68 ± 12.6 yrs, p=0.005), lower serum concentrations of phosphate (1.2 ± 0.36 vs 1.51 ± 0.46 mmol/l, p=0.039) and lower phosphorous dietary intake (842 ± 449 vs 736 ± 549 mg/24 hours, p=0.033) compared with the other 87 patients. Serum FGF23 was not significantly different in diabetic patients (n=29, 1471 ± 1859 vs 2202 ± 3885 pg/ml), patients taking calcitriol (n=50, 1908 ± 3852 vs 2065 ± 2916 pg/ml) or cinacalcet (n=13, 2368 ± 2847 vs 1924 ± 3504 pg/ml) and patients with polycystic kidney disease (n=11, 3305 ± 6978 vs 1815 ± 2680 pg/ml) compared with patients without these characteristics.
Patients were stratified according to tertiles of baseline serum FGF23 (Table 10); patients in the highest tertile were younger than patients in the lowest tertile and had higher baseline serum phosphate than patients in the other tertiles. At the second control, two years after the baseline, patients in the highest tertile at baseline also showed higher serum calcium and creatinine than patients in the lowest tertile and higher FGF23 than patients in the other two tertiles. Linear regression analysis (Table 12, upper part) using baseline variables showed that serum FGF23, expressed as $\log_{10}$, was positively associated with serum phosphate, phosphate intake and serum calcium (Figure 14, panel A and B).

	Tertiles of baseline serum FGF23			
	The lowest tertile	**The middle tertile**	**The highest tertile**	**All patients**
FGF23 (pg/ml)	20-240	274-1340	1406-23388	20-23388
N (M/F)	32 (18/14)	33 (21/12)	32 (26/6)	97 (65/32)
Age (yrs)	73 ± 7	69 ± 14.2	64 ± 13.6§	68 ± 12.3
Dialysis vintage (mo)	65 ± 49.1	60 ± 52.6	57 ± 69.7	61 ± 57.3
Weight (kg)	67 ± 15.9	69 ± 13.8	69 ± 14.2	68 ± 14.5
Baseline control				
Calcium (mmol/l)	2.17±0.18	2.26±0.22	2.20±0.16	2.21±0.19
Phosphate (mmol/l)	1.29±0.4	1.38±0.39	1.78±0.45*	1.48±0.46
Creatinine (mg/dl)	8±2.26	8.4±2.51	9.1±2.63	8.5±2.48
25(OH)D (ng/ml)	11±3.7	12±5.6	15±7	13±5.9
$1,25(OH)_2D$ (pg/ml)	8±4.9	9±5.9	12±12.5	10±8.4
PTH (pg/ml)	72±53.5	106±87.7	94±58.6	92±70.1
FGF23 (pg/ml)	122±82.6	677±351.4	5194±4463°	1984±3414
	Second control, 2 years after the baseline			
Calcium (mmol/l)	2.14±0.14	2.19±0.18	2.25±0.17†	2.19±0.17
Phosphate (mmol/l)	1.23±0.43	1.53±0.53	1.57±0.47	1.45±0.49
Creatinine (mg/dl)	8±1.69	9.4±2.24	9.8±2.48‡	9.02±2.27
25(OH)D (ng/ml)	17±8.0	16±7.4	17±11.0	17±8.8^
$1,25(OH)_2D$ (pg/ml)	11±7.3	10±5.9	12±18.5	11±12.6
PTH (pg/ml)	86±619.6	126±72	180±165.8.6	139±120.7¶
FGF23 (pg/ml)	378±82.6	687±695.7	4497±4077°	1845±3027

§ p=0.035 vs the lowest tertile
* p<0.001 vs the lowest and the middle tertile
° p=0.0001 vs the lowest and the middle tertile
† p=0.043 vs the lowest tertile
‡ p=0.006 vs the lowest tertile
^ p=0.002 vs values at baseline control in all patients
¶ p=0.03 vs values at baseline control in all patients

Table 10: Characteristics of HD patients in tertiles of serum FGF23 measured at baseline control. Variables were measured at baseline and at a second control, two years after the baseline.

Serum FGF23 two years after the baseline control

Serum values of PTH and 25(OH)D significantly increased two years after the baseline control in our cohort (Table 9) and the number of patients taking cholecalciferol supplements was higher compared to baseline (n=34 at the second control vs n=7 at baseline; chi-square=28, DF=1, p=0.0001).
Considering patients in serum FGF23 tertiles and variables at the second control (Table 11), patients in the highest tertile showed higher serum calcium than patients in the other two tertiles and higher serum creatinine than patients in the lowest tertile. Subjects in the lowest tertile had shorter dialysis vintage and lower phosphatemia than patients in the other tertiles. Linear regression analysis (Table 12, lower part), considering variables measured at the second control, showed that serum FGF23 (expressed as $\log_{10}$), was positively associated with phosphatemia and calcemia (Figure 14, panel C).

	Tertiles of serum FGF23 at the second control		
	The lowest tertile	**The middle tertile**	**The highest tertile**
FGF23 (pg/ml)	15-232	244-1212	1231-15032
N (M/F)	32 (19/13)	33 (19/14)	32 (27/5)
Age (yrs)	71±7.9	69±13.9	65±13.7
Dialysis vintage (months)	84±54.8*	49±51.1	50±60.2
Weight (kg)	71±11.5	64±11.2	72±15.6§
Calcium (mmol/l)	2.14±0.14	2.19±0.18	2.25±0.17†
Phosphate (mmol/l)	1.23±0.43	1.53±0.53	1.57±0.47
Creatinine (mg/dl)	8±1.69	9.4±2.24	9.8±2.48‡
25(OH)D (ng/ml)	17±8.0	16±7.4	17±11.0
$1,25(OH)_2D$ (pg/ml)	11±7.3	10±5.9	12±18.5
PTH (pg/ml)	86±619.6	126±72	180±165.8.6
FGF23 (pg/ml)	378±82.6	687±695.7	4497±4077°

* p=0.039 vs the highest tertile and p=0.031vs the middle tertile
§ p=0.026 vs the middle tertile
† p=0.027 vs the lowest tertile and p=0.012 vs the middle tertile
‡ p=0.0001 vs the highest and p=0.001 vs the middle tertile
° p=0.035 vs the lowest tertile
** p=0.0001vs the lowest and the middle tertile

Table 11: Characteristics of HD patients divided in tertiles of serum FGF23 measured two years after the baseline control.

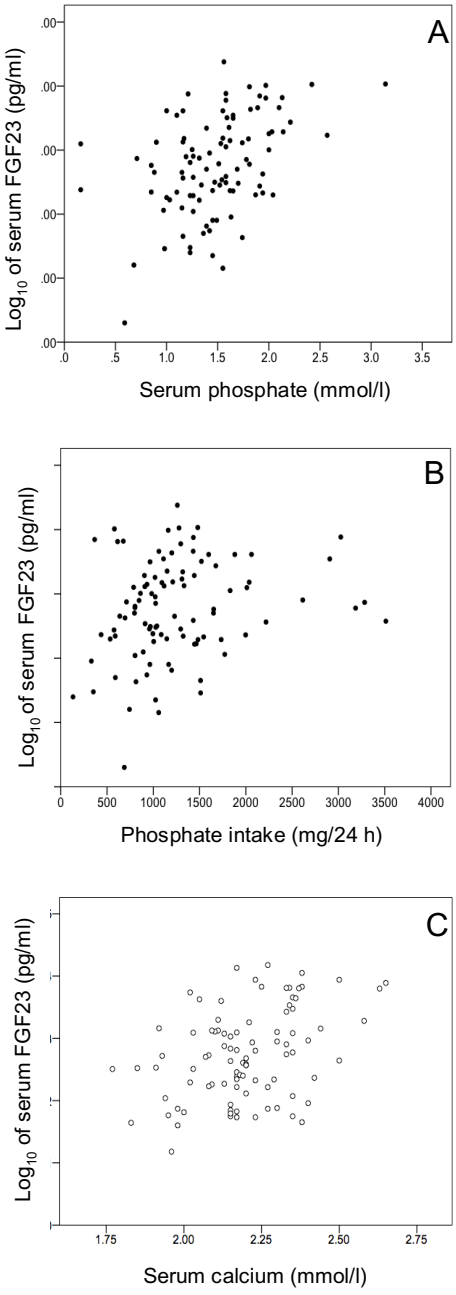


Figure 14: Multiple regression analysis in 97 HD patients showed a positive relation between serum FGF23 (expressed as logarithm) and (A) serum phosphate and (B) phosphate intake. A positive relation with (C) serum calcium was found at the second control performed two years after the baseline in the same population.

Dependent variables	*Independent variables*	*Beta*	*Cumulative r*	r^2	*p*
Log_{10} serum FGF23 at **baseline control**	Serum Phosphate	0.475	0.424	0.18	0.0001
	Phosphate intake	0.288	0.511	0.082	0.002
	Serum Calcium	0.176	0.541	0.031	0.047
Log_{10} serum FGF23 at **second control**	Serum Phosphate	0.524	0.429	0.184	0.0001
	Serum Calcium	0.493	0.647	0.234	0.0001

Table 12: Variables associated with serum concentrations of FGF23, expressed as log_{10}, in 97 HD patients at baseline (upper part) and at the second control two years after the baseline (lower part).

Patients increasing serum FGF23 at the second control

Ten patients (10.3%) shifted their serum FGF23 value from the lowest or the middle tertile at baseline to the highest tertile at the second control (group 1); 22 patients (22.7%) were in the highest tertile of serum FGF23 at both controls (group 2); the other 65 patients (67%) were in the lowest or the middle tertile of serum FGF23 at the second control irrespectively of the tertile they were at baseline (group 3) (Table 13). Serum FGF23 was significantly different in these three patients groups, the highest values in patients of the group 2 and the lowest in those of the group 3; serum FGF23 significantly increased in patients of the group 1 (1620 ± 3060 vs 2788 ± 3062 pg/ml, p=0.001; Student's t test for paired data) and decreased in patients of the group 3 (766 ± 1408 vs 338 ± 302 pg/ml, p=0.015) from the first to the second control. Serum calcium was significantly higher in patients of the groups 1 and 2 than those in the group 3; age and serum phosphate were lower and serum PTH was higher in patients of the group 2 than patients in the group 3 (Table 13).

	Tertiles of serum FGF23 at the baseline and at the second control		
Tertile at the baseline **Tertile at the second control**	**Group 1** **The lowest or the middle** **The highest**	**Group 2** **The highest** **The highest**	**Group 3** **Any** **The lowest or the middle**
N (M/F)	10 (8/2)	22 (19/3)	65 (38/27)
Age (yrs)	72 ± 11.3	62 ± 13.7*	70 ± 11.3
Dialysis vintage (months)	41 ± 44.6	54 ± 66.7	66 ± 55.5
Weight (kg)	76 ± 15.1	70 ± 15.3	66 ± 13.8
Calcium (mmol/l)	2.32 ± 0.17	2.26 ± 0.18	2.16 ± 0.15‡
Phosphate (mmol/l)	1.43 ± 0.23	1.72 ± 0.43°	1.38 ± 0.51
Creatinine (mg/dl)	8.75 ± 1.49	10 ± 2.86	8.76 ± 2.09
25(OH)D (ng/ml)	14 ± 4.6	14 ± 5.7	18 ± 9.9
$1,25(OH)_2D$ (pg/ml)	12 ± 9.2	13 ± 15.9	10 ± 6.7
PTH (pg/ml)	99 ± 58.5	232 ± 316.8¶	120 ± 75.2
FGF23 (pg/ml)	2788 ± 3062†	5914 ± 3623§	338 ± 302
Calcium intake (mg/24h)	722 ± 938	814 ± 502	662 ± 492
Phosphate intake (mg/24h)	1161 ± 817	1462 ± 646	1187 ± 595

* p=0.02 vs patients in group 3
‡ p=0.045 vs patients in group 2 and p=0.031 vs group 1
° p=0.023 vs patients in group 3
¶ p=0.019 vs patients in group 3
† p=0.001 vs patients in groups 3 and p=0.0001 vs patients in group 2
§ p=0.0001 vs patients in groups 1 and 3

Table 13: Characteristics of patients in tertiles of serum FGF23 at the second control, two years after the baseline. Group 1 included patients in the lowest or middle tertile at baseline and in the highest tertile at the second control. Group 2 included patients in the highest tertile at both controls. Group 3 consisted of patients in lowest or the middle.

Sclerostin in pre-dialysis and HD patients

To investigate the evolution of sclerostin values in different CKD stages, we analysed 86 pre-dialysis patients and 209 HD patients.

Serum sclerostin increased with the worsening of kidney function (p=0.0001, one-way ANOVA, Table 14). The percentage of patients with serum sclerostin above the upper limit of the normal range (>853 pg/ml) was 16%, 34.6%, 80% and 94.2% in patients with CKD stage from III to V and HD, respectively (chi-square=166, df=3, p=0.0001). Similarly, serum phosphate, FGF23 and PTH were significantly higher, whereas body weight, serum calcium, 25(OH)D and $1,25(OH)_2D$ were significantly lower in dialysis patients than in stage III-V CKD patients (p=0.0001 for all variables, one-way ANOVA, Table 14). Kauppila aorta calcification score increased from patients with stage III CKD to those in HD treatment (p=0.011, Table 13). Lumbar-spine z-score was lower in HD patients than stage III and V CKD patients (Tukey test, Table 14).

	CKD stage 3	CKD stage 4	CKD stage 5	CKD stage 5d
N (M/F)	50 (37/13)	26 (14/12)	10 (7/3)	209 (139/70)
Age (yrs)	69±10.6	71±7.2	70±7.8	67±14.1
Weight (kg)	77±15.2	73±13.7	78±10.4	66±13.6[a]
Creatinine (mg/dl)	1.62±0.25	2.56±0.46	4.57±0.95	8.33±2.36
eGFR (ml/min)	41±8.7	22±3.9	12±2.5	-
Calcium (mmol/l)	2.33±0.11	2.36±0.12	2.27±0.10	2.21±0.17[b]
Phosphate (mmol/l)	1.06±0.17	1.17±0.22	1.35±0.25	1.48±0.55[c]
ALP (mU/l)	101±49	104±61	87±45	119±73
CRP (U/l)	6±8.8	3.6±2.4	8.9±9.3	9.3±16.7
PTH (pg/ml)	38±23.7	38±19.8	111±114.5[d]	87±94.5[e]
FGF23 (pg/ml)	52±26	199±335	401±793	2088±3503[f]
log_{10} FGF23 (pg/ml)	1.64±0.29	2.1±0.35 [g]	2.29±0.42[h]	2.79±0.76[i]
25(OH)D (ng/ml)	18±8.9	20±10.0	16±7.0	13±10.0[l]
$1,25(OH)_2D$ (pg/ml)	31±13.5 [m]	20±6.6	15±8.6	10±10.1[n]
Sclerostin (pg/ml)	606±261	753±262	1129±451	2381±1243[o]
KAU score (0-24)	8.4±6.2[p]	10.5±6.2	13.3±7.5	13.7±6.9
Lumbar spine BMD z-score	0.636±1.80	0.282±1.79	0.98±2.019	0.358±1.731[q]

[a] p=0. 0.0001 vs patients with CKD stage 3, p=0.013 vs patients with CKD stage 4 and p=0.007 vs patients with CKD stage 5
[b] p=0.0001 vs patients with CKD stage 3 and 4
[c] p=0.0001 vs CKD patients in stage 3 and p=0.002 vs CKD patients in stage 4
[d] p=0.015 vs CKD patients in stage 3 and p=0.022 vs CKD patients in stage 4
[e] p=0.0001 vs patients with CKD stage 3 and p=0.005 vs CKD patients in stage 4
[f] p=0.0001 vs patients with CKD stage 3 and p=0.002 vs patients with CKD stage 4
[g] p=0.004 vs patients with CKD stage 3
[h] p=0.005 vs patients with CKD stage 3, p=0.02 vs patients with CKD stage 6
[i] p=0.0001 vs patients with CKD stage 3 and 4, p=0.02 vs patients with CKD stage 5
[l] p=0.011 vs patients with CKD stage 3, p=0.001 vs patients with CKD stage 4
[m] p=0.0001 vs patients with CKD stage 4, 5 and 5d
[n] p=0.0001 vs patients with CKD stage 3 and 4
[o] p=0.0001 vs patients with CKD stage 3, 4 and 5
[p] p=0.042 vs patients with CKD stage 5 and p=0.002 vs patients with CKD stage 5d
[q] p=0.019 vs patients with CKD stage 3 and p=0.043 vs patients with CKD stage 5

Table 14: Characteristics of participants according to their CKD stage; analysis was performed using one-way ANOVA and LSD test for multiple comparison between groups.

Serum sclerostin was not different in diabetic or non-diabetic patients either with stage 3-5 CKD (748±312 [n=35] vs. 686±341 [n=51] pg/ml; p=0.39) or in HD (2626±1230 [n=65] vs. 2356±1248 [n=165] pg/ml; p=0.16), whereas it was lower in HD patients taking cinacalcet than patients not-taking cinacalcet (1850±847 [n=23] vs 2446±1270 [n=186] pg/ml; p=0.005). Patients with stage 3-5 CKD treated with calcitriol had lower eGFR and higher serum values of sclerostin and FGF23 (expressed as $\log_{10}$) than patients not-taking calcitriol (Table 15). Hemodialysis patients taking calcitriol showed higher serum $1,25(OH)_2D$ compared with patients not-treated with calcitriol.

	Pre-dialysis CKD		HD Patients	
	Calcitriol	No Calcitriol	Calcitriol	No Calcitriol
N (M/F)	26 (16/10)	60 (42/18)	108 (67/41)	101 (72/29)
Age (yrs)	70±7.6	70±10	67±13.4	67±14.9
Weight (kg)	77±14.6	75±14.2	68±14	64±12.9
Creatinine (mg/dl)	2.61±1.06	2.09±1.01[a]	8.27±2.30	8.39±2.42
eGFR (ml/min)	26±11	34±13.8[b]	-	-
Calcium (mmol/l)	2.31±0.11	2.32±0.14	2.21±0.16	2.22±0.18
Phosphate (mmol/l)	1.13±0.19	1.13±0.23	1.42±0.58	1.55±0.52
ALP (mU/l)	93±42	104±57	125±73	113±73
PTH (pg/ml)	48±33	45±52.2	76±60.3	97±117.2
FGF23 (pg/ml)	179±335	119±340	1806±3362	2365±3631
$\log_{10}$ FGF23 (pg/ml)	2.02±0.4	1.78±0.4[c]	2.72±0.77	2.86±0.76
25(OH)D (ng/ml)	17±6.9	19±9.9	13±13	13±6.2
$1,25(OH)_2D$ (pg/ml)	24±9.4	27±13.9	12±12.9	8±6.1[d]
Sclerostin (pg/ml)	841±349	655±306[e]	2366±1159	2396±1333
KAU score (0-24)	11.3±6.7	8.9±6.9	14.5±8	13.2±6.5
Lumbar spine BMD z-score	0.778±1.798	0.5±1.834	0.867±1.474	0.037±1.84

[a] p=0.039
[b] p=0.007
[c] p=0.014
[d] p=0.007
[e] p=0.015

Table 15: Characteristics of patients with stage III-V CKD and stage Vd CKD taking or not-taking calcitriol. Thirty-one hemodialysis patients underwent DXA and lateral lumbar Radiography: n=12 were not taking calcitriol and n=19 were treated with calcitriol.

Considering stage III-V CKD patients, serum sclerostin was negatively correlated with eGFR and serum $1.25(OH)_2D$ (r=-0.440, p=0.0001) and positively with age (r=0.221, p=0.04), serum phosphate (r=0.268, p=0.014), lumbar-spine BMD z-score and serum FGF23 (r=0.430, p=0.0001) as absolute value and log_{10} (Figure 15). In HD patients, serum sclerostin was positively correlated with age (r=0.259, p=0.0001) and serum creatinine, and negatively with serum PTH and lumbar-spine BMD z-score (n=32).

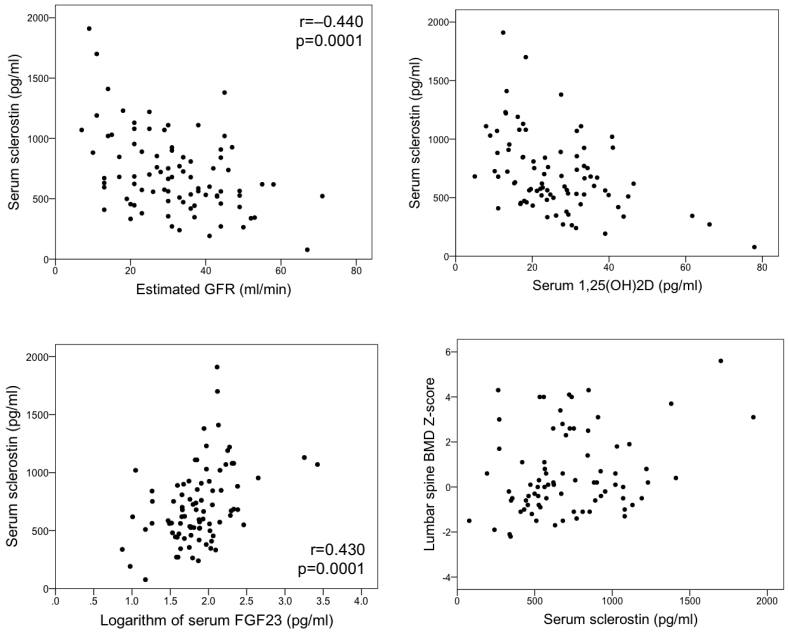


Figure 15: Linear correlations of serum sclerostin with age, eGFR, serum phosphate, serum $1,25(OH)_2D$, log_{10} of serum FGF23 and BMD z-score in 86 CKD patients.

Multivariate association analysis

Multiple stepwise regression analysis in stage III-V CKD patients (Table 16) showed that serum sclerostin was negatively associated with GFR and positively with body weight in a model including age, body weight, GFR, serum concentrations of calcium, phosphate, $1,25(OH)_2D$, PTH and FGF23 as independent variables. Excluding GFR from this model, serum sclerostin resulted negatively associated with serum $1,25(OH)_2D$ and positively with FGF23 in stage III-V CKD patients, and negatively associated with serum $1,25(OH)_2D$ in stage III-V CKD patients not-taking calcitriol.

In HD patients, the same multiple stepwise regression model used in stage III-V CKD patients, but including dialysis vintage and serum creatinine in the place of GFR (Table 16), showed that serum sclerostin was positively associated with age and serum creatinine and negatively associated with serum PTH either in all patients or in patients not taking calcitriol.

Variables predictive of lumbar spine BMD (as z-score) were also evaluated (Table 16).

	Beta	*Cum. R*	*R^2 change*	*F change*	*p*
Patients with stage 3-5 CKD (n=86)					
Sclerostin	0.268	0.268	0.07	5.5	0.022
Dialysis patients (n=32)					
Sclerostin	-0.598	0.495	0.245	8.8	0.006
Calcium	0.355	0.601	0.116	4.7	0.039

Table 16: Variables associated with z-score BMD studied using linear regression model in stage III-V CKD patients and dialysis patients. The model included age, body weight, eGFR or serum creatinine in dialysis patients, serum concentrations of calcium, phosphate, $1,25(OH)_2D$, PTH and FGF23 (as log_{10}) as independent variables.

BMD z-score was positively associated with serum sclerostin in stage III-V CKD patients, whereas BMD z-score was negatively associated with serum sclerostin and positively with serum calcium in HD patients (Figure 16).

The Kauppila score showed no associations with other variables in hemodialysis patients; age was the only associated variable in stage III-V CKD patients.

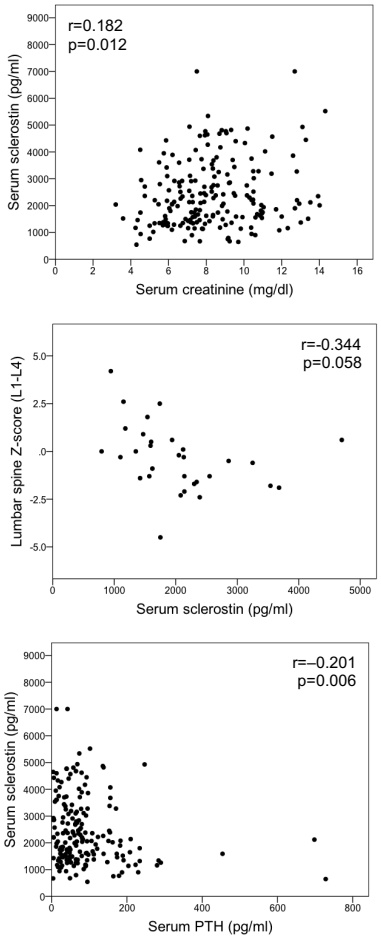


Figure 16: Linear correlations of serum sclerostin with age, serum creatinine and PTH and BMD z-score in 205 hemodialysis patients.

Genetic Analysis

Genetic Assessment

We used Illumina Mega-Ex Array (~2M SNPs), to investigate any potential genetic marker predisposing to cardiovascular disease in HD patients.
From initial dataset of 245 genotyped individuals, we removed the genetically close relatives and we obtained a dataset of 208 unrelated individuals that we used for the analysis. At the baseline, from 208 subjects we defined a group of 135 subjects with previous cardiovascular events and a group of 73 subjects without cardiovascular events at the recruitment. The fixation index (Fst) was estimated as a measure of the genetic differentiation. Then we selected a total of 3218 markers with Fst over the 99th percentile of the genome-wide distribution (which represents the most differentiated SNPs between the CV1 group vs CV0) (Figure 17).

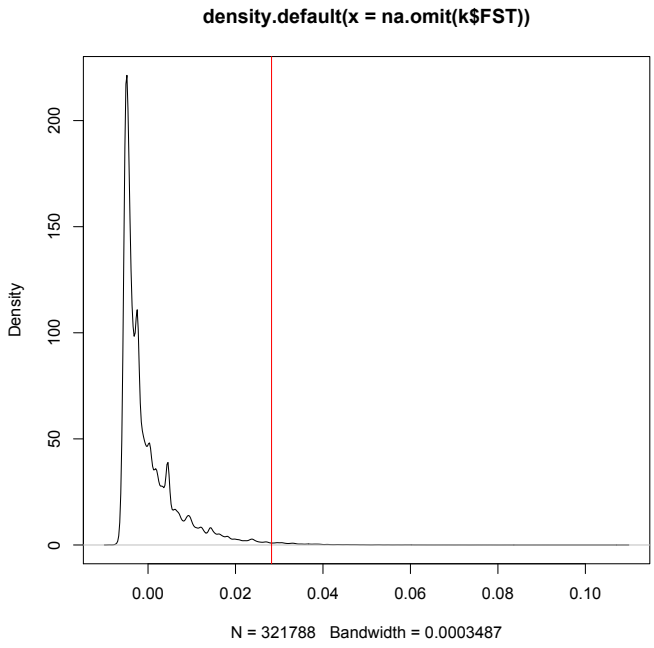


Figure 17: Fst distribution in the analysed subjects. Cut-off >99th percentile (red line).

Gene ontology analysis

IPA (Ingenuity Pathway Analysis) analysis was performed to identify possible functional roles of the 3218 selected variants. We identified a total of 12 disease pathways with a p-value >7.5-log, among which cardiovascular diseases scored in the top ten. We focused on the disease categories from the cardiovascular diseases pathway and identified 4 enriched categories with a p-value >7.5-log (Figure 18) which are the coronary artery disease, coronary disease, occlusion of artery and atherosclerosis.

Gene ontology analyses using Panther analysis identified hearth development pathway as the one with the highest value of gene enrichment (3.05 fold, p-value = $2.65*10^{-2}$) (Figures 18, 19).

Figure 18: Ingenuity Pathway Analysis (IPA): enriched categories among cardiovascular diseases that are enriched in CV1 vs CV0. Cut-off = 7.5 –log(p-value).

HUMAN|HGNC=4324|UniProtKB=P43220	GLP1R	Glucagon-like peptide 1 receptor	G-protein coupled receptor(PC00197)
HUMAN|HGNC=23647|UniProtKB=Q9BY15	ADGRE3	Adhesion G protein-coupled receptor E3	G-protein coupled receptor(PC00197)
HUMAN|HGNC=1749|UniProtKB=Q9Y6N8	CDH10	Cadherin-10	cadherin(PC00069)
HUMAN|HGNC=9609|UniProtKB=P49190	PTH2R	Parathyroid hormone 2 receptor	G-protein coupled receptor(PC00197)
HUMAN|HGNC=8005|UniProtKB=O60462	NRP2	Neuropilin-2	apolipoprotein(PC00219)
HUMAN|HGNC=13838|UniProtKB=Q86SQ6	ADGRA1	Adhesion G protein-coupled receptor A1	G-protein coupled receptor(PC00197)
HUMAN|HGNC=16700|UniProtKB=Q8WW38	ZFPM2	Zinc finger protein ZFPM2	nucleic acid binding(PC00171)
HUMAN|HGNC=13830|UniProtKB=Q9UHC6	CNTNAP2	Contactin-associated protein-like 2	apolipoprotein(PC00219)
HUMAN|HGNC=10807|UniProtKB=Q92629	SGCD	Delta-sarcoglycan	cytoskeletal protein(PC00085)
HUMAN|HGNC=23111|UniProtKB=Q6V1P9	DCHS2	Protocadherin-23	cadherin(PC00069)
HUMAN|HGNC=1760|UniProtKB=Q9HBT6	CDH20	Cadherin-20	cadherin(PC00069)
HUMAN|HGNC=1763|UniProtKB=P55283	CDH4	Cadherin-4	cadherin(PC00069)
HUMAN|HGNC=13733|UniProtKB=Q9H251	CDH23	Cadherin-23	cadherin(PC00069)
HUMAN|HGNC=14075|UniProtKB=Q96LD1	SGCZ	Zeta-sarcoglycan	cytoskeletal protein(PC00085)
HUMAN|HGNC=8008|UniProtKB=Q9ULB1	AC007682	Neurexin-1	apolipoprotein(PC00219)
HUMAN|HGNC=13839|UniProtKB=Q8IWK6	ADGRA3	Adhesion G protein-coupled receptor A3	G-protein coupled receptor(PC00197)

Figure 19: Panther analysis: genes involved in the heart development.

The significant results from both IPA and Panther resulted in the identification of 2 genes: Glucagon-like peptide 1 receptor (GLP1R) and sarcoglycan delta (SGCD) (Figure 20).

The markers found in these analyses were further confirmed using the dataset also with related individuals, as the markers found in GLP1R and SGCD over the 99th percentile of Fst distribution of the whole dataset.

IPA-CVD p>10-8				PANTHER
coronary artery disease	coronary disease	occlusion of artery	atherosclerosis	HEART DEV
ADARB2	ADARB2	ADARB2	ADARB2	GLP1R
ASTN2	ASTN2	ASTN2	ASTN2	ADGRE3
BMPR1B	BMPR1B	BMPR1B	BMPR1B	CDH10
C6orf10	C6orf10	C6orf10	C6orf10	PTH2R
C8orf34	C8orf34	C8orf34	C8orf34	NRP2
CACNA1C	CACNA1C	CACNA1C	CACNA1C	ADGRA1
CACNA1D	CACNA1D	CACNA1D	CACNA1D	ZFPM2
CACNA2D1	CACNA2D1	CACNA2D1	CACNA2D1	CNTNAP2
CNTN5	CNTN5	CBLB	CD44	SGCD
CNTN6	CNTN6	CD44	CNTN5	DCHS2
COL5A2	COL5A2	CNTN5	CNTN6	CDH20
CSMD1	CSMD1	CNTN6	COL5A2	CDH4
CSMD2	CSMD2	COL5A2	CSMD1	CDH23
CTNNA3	CTNNA3	CSMD1	CSMD2	SGCZ
F2R	DPP6	CSMD2	CTNNA3	AC007682
FAM107B	F2R	CTNNA3	CYP19A1	ADGRA3
FRMD4A	FAM107B	CYP19A1	F2R	
GABRB3	FRMD4A	F2R	FAM107B	
GBE1	GABRB3	FAM107B	FRMD4A	
GLP1R	GBE1	FRMD4A	GABRB3	
GRIA1	GLP1R	GABRB3	GBE1	
GUCY1A2	GRIA1	GBE1	GHR	
HRH2	GUCY1A2	GHR	GLP1R	
IL1R1	HRH2	GLP1R	GRIA1	
INSR	IL1R1	GRIA1	GRIN2A	
ITGA8	INSR	GRIN2A	GRIN2B	
MECOM	ITGA8	GRIN2B	GUCY1A2	
MICAL2	KCNJ2	GUCY1A2	HRH2	
MSRA	MECOM	HRH2	IL1R1	
MTHFD1L	MICAL2	IL1R1	INSR	
NOS1	MSRA	INSR	ITGA8	
NOS2	MTHFD1L	ITGA8	MECOM	
PARVA	NEBL	MECOM	MICAL2	
PDE10A	NOS1	MICAL2	MMP2	
PDE3A	NOS2	MMP2	MSRA	
PON1	PARVA	MSRA	MTHFD1L	
PRKCH	PDE10A	MTHFD1L	NOS1	
PRKG1	PDE3A	NOS1	NOS2	
RBFOX1	PON1	NOS2	PAPPA	
SCN10A	PRKCH	PAPPA	PARVA	
SCN9A	PRKG1	PARVA	PDE10A	
SGCD	RBFOX1	PDE10A	PDE3A	
SNX29	SCN10A	PDE3A	PON1	
TKT	SCN9A	PON1	PRKCH	
VEPH1	SGCD	PRKCH	PRKG1	
WWOX	SLC12A1	PRKG1	RBFOX1	
	SNX29	RBFOX1	RUNX2	
	TKT	RUNX2	SCN10A	
	VEPH1	SCN10A	SCN9A	
	WWOX	SCN9A	SGCD	

Figure 20: Selection of the genes common to the selected IPA CVD pathways and Panther – Heart development process.

Tree Regression analysis

We used a regression analysis to investigate any correlation between the analyses SNPs belonging to SGCD and GLP1R and the cardiovascular events and CV risk factors at the baseline. We found GLP1R rs10305445-A allele is linked to a higher number of subjects with previous CV events (Figure 21). We also found a significant relationship of SCGD rs145292439-A allele with lower number of subjects with previous CV events and higher level of HDL (Figure 22), suggesting a protective effect of the allele. Furthermore, regression tree analyses, using also age as covariate, identified that individuals with more than 59 years and that are homozygous carriers of the G allele in SCGD had a higher risk respect to individuals that are carrier of the A allele (Figure 22), confirming the age effect. The regression analysis at 5 years follow up did not identify any significant genotype-phenotype effect.

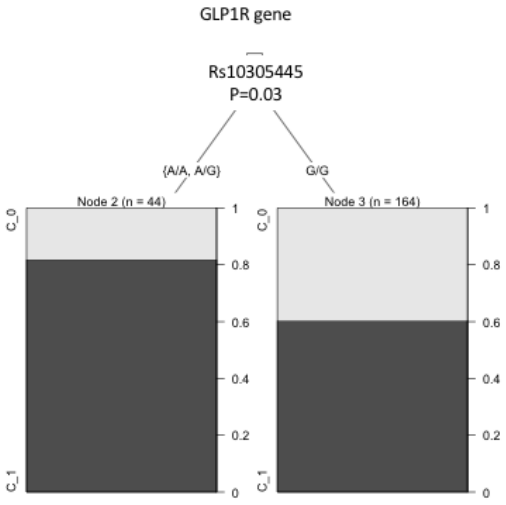


Figure 21: GLP1R rs10305445 resulted statistically associated with CVD in HD patients.

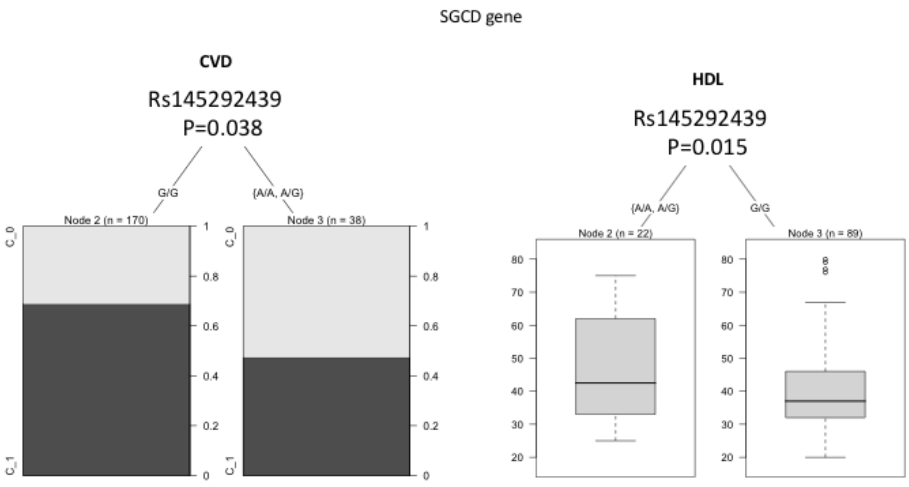


Figure 22: SCGD rs145292439 resulted significantly associated with CVD and HDL level in HD patients.

SNP function prediction analysis

The next step was to understand what could be the functional mechanism. To define the functional role of the two SNPs, the GTEx database was interrogated and "quantitative trait loci expression" (eQTL) for GLP1R rs10305445 in whole blood was found (Figure 23). The rs10305445 variant resulted in a significant increase in GLP1R gene expression in blood measured with GTEx (effect size 0.52, p-value = $1.3 * 10^{-6}$). We can hypothesize that we can have similar effects in our population. Instead, For SGCD rs 145292439 an eQTL was not found.

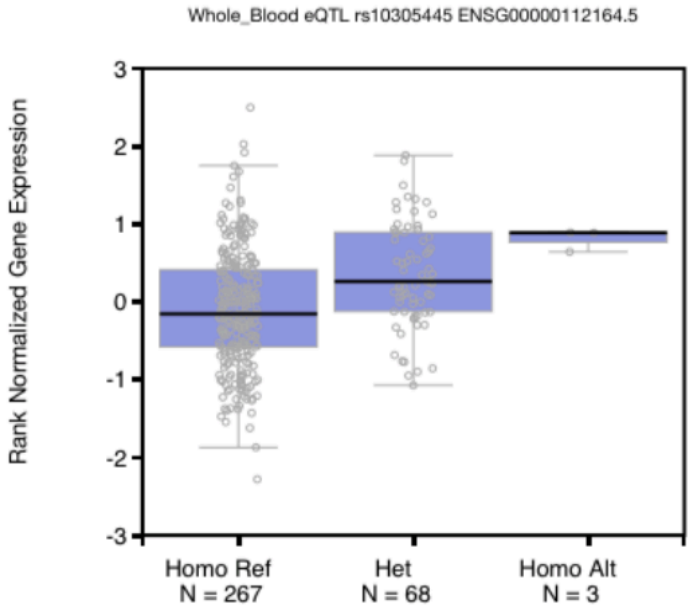


Figure 23: eQTL analysis from GTEx database demonstrated an effect of rs10305445 on the GLP1R expression in the general population. Homo Alt = Alternative (allelic variant).

Survival analysis

Occurrence of cardiovascular events was analysed with Cox regression in patients with different genotype at rs10305445 and rs145292439 during a 5-years follow-up. Odds ratio of cardiovascular events was increased in homozygous patients for the minor allele A (n=9) at rs10305445 than homozygous patients for the major allele G (OR=3.3, 95%CI 1.4-7.7; p=0.006; Figure 24, upper part). These findings were confirmed when we considered tertiles of age, body weight, serum phosphate, calcium and PTH and dialysis vintage in the analysis (AA patients: HR=2.8, 95%CI 1.1-6.7, p=0.026; GA patients, HR=1.4, 95%CI 0.8-2.5, p=0.23; GG as the reference group). The distribution of cardiovascular events during the follow-up was

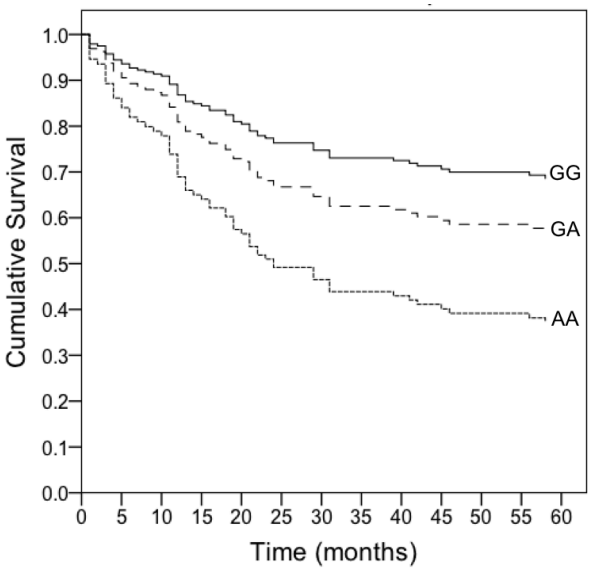
not related with genotypes at rs145292439 (AA OR=1.2, 95%CI 0.4-4.1, p=0.72, GG as the reference group).

Cox regression showed that cardiovascular events were more frequent in AA patients for rs10305445 in patients with a clinical history of cardiovascular events before (n=161) (AA patients: OR=2.4, 95%CI 1-5.6, p=0.043; GA patients, OR=1.1, 95%CI 0.6-1.8, p=0.86; GG as the reference group). There was no association in patients without previous events (Figure 24, lower part). The distribution of cardiovascular events during the follow-up was not related with genotypes at SCGD rs145292439 (AA OR=1.2, 95%CI 0.4-4.1, p=0.72, GG as the reference group; data not shown).

Table 17 shows the genotypes distribution in our population and the combination of the 2 SNPs that demonstrated bigger percentage of subjects carrying the GLP1R rs10305445 allele A (n carriers=44) compared to SCGD rs145292439 allele A (n carrier=38), which allele frequencies are ~0.11 and ~0.09 respectively in our sample.

	Table Genotype combination				
		GLP1R SNP (columns) rs10305445			**total**
SCGD SNP (rows) rs145292439	**0/0**	**A/A**	**A/G**	**G/G**	
0/0	0	0	0	0	**0**
A/A	0	0	1	2	**3**
A/G	0	1	5	29	**35**
G/G	0	5	32	133	**170**
total	**0**	**6**	**38**	**164**	**208**

Table 17: SNP genotypes combination at GLP1R rs10305445 and SCGD rs145292439 (0/0=missing data).



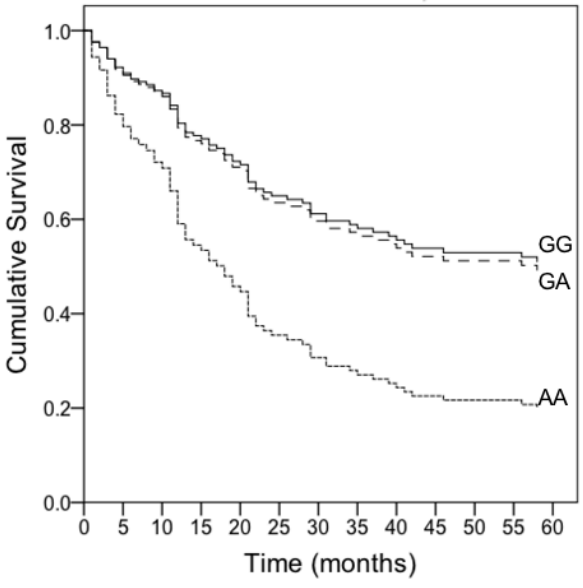


Figure 24: Cox regression analysis of cardiovascular events during a 5-yrs follow-up in 245 (upper part) and in 161 hemodialysis patients with a history of cardiovascular events (lower part) divided according to their genotype at rs10305445 (G>A).

DISCUSSION

FGF23, cardiovascular risk and dietary phosphate intake in CKD patients

Chronic Kidney Disease is a pathological condition that increases the risk of cardiovascular events, which, in turn, increase with the progressive worsening of the renal function. In fact, cardiovascular disease is the leading cause of morbidity and mortality in CKD patients, with a reported worse prognosis than the general population[67].

Such unfavourable risk profile is justified by the simultaneous presence of classical risk factors of cardiovascular disease and peculiar factors related to nephropathy, among which there are the mineral metabolism disorders. Previous studies have largely demonstrated that CKD patients have an altered phosphate homeostasis which predisposes to hyperphosphatemia. This defect is secondary to the reduction of the number of nephrons and the glomerular filtration, which makes the kidney unable to adequately respond to the maintenance of a correct phosphate balance[62].

Following the increase in phosphatemia, in the very early stages of renal pathology, an adaptive increase of serum PTH and FGF23 is observed to have an hyperphosphatemic effect[117,121]. The increase of FGF23 seems to be the earliest alteration in the course of CKD, causing the reduction of circulating levels of 1,25 $(OH)_2D$[157,99]. When eGFR values are less than 30ml/min/1.73m^2, the adaptive mechanisms can no longer maintain normal phosphate values due to the excretory inability of the kidney. The serum phosphate, PTH and FGF23 levels increase with the progression of the disease secondarily to several causes[117].

Phosphate retention, with or without increased phosphatemia, promotes the precipitation of calcium-phosphate salts in soft tissues. The resulting calcification of arterial walls causes significant cardiovascular damage that explains the increased cardiovascular morbidity and mortality in CKD patients[85,18]. Furthermore, phosphate retention induces bone damage

through PTH and FGF23 stimulation[113]. Increased tissue and cell phosphate content can stimulate myocardial hypertrophy by increasing FGF23[53].

The reduction of the burden of dietary phosphate favourably influences the clinical progress of the patients because it is followed by a reduction of FGF23 and a reduction of phosphatemia[156].

In this study, we analysed CKD patients at different stages of development, from the third to the fifth dialysis stage, with the aim of investigating the correlation between the phosphate intake with the diet and the serum levels of FGF23 and their influence on the risk of cardiovascular events.

This nutritional study was included in a large 5-year follow-up project that allowed us to correlate dietary phosphate intake with the risk of cardiovascular events by analysing the patient's clinical history.

The results were presented by comparing two subgroups of CKD patients: those who were in a pre-dialysis stage and were following conservative therapy and patients with end-stage renal function on hemodialysis treatment three times a week.

From the analysis of the biochemical parameters, all the recruited subjects showed a reduction of the calcemia and the concentration of 1,25 $(OH)_2D$, associated with an increase in phosphate, FGF23 and PTH concentration. These data are in agreement with the condition of different renal failure. The most statistically significant alteration was found in FGF23 levels. Indeed, other studies in the literature have confirmed FGF23 to be one of the first factors to undergo alterations in its serum levels during CKD, although the molecular mechanism underlying this alteration is not completely clear.

HD patients follow a richer diet in proteins, lipids, complex carbohydrates and sodium compared to patients in pre-dialysis stages, according to the nutritional guidelines[128]. The protein requirement of HD patients is 1.2 g/kg of body weight, without markedly increasing the phosphate intake. In fact, to control phosphatemia in uremic patients, the use of phosphate binders, which reduce intestinal absorption, is authorized. In addition, the energy

demand from carbohydrates and lipids is also higher. Therefore, HD patients follow a less restrictive diet than pre-dialysis patients. Despite this, our HD patients' BMI is lower than pre-dialysis patients' ones.

The recruited patients were divided into two groups (pre-dialysis and HD) and stratified in relation to the amount of phosphate they introduced with the diet. The discriminant levels were 700 mg/day and 1200 mg/day, which are respectively the amount recommended by the LARNs and the average quantity normally assumed by the western population.

In particular, the dietary phosphate intake derives above all from animal foods, mainly meat and cheese, that must be reduced in the diet of pre-dialysis patients to obtain a phosphate intake of less than 700 mg/day and a protein intake ranging from 0.3 to 0.8 g/kg of body weight (hypoprotein diet); whereas, in HD patients a protein intake of about 1.2 g/day is recommended, the phosphate contribution is also higher (on average between 700 and 1200 mg/day), as showed from our data.

In HD patients with phosphate intake greater than 700 and 1200 mg/day, higher FGF23 values were observed compared to patients with lower phosphate intake. Together with the results of multiple regression analysis, such data showed that the low-phosphate diet is able to limit the production of FGF23 in dialysis patients. Such result was not observed in pre-dialysis patients due to the different phosphate management in the pre-dialysis phases, which still highlights the renal contribution to phosphate excretion, even if reduced.

The reduced phosphate diet is, therefore, recommended for CKD patients, but there are controversial and not entirely clear data about its influence on FGF23 secretion in the current literature.

Several epidemiological studies have observed a relationship between phosphatemia and cardiovascular morbidity in CKD patients and in patients with heart disease and normal renal function[180]. In our population, the cardiovascular risk increased with phosphatemia values greater than

1mmol/l. In addition to phosphatemia, the serum values of circulating FGF23 above 50 pg/ml were also associated with an increased cardiovascular risk. This data has already been acquired in the medical literature, even if with some controversies[106,187,206].

What has been poorly evaluated in the literature is the relationship between dietary intake of phosphate and cardiovascular morbidity[216]. This relationship was explored in our series, considering HD patients and those in conservative therapy, separately. The considered cardiovascular events were: all-causes death, heart failure, stroke, myocardial infarction, transient ischemic attacks and acute peripheral coronary occlusion.

The number of acute events was higher in patients on conservative therapy with an intake greater than 700 mg/day. The analysis of the onset of acute cardiovascular events, in the 5-year follow-up through survival curves, also identified a higher frequency of events in pre-dialysis patients with phosphate intake greater than 700 mg/day. In HD patients, a specific role of phosphate in the determination of cardiovascular risk has not been observed.

Finally, through a Cox analysis, the role of the factors determining cardiovascular morbidity was studied, including also dietary phosphate. The results showed as determinants: diabetes mellitus (OR 2.8), the clinical history of cardiovascular events (OR 3,4), the levels of C-reactive protein (OR 1.04) and an increased phosphate intake of 1200 mg/day (OR 3.8). The results showed that patients with phosphate intake above 1200 mg/day had a 4-fold higher cardiovascular risk than those with an intake of less than 700 mg/day.

FGF23 in HD patients

The largest proportion of HD patients presented high or very high serum values of FGF23 that were positively associated with serum phosphate. Moreover, FGF23 values were positively correlated with phosphate intake at baseline control and with serum calcium at a second control, two years after the baseline[171,204]. At the second control, serum calcium was significantly higher in patients in the highest tertile of serum FGF23, suggesting that an increase of serum FGF23 occurred in patients with higher values of serum calcium, although in the normal range. Accordingly, patients who shifted to the highest tertile of serum FGF23 during the follow-up or who were in the highest FGF23 tertile at both the baseline and the second control had higher serum calcium than patients who were not in the highest tertile at the second control.

Phosphate intake and serum phosphate were independently associated with serum FGF23 in HD patients at baseline control and might independently stimulate FGF23 production in osteocytes and osteoblasts[159,48]. The association with these two variables is consistent with the current knowledge about the FGF23 role in phosphate homeostasis and serum phosphate control[153], although the pathway used by phosphate to affect FGF23 still remains unclear[81,216]. Moreover, the independent effect of dietary phosphate and serum phosphate suggests that intestine might have a sensing mechanism for dietary phosphate which, although not yet identified, might directly regulate FGF23 production[153].

In addition to phosphate, our findings suggest that calcium could sustain FGF23 secretion in HD patients[171,160,35,170,166,69]. A positive relationship was observed between serum calcium and FGF23 in rats fed with a diet deficient or enriched of calcium and vitamin D or after calcium infusion[35,69]. This association was not explained by the increase of serum phosphate, $1,25(OH)_2D$ or PTH. Moreover, in humans with normal renal function, dietary

calcium intake was associated with an increase of serum FGF23[204]. In this context, hypocalcemia was observed to blunt FGF23 response to serum phosphate in animal models, probably to avoid an excessive FGF23 production that could inhibit $1,25(OH)_2D$ synthesis and aggravate hypocalcemia[171,160]. In particular, FGF23 activation by calcium was not mediated by calcium-sensing receptor and was inhibited by calcium-channel blockers[160,35]. It was explained with a stimulation of the FGF23 gene promoter transcription by calcium ions that was observed in cultured mice osteoblasts and intestinal epithelial cells[35,170]. The positive association between FGF23 secretion and serum calcium may lead to the positive effect of reducing phosphate excretion threshold in case of serum calcium increase in order to protect soft tissues against calcification risk. This relationship is particularly important in HD patients, because the increase of serum calcium may influence their cardiovascular prognosis and amplify the negative effect of phosphate and FGF23 on myocardial and vascular condition[194]. It is noteworthy that serum calcium values associated with the increase of serum FGF23 were still in the normal range.

The evaluation of the role of serum calcium on FGF23 is complicated by an independent stimulatory effect of PTH on FGF23 that was observed in infusion studies in osteocyte coltures[170], mice, healthy volunteers and dialysis patients[145,166,106,21]. A positive relationship between serum FGF23 and PTH was found in patients with CKD and secondary hyperparathyroidism[166], but may vary according to factors like acidosis, circulating Klotho, iron storage and leptin[145]. This variability may explain the fact that our findings did not evidence a clear contribution of PTH to FGF23 production.

Sclerostin in CKD patients

Serum sclerostin was investigated in patients at different stages of CKD to study its involvement in calcium-phosphate metabolism, bone disease and vascular calcification[45,22,84,213]. Serum values of sclerostin were significantly increased in CKD patients and its increase was proportional to the renal function decline[23]; in particular, it was related with different variables in pre-dialysis and HD patients. Multivariate analysis showed that age, serum creatinine and serum PTH were associated with serum sclerostin in HD patients. On the contrary, eGFR was the main determinant of serum sclerostin in pre-dialysis stage 3-5 CKD patients, in whom serum $1,25(OH)_2D$ and FGF23 were also independently associated with serum values of sclerostin when the effect of eGFR was not accounted.

Despite the negative association between serum sclerostin and $1,25(OH)_2D$, pre-dialysis patients undergoing calcitriol therapy had higher serum concentrations of sclerostin[172].

The negative association between serum sclerostin and $1,25(OH)_2D$ in pre-dialysis CKD patients may reflect the inhibitory activity of sclerostin on the activity of the renal 1-alpha-hydroxylase of 25(OH)D in CKD patients[12]. Therefore, sclerostin may contribute to decrease $1,25(OH)_2D$ serum levels in CKD by adding its inhibitory effect on $1,25(OH)_2D$ synthesis to that of FGF23[133]. At the opposite, calcitriol may maintain its stimulatory activity on sclerostin gene (*SOST*) transcription and sclerostin synthesis, in addition to that of FGF23, by osteocytes in CKD patients[172,210].

Therefore, the therapeutic use of calcitriol may enhance the early increase of serum sclerostin and FGF23 in the course of CKD and may trigger low turnover bone disease especially in case of excessive inhibition of PTH production[45]. Specific studies observed that treatment with analogues of calcitriol, like paricalcitol and doxercalciferol, significantly increased skeletal expression of sclerostin in bone tissue samples from HD patients and serum

sclerostin in patients with stage 3 and 4 CKD[198,151]. At the opposite of what was observed in pre-dialysis CKD patients, the negative relationship between serum sclerostin and $1,25(OH)_2D$ was not detected in our hemodialysis patients probably because of their very low values of serum $1,25(OH)_2D$, which are significantly increased by calcitriol supplementation.

The increase of serum sclerostin with ageing has been previously observed in CKD patients and explained by the altered renal function in the elderly, even though the presence of this relationship in HD patients suggests the intervention of other factors[44]. The potential inhibitory role of PTH on sclerostin production emerged in HD patients, who had lower serum sclerostin when treated with cinacalcet.

Serum sclerostin was positively related to bone mass density (BMD) in patients not-undergoing HD as observed in previous studies[84,142,163]. This positive relationship stands in apparent contradiction with the inhibitory effect of sclerostin on osteoblast bone formation and could be explained by a larger number of osteocytes in patients with higher bone mass. Conversely, this relationship was negative in HD patients at the opposite of what observed in previous studies, but in apparent agreement with the known sclerostin activity[84,10,142,163,119]. Probably, the higher levels of serum sclerostin in HD patients may be crucial to address bone metabolism to low turnover and to invert the relationship between sclerostin and BMD. Unfortunately, only 31 patients with good clinical condition were available to test DXA; therefore, our findings have to be confirmed in a larger patient sample.

The present study quantified calcification of lumbar aorta using Kauppila scoring method that may be employed in the clinical practice in the place of the more accurate, but more expensive, scoring methods measuring coronary and aorta calcification with computed tomography[95]. Aorta calcification score was not related with sclerostin serum levels in our patients. This association was observed in a French study, but not found in another French study, both conducted in HD patients[20,41]. Opposite

associations between serum sclerostin and vascular calcification were also observed in diabetic patients[163,41]. Therefore, the association of sclerostin with arterial calcification remains to be clarified and longitudinal studies will be useful to define it[119].

Genetic and nutritional markers of cardiovascular risk in CKD patients

Studies have undergone further impetus from the evolution of methods and technologies applied to genetics. The nutritional aspect remains of great importance for both CKD and cardiovascular disease. The study aimed to investigate the relationships between nutritional and genetic factors that can contribute to increase the risk of cardiovascular events in CKD patients. As for the nutritional aspect, the results are derived from a population of CKD patients both in dialysis and in conservative therapy. In selected individuals, with the complete data of 68 studied variables, we obtained some significant data that confirmed what is already known in the literature[58,90,101]. A positive correlation was found between the progression of CKD and dialysis, the duration of dialysis (in terms of the number of months), the blood values of creatinine and the intake of vitamin E.

The correlation was negative between the CKD progression and the decrease in serum calcium concentration, the intake of beta-carotene with diet, water and weight gain. The most representative data concern the intake of vitamin E, beta-carotene and calcium. Higher vitamin E intake was seen in patients receiving dialysis than patients on conservative treatment. Although no indications of dietary type have been given to patients, this data is to be considered positive for the known antioxidant effect of vitamin E[40,7]. Oxidative stress increases in CKD patients due to the pathology itself, which causes an increase in toxins around leaking and this is considered a possible aggravating factor in the risk of cardiovascular disease[90]. CKD patients also accelerate the evolution of the atherosclerotic process, especially for

hypertension, due to sodium retention, and with the increase of pro-oxidative components (age, diabetes, hypertension, inflammation, incompatibility of dialysis membranes) and reduced antioxidant defences. Among the most promising therapeutic approaches, the use of vitamin E[40] seems to be important; in fact, antioxidant therapy can reduce cardiovascular morbidity and mortality in individuals with CKD[90].

Beta-carotene intake is much lower in dialysis patients than in conservative therapy.

It seems that an integration of beta-carotene alone is not significantly associated with a lower risk of CKD, but, associated with other antioxidant elements in the diet, may instead have a positive influence on the progression of kidney disease[7].

With a regression analysis, we have seen how serum calcium decreases as vegetable protein intake increases, probably because fibres decrease intestinal calcium absorption. This is an important element to be evaluated in the dietetic approach of these patients. Moreover, the presence and extent of calcium in the coronary arteries are associated with an increased risk of cardiovascular events[55].

The genetic component appears to play a significant role in the evolution and progression of cardiovascular disease in CKD patients.

In this study, 245 hemodialysis patients were selected for the GWAS and divided into two groups: a group that had previous cardiovascular events (n=84) and a group that had no cardiovascular events (n=161). All the patients were characterized for clinical parameters of CKD and cardiovascular disease. Previous GWASs investigated genes associated with the CKD and dialysis[100,70,68] and, in our knowledge, no whole genome variant array was performed to analyse CVD in hemodialysis patients.

After having performed the SNPs genotyping with the Illumina Mega EX Array method, the Fst distribution was used with a cut off > of the 99th

percentile to identify all the variants considered different in the two groups of patients.

Thanks to the use of bioinformatics programs such as IPA and PANTHER, a systemic approach was used to identify and define the pathways. IPA has identified SNPs associated primarily with certain categories of diseases including cardiovascular disease. Within cardiovascular diseases, the most frequently identified ones, always identified with SNPs, are coronary artery disease, coronary disease, occlusion of the arteries and atherosclerosis.

With PANTHER, another tool that investigates biological processes, 16 genes have been identified in the heart development pathway of SNPs present in our population. We therefore see a genetic enrichment of 3.05 times which demonstrates, in our population, a stronger association with this phenotype than to the general population.

Comparing the results of the two instruments, only two genes, SCGD and GLP1R, were present in all the groups.

With the linear regression analysis, two allelic variants of the cardiovascular risk-related genes have been identified: GLP1R rs10305445 and SCGD rs145292439.

In the literature, variants of the GLP1R gene related to cardiovascular risk have been described and some of them appear to be protective for coronary heart disease[181]. This effect appears to be mediated by lower fasting glucose and a lower risk of type 2 diabetes. GLP1R plays a major role in the known effects of incretins, which not only increase the secretion of postprandial insulin, but also give cardio-protection and increase cardiac output, reduce gastric emptying and glucose production by the liver[88].

It is interesting to note from the literature that some missense variants of the GLP1R gene have a lower response to some categories of anti-diabetic drugs[3]. For example, one study reports a reduced response to gliptins, drugs commonly used in the treatment of diabetes, which act by increasing the level of incretins (including GLP1) in patients with GLP1R variant

rs6923761[88]. Such finding has a strong translational value, because a preventive genetic analysis could allow us to customize anti-diabetic drug therapy to each specific patient, excluding those drugs that are certainly ineffective.

In the case of the present study, the results suggest that the identified gene variant, GLP1R rs10305445, may increase cardiovascular risk. This result increases the available information but needs further research.

The other allelic variant of the SGCD gene, rs145292439, the A/G and A/A genotype is associated with a lower frequency of cardiovascular events and a higher level of HDL cholesterol.

Therefore, it seems that variant A of this SNP has a protective role against cardiovascular event in the categories identified in this study.

However, most of the studies in literature correlate this variant to hypertrophic cardiomyopathy and there is no study that concerns an association with coronary artery disease of an ischemic type[200]. Furthermore, no associations with CKD were found.

CONCLUSIONS

CKD patients at different stages suffer of high risk of cardiovascular mortality, which is higher than the age-matched general population. In fact, CVD is the first cause of death in CKD patients, because of the simultaneous presence of classical factors and the ones specific of renal disease, among which there are metabolic mineral disorders. The long-term complications include hyperparathyroidism and hyperphosphatemia that are linked to an increase of FGF23 serum level.

The current study is called "The Cardiovascular Mortality project: study of functional markers" (CREMA) and aimed to investigate cardiovascular morbidity and mortality by identifying the genetic, biochemical and functional markers of arterial damage in relation to the diet. Recruited patients were followed for a 5-year follow-up period. The main purpose of the study was to identify the genetic factors predisposing the risk of occurrence of cardiovascular events in CKD and, together with these, the nutritional and biochemical factors that could influence the development and progression of cardiovascular events.

Our findings suggest that the functional role of FGF23 appears complex because of their multiple interrelationships with the other variables of calcium-phosphate metabolism. In this context, serum calcium may be associated with FGF23 production in HD patients and might modulate the effect of phosphate on FGF23 synthesis.

In particular, in HD patients serum calcium should be maintained in the lower concentrations of the normal range to prevent vascular calcification and to minimize the negative effect of FGF23 and phosphate on the cardiovascular system. In conclusion, these results suggest that the daily dietary intake of phosphate is an important factor in determining cardiovascular risk in CKD patients.

Moreover, our findings suggest that sclerostin may act as a main determinant of $1,25(OH)_2D$ production in CKD patients. Its inhibitory effect on $1,25(OH)_2D$ synthesis may be the most relevant aspect in its relationship with $1,25(OH)_2D$

in CKD patients, although calcitriol maintains its stimulatory activity on sclerostin production in these patients. The fact that supplementation with calcitriol or analogues of vitamin D may stimulate sclerostin production have to be considered to avoid skeletal or cardiovascular complications in CKD patients. Serum sclerostin may also predict bone mass in CKD patients, but the mechanism of this association needs to be furtherly clarified.

From this preliminary analysis of CREMA study, we can conclude that genetic factors may contribute more than diet to cardiovascular disease in CKD patients and we can speculate a role of *GLP1R* as a genetic risk marker and *SGCD* as a protective marker for cardiovascular events. The mechanisms hidden under these allelic variants influencing the specific metabolic pathways associated with cardiovascular disease in CKD still remain to be investigated. Both SNPs are no-coding intronic variants and their effect on the gene function is not known. We explored preliminary predictive tools (Regulome[182], HaploReg[205] and Match[64]) to identify functional effects on the gene function (gene transcription and epigenetic mechanisms) but we did not find any significant and consistent result (data not shown). An *in-vitro* model to test the two variants will be useful to better characterize them.

Moreover, further studies are needed to evaluate the impact of vitamin E and beta-carotene on CKD.

One limitation of our study is indeed the sample size. For this reason, we opted for a population-based approach to discover variants that could affect the number of cardiovascular events in our sample.

Further studies should be focused to increase sample size and to the use of whole genome sequencing to discover variants affecting cardiovascular risk in HD patients or other categories of patients at risk of CVD such as, for example, patients with hypertension, obesity or diabetes.

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APPENDIX

Annex 1 - Food-frequency questionnaire to evaluate calcium, phosphate and Vitamin D intake.

Nome:
Cognome:
Età:
Codice:

1) Quanto latte beve al giorno?

□Una tazza da 100ml □Una tazza da 250ml □Una tazza da 500ml □Mai

Quante volte a settimana?.....

2) Mangia yogurt? Sì□ No □

quanto in un giorno? □un vasetto da 125g □uno da 250g □uno da 500gr

quante volte a settimana?.....

Di che tipo? □frutta □bianco □ai cereali □con la frutta secca (noci, mandorle)

3) Mangia formaggi? Sì □ No □

quante volte a settimana?.....

Di che tipo?

3.1) Formaggi grassi (Parmigiano, Grana, Gorgonzola,Taleggio, Fontina, Crescenza etc)? Sì □ No□

- *quanto in un giorno?* □Porzione piccola 40g □porzione media 80g □porzione grande 120g
- *quante volte a settimana?.....*

3.2) Formaggi semi-grassi (Emmenthal,Provolone,Caciotta,Mozzarella)? Sì□ No□

- *quanto in un giorno?* □Porzione piccola 40g □porzione media 80g □porzione grande 120g
- *quante volte alla settimana?.....*

3.3) Formaggi leggeri (ricotta, latticini freschi, caprino, stracchino)? Sì □ No □

- *quanto in un giorno?* □Porzione piccola 40g □porzione media 80g □porzione grande 120g
- *quante volte alla settimana?.....*

3.4) Usa burro? Sì□ No□

- *quanto?* □50g □100g □200g

- *quante volte a settimana?.....*

4) Mangia pasta o riso? Sì□ No□

- *di che tipo?* □Di grano duro □integrale

- *quanto?* □porzione piccola 50g □porzione media 100g □porzione grande 200g

- *quante volte a settimana?.....*

4.1) Quanti cucchiaini di parmigiano o grana mette sulla pasta?

□Nessuno □1 cucchiaino (5g) □2 cucchiaini (10g) □3 cucchiaini (15g)

5) Consuma pane o prodotti da forno (crackers, grissini, taralli, biscotti)?

Sì □ No□

- che tipo di pane? □Di grano tenero "00" □di grano duro □integrale

- *quanto in un giorno?* □50g (un panino piccolo/2 fette di pane) □100g (2 rosette/4 fette di pane) □200g

- *quante volte a settimana?.....*

6) Consuma patate? Sì□ No□

- *quanto?* □200g (2 patate) □400g (4 patate) □600g (6 patate)

- *quante volte a settimana?.....*

7) Mangia carne o pesce? Sì□ No□

- *quanto?* □porzione piccola 100g □porzione media 150g □porzione grande 200g

- *quante volte a settimana?.....*

8) Mangia carne conservata (prosciutto cotto, crudo, bologna, coppa, salame, speck etc.)? Sì□ No□

- *di che tipo?* □Al banco □in busta

- *quanto?* □porzione piccola 100g □porzione media 150g □porzione grande 200g

- *quante volte a settimana?.....*

9) Mangia uova? Sì□ No□

- *quante uova a settimana?.....*

10) Mangia legumi (fagioli, piselli, ceci, lenticchie..)? Sì□ No□

- *quanto?*

□Porzione piccola (80g cotti o 1/3 di una tazza crudi)

□Porzione media (150g cotti o mezza tazza crudi)

□Porzione grande (250g cotti o 1 tazza crudi)

- *quante volte a settimana?.....*

11) Consuma verdura? Sì□ No□

- *quanto?* □porzione piccola 100g □porzione media 200g □porzione grande 300g

- *quante volte a settimana?.....*

12) Mangia frutta fresca? Sì□ No□

- *quanto al giorno?* □1 frutto □2 frutti □3/4 frutti

- *quante volte a settimana?.....*

13) Mangia frutta secca (mandorle, noci, arachidi, fichi secchi, nocciole, albicocche secche)? Sì□ No□

- *quanto?* □40g □80g □120g

- *quante volte a settimana?.....*

14) Mangia gelato (fatto con il latte)? Sì□ No□

- *quanto?* □un cono/coppetta piccola (1 gusto) □cono/coppetta media (2 gusti) □cono/coppetta grande (3 gusti)

- *quante volte a settimana?.....*

15) Mangia cioccolato bianco o al latte? Sì□ No□

- *quanto?* □25g (5 quadratini) □50g (10 quadratini) □100g (1 tavoletta)

- *quante volte a settimana?.....*

16) Quanta acqua beve in un giorno?

□500ml □750ml □1 L □1,5L □2L □2,5L

- di che tipo? □rubinetto □oligominerale □ricca in calcio (Sangemini, Lete, Ferrarelle)

17) Beve bevande a base di succo di pompelmo, di arancio o coca-cola?

Sì□ No□

- *quanto?* □1 bicchiere □2 bicchieri □3/4 bicchieri

- *quante volte a settimana?.....*

Annex 2 - 24-hours Dietary Recall

Istruzioni necessarie per una corretta compilazione del diario alimentare

l- Registrare i cibi e le bevande con il loro nome abituale;
2- Precisare se il cibo è fresco, in scatola, congelato, essiccato, condensato, evaporato, affumicato, sott'olio, sott'aceto e se viene consumato crudo bollito, arrostito, fritto, alla griglia etc.
3- Precisare ogni altra informazione utile per determinare il valore nutritivo di ogni cibo consumato (ad esempio indicare il nome commerciale del cibo e delle bevande)
4- Registrare separatamente su ogni rigo il cibo consumato;
5- Indicare separatamente il tipo e la quantità di condimento utilizzato per ogni singola pietanza
6- Misurare la quantità di cibo consumata con la bilancia al netto degli scarti (cioè la carne senza ossa, la frutta senza buccia, etc.);
7- Quando possibile specificare la ricetta e gli ingredienti della pietanza.
8- E' possibile avvalersi di misure di capacità standard, ad esempio: 1 cucchiaino da tè o da caffè di zucchero; 1 cucchiaio da minestra di olio; 1 panino tipo rosetta; 1 bicchiere di vino da pasto; 1 lattina di birra etc.

PAZIENTE

NOME

COGNOME

DIALISI O TERAPIA CONSERVATIVA

CODICE IRC

LUOGO

DATA

COLAZIONE

Luogo di consumo _____

Descrizione alimenti
Peso in gr o

porzione
(indicare anche quantità di zucchero o dolcificante)

_____

_____

_____

SPUNTINO

Luogo di consumo _____

Descrizione alimenti
Peso in gr o porzione
(indicare anche quantità di zucchero o dolcificante)

_____

_____

PRANZO

Luogo di consumo_____

Descrizione alimenti
Peso in gr o porzione
Pane

_____

_____

_____

Primo piatto

_____

_____

_____

Secondo piatto

_____

_____

_____

Contorno

_____

_____

_____

Frutta e/o dessert

_____

_____

_____

Bevande

_____

_____

Caffé sì no

Zucchero _____ Dolcificante_____

MERENDA

Luogo di consumo _____

Descrizione alimenti
Peso in gr o porzione
(indicare anche quantità di zucchero o dolcificante)

_____

_____

_____

CENA

Luogo di consumo _____

Descrizione alimenti
Peso in gr o porzione
Pane

_____

_____

_____

Primo piatto

_____

_____

_____

Secondo piatto

_____

_____

_____

Contorno

_____

_____

Frutta e/o dessert

_____

_____

Bevande

_____

Caffé sì no

Zucchero _____ Dolcificante_____

DOPO CENA

Luogo di consumo_____

Descrizione alimenti
Peso in gr o porzione
(indicare anche quantità di zucchero o dolcificante)

_____

_____

SCIENTIFIC PRODUCTS

1. La Corte E, Younus I, **Pivari Francesca**, Selimi A, Ottenhausen M, Forbes JA, Pisapia DJ, Dobri GA, Anand VK, Schwartz TH. **BRAF V600E mutant papillary craniopharyngiomas: a single-institutional case.series.** Pituitary. 2018 Sep 5. doi: 10.1007/s11102-018-0909-z.

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PUBLISHED ABSTRACTS

1. M. Cozzolino, P. Ciceri, F. Elli, S. Cannizzo, A. Mingione, E. Ottaviano, **Francesca Pivari**, A. D'Arminio and G. Marchetti. **Iron citrate inhibits phosphate-induced early apoptosis by preventing phosphatidylserine translocation and mitochondrial depolarization.** ERA-EDTA 54th Annual Congress, June 3-6, 2017 – Madrid (Spain). Nephrology Dialysis Transplantation, 32(): iii233–iii235, MAY 2017. DOI: 10.1093/ndt/gfx147.SP364.

2. Annalisa Terranegra, Caterina Brasacchio, Alessandra Mingione, Elena Dogliotti, **Francesca Pivari**, Teresa Arcidiacono, Lorenza Macrina, Giuseppe Vezzoli, Laura Soldati. **Calcium and Phosphate Intake May Influence Bone Remodeling Marker in Hemodialysis Patients.** Qatar Foundation Annual Research Conference Proceedings, March 21, 2016 – Qatar. DOI: 10.5339/qfarc.2016.HBPP2508.

3. Annalisa Terranegra, Alessandra Mingione, Chiara Verdelli, Caterina Brasacchio, **Francesca Pivari**, Elena Dogliotti, Sabrina Corbetta, Laura Soldati. **Filamin A is Reduced and Contributes to CASR Sensitivity in Human Parathyroid Tumors**. Qatar Foundation Annual Research Conference Proceedings, March 21, 2016 – Qatar. DOI: 10.5339/qfarc.2016.HBPP2033.

4. Annalisa Terranegra, Alessandra Mingione, Caterina Brasacchio, Katia Maruca, Silvia Capelli, Elena Dogliotti, **Francesca Pivari**, Mariangela Cisternino, Stefano Mora, Laura Soldati**. Autosomal Dominant Hypocalcemia due to a Truncation in the C-Tail of the Calcium-Sensing Receptor.** Qatar Foundation Annual Research Conference Proceedings, March 21, 2016 – Qatar. DOI: 10.5339/qfarc.2016.HBPP2064.

5. Annalisa Terranegra, Elena Dogliotti, Caterina Brasacchio, Costanza Conti, Monica Lazzaroni, Alessandra Mingione, **Francesca Pivari**, Cristina Barlassina, Daniele Cusi, Laura Soldati. **Nutritional and Genetic Determinants of Cardiovascular Risk.** Qatar Foundation Annual Research Conference Proceedings, March 21, 2016 – Qatar. DOI: 10.5339/qfarc.2016.HBPP2026.

ABSTRACTS, ORAL & POSTER PRESENTATIONS

1. Oral presentation: How Mediterranean is our diet? Validation of a short Mediterranean diet questionnaire. International Nutrition and Diagnostics Conference (INDC), Praga, 21-25 settembre 2018.

2. Poster: Cardiovascular risk and nutritional status in CKD patients. International Nutrition and Diagnostics Conference (INDC), Praga, 21-25 settembre 2018.

3. Poster: The impact of nutritional and genetic characteristics on CKD patients' cardiovascular risk. International Nutrition and Diagnostics Conference (INDC), Praga, 21-25 settembre 2018.

4. Oral presentation: Curcumina, un promettente nutraceutico in Oncologia. Convegno D.I.E.T. DIET for IMMUNOTHERAPY ENHANCEMENT against TUMORS, DiSS – Università degli Studi di Milano, 25 maggio 2018.

5. Poster: How Mediterranean is our diet? Validation of a short Mediterranean diet questionnaire. Convegno Progetto D.I.E.T. DIET for IMMUNOTHERAPY ENHANCEMENT against TUMORS, Università Tor Vergata di Roma, 23 maggio 2018.

6. Oral presentation: Human uremic serum stimulates calcification in an in vitro model of VSMCs: molecular pathways and clinical correlations. III Congresso DiSS, 13 novembre 2017.

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