METABOLIC AND NON-TRADITIONAL RISK FACTORS OF DEATH AND CARDIOVASCULAR EVENTS IN AGING PATIENTS WITH END-STAGE RENAL DISEASE

Metabolic and non-traditional risk factors of death and cardiovascular events in aging patients with end-stage renal disease

Metabole en niet-traditionele risicofactoren voor sterfte en cardiovasculaire voorvallen bij ouder wordende patiënten met nierziekte in het eindstadium

Proefschrift

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A chi non ha mai smesso di credere in me.
A chi ha fatto in modo che anch'io ci credessi.

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Chapter 1

General Introduction

GENERAL INTRODUCTION

Recent progress in the field of medicine (both for the prognosis and the diagnosis of diseases), together with improved therapeutic resources, resulted in a significant lengthening of the life span. However, the main implication of a better life expectancy is the concurrent increase in the prevalence of chronic diseases, typically associated with age. This is particularly true for Chronic Kidney Disease (CKD), whose prevalence has recently reached 13% worldwide [1]. Furthermore, the mean age of patients affected (from stage G1 to G5-D) has been increasing, as expected.

Patients with End-Stage Renal Disease (ESRD) have an almost uniquely high risk of death and cardiovascular disease, with a rate of incident cardiovascular events strongly associated with the level of renal function both in community-based studies and in selected populations with established cardiovascular disease [2].

As an additional complication, contrarily to the general population, where as much as the 75% of excess risk for coronary heart disease could be explained by classical, Framingham risk factors [3], the excess of risk of cardiovascular diseases (CVD) in elderly CKD patients it is not so easy to explain, probably because the burden of comorbidities is similar in these patients, and this makes it difficult to stratify the risk according to known, classical risk factors. Furthermore, it is estimated that, as renal function deteriorates, the risk increases linearly, making chronic renal insufficiency a strong 'risk amplifier'. For this reason, several efforts have been made to discover non-classical (i.e. uremic) risk factors. Positive sodium balance, responsible of volume expansion and pressure burden on the left ventricle, anaemia, high calcium–phosphate product, inflammation, hyperhomocysteinemia, and impaired nitric oxide (NO) synthesis, due to accumulation of NO synthase inhibitors, all might contribute to the increase in cardiovascular risk in patients with ESRD [4].

In this new category of chronic patients, elderly and with a high prevalence of risk factors, it appears tremendously challenging to be able to stratify the risk of death and cardiovascular (CV) events.

More recently, novel risk factors, such as inflammation and endothelial dysfunction markers have been recognized as potential risk factors for cardiovascular morbidity and mortality in ESRD and dialysis patients [5–9]. Following this new trend, my research group has dedicated the last years to the identification of emerging risk factors to be used for risk stratification in CKD population.

This approach has already led to the finding that asymmetric dimethylarginine (ADMA) an established risk factor for cardiovascular disease and all-cause mortality in general population [10], in patients with coronary artery disease [11, 12] and CKD [13–15] interacts with uric acid in predicting CKD progression [16]. ADMA levels are strongly and positively associated with sympathetic nerve activity in CKD patients,

suggesting that they may share a pathway leading to renal disease progression, proteinuria, and left ventricular (LV) concentric remodelling in CKD patients [17].

The aim of this thesis is to describe novel metabolic and clinical factors in an aging population with CKD, in order to show their crucial role for risk stratification and risk monitoring in end-stage renal disease patients, and to underlie their role in the high risk of death and cardiovascular events in the dialysis population.
Metabolic and clinical factors are described in chapter 2, whereas in chapter 3 novel instruments for risk stratification are reported, as detailed in the next paragraphs.

Chapter 2.1 describes how paricalcitol, an analogue of Vitamin D, raises serum sclerostin levels. Sclerostin is an osteocyte glycoprotein that reduces bone formation, whose synthesis in osteocytes is tightly regulated by mechanical loading, cytokines, parathormon (PTH) and calcitonin and, in primary osteoblasts in culture, by $1,25(OH)_2$ Vitamin D. It is also described in what extent the effect of paricalcitol on sclerostin is modified by circulating pentosidine, one of the Advanced Glycosylation End Products (AGEs).

In chapter 2.2 two metabolic biomarkers are described: Alkaline Phosphatase (Alk-Phos) and γ-Glutamyl-Transpeptidase (GGT). AlkPhos is an enzyme which catalyses the hydrolysis of pyrophosphate, the main calcification inhibitor, and its association with the risk of death in ESRD is well documented. GGT is now regarded as one of the most robust indicators of whole body oxidative stress and a strong predictor of mortality in the same population. The effect modification of GGT on the link AlkPhos - all-cause/cardiac mortality is described in this chapter.

In the second part of this thesis (Chapter 3), novel instruments for risk stratification are discussed. In Chapter 3.1 it is reported how self-reported snoring amplifies the risk of death and cardiovascular events in ESRD patients on dialysis with heart failure. This questionnaire was validated for its capability of predicting sleep disordered breathing (SDB), showing a high reliability for excluding SDB.

Chapter 3.2 describes the diagnostic reliability of pulmonary crackles and peripheral oedema as a clinical sign of pulmonary congestion as compared with Ultrasound B-lines (US-B lines), a well-validated measure of pulmonary water in patients with cardiovascular disease and in intensive care patients, as well as a strong prognostic factor for death and cardiovascular events in ESRD.

In Chapter 3.3 the prognostic value of the components of the Rand- QoL Short Form 36 (SF36) for mortality has been measured in a large cohort of very old haemodialysis patients. Among all domain of the SF36, I found that the physical activity component is the one holding the highest prognostic value. Furthermore, physical function held a

robust risk reclassification ability, i.e. the ability to correctly reclassify high risk patients versus low risk patients as identified by standard risk factors.

Finally, in chapter 3.4, the relationship between physical performance, as assessed by the Six-Minutes Walking Test, and mortality, cardiovascular events and hospitalizations in dialysis patients has been analysed. The results of this analysis suggest that baseline physical performance is a strong predictor of adverse clinical outcomes in this population.

I have performed these analyses within the framework of four studies.

The PENNY (Paricalcitol and Endothelial Function in Chronic Kidney Disease Patients) Study is a double-blind, randomized trial (ClinicalTrials.gov identifier: NCT01680198) performed in Reggio Calabria, Italy. Inclusion criteria were parathormone > 65 pg/ml, serum total calcium between 2.2 and 2.5 mmol/L, phosphate levels between 2.9 mg/dL and 4.5 mg/dL, negative serum pregnancy test for female subjects of childbearing potential. Exclusion criteria were treatment with vitamin D compounds or anti-epileptic drugs, cancer, symptomatic cardiovascular disease or liver disease. Patients who met the inclusion criteria were randomized (1:1) to receive 2 µg paricalcitol once daily or matching placebo for 12 weeks after a 2-week run-in. The dose of paricalcitol was adjusted on the basis of serum parathormone and calcium and the maximum dose allowed was 2 µg daily. No vitamin D compounds were allowed during the trial. The study enrolled 88 patients with CKD stage 3 to 4. Primary outcome was endothelial function measurement at 12 weeks from baseline. Secondary outcomes were endothelial function, plasma/serum and genetic biomarkers of bone mineral disorders in CKD (BMD-CKD) and renin angiotensin-aldosterone system (RAS) at 12 weeks from baseline.

The PROGREDIRE (Prospective Registry of The Working Group of Epidemiology of Dialysis Region Calabria) study is a multicentre, cohort study involving 35 dialysis units in two regions in Southern Italy (Calabria and Sicily). No inclusion or exclusion criteria were applied. In total, 1189 dialysis patients were enrolled. Primary aim of this study was to investigate new biomarkers of cardiovascular risk in dialysis patients.

The LUST (Lung Water by Ultrasound Guided Treatment in Haemodialysis Patients) study is a European, multicentre, open, randomized, controlled trial aimed at assessing the usefulness of US-B lines in preventing adverse clinical outcomes (mortality, cardiovascular events, hospitalizations, progression of LVH and LV dysfunction) in haemodialysis patients at high cardiovascular risk (ClinicalTrials.gov Identifier: NCT02310061). The study is currently ongoing, and the number of patient enrolled so far is 347. Inclusion criteria are age > 18 years, dialysis vintage > 3 months, a history of myocardial infarction with or without ST elevation or unstable angina, acute coronary syndrome, documented by ECG recordings and cardiac troponins, or stable angina pectoris with documented coronary artery disease by prior coronary angiography or ECG or dyspnoea class III-IV NYHA. Exclusion criteria are cancer or other advanced non cardiac disease or comorbidity imposing a very poor

short-term prognosis, active infections or relevant inter-current disease, inadequate lung scanning and echocardiographic studies. Patients who met the inclusion criteria were randomized to extra-vascular lung water measurements by ultrasound or standard protocol of fluid management in haemodialysis.

The EXCITE (EXerCise Introduction To Enhance Performance in Dialysis) study is a multicentre, randomized, controlled trial performed in Italy on the effectiveness of exercise in improving physical performance and the quality of life (primary outcome) and in reducing adverse clinical outcomes (mortality, cardiovascular events and hospitalizations) (secondary outcome) in dialysis patients (ClinicalTrials.gov Identifier: NCT01255969). Inclusion criteria were dialysis vintage >6 months, age>18 years, stable clinical conditions. Exclusion criteria were physical or clinical limitations to deambulation. The intervention consisted in a personalised exercise program to be performed at home. The total number of patients who participated in this study is 296.

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Chapter 2

Novel metabolic and clinical factors in patients with End-Stage Renal Disease

Chapter 2.1

Active Vitamin D treatment in CKD patients raises serum sclerostin and this effect is modified by circulating pentosidine levels

ABSTRACT

$1,25(OH)_2$Vitamin D increases the expression of sclerostin gene. Whether vitamin D receptor activation (VDRA) influences serum sclerostin in Chronic Kidney Disease (CKD) and whether compounds interfering with VDRA like Advanced Glycosylation End Products (AGEs) may alter the sclerostin response to VDRA is unknown.

Eighty-eight stage G3-4 CKD patients randomly received 2 µg paricalcitol (PCT) /day (n=44) or placebo (n=44) for 12 weeks. Sclerostin, a major AGE compound like pentosidine and bone mineral disorder biomarkers were measured at baseline, at 12 week and 2 weeks after stopping the treatments.

At baseline, in the whole study population sclerostin correlated with male gender (P=0.002), Body Mass Index (BMI) (p<0.001), waist circumference (P<0.001), serum pentosidine (p=0.002) and to a weaker extent with diabetes (P=0.04), $1,25(OH)_2$Vitamin D (r=0.22, P=0.04) and serum phosphate (r=-0.26, P=0.01).

Sclerostin increased during PCT treatment (average +15.7 pg/ml, 95% CI: -3.0 to +34.3) but not during placebo (P= 0.03) and the PCT effect was abolished 2 weeks after stopping this drug. The increase in sclerostin levels induced by PCT was modified by prevailing pentosidine levels (P=0.01) and was abolished by statistical adjustment for simultaneous changes in PTH but not by FGF23 changes.

VDRA by paricalcitol causes a moderate increase in serum sclerostin in CKD patients. Such an effect is abolished by adjustment for parathormon (PTH) suggesting that it may serve to counter PTH suppression. The sclerostin rise by PCT is attenuated by pentosidine, an observation in keeping with in vitro studies showing that AGEs alter the functioning of the VDRA.

Keywords: vitamin D, sclerostin, pentosidine, chronic kidney disease

INTRODUCTION

Sclerostin is an osteocyte glycoprotein with a C-terminal cysteine knot-like (CTCK) domain [1]. This glycoprotein reduces bone formation by binding to low-density lipoprotein receptor-related protein 5 and 6 (LRP5 and LPR6), a fundamental pathway for bone formation [2]. The synthesis of this glycoprotein in osteocytes is a process tightly regulated by mechanical loading [3], cytokines [4, 5], parathormon (PTH) [6] and calcitonin [7] and studies in primary osteoblasts in culture show that $1,25(OH)_2$ Vitamin D (VD) dose-dependently increases the expression of sclerostin gene [8]. Studies with inactive forms of vitamin D in healthy elderly men [9] and in vitamin D deficiency/insufficiency [10] show that vitamin D may induce mild to moderate increases in serum sclerostin in these populations. Bone mineral balance has a peculiar hormonal setting in CKD and the response of the vitamin D receptor to active vitamin D is altered in this condition [11]. However, to our knowledge there is no intervention study testing the sclerostin response to vitamin D compounds in CKD.

Advanced Glycosylation End Products (AGEs) interfere with normal osteoblast development [12] and function [13] and inhibit osteoclastic differentiation [14]. AGEs are markedly increased both in type 2 diabetes [15] and in Chronic Kidney Disease (CKD) patients [16] and pentosidine, a major AGE [17], is an inverse correlate of bone turnover in advanced CKD [33] and predicts the risk of fracture in type-2 diabetes [19, 20]. Of note, due to their pro-oxidant ability at cell level [21] and the strong influence of pentosidine, on bone cell function [13] these compounds may in theory affect sclerostin expression in the same cells. AGEs directly alter the functioning of the vitamin D receptor [22]. Furthermore, in an animal model overexpressing a major anti-oxidant enzyme, para-oxonase, sclerostin gene expression is markedly reduced [23].

We have recently performed a clinical trial testing the effect of an activated form of vitamin D (paricalcitol) on vascular function in CKD patients [24]. During this trial we created a Biobank aimed at exploring the relevance of vitamin D receptor activation (VDRA) for the CKD-related bone mineral disorder (CKD-BMD) (clinicaltrials.gov identifier: NCT01680198). Herein we report the effect of VDRA by paricalcitol (PCT) on serum sclerostin and other mineral-bone disorder (MBD) biomarkers as tested in the setting of the same double-blind randomized trial. Given the peculiar relevance of AGEs on oxidative stress [25] in CKD patients and on the effects of pentosidine on bone cell functioning [13], we also tested whether this AGE compound which we previously associated with bone turnover in CKD [18], may influence the sclerostin response to PCT in CKD patients.

METHODS

The study protocol was approved by the ethics committee of our hospital, and all patients provided written informed consent. The protocol of the PENNY trial as well as

the CONSORT flow diagram were reported into detail in the source study [20]. In brief, PENNY enrolled stage G3-4 CKD patients with age ranging between 18 and 80 years, PTHi > 65 pg/ml, serum total Calcium (Ca) between 2.2 and 2.5 mMol/L and Phosphate levels between 2.9 mg/dl and 4.5 mg/dl who were not being treated with vitamin D compounds or anti-epileptic drugs, without neoplasia or symptomatic cardiovascular disease or liver disease. After baseline measurements, CKD patients were randomized (double blinded) to receive 2 μg PCT capsules (or matching placebo) daily, for 12 weeks. This dose was adjusted on the basis of serum PTHi and Ca and the maximum dose allowed was 2μg daily.

Biochemical measurements and GFR

Serum calcium, phosphate, glucose, lipids were measured in the routine clinical pathology laboratory at our institution. Plasma PTH was measured by IRMA (DiaSorin Stillwater, MN, USA, normal range 13-54 pg/ml); 1.25 OH_2 VD by RIA (Immunodiagnostic Systems, Boldon, UK, normal range 18.1-70.6), and FGF23 by ELISA (Kainos Laboratories, Bunkyo, Tokyo, Japan, normal range: 8.2–54.3 pg/mL). Serum creatinine was measured by the Roche enzymatic, IDMS calibrated, method and serum cystatin C by the Siemens Dade Behring kit which is traceable to the International Federation of Clinical Chemistry Working Group for Standardization of Serum Cystatin C and the GFR was calculated by the CKD-Epi Creatinine-Cystatin formula [26]. Sclerostin was measured by ELISA (R&D Systems, Ltd., Abingdon, United Kingdom, normal range: 131 – 1156 pg/ml). Pentosidine was measured by EIA (Cusabio, Wuhan, Hubei Province, P.R.China, normal range: 36.6 – 60.3).

Statistical analysis

Data are reported as mean ± standard deviation (normally distributed data), median and inter-quartile range (non-normally distributed data) or as percent frequency. Comparisons between groups were made by independent T-Test, Mann-Whitney Test, or Chi Square test, as appropriate. Correlates of sclerostin, pentosidine and of PCT-induced changes in serum sclerostin were identified by standard correlation analysis and multiple regression analysis was used to determine the independent correlates of sclerostin. The effect of paricalcitol on serum sclerostin levels was analysed by comparing the changes in sclerostin in paricalcitol and in placebo treated patients by using the T-Test for independent observations. The influence of seasons on the sclerostin response to PCT was assessed creating 3 dummy variables for spring, summer and autumn (winter was considered as the reference season). The effect modification by pentosidine on the paricalcitol-induced changes in circulating sclerostin was assessed including the interaction term (pentosidine*PCT) in unadjusted and adjusted regression models. Data analysis was performed by SPSS for Windows (version 20.0, Chicago, Illinois, USA) and STATA (version 11, College Station, Texas, USA).

RESULTS

Serum samples for sclerostin measurement were available in all patients who participated into the PENNY trial both at baseline and after 12 weeks treatment with PCT or placebo. The main baseline characteristics of the whole study cohort and of the patients as randomized to PCT and placebo are reported in Table 1. Overall, patients randomized to the active and control group were similar for demographic, clinical and biochemical characteristics and the diagnosis of renal disease [20]. The eGFR tended to be higher in patients randomized to PCT (P=0.06) (Table 1), whereas FGF-23 tended to be higher in the placebo group (P=0.07). Sclerostin at baseline (Table 1) was by 9% higher in the placebo group (average 155 pg/ml) as compared to the paricalcitol group (141 pg/ml) but the difference was largely non-significant (P=0.15). No differences were noticed as for the use of antihypertensive drugs (P ranging from 0.19 to 1.00), statins (P=0.52), hypoglycaemic agents (P=0.11), insulin (P=0.76), antiplatelet drugs (P=0.39), nitrates (P=0.40), proton pump inhibitors (P=0.20), iron preparations (P=0.75) and Erythropoietin Stimulating Agents (P=0.20), whereas calcium binders were more frequently prescribed to patients randomized to the placebo group (P<0.01). In both groups all treatments were maintained unchanged across the trial.

Descriptive analysis of functional correlates of sclerostin at baseline
In the whole study population baseline sclerostin was significantly higher in men (158.3 pg/mL, IQR: 132.9 pg/mL – 244.6 pg/mL) than in women (114.1 pg/mL, IQR: 82.8 pg/mL – 186.9 pg/mL) (P=0.002) and correlated directly with age (r= 0.23, P=0.03). Sclerostin was also directly related with major anthropometric parameters including height (r= 0.36, P=0.001), weight (r= 0.42, P=0.001) and waist (r= 0.35, P=0.001) circumference (Fig. 1) as well as with serum cholesterol (r= -0.28, P=0.008), diabetes (diabetic patients: 205.4 ± 117.3 pg/mL, non-diabetic patients: 156.2 ± 78.5 pg/mL; P=0.04) and with serum pentosidine (r=0.33 P=0.002) (Fig.2, upper panel). Of note the correlation between pentosidine and sclerostin remained highly significant (β: 0.31, P=0.001) in analyses adjusting for age, gender, height, weight and diabetes
Among MBD biomarkers, sclerostin associated in an inverse fashion with serum phosphate (r= -0.26, P=0.01) and directly with $1.25OH_2VD$ (r= 0.22, P=0.04) (Fig.1), but was independent of serum calcium (r=-0.10, P=0.53) and PTH (r=-0.16, P=0.14).

Tab. 1 *Main demographic, anthropometric, and clinical characteristics in patients as divided according to randomization group.*

	Paricalcitol group (n=44)	Placebo group (n=44)	P
Age (years)	63±11	62±12	0.65
Male sex (%)	59%	70%	0.27
Current smokers (%)	12%	19%	0.37
Past smokers (%)	45%	41%	0.66
BMI (kg/m²)	29±5	29±5	0.66
Systolic/Diastolic BP (mmHg)	123±16/73±9	129±21/73±11	0.16/0.81
Heart rate (beats/min)	67±8	68±10	0.64
Glucose (mg/dL)	107±46	109±32	0.84
Cholesterol (mg/dL)	164±41	162±43	0.84
Pentosidine (pmol/ml)	43.6(31.2-108.9)	44.1(31.2-99.5)	0.87
eGFR$_{Cyst}$ (ml/min/1.73m²)	34±12	29±13	0.06
Haemoglobin (g/dL)	12±2	12±2	0.49
Calcium (mMol/L)	2.25±0.12	2.21±0.10	0.16
Phosphate (mMol/L)	1.20±0.19	1.23±0.16	0.29
PTH (pg/mL)	102 (81-146)	102 (85-154)	0.70
FGF-23 (pg/mL)	64.7 (52.7-81.2)	78.0 (53.7-103.1)	0.07
1.25 OH Vit. D (pg/mL)	39±16	36±16	0.32
Sclerostin (pg/mL)	141.0(93.5-189.7)	155(117.4-229.2)	0.15

Fig. 1. *Main correlates of Ln baseline sclerostin.*
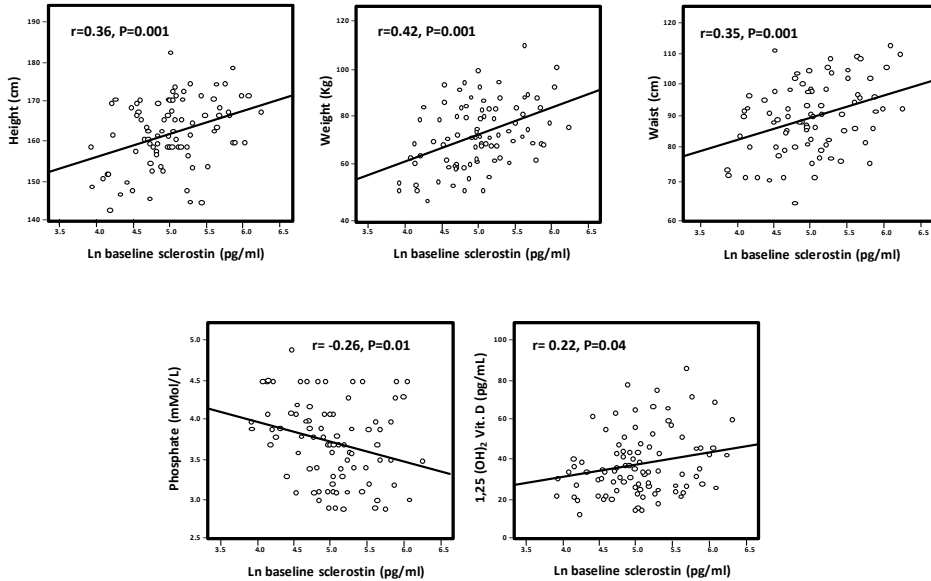
Effect of paricalcitol treatment on serum sclerostin

As alluded to before, serum sclerostin levels at baseline were by the 9% higher in the placebo arm (Table 1). After 12 weeks of treatment sclerostin rose in the PCT arm [from 166.7 pg/ml, 95% CI: 134.2 – 199.2 pg/ml, to 182.4 pg/ml, 95% CI: 148.2 – 216.6 pg/ml (+15.7 pg/ml)] but not in the placebo arm [from 180.4 pg/ml, 95% CI: 154.5 – 206.3 pg/ml, to 167.3 pg/ml, 95% CI: 139.4 – 195.2 pg/ml (-13.1 pg/ml) (between groups difference P= 0.03)]. Adjustment for baseline eGFR, and baseline sclerostin did not modify this difference which remained significant (P=0.04). The rise in sclerostin induced by PCT was independent of simultaneous FGF23 changes (after adjustment for FGF23 variation, P=0.04). Adding into the model PTH changes the effect of PCT on sclerostin became largely non-significant (p=0.73), suggesting a role of PTH as mediator of the PCT-induced effect on sclerostin. Changes in serum sclerostin induced by PCT (expressed in relationship to placebo) at 12 weeks (+25%) and two weeks after stopping the treatments are reported in Fig.3. The effect of PCT on sclerostin was almost entirely abolished 2 weeks after stopping this drug (P>0.13) (Fig. 3).

Fig. 2. *Upper side: correlation between Ln baseline sclerostin and pentosidine. Lower side: effect modification of pentosidine on the link paricalcitol – sclerostin.*
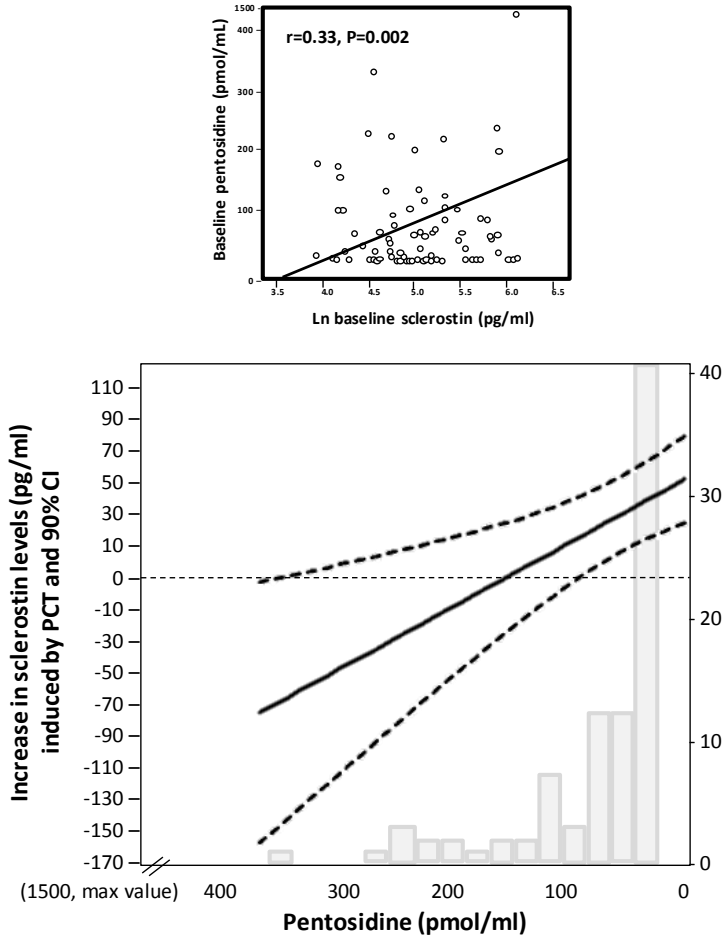
As expected, PCT treatment suppressed PTH (PCT -75.1 pg/ml, 95% CI from -90.4 to -59.8 pg/ml; Placebo +20.5 pg/ml, 95% CI from 4.8 to 36.3 pg/ml; P<0.001), raised FGF23 (PCT +107.0 pg/ml, 95% CI from 43.8 to 170.1 pg/ml; Placebo -20.2 pg/ml, 95% CI from -63.9 to 23.5 pg/ml; P=0.001) and reduced $1.25OH_2VD$ levels (PCT -24.3 pg/ml 95% CI from -30.0 to -18.6 pg/ml; Placebo: -5.5 pg/ml, 95% CI from -9.8 to -1.1 pg/ml; P<0.001). No effect of seasons was found on the rise in serum sclerostin induced by PCT (P for the effect modification by season > 0.38).

Fig. 3. *Changes in serum sclerostin induced by paricalcitol   expressed as paricalcitol/placebo ratio.*

Changes in serum sclerostin induced by paricalcitol expressed as paracalcitol/placebo ratio (%)
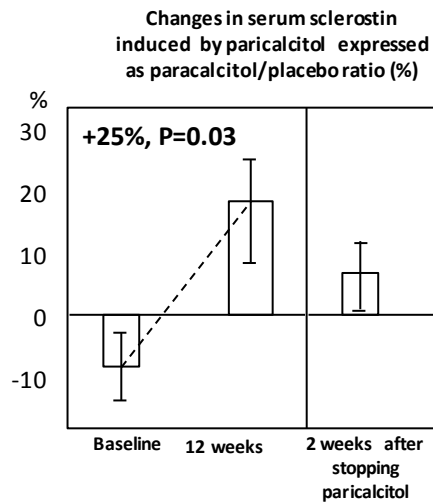
Pentosidine: an effect modifier of the effect of paricalcitol on sclerostin
Baseline levels of pentosidine were similar in the study arms (Table 1). After 2 weeks of PCT treatment no significant difference in pentosidine levels were found among the groups [active arm: from 110.7 pmol/ml, 95% CI: 36.5 – 184.8 pmol/ml, to 116.6 pmol/ml, 95% CI: 70.4 – 162.7 pmol/ml (+5.9 pmol/ml); placebo arm: from 74.8 pmol/ml, 95% CI: 56.3 – 93.3 pmol/ml, to 89.8 pmol/ml, 95% CI: 65.7 – 113.9 pmol/ml (+15.0 pmol/ml), P=0.74)]. However, baseline pentosidine was a strong modifier of the effect of PCT on sclerostin levels in unadjusted and adjusted analyses. Indeed in a model including pentosidine, PCT-treatment and their interaction term, the increase in sclerostin levels was progressively reduced with increasing levels of pentosidine [Pentosidine x PCT-treatment, regression coefficient: -0.39 (95% CI: -70 to -0.08); P for the effect modification: 0.02] (Fig. 2, lower panel) and this effect modification became stronger after adjustment for baseline eGFR and baseline sclerostin (Pentosidine x PCT-treatment, regression coefficient: -0.39 (95% CI: -70 to -0.09); P=0.01]. No effect modification of the sclerostin response to PCT by other variables was registered (data not shown).

DISCUSSION

In the setting of a randomized clinical trial, we found that vitamin D receptor activation by paricalcitol raised serum sclerostin levels, independently of eGFR, baseline sclerostin and FGF23. Such an effect was abolished by statistical adjustment for simultaneous changes in serum PTH suggesting that PTH may mediate the paricalcitol-induced sclerostin rise. Of note, the same effect was attenuated by pentosidine, an advanced glycosylation end product. Furthermore, we specifically confirm in CKD patients that sclerostin levels go along with major anthropometric measures like height, weight, the BMI and waist circumference and associate with $1,25 (OH)_2$ vitamin D and serum phosphate in this population.

A close inter-relationship between $1,25(OH)_2VD$ and the sclerostin gene has been described in experimental models. In the knockout model for sclerostin gene the renal expression $25(OH)VD-1\alpha$ hydroxylase is enhanced [9], a phenomenon attributable to removal of the restraining effect of sclerostin on the expression of this gene (ibidem), Conversely, $1,25(OH)_2VD$ stimulates sclerostin expression in bone cells in culture with intact sclerostin gene [8], an observation in line with the direct correlation between $1,25(OH)_2VD$ levels and serum sclerostin in CKD patients in the present study. Along with the hypothesis that active vitamin D stimulates sclerostin synthesis and secretion, we found that vitamin D receptor activation by paricalcitol raises serum sclerostin in CKD patients, an effect that was independent of age, gender and the severity of renal dysfunction. This observation is in keeping with the STOP/IT trial in elderly healthy men [9] where a calcium and vitamin D (700 UI/day) association produced a 13% increase in serum sclerostin which was sustained up to 2-years. Similarly, in an uncontrolled study testing a high intramuscular dose (300.000 UI) of vitamin D in patients with vitamin D insufficiency/deficiency a mild (+8%) but significant increase in serum sclerostin was registered at 3-month [10]. Accordingly, in a study in paediatric dialysis patients the bone expression of sclerostin rose during therapy with doxercalciferol [27]. However, circulating sclerostin was not measured in this study.

Sclerostin shows a progressive increase as the GFR declines in CKD patients [28], correlates inversely with PTH [29], and is considered a potentially relevant player in the bone mineral disorder in this condition (reviewed by Evenepoel et al., [30]). Of note, in the present study we show that serum sclerostin levels in CKD patients coherently associate in a direct fashion with fundamental anthropometric metrics like weight, height, and the BMI and waist circumference as well as with diabetes and high cholesterol. These findings extend to CKD observations in pre-diabetes and type-2 diabetes in previous studies in other populations [31, 32] and suggest that, like other biomarkers of bone mineral disorders, including FGF23 [33], vitamin D [34] and PTH [35], sclerostin levels associate with body and fat mass and carbohydrate metabolism.

Circulating 1,25(OH)$_2$VD levels decline as renal function deteriorates, a phenomenon which goes along with a rise in FGF23 [36]. In this regard, the moderate (+25%) paricalcitol-induced increase in serum sclerostin in this randomized trial can be seen as a counter-regulatory phenomenon aimed at countering the strong PTH suppression and the marked FGF23 increase induced by this drug. In this regard we found that the sclerostin rise by PCT was   abolished when we adjusted the analysis for PTH changes but not by FGF3 changes. Such specificity would support the contention that sclerostin changes in response to PCT treatment mainly serve to counter the PTH suppressing effect of this drug rather than the concomitant, marked rise in serum FGF23.

Uremic toxins impair the response to activated vitamin D in patients with renal failure [37]. In particular, reactive carbonyl compounds [38] which generate pentosidine and other AGEs [39]   alter the vitamin D receptor functioning [22]. In this regard the attenuation of the sclerostin rise produced by pentosidine we found in the present study may depend on altered vitamin D receptor functioning in patients with relatively higher levels of this AGE.

Our study has strengths and limitations. The fact that we tested our working hypothesis in the context of a randomized placebo-controlled clinical trial is strength. However, the trial was too small to allow analyses based on clinical end-points like fractures of cardiovascular events. The rise in serum sclerostin induced by paricalcitol we observed was of moderate degree and the possible implications of this phenomenon remain unclear in the present knowledge scenario where the clinical significance of alterations in serum sclerostin are still largely undefined [30]. Furthermore, even though sound biological underpinnings exist to interpret the attenuation of the sclerostin rise by pentosidine in the present study, experiments are needed to confirm that this AGE compound interferes with the sclerostin rise induced by vitamin D receptor activation.

In conclusion, vitamin D receptor activation by paricalcitol causes a moderate increase in serum sclerostin in CKD patients which goes along with a direct association of 1,25(OH)$_2$Vitamin D with the plasma concentration of sclerostin in this population. The sclerostin rise by PCT is attenuated by pentosidine, an observation in keeping with in vitro studies showing that AGEs alter the functioning of the vitamin D receptor.

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Chapter 2.2

Oxidative stress amplifies the alkaline phosphatase-dependent risk for mortality in ESRD patients on dialysis

ABSTRACT

Alkaline phosphatase (Alk-Phos) is a powerful predictor of death in patients with end-stage renal disease (ESRD) and oxidative stress is a strong inducer of Alk-Phos in various tissues. We tested the hypothesis that oxidative stress - as estimated by a robust marker of systemic oxidative stress like γ-Glutamyl-Transpeptidase (GGT) levels- may interact with Alk-Phos in the high risk of death in a cohort of 993 ESRD patients maintained on chronic dialysis.

In fully adjusted analyses the HR for mortality associated to Alk-Phos (50 IU/L increase) was progressively higher across GGT quintiles, being minimal in patients in the first quintile (HR: 0.89, 95% CI: 0.77-1.03) and highest in the GGT fifth quintile (HR: 1.13, 95% CI: 1.03-1.2) (P for the effect modification = 0.02). These findings were fully confirmed in sensitivity analyses excluding patients with pre-existing liver disease, excessive alcohol intake or altered liver disease biomarkers.

GGT amplifies the risk of death associated to high Alk-Phos levels in ESRD patients. This observation is compatible with the hypothesis that oxidative stress is a strong modifier of the adverse biological effects of high Alk-Phos in this population.

Keywords: dialysis, alkaline phosphatase, oxidative stress, γ-Glutamyl-Transpeptidase

INTRODUCTION

Tissue nonspecific alkaline phosphatase (Alk-Phos) is an enzyme highly represented in the bone and in the liver and the measurement of the activity of this enzyme is a time-honoured biomarker applied for the diagnosis and the clinical monitoring of bone and liver diseases [1]. Alk-Phos catalyses the hydrolysis of pyrophosphate, the main calcification inhibitor, and seminal studies in patients with end-stage renal disease (ESRD) documented that circulating Alk-Phos activity is robustly related to the risk of death [2-4]. In ESRD patients Alk-Phos mainly reflects increased bone turnover [1] triggered and maintained by secondary hyperparathyroidism and modulated by several other factors among which oxidative stress [5] plays a relevant role. Oxidative stress is notoriously pervasive in ESRD patients [6, 7]. Among biomarkers of oxidative stress γ-Glutamyl-Transpeptidase (GGT) is now regarded as one of the most robust indicators of whole body oxidative stress [8, 9]. High levels of GGT predict mortality in ESRD patients [10, 11] and in the general population [12] being associated with a high risk for coronary heart disease [12, 13] and heart failure [14]. Of note, oxidative stress is a powerful inducer of Alk-Phos in vascular and bone cells [15] and is a key to vascular calcification [16]. Even though the predictive power of Alk-Phos for adverse clinical outcomes has been previously confirmed in ESRD [17-23], the possible interaction between Alk-Phos with biomarkers of oxidative stress like GGT has not been investigated so far. As oxidative stress and mineral metabolism are intimately related phenomena in ESRD [5], we investigated if GGT modifies the association between Alk-Phos and all-cause and cardiac mortality in a sizable cohort of patients with ESRD maintained on chronic dialysis.

METHODS

The study protocol was approved by the ethical committee of our institution. All participants gave their informed consent before enrolment.

Study population
The study population is part of a cohort of 1189 dialysis patients enrolled in the PROGREDIRE (Prospective Registry of The Working Group of Epidemiology of Dialysis Region Calabria), a cohort study involving 35 dialysis units in two regions in Southern Italy (Calabria and Sicily). We included in this analysis 993 patients in which both Alk-Phos and GGT measurements were available. Patients where Alk-Phos and GGT were not available (n=196, 16%) did not differ from those included in the study for any of the main demographic, clinical and biochemical characteristics listed in Table 1.
Patients had been on regular dialysis [haemodialysis (HD) or peritoneal dialysis (PD)] for a median time of 3.0 years (inter-quartile range: 1.8-4.4 years). HD patients (n=932) were being treated with standard bicarbonate dialysis with non-cellulosic membrane filters of various type. PD patients (n=61) were either on 4 standard

exchanges day or on continuous cycling peritoneal dialysis. Six hundred and thirty-four patients were treated with various anti-hypertensive drugs (271 on monotherapy with ACE inhibitors, calcium channel blockers, α- and β-blockers, vasodilators, diuretics or other drugs, 194 on double therapy, 92 on triple therapy and 77 patients on quadruple or quintuple therapy with various combinations of these drugs). The main demographic, somatometric, clinical and biochemical characteristics of the study population are detailed in Table 1.

Laboratory measurements

Blood sampling was performed at baseline after an overnight fast. For HD patients, blood was always drawn during a mid-week day (brief dialysis interval). Alk-Phos, GGT, cholesterol, albumin, calcium, phosphate, C-Reactive Protein (CRP), haemoglobin, Glutamic-Oxaloacetic Transaminase (GOT) and Glutamic-Pyruvic Transaminase (GPT) measurements were made using standard methods in the routine clinical laboratory. In our laboratory the normal range of Alk-Phos was 30 to 120 UI/L and that of GGT 0-45 UI/L.

Study end-points

Mortality, fatal and non-fatal cardiac events were the main study end-points. Cardiac events were classified as follows: myocardial infarction confirmed by serial changes of ECG and cardiac biomarkers; ECG-documented angina episodes; ECG-documented arrhythmia; unexpected, sudden death highly suspected as of cardiac origin. De novo chronic heart failure (CHF) was defined as CHF in a patient without CHF at baseline. To be classified as having CHF patients had to show mild or more severe dyspnoea during ordinary activities (NYHA class II or higher) plus evidence of anatomical/functional left ventricular (LV) disease on echocardiography. Each cause of death was assessed by 3 independent physicians. In doubtful cases, diagnosis was attributed by consensus. During the review process, involved physician used all available medical information, including hospitalization forms and medical records. In case of death occurred at home, family members and/or general practitioners were interviewed to better understand the circumstances which led to death.

Statistical analysis

Data were expressed as mean ± standard deviation (normally distributed data), median and interquartile range (non-normally distributed data) or as per cent frequency (categorical data). Comparisons among groups were made by one-way ANOVA, Kruskal-Wallis or Chi Square test, as appropriate. Regression analysis was performed to investigate the relationship between Alk-Phos, GGT and markers of liver function and bone mineral metabolism. Due to the non-normal distribution of both Alk-Phos and GGT both variables were log-transformed before analysis.

Survival analyses were performed by using both univariate and multivariate Cox regression analyses, including Alk-Phos, GGT and their interaction term as well as

traditional [age, gender, current smoking, diabetes, cholesterol, arterial pressure and antihypertensive treatment and cardiovascular comorbidities], inflammation and nutritional status [CRP, BMI, albumin] and ESRD-related risk factors [dialysis vintage, haemoglobin]. ALT, AST, HbsAg, HCV, alcohol consumption and pre-existing liver disease were always included into the multivariate models. The hazard ratios of alkaline phosphatase across GGT categories were calculated by the standard linear combination method. The best functional form of GGT (i.e. quintiles) was chosen by analysing the Martingale residuals in Cox's regression analysis [24]. Multivariate models were built as previously described. Statistical analysis was performed by using standard statistical packages (SPSS for Windows, Version 20, Chicago, Illinois, USA; STATA for Windows, Version 13, College Station, Texas, USA).

RESULTS

The main baseline characteristics of the study population are reported in Table 1. Both Alk-Phos and GGT distributions were right-skewed and the median value of the two biomarkers was 89 UI/L and 20 UI/L respectively (Fig. 1). Two hundred and seventy-one patients (27%) had Alk-Phos exceeding the upper limit of the normal range of this biomarker (120 UI/L) and 83 (17%) had GGT greater than 45UI/L (the upper limit of the normal range). Sixty-three per cent of patients were males and mean age was 65 years. Diabetics were 28%. Alk-Phos levels were higher in female patients (median 97 UI/L, IQR: 74-140 UI/L) than in male patients (median 85 UI/L, IQR: 64-116 UI/L). Patients with higher levels of Alk-Phos had been on dialysis for longer time and had higher CRP levels. Conversely, calcium and phosphate levels showed an opposite trend (Table 1).

Correlates of Alkaline Phosphatase and γ-Glutamyil-Transpeptidase
Alk-Phos showed a direct, highly significant association with GGT (r=0.26, P<0.001) (Fig. 1).
Furthermore, Alk-Phos was directly associated with GOT (r=0.13, P<0.001), GPT (r=0.14, P<0.001) and Parathyroid Hormone (PTH) (r=0.38, P<0.001), and correlated inversely with calcium (r=-0.13, P<0.001) and phosphate (r=-0.16, P<0.001).
The same variables, except PTH, were associated to GGT [GGT vs GOT (r=0.40, P<0.001); GGT vs GPT (r=0.41, P<0.001); GGT vs calcium (r=-0.08, P=0.01); GGT vs phosphate (r=-0.14, P<0.001)].

Table 1. *Main demographic, somatometric and clinical characteristics in the whole study population and in patients as divided according to alkaline phosphatase quartiles.*

	Whole group (n=993)	Alk-Phos <median value (n=497)	Alk-Phos >median value (n=496)	P for linear trend
Age (years)	65±14	65±14	65±13	0.93
BMI (kg/m²)	25±5	25±5	25±5	0.74
Male sex n. (%)	624(63)	343(69)	281(57)	**<0.001**
Current smokers n. (%)	149(15)	78(16)	71(14)	0.54
Past smokers n. (%)	370(37)	202(41)	168(34)	**0.03**
Diabetics n. (%)	272(28)	127(26)	145(30)	0.14
On anti-hypertensive treatment n. (%)	634(64)	320(64)	314(63)	0.72
Dialysis vintage (months)	45(21-85)	38(19-76)	52(26-96)	**<0.001**
With cardiovascular comorbidities* n. (%)	533(54)	257(52)	276(56)	0.21
Systolic Blood Pressure (mmHg)	135±22	135±22	135±23	0.99
Diastolic Blood Pressure (mmHg)	74±12	74±12	73±11	0.09
Pulse Pressure (mmHg)	74±11	73±10	74±11	0.13
Cholesterol (mg/dL)	156±40	155±39	156±41	0.61
Haemoglobin (g/dL)	11.3±1.5	11.3±1.4	11.3±1.5	0.96
Albumin (g/dL)	3.9±0.5	3.9±0.5	3.9±0.5	0.87
CRP (mg/L)	5.0(3.0-13.0)	4.1(2.9-12.0)	5.7(3.0-14.0)	**0.02**
Calcium (mg/dL)	9.1±0.9	9.2±0.9	9.0±0.9	**0.001**
Phosphate (mg/dL)	5.0±1.6	5.2±1.6	4.9±1.6	**0.001**

*Cardiovascular comorbidities: The presence, at baseline, of at least one of these comorbidities: angina, arrhythmia, myocardial infarction, coronary surgery, angioplasty, other heart surgery, claudicatio intermittens, amputations, peripheral surgery, stroke, TIA and pre-existing chronic heart failure.
Data are expressed as mean ± SD. median and inter-quartile range or as percent frequency, as appropriate.

Fig. 1 *Distribution of Alk-Phos, GGT and their correlation in the study population.*
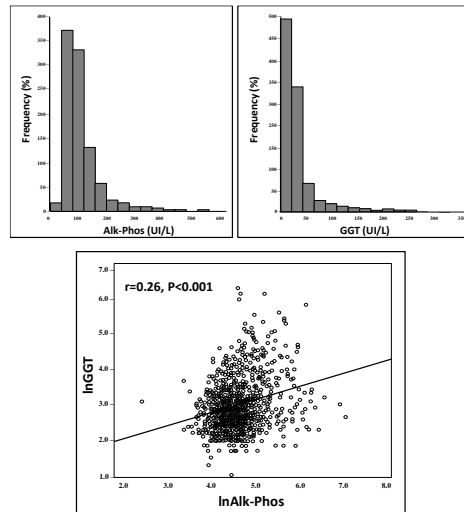
Survival analysis – all cause death

During a median follow-up of 3.0 years (interquartile range: 1.8-4.4 years), 405 patients died. In a basic model including Alk-Phos, GGT and their interaction term, GGT significantly amplified the risk of death across progressively increasing Alk-Phos levels (P for the effect modification=0.004)

(Table 2, crude analysis). These results were confirmed in fully adjusted analyses, where the risk associated to 50 UI/L increase of in Alk-Phos for all-cause mortality was progressively higher from the first to the fifth quintile (1st quintile: HR: 0.89, 95% CI: 0.77-1.03; 2nd quintile: HR: 0.95, 95% CI: 0.85-1.05; 3rd quintile: HR: 1.01, 95% CI: 0.94-1.08; 4th quintile HR: 1.07, 95% CI: 1.01-1.14, 5th quintile HR: 1.13, 95% CI: 1.03-1.2) (P for the effect modification = 0.02). (Table 2; Fig. 2). Exclusion of heavy drinkers (n=23) and of patients affected by chronic liver diseases (n=68) only modestly reduced the HR of the Alk-Phos-GGT interaction (HR: 1.06, 95% CI: 1.01-1.12).

Table 2. *Crude and adjusted Cox regression analyses showing the effect modification of γ-Glutamyl-Transpeptidase on alkaline phosphatase for all-cause mortality. The criteria for building these models are detailed in the Methods.*

Variables (units of increase)	Crude analysis	Fully adjusted analysis
	Fig. 2A Left side	*Fig.2A Right side*
Alk-Phos (50UI/L)	0.80 (0.67 – 0.96), P=0.02	0.84 (0.69 – 1.02), P=0.08
GGT (quintiles)	0.96 (0.84 – 1.09), P=0.52	0.97 (0.85 – 1.12), P=0.72
Alk-Phos*GGT (50UI/L*quintiles)	1.08 (1.02 – 1.13), P=0.004	1.06 (1.01 – 1.12), P=0.02
Age (1 year)		1.05 (1.04 – 1.06), P<0.001
Gender (0=female; 1=male)		0.97 (0.77 – 1.21), P=0.77
Current smoking (0=no; 1=yes)		0.93 (0.67 – 1.29), P=0.66
Diabetes (0=no; 1=yes)		1.29 (1.03 – 1.62), P=0.03
Systolic blood pressure (1 mm Hg)		1.00 (0.99 – 1.00), P=0.67
CV comorbidities[a] (0=no; 1=yes)		1.55 (1.24 – 1.94), P<0.001
Antihypertensive treatment (0=no; 1=yes)		1.12 (0.90 – 1.39), P=0.98
Dialysis vintage (1 month)		1.00 (1.00 – 1.00), P<0.001
Cholesterol (1 mg/dL)		1.00 (1.00 – 1.00), P=0.002
Hb (1g/dL)		0.93 (0.86 – 0.99), P=0.04
Phosphate (1 mg/dL)		1.01 (0.94 – 1.08), P=0.80
Albumin (1 g/dL)		0.70 (0.55 – 0.88), P=0.002
CRP (1 mg/L)		1.00 (1.00 – 1.00), P=0.56
Body Mass Index (BMI) (1 Kg/m^2)		0.99 (0.97 – 1.02), P=0.46
GOT (1 UI/L)		1.01 (0.99 – 1.03), P=0.45
GPT (1 UI/L)		1.00 (0.99 – 1.01), P=0.98
Bilirubin (1 mg/dL)		0.91 (0.54 – 1.51), P=0.70
HbsAg (0=no; 1=yes)		0.73 (0.37 – 1.44), P=0.37
HCV (0=no; 1=yes)		0.81 (0.56 – 1.16), P=0.25
Cirrhosis/hepatitis (0=no; 1=yes)		1.26 (0.71 – 2.24), P=0.44
Current alcohol consumption (0=no; 1=yes)		1.12 (0.88 – 1.42), P=0.34

Data are expressed as hazard ratio, 95% confidence interval (CI) and P values.
[a] CV comorbidities were defined as in Table I

Fig. 2. *Effect modification by γ-Glutamyl-Transpeptidase on the relationship between alkaline phosphatase and all-cause mortality. The HR in this graph represents the risk for all-cause death due to alkaline phosphatase across γ-Glutamyl-Transpeptidase levels.*
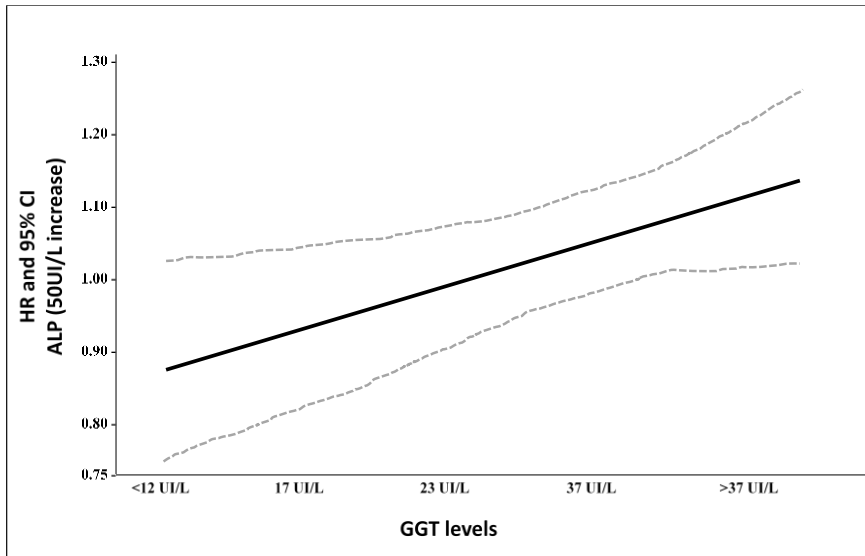
DISCUSSION

In this study GGT, a systemic marker of oxidative stress, emerged as a coherent amplifier of the death risk portended by high Alk-Phos in ESRD patients on dialysis. This interaction was largely independent of liver disease and alcohol intake and was confirmed in sensitivity analyses excluding patients with pre-existing liver disease or self-reported high alcohol intake. Overall, these findings suggest that systemic oxidative stress, as estimated by GGT, plays a relevant role in predicting the risk for major clinical outcomes portended by increased alkaline phosphatase.

Alk-Phos is an established predictor of death in ESRD patients on haemodialysis. Several studies reported a linear association between Alk-Phos levels and mortality in ESRD [2-4, 17-23]. Additional studies focusing on pre-dialysis CKD patients showed that such a link is not peculiar to the end-stage phase of CKD [25-27]. Furthermore, observational studies in various communities documented that Alk-Phos is a quite strong risk factor for death and cardiovascular events in the general population [28]. This enzyme is ubiquitous and located at cell surface and it is directly involved in glutathione catabolism, the main anti-oxidant system in humans [29, 30]. Circulating levels of Alk-Phos in ESRD in patients without obvious liver disease mainly reflect bone turnover [31]. In this regard it is worth mentioning that in vitro experiments in

vascular and bone cells, oxidative stress is a strong inducer of alkaline phosphatase and a key event promoting the transition of the vascular cells phenotype into calcifying cells [15]. Alk-Phos is seen as a host defence molecule that is part of the innate immune response to bacterial agents. Indeed, this enzyme is potently induced by IL-6, TNF-α and bacterial lipopolysaccharide, all factors typically associated with inflammation and high oxidative stress [32]. Thus the hypothesis that oxidative stress may interact with Alk-Phos in organ damage and ultimately in major clinical outcomes is biologically well founded. However, in no study such an interaction was formally investigated. The issue is of particular relevance in ESRD because high levels of oxidative stress are a hallmark in these patients [6, 7]. Various biomarkers of oxidative stress are currently applied in clinical research [33-36] and among these GGT is seen as the one that better captures whole body oxidative stress [8]. Of note, in previous studies in ESRD, GGT exhibited a much stronger link with mortality [10] than other oxidative stress biomarkers tested in the same population. With this background in mind we set out to make a detailed analysis of the interaction between GGT and alkaline phosphatase for risk of death, the most solid outcome measure in clinical studies. Along with the working hypothesis we found that GGT is a relevant modifier of the risk of Alk-Phos for mortality. Indeed, in a crude analysis the interaction term indicated that a fixed increase in Alk-Phos levels (50 UI/L) produced a stepwise increase in the risk of death across GGT quintile (HR = 1.08 per GGT quintile). Importantly, this interaction was unmodified by adjustment for a comprehensive series of risk factors for mortality, including CV comorbidities, clinical risk factors, BMI, risk factors peculiar to ESRD like haemoglobin, C-reactive protein, serum albumin and phosphate and biomarkers of liver disease and alcohol intake. The fact that the interaction was independent of concomitant liver disease indicates that the effect modification of GGT on the Alk-Phos - death relationship does not represent a mere effect of liver damage but a more general phenomenon, more likely oxidative stress [8].

Our study has limitations. First, our observations are limited to a single ESRD cohort of Caucasian patients. Therefore, confirmation in a second ESRD cohort and in studies in other ethnicities is still needed for establishing the external generalizability of our findings. Second, even though we did a comprehensive adjustment for a long list of potential confounders, confounding for unmeasured and/or unknown risk factors remains possible, an issue that can be solved only by a clinical trial. In this regard it is worth mentioning that drugs that reduce serum Alk-Phos are being investigated and that one of these drugs has produced a significant reduction in vascular calcification and bone loss in ESRD patients [37]. Our findings suggest that the protective effect of this drug may be attenuated in patients with high GGT, hypothesis which may be formally tested in secondary analyses of the same trial.

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Chapter 3

Novel instruments for risk stratification in patients with End-Stage Renal Disease

Chapter 3.1

Snoring amplifies the risk of heart failure and mortality in dialysis patients

ABSTRACT

Snoring, an indicator of sleep disordered breathing (SDB), associates with all-cause and cardiovascular (CV) mortality in high risk conditions such as chronic heart failure (CHF). Because SDB and HF are exceedingly frequent in end-stage renal disease (ESRD), we hypothesized that SDB as detected by snoring may impact upon the relationship between chronic CHF and all-cause and CV mortality in these patients.

We tested this hypothesis in a cohort of 827 ESRD patients, followed up for 2.3 years. In this population, snoring was a strong modifier of the risk of CHF for all-cause and CV death. In fully adjusted Cox models, the hazard ratio (HR) associated to CHF for the study outcomes was highest in heavy snorers [all-cause death: HR: 2.6 (95% CI: 1.6-4.3, P<0.001); CV death: HR: 4.0 (95% CI: 2.1-7.6), P<0.001], intermediate in moderate snorers [all-cause death: HR: 1.6 (95% CI: 1.1-2.2, P=0.01); CV death: HR: 1.8 (95% CI: 1.2-2.8, P=0.01) and lowest and not significant in non-snorers [all-cause death: HR: 0.9 (95% CI: 0.6-1.6, P=NS); CV death: HR: 0.8 (95% CI: 0.4-1.6, P=NS)].

Snoring is a strong and independent effect modifier of the relationship between CHF and all-cause and CV mortality in ESRD. Since SDB and snoring are in part attributable to reversible pharyngeal oedema, intensified surveillance and treatment of CHF snorers on dialysis may translate into better clinical outcomes in this very high risk population, an issue which remains to be tested in specifically designed clinical trials.

Keywords: dialysis, heart failure, sleep-disordered breathing, snoring

INTRODUCTION

Patients with end-stage renal disease (ESRD) have an almost uniquely high risk of death and cardiovascular disease. Hearth failure (HF) has a 30% prevalence in this population [1], being at least 6 times higher than in the coeval general population [2]. Sleep disordered breathing (SDB) is a common disorder in chronic renal disease (CKD) patients [3], with a 75% prevalence in symptomatic ESRD patients [4], and nocturnal hypoxia, the most serious consequence of SDB, is a strong, independent predictor of cardiovascular events in the dialysis population [5]. HF and SDB are often associated in patients with heart disease and the concomitant presence of these disorders doubles the risk of death in these patients [6]. The issue whether SDB and HF in ESRD patients represent complementary or interacting risk factors for the risk of death and cardiovascular complications has never been previously investigated. The question is of relevance because SDB in ESRD and in HF has been in part attributed to pharyngeal oedema [7,8], an alteration which can be reversed by appropriate volume fluid subtraction.

State of the art polysomnography is a time consuming and costly procedure and, mostly due to this limitation, large scale cohort studies on respiratory disorders during sleep are problematic in ESRD. Snoring is a main feature of SDB [9] and a fundamental item in scoring systems applied for the screening of SDB [10,11] and it is therefore an interesting surrogate of SDB which may be applied in large scale epidemiological studies. With this background in mind we preliminarily validated a brief questionnaire about self-reported snoring in CKD patients and then investigated the hypothesis that snoring may amplify the risk of death and cardiovascular events in ESRD patients with HF by testing the interaction between this disturbance and HF in a large, well characterized, cohort of ESRD patients.

METHODS

The study protocol was approved by the ethical committee of our institution. All participants gave the informed consent before enrolment.

Patients
The study population is part of a cohort of 1189 dialysis patients enrolled in the PROGREDIRE (Prospective Registry of The Working Group of Epidemiology of Dialysis Region Calabria), a cohort study involving 35 dialysis units in two regions in Southern Italy (Calabria and Sicily). In this population detailed information about snoring habits and heart failure was available in a subgroup of 827 patients. The remaining patients did not reply to the questionnaire (n=82) or lacked accurate assessment of heart function status (n=220) or both (n=60). Patients included in the present study cohort had been on regular dialysis (HD or PD) for a median time of 3.8 years (inter-quartile range: 1.8-7.2). Haemodialysis patients (n=773) were being treated with standard

bicarbonate dialysis with non-cellulosic membrane filters of various type. PD patients (n=54) were either on 4 standard exchanges day or on continuous cycling peritoneal dialysis. Five hundred and eleven patients were treated with various anti-hypertensive drugs (226 on mono-therapy with ACE inhibitors, calcium channel blockers, α- and β-blockers, vasodilators, diuretics or other drugs, 172 on double therapy, 76 on triple therapy and 37 patients on quadruple or quintuple therapy with various combinations of these drugs). The main demographic, somatometric, clinical and biochemical characteristics of the study population are detailed in Table 2.

Laboratory measurements
Blood sampling was performed after an overnight fast. For haemodialysis patients, blood was always drawn during a mid-week day (brief dialysis interval). Serum cholesterol, albumin, calcium, phosphate, C-Reactive Protein (CRP) and haemoglobin measurements were made using standard methods in the routine clinical laboratory.

Clinical assessment of heart failure
Chronic Heart Failure (CHF) was assessed at baseline and to be classified as having CHF patients had to show mild or more severe dyspnoea during ordinary activities (NYHA class II or higher) plus evidence of anatomical/functional left ventricular (LV) disease i.e. echocardiographic documentation of a low ejection fraction (less than 50%) and/or diastolic dysfunction and/or eccentric or concentric left ventricular hypertrophy. NYHA criteria for the functional classification of heart failure in dialysis patients were specifically validated in this population in a previous study by our group [12] and in this study we showed that the inter-observer agreement of this classification in the ESRD population is fairly good. Extending to all participating centres the standard approach at our central echocardiography laboratory [13], echocardiography was performed while patients were in stable condition during the dialysis interval rather than in coincidence of acute, decompensated heart failure.
De novo CHF was defined as CHF in a patient without CHF at baseline and decompensated HF was defined according to Foley et al. [14], i.e. severe dyspnoea in addition of at least two of the following conditions, raised jugular venous pressure, bibasilar pulmonary crackles, pulmonary venous hypertension or interstitial oedema at chest X ray requiring hospitalization or extra-dialyses.

Self-reported snoring
Self-reported information about snoring was obtained at the enrolment by a specific, simple questionnaire based on two questions. The first one "Do you snore?" contemplated "yes" or "no" as possible options. The second one was "If yes, do you snore often or sometimes?" Patients were classified as non-snorers in case of negative response to the first question, mild to moderate snorers in case they answered "sometimes" to the second question, and heavy snorers in case they answered "often" to the same question. Patients were instructed to ask their partners about

their sleeping habits and about they being snorers or non-snorers before answering the questionnaire. This questionnaire was validated in a series of 45 CKD patients, the vast majority of whom (67%) had stage 4 or 5 CKD. These patients responded to the questionnaire and underwent polysomnography studies according to the protocol applied at our research centre [15]. To define a patient as affected by SDB, he or she had to have at least 5 episodes of apnoea or hypopnea per hour on polysomnography testing [16]. The average age in this series of patients (males 46%) was 49 ± 15 years. The proportion of diabetics was 9%. Thirty-six per cent were past smokers and 15% current smokers.

Study end-points
Mortality, fatal and non-fatal cardiovascular events were the main study end-points. Cardiovascular events were classified as follows: stroke (ischaemic or haemorrhagic), documented by computed tomography, magnetic resonance imaging and / or clinical and neurological evaluation; transient ischaemic attacks (TIA); myocardial infarction confirmed by serial changes of ECG and cardiac biomarkers; ECG-documented angina episodes; de novo chronic heart failure and acute decompensated heart failure as defined before; ECG documented arrhythmia; peripheral ischemia or amputations; unexpected, sudden death highly suspected as of cardiac origin. Each cause of death was assessed by 3 independent physicians. In doubtful cases, diagnosis was attributed by consensus. During the review process, involved physician used all available medical information, including hospitalisation forms and medical records. In case of death occurred at home, family members and/or general practitioners were interviewed to better understand the circumstances which led to death.

Statistical analysis
Data were expressed as mean ± standard deviation (normally distributed data), median and inter-quartile range (non-normally distributed data) or as per cent frequency (categorical data). Comparisons among groups were made by one-way ANOVA, Kruskal-Wallis or Chi Square test, as appropriate. Survival analyses were performed by using both univariate and multivariate Cox regression analyses, including as covariates traditional risk factors, [age, gender, current/past smoking, diabetes, cardiovascular comorbidities, cholesterol, arterial pressure], BMI, anti-hypertensive treatment, risk factors related to CKD-5D [dialysis vintage, haemoglobin, albumin, phosphate] and CRP. The effect modification by snoring on the risk of HF for all-cause and CV death was investigated by including into the same Cox model snoring, HF and their interaction term (snoring*HF) [17]. The hazard ratios of HF across snoring categories was calculated by the standard linear combination method. To account for over-fitting in multivariate Cox models for CV death, in which the number of events for each variable in the model was below 10, the shrinkage method was applied [18].

The predictive value of snoring was also investigated by analysing the area under receiving operating characteristic (ROC) curves. In this analysis, three categories of snoring (0=non-snorers, 1=mild to moderate snorers, 2=heavy snorers) were used to predict the presence or absence of SDB as defined by an apnoea-ipopnaea index lower than five [16]. Sensitivity, specificity, positive and negative predictive power were also calculated [19]. Statistical analysis was performed by using standard statistical packages (SPSS for Windows, Version 20, Chicago, Illinois, USA; STATA for Windows, Version 13, College Station, Texas, USA).

RESULTS

Questionnaire Validation
On discriminant analysis (ROC curve), snoring (either moderate or severe) had a reasonably high discriminatory power (area under the curve: 0.75, CI: 0.63-0.87, P=0.001) for SDB as assessed by the golden standard (polysomnography) (Figure 1). The sensitivity of snoring for the diagnosis of SDB was 100%, and the specificity was 39%. Positive and negative values were 38% and 100% (Table 1). Thus snoring in CKD is highly reliable for excluding SDB but, notwithstanding perfect sensitivity (100%), due to the fact that 38% (n=20) of self-reported snorers were negative for SDB at polysomnography, the predictive value of snoring was limited (38%). Vice-versa, notwithstanding low specificity (39%), because all non-snorers tested negative for SDB at polysomnography, the negative predictive value of snoring was 100%.

Fig. 1. *ROC curve analysis showing the discrimination power of snoring in diagnosis of SDB.*
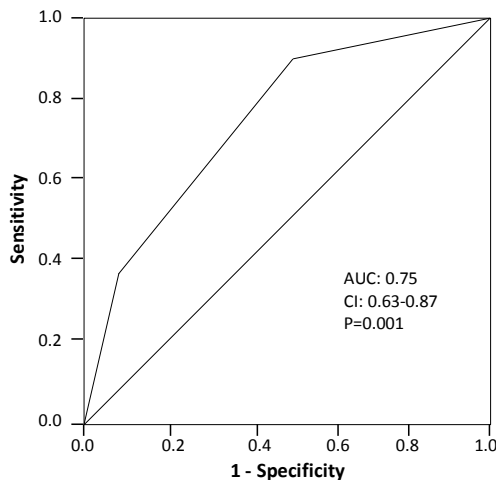
Table 1. *Diagnostic value of the snoring questionnaire for Sleep Disordered Breathing (SDB).*

		Polysomnography		Total
		SDB +	SDB -	
Questionnaire	**Snorers**	12	20	32
	Non-snorers	0	13	13
	Total	12	33	45

	Value (95% CI)
Sensitivity	100 (90-100)
Specificity	39 (26-55)
Positive predictive power	38 (24-53)
Negative predictive power	100 (90-100)

Cohort study

The main baseline characteristics of the study cohort are reported in Table 2. One hundred and thirty-two patients were affected by HF at baseline, corresponding to 16% of overall population. On the basis of self-reported snoring, 194 patients were classified as heavy snorers (24%), 308 as mild to moderate snorers (37%) and 325 patients as non-snorers (39%). Twenty-nine per cent of patients were diabetics. Body Mass Index (BMI) was significantly higher in snorers than in non-snorers. Furthermore, snorers were more frequently male and former smokers and diabetics as compared to non-snorers. Non-snorers, mild to moderate snorers and heavy snorers did not differ for age, use of anti-hypertensive treatment, dialysis vintage, cardiovascular comorbidities, systolic and diastolic blood pressure, pulse pressure, cholesterol, haemoglobin, albumin, CRP, calcium and phosphate levels.

HF, snoring, all cause and CV death

The median follow-up was 2.3 years (interquartile range: 1.8-2.9 years). Two hundred and thirty-three patients died, 127 of whom of cardiovascular causes. At univariate analysis, heart failure significantly predicted both all cause (HR: 2.06, 95% CI: 1.54-2.76, P<0.001) and cardiovascular mortality (HR: 2.53, 95% CI: 1.74-3.68, P<0.001), while snoring failed to predict these outcomes (all-cause mortality HR: 1.08, 95% CI: 0.92-1.27, P=0.37; CV mortality HR: 1.14, CI: 0.92-1.42), p=0.22). However, snoring was a strong effect modifier of HF for all-cause (HR: 1.50, CI: 1.02-2.19, P=0.04) and CV death (HR: 1.97, CI: 1.20-3.23, P=0.01), and for this reason we built multivariate models including this interaction term. Confirming unadjusted analysis, in fully adjusted analyses the risk of HF for all-cause and CV mortality was lowest in non-snorers, intermediate in moderate snorers and highest in heavy snorers (Supplementary Table 1 and 2; Figure 2).

Table 2. *Main demographic, somatometric and clinical characteristics in the whole study population and in patients as divided according to snoring habits.*

		Snoring habits			
	Whole group (n=827)	Non-snorers (n=325)	Moderate snorers (n=308)	Heavy snorers (n=194)	P for linear trend
Age (years)	65±14	64±15	64±14	66±11	0.23
BMI(kg/m²)	25±5	24±4	25±4	27±5	**<0.001**
Male sex n. (%)	525(64)	196(60)	188(61)	141(73)	**0.01**
Current smokers n. (%)	124(15)	41(13)	45(15)	38(20)	0.10
Past smokers n. (%)	301(36)	110(34)	106(34)	85(44)	**0.05**
Diabetics n. (%)	240(29)	84(26)	85(28)	71(37)	**0.03**
On anti-hypertensive treatment n. (%)	509(62)	205(63)	194(63)	110(57)	0.28
Dialysis vintage (months)	45(21-86)	42(20-94)	47(21-83)	47(23-85)	0.99
With cardiovascular comorbidities* n. (%)	444(54)	164(51)	171(56)	109(56)	0.32
Systolic Blood Pressure (mmHg)	135±23	134±23	135±22	136±23	0.62
Diastolic Blood Pressure (mmHg)	73±12	74±12	73±12	73±12	0.72
Pulse Pressure (mmHg)	62±19	61±19	62±18	63±18	0.32
Cholesterol (mg/dL)	156±41	155±39	155±41	161±44	0.18
Haemoglobin (g/dL)	11.4±1.4	11.3±1.4	11.4±1.5	11.3±1.5	0.95
Albumin (g/dL)	3.9±0.5	3.9±0.6	3.9±0.5	4.0±0.4	0.38
CRP (mg/L)	5.0(3.0-12.8)	4.0(2.9-11.0)	5.0(2.9-12.8)	6.0(3.0-16.0)	0.16
Calcium (mg/dL)	9.1±0.9	9.2±0.8	9.1±0.9	9.1±0.9	0.35
Phosphate (mg/dL)	5.0±1.5	4.8±1.5	5.1±1.5	5.0±1.5	0.06

*Cardiovascular comorbidities: The presence, at baseline, of at least one of these comorbidities: angina, arrhythmia, myocardial infarction, coronary surgery, angioplasty, other heart surgery, claudicatio intermittens, amputations, peripheral surgery, stroke, TIA and pre-existing chronic heart failure.
Data are expressed as mean ± SD. median and inter-quartile range or as percent frequency, as appropriate.

Supplementary Table 1. *Crude and adjusted Cox regression analyses showing the effect modification of snoring on chronic heart failure (CHF) for all-cause mortality. The criteria for building these models are further detailed in the Methods.*

Variables (units of increase)	Crude analysis	[b]Adjusted analysis
	Fig. 2 Left side	*Fig. 2 Right side*
CHF (0=no; 1=yes)	1.40 (0.87 – 2.26), P=0.17	0.93 (0.56 - 1.55), P=0.79
Snoring (0=no; 1=sometimes; 2=often)	0.97 (0.81 – 1.18), P=0.09	0.96 (0.79 - 1.17), P=0.66
CHF*Snoring	1.50 (1.02 – 2.19), P=0.04	1.67 (1.14 - 2.44), P=0.01
Age (1 year)		1.05 (1.03 - 1.06), P<0.001
Gender (0=female; 1=male)		1.00 (0.72 - 1.40), P=0.98
[a]Current or past smoking (0=no; 1=yes)		1.27 (0.93 - 1.73), P=0.13
Diabetes (0=no; 1=yes)		1.18 (0.88 - 1.58), P=0.27
Systolic blood pressure (1 mm Hg)		1.00 (0.99 - 1.00), P=0.53
Anti-hypertensive treatment (0=no; 1=yes)		0.93 (0.70 - 1.25), P=0.63
CV comorbidities[b] (0=no; 1=yes)		1.36 (0.99 - 1.88), P=0.06
Dialysis vintage (1 month)		1.02 (1.00– 1.04), P=0.01
CRP (1 mg/L)		1.00 (0.99 - 1.00), P=0.73
Phosphate (1 mg/dL)		1.03 (0.94 - 1.13), P=0.52
Cholesterol (1 mg/dL)		1.00 (1.00 – 1.00), P=0.02
Haemoglobin (1 g/dL)		0.92 (0.83 - 1.01), P=0.09
Albumin (1 g/dL)		0.66 (0.49 - 0.90), P=0.01
BMI (1 Kg/m^2)		0.98 (0.95 - 1.02), P=0.34

Supplementary Table 2. *Crude and adjusted Cox regression analyses showing the effect modification of snoring on chronic heart failure (CHF) for CV mortality. The criteria for building these models are further detailed in the Methods.*

Variables (units of increase)	Crude analysis	Adjusted analysis	Adjusted analysis after shrinkage
	Fig. 3 Left side	*Fig. 3 Middle side*	*Fig. 3 Right side*
CHF (0=no; 1=yes)	1.28 (0.66 - 2.47), P=0.47	0.81 (0.41 - 1.62), P=0.55	0.84 (0.42 - 1.68), P=0.62
Snoring (0=no; 1=sometimes; 2=often)	0.94 (0.73 - 1.23), P=0.66	0.89 (0.67 - 1.17), P=0.41	0.91 (0.69 - 1.20), P=0.49
CHF*Snoring	1.97 (1.20 - 3.23), P=0.01	2.22 (1.34 - 3.67), P=0.002	1.93 (1.17 - 3.20), P=0.01
Age (1 year)		1.04 (1.02 - 1.06), P<0.001	1.04 (1.02 - 1.05), P<0.001
Gender (0=female; 1=male)		1.23 (0.78 - 1.96), P=0.37	1.19 (0.75 - 1.89), P=0.46
[a]Current or past smoking (0=no; 1=yes)		1.14 (0.75 - 1.72), P=0.55	1.11 (0.73 - 1.68), P=0.63
Diabetes (0=no; 1=yes)		1.30 (0.89 - 1.92), P=0.18	1.24 (0.84 - 1.83), P=0.27
Systolic blood pressure (1 mm Hg)		1.01 (1.00 - 1.01), P=0.22	1.00 (1.00 - 1.01), P=0.30
Anti-hypertensive treatment (0=no; 1=yes)		0.88 (0.59 - 1.30), P=0.51	0.90 (0.60 - 1.33), P=0.59
CV comorbidities[b] (0=no; 1=yes)		1.68 (1.07 - 2.65), P=0.03	1.53 (0.97 - 2.42), P=0.07
Dialysis vintage (1 month)		1.02 (1.00 - 1.04), P=0.21	1.00 (1.00 - 1.00), P=0.30
CRP (1 mg/L)		1.00 (0.99 - 1.00), P=0.86	1.00 (1.00 - 1.00), P=1.00
Phosphate (1 mg/dL)		1.03 (0.91 - 1.17), P=0.67	1.02 (0.90 - 1.16), P=0.73
Cholesterol (1 mg/dL)		0.99 (0.99 - 1.00), P=0.004	0.99 (0.99 - 1.00), P=0.03

Data are expressed as hazard ratio, 95% confidence interval (CI) and P values.

[a]The inclusion of current smoking instead of current or past smoking into the Cox models did not materially affect the strength of the associations of each predictor and the occurrence of study outcomes (data non shown).

[b] CV comorbidities, except CHF which was herein considered as a separate covariate, were defined as in Table II

Fig. 2 *Crude and Adjusted Cox regression analysis showing that the increasing risk of HF for all-cause and CV mortality across snoring categories.*

All cause mortality
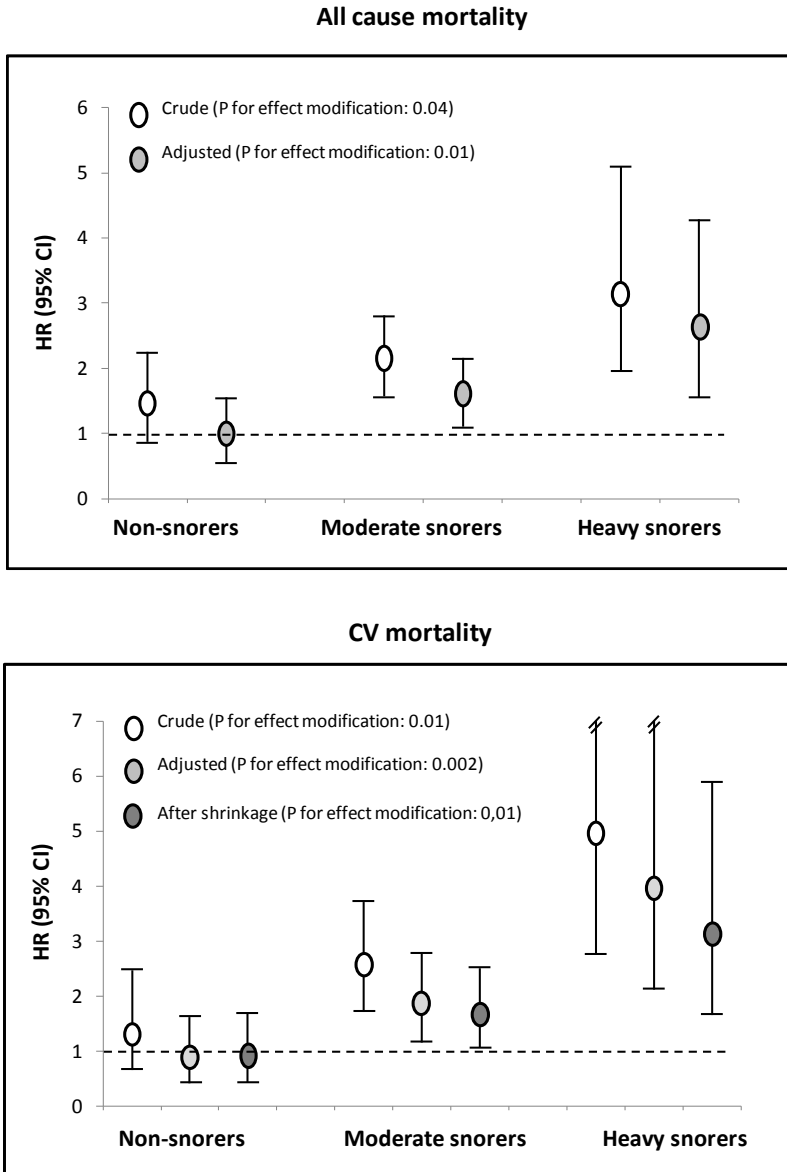
CV mortality

DISCUSSION

This study shows that snoring, a key symptom and sign of sleep disordered breathing, is an amplifier of the risk for all cause and cardiovascular mortality in ESRD patients on chronic dialysis with heart failure. Furthermore, in dialysis patients with chronic heart failure there is a dose-response relationship between the intensity of snoring and the independent risk for all-cause and cardiovascular death, which was respectively about 2 and 3 times higher in heavy snorers than in non-snorers.

Snoring is considered as a useful surrogate marker of SDB in large scale epidemiological and clinical studies [20-23]. However, until now self-reported snoring has not been validated as surrogate marker of SDB in the CKD population. In the validation part of this study we found that self-reported snoring had a fair discriminant power for the identification of CKD patients with SDB (area under ROC curve 75%) and perfect negative predictive power. However, the positive predictive power of this surrogate (38%) is limited, indicating that it may misclassify as affected by SDB some patients who are actually not affected by SDB. Notwithstanding this limitation, the fairly high discrimination power and the ability to exclude SDB makes snoring an acceptable surrogate of SDB in large scale studies in CKD like our study.

Several population-based studies and observations in patients with pre-existing CV disease documented that snoring per se has a limited prognostic impact upon health outcomes. Heavy snoring predicts short-term but not long-term mortality in patients with myocardial infarction [24]. Loud snoring associates with an increased risk for cardiovascular disease and more intensive use of health-care resources [25] as well as with the severity of carotid atherosclerosis [26]. However, in patients with established cerebro-vascular disease there is no such an association [27]. In line with these studies [24,27], we found that self-reported snoring per se fails to predict both all cause and CV mortality in ESRD patients. However, this surrogate of SDB emerged as a strong modifier of the link between heart failure and all cause and CV death. Indeed, in adjusted models, the risk of all-cause and CV death in ESRD patients with heart failure was 2 times and 4 times higher in heavy snorers than in non-snorers, respectively. Importantly, the effect modification by snoring showed a dose-response relationship (Fig 2). This observation has biological plausibility, as it likely reflects amplification of background sympathetic nervous system over-activity driven by nocturnal hypoxemia [5,28-29].

Sympathetic over-activity is a pervasive non-traditional risk factor in CKD patients [3]. Sympathetic activity as measured by plasma norepinephrine predicts mortality and cardiovascular outcomes independently of other risk factors in dialysis patients [30] and studies in pre-dialysis CKD patients by state of art sympathetic neurography fully confirm that high sympathetic activity is a strong and independent predictor of adverse clinical outcomes in CKD [31]. Sympathetic over-activation secondary to hypoxemia increases afterload and worsens left ventricular failure in patients with a pre-existing HF [32]. Furthermore, SDB – a well-known trigger of nocturnal

hypertension [33, 34] – induces cyclical episodes of bradycardia/tachycardia, leading to ventricular tachycardia and sudden death during sleep [35].

Our study has some limitations. First, the observational nature of our study prevents causal interpretations about the effect of snoring on mortality in ESRD patients with HF. Second, our study was based on self-reported snoring, which only in part coincides with SDB as diagnosed by polysomnography. Thus due the lack of precision of the questionnaire, snoring might not adequately reflect the underlying strength of the interaction between SDB and CHF for the risk of all-cause and cardiovascular mortality. Snoring and SDB are modifiable risk factors in ESRD [9,36] and in HF as well [37] and evidence has been produced that UF intensification during nocturnal dialysis in ESRD [38] or treatment with diuretics in HF [7,39] attenuate SDB. Clinical trials are needed to verify whether intensified surveillance and treatment (e.g. UF intensification) of ESRD snorers affected by heart failure may translate into better clinical outcomes.

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Chapter 3.2

The agreement between auscultation and US-B lines in the LUST study: a pre-planned analysis

ABSTRACT

Accumulation of fluid in the lung is the most concerning sequela of volume expansion in end-stage renal disease (ESRD) patients. Lung auscultation is recommended to detect and monitor pulmonary congestion but its reliability in ESRD is unknown.

In a subproject of the ongoing "Lung water by Ultra-Sound (US) guided Treatment to prevent death and cardiovascular complications in high risk ESRD patients with cardiomyopathy" (LUST) trial we compared a lung US guided ultrafiltration prescription policy vs standard care in high risk haemodialysis patients. The reliability of peripheral oedema was tested alongside. This study was based on 1106 pre- and post-dialysis lung US studies (in 79 patients) simultaneous with standardized lung auscultation (crackles at the lung bases) and quantification of peripheral oedema.

Lung congestion by crackles or oedema or a combination thereof poorly reflected the severity of congestion as detected by US-B lines in various analyses including standard regression analysis weighting for repeated measures in individual patients (shared variance 12% and 4% respectively) and kappa statistics (k ranging from 0.00 to 0.16). In general, auscultation had very low discriminatory power for the diagnosis of mild (area under the Receiver Operating Curve, ROC= 0.61), moderate (0.65) and severe (0.68) lung congestion and the same was true for peripheral oedema (ROC 0.56 or lower) and the combination of the two physical signs.

Lung crackles, either alone or combined with peripheral oedema, reflect very poorly interstitial lung oedema in ESRD patients. These findings reinforce the rationale underlying the LUST trial, a trial adopting US-B lines as an instrument to guide interventions aimed at mitigating lung congestion in high risk haemodialysis patients.

Keywords: lung ultrasound, pulmonary crackles, peripheral oedema, end-stage renal disease, chronic kidney disease

INTRODUCTION

In end-stage renal disease (ESRD) patients, accumulation of fluid in the lung is the most concerning consequence of volume expansion and the risk for pulmonary oedema is very high in this population [1, 2]. Systematic application of lung auscultation for the detection of crackles at the bases of the lungs is recommended in clinical practice both in individuals with suspected heart failure [3] and in ESRD patients [4]. However, the reliability of auscultation for the diagnosis of lung congestion has never been assessed in this population.

Lung water can be reliably estimated in clinical practice by applying lung ultrasound (US) [5-8]. Importantly, lung US has been well validated as a measure of pulmonary water in patients with cardiovascular disease [6] where it has virtually no bias when tested against thermodilution and shows reproducibility even higher than that of this gold standard in patients with heart disease. Furthermore, the same technique has also been extensively validated in intensive care patients where it holds very high discriminatory power for identifying moderate and severe congestion (areas under the ROC curves of 0.94 and 0.96, respectively) [9]. In ESRD the technique shows high intra and inter-observer reproducibility and high reproducibility also when assessed with diverse echo-tomography machines [10]. In ESRD lung congestion as detected by lung US holds strong prognostic power for death and cardiovascular events independently of traditional and ESRD specific risk factors [11, 12].

This study was performed within the frame of the "Lung water by Ultra-Sound guided Treatment to prevent death and cardiovascular complications in high risk ESRD patients with cardiomyopathy" (LUST) [13], an ongoing clinical trial testing the usefulness of systematic application of lung US in the clinical care of high risk haemodialysis patients. In this LUST sub-project we have adopted lung US as a reference method for testing the diagnostic reliability of pulmonary crackles as a clinical sign of pulmonary congestion and prospectively collected serial, well-standardized [14], pulmonary auscultation data alongside with measurements of lung water by US. The diagnostic value of pulmonary auscultation for detecting moderate to severe degrees of lung congestion was assessed by comparing over 1000 paired measurements of lung water by US with simultaneous standardized auscultation of the thorax. Since pitting oedema is frequent in patients with pulmonary crackles attributable to heart failure and/or volume overload, a secondary aim of this study was that of assessing whether the combined detection of lung crackles and peripheral oedema may improve the diagnostic performance of these physical signs for lung congestion.

METHODS

The study protocol was approved by the ethical committees of the Renal Units participating in the LUST trial. All participants gave informed consent before enrolment.

Patients
The LUST Study is a multicentre open randomized controlled trial aimed at assessing the usefulness of US-B lines in preventing adverse clinical outcomes (mortality, cardiovascular events, hospitalisations, progression of LVH and LV dysfunction) in dialysis patients at high cardiovascular risk. This trial is registered in ClinicalTrials.gov (Identifier: NCT02310061). The inclusion criteria for the enrolment in the LUST study are age > 18 years; dialysis vintage > 3 months; a history of myocardial infarction with or without ST elevation or unstable angina, acute coronary syndrome, documented by ECG recordings and cardiac troponins, or stable angina pectoris with documented coronary artery disease by prior coronary angiography or ECG or dyspnoea class III-IV NYHA. Patients with cancer or other advanced non cardiac disease or comorbidity (e.g. end-stage liver failure) imposing a very poor short-term prognosis; active infections or relevant inter-current disease; inadequate lung scanning and echocardiographic studies were excluded from the trial. For the scope of the present study we focused on patients randomized into the active arm (no pre- and post-dialysis lung US is contemplated in patients in the control arm in the LUST trial). This subproject included 79 patients with simultaneous pre- and post-dialysis ultrasound B (US-B) lines, peripheral oedema and pulmonary crackles measurements. The total number of paired US lung scan and lung auscultation records was 1106 (on average 14 per patient) over an observation period of 11 months.

US-B lines measurement
US-B lines are the sonographic equivalent of classical B lines detected in standard chest X-ray in patients with lung congestion/oedema [15]. US-B lines measurements have a high inter-observer reliability (concordance index 0.96) as well as a high inter-probe concordance (concordance index 0.99) in haemodialysis patients [10]. US-B lines assessment was made immediately before and after dialysis, in supine position. Scanning of the anterior and lateral chest was performed on both sides of the chest, from the second to the fourth (on the right side to the fifth) intercostal space, at parasternal to mid-axillary lines, as previously described [10]. US-B lines were recorded in each intercostal space and were defined as a hyperechoic, coherent US bundle at narrow basis going from the transducer to the limit of the screen. The sum of US-B lines produces a score reflecting the extent of LW accumulation [5, 16, 17]. Detailed description of the technique is available in a 2-min movie in YouTube (The incredible ULCs. Available at http://www.youtube.com/watch?v=7y_hUFBHStM. Accessed: 22nd February 2016). Lung congestion was categorized according to Frassi

et al., [18] as absent <5 US-B lines; mild: >5 <15; moderate: >15 and <30 US-B lines; severe >30 US-B lines. All nephrologists participating into the LUST study were trained by a specific WEB-based educational program [19] and certified by the validation centre at the IFC-CNR in Pisa. To be certified, assessors had to have an 85% or higher concordance in the assessment of lung US scans with the expert trainer at the validation centre.

Clinical evaluation of volume status, lung auscultation
In all patients included into the active arm of the LUST trial, a standard pre- and post-dialysis clinical evaluation of volume status was done immediately before the US-B lines measurements. Information about blood pressure (BP) and BP changes over time, peripheral oedema, presence/absence of dyspnoea, crackles on lung auscultation, inter-dialysis body weight gain and body weight trajectory data over time was collected. Lung auscultation was carefully done in anterior and posterior basilar sites in each hemi-thorax in seating position. Patients were asked to perform periodic slow deep respirations. To evaluate crackles, the following scale (adapted from [14]) was used: 1 = No crackles; 2 = I am uncertain about the presence of fine crackles; 3 = Definite fine crackles at lung bases; 4 = Moderate crackles; 5 = Bilateral, diffuse crackles. For clinical oedema, the following scale was used: 1 = No clinical oedema; 2 = Slight pitting (2 mm depth) with no visible distortion; 3 = Somewhat deeper pit (4 mm) with no readily detectable distortion; 4 = Noticeably deep pit (6 mm) with the dependent extremity full and swollen; 5 = Very deep pit (8 mm) with the dependent extremity grossly distorted [20].

Statistical analysis
Data are expressed as mean ± standard deviation (normally distributed data), median and inter-quartile range (non-normally distributed data) or per cent frequency (categorical data). The correlation between US-B lines and pulmonary crackles/peripheral oedema was assessed by using the Pearson's correlation coefficient and the shared variance was calculated by squaring the same correlation coefficient. To account for the fact that US-B lines were repeated measurements in the same patients we performed weighted regression analyses [21]. The discrimination power of crackles and peripheral oedema for lung congestion as detected by US-B lines was investigated by analysing the area under receiving operating characteristic (ROC) curve. In this analysis, five categories of crackles [14] or peripheral oedema [20] (as described before) were used to predict the presence of lung congestion as assessed by lung US (mild: >5 <15; moderate: >15 and <30 US-B lines; severe >30 US-B lines) [18]. Sensitivity, specificity, positive and negative predictive value of crackles and oedema were also calculated. Pre- and post-dialysis variations of US-B lines were compared by the Wilcoxon test and the relationship between pre- to post-dialysis changes in crackles and in peripheral oedema were investigated by using the Wilcoxon test for dependent variables. The agreement

between US-B lines and crackles/oedema was also described by using the Cohen's kappa coefficient. Statistical analysis was performed by using standard statistical packages (SPSS for Windows, Version 20, Chicago, Illinois, USA; MedCalc Software, Version 15, Ostend, Belgium).

RESULTS

The main demographic, anthropometric, clinical and biochemical characteristics of the study population at baseline are detailed in Table 1. The categorization of patients refers to the baseline assessment of US-B lines; the number of paired US-B lines reported in Table 1 refers to the number of measurements falling in each category. Mean age was 72 years, 65% of patients were male, 20% were current smokers. Thirty-seven per cent of patients were diabetics and all patients had cardiovascular comorbidities. No differences in the other clinical data were found. These patients had been on regular haemodialysis for a median time of 52 months (inter-quartile range: 30-113) and were being treated with thrice weekly haemodialysis with various haemodialysis filters. Fifty-six per cent of patients were treated with various anti-hypertensive drugs (32% of them on mono-therapy with calcium channel blockers, ACE inhibitors, sartans, alpha or beta blockers, clonidine, furosemide, 36% on double therapy, 18% on triple therapy and 14% patients on multiple therapy with various combinations of these drugs).

Association of crackles and peripheral oedema with lung congestion as measured by US-B lung
No patient had intercurrent inflammatory or infectious bronco-pulmonary disease when simultaneous assessment of US-B lines and crackles was done. Overall 1106 paired assessments (in 79 patients) of lung congestion by auscultation and by lung US were performed. Thus on average each patient had 14 paired assessments. In 144 assessments (13%) there was evidence of moderate congestion (>15 <30 US-B lines) and in 118 assessments (11%) severe lung congestion (>30 US-B lines) was documented. Overall, in the vast majority (61%) of these assessments, evidence of moderate or severe lung congestion by US was not accompanied by the presence of crackles. In severe lung congestion (as defined by US) the prevalence of crackles (49%) was higher (P=0.003) than that registered in patients with moderate (by US) congestion (31%). Table 2 shows the number of US B lines across crackles-number strata of increasing severity.
By the same token, peripheral oedema was conspicuously absent in as much as 87% and the 80% of assessments where lung US indicated moderate and severe lung congestion respectively. The severity of lung congestion by crackles correlated very weakly with the severity of lung congestion as detected by US-B lines (shared variance 12%) (Fig. 1 A). The correlation between peripheral oedema with the number of US-B lines was even weaker and the shared variance was minuscule being a mere 4% (Fig. 1

B). The combination of crackles and oedema (i.e. the sum of the two scores) did not improve the degree of the association of these signs with US-B lines (shared variance 10%).

Post-dialysis changes of US-B lines, pulmonary crackles and peripheral oedema.
Lung congestion as detected by lung US was modified by dialysis treatment. The median number of US-B lines before dialysis was 9 (IQR: 5-19) and fell to 5 (IQR: 2-10, P<0.001) after dialysis. In detail, the number of US B lines decreased in the 79% of patients and did not change in the 21% of patients (i.e. remained exactly the same or changed by 2 US-B lines at most). Before dialysis, in 39 patients out 79 (49% of patients) and in a total of 439 assessments in the same patients (79% of assessments) no crackles were detected. Similarly, in 31 patients out of 79 patients (39% of patients) and in 495 assessments (90% of assessments) in the same patients no peripheral oedema was registered. In the remaining assessments, i.e. those where these alterations were noted, both crackles [from a median pre-dialysis score of 2.00 (IQR: 1.00-2.25) to a post-dialysis score of 1 (IQR: 0.00-2.00), P<0.001)] and peripheral oedema [from 1.00 (IQR: 1.00-1.25 to 1.00 (IQR 1.00-1.00, P<0.001] reduced after dialysis. After dialysis the degree of association of crackles and peripheral oedema with US-B lines remained very poor (shared variance 13% and 8% respectively).

Table 1: *Main demographic, anthropometric and clinical characteristics in patients as divided according to US-B lines number.*

	US-B Lines number				
	<5	>5 <15	>15 <30	> 30	P for linear trend
Number of patients	22	35	16	6	
Number of paired US-B lines crackles/oedema measurements	391	453	144	118	
Age (years)	71±11	74±13	71±13	68±10	0.57
BMI (kg/m^2)	27±5	26±4	24±4	28±4	0.26
Male sex (%)	73	71	50	33	0.15
Smokers (%)	5	31	19	33	0.12
Diabetics (%)	43	33	38	67	0.48
On anti-hypertensive treatment (%)	67	59	73	50	0.71
Dialysis vintage (months)	33(10-71)	57(34-282)	84(33-150)	55(39-85)	0.12
History of myocardial infarction (MI) or stable angina pectoris with documented coronary artery disease, without Heart Failure (HF) n. (%)	13 (59)	19 (54)	6 (38)	3 (50)	0.60
Heart Failure, NYHA class III-IV without a history of myocardial infarction and/or coronary heart disease n. (%)	3 (14)	2 (6)	5 (31)	1 (17)	0.11
History of MI/coronary heart disease and HF n. (%)	6 (27)	14 (40)	5 (31)	2 (33)	0.79
Systolic Blood Pressure (mmHg)	139±28	138±24	145±30	145±21	0.84
Diastolic Blood Pressure (mmHg)	70±13	69±17	67±12	72±13	0.86
Pulse Pressure (mmHg)	69±27	70±22	78±29	73±16	0.69
Cholesterol (mg/dL)	166±42	165±48	166±50	149±31	0.92
Haemoglobin (g/dL)	11.1±1.2	11.2±1.3	11.0±0.9	10.0±0.9	0.24
Albumin (g/dL)	4.0±0.7	3.7±0.3	4.0±0.8	3.4±0.5	0.15
Calcium (mg/dL)	8.8±0.4	8.8±0.8	8.8±0.8	9.2±0.8	0.66
Phosphate (mg/dL)	4.9±1.8	5.2±1.4	4.8±1.1	5.6±2.3	0.71
III-IV NYHA Class n. (%)	6 (27)	9 (26)	5 (31)	3 (50)	0.67

Table 2: *US-B lines number across lung crackles strata of increasing severity*

		US-B lines number				Total
		<5	>5 <15	>15 <30	> 30	
Crackles category	**0**	370	388	88	60	906
	1	13	35	7	6	61
	2	6	26	24	37	93
	3	1	3	3	4	11
	4	1	14	9	11	35
Total		391	466	131	118	1106

Fig. 1: *Correlation between the severity of lung congestion as detected by US-B lines with pulmonary crackles (A) and peripheral oedema (B) respectively.*
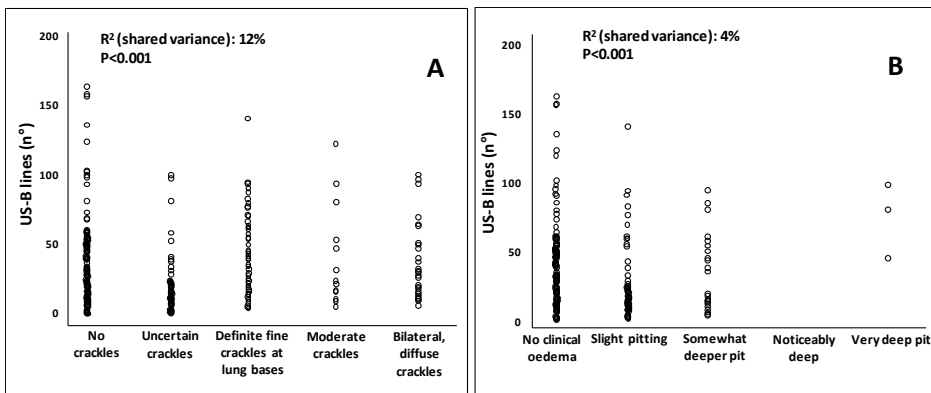
Agreement between US-B lines and clinical examination

In line with the previous analysis showing a modest shared variance between US-B lines and pulmonary crackles/peripheral oedema respectively (see above) we found a poor agreement (by the K weighted statistics) between US-B lines and pulmonary crackles either in analyses considering the average number of USB lines and the average grading of crackles and peripheral oedema across the observation period or the whole series of measurements considered individually (Table 3)

Table 3: *Agreement [Weighted Kappa (95% CI)] between US-B lines and pulmonary crackles, peripheral oedema and a combination thereof considering the individual average number of USB lines in the 79 patients and the simultaneous average grading of crackles and peripheral oedema or the whole series of measurements considered one by one (n=1106).*

	Individual average US B lines values (n=79 patients)	Whole series of US B lines (n=1106)
Pulmonary crackles	0.10 (0.01 to 0.20)	0.16 (1.13 to 1.20)
Peripheral oedema	-0.01 (-0.11 to 0.09)	0.02 (-0.01 to 0.04)
Crackles / Oedema	-0.00 (-0.02 to 0.01)	0.07 (0.05 to 0.09)

Discrimination analysis and standard diagnostic tests

On discriminant analysis (ROC curve), crackles had a limited discriminatory power for the diagnosis of mild, moderate or severe lung congestion as assessed by US (mild lung congestion: AUC 0.61, CI: 0.57-0.64, P<0.001; moderate congestion: AUC 0.65, CI: 0.61-0.70, P<0.001; severe congestion: AUC 0.68, CI: 0.62-0.74, P<0.001). Peripheral oedema had virtually no discriminatory power (mild lung congestion: AUC 0.51, CI: 0.48-0.55, P=0.54, moderate congestion: AUC 0.54, CI: 0.50-0.58, P=0.05; severe congestion: AUC 0.56, CI: 0.50-0.62, P=0.03). The use of a composite score (the sum of crackles and oedema) failed to materially increase the discrimination power of crackles and peripheral oedema considered as separated physical signs (mild lung congestion: AUC 0.60, CI: 0.57-0.64, P<0.001; moderate congestion: AUC 0.65, CI: 0.61-0.70, P<0.001; severe congestion: AUC 0.68, CI: 0.62-0.74, P<0.001). The sensitivity, specificity, positive and negative predictive value of crackles and peripheral oedema for lung congestion are reported in Table 4. In general, both clinical signs had very low sensitivity but high specificity for lung congestion. However, the false negative rate was exceedingly high both for lung crackles and peripheral oedema ranging from 69% to 99% (Table 4). The likelihood ratios clearly indicated that crackles and peripheral oedema had modest value for ruling in or ruling out lung congestion.

DISCUSSION

This study shows that two time honoured clinical signs like lung crackles and peripheral oedema which are universally applied to detect and monitor volume excess and/or fluid translocation to tissues and organs in disease states, including ESRD, have an unsuspectedly low sensitivity for detecting interstitial oedema in a most critical organ like the lungs in this population. These findings have potential implications for clinical practice and suggest that these clinical signs only remotely reflect the degree of lung congestion as measured by an objective, well validated method like lung US scanning.

Table 4: *Diagnostic value of pulmonary crackles and peripheral oedema for the diagnosis of moderate and severe lung congestion in the 1106 paired measurements (in 79 patients) of these clinical signs and simultaneous US-B lines. The positive Likelihood ratio (i.e. sensitivity/ false positive rate) of peripheral oedema could not be calculated because the corresponding false positive rates were 0.*

	Pulmonary crackles			Peripheral oedema			Crackles and oedema		
	Mild lung congestion	Moderate lung congestion	Severe lung congestion	Mild lung congestion	Moderate lung congestion	Severe lung congestion	Mild lung congestion	Moderate lung congestion	Severe lung congestion
Sensitivity	172/644 (27%)	27/249 (11%)	11/118 (9%)	67/644 (10%)	3/249 (1%)	3/118 (3%)	200/644 (31%)	44/249 (18%)	15/118 (13%)
False Positive Rate	28/462 (6%)	19/857 (2%)	24/988 (2%)	39/462 (8%)	0/857 (0%)	0/988 (0%)	54/462 (12%)	25/857 (3%)	31/988 (3%)
Specificity	434/462 (94%)	838/857 (98%)	964/988 (98%)	423/462 (92%)	857/857 (100%)	988/988 (100%)	408/462 (88%)	832/857 (97%)	957/988 (97%)
False Negative Rate	472/644 (73%)	222/249 (89%)	107/118 (91%)	577/644 (90%)	246/249 (99%)	115/118 (97%)	444/644 (69%)	205/249 (82%)	103/118 (87%)
Positive Predictive Value	172/200 (86%)	27/46 (59%)	11/35 (31%)	67/106 (63%)	3/3 (100%)	3/3 (100%)	200/254 (79%)	44/69 (64%)	15/46 (33%)
Negative Predictive Value	434/906 (48%)	838/1060 (79%)	964/1071 (90%)	423/1000 (42%)	857/1103 (78%)	988/1103 (90%)	408/852 (48%)	832/1037 (80%)	957/1060 (90%)
Positive Likelihood Ratio	4.41	4.89	3.84	1.23	---	---	2.66	6.06	4.05
Negative Likelihood Ratio	0.78	0.91	0.93	0.98	0.99	0.97	0.78	0.85	0.90

Lung auscultation is a cornerstone of physical examination. This procedure provides important clinical information about the respiratory system. Even though expertise in auscultation requires standardization and specific training, the technique is simple, low cost and widely available and can be repeated whenever required to monitor patients [22]. In patients with heart failure, the presence of crackles is considered indicative of pulmonary congestion secondary to left ventricular dysfunction and/or volume overload and this physical sign guides physicians to implement or to change therapy in patients with heart disease [23]. The presence of ankle oedema and basal lung crackles helps to identify patients with suspected heart failure who should be referred for echocardiography [3]. However, when compared to imaging techniques, lung auscultation is notoriously insensitive to capture an increased quantity of fluid in the lungs in patients with acute heart failure [24] and in ambulatory patients with chronic heart failure [25, 26] where the technique has both low sensitivity and low specificity as well [25]. Furthermore, in the acute care setting the diagnosis of

interstitial oedema by auscultation is substantially inferior to that of chest radiography and lung US [27].

ESRD patients maintained on chronic dialysis have an exceedingly high risk of hospitalisation and death for pulmonary oedema [1] and the differentiation of congestive heart failure from volume expansion may be problematic in these patients [28]. Lung congestion is an insidious phenomenon which builds up gradually over weeks before frank, symptomatic pulmonary oedema [29]. Furthermore, the degree of lung congestion only weakly associates with estimates of volume excess in ESRD like total body water by BIA and inter-dialysis weight gain/ultrafiltration volume [10]. Subclinical congestion is of peculiar relevance because these patients have increased alveolo-capillary permeability [30] which makes them vulnerable to volume overload which gradually builds up during the dialysis interval. In this study the median number of US-B lines before a regular haemodialysis session was 9 and the interquartile range spanned from 5 to 19 lines. These estimates underlie a median accumulation of water in the lungs of about 1.2 Litres in a range comprised between 0.5 and about 2.2 L, which is a substantial degree of congestion [16]. Of note the majority of these ESRD patients had no or very mild effort dyspnoea indicating that clinical symptoms may be conspicuously absent even at relevant levels of water accumulation in the lung. At peak of volume expansion (i.e. before dialysis) crackles and peripheral oedema were relatively rare being present just in 21% and 10% of cases respectively. The agreement between US-B lines and pulmonary crackles or between the same parameter with peripheral oedema or a combination of crackles/oedema was poor. As one may expect from the relatively rare occurrence and the low sensitivity of lung crackles and peripheral oedema, the specificity of these signs for the diagnosis of lung congestion was very high indeed but this high negative diagnostic power was counterbalanced by almost equally high false negative rates. Considering crackles and oedema in aggregate (which conforms to clinical practice and maximizes the discriminant power for lung congestion of these signs) the combination of these two signs showed a satisfactory to low positive predictive value [see Table 4, ranging from 79% (mild congestion) to 33% (severe congestion)] and a high to moderate negative predictive power [ranging from 90% (severe congestion) to 48% (severe congestion)]. However, this alteration went unnoticed in a substantial proportion of patients without crackles and/or peripheral oedema (false negative rate ranging from 69% to 87%). Overall the discriminant ability of crackles and peripheral oedema for the diagnosis of moderate and of severe lung congestion ranged from 0.54 to 0.68 which is a poor discrimination power [31]. Findings in this study support the contention that crackles and oedema provide only modest information on interstitial oedema in a critical organ like the lung and reinforce the rationale underpinning the "Lung water by Ultra-Sound guided Treatment to prevent death and cardiovascular complications in high risk ESRD patients with cardiomyopathy" (LUST) [13]. Lung US is a highly reliable, low-cost, easy to learn technique which can be performed with whatever echotomographic machine, from a hand-held one to echocardiography machines and machines applied for the

sonography of abdominal organs [8]. The ongoing LUST study will establish whether systematic application of lung US may help preventing excessive water accumulation in the lung and ultimately improve clinical outcomes in high risk dialysis patients.

This study has limitations. First, we repeatedly measured US-B lines and quantified crackles and peripheral oedema in patients in the active arm of a clinical trial enrolling patients at high cardiovascular risk. Therefore, our results cannot be generalized to the whole dialysis population. Yet it is precisely in this population that the risk of lung congestion is highest. Second, we focused on lung auscultation as traditionally performed by standard stethoscopes by trained physicians. Novel acoustic devices for use at the bedside are being proposed - e.g. electronic stethoscopes synchronized with small recorders in the form of smartphone applications - to record lung sounds and to enhance the usefulness of auscultation [22]. Therefore, the value of these novel devices has potential for the detection of lung congestion far superior to conventional auscultation. Finally, although the measurements of crackles and peripheral oedema in LUST were well-standardized and performed with a high degree of attention by physicians, we have not specifically measured the inter-observer variability of these clinical signs in LUST.

In conclusion, two classical physical signs like lung crackles and peripheral oedema have a very low sensitivity for detecting interstitial lung oedema in the ESRD patients. These findings reinforce the rationale underlying the LUST trial, a trial testing US-B lines as an instrument to guide interventions aimed at mitigating lung congestion in high risk haemodialysis patients.

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Chapter 3.3

Physical functioning and mortality in very old patients on dialysis

ABSTRACT

Old patients with end-stage renal disease (ESRD) represent an increasing segment of the ESRD population maintained on chronic dialysis treatment. Quality of life (QoL) is notoriously poor in ESRD but relationship between QoL and mortality has not been investigated in the old dialysis population.

Objective of this study was to investigate the relationship between QoL and mortality in the old dialysis population.

We measured Quality of Life by the Rand- QoL Short Form 36 in a cohort study including 253 very old patients (age > 75 years) on chronic dialysis.

In multivariate statistical models including a series of demographic and clinical variable physical function and general health maintained an independent relationship with survival (P<0.01). In analyses testing the prognostic value of these two SF36 components physical functioning was the component adding the highest explanatory power to standard demographic and clinical risk factors (+5.9%). Furthermore, the same parameter increased by 4.8% the discriminant power by the Harrell's C Index, improved risk reclassification by the 21% (P=0.003) and model calibration by the 59%.

Conclusions: In the very old dialysis population the physical function component of the SF36 is the QoL component holding the highest predictive power for mortality among the eight components of this instrument. As the discrimination power and risk reclassification ability by physical functioning is of degree relevant for clinical practice, such a measure has potential for refining prognosis and informing exercise programs in this population.

Keywords: Quality of Life, Physical Function, mortality, end-stage renal disease, dialysis

INTRODUCTION

Old patients with end-stage renal disease (ESRD) constitute a frail population with multiple comorbidities and a high risk of cardiovascular morbidity and mortality [1]. In Europe, between 2001 and 2011 the incidence rate of ESRD in the age stratum 75-84 years has increased from approximately 450 patients per million population (pmp) to approximately 530 patients pmp and in the oldest age stratum (> 85 years) from about 180 pmp to about 280 pmp [2]. In 2014 approximately 20% ESRD patients across Europe were older than 75 years [3]. On the other hand, analyses in the USA Renal Data System (USRDS) document a coherent, progressive aging of the dialysis population [4]. Thus, the ESRD burden of the old population is an issue of major clinical and public health relevance both in Europe and in the USA.

Quality of life (QoL) is notoriously poor in ESRD patients and the problem is of particular concern in older people [5] where cognitive and [6] physical impairment are quite common [5].

The RAND QoL Short Form 36 (SF-36) represents an efficient, easy to administer, tool for assessing QoL [7, 8]. SF-36 incorporates eight QoL components which explore both emotional and physical well-being [7]. This instrument has been specifically validated in ESRD patients [9] and the physical and mental components of SF36 associated with the risk of death independently of other risk factors in an international study in middle-age dialysis patients in five European countries (France, Germany, Italy, Spain, and the United Kingdom), Japan and United States [10]. However, the relationship between the eight components of SF-36 and mortality has never been investigated in the old ESRD population. This issue is of relevance because the identification of the components that better capture the risk of mortality in older people may be useful for profiling interventions in these frail, high risk patients.

With this background in mind we investigated the relationship between the eight QoL components of the SF36 and mortality in a cohort of dialysis patients older than 75 years. We specifically focused in this population because very old patients represent the most vulnerable population of patients with chronic diseases [9] and because the incidence rate of ESRD in the very old people has grown at an accelerated rate both in the dialysis population of the United States Renal Data System (USRDS)[4] and in the European Renal Association, European Dialysis and Transplantation (ERA-EDTA) Registry [11] where about 20% of patients are older than 75 years [3].

METHODS

The study protocol had the approval of the ethical committee of our institution. All participants gave their informed consent before enrolment.

Study population

In this analysis we included 253 dialysis patients aged more than 75 years who completed the Rand- QoL Short Form (SF36) questionnaire among the 1189 composing the whole PROGREDIRE (Prospective Registry of The Working Group of Epidemiology of Dialysis Region Calabria) cohort, a study involving 35 dialysis units in two regions in Southern Italy (Calabria and Sicily) [12]. Patients had been on regular dialysis (HD or PD) for a median time of 43 months (inter-quartile range: 18-75). Haemodialysis patients (n=245) were being treated with standard bicarbonate dialysis with non-cellulosic membrane filters of various type. PD patients (n=8) were either on 4 standard exchanges day or on continuous cycling peritoneal dialysis. A hundred and forty-seven patients were treated with various anti-hypertensive drugs (66 on mono-therapy with ACE inhibitors, calcium channel blockers, α- and β-blockers, vasodilators, diuretics or other drugs, 48 on double therapy, 22 on triple therapy and 11 patients on quadruple or quintuple therapy with various combinations of these drugs). The main demographic, somatometric, clinical and biochemical characteristics of the study population are detailed in Table 1.

Questionnaires
The SF-36 questionnaire [8] contains 36 short questions, that can be grouped into 8 components: Physical Function (PF, 10 questions), Role Limitation due to Physical Health (RLPH, 4 questions), Role Limitation due to Emotional Problems (RLEP, 3 questions), Energy/Fatigue (EF, 4 questions), Emotional Well-being (EW, 5 questions), Social Functioning (SF, 2 questions), Pain (P, 2 questions), General Health (GE, 5 questions), and one question about any change in the health status. The validated, Italian version of SF36 [13] was administered to the enrolled patients at baseline. Patients unable to fill the questionnaire in by themselves were helped by a trained nurse.

Statistical analysis
Data were expressed as mean ± standard deviation (normally distributed data), median and inter-quartile range (non-normally distributed data) or as per cent frequency (categorical data). Correlates of the 8 components of SF36 were found by using the Spearman's Rho (ρ). Linear regression analysis was applied to investigate the explained variability of physical functioning. Survival analyses were performed by using both univariate and multivariate Cox regression analyses, including as covariates traditional [age, gender, current smoking, diabetes, cardiovascular comorbidities, cholesterol, arterial pressure, antihypertensive use, BMI], inflammatory [CRP, albumin], dialysis-related risk factors [dialysis vintage, haemoglobin, phosphate]. The prognostic power of the components of SF36 was assessed by the explained variation in mortality (R2) [14]; the discrimination power (Harrell's C Index) [15]; risk reclassification (Net Reclassification Index, NRI) [16] and calibration by the May-Hosmer test [17]. Multivariate logistic regression models, including the set of covariates listed above, were used to compute the predicted probability of death. The

33rd and 66th percentile of the death probability were chosen as cut-offs for NRI. In each of the prognostic tests listed above we compared a basic model, including Framingham risk factors, inflammatory and dialysis-related factors, with a full model, including the same variables of the base model and, in turn, each of the eight components of SF36. Statistical analysis was performed by using standard statistical packages (SPSS for Windows, Version 22, Chicago, Illinois, USA; STATA for Windows, Version 13, College Station, Texas, USA).

RESULTS

Among a total of 297 patients > 75 years, 253 underwent the SF36 questionnaire. Patients who underwent the questionnaire did not differ from people not included in this analysis for the main demographic, somatometric and clinical characteristics.
The main baseline characteristics of the study population are reported in Table 1. Fifty-six per cent of patients were males; mean age was 81 years. Diabetics were 30% and 63% of patients had cardiovascular co-morbidities.

Correlation analysis
As expected, the eight components of the SF36 were strongly associated to each other, with ρ values ranging from 0.24 to 0.53 (P<0.001). Six SF-36 components (Physical Function, Role Limitation due to Emotional Problems, Energy/Fatigue, Emotional Wellbeing, Social Functioning, Pain) were directly associated with gender [ρ ranging from 0.16, P=0.01 (Role Limitation due to Emotional Problems) to 0.24 (Pain), P<0.01]. All SF36 components [ρ ranging from = 0.28, P<0.001(physical function) to -0.13, P=0.05 (Pain)] but Role Limitation due to Physical Health associated with serum albumin. Physical function was the only component which associated with age [ρ = -0.16, P=0.01)], whereas the same parameter and Role Limitation due to Physical Health correlated with background cardiovascular comorbidities [Physical Function: ρ = -0.20, P=0.002; Role Limitation due to Physical Health: ρ = -0.14, P=0.03]. Pain (ρ = -0.18, P=0.005) and General health (ρ = -0.14, P=0.02) but no other SF36 component were related with dialysis vintage. No association was found between SF-36 components and diabetes.

Table 1 *Main demographic, somatometric and clinical characteristics in the whole study population.*

	Study cohort N=253
Age (years)	81±4
BMI (kg/m^2)	25±4
Systolic/Diastolic Blood Pressure (mmHg)	135±24/70±11
Diabetics n. (%)	75(30)
Dialysis vintage (months)	42(18-75)
With cardiovascular comorbidities n. (%)	*158(63)*
Coronary Heart Disease [a] n. (%)	56(22)
Heart Failure	42(17)
Cerebrovascular Disease [b] n. (%)	37(15)
Peripheral Vascular Disease n. (%)	67(27)
On anti-hypertensive treatment n. (%)	147(58)
Cholesterol (mg/dL)	154±42
Haemoglobin (g/dL)	11.0±1.5
Albumin (g/dL)	3.8±0.4
C-Reactive Protein (mg/L)	6.2(3.0-16.1)
Calcium (mg/dL)	9.1±0.9
Phosphate (mg/dL)	4.6±1.5
PTH (pg/ml)	180(93-337)
SF-36 components	
Physical function	20 (0-50)
Role Limitation due to Physical Health	0 (0-100)
Role Limitation due to Emotional Problems	33 (0-100)
Energy/Fatigue	35 (20-50)
Emotional Well-being	48 (32-64)
Social Functioning	50 (38-75)
Pain	45 (23-78)
General Health	25 (15-40)

Data are expressed as mean ± SD. median and inter-quartile range or as percent frequency, as appropriate.
[a]Past myocardial Infarction or angina, coronary angioplasty or surgery [b]Stroke or transient ischaemic attack

Survival analysis

During a median follow-up of 2.2 years (interquartile range: 1.4-3.2 years), 154 patients died.

Among SF36 components, physical function, energy/fatigue and general health predicted survival (P<0.015). In adjusted models, including traditional, inflammatory and dialysis related risk factors, only physical functioning and general health remained significantly associated to the outcome (P<0.01) (Table 2).

Prognostic power of the SF36 domains

Prognostic analyses were focused on physical functioning and general health, i.e. the two components that were independently related to death risk (see Table 2). Physical functioning added a higher explanatory power (+5.9%, P for the overall fitting <0.001) to a clinical model formed by established predictors of mortality in dialysis patients (age, gender, current smoking, diabetes, cardiovascular comorbidities, cholesterol, arterial pressure, antihypertensive use, BMI, CRP, albumin, dialysis vintage, phosphate and haemoglobin) as compared to general health (2.5%, P for the overall fitting = 0.01). The gain in discriminating power (Harrell's C Index) was 4.8% for physical functioning, and 3.2% for general health. Physical functioning improved model calibration by the 59%, whereas the addition of general health improved the overall fitting only by the 20%. Furthermore, physical functioning was also more powerful for correctly reclassifying the risk categories (Net Reclassification Improvement +21%, P=0.002) than general health (+18%, P=0.002). The combination of physical activity and general health added a 5% discriminant power for mortality, which is a gain of the same order achieved by the physical functioning component alone (+4.8%, see above). The risk reclassification and calibration improvement (26% and 36 % respectively) achieved by the combination of these QoL components were again of the same order or lower than that of physical functioning per se (21% and 59%, see above). By the same token, the Integrated Discrimination Index (IDI) of physical functioning (5.3%, P<0.001) was higher than that of general health (3.1%, P=0.004) and the combination of these two SF36 components just to a modest extent increased the IDI from 5.3% (physical functioning alone) to 6.2%.

Table 2 *HR of the eight SF-36 components for all-cause mortality in univariate and multivariate models. Multivariate models are adjusted for traditional [age, gender, current smoking, diabetes, cardiovascular comorbidities, cholesterol, arterial pressure, antihypertensive use, BMI], inflammatory [CRP, albumin], dialysis-related risk factors [dialysis vintage, haemoglobin, phosphate]. See text for details.*

	Univariate analysis HR (95%CI), P	Multivariate analysis HR (95%CI), P
Physical Function	0.87 (0.81-0.93), P<0.001	0.88 (0.81-0.95), P=0.001
Role Limitation due to Physical Health	0.99 (0.96-1.03), P=0.72	1.00 (0.97-1.04), P=0.82
Role Limitation due to Emotional Problems	1.002 (0.97-1.04), P=0.90	1.00 (0.97-1.03), P=0.98
Energy/Fatigue	0.90 (0.83-0.98), P<0.02	0.92 (0.85-1.01), P=0.07
Emotional Well-being	0.95 (0.88-1.02), P=0.15	0.95 (0.88-1.02), P=0.15
Social Functioning	0.96 (0.91-1.01), P=0.13	0.96 (0.90-1.02), P=0.14
Pain	0.99 (0.94-1.05), P=0.78	1.00 (0.95-1.06), P=0.99
General Health	0.88 (0.80-0.96), P=0.005	0.88 (0.80-0.97), P=0.01

DISCUSSION

This analysis in very old ESRD patients focusing on the prognostic power for mortality by the quality of life components of the SF36 questionnaire indicates that physical activity is the component holding the highest predictive power for this outcome.

Measuring QoL in older people is important for the identification of possible areas of intervention aimed at alleviating the multiple comorbidities and disabilities burden of the aged population [18]. Furthermore, the identification of the QoL components more strongly related to the risk of death may help to define priority areas for intervention in the scenario characterized by chronic diseases of the old population. Even though QoL has received substantial attention in ESRD [19], there is no detailed analysis of the relationship between individual components of QoL and mortality in old and very old ESRD patients. The issue is important for at least three reasons. First because risk factors for death in older people differ from those in the middle aged population [20]. Second, because life priorities and health perception in older people [21] do not coincide with those in other age-strata. Third, because ESRD is a condition with a peculiar series of risk for mortality [22] and because this condition is characterized by disability and poor QoL [19].

As previously alluded to, the SF-36 is an instrument well suited for the assessment of QoL in chronic conditions and this instrument has been specifically validated in the ESRD population [9, 13]. This questionnaire is a reduced version of a much broader QoL instrument which was specifically produced for large surveys and for clinical practice and it can be completed in 10 minutes or less. In the present study in ESRD patients the physical functioning component of SF-36 was the one holding the highest prognostic power in that it added about the 5% discriminant ability to prediction made on a simple model based on demographic and clinical variables. Furthermore, physical function held a robust risk reclassification ability (+21%), i.e. the ability to correctly reclassify high risk patients versus low risk patients as identified by standard risk factors. Besides physical functioning, only the general health component of SF36 was related to mortality but the prognostic power for mortality of this component was inferior to that by physical function and the combination of this component with physical function added just a small additional prognostic value as compare to physical functioning alone. Even though an assessment of QoL in clinical practice at least once a year is formally recommended by the Centre for Medicare and Medicaid Services [19] QoL is still not measured in clinical practice in most dialysis centres worldwide. Comprehensive assessment of QoL by the SF36 provides relevant information on the psychological and physical dimensions of QoL which cannot be easily surrogated by minimalist approaches to the problem. In this respect our findings indicate that assessment of physical function only may suffice to refine prognosis in ESRD. The physical function component of SF36 demands no more than 2 minutes to be assessed and therefore it appears ideally suited for application in clinical practice, at least for the scope of refining prognosis in a high risk population

like very old patients maintained on chronic dialysis treatment. Furthermore, knowledge of physical function at patient level may be useful for designing physical rehabilitation interventions in this high risk, sedentary population [23].

Our study has limitations. First, our cohort was relatively small and composed by very old Caucasian dialysis patients. Therefore, findings in the present study should be confirmed in larger cohorts and in other ethnicities. Second, although findings in our study were robust and internally consistent, the lack of a validation cohort reduces generalizability of our results.

The physical function component of the SF36 is the QoL component holding the highest predictive and discrimination power for mortality among the eight components of this instrument and the top rank predictor among all risk factors currently applied to model survival in the dialysis population. The prognostic value of this component is of magnitude that it may have potential for refining risk stratification in clinical practice in a high risk population like very old dialysis patients.

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Chapter 3.4

Physical performance and clinical outcomes in dialysis patients: a secondary analysis of the EXCITE trial

ABSTRACT

Scarce physical activity predicts shorter survival in dialysis patients. However, the relationship between physical (motor) fitness and clinical outcomes has never been tested in these patients.

We tested the predictive power of an established metric of motor fitness, the Six-Minute Walking Test (6MWT), for death, cardiovascular events and hospitalisation in 296 dialysis patients who took part in the trial EXCITE (ClinicalTrials.gov Identifier: NCT01255969).

During follow up 69 patients died, 90 had fatal and non-fatal cardiovascular events, 159 were hospitalized and 182 patients had the composite outcome. In multivariate Cox models - including the study allocation arm and classical and non-classical risk factors - an increase of 20 walked metres during the 6MWT was associated to a 6% reduction of the risk for the composite end-point (P=0.001) and a similar relationship existed between the 6MWT, mortality (P<0.001) and hospitalisations (P=0.03). A similar trend was observed for cardiovascular events but this relationship did not reach statistical significance (P=0.09).

Poor physical performance predicts a high risk of mortality, cardiovascular events and hospitalisations in dialysis patients. Future studies, including phase-2 EXCITE, will assess whether improving motor fitness may translate into better clinical outcomes in this high risk population.

Keywords: Physical activity, 6MWT, clinical outcomes, dialysis

INTRODUCTION

Physical activity and physical performance are notoriously poor in patients with end-stage renal disease (ESRD) [1], a population with an extremely high risk of death and cardiovascular events [2]. Even though representing strictly related phenomena, physical activity and physical performance are separated entities with different metrics. Physical activity, i.e. physical engagement in daily activities, is a well-established predictor of mortality and cardiovascular events in the general population [3] and in pathological conditions such as diabetes [4] and coronary artery disease [5] and in end-stage renal disease (ESRD) as well [6-9]. To our knowledge, the relationship between actual physical performance, i.e. the objectively measured ability to perform well standardized physical efforts, and clinical outcomes in ESRD has been investigated just in a small study with a very limited number of major clinical events (just 21 deaths) [10]. The Six-Minute Walking Test (6MWT) is an established test to assess physical performance in frail elderly patients [11], and this test has been applied in clinical studies in various conditions, such as heart failure [12, 13] and chronic obstructive pulmonary disease (COPD) [14].
The EXCITE (EXerCise Introduction To Enhance Performance in Dialysis) study, is a large, multicentre, randomized trial whose phase – 2 (clinical outcomes and hospitalisation) is still in progress. This study tests the effectiveness of an easy-to-implement program of physical training in dialysis patients. We have taken the opportunity of the EXCITE study to investigate the relationship between actual physical performance, as assessed by the Six-Minutes Walking Test, on mortality, cardiovascular events and hospitalisations in dialysis patients.

METHODS

The study protocol was approved by the ethical committee of our institution. All participants gave informed consent before enrolment.

Patients
The EXCITE Study is a multicentre randomized controlled trial on the effectiveness of exercise in improving physical performance and the quality of life (phase-1) and in reducing adverse clinical outcomes (mortality, cardiovascular events and hospitalisations) (phase-2) in dialysis patients. This trial is registered in ClinicalTrials.gov (Identifier: NCT01255969). In this secondary analysis, we included 296 dialysis patients who performed the 6MWT at baseline. These patients had been on regular dialysis (HD or PD) for a median time of 44 months (inter-quartile range: 26-83). Haemodialysis patients (n=247) were being treated with standard bicarbonate dialysis with non-cellulosic membrane filters of various type. PD patients (n=49) were either on 4 standard exchanges day (n=11) or on continuous cycling peritoneal dialysis (n=38). Two hundred and six patients were treated with various anti-hypertensive

drugs (76 on mono-therapy with calcium channel blockers, ACE inhibitors, sartans, alpha or beta blockers, clonidine, furosemide, 65 on double therapy, 44 on triple therapy and 21 patients on quadruple and quintuple therapy with various combinations of these drugs). The main demographic, somatometric, clinical and biochemical characteristics of the study population are detailed in Table 1.

Laboratory measurements
Blood sampling was performed after an overnight fast. In haemodialysis patients, blood was always drawn in the morning hours (8 am – 12 am) during a mid-week day (brief dialysis interval). Serum cholesterol, albumin, calcium, phosphate, C-Reactive Protein (CRP) and haemoglobin measurements were made using standard methods in the routine clinical laboratory.

Six-Minute Walking Test
Physical performance was assessed at baseline with the Six-Minute Walking Test (6MWT). This test consists in a 6 minute-walk along a marked walkway on a hard, flat surface, at the maximum speed that each patient can maintain. The goal of this test is to walk as far possible in six minutes. During the walk, the patient is allowed to stop and rest whenever he/she wants, and the number of interruptions are carefully recorded by an operator. At the end of the test, the fatigue perceived by the patient is classified by the Borg Scale, a simple method that allows to rate the perceived exertion by using a scale from 0 (no exertion) to 10 (maximum exertion).

Study end-points
In this secondary analysis of EXCITE, a composite end-point including mortality, fatal and non-fatal cardiovascular events and hospitalisations was the main study end-point. Cardiovascular events were classified as follows: stroke (ischaemic or haemorrhagic) documented by computed tomography, magnetic resonance imaging and / or clinical and neurological evaluation; transient ischaemic attacks (TIA); myocardial infarction confirmed by serial changes of ECG and cardiac biomarkers; ECG-documented angina episodes; heart failure, diagnosed according to criteria by the AHA guideline [15]; ECG documented arrhythmia; peripheral ischemia or amputations; unexpected, sudden death highly suspected as of cardiac origin. Hospitalisations were classified in cardiovascular and non-cardiovascular using information included in the hospital records. Cause of death was assessed by 3 independent physicians. In doubtful cases, diagnosis was attributed by consensus. During the review process, involved physician used all available medical information, including hospitalisation forms and medical records. In case of death occurred at home, family members and/or general practitioners were interviewed to better understand the circumstances surrounding death.

Statistical analysis

Data were expressed as mean ± standard deviation (normally distributed data), median and inter-quartile range (non-normally distributed data) or as per cent frequency (categorical data). The Person correlation coefficient was used to describe correlates of 6MWT variable.

The independent correlates of 6MWT were identified by correlation analysis and by multiple linear regression. Tested variables included age, gender, smoking, cardiovascular comorbidities, diabetes, antihypertensive therapy, Body Mass Index (BMI), dialysis vintage, systolic and diastolic blood pressure, cholesterol, albumin, C-reactive protein, calcium, phosphate, haemoglobin and NYHA class. All variables which correlated with 6MWT (with P<0.05) were jointly introduced into the same model. Survival analyses were performed by using bivariate and multivariate Cox regression models. In close parallelism with the strategy used for the identification of independent correlates of 6MWT (see above), in the multiple Cox Regression model we included all univariate correlates of the combined end point (with P < 0.05). Statistical analysis was performed by using standard statistical packages (SPSS for Windows, Version 20, Chicago, Illinois, USA; STATA for Windows, Version 13, College Station, Texas, USA).

RESULTS

The flow-chart describing the recruitment basis of the study population and the subsequent selection process, from eligibility to randomisation, is reported in Fig. 1 and the baseline characteristics of patients randomised to the study intervention are described in Table 1. Enrolled patients had a mean age of 65 year. Sixty-eight of them were male, 17% were current smokers, 26% were past smokers. Twenty-one per cent of patients were diabetics and 76% had cardiovascular comorbidities.

Correlation analyses

Baseline 6MWT, expressed as number of meters walked in 6 minutes, significantly correlated with age (ρ= -0.57, P<0.001), gender (ρ= 0.21, P=0.001), cardiovascular comorbidities (ρ= -0.24, P<0.001), diabetes (ρ= -0.20, P=0.001), diastolic blood pressure (ρ= 0.38, P<0.001) albumin (ρ= 0.22, P=0.001), phosphate (ρ= 0.22, P<0.001), CRP levels (ρ= -0.16, P=0.03) and NYHA class (ρ= -0.21, P<0.001). In a multiple linear regression analysis including all univariate correlates of 6MWT, only age (beta= -0.55), gender (beta= 0.16), and cardiovascular comorbidities (beta= -0.17) maintained an independent association with 6MWT (P<0.02).

Fig. 1. *Flow diagram of the patients enrolled into the study (patients randomised to the physical exercise program or to standard care).*


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│        714 patients assessed for eligibility │
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                        │        │ 180 patients refused the      │
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   │ Withdrew consent before starting          │
   │ the interventions n=16                     │
   │ Deaths before starting the                 │
   │ interventions n=4                          │
   │ Transplantation before starting the        │
   │ interventions n=1                          │
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   │    296 started the study interventions     │
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Survival analyses

The median follow-up was 3.3 years (interquartile range: 2.7-3.5 years). During this period, 69 patients died, 90 had fatal or non-fatal cardiovascular events, 159 were hospitalized. Overall, 182 patients had the composite end-point death/cardiovascular events/hospitalisations. In a bivariate Cox regression model, including the allocation arm as covariate, an increase of 20 meters walked during the 6MWT significantly ($P<0.001$) reduced the risk of the composite end-point by 6%. Similar results were obtained in bivariate analyses of the individual end-points. In these models, an increase of 20 meters significantly reduced all-cause death by 12% ($P<0.001$), fatal and

non-fatal cardiovascular events by 7% (P<0.001), and all-cause hospitalisations by 4% (P=0.002).

Table 1 *Main demographic, somatometric and clinical characteristics in the study population and correlates of Six-Minute Walking Test (6MWT).*

	Whole group (n=296)	6MWT correlation coefficient (P)
Age (years)	65±13	**-0.57 (<0.001)**
BMI (kg/m^2)	25±5	-0.04 (0.63)
Male sex n. (%)	201 (68)	**0.21 (<0.001)**
Current smokers n. (%)	48 (17)	-0.04 (0.52)
Past smokers n. (%)	72 (26)	
Diabetics n. (%)	60 (21)	**-0.20 (0.001)**
On anti-hypertensive treatment n. (%)	206 (73)	0.004 (0.95)
Dialysis vintage (months)	44 (26-83)	-0.03 (0.58)
With cardiovascular comorbidities* n. (%)	226 (76)	**-0.26 (<0.001)**
Systolic Blood Pressure (mmHg)	128±20	0.07 (0.27)
Diastolic Blood Pressure (mmHg)	69±11	**0.38 (<0.001)**
Cholesterol (mg/dL)	164±38	-0.08 (0.23)
Haemoglobin (g/dL)	11.0±1.9	0.11 (0.07)
Albumin (g/dL)	3.9±0.4	**0.22 (0.001)**
hsCRP (mg/L)	0.7(0.4-2.6)	**-0.16 (0.03)**
Calcium (mg/dL)	8.7±1.4	-0.06 (0.31)
Phosphate (mg/dL)	4.9±1.5	**0.22 (<0.001)**
NYHA Class 0 n. (%)	124 (44)	**-0.21 (<0.001)**
NYHA Class 1 n. (%)	95 (34)	
NYHA Class 2 n. (%)	40 (14)	
NYHA Class 3 n. (%)	20 (7)	

* Angina, arrhythmia, myocardial infarction, coronary surgery, angioplasty, other heart surgery, claudicatio, amputations, peripheral surgery, stroke, TIA, heart failure.
Data are expressed as mean ± SD. median and inter-quartile range or as percent frequency, as appropriate.

The relationship between physical performance and the combined end-point was confirmed in a model adjusting for age, gender, systolic blood pressure, cholesterol, diabetes, smoking, cardiovascular comorbidities and allocation arm (HR: 0.94, CI: 0.91-0.98, P=0.001) (Fig. 2). By the same token, physical performance by 6MWT predicted all-cause death (HR: 0.89, CI: 0.84-0.94, P<0.001) and hospitalisations (HR: 0.96, CI: 0.92-0.99, P=0.03). A similar trend was observed for CV events, but this relationship did not reach statistical significance (HR: 0.96, CI: 0.91-1.01, P=0.10). Forcing risk factors peculiar to ESRD (haemoglobin, albumin and phosphate) into the model did not modify these relationships (Fig. 2).

Fig. 2. *Hazard ratio and 95% CI associated to an increase of 20 walked metres during the six-minute walking test (6MWT) for the composite end-point (A), all-cause mortality (B), fatal and non-fatal cardiovascular (CV) events (C) and hospitalisations (D).*
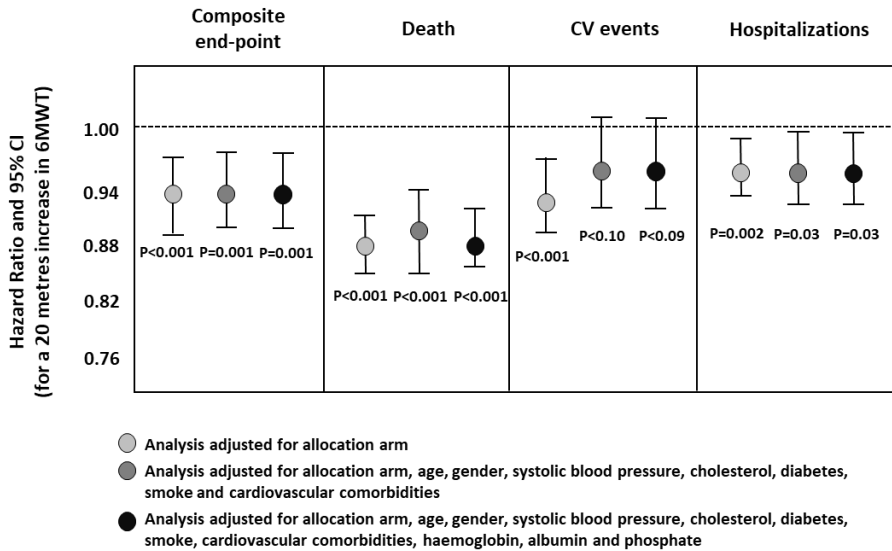
DISCUSSION

This study shows that 6MWT, a test commonly used to measure exercise capacity and motor fitness, predicts the risk for mortality, cardiovascular events and hospitalisations in chronic renal disease patients on dialysis.

Physical activity, either measured by questionnaires [16, 9] or by accelerometers [17] or pedometers [18], is about 50% less in dialysis patients than in age and sex matched individuals. In the Dialysis Outcomes and Practice Patterns Study (DOPPS), self-reported activity was an independent predictor of death and exercising at least once a week predicted a 27% risk reduction [19]. Similar results emerged from the Wave 2 study of the United States Renal Data System (USRDS) [7] and in a study based on accelerometry [8].

While physical activity estimates engagement in daily activities, metrics of physical performance provide objective measures of motor fitness. As such, metrics of physical performance looking at walking speed like Six-Minute Walking Test (6MWT), measure cardio-respiratory endurance, muscle endurance and strength as well as balance and

coordination. The 6MWT has been applied in the whole age spectrum [19, 20] at population level and in several conditions, including chronic congestive heart failure [13, 21] and other cardiac conditions [22], COPD [14] and in hereditary diseases like cystic fibrosis [20]. This test has prognostic relevance because it predicts clinical outcomes in several categories of patients including elderly patients undergoing coronary artery bypass grafting [23] and patients with chronic heart failure treated with cardiac resynchronization [24]. Whether this test of motor fitness predicts mortality in dialysis patients has never been investigated. In this study, we found coherent correlations between physical performance (6MWT) and some factors which have an obvious influence on health status, such as age, gender, cardiovascular comorbidities, NYHA class, diabetes, diastolic blood pressure and other parameters [25-29]. However, only age, gender and cardiovascular comorbidities maintained an independent association with the 6MWT suggesting that these factors are major determinants of motor fitness in dialysis patients. According to our working hypothesis that physical performance measured by the 6MWT holds prognostic value in dialysis patients, we found that this test is a strong predictor of mortality, cardiovascular events and hospitalisations in this population. More specifically, in adjusted analyses, we observed a reduction of 6% in the combined outcome for each increase of 20 walked meters, and a reduction of 12% and 4% for all cause death and hospitalisation. Thus our data extend to the dialysis population observations made in other conditions [23, 24] and underscore the relevance of objective measures of motor fitness in assessing the overall risk profile of dialysis patients.

Future studies, including phase-2 of EXCITE, will assess whether interventions aimed at improving physical fitness may translate into better clinical outcomes, including better physical performance, longer survival and reduced rate of cardiovascular events and hospitalisations in dialysis patients.

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Chapter 4

Discussion

Studies included in this thesis describe novel metabolic and clinical factors in an aging population with CKD, showing their crucial role for risk stratification and risk monitoring in end-stage renal disease patients, underling their role in the high risk of death and cardiovascular events in the dialysis population. The analyses reported have been performed in different frames (clinical trials or observational studies), involving aging CKD patients or ESRD patients, thus demonstrating the importance of emerging risk factors across different stages of renal disease.

• The PENNY (Paricalcitol and Endothelial Function in Chronic Kidney Disease Patients) Study, a double-blind, randomized trial performed in Reggio Calabria, Italy, which enrolled CKD patients with parathormone > 65 pg/ml, serum total calcium between 2.2 and 2.5 mmol/L, phosphate levels between 2.9 mg/dL and 4,5 mg/dL

• The PROGREDIRE (Prospective Registry of The Working Group of Epidemiology of Dialysis Region Calabria) study, a multicentre, cohort study involving patients in dialysis in two regions in Southern Italy (Calabria and Sicily).

• The LUST (Lung Water by Ultrasound Guided Treatment in Haemodialysis Patients) study is a European, multicentre, open, randomized, controlled trial, which enrolled dialysis patients with at high cardiovascular risk.

• The EXCITE (EXerCise Introduction To Enhance Performance in Dialysis) study, a multicentre, randomized, controlled trial performed in Italy, which enrolled dialysis patients with stable clinical conditions.

Effect of paricalcitol on sclerostin and its interaction with circulating pentosidine levels

My findings confirm, in a cohort of elderly CKD patients, that sclerostin correlates inversely with eGFR [1] and PTH [2] and show a direct association with anthropometric measurements like weight, height, BMI and waist circumference as well as with diabetes and high cholesterol. This suggests that, similarly to other biomarkers of bone mineral disorders, including FGF23 [3], vitamin D [4] and PTH (5), sclerostin levels associate with body and fat mass and carbohydrate metabolism.

Experimental models [6, 7] and clinical trials [7-9], describing the increasing of sclerostin levels after Vitamin D administration, demonstrate the close link between 1,25(OH)$_2$ Vitamin D and sclerostin. I confirmed, in a randomised controlled trial setting, that vitamin D receptor activation by paricalcitol raises serum sclerostin in Chronic Kidney Disease (CKD) patients, and that this effect was independent of age, gender and severity of renal dysfunction.

The paricalcitol-induced increase of sclerostin goes along with the parallel increase of FGF23 and a dramatic decrease of parathormon (PTH), suggesting an underlying effect of a regulatory mechanism aimed at countering the strong PTH suppression and the marked FGF23 increase induced by this drug. However, looking at my data, as sclerostin rise by PCT was abolished after adjustment for PTH changes but not for

FGF3 changes, it appears more likely that changes in sclerostin in response to PCT treatment mainly serve to counter the PTH suppressing effect of this drug rather than the concomitant, marked rise in serum FGF23.

Notably, the sclerostin rise by PCT is attenuated by pentosidine, a member of the Advanced glycation end-products (AGEs) family. As it is known by in vitro studies that AGEs alter the functioning of the vitamin D receptor, the reduced increase can easily be explained by altered vitamin D receptor functioning in patients with relatively higher levels of this AGE.

Gamma GT amplifies the alkaline phosphatase- dependent risk for mortality.

My study suggests a new, possible mechanism underlying the high mortality rate in elderly dialysis patients. I found that γ-Glutamyl-Transpeptidase (GGT), a marker of oxidative stress, emerged as a strong amplifier of the death risk portended by high alkaline phosphatase (Alk-Phos) in End-Stage Kidney Disease (ESRD) patients on dialysis. This interaction was largely independent of liver disease and alcohol intake and was confirmed in sensitivity analyses excluding patients with pre-existing liver disease or self-reported high alcohol intake. Among risk factors in this population, oxidative stress is considered a hallmark [10, 11] and for this reason numerous biomarkers have been investigated to detect this condition [12-15]. Among these, GGT is able to capture the oxidative stress of the entire body [16], in addition to being a well-established risk factor for mortality in dialysis patients. It is worth to remember that these fragile patients are also affected by mineral-bone disorder, a systemic disease which impairs bone metabolism and predisposes to vascular calcifications. Alk-Phos, another strong predictor of death in CKD [17-19] and ESRD [20-29] patients and in the general population [30], is an enzyme that, in patients without obvious liver disease, mainly reflect bone turnover [31] and it is directly involved in glutathione catabolism [32, 33]. Notably, in vitro experiments in vascular and bone cells shows that oxidative stress is a strong inducer of alkaline phosphatase, promoting cell calcification [34].

Looking at this data, the hypothesis that oxidative stress may interact with Alk-Phos in organ damage and ultimately in major clinical outcomes is biologically well founded. However, in no study such an interaction was formally investigated. I found that a fixed increase in Alk-Phos levels (50 UI/L) produced a stepwise increase in the risk of death across GGT quintile (HR = 1.08 per GGT quintile). Importantly, this interaction was unmodified by full adjustment for a comprehensive series of risk factors for mortality. This interaction was independent of concomitant liver disease, indicating that it does not represent a mere effect of liver damage but more likely a consequence of oxidative stress [16].

Overall, these findings suggest that systemic oxidative stress, as estimated by GGT, plays a relevant role in predicting the risk for major clinical outcomes portended by increased alkaline phosphatase.

Snoring amplifies the risk of mortality driven by heart failure.

One of the difficulties in finding new tools for risk stratification in elderly, frail patients such as those on dialysis is that, even though highly effective, they are too often scarcely applicable. For this reason, the usefulness of a simple 2-question questionnaire about snoring habits in detecting Sleep Disordered Breathing (SDB) in this population assumes a greater value.

Even though snoring is considered a useful surrogate marker of SDB in large scale epidemiological and clinical studies [35-38] this is the first time that self-reported snoring is validated in CKD population. Even though it tends to misclassify as affected some patients who are actually not affected by SDB, notwithstanding this limitation it has the ability to exclude SDB, and this makes snoring an acceptable surrogate of SDB in large scale studies in CKD like my study.

However, self-reported snoring is not just a surrogate of SDB. Scarcely useful in predicting all-cause and cardiovascular mortality, as reported in literature [39, 40] and as described in this thesis, it emerged as a strong modifier of the link between heart failure and all cause and cardiovascular (CV) death. I found that the risk of all-cause and CV death in ESRD patients with heart failure was 2 times and 4 times higher in heavy snorers than in non-snorers, respectively, and this effect modification showed a dose-response relationship.

From a biological point of view, my findings are plausible, as they likely reflect the amplification of background sympathetic nervous system over-activity, extremely common in dialysis patients, driven by SDB and consequent nocturnal hypoxemia [41-43].

The agreement between auscultation and US-B lines in dialysis patients.

Dialysis patients have an exceedingly high risk of hospitalisation and death for pulmonary oedema [44], caused either by congestive heart failure or volume expansion. The correct diagnosis may be problematic in these patients [45], and so far it has been mainly based on lung auscultation (indicative of pulmonary congestion secondary to left ventricular dysfunction and/or volume overload) and peripheral oedema examinations (indicative of heart failure) universally applied to detect and monitor volume excess. However, the performance of clinical examination in comparison with new techniques, like lung ultrasound (US) scanning, to detect pulmonary oedema had never been tested so far. Surprisingly, I found that these honoured clinical signs have an unsuspectedly low sensitivity for elderly dialysis patients, suggesting that they only remotely reflect the degree of lung congestion as measured by an objective, well validated method.

In fact, despite the fact that all patients described in my study had a substantial degree of congestion, at peak of volume expansion crackles and peripheral oedema were present just in 21% and 10% of cases respectively, with an agreement between US-B lines and pulmonary crackles, peripheral oedema or a combination of them

consequently poor. Both lung crackles and peripheral oedema, taken alone or aggregate, had a satisfactory specificity or the diagnosis of lung congestion, counterbalanced by a pitifully inadequate sensibility. It follows that pulmonary congestion is unnoticed in a substantial proportion of patients without crackles and/or peripheral oedema. This makes universally recognised biomarkers of volume overload unable to detect subclinical congestion, condition of peculiar relevance because the consequently increased alveolo-capillary permeability [46] makes them vulnerable to volume overload which gradually builds up during the dialysis interval, paving the way to the use, in the clinical environment, of new, more trustable, tools for the detection of pulmonary congestion.

The physical function component of the SF36 holds the highest predictive power in elderly patients on dialysis.

As previously reported in previous chapters and in the current one, questionnaires are invaluable tools to assess several conditions in elderly dialysis patients. As physical activity is notoriously low in this population, it is not surprisingly how many questionnaires have been validated to measure this aspect of quality of life in these patients. Among available questionnaires, the RAND QoL Short Form 36 (SF-36) has been specifically validated in the ESRD population [47, 48]. This instrument incorporates various QoL domains which capture emotional and physical well-being, providing information potentially useful to guide clinical decisions in patients with CKD on dialysis, including the elderly. The physical function component of SF-36 was the one holding the highest prognostic power in that it added about the 5% discriminant ability to prediction made on a simple model based on demographic and clinical variables. Furthermore, physical function held a robust risk reclassification ability (+21%), i.e. the ability to correctly reclassify high risk patients versus low risk patients as identified by standard risk factors. Besides physical functioning, only the general health component of SF36 was related to mortality but the prognostic power for mortality of this component was inferior to that by physical function and the combination of this component with physical function added just a small additional prognostic value as compare to physical functioning alone. Taken together, these results indicate that the prognostic value of this component is of magnitude that it may have potential for refining risk stratification in clinical practice in a high risk population like very old dialysis patients.

Physical performance and clinical outcomes in dialysis patients.

In dialysis patients not only physical activity, i.e. the engagement in daily activities, but also physical performance, i.e. the objective measures of motor fitness, is notoriously scarce. While physical activity can be effectively measured by questionnaires, as reported in literature [49, 50] and in chapter 3.2, physical performance can be easily measured by using the Six-Minute Walking Test (6MWT), which is widely used to measure cardio-respiratory endurance, muscle endurance and strength as well as

balance and coordination in several conditions. I discovered that 6MWT, which predicts clinical outcomes in several categories of patients [51, 52], predicts the risk for mortality, cardiovascular events and hospitalisations in chronic renal disease patients on dialysis.

As expected, the distance covered during the test correlates with some factors which have an obvious influence on health status, such as age, gender, cardiovascular comorbidities, NYHA class, diabetes, diastolic blood pressure and other parameters [53-57]. However, only age, gender and cardiovascular comorbidities maintained an independent association with the walking distance, suggesting that these factors are major determinants of motor fitness in dialysis patients. According to my working hypothesis that physical performance measured by the 6MWT holds prognostic value in dialysis patients, I found that this test is a strong predictor of mortality, cardiovascular events and hospitalisations in this population. More specifically, in adjusted analyses, I observed a reduction of 6% in the combined outcome for each increase of 20 walked meters, and a reduction of 12% and 4% for all cause death and hospitalisation. Thus my data extend to the elderly dialysis population observations made in other conditions [51, 52], highlighting the importance of physical performance in risk stratification in dialysis patients.

Future perspectives

The main limitation of the PROGREDIRE study is its observational nature, which prevents causal interpretations about the described associations. Second, even though I did a comprehensive adjustment for a long list of potential confounders, confounding for unmeasured and/or unknown risk factors remains possible, an issue that can be solved only by a clinical trial. The study based on self-reported snoring may suffer from the lack of precision of the questionnaire, so snoring might not adequately reflect the underlying strength of the interaction between SDB and CHF for the risk of all-cause and cardiovascular mortality. For what concerns the PENNY study, even though my working hypothesis was tested in the context of a randomized placebo-controlled clinical trial, it was too small to allow further analyses based on harder clinical end-points.

In the LUST study, I repeatedly measured US-B lines and quantified crackles and peripheral oedema in patients in the active arm of a clinical trial enrolling patients at high cardiovascular risk. Therefore, my results cannot be generalized to the whole dialysis population. Second, I focused on lung auscultation as traditionally performed by standard stethoscopes by trained physicians. Novel acoustic devices may enhance the usefulness of auscultation.

A future challenge will be to plan specific interventional studies in order to confirm the usefulness of the markers described in this thesis in dialysis patients. The ongoing LUST study will establish whether systematic application of lung US may help preventing excessive water accumulation in the lung and ultimately improve clinical outcomes in high risk dialysis patients. Future studies, including phase-2 of EXCITE,

will assess whether interventions aimed at improving physical fitness may translate into better clinical outcomes, including better physical performance, longer survival and reduced rate of cardiovascular events and hospitalisations in dialysis patients.

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SUMMARY

The data analysis of four different databases, one (PENNY) including patients affected by CKD and three (PROGREDIRE, EXCITE, LUST), including patients with ESRD on dialysis, allowed me to explore novel metabolic/clinical factors and novel instruments for risk stratification in patients with End-Stage Renal Disease.

I found that paricalcitol, an activator of the Vitamin D receptor, increases serum levels of sclerostin, an osteocyte glycoprotein involved in the bone metabolism, and this increase goes along with the rise in FGF23 and the suppression of PTH. Such an effect is attenuated by pentosidine, a member of the AGEs family, suggesting, as a possible explanation, that patients with high levels of this AGEs have altered vitamin D receptor functioning.

Furthermore, I found that systemic oxidative stress, as estimated by GGT, interacts with Alkaline Phosphatase in predicting the risk of death in dialysis patients, and this interaction is independent of concomitant liver disease. This suggests that this effect modification is not a mere effect of liver damage but it is probably a direct consequence of oxidative stress.

Among novel risk factors, in spite of being unable to predict mortality in dialysis patients, snoring emerged as a strong modifier of the link between heart failure and all cause and CV death. From a biological point of view, these findings likely reflect the amplification of background sympathetic nervous system over-activity, extremely common in dialysis patients, driven by SDB and consequent nocturnal hypoxemia.

I established for the first time that lung auscultation (indicative of pulmonary congestion) and peripheral oedema examinations (indicative of heart failure) universally applied to detect and monitor volume excess in dialysis patients have an unsuspectedly low sensitivity for elderly dialysis patients to detect pulmonary oedema in comparison with an objective, well validated method, lung US scanning, paving the way to the use, in the clinical environment, of new, more trustable, tools for the detection of pulmonary congestion.

The RAND QoL Short Form 36 (SF-36) represents an efficient, easy to administer, tool for assessing quality of life in CKD patients. I found that two of the SF-36 domains predict mortality in fully adjusted analyses, but the physical activity component of SF-36 is the domain with the highest prognostic power for mortality in this population.

While physical activity can be effectively measured by questionnaires, physical performance can be easily measured by using the Six-Minute Walking Test (6MWT). I discovered that 6MWT predicts the risk for mortality, cardiovascular events and hospitalisations in dialysis patients. Thus my data highlights the importance of physical performance in risk stratification in dialysis patients.

SAMENVATTING

De analyse van vier verschillende databases, één (PENNY) die includeert patiënten met chronische nierinsufficiëntie en drie (PROGREDIRE, EXCITE, LUST), die includeren patiënten met terminale nierinsufficiëntie (dialyse), stelde mijn in staat om nieuwe metabole, klinische factoren en nieuwe instrumenten voor risicostratificatie te onderzoeken in patiënten met nierinsufficiëntie.

Ik ontdekten dat paricalcitol, een activator van de vitamine D-receptor, de serumspiegels van sclerostin verhoogt, een osteocyt glycoproteïne dat betrokken is bij het botmetabolisme, en deze toename gaat gepaard met de toename van FGF23 en de onderdrukking van PTH. Een dergelijk effect wordt verzacht door pentosidine, een lid van de AGEs-familie, wat suggereert, als een mogelijke verklaring, dat patiënten met hoge niveaus van deze AGE's een gewijzigde werking van de vitamine D-receptor hebben.

Verder vonden Ik dat systemische oxidatieve stress, zoals geschat door GGT, interageert met Alkaline Phosphatase in het voorspellen van het risico op overlijden bij dialysepatiënten, en deze interactie is onafhankelijk van leverziekte. Dit suggereert dat deze effectmodificatie niet louter een effect is van leverschade, maar waarschijnlijk een direct gevolg is van oxidatieve stress.

Onder nieuwe risicofactoren, ondanks het niet kunnen voorspellen van mortaliteit bij dialysepatiënten, ontstond snurken als een sterke modifier van het verband tussen hartfalen en alle oorzaken en CV-sterfte. Vanuit een biologisch gezichtspunt weerspiegelen deze bevindingen waarschijnlijk de amplificatie van overactieve activiteit van het sympathisch zenuwstelsel, zeer gebruikelijk bij dialysepatiënten, aangedreven door slaapapneustoornissen en als gevolg daarvan nachtelijke hypoxemie.

In hoofdstuk 3.2 hebben wij vastgesteld de diagnostische betrouwbaarheid van de auscultatie van de longen (indicatief voor pulmonale congestie) en het onderzoek van perifeer oedeem (indicatief voor hartfalen) in vergelijking met Ultrasound B-lines (US-B lines) een goed gevalideerde meting bij patiënten met hart- en vaatziekten en bij intensive care-patiënten, evenals een sterke prognostische factor voor overlijden en cardiovasculaire events in einde stadium nierziekte.

In hoofdstuk 3.3 hebben wij de prognostische waarde van De RAND QoL Short Form 36 (SF-36) instrument voor het beoordelen van de kwaliteit van leven bij CKD-patiënten. Ik ontdekten dat twee van de SF-36-domeinen mortaliteit voorspellen, het fysieke domein van de SF-36 heeft de sterkste prognostische waarde voor mortaliteit in deze populatie.

Tenslotte in hoofdstuk 3.4 behandelen wij onze bevindingen over de prognostische waarde van de Six-Minutes Walking Test het risico op cardiovasculaire morbiditeit, hospitalisatie en mortaliteit, voorspelt bij dialysepatiënten. Onze gegevens benadrukken dus het belang van fysieke prestaties in risicostratificatie bij dialysepatiënten.

LIST OF PUBLICATIONS

Torino C, Mattace-Raso F, van Saase JL, Panuccio V, Tripepi R, Vilasi A, Postorino M, Tripepi GL, Mallamaci F, Zoccali C, Progredire Study Group. Physical functioning and mortality in very elderly patients on dialysis. Arch Gerontol Geriatr. 2019 Jul 27;85:103918.

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Research Skills
- Biomedical English

In-dept Courses
- Intervention Research and Clinical Trials
- Advanced Topics in Clinical Trials
- Principles of Genetic Epidemiology
- Case-control Studies
- Cohort Studies
- Survival Analysis
- Diagnostic Research
- Advanced Topics in Decision Making in Medicine
- Conceptual Foundation of Epidemiologic Study Design
- Causal Inference
- Markers and Prognostic Research
- Regression Analysis for Clinicians
- Advanced Analysis of Prognosis Studies
- Mendelian Randomisation
- ESP66 Logistic Regression
- Pharmaco-epidemiology and Drug Safety
- Repeated Measurements

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Pre-congress Course in Clinical Statistics. 33° Biennal Congress of the Nordic Society of Nephrology, Stavanger (Norway), August 26th – 29th, 2015.
Survival analysis: the Kaplan-Meier method and Cox regression analysis - Pre-congress educational course. 57° Congresso della Società Italiana di Nefrologia, held in Milano, October 12th – 15th, 2016

International conferences
50th ERA EDTA Congress, Istanbul (Turkey), 18-21 May 2013
31st Annual Meeting of the International Society of Blood Purification, Bologna (Italy), 12-14 September 2013
51st ERA EDTA Congress, Amsterdam (The Netherlands), 31 May - 3 June 2014
52nd ERA EDTA Congress, London (UK), 28-31 May 2015
33° Biennal Congress of the Nordic Society of Nephrology, Stavanger (Norway), 26-29 August 2015
53rd ERA EDTA Congress, Wien (Austria), 21-24 May 2016
54th ERA EDTA Congress, Madrid (Spain), 3-6 June 2017

Other
Reviewer for:
- PLOS One
- CJASN
- Nephrology, Dialysis and Transplantation
- Journal of Nephrology
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- British Journal of Nutrition
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Claudia Torino was born in Reggio Calabria (Italy) on April 6th 1978. She obtained the University Degree of Doctor in Biological Science at University of Messina in November 2002. After that, she achieved the first PhD in Pathology of Cell Proliferation and Differentiation (April 2009) and a Specialization Diploma in Clinical Pathology (October 2010) at University of Messina. Presently she is Researcher at the National Research Council, Institute of Clinical Physiology (CNR-IFC) of Reggio Calabria (Italy). Her areas of expertise are study design, prospective studies, case-control studies, cross-sectional studies, clinical trial, biostatistics, epidemiology, prognostic biomarkers, diagnostic biomarkers in chronic renal failure, cardiovascular risk. She is Referent of the Bioinformatics Laboratory, and Member of the Working Group aimed at carrying out research activities "Drawing up of research protocols, submission to calls for tenders in the National and European sphere, financial management of projects", both set up at the CNR-IFC. She is also responsible for the monitoring of registries and clinical studies performed at CNR-IFC, Reggio Calabria, and Scientific Referent of National and International projects.