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New insight in primary and secondary osteoporosis: Potential new genetic and biochemical markers

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1. Introduction

1.1 Osteoporosis and CKD-MBD

Chronic kidney disease (CKD) and osteoporosis are among the main public health problems that occur in the elderly population.

Osteoporosis is a systemic chronic disease, defined as a skeletal disorder characterized by low bone mass and deterioration of its micro-architecture, causing bone fragility and increased risk of fracture.

The pathogenesis of osteoporosis resides in an imbalance of bone remodeling.

Physiological bone remodeling is a process responsible for bone resorption operated by osteoclasts and bone formation operated by osteoblasts, this process is necessary to maintain mineral homeostasis.

There are various forms of osteoporosis; the two main types of osteoporosis common in adults are primary and secondary osteoporosis.

Primary osteoporosis is the result of a para-physiological process, and appears with menopause (postmenopausal) or aging.

Various drugs and diseases, such as metabolic endocrine diseases, rheumatic diseases, and especially chronic kidney disease, may cause secondary osteoporosis.

Chronic kidney disease (CKD) is a condition characterized by a progressive deterioration of renal function with increasing risk of

kidney failure and end-stage renal disease (ESRD), the disease stage where dialysis and transplantation are needed [1].

Kidney finely regulates the homeostasis of calcium and phosphate, so the loss of functionality determines an imbalance of mineral metabolism.

Patients with chronic renal failure develop with high frequency disorders of mineral metabolism and bone diseases that affect both the structure and the mineral composition of the bone tissue.

Osteoporosis in fact can coexist with chronic kidney disease.

The term CKD-MBD (Chronic Kidney Disease - Mineral Bone Disorder), used by the KDIGO guidelines since 2005, indicates one of the many complications associated with chronic kidney disease [2].

CKD-BMD syndrome is characterized by three components: an abnormal serum mineral levels (Calcium, Phosphorus); abnormalities in Parathyroid Hormone (PTH), FGF23 and Vitamin D levels; abnormality in the soft tissue calcification including vascular calcification.

Hyperphosphatemia and secondary hyperparathyroidism are common conditions in dialyzed patients with abnormal bone mineralization and vascular calcification [3].

It has been proved that in almost all patients with CKD on dialysis (Stage 5) and in most patients at stage 3-5, bone changes are present [4].

Both osteoporosis and CKD-MBD syndrome are conditions that increase bone fragility and susceptibility to fractures, in addition several studies have shown that patients with ESDR have a greater risk of fracture than the general population.

Metabolism abnormalities, bone abnormalities and vascular calcifications in CKD-MBD are related, together contribute to mortality and morbidity of patients with chronic renal failure.

The pathogenic mechanisms of osteoporosis in the general population are different from those established in the CKD-MBD syndrome, it remains to be clarified whether osteoporosis in CKD patients shares the same pathogenic mechanism of primary osteoporosis, or if the osteoporosis is secondary to CKD-MBD, or is it secondary to some clinical condition other than CKD-MBD [5].

However, it is important to highlight that both conditions share many of the factors involved in their pathogenesis.

CKD-MBD syndrome and primary osteoporosis are multifactorial diseases, in addition to hormonal and environmental factors, genetic factors play a significant role in their etiology [6-7].

It is well known that mineral and bone homeostasis is controlled in particular by three hormones: PTH, Calcitonin and 1,25-$(OH)_2$ Vitamin D, but several studies in recent years have highlighted new regulation mechanisms of mineral metabolism.

A fundamental role in mineral metabolism and bone remodeling has been attributed to FGF23 / Klotho axis and RANK / RANKL / OPG regulatory pathway.

1.2 RANK/RANKL/OPG pathway

Bone is a metabolically active tissue: in the bone, there is a continuous remodeling process, that consists in a dynamic bone resorption by osteoclasts and bone formation by osteoblasts maintaining a normal bone mass.

Bone remodeling is finely tuned by different factors regulating osteoclasts and osteoblasts activity.

In the middle to late 1990s the discovery and characterization of a signaling system involving receptor activator of nuclear factor NF-kB ligand (RANKL), its receptor RANK, and its decoy receptor osteoprotegerin (OPG) has led to a novel concept of bone metabolism.

This cluster of cytokines is essential for normal osteoclasts differentiation [8] and the alterations in the RANK/RANKL/OPG signaling pathway play a major role in the primary osteoporosis development.

RANKL (RANK-Ligand) is a cytokine belonging to the Tumor Necrosis Factor (TNF) family, produced by osteoblasts, which is able to bind to a specific receptor RANK, expressed both on osteoclast progenitor cells, and on mature osteoclasts.

The interaction of RANKL with the NF-kB activating receptor (RANK), determines osteoclasts differentiation; this mechanism is essential for the formation, function and survival of the osteoclasts and it is a necessary condition in order to carry out the osteoclast mediated bone resorption activity [9].

Osteoprotegerin (OPG) is a soluble factor produced by osteoblasts, structurally similar to RANK.

The OPG acts as a "decoy" receptor, binding RANKL in competition with RANK and so blocking RANKL activation.

The balance between RANKL and OPG underlies the dynamics of bone remodeling. They have a central role in bone remodeling [10] and a close association between this pathway and osteoporosis has been reported [11]. Many studies confirm the role of this pathway in the pathogenesis of osteoporosis, with enhanced RANKL expression and increased RANK responsiveness of osteoclasts [12].

In relation to CKD-MBD development, several humoral factors are involved.

The OPG/RANK/RANKL cytokine system appears to mediate the effect of many bone turnover factors, contributing to the pathogenesis of renal bone disease [13].

In CKD patients, the most critical factor determining bone turnover is unpaired parathyroid function, the altered levels of PTH and 1,25 dihydroxyvitamin D3 and accumulation of some circulating factors in the uremic serum.

Serum calcium level response to PTH is less efficient than in normal subjects, high PTH levels are needed, about three to four times higher than normal, to overcome skeletal resistance to PTH and maintain a normal rate of bone formation [14-16].

Several factors that induce bone resorption and hypercalcaemia, such as parathyroid hormone (PTH), prostaglandin E2 (PGE2), and 1,25 vitamin D3, IL6 and IL17 may up-regulate the expression of the RANKL gene in osteoblasts and bone stromal cells by improving the production of RANKL [17-20].

The observation that PTH acts both stimulating RANKL production and inhibiting the production of OPG, suggests that the RANKL/

OPG system may be involved in turn in counteract PTH effect in dialysis patients [21].

Despite these physio-pathological pictures, studies in patients with chronic kidney disease and on dialysis, have reported controversial results concerning the RANK / RANKL / OPG system in bone disorders associated with kidney disease.

Several studies have evaluated serum levels of RANKL and OPG in patients with CKD and hemodialysis. Some studies have reported concentrations of OPG higher in dialysis patients [22], while the concentrations of RANKL in some studies were normal, higher or lower, compared to the general population [23-25].

Some study showed that serum levels of OPG in CKD-MBD were increased independently to serum PTH levels [26], while another study showed that circulating OPG in patients on dialysis is dependent on PTH concentration [27].

In this view, in recent years, numerous candidate genes have been studied as genetic risk factors for bone mineral density (BMD) reduction and spontaneous fractures [28].

It is not surprising that particular attention was given to receptor activator of nuclear factor kappa-B (RANK), RANK ligand (RANKL) and osteoprotegerin (OPG) gene polymorphisms.

1.3 RANK/RANKL/OPG gene polymorphisms

Some polymorphisms (DNA sequence variations that commonly occur within a population) in the RANK, RANKL and OPG genes

seem to influence their expression [29-30] and can modify the RANKL / OPG ratio affecting bone remodeling process and consequent alteration of bone mineral density [29].

Association between polymorphic variants of the RANK / RANKL / OPG system with development of osteoporosis and the consequent risk of fractures have been reported in many studies [31-35].

In particular, some polymorphisms of the RANKL (rs2277438) and RANK (rs3018362) genes are associated with a low BMD [36] and an increased risk of fracture [30] in the general population.

Similarly, the relationship between polymorphisms of the OPG gene, bone mineral density and osteoporosis have been evaluated in many studies, with conflicting results.

In 2013, Guo et al. [37] published a meta-analysis showing a significant association between G1181C polymorphism (rs2073618) and osteoporosis, individuals with the C allele have a reduced risk of osteoporosis.

On the other hand, very few data have been published on the possible role of RANKL, OPG and RANK polymorphisms in CKD-BMD patients.

1.4 FGF23/Klotho axis

Bone can be considered as an endocrine organ, it produces at least two hormones, fibroblast growth factor 23 (FGF23) and osteocalcin; The FGF23/Klotho axis, alongside the classic homeostasis mechanisms, has been recently enlighten as a new regulation pathway of mineral and bone metabolism [38].

Klotho plays an important role not only in calcium and phosphate regulation, but also in aging processes and in the age-related diseases [39-41].

Fibroblast growth factor 23 (FGF23) is a bone-derived hormone, secreted mainly by osteocytes and osteoblasts, [42].

Nowadays, this bone-derived hormone is considered as the principal regulator of phosphatemia; FGF23 induces phosphaturia and inhibits calcitriol synthesis in the kidney, therefore maintaining systemic phosphate homeostasis [43-45].

In the kidney, FGF23 exerts its hypophosphatemic effect, by inhibiting sodium phosphate co-transporters at the proximal tubular level [46].

Additionally, FGF23 also down-regulates renal proximal tubular expression of 1α-hydroxylase, thus reducing the levels of 1,25 dihydroxyvitamin D [47].

The direct action of FGF23 leads to increased urinary excretion of calcium and phosphorus, while indirectly suppresses their intestinal absorption by lowering the circulating levels of 1,25 $(OH)_2$ vitamin D. The net effect of these hormonal actions, is to lower circulating phosphate concentrations, safeguarding against the deleterious effects of hyperphosphatemia.

Moreover, FGF-23 directly suppresses PTH mRNA in vitro and decreases serum PTH in vivo [48].

FGF23 exerts its pleiotropic biological actions by binding, and activating cell surface FGF receptors (FGFRs) [49].

The affinity of FGF23 for its ubiquitous receptors is quite low, it was shown that this affinity is enhanced by the co-receptor α-Klotho [50].

The Klotho gene was identified in 1997, originally it was identified as an aging suppressor.

Inactive Klotho gene induces a premature aging-like syndrome and multiple organ dysfunction in transgenic mice [51] whereas overexpression extends mice life span [52].

Alpha-Klotho is a transmembrane protein mainly expressed in the kidney, in particular in distal tubular cells [53].

Experimental study have demonstrated that in the kidney, Klotho acts as an obligate co-receptor for FGF23, enhancing and conferring organ specificity to FGF23 hypo-phosphatemic effect [54].

Klotho can be enzymatically cleaved and released as a soluble form in different biological fluids [55]; Klotho protein is present in cerebrospinal fluid, blood, and urine of mammals.

Alpha-Klotho circulating can activate FGF-23 signal transduction, but at a lower level than the membrane-bound form.

Moreover, soluble Klotho has a paracrine function and FGF23 independent renal and extra-renal effects [56].

In particular, the soluble form of Klotho has the ability to inhibit the expression of sodium-phosphate cotransporter NaPi2a in the proximal tubules, thus inducing a phosphaturic effect enhancing the effect of FGF23 [57].

Moreover, soluble Klotho leads to an increase reabsorption of calcium inducing the activation of TRPV5 ion channels at the distal tubules levels [58].

All these evidences emphasize the role of the Klotho isoforms in the mineral and bone homeostasis regulation.

Mice lacking of FGFG 23 and/or Klotho have a similar phenotype, characterized by increases in phosphorus, calcium and vitamin D

serum levels, aging phenotype with osteopenia, vascular calcifications, and a shortened life span [59].

1.5 FGF23 and Klotho in CKD-MBD

In patients with CKD, hyperphosphatemia is often observed, due to an altered renal excretion of phosphorus, associated with renal impairment and decrease in number of physiologically active nephrons, leading to a profound imbalance of mineral homeostasis.

Several studies in CKD animal models and patients, reported that serum FGF23 levels increase in early stages of disease, preceding changes in calcium, phosphorus, or PTH levels [60]. Elevated levels of FGF23 are also consistently associated with lower estimated glomerular filtration rate (eGFR).

The increase in FGF23 can be considered an early adaptation mechanism to mineral metabolism changes during CKD, this increase of FGF23 seems to prevent the development of hyperphosphatemia at least in part by enhancing urinary phosphate excretion.

FGF23 production of by osteocytes might be the triggering that subsequently leads to overt CKD-MBD symptoms.

In patients with CKD the increase of FGF23 was also associated with the decrease of $1,25(OH)_2D$ [61], resulting in increased secretion of PTH in order to maintain normal serum Calcium level, but at the same time leading to a high bone turnover [62].

Klotho protein is essential for FGF23 function, but in CKD there is a contemporaneous increase of FGF23 levels and a decreased expression of Klotho protein [63].

Rotondi et al. [64] have reported that soluble plasma Klotho levels were significantly decreased in the early stages of CKD; moreover, they have found reduced levels of this protein since stage 2 of CKD. Klotho protein levels decreased with disease progression [65], thus causing resistance to FGF-23 in spite of rising levels of circulating FGF-23 and PTH [66].

In CKD patients, the level of FGF23 have a good clinical relationship with fractures, heart failure, and progression of kidney functional impairment [67].

In addition, a recent meta-analysis of seven studies, including ESRD patients has reported an association between FGF23 and increased mortality [68].

All together evidences are suggesting that FGF23 and Klotho may play a role in the development of CKD-MBD syndrome, although it is no clear what initial trigger causes the early increase of FGF23.

1.6 Klotho and FGF23 gene polymorphisms

Several studies in humans have highlighted the association between some polymorphic variants (SNPs) of the Klotho gene and various pathologies such as osteoporosis, stroke, essential hypertension, coronary artery disease and some features such as bone mineral density [68-69].

Some polymorphisms of the Klotho gene have been shown to be associated with the severity of renal impairment and the parameters of aging in patients with CKD [70-72].

In particular, Friedman DJ et al., [73] reported in their studies, an association between Klotho gene polymorphism (rs577912) and an increased risk of mortality in patients with chronic renal disease on hemodialysis.

Polymorphisms influencing Klotho gene expression might affect FGF-23 functional efficiency and in turn the risk of mortality and morbidity in CKD.

Similarly studies about some gene variants of FGF23, have suggested their potential role as risk factors for renal failure, cardiovascular insufficiency and stroke [74]. FGF23 polymorphisms as rs7955866 might affect biological proprieties and its interaction with FGF receptors and Klotho allows one to hypothesize a role in phosphate homeostasis. FGF23 rs7955866A variant is associated to an inhibition of sodium-phosphate cotransporters in the proximal tubule leading to an increased loss of urine phosphate [75].

1.7 IL-6 in primary osteoporosis and CKD-MBD

The immune system plays a critical role in the pathophysiology of osteoporosis, immune system cells and osteoclasts share many regulatory factors, including growth factors, transcription factors, and cytokines.

Some inflammatory cytokines such as IL-1, IL-6 and TNFα, can directly contribute to bone loss and pathogenesis of osteoporosis,

they can potentially up-regulate RANKL expression on osteoblasts, influence RANKL "signaling", and stimulate bone resorption [76], in addition pro-inflammatory cytokines suppress osteoblastogenesis.

It is know that Klotho deficiency is associated with oxidative stress and inflammation in experimental kidney disease models. Liu et al. reported that Klotho is involved in regulation of IL-6 expression in vitro and in vivo [77-78].

Moreover, Klotho deficiency might contribute to increased oxidative stress and inflammation, although there are not so many studies that identify the relationship between klotho, inflammation and hemodialysis patients.

The IL-6 expression is regulated, in women with primary osteoporosis, the physiological reduction of estrogen levels leads to an increase of IL-6 levels, with possible effects on bone metabolism. Inflammation and pro-inflammatory cytokines also play a role in chronic renal disease [79]; in CKD patients, especially among dialysis patients, there is an increase in oxidative stress and inflammation [80]. -

In these subjects, the increase in IL6 levels is associated with a higher risk of inflammation, atherosclerosis, cardiovascular events and mortality [81-84].

Furthermore, in these patients it was observed that biomarkers of inflammation (IL-1β, IL-1 receptor antagonist, IL-6, TNF-α, CRP, and fibrinogen) were inversely associated with residual kidney functionality [85].

The gene encoding IL-6 is located on chromosome 7 (7p21-24), single nucleotide polymorphisms in the IL-6 gene promoter region are associated with different circulating IL-6 levels [86].

The most studied polymorphism is the SNP -174 G / C of IL-6 (rs1800795), the CC genotype has been found associated with low BMD and bone mass loss, in osteoporosis [87].

However, published studies on the role of these SNPs in association with genetic predisposition to chronic kidney disease have provided conflicting data.

Two studies in particular showed that there is a link between patients with IL-6 GC genotype and terminal renal failure (ESRD) but there are no data on the role of this SNP in CKD-MBD [88-89].

2. Aim of the thesis

The mineral and bone metabolism is regulated by several factors, including the RANK / RANKL / OPG pathway and Klotho/FGF23 axis, these regulatory mechanisms, alongside the PTH / calcitonin / vitamin D axis, play a central role in tissue bone loss, both in patients with primary osteoporosis and in CKD-MBD.

Pro-inflammatory cytokines also play a central role both in primary osteoporosis and in bone complications of chronic renal disease.

CKD patients in particular End Stage Renal Disease dialyzed patients are in a chronic inflammation state characterized by an up-regulation of proinflammatory cytokines.

Primitive osteoporosis and CKD-MBD are multifactorial diseases, in which several factors are involved, in addition to hormonal and environmental factors, genetic factors play a central role.

Several studies in the literature, have tried to relate the role of polymorphisms of the genes involved in the homeostasis of mineral and bone metabolism with bone loss, but few and contradictory studies are available particularly for CKD-MBD.

The aim of this research was to assess the effect of polymorphisms of genes playing key roles in the regulation of calcium and phosphate metabolism at bone and kidney level, on risk of osteoporosis in the CKD-MBD syndrome.

For this purpose, the differences in the genetic background of subjects with CKD-MBD syndrome compared to healthy subjects and patients with primary osteoporosis were evaluated.

The presence and contribution of the following polymorphic variants were considered: rs7955866 of FGF23; rs577912 of Klotho1; rs3018362 of RANK; rs2277438 of RANKL; rs2073618 of OPG; rs1800795 of IL-6.

These allelic variants, in fact alone or interacting with each other, can potentially depict differences in the genetic risk profiles associated to primary osteoporosis and CKD-MBD syndrome.

3. Experimental section: materials and methods

3.1 Subjects

A total of 349 subjects of west Sicily ancestry were recruited for this study. One hundred and fifty-four patients: 94 (age 69±15, female 45%) with chronic kidney disease - mineral bone disorder (CKD-MBD) on dialysis and 60 (age 69±08, female 59%) with primary osteoporosis, confirmed by radiography or DEXA; blood samples were collected respectively from Unit of Nephrology and Hypertension, University of Palermo and from Department of Internal Medicine and Geriatrics.

One hundred ninety-five healthy subjects (age 58±18, female 52%) were enrolled as a control group.

This study received approval from local ethic committee and all participants gave their informed consent. Data were encoded to ensure privacy protection of patients and controls. All laboratory procedures were performed without knowledge about nature of material.

3.2 DNA extraction

DNA from subjects was extracted from peripheral blood and it were collected in EDTA sterile tubes using a salting out protocol.

Briefly, after 3-4 washings with SE 10x buffer (0,075M NaCl, 0,025M EDTA, pH 8) at 4100 rpm for 10 minutes, the hemolyzed

was incubated at 37 °C over-night, under continuous stirring, in a solution containing SE1x, 10% SDS and PtK (200 mg/ml).

Subsequently, the samples were treated with 200 µl of a supersaturated solution of NaCl 6M, which reduces the solubility of the proteins and facilitates their precipitation, and then agitated for 30 ".

After centrifugation at 13,000 rpm for 5 minutes, the supernatant containing DNA was recovered, transferred to a clean eppendorf tube, centrifuged until debris and impurities were completely removed.

Finally, the nucleic acid present in the supernatant was precipitated with the addition of a volume of cold absolute ethanol.

The quality of the extracted DNA, is checked at the spectrophotometer, measuring the absorbance at 260 and 280 nm and calculating the ratio, or using agarose gel electrophoresis, in TBE 1x buffer (TRIS 0.9, Boric Acid 0, 8 M, EDTA 0, 02 M) for 20 minutes to 80 volts.

3.3 SNP Genotyping: The KASPar Assay

Dedicated and pre-made competitive allele specific PCR (Polymerase Chain Reaction) assays (KASPar), developed by KBioscience (England) were used to perform the Allelic Discrimination tests for the typing of the following polymorphisms: FGF23 (rs7955866), Klotho1 (rs577912), RANK (rs3018362), RANKL (rs2277438), OPG (rs2073618);and IL6 (rs1800795).

In details, KASPar SNP genotyping method is an end-point PCR, based on a fluorescent FRET (Fluorescent Resonance Energy Transfer) system.

A mixture of unlabeled primers are specifically designed on the sequence of interest and it consists of:

1) two allele-specific forward primers, having a terminal common tag at the 5'end;

2) a common reverse primer;

3) two small oligonucleotides complementary to the 5' tag regions of the allele-specific forward primers, labeled with two different fluorophores (FAM, 6 carboxy-fluorescein; VIC, 2'- chloro-7'-phenyl-1,4-dichloro-6 carboxy-fluorscein).

4) two small oligonucleotides labeled with quenchers at the 3' end, complementary to those described in point 3 (Fig. 1).

KBioscience (England) specifically designed unlabeled primers on demand.

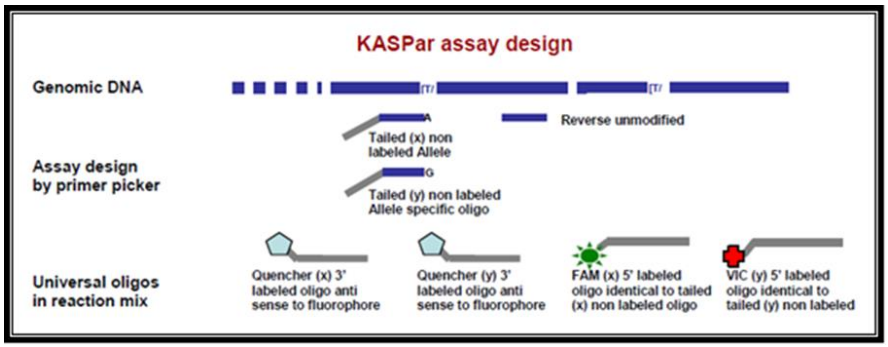

Fig 1: KASPar mix components

With the advancing of PCR cycles the labeled oligonucleotides bind, in a competitive way, the tag regions of forward primers rather than the quenching tags and will remain incorporated in the new synthesis molecules, releasing an increasing signal.

Briefly, 10 ng of DNA for each sample were used in an end-point KASPar PCR reaction, containing optimized master mix and the specific primer assay mix for each SNP, according to manufactory protocol in a final volume of 8 µl. Then, the amplification reactions were performed in the Master Cycler Gradient (Eppendorf, Germany), using a standard program consisting of the following cycles:

1) 94 °C for 15';

2) 94 °C for 20";

3) Touchdown 61-55°C per 60";

4) Repeating steps 2 and 3 for 10 cycles (Drop - 0.6°C/for each annealing cycle);

4) 94 °C for 20";

5) 55 °C per 60";

6) Repeating steps 4 and 5 for 26 cycles.

Finally, plates were scanned at the temperature of 4°C in a 7300 Real-Time ABI Prism PCR System (Applied Biosystems, USA) to register the fluorescence emission for each well. Then samples were graphically grouped by the SDS software vs.1.3 (Applied Biosystem,

USA) in three genotypic clusters (homozygous and heterozygous subjects), easily recognizable in the Allelic Discrimination plot on the basis of the two probe's fluorescence intensity emission.

3.4 Statistical Analysis

Allele and genotype frequencies were evaluated by gene count.
Data were tested for goodness of fit between observed and expected genotype frequencies according to Hardy-Weinberg equilibrium, by Chi square test.
Significant differences in homozygous and heterozygous genotype distributions among groups were calculated by using Chi square test and appropriate tables. Multiple logistic regression models were applied using dominant (major allele homozygotes vs. heterozygotes plus minor allele homozygotes) and recessive (major allele homozygotes plus heterozygotes vs. minor allele homozygotes) models. Odds ratios (OR), 95% confidence intervals (95% C.I.), and p-values were determined. A $p<0.05$ was considered statistically significant. Data were analyzed with GraphPad InStat software version 3.06 (San Diego California USA).

4. Results

Patients and control groups were typed for rs7955866 SNP of FGF23, rs577912 of Klotho1, rs3018362 of RANK, rs2277438 of RANKL, rs2073618 of OPG and rs1800795 of IL6.

The analysis of allelic frequencies has highlighted that the frequency of IL6 C allele (rs1800975) was significantly higher in controls than in cases (Tables 1-2).

The distribution of genotype frequencies between patient affected by primary osteoporosis and healthy control showed a statistically significant difference for SNP rs1800795 of IL-6, in particularly the C allele positive genotypes have a higher frequency in controls than in cases as shown in table 1.

Moreover using dominant (major allele homozygotes vs. heterozygotes plus minor allele homozygotes) model the possible protective role of this allele was confirmed (**IL-6 C/* Vs GG**: OR= 0.33 C.I: 0.15-0.71 P=0.004),

Similarly, comparing genotype distributions and allele frequencies of CKD-MBD patients and healthy control (Table 2), significant differences were observed. Dominant model: IL-6 C / * Vs GG: OR = 0.4; C.I = 0.23-0.68 P = 0.0007.

Together these data indicate a protective role of C allele of IL-6 rs1800795, associated to low production of this pro-inflammatory cytokine.

In addition, no statistically significant differences were found in the analysis of genotype distribution between patient affected by primary osteoporosis and CKD-MBD patients (Data not shown).

IL-6 (rs1800975)		OSTEOPOROTIC PATIENTS n/ (%)	HEALTHY CONTROLS n/ (%)	O.R	C.I.	p
	GG	29 (74%)	95(49%)	3.05	1.41-6.61	**0.0045**
	CG	10 (26%)	76 (39%)	0.54	0.25-1.17	0.1457
	CC	0	24 (12%)	0.09	0.005-1.49	**0.0177**

Table 1: Frequencies of polymorphism of IL-6 in patients affected by primary osteoporosis and healthy controls

IL-6 (rs1800975)		CKD-MBD PATIENTS n/ (%)	HEALTHY CONTROLS n/ (%)	O.R	C.I.	p
	GG	60 (70%)	95(49%)	2.53	1.47-4.36	**0.0007**
	CG	21 (25%)	76 (39%)	0.513	0.29-0.91	**0.0285**
	CC	4(5%)	24 (12%)	0.35	0.12-1.05	**0.0536**

Table 2: Frequencies of polymorphism of IL6 in CKD-MBD patients and in healthy controls.

As shown in Table 3, the analysis of the polymorphisms in RANK / RANKL / OPG genes, performed between CKD-MBD subjects and population control showed a different distribution of some RANK genotypes.

In particular, the G allele of RANK has a higher frequency in control population compared to CKD-MBD patients. In addition applying

dominant model (major allele homozygotes vs. heterozygotes plus minor allele homozygotes), the significantly increase of rs3018362 A positive genotype frequency might indicate this allele of RANK associated to increased osteoclast activity as one of genetic risk factor for CKD-MBD (A/* Vs GG OR= 1.88 C.I.= 1.07-3.30 p = 0.0341).

On the other hands, no statistically significant difference for this polymorphism was found in comparing genetic background between patients affected by primary osteoporosis and population control, and between osteoporotic and CKD-MBD patients (Data not shown).

Similarly, no significant results were observed for the rs2277438 polymorphism of RANKL gene (Data not shown).

RANK (rs3018362)		CKD-MBD PATIENTS n/ (%)	HEALTHY CONTROLS n/ (%)	O.R	C.I.	p
	GG	31 (39%)	71 (54%)	0.53	0.30-0.94	**0.0341**
	AG	45 (55%)	52 (39%)	0.52	0.30-0.91	**0.024**
	AA	5 (6%)	9 (7%)	0.89	0.29-2.78	1

Table 3: Genotype frequencies of RANK gene single nucleotide polymorphisms in CKD-MBD patients and in healthy controls.

Next, comparing genotype distributions and allele frequencies of the patients affected by primary osteoporosis and controls, significant differences were observed only for OPG rs2073618 polymorphism, as shown in tables 4a, 4b and 4c.

OPG (rs2073618)		OSTEOPOROTIC PATIENTS n/ (%)	HEALTHY CONTROLS n/ (%)	O.R	C.I.	p
	GG	23 (45%)	35 (29%)	1.9	1.00-3.88	**0.054**
	CG	24 (47%)	59 (50%)	0.9	0.47-1.74	0.8672
	CC	4 (8%)	25 (21%)	0.32	0.11-0.97	**0.0447**

Table 4a: Genotype frequencies of OPG gene single nucleotide polymorphisms in patients affected by primary osteoporosis and in healthy controls.

OPG (rs2073618)		OSTEOPOROTIC PATIENTS n/ (%)	CKD-MBD PATIENTSS n/ (%)	O.R	C.I.	p
	GG	23 (45%)	28 (35%)	1.37	0.62-2.08	0.4674
	CG	24 (47%)	31 (39%)	1.50	0.73-3.07	0.2775
	CC	4 (8%)	20 (26%)	0.25	0.08-0.79	**0.0191**

Table 4b: Frequencies of polymorphism of OPG in patients affected by primary osteoporosis compared to CKD-MBD patients. (OPG C/* vs GG (P=0,0191 OR =0.25 C.I =0.08-0.79)

OPG (rs2073618)	MODEL	O.R	C.I	p
	OPG C/* Vs GG			
	Osteoporosis Patients Vs Healthy Controls	0.51	0.26-0.99	**0.054**
	Osteoporosis Vs CKD-MBD patients	0.67	0.33-1.37	0.2775
	OPG G/* Vs CC			
	Osteoporosis Patients Vs Healthy Controls	1.97	1.00-3.88	**0.054**
	Osteoporosis Vs CKD-MBD patients	1.49	0.72-3.07	0.277

Table 4c: Multiple logistic regression analyses of dominant (major allele homozygotes versus heterozygotes plus minor allele homozygotes) and recessive (major allele homozygotes plus heterozygotes versus minor allele homozygotes) models applied to osteoporotic patient group compared with healthy control and CKD-MBD patients.

In detail, comparing patients affected by primary osteoporosis and healthy controls, C allele is more represented in healthy controls than in osteoporotic patients, thus C allele seems to play a protective role in subjects with primary osteoporosis, as shown in Tables 4a and 4c. Table 4b, shows the comparison between patients with primary osteoporosis and CKD-MBD patients, the latter have a higher frequency of the C allele, confirming that the reduction of C allele frequency characterize only primary osteoporosis patient group suggesting a different role of rs2073618 genotypes in primary and secondary osteoporosis. Accordingly, no statistically significant differences were observed comparing CKD-MBD patients and healthy controls (Data not shown).

The analysis of FGF23 (rs7955866) and Klotho (rs577912) gene SNPs does not allow finding significant differences in genotype

frequencies among the three populations examined (Data not shown). Considering that non-Mendelian multifactorial pathologies are characterized by complex genotype interactions that might influence the disease phenotypes, we searched association between complex combined genotypes with susceptibility or protection against primary osteoporosis or CKD-MBD.

For this analysis genotypes of the RANK / RANKL / OPG pathway and those of the FGF23 / Klotho axis and IL-6 were clustered, as reported in the table 5, and frequency of all possible complex genotype combination were compared among the different subject groups.

GENES	SNPs	RISK GENOTYPE	NO RISK GENOTYPE
IL-6	rs1800795	GG	C/*
KLOTHO	rs577912	CC	G/*
FGF23	rs7955866	GG	A/*
RANK	rs3018362	A/*	GG
RANKL	rs2277438	G/*	AA
OPG	rs2073618	GG	C/*

Table 5: Classification of risk genotype

Table 6 shows the odds ratio of the combined genotypes identified as relevant by statistical analyses.

Our results indicated that the cotemporaneous presence of Klotho rs577912CC (associated to a Klotho production reduced respect A positive genotypes) and IL-6 rs1800795GG (associated to a IL-6 synthesis increased respect C positive genotypes) combined genotype is statistically significant susceptibility factor both for CKD-MBD syndrome and primary osteoporosis.

Conversely, the cotemporaneous presence of Klotho A positive genotypes and IL-6 C positive genotypes seems to have protective role at least for CKD-MBD subjects.

The analysis of the combined RANK, RANKL and OPG genotypes showed that the presence of rs3018362GG genotype of RANK gene (considered as associated to a reduced production of the cytokine respect to A positive genotypes) [30, 36], might be protective against primary osteoporosis even if the heterozygous, potentially risk associated rs2277438AG genotype is expressed at RANKL gene. In addition a possible but not statistically significant protective effect of this genotype against primary osteoporosis seems to be identified in combination with OPG rs2073618GC potentially protective genotype (C allele actually seem to be associated to an increased OPG production respect A allele) [37].

SNP allele combinations	CKD-MBD patients Vs Healthy controls			Osteoporotic patients Vs Healthy Controls		
	O.R.	CI	p	O.R	CI	p
Kloto rs 577912 CC IL6 rs1800795 GG	2.06	1.18-3.59	**0.0115**	3.31	1.54-7.13	**0.0022**
Kloto rs 577912 AC IL-6 rs1800795 CG	0.24	0.07-0.85	**0.018**	--	--	--
RANK rs 3018362 GG RANKL rs2277438 AG	--	--	--	0.15	0.02-1.16	**0.00406**
RANK rs 3018362 GG OPG rs2073618 GC	--	--	--	0.39	0.15-1.00	0.0636
RANK rs 3018362 GG RANKL rs2277438 AA	0.56	0.31-1.04	0.07	--	--	--

Table 6: Odds ratio of SNP combined genotype identified as relevant by statistical analyses.

5. Discussion and conclusions

Disorders of mineral metabolism and bone diseases are common complications in patients with chronic kidney disease, especially in that undergoing hemodialysis therapy.

Subjects that develop bone disease associated chronic kidney failure are classified in Chronic Kidney Disease - Mineral Bone Disorder (CKD-MBD) syndrome, a systemic disorder that occurs in chronic kidney disease characterized by osteoporosis and vascular calcifications causing an increased mortality and morbidity rate [90]. The pathogenesis of alteration of bone metabolism in patients with CKD has not been fully explained.

Several studies have shown that genetic factors play an important role in the pathogenesis of osteoporosis and CKD-MBD [91-94]. Over the last decades, numerous candidate genes have been investigated, and linked with bone density and risk of fracture in osteoporosis patients, however few data are available about CKD-MBD.

In this study, we analyzed genetic polymorphisms in the pathogenetic mechanisms of primary osteoporosis and CKD-MBD syndrome aiming to find markers useful to better define osteoporosis and CKD-MBD genetic risk and design possible new prevention and therapeutic strategies.

Our results shown that, IL-6 rs1800975C allele is more represented in our controls population than in osteoporotic patients and CKD-MBD suggesting a protective role of this genotype.

This result is in agreement with previous data by Ferrari SL et al. [87] that reported a reduced risk for osteoporosis in women carrier of C allele and association of CC genotype with a high bone mineral density and a low decrease in bone mass, in osteoporosis.

As well known C allele is associated with a lower production of IL6, so it is not surprising that a genetically determined reduced production of the pro-inflammatory cytokine IL-6 implied in osteoclast activation [76] might be protective against primary osteoporosis. It seems of interest, and at best of our knowledge for the first time, that a similar protective effect have been found in CKD-MBD patients by our analysis.

The CC genotype of Klotho was associated with a decrease in Klotho mRNA levels, and was also associated with risk of mortality in subjects on dialysis [95]

We were not able to demonstrate any association of Klotho rs577912 polymorphisms and primary osteoporosis or CKD-MBD.

Moreover, in spite of well-known role of FGF23/ Klotho axis in pathogenesis of CKD and CKD-MBD [63] no association of FGF23 rs7955866 SNP genotypes alone or in combination with Klotho rs577912 polymorphisms with primary osteoporosis or CKD-MBD was been found.

Conversely, analyzing combined genotypes, we demonstrated that the presence of Klotho rs577912CC and IL-6 rs1800975GG genotype might be a susceptibility factor for both primary osteoporosis and CKD-MBD syndrome.

It is known that the expression of IL-6 is down regulated by Klotho [78], so a genetically determined reduction of Klotho levels in subjects lacking the rs1800975C allele of IL-6 might be considered a

worse genetic milieu that might facilitate bone loss particularly in CKD subjects.

These data were confirmed by the analysis of the combined genotypes, our results suggest that the presence of Klotho CC- IL6 GG genotype might be a susceptibility factor for primary osteoporosis and CKD-MBD syndrome.

The results obtained from the genotyping of polymorphisms in RANK/RANKL/OPG genes, showed a different distribution of the RANK genotypes, between CKD-MBD patients and healthy control, although the specific function of the SNPs genotyped is not known

In particular, our study showed that the allele A of RANK (rs3018362), is more represented in the CKD-MBD patients than in the control population. These data suggests that the allele A of RANK could increase the effect of the protein on the osteoclasts.

Actually, some studies have shown that the minor allele of SNP rs3018362 is associated with a low frequency of BMD [96], although other studies have not reported the same association [97].

The TNFRSF11B gene, coding for OPG has been considered as candidate gene in the pathogenesis of osteoporosis.

OPG gene is located at 8q24.12 and it contains several polymorphisms, including OPG 1181 G>C (rs2073618), resulting in changes form Lys3Asn.

In this study, the comparison between the genetic background of subjects with primary osteoporosis and control population showed that the allele C of OPG (rs2073618) could play a protective role. Accordingly, it was proven that rs2073618 is functionally relevant; patients carrying C allele of OPG show a higher average protein concentration. [98].

Instead, the comparison between subjects with dialysis and population control, showed no statistically significant differences for this SNP, so the protective role of this allele is not confirmed in subjects with CKD-MBD.

Therefore, the role of OPG rs2073618 in pathogenesis of CKD-MBD might be marginal and other OPG SNP should be studied.

The data obtained agrees with the literature data that highlighted a reduced risk of osteoporosis for the carrier women of the C allele of OPG [37].

Several studies show conflicting data concerning the role of this SNP of OPG in association with osteoporosis, even though some of these studies have suggested a possible role of this polymorphism as a genetic factor in osteoporosis [99].

The analysis of RANK / RANKL combined genotypes in patients with CKD showed that the presence of rs3018362GG genotype of RANK gene seems to have a protective role even if the heterozygous, potentially risk associated rs2277438AG genotype is expressed at RANKL gene.

Analysis of the combined RANK/OPG genotypes do not allow drawing substantial conclusions as the statistics do not produce significant results. The rough marginal significance of the possible protective role of the contemporaneous presence of RANK rs3018362GG and OPG rs2073618GC observed for primary osteoporosis, might confirm the above reported data.

In conclusion, the comparison of the genetic background of patients with primary osteoporosis and that of subjects with CKD-MBD, has shown that some polymorphisms of IL-6, Klotho, RANK, RANKL

alone or combined in complex genotypes might have a role in genetic susceptibility or protection against CKD-MBD.

These results might open new perspectives for the analysis of CKD-MBD susceptibility factors and prevention.

Actually, these findings, which need certainly to be confirmed, might prompt studies on pharmacological strategies to prevent CKD-MBD in predisposed subjects.

Further evaluations are warranted, in order to identify the biological mechanisms by which these polymorphisms influence osteoporotic phenotype in dialyzed patients.

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