UNIVERSITÀ DEGLI STUDI DI MILANO

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# *In vitro* and *in vivo* approaches to study oxidative stress, anemia and dysbiosis in Chronic Kidney Disease

BIO/06


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# 1. INTRODUCTION

Kidneys have many essential functions, including depurative functions and hormone production. The depurative functions comprehend the elimination of blood waste products and foreign substances (primarily urea, creatinine, uric acid and drugs); the maintenance of electrolyte homeostasis (including sodium, chlorine, potassium, phosphorus, calcium and magnesium), by modulating their elimination through the urine; the water balance, by regulating the amount of urine produced daily, so as to ensure a balance between the water inlet (with drinks and food or deriving from metabolic production) and the outlets (urine, feces, breathing, sweating). Because they control water and salts balance, kidneys regulate extracellular fluid volume and plasma osmolality. Ensuring adequate volume for the cardiovascular system and producing vasoactive substances (via the renin-angiotensin-aldosterone system), kidneys exert major control over vascular smooth muscle, influencing peripheral vascular resistance and therefore systemic arterial blood pressure. Kidneys are also the site of production of various hormones: renin, involved in the control of blood pressure; erythropoietin, stimulator of hematopoiesis; vitamin D (not properly produced by the kidney, but converted here into its metabolically active form), essential for a proper bone calcification. In addition, kidneys are responsible for the regulation of acid-base balance and they are a site for gluconeogenesis [Eaton et al., 2016].

All these functions can be compromised if kidneys are damaged, as in patients with chronic kidney disease.

Chronic kidney disease (CKD) is a non-communicable disease characterized by a glomerular filtration rate reduction and/or an increased urinary albumin excretion. This clinical condition is determined when kidneys are damaged and their functional capacities are irreparably compromised, independently from the disease onset cause. CKD has a world prevalence of 8–16% and the WHO declared it as a public health problem which is continually increasing [Jha et al., 2013; Hill et al., 2016].

Kidney functions loss results in a complex picture with numerous alterations, often a prelude to comorbidities. In fact, the condition of renal impairment predisposes to a series of complications, including an increased mortality from multiple causes and cardiovascular diseases, progression of kidney disease, acute renal damage, cognitive decline, anemia, disorder in the balance of minerals and in bone health, fractures [Hannedouche et al., 2018; Fujii et al., 2017; Baluarte et al., 2017]. It has consequently an important weight in national public expenditure [Mushi et al., 2015]. An early screening and the implementation of targeted interventions, including pharmacological and

nutritional interventions, could prevent this pathology or slow down its progression [Couser et al., 2011]. For these reasons, it would be desirable to include strategies to reduce CKD burden in national programs for non-communicable diseases and continuing to study the better targeted interventions to increase the years and the quality of life of CKD patients.

## 1.1 Chronic kidney disease stages

Each kidney is made up of approximately 2 million of nephrons. Nephrons are the kidney functional units and they filter around 60 ml of blood per minute. In the renal glomeruli the blood is filtered through a dense network of capillaries, forming the so called pre-urine. Pre-urine passes through the renal tubules, where it undergoes various transformations both in composition and in volume, until it assumes the characteristics of the proper urine. Urine is then conveyed through the ureter to the bladder [Eaton et al., 2016].

Since a healthy subject normally has two kidneys (although having only one kidney does not compromise the quality of life), the overall glomerular filtration rate (GFR, the rate of blood cleaned by the kidneys per unit of time) that is reached is 120 ml per minute.

CKD is diagnosed when there's a decreased kidney function shown by a GFR less than 60 ml / min (established for a reference man with 1.73 $m^2$ body surface area), or markers of kidney damage, or both, of at least 3 months duration [Webster et al., 2017]. The value of 60 ml / min represents the reference GFR from which a healthy adult can differ with an inter-individual coefficient of variation from 15% to 20%, according to various parameters including age, sex and body weight [National Kidney Foundation 2002]. In 2002, the National Kidney Foundation Kidney Disease Outcome Quality Initiative (NKF-K / DOQI) proposed to categorize CKD in five stages, based essentially on the estimated GFR (Table 1.). Highest stadiums correspond to lowest GFR levels [National Kidney Foundation., 2002].

| Stage | Description | GFR (mL/min/1.73 $m^2$) |
|---|---|---|
| 1 | Kidney damage with normal or ↑ GFR | ≥90 |
| 2 | Kidney damage with mild ↓ GFR | 60–89 |
| 3 | Moderate ↓ GFR | 30–59 |
| 4 | Severe ↓ GFR | 15–29 |
| 5 | Kidney failure | <15 (or dialysis) |

*Table 1. Stages of CKD, K / DOQI classification [National Kidney Foundation, 2002].*

This CKD staging system defines the pathology degree and the stratification of patients according to different risk categories, but it has some limitations. In particular, the range of GFR values included in stage III (30-59 ml / min) appears wide and it includes a large number of patients with extremely variable prognosis. Indeed, some authors have proposed a subdivision of stage III into two subgroups, 3a (GFR: 45-59 ml / min) and 3b (GFR: 30-44 ml / min) [Kirsztajn et al., 2009]. The problem of an inaccurate classification becomes more relevant when referred to patients of advanced age with mild renal dysfunction. In the K / DOQI classification there is no stratification by age and the progressive reduction of the GFR due to the physiological aging process is not taken into account [Gambaro et al., 2010]. In addition, surveys made on the general population often evaluate the GFR with one single serum creatinine measurement. This aspect determines a classification without taking into account the criterion of chronicity indicated by the K / DOQI guidelines for the definition of CKD: these guidelines suggest to confirm the reduction of the GFR value with two measurements at a distance of at least three months.

Considering these limits and given that several studies have shown that albuminuria can also be considered a powerful predictor of mortality and worsening of renal function independently of the GFR itself, the organization Kidney Disease Improving Global Outcomes (KDIGO) developed a risk table based on both parameters (Table 2). The previous classification was thus modified by adding albuminuria ranges to the GFR ranges, dividing stage 3 into 3a and 3b and emphasizing the prognosis [KDIGO, 2012].

**Prognosis of CKD by GFR and albuminuria category**

| Prognosis of CKD by GFR and Albuminuria Categories: KDIGO 2012 | | | | Persistent albuminuria categories Description and range | | |
|---|---|---|---|---|---|---|
| | | | | A1 | A2 | A3 |
| | | | | Normal to mildly increased | Moderately increased | Severely increased |
| | | | | <30 mg/g <3 mg/mmol | 30-300 mg/g 3-30 mg/mmol | >300 mg/g >30 mg/mmol |
| GFR categories (ml/min/ 1.73 m²) Description and range | G1 | Normal or high | ≥90 | | | |
| | G2 | Mildly decreased | 60-89 | | | |
| | G3a | Mildly to moderately decreased | 45-59 | | | |
| | G3b | Moderately to severely decreased | 30-44 | | | |
| | G4 | Severely decreased | 15-29 | | | |
| | G5 | Kidney failure | <15 | | | |

Green: low risk (if no other markers of kidney disease, no CKD); Yellow: moderately increased risk; Orange: high risk; Red, very high risk.

*Table 2. Stages of CKD, KIDGO classification according to GFR and albuminuria values [KDIGO, 2012].*

The GFR reduction is associated with a wide range of complications, which can show up in abnormal values of laboratory parameters and in numerous and heterogeneous symptoms, as a decrease in urine volume, water retention, drowsiness, mental confusion, nausea, vomit, loss of appetite, hypertension, fatigue etc. These symptoms are summarized in Figure 1 [Webster et al., 2017].

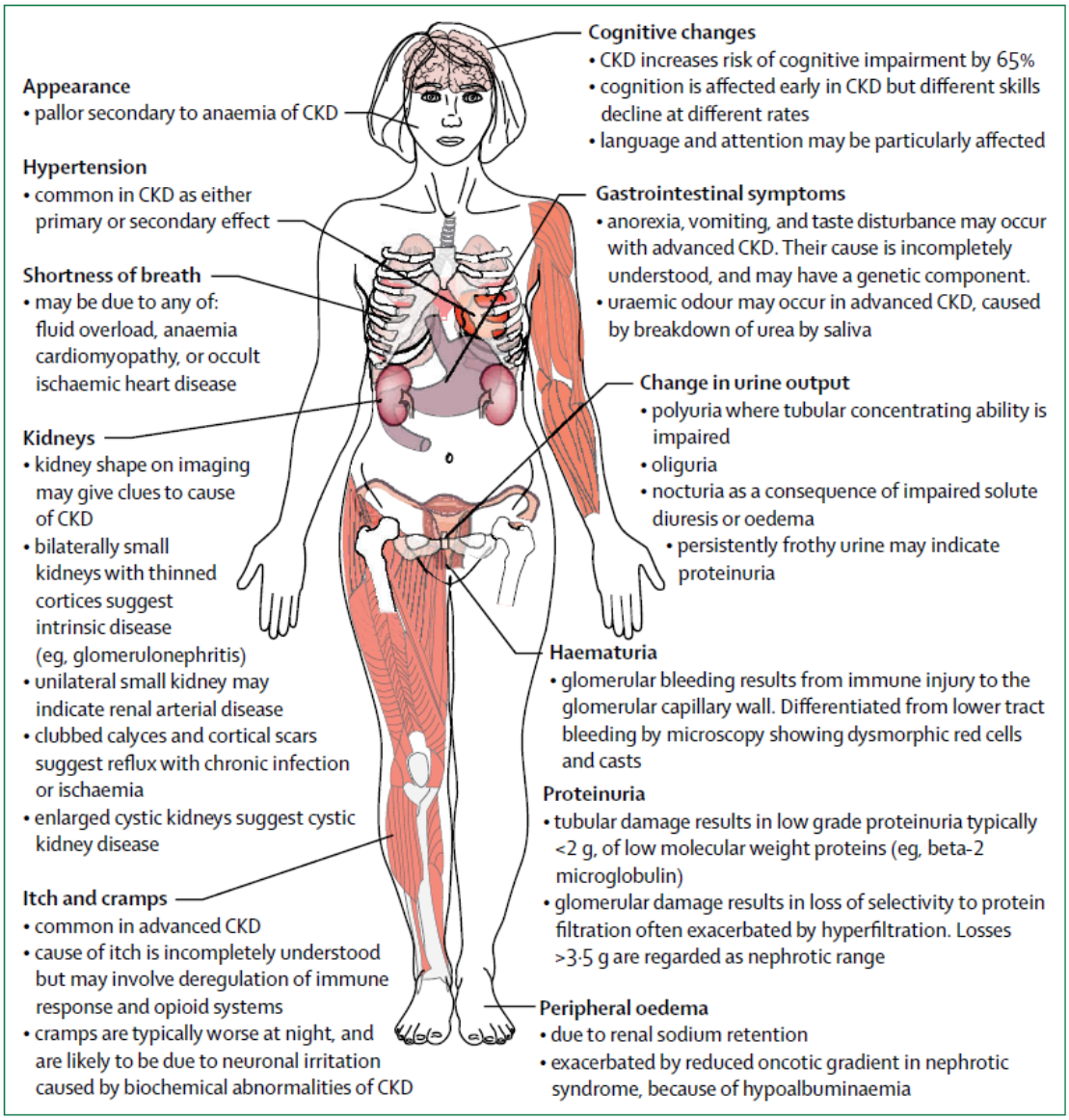


*Figure 1. Symptoms and signs of CKD [Webster et al., 2017].*

The severity of complications increases in parallel with the GFR decline. The progressive worsening of the health status of CKD patients means that they have to follow stage-specific prescriptions and therapies, that become more and more articulated with the passage from one stage to the next one (Figure 1). The V stadium, reached when the GFR decreases under 15 mL/min, is called End-stage Renal Disease (ESRD). At this stadium, the patient needs to start a renal replacement therapy (RRT), the hemodialysis (HD) or the peritoneal dialysis (PD), because at this point kidney function is no longer able to sustain life over the long term. Furthermore, the patient has to follow a pharmacological therapy and a specific diet, in order to prevent and correct malnutrition and metabolic alterations, common features of this stadium [Fondazione italiana del rene, 2006; Fouque et al., 2007].

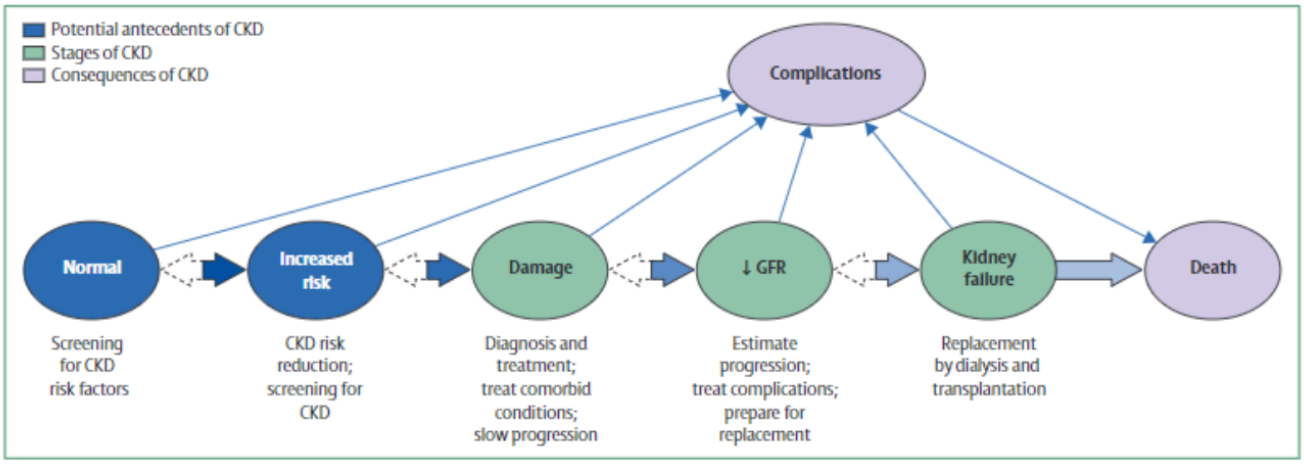


*Figure 2. Continuum of development, progression, and complications of CKD and strategies to improve outcomes [Levey et al., 2012].*

## 1.2 Epidemiology

CKD incidence and prevalence vary among countries because of differences in monitoring diseases rates and availability of government-sponsored treatment. According to a study published on The Lancet by Levey et colleagues in 2012, in many countries CKD incidence is around 200 cases per million per year, with spikes of 400 cases per million in the USA, Taiwan, and some regions in Mexico [Levey et al., 2012]. CKD prevalence has been studied by Hill and colleagues, who published in 2016 the first meta-analysis of CKD prevalence globally on Plos One. They tried to determine CKD prevalence by stage, geographical location, gender and age using only 'High' quality studies and studies with a consistent number of participants. They concluded that CKD has a high global prevalence between 11 to 13%, with the majority stage 3

(Table 3) [Hill et al., 2016].

| | Stage 1 to 5 | | Stages 3 to 5 | |
|---|---|---|---|---|
| | N* | Prevalence (%) | N* | Prevalence (%) |
| S Africa, Senegal, Congo | 5,497 | 8.66 (1.31, 16.01) | 1,202 | 7.60 (6.10, 9.10) |
| India, Bangladesh | 1,000 | 13.10 (11.01, 15.19) | 12,752 | 6.76 (3.68, 9.85) |
| Iran | 17,911 | 17.95 (7.37, 28.53) | 20,867 | 11.68 (4.51, 18.84) |
| Chile | 0 | NONE | 27,894 | 12.10 (11.72, 12.48) |
| China, Taiwan, Mongolia | 570,187 | 13.18 (12.07, 14.30) | 62,062 | 10.06 (6.63, 13.49) |
| Japan, S Korea, Oceania | 654,832 | 13.74 (10.75, 16.72) | 298,000 | 11.73 (5.36, 18.10) |
| Australia | 12,107 | 14.71 (11.71, 17.71) | 896,941 | 8.14 (4.48, 11.79) |
| USA, Canada | 20,352 | 15.45 (11.71, 19.20) | 1,319,003 | 14.44 (8.52, 20.36) |
| Europe | 821,902 | 18.38 (11.57, 25.20) | 2,169,183 | 11.86 (9.93, 13.79) |

*N is number of participants in the sample estimate.

*Table 3. Mean prevalence of CKD split by geographical region with 95% Confidence Intervals [Hill et al., 2016].*

These results are in agreement with another study published on the Lancet which reported a CKD world prevalence of 8–16% [Jha et al., 2013]. So CKD has an important global prevalence and the WHO declared it as a public health problem which is continually increasing [Hill et al., 2016]. According to the 2010 "Global Burden of Disease study", in 1990 CKD was ranked 27th on the list of diseases causing the highest number of deaths worldwide. In 2010 it rose to eighteenth place (with a rate of 16.3 per 100,000 deaths per year) [Lozano et al., 2012].

The 2008 CARHES epidemiological study (Cardiovascular risk in Renal patients of the Italian Health Examination Survey) of the Italian Society of Nephrology, in collaboration with the Istituto Superiore di Sanità and the National Association of Hospital Cardiologists, made available for the first time the data of CKD prevalence on a national scale. The prevalence of CKD was 7.5% in men and 6.5% in women with a higher prevalence of the initial stages 1 and 2 (about 60%), compared to stages 3-5 (equal to 40%) [Mennini et al., 2013; Pontoriero et al., 2007] (Table 4).

| CKD stages | **Total prevalence (%)** | Male prevalence (%) | Female prevalence (%) |
|---|---|---|---|
| 1 | 2.6 (2.3-3.0) | 2.7 (2.2-3.2) | 2.6 (2.1-3.2) |
| 2 | 1.5 (1.3-1.8) | 2.1 (1.7-2.6) | 0.9 (0.6-1.3) |
| 3a | 2.1 (1.8-2.5) | 2.0 (1.5-2.4) | 2.3 (1.8-2.8) |
| 3b | 0.5 (0.4-0.7) | 0.5 (0.3-0.7) | 0.5 (0.3-0.8) |
| 4 | 0.2 (0.1-0.3) | 0.2 (0.1-0.4) | 0.1 (0.0-0.3) |
| 5 | 0.1 (0.0-0.2) | 0.1 (0.0-0.3) | 0.1 (0.0-0.3) |
| **All stages** | 7.1 (6.5-7.7) | 7.5 (6.7-8,4) | 6.5 (5.8-7.4) |

*Table 4. CKD prevalence (%) in Italy by stages and gender [Conte et al., 2013].*

On the basis of these data, it has been possible to highlight how CKD in Italy is characterized by a lower prevalence compared to other western countries, but it is accompanied by a higher cardiovascular profile risk which is, at least in part, attributable to the average advanced age. A data of great interest is represented by the greater prevalence of the earlier stages of CKD (stages 1-2) than the more advanced ones (stages 3-5). The need to study this subgroup of patients therefore emerges with a twofold purpose: on the one hand, to slow down the progression of the disease and, on the other hand, to eventually identify glomerular diseases or nephropathies able to cause kidney damages that are reversible if early diagnosed and treated [De Nicola et al., 2011; Couser et al., 2011].

## 1.3 Etiopathogenesis

Diabetes and hypertension are the leading causes of CKD onset in all developed countries and in many developing countries, together with old age, obesity, and cardiovascular diseases, with diabetic glomerulosclerosis and hypertensive nephrosclerosis as the presumed pathological entities. On the contrary, glomerulonephritis and tubulointerstitial diseases are more common in other countries such as Asia and sub-Saharan Africa (Figure 2) [Levey et al., 2012]. To a large extent, these differences are attributable to the different prevalence of lifestyle-related chronic diseases and infectious diseases, respectively in rich and poor countries: in low-income countries infectious diseases continue to be prevalent [Ayodele et al., 2010]. Some genetic factors can also contribute to CKD onset: variants of the MYH9 and APOL1 genes are associated with CKD in African subjects [Jha et al., 2013].

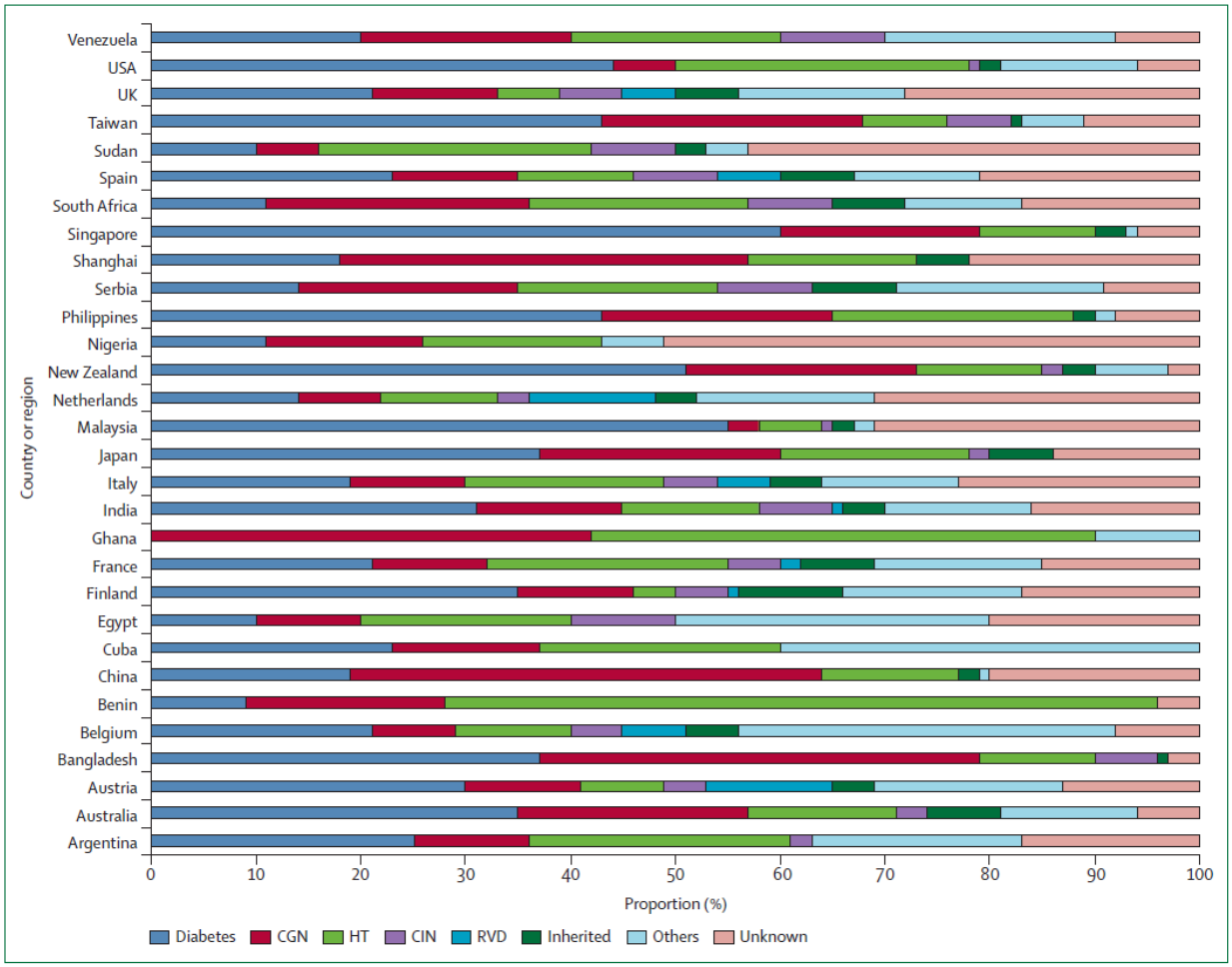


*Figure 3. Distribution of causes of chronic kidney disease worldwide*

*CGN=chronic glomerulonephritis. HT=hypertensive nephrosclerosis. CIN=chronic interstitial nephritis. RVD=renovascular disease.*

Kidney damages, often irreversible, are therefore secondary to numerous and heterogeneous morbid events. Some of them specifically affect the kidneys (e.g. glomerulonephritis, polycystic kidney and kidney bacterial infections), while others interest the cardiovascular system and affect the kidneys as they are richly vascularized organs (e.g. hypertension, dyslipidemia, diabetes and metabolic syndrome). Other risk factors are related to the lifestyle, such as cigarette smoking [Jha et al., 2013].

In addition to the pathologies listed above, there are many other risk factors for CKD, with a highly varied nature, for example:

- Use of medical herbs: traditionally used by rural populations in Africa and Asia, today they have become popular also in developed countries. Their nephrotoxic effects can derive from their potential intrinsic toxicity, possible contamination with toxic compounds (such as some heavy metals) or from drug interactions. Some edible plants that have been associated with

chronic kidney injury are *Larrea tridentata*, a Native American Shrub, commonly used to make tea (associated with renal cysts and renal cell carcinoma); *Glycyrrhiza glabra,* liquorice, which can cause hypokalaemic nephropathy; *Salix daphnoides*, commonly named Willow bark, implicated in the causation of renal papillary necrosis etc [Jha et al., 2010].

- Infections (as HIV, hepatitis B and C): they often cause serious damages also to the kidneys; antiretroviral therapies also have nephrotoxic effects including crystal deposition, tubular dysfunction, and interstitial nephritis.
- Water consumption: water can be contaminated with heavy metals or organic compounds washed out of the soil (including pesticides) or it can carry some pathogens (of schistosomiasis, leptospirosis, malaria etc), thus triggering events that can lead to the onset of kidney damages implicated in geographically localized epidemics [Webster et al., 2017].
- Increased use of nephrotoxic agents (e.g. analgesics and radiological contrast media): radiologic contrast media can cause acute renal failure and accelerate progression to ESRD [Muntner et al., 2003].

Besides known etiologies, CKD has also many unknown causes, especially in low- and middle-income countries. CKD of uncertain etiology appears to be growing, particularly among agricultural communities and most conspicuously, among young male farmers. For these reasons, many epidemiological studies, sponsored also by the World Health Organization, are trying to understand this phenomenon, to better prevent CKD [Lunyera et al., 2016].

## 1.4 Conservative therapy, renal replacement therapy and kidney transplantation

In most cases CKD progression is gradual, so CKD patient at stage 1-4 initially has to start the so called "conservative therapy". This therapy aims at slowing down kidney failure and it comprehends a pharmacologic therapy, a diet therapy and a more healthy lifestyle [Sabatino et al., 2017; KDIGO 2012]. When a patient shows symptoms or signs attributable to kidney failure (serositis; acid-base or electrolyte abnormalities; pruritus; inability to control volume status or blood pressure; a progressive deterioration in nutritional status; cognitive impairment) KIDGO guidelines recommend to start the renal replacement therapy (RRT) [KDIGO 2012]. RRT consists in the hemodialysis (HD) or peritoneal dialysis (PD), two equivalent systems which replace kidneys depurative function. Normally RRT starts when the GFR ranges between 5 and 10 ml/min/1.73 $m^2$.

At this point, kidney function is no longer able to sustain life, so the RRT becomes necessary lifetime or until a renal transplant occur.

HD and PD have many side effects both during and immediately after the treatment (as fatigue, cramping, post-dialysis dizziness, headache, pruritus, back pain, nausea and vomiting [Morfin et al., 2016]), both chronically since they induce an increased oxidative stress and inflammation, with a consequent increased risk in comorbidities, first cardiovascular diseases (CVD) [Liakopoulos et al., 2019; Li et al., 2017]. The CVD risk for a 40-year-old dialysis patient is the same as for an 80-year-old individual without renal disease [Foley et al., 1998]. So, despite recent advances in dialysis technology, solutes and membranes, dialysis patients have a high risk for premature death, mainly dependent by CVD and infectious complications [Stenvinkel et al., 1999].

Globally, the prevalence of ESRD patients treated with either hemodialysis or peritoneal dialysis is around 280 per million people, while the prevalence of who received a kidney transplant is 65 per million people [Webster et al., 2017]. Kidney transplantation is associated with a marked reduction of mortality risk and cardiovascular diseases and it substantially increases quality of life [Tonelli et al., 2011], but among ESRD patients on dialysis who are actively waiting for a transplant, only 25% receive a kidney, whereas 6% die while waiting, each year [US Organ Procurement and Transplantation Network, 2015].

## 1.5 Socio-economic effects and economic implications

CKD risk is influenced by the economic level of each country. Poverty is related to an increased exposure to various risk factors that predispose to CKD onset and that accelerate its progression and, at the same time, correlates with a lower chance of accessing dialysis.

Chronic renal failure is a pathology with a significant economic weight both for the state and the patient. In developed countries, around 2-3% of the public health expenditure is used to finance RRT, although ESRD patients represent only 0.1-0.2% of the total population. In the majority of poor or developing countries, the impossibility to access to health insurance makes the expenses for the patients so heavy to become impossible to sustain (to give an example, a single dialysis session costs around 20-60 dollars in India and about 100 dollars in China and Nigeria) [Jha et al., 2008]. Recently, a systematic review reported that the annual cost per HD patient ranges from $ 3,424 to $42,785, while per PD patient ranges $ 7,974 to $47,971 [Mushi et al., 2015]. In Italy, the direct cost of each dialysis patient is estimated to be minimum € 29,800 per year (in the case of peritoneal dialysis) and maximum € 43,800 per year (for patients undergoing hemodialysis) [Mennini et al., 2013] . It has been calculated that a 5 years delay of renal failure progression from stage 3 to stage 4

for 10% of subjects (therefore delaying the start of dialysis by 5 years), would allow the National Health System to save 2.5 billion euros [Pontoriero et al., 2007].

In this context, the primary importance of prevention emerges clearly: the control of blood pressure, blood glucose, lipid profile, acidosis and other strategies aimed at prevent and early detect CKD, slowing down CKD progression and minimizing the need for RRT, should not be overlooked [Couser et al., 2011].

## 1.6 CKD comorbidities

In parallel to CKD progression, there is the development of the so called "uremic phenotype": complications seriousness increases in parallel with the decline of GFR. The uremic patient shows an increased mortality risk for multiple causes and is progressively more exposed to comorbidities as cardiovascular diseases (CVD), renal failure progression, acute kidney injury (AKI), protein energy wasting, insulin resistance, sympathetic overactivity, acidosis, endothelial dysfunction, cognitive decline, bones disorder, minerals balance disorder, hospitalization, anemia, dysbiosis, oxidative stress, chronic inflammation etc [Murabito et al., 2018].

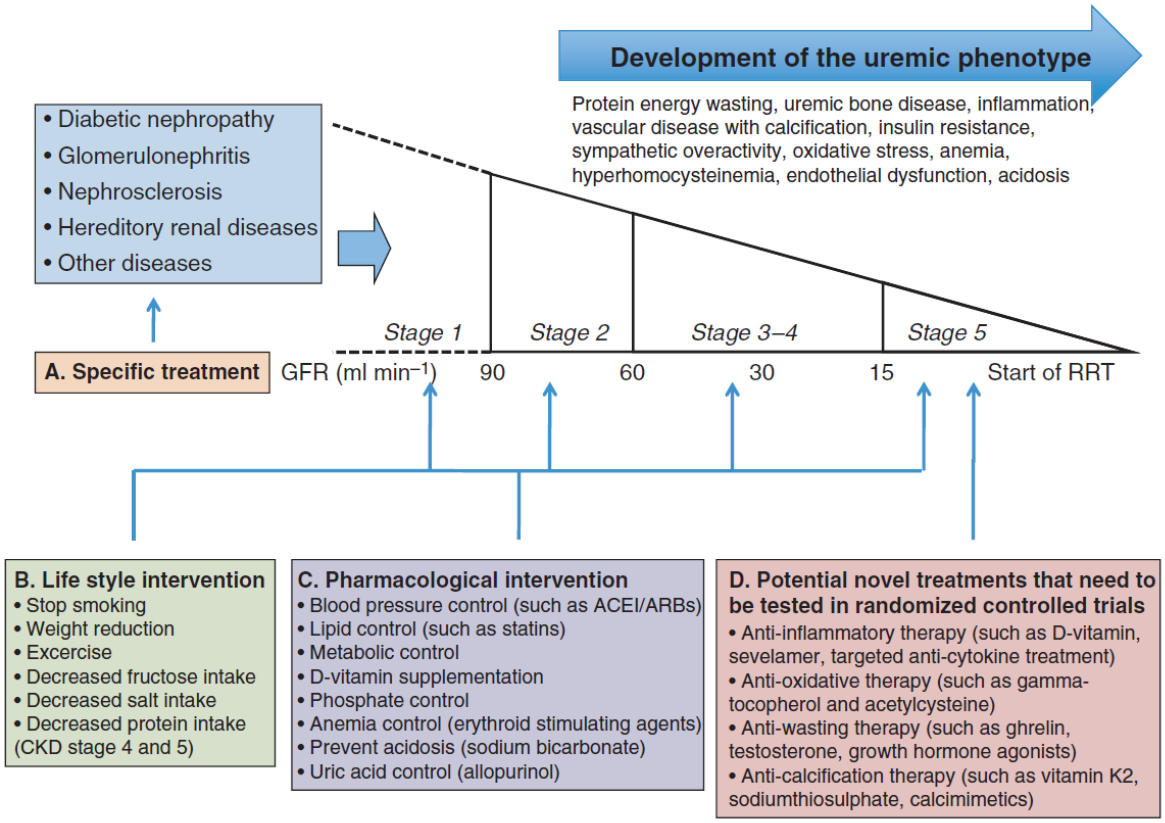


*Figure 4. Development of the uremic phenotype, complications and treatments. ACEI; angiotensin-converting enzyme inhibitor; ARBs, angiotensin receptor blockers; RRT, renal replacement therapy; GFR, glomerular filtration rate [Stenvinkel et al., 2010].*

Cardiovascular diseases show a very close correlation with CKD; subjects with renal insufficiency are included in the population group at the highest risk for developing cardiovascular pathologies. ESRD subjects have a cardiovascular mortality from 10 to 30 times greater than healthy subjects (compared at the same age, gender and ethnicity) [Jha et al., 2013]. People with a GFR less than 60 ml / min have 57% higher cardiovascular mortality and 33% higher risk of having non-fatal myocardial infarction compared with people without CKD [Di Angelantonio et al., 2007; Matsushita et al., 2010]. At the same time, people with micro-albuminuria have a cardiovascular mortality increase around 63% and their myocardial infarction risk is increased by 48% [Perkovic et al., 2008; Gansevoort et al., 2011]. This close positive relationship between CKD and CVD triggers to a vicious cycle that contributes to an increased premature mortality. In patients with CKD, the remodeling of myocardium and blood vessels leads to several cardiovascular complications such as cardiomyopathy, atherosclerosis, arterial stiffness, calcification, and subsequent ischemic heart disease, heart failure, cerebrovascular and cardiovascular death, and progression of renal disease [Chen et al., 2018]. Recent studies have highlighted how CVD risk factors in subjects with renal insufficiency are different from that of the general population: in addition to the traditional Framingham risk factors, anemia, inflammation, oxidative stress, metabolic alterations, sympathetic overactivity, electrolyte disturbances and vascular calcification, appear not only more frequent in these subjects, but also closer related to CVD [Stenvinkel et al., 2008; Stevinkel et al., 2010].

CKD is also the most common condition associated with acute kidney injury (AKI). AKI is characterized by a rapid reduction in kidney function, with an increased risk of death. Both with regard to CVD and AKI, the relationship with CKD is bi-directional, given that the former is an important risk factor for the latter and vice versa [Negi et al., 2018; Stevinkel et al., 2010] .

CKD is associated with an increased risk of developing complications after infections. Infections in CKD patients, and especially in dialysis patients, occur with rates 3 or 4 times higher than in the general population, probably as a result of immune system alterations. Infections (commonly at the urinary tract, pneumonia or sepsis, but also catheter-related bloodstream infections, access site infections, thrombosed IV fistulas and grafts, and episodes of peritonitis in PD patients) are important causes of morbidity and mortality in patients with renal insufficiency and represent the second main cause of death after CVD [Nassar et al., 2013; KDIGO, 2012].

From these premises, it appears evident that CKD comorbidities are multiple and heterogeneous; over time they have acquired increasing attention since in most cases these complications feed each other and worsen CKD progression. In the next chapters we will focus on three comorbidities extremely common in CKD patients: oxidative stress and inflammation [Jofrè et al., 2006; Kalantar-

Zadeh et al., 2003; Colombo et al., 2015], anemia [Pisani et al., 2015; Babitt at al., 2012], dysbiosis and uremic toxins [Cigarran Guldris et al., 2017; Al Khodor et al., 2017].

### 1.6.1 Oxidative stress and inflammation

Chronic inflammation and oxidative stress represent two complications, strongly correlated among them, that in turn worsen health status, causing 10- to 100-fold increase in CVD and all-cause mortality in HD patients (compared with controls) [Colombo et al., 2015]. They play a major role in CKD progression and its complications onset [Himmelfarb et al., 2002; Vaziri et al., 2004; Cachofeiro et al., 2008]. On the one hand, oxidative stress triggers the activation and recruitment of immune cells by activating the redox-sensitive nuclear factor kappa B (NF-κB), which is the master regulator of pro-inflammatory cytokines and chemokines, thereby promoting inflammation. On the other hand, inflammation initiates or amplifies oxidative stress via production of reactive oxygen, nitrogen, and halogen species, by the activated immune cells. Recently, it has been demonstrated a CKD-induced impairment of the nuclear factor-erythroid-2-related factor 2 (Nrf2) pathway. This transcription factor regulates the expression of antioxidant and cytoprotective enzymes and substrates; its CKD-induced impairment plays a major role by disabling the natural antioxidant response to oxidative stress and inflammation [Vaziri et al., 2012].

Oxidative stress and inflammation increase together with uremia and CKD progression. Therefore it could be speculated that uremia is a pro-oxidant condition per se: some authors think that uremia is an increase oxidative stress status [Himmelfarb et al., 2003], while others consider uremia the triggering event which is responsible for the unbalance between pro- and anti-oxidant factors [Floccari et al., 2005]. This unbalance depends both by an increased ROS production and a reduced antioxidant capability, typical of uremic patients [Wu et al., 2005].

Nevertheless, also other factors contribute to exacerbate oxidative stress and inflammation problems, as aging, hypertension, diabetes, obesity and low antioxidant level, common features in CKD patients, especially at stages 4-5. Even iron treatment (prescribed to correct anemia) and the dialysis treatment increase oxidative stress and inflammation level: the first, because iron can act as an oxidative molecule; the second, because the blood contact with dialysis membrane stimulates monocytes and polymorphonuclear leukocytes to produce reactive oxygen species (ROS) [Colombo et al., 2015; Wu CC., 2005; Himmelfarb et al., 2002; Çakatay et al., 2005; Ramakrishna and Jailkhani, 2007; Pandey et al., 2010].

So oxidative stress and inflammation represent a redundant replay to many factors that stimulate chronically CKD patient immune system and endothelium, as summarized in Table 5.

| OXIDATIVE STRESS AND INFLAMMATION RISK FACTORS | |
|---|---|
| CKD OR REDUCED GFR | - Reduces pro-inflammatory cytokines clearance<br>- Volume overload<br>- Increased oxidative stress<br>- Reduced antioxidant level<br>- Protein-energy malnutrition<br>- Chronic exposure to uremic toxins |
| COMORBIDITIES | - Increased comorbidities prevalence (CVD, diabetes, hypertension etc.)<br>- Inflammatory diseases which involved kidneys (LES, AIDS)<br>- Altered calcium-phosphorus metabolism<br>- Infections (Helicobacter pylori, Chlamydia pneumoniae, arterio-venous fistula infection etc.,)<br>- Intestinal dysbiosis<br>- Altered adipose tissue metabolism |
| DIALYSIS TREATMENT | - Blood contact with dialysis membrane<br>- Exposition to contaminated dialysate<br>- Contaminants back-filtration or back-diffusion<br>- Foreign body in the arterio-venous grafts<br>- Catheters |

*Table 5. Causes of increased oxidative stress and inflammation in CKD and ESRD patients [Jofrè et al., 2006; Kalantar-Zadeh et al., 2003; Rapa et al., 2019].*

Inflammation is a common feature of CKD patients: it is highly prevalent in patients at stages 3-5, with about 50% of patients at stages 3-4 with C-reactive protein (CRP) levels >2.1 mg/l [Eustace et al., 2004]. At the ERSD, this phenomenon seems to interest more European and North American dialysis patients (who have CRP levels >5 mg/l) than Asian dialysis patients [Kaizu et al., 2003]. It is worth pointing out that, in the toxic uremic milieu, persistent inflammation not only has proatherogenic effects and contributes to CKD progression, but it can act as a catalyst and magnifies the risk of poor outcome through the self-enhancement of the inflammatory cascade and the exacerbation of the wasting and the vascular calcification processes [Carrero et al., 2009]; it promotes insulin resistance, oxidative stress, endothelial dysfunction, mineral and bone disease, anemia and erythropoietin (Epo) resistance [Rapa et al., 2019].

Increased oxidative stress level in CKD patients compared to healthy subjects is well documented too [Handelman et al., 2001; Oberg et al., 2004; Nguyen-Khoa et al., 2001; Miyata et al., 2001]. Uremic patients are characterized by an accumulation of reactive aldehydes and oxidized thiols, with a consequent reduction of reduced thiols, that are antioxidant molecules [Himmelfarb et al., 2003]. Oxidative stress does not involved only the main plasma proteins, but also proteins that are present in the plasma in small amount, which become susceptible to modifications, as carbonylation [Pavone et al., 2011]. A high oxidative stress level results in the oxidation of biological molecules like lipids, proteins, and DNA. In the CKD context, it has been associated with an acceleration in kidney failure and with a plethora of complications such as hypertension, atherosclerosis, diabetes, inflammation, and anemia [Daenen et al., 2019; Rapa et al., 2019].

If inflammation parameters (such as white blood cells, CRP, interleukins, adipokines, tumor necrosis factor alpha etc) have been monitored since a long time in HD patients, oxidative stress parameters have emerged only in the last years as a cheap and simple tool suitable for large-scale clinical studies that reveals additional information on patient health status and that paves the way for the development of new therapies. Among these oxidative stress biomarkers there are GSH/GSSG ratio (reduced glutathione/glutathione disulphide ratio) [Giustarini et al., 2017], diTyr (protein-bound dityrosine) [Colombo et al., 2017], PCO (carbonylated proteins) [Colombo et al., 2017], PTI (protein thiolation index) [Giustarini et al., 2012], PSH (protein sulfhydryl groups) [Dalle Donne et al., 2008], AOPPs (advanced oxidation protein products) [Zhou et al., 2012].

Overall it appears that oxidative stress and inflammation have crucial roles in CKD and even more in ESRD [Colombo et al, 2015]. They could represent a silent culprit of other commonly observed pathophysiologic alterations in CKD, so it should be useful to monitor regularly inflammation and oxidative stress biomarkers, to deepen their causes and consequences and to outline therapeutic strategies aimed at reducing them. We evaluated oxidative stress biomarkers both in studies *in vitro* (performed with endothelial cells exposed to uremic toxins) and in studies *in vivo* (carried out analyzing plasma samples of ESRD patients and healthy subjects).

### 1.6.2 Anemia

Anemia is defined as the decrease of blood hemoglobin (Hb) value under 12-13 g/dL in adults. Anemia (typically normocytic, normochromic and hypoproliferative) is a common feature of CKD, associated with poor outcomes such as a reduced quality of life, increased incidence of CVD, higher rates of hospitalization, cognitive impairment, and mortality [Tsagalis et al., 2011; Lefebvre et al., 2006; Locatelli et al., 2004]. It appears at stadium 3 and it worsens at stadiums 4 and 5, so it's prevalent in ESRD patients [Fondazione italiana del rene, 2006]. In the latter, principal causes of anemia are primarily reduced erythropoiesis and secondarily the shortened red blood cell survival.
Reduced erythropoiesis is due first to a reduced erythropoietin (EPO) amount, whose production decreases along with the renal function decline. EPO is a glycoprotein hormone produced by the interstitial fibroblasts around peritubular capillaries and proximal convoluted tubules in the kidneys; it represents the main stimulus for red blood cells production in the bone marrow and it controls hemoglobin homoeostasis. Other factors influence negatively erythropoiesis: iron deficiency (necessary for the hemoglobin synthesis); folate and vitamin B12 deficiency (needed for DNA synthesis); aluminum toxicity (prevalent in patients who swallow antacid to correct phosphoremia); inflammation (which inhibits EPO production and impairs the growth of erythroblast). The

secondary cause of anemia is the shortened red blood cell survival; it's chiefly due to some uremic toxins that induce hemolysis. The red blood cells medium life in patients with ESRD is 60-90 days (instead of 120 days in healthy subjects) [Tsagalis et al., 2011].

Recent studies concentrated on the role of disordered iron homeostasis as a major contributor to anemia onset: iron deficiency occurs in more than 50% of patients with non-dialysis-dependent CKD and in a greater percentage of patients receiving dialysis. CKD patients have increased iron losses, because of chronic bleeding, frequent phlebotomy and blood trapping in dialysis apparatus [Fishban et al., 2018]. They also have impaired dietary iron absorption and impaired iron release from body store. These last two features seem to be due to hepcidin excess, the main hormone responsible for systemic iron homeostasis, whose excess depends on its reduced renal clearance and its increased expression induced by inflammatory cytokines. Last, many ESRD patients receive erythropoiesis stimulating agents (ESAs, that are erythropoietin and its synthetic derivatives as epoetin alfa, epoetin beta, darbepoetin alfa, methoxy polyethylene glycolepoetin beta [Webster et al., 2017]), which deplete the circulating iron pool by increasing erythropoiesis [Babitt et al., 2012; Fishbane at al., 2014]. All together these factors lead to an iron deficiency that plays a crucial role in the genesis of CKD-related anemia and that makes iron replacement a cornerstone of the treatment of anemia in dialysis patients. Erythropoiesis, in fact, is limited by low iron availability and so iron deficiency should be corrected before initiating ESAs [KDIGO, 2012].

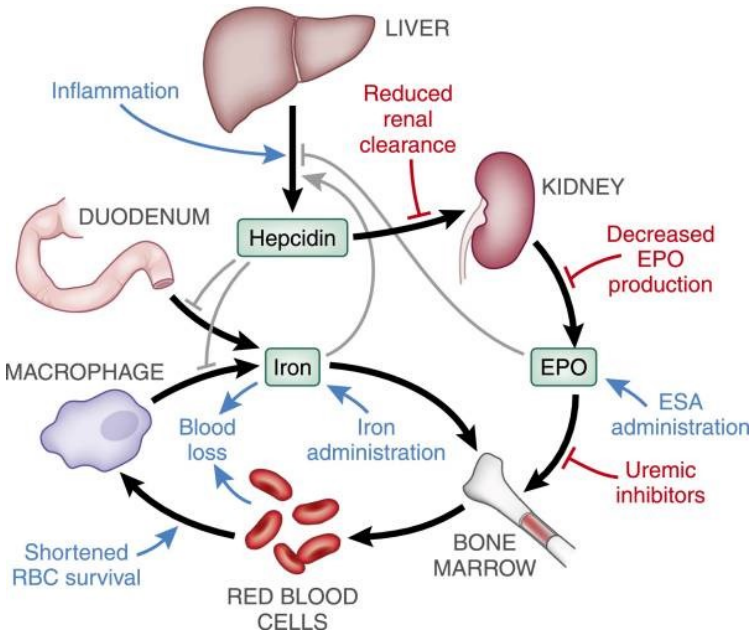


*Fig. 5 Schematic representation of the mechanisms underlying anemia of CKD [Babbit et al., 2012]*

The optimum route of iron administration is still controversial.

Intravenous iron administration is prevalent in hemodialyzed patients, because it corrects anemia and replenishes iron stores more effectively than oral treatment and it reduces the need for ESAs [Wingard et al., 1995; Albaramki et al., 2012; Shepshelovich et al., 2016]. It confers a greater increase in hemoglobin, but it has many side effects. In fact, it can promote allergic reactions (also severe anaphylactic reactions), infections (by supplying iron to pathogenic bacteria) and may cause endothelial damages [Del Vecchio et al., 2010; Webster et al., 2017]. In addition, since it has a high reactivity with oxygen (as described by the Haber-Weiss and Fenton reactions), iron generates oxidative stress, enhancing atherosclerosis and other complications [Agarwal et al., 2004] and may increase inflammation state, aggravating functional iron deficiency [Brewster et al., 2004]. The main concern about the use of intravenous iron and the risk of iron overload is the possible effect on mortality, in fact some recent studies showed an increased mortality in patients treated with higher doses of intravenous iron [Kalantar-Zadeh et al., 2005; Bailie et al., 2015].

On the other hand, oral iron has advantages such as low cost and easy administration, but it shows poor gastrointestinal absorption and side effects such as nausea, vomiting, diarrhea and abdominal pains [Agarwal et al., 2006].

Sucrosomial iron (Sideral® Forte) is a new generation oral iron that appears a promising strategy to administrate iron because it shows high gastrointestinal absorption and high bioavailability and, at the same time, low incidence of side effects. It is a preparation of ferric pyrophosphate conveyed within a phospholipid membrane associated with ascorbic acid. According to Pisani and colleagues, liposomal iron determines a significant increase in hemoglobin, it has less gastrointestinal side effects and it doesn't increase inflammation biomarkers [Pisani et al., 2015]; in addition, its absorption isn't influenced by hepcidin levels [Gómez-Ramírez et al., 2018]. Moreover, some recent studies pointed out that liposomal iron seems to have an anti-inflammatory action, since it decreased inflammation biomarkers in patients suffering from chronic inflammatory diseases [Giordano et al., 2015]. So, in these years my group, together with a group of nephrologists who work at the Humanitas Clinical Centre (Rozzano, MI), examined the capabilities of this kind of oral iron in anemia correction and its relationship with inflammation and oxidative stress biomarkers in hemodialyzed patients. The aim was to asses if liposomal iron could represent a safe and efficacious alternative to other form of iron supplementation and if it could ameliorate HD patients life avoiding side effects and improving oxidative stress and inflammation level.

### 1.6.3 Dysbiosis and uremic toxins

Dysbiosis is another feature of CKD patients. CKD and ESRD patients show a quantitative and qualitative alteration of the intestinal microflora (dysbiosis). Many recent studies suggest that toxic

products generated by a dysbiotic gut microbiome may contribute to CKD progression and to CKD-related complications [Ramezani et al., 2014].

The germs that inhabit our body are called the microbiota and their collective genomes, the microbiome. Microbiota is composed by more than 100 trillion microbial cells ($10^{14}$), representing 10 times the number of cells that make up our body. It constitutes a true ecosystem which performs so many functions that it should be considered as a metabolically active endogenous "organ" in itself [O'Hara et al., 2006]. In fact, it plays important roles in metabolic, nutritional, physiological and immunological processes. In particular, it is responsible for some complementary metabolic activities, such as the breakdown of undigestible plant polysaccharides, the synthesis of certain vitamins and amino acids and the metabolism of bile acids [Ramezani et al., 2014]. Microbiota is also involved in the maturation of the immune system in infancy and in its homeostasis during life, reducing allergic responses to food and environmental antigens [Hevia et al., 2015]. For these reasons, microbiota has a huge impact on human well-being, both in health and in disease [Pflughoeft et al., 2012].

The concentration of germs in the digestive tract gradually increases from the stomach to the colon. Different types and amount of bacteria characterize each intestinal tract. Overall, adult gut is dominated by two bacterial phyla, Firmicutes and Bacteroidetes; other phyla are present in smaller proportions [Eckburg et al., 2005]. The composition of the gut microbiota is affected by many factors, so that it differs from one person to another one and it changes in the same person through life. Some of these factors are host genetic, geographical origin and location, early microbial exposure, age, lifestyle, eating habits, antibiotics or probiotics intake etc [Al Khodor et al., 2017].

From the early stages of CKD, there's a change in the composition and structure of the microbiota. The duodenum and the jejunum, normally lightly colonized in healthy people, become intensely colonized by aerobic and anaerobic bacteria in uremic patients [Simenhoff et al., 1978]. Uremic patients show a higher number of Enterobacteria and *Enterococci* and a lower number of Lactobacillaceae and Prevotellaceae families [Vaziri et al, 2013]. In particular, hemodialyzed patients present an overgrowth of aerobic bacteria, with the number of Enterobacteria and *Enterococci* species approximately 100 times higher than in healthy subjects. Regarding anaerobic bacteria, HD patients have a significantly lower number of *Bifidobacteria* and higher *Clostridium perfringens* [Hida et al., 1996].

Gut microbiota and CKD have a bidirectional relationship: kidney disease may disrupt microbiota balance and at the same time the unbalanced microbiota affects kidney disease progression.

Dysbiosis may depend on uremia per se. Kidney dysfunction leads to urea secretion into the gastrointestinal tract. Urea is then hydrolyzed by gut microbes, resulting in ammonia formation.

Large quantities of ammonia affect the growth of commensal bacteria [Kang et al., 1993]. Other factors, associated with CKD and potential causes of dysbiosis, are the decreased consumption of dietary fiber, frequent use of antibiotics, slow colonic transit, metabolic acidosis and intestinal wall edema [Ramezani et al., 2014]. Also treatment with oral iron can modify the microbiota [Werner et al., 2011].

In turn, dysbiosis implies many consequences that contribute to chronic kidney disease progression. First, dysbiosis contributes to modifying intestinal barrier permeability. In CKD patients, high urea levels and increased number of bacteria with urease result in a raised ammonium production, which in turn induces a change in intestinal lumen pH that affects the tight junctions of the enterocytes. In fact, CKD subjects show a reduction in tight junction proteins (such as claudin-1, occluding and ZO-1) [Vaziri et al. 2012]. Also the reduced number of short-chain fatty acids (SCFAs) producing bacteria is associated with modifications in gut environment which result in a loss of epithelial barrier integrity [Felizardo et al., 2019]. The consequence is an increased permeability of the intestinal barrier that leads to the translocation of bacteria, endotoxins and gut-derived uremic toxins across the intestinal wall [Goncalves et al., 2006]. This infiltration stimulates immune system cells to become activated and to synthesize and secrete a variety of effector molecules, which cause an inflammatory response, exacerbating chronic inflammation and oxidative stress problems [Wang et al., 2012]. By this way, the gut dysbiosis is associated with an altered activity of the autonomic nervous system, vascular tonus and neuroinflammation. For these reasons, the microbiota is now emerging as a modifiable non-traditional risk factor in nephrology for cardiovascular and neuropsychiatric disorders, besides CKD progression [Cosola et al., 2019].

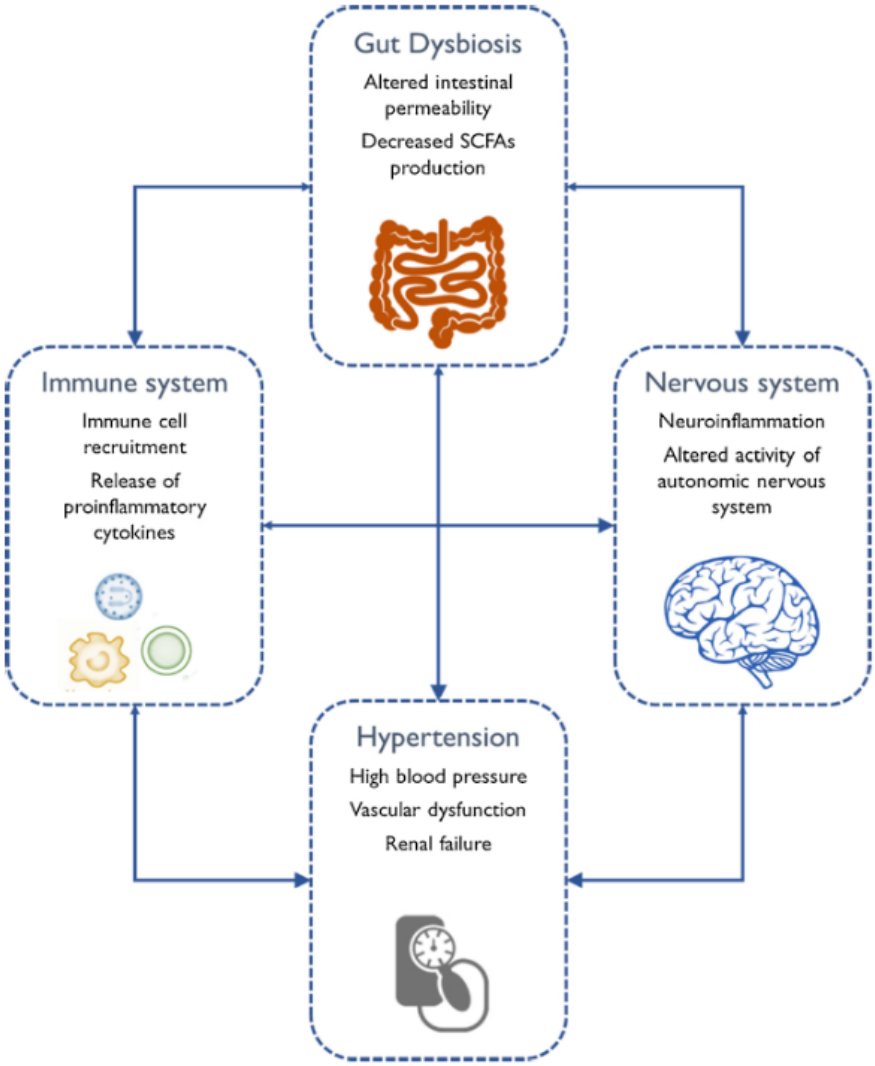


*Fig. 6 Correlation between dysbiosis, immune, nervous and cardiovascular systems [Cosola et al., 2019].*

Second, the microbiota produces uremic toxins (UTs). In the colon, protein fermentation performed by intestinal bacteria generates several metabolites including ammonium, amines, thiols, phenols and indoles. Normally, these molecules are in part eliminated through faeces and in part cleared by the kidneys, so they accumulate in CKD and are called uremic toxins (even if their metabolism or production do not depend on urea metabolism) [Evenepoel et al., 2009]. Some microbiota primary actions include the deamination and decarboxylation of amino acids. These processes lead to the release of hydrogen sulphide from sulphur-containing amino acids (methionine and cysteine), and to the production of ammonia, SCFAs or phenolic compounds (indole and p-cresol) from aromatic amino acids (tyrosine, phenylalanine, and tryptophan). In addition, the decarboxylation of amino acids also results in formation of different amines which serve as precursors for nitrosamines

[Vaziri et al., 2016]. A metabolomic characterization study on ESRD patients showed an expansion of bacterial families possessing urease, uricase, indole and p-cresol forming enzymes, in parallel with a reduction of families possessing butyrate forming enzymes [Wong et al., 2014]. So ESRD microbiota shows a shift towards the proteolytic metabolism, which results in an increased production of UTs.

Uremic toxins are usually classified according to their physicochemical characteristics, which determine their clearance during dialysis, in three categories: small water-soluble molecules (molecular weight (MW) <500 Da), larger 'middle molecules' (MW >500 Da), and protein-bound molecules [Duranton et al., 2012].

Uremic toxins exert their side effects not only in the gut, but also, thanks to the systemic translocation through the leaky gut epithelial barrier, in multiple organs: they contribute to kidney fibrosis and CKD progression; increase CVD risk and CVD mortality; they lead to leukocytes dysfunction and consequent increase hospitalization for infections; exacerbate anemia; uremic toxins cause adipocytes dysfunction, contributing to insulin resistance onset; and they increase bone diseases risk (*Figure 7*) [Wei Ling Lau et al., 2018].

The mechanisms through which UTs cause these multiorgan dysfunctions involved inflammatory, oxidative stress, and apoptosis pathways [Rapa et al., 2020]. Uremic toxins induce inflammation in the systemic circulation: they contribute to an altered immune response [Adesso et al., 2013]; they have a positive correlation with inflammation biomarkers as IL-6, TNF-alpha, CRP levels, iNOS and COX-2 and transcription factors attendee to inflammation pathways as NF-kB [Stockler-Pinto et al., 2016; Stockler-Pinto et al., 2018]. As described in the chapter 1.6.1, inflammation and oxidative stress are strongly correlated among them, so uremic toxins exacerbate also the oxidative stress problem: they increase ROS production [Adesso et al., 2013; Stockler-Pinto et al., 2016] and oxidant markers level [Xu et al., 2015]; at the same time, they seem able to downregulate enzymes with cytoprotective and antioxidant activities, as Nrf2 [Adesso et al., 2018].

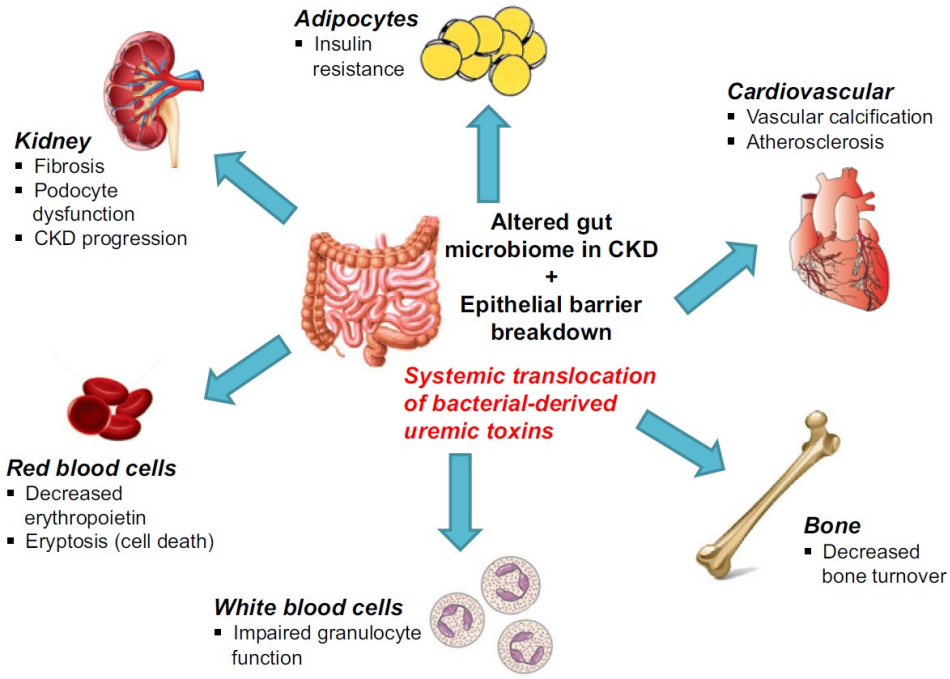


*Fig. 7 Multiorgan dysfunctions promoted by the gut-derived uremic toxins via inflammatory, oxidative stress, and apoptosis pathways [Wei Ling Lau et al., 2018]*

Urea, a water-soluble molecule, is the uremic toxin with the highest concentration [Lau et al., 2017]. It is produced in the hepatocytes during the urea cycle, which converts toxic ammonia, derived from protein catabolism, into urea, subsequently excreted through the urine [Barmore et al., 2020]. The biologic activities of urea appear to be relatively limited when compared to the other UTs [Vanholder et al., 2001]; for this reason, some studies have questioned urea pathogenicity, while others have shown its direct and indirect side effects. In this regard, urea is reported to exert toxic effects on the gastrointestinal tract, kidneys, adipocytes, blood components, and on the cardiovascular system (CVS) [Lau et al., 2017].

For the biological and clinical consequences of their accumulation, p-cresyl sulfate (PCS) and indoxyl sulfate (IS) are the most studied UTs [Vanholder et al., 2014; Meijers et al., 2009; Yu et al., 2011; Yisireyili et al., 2013; Lin et al., 2015].

IS is a protein-bound uremic toxin. It derives from dietary tryptophan conversion into indole, operated by the gut microbiota. Indole then is absorbed into the blood circulation, through which it reaches the liver, where it is oxidized and sulfated to form IS [Leong et al., 2016]. Normally IS is cleared through renal tubular secretion, while in CKD patients it accumulates in blood and tissues, at least 90% bound to plasma proteins [Eloot et al., 2016].

Also PCS is a uremic toxin highly conjugated with proteins. It is product by intestinal bacteria which metabolize tyrosine and phenylalanine, two essential amino acids findable mainly in protein-rich food [Yavuz et al., 2005].

Both IS and PCS are associated with CKD progression, cardiovascular complications, alteration of bone-mineral metabolism, insulin resistance, anemia, inflammation and oxidative stress [Cigarran Guldris et al., 2017; Liu et al., 2018].

IS and PCS have strong and evident pro-oxidative [Itoh et al., 2012; Bolati et al., 2013], pro-inflammatory [Sun et al., 2013] and pro-fibrotic effects [Bolati et al., 2011; Sun et al., 2012], shown through *in vitro* and *in vivo* experiments. All these data seem to provide the molecular basis for their role as co-promoters of CV and renal damage progression. It is worth considering that these intestinal-derived protein-bound UTs are not efficiently removed by the dialytic treatment and that this aspect contributes to additionally increased CV risk in ESRD [Tammy et al., 2014; Shafi et al., 2015]. Nowadays 30 colon- derived UTs have been identified (including IS, PCS, indoxyl glucuronide, indoleacetic acid (IAA), p-cresyl glucuronide, phenyl sulphate, phenyl glucuronide, phenylacetic acid, phenyl acetyl glutamine, hippuric acid and 3-carboxy-4- methyl-5-propyl-2-furanpropionic acid [Aronov et al., 2011]), but also other microbial metabolites, such as Trimethylamine-N-Oxide (TMAO), Nitric Oxide (NO) and Short-Chain Fatty Acids (SCFA) were shown to exert a direct effect or an association with CV risk [Cosola et al., 2018; Castillo-Rodriguez et al., 2018].

In the last years, many studies have explored various ways to reestablish symbiosis. These ones include food supplements (prebiotics, probiotics and symbiotics) and fecal microbiota transplantation, to restore a more balanced gut microbiome, and adsorptive therapies, to enhance the disposal of uremic toxins [Al Khodor et al, 2017; Felizardo et al., 2019]. Seen the promising results, microbiota manipulation is now emerging as a promising tool to include in the nutritional management of CKD [Cosola et al., 2018]

Despite the progress in microbiota management, inflammation, together with oxidative stress, persist in CKD patients, with all their consequences. Accumulating evidences over recent years have spotlighted right the gastrointestinal tract as a major source of chronic inflammation in CKD [Lau WL et al., 2015]. So in these years we have carried on *in vitro* studies with the aim to better understand the effects of single uremic toxin exposure, in order to enrich fundamental research knowledge, essential to assume which uremic toxin has the more detrimental side effects and how it exerts its effects. These knowledges may constitute the basis also to hypothesize which treatment is the best to counteract uremic toxicity.

Overall, my PhD project focused on CKD complications (oxidative stress, chronic inflammation, anemia and UTs derived from dysbiosis) and it aimed at deepen the knowledge about these problems, in order to better understand their relevance in uremic patients management and to provide starting points for targeting interventions.

## 2. STUDIES AIM AND THESIS STRUCTURE

During my PhD studies I have investigated some CKD comorbidities both with *in vitro* and *in vivo* approaches. More in detail, regarding *in vivo* studies, we measured oxidative stress biomarkers in a population of ESRD patients before and after the hemodialysis treatment, comparing the results with a population of healthy subjects; we evaluated oxidative stress biomarkers in the plasma of HD patients before, during and after two type of iron treatments. Regarding *in vitro* experiments, we focused on two uremic toxins, urea and indoxyl sulphate, and we evaluated their effects on a human endothelial cell line (Human Microvascular Endothelial Cells 1, HMEC-1).

These studies resulted into five publications, which constitute the following five chapters of my thesis:

- **Chapter 1:** As reported in the introduction, CKD patients, and even more ESRD patients, experience oxidative stress. Since the hemodialysis treatment itself is among the causes of the increased oxidative stress level, we evaluated its effects in a group of HD patients immediately before and after a single dialysis session (performed with different dialysis filters), measuring the formation of protein carbonyls (PCOs). PCOs concentration is the oxidative stress biomarker most commonly used to assess protein oxidation; carbonylation is an irreversible and stable modification, which points out a condition of oxidative stress quite severe. In addition, since diabetic nephropathy is the most common cause of ESRD onset in developed and developing countries and seen the differences between males and females in the epidemiology, pathophysiology, clinical manifestations, and outcomes for several diseases, with this study we aimed also at clarifying the influence of diabetes mellitus and sex on plasma proteins oxidation.

  *Title:* Plasma Protein Carbonylation in Haemodialysed Patients: Focus on Diabetes and Gender.
  *Authors:* Colombo, G., Reggiani, F., Cucchiari, D., Astori, E., Garavaglia, M., & Portinaro, N. et al.
  *Journal:* Oxidative Medicine And Cellular Longevity, 2018, 1-12. doi: 10.1155/2018/4149681

- **Chapter 2:** In this study, we focused on oxidative stress and inflammation, two CKD complications strongly correlated among them. In this case, we measured advanced oxidation protein products (AOPP) in the plasma of patients undergoing HD. AOPP are the most commonly used biomarker of oxidative stress in ESRD patients on HD. Their production, which can depend both by myeloperoxidase (MPO)-dependent and MPO-independent mechanisms, is irreversible. In addition, AOPP are proinflammatory mediators, so they could represent a bridge between oxidative stress and inflammation. Seen these premises, we evaluated a potential correlation between AOPP level, urea and creatinine; AOPP and other oxidative stress biomarkers (as PCO and protein-bound di-tyrosines); AOPP level and inflammation biomarkers (C-reactive protein concentration and white blood cells count).

  *Title:* Advanced oxidation protein products in nondiabetic end stage renal disease patients on maintenance haemodialysis.
  *Authors:* Colombo G, Reggiani F, Astori E, Altomare A, Finazzi S, Garavaglia ML, Angelini C, Milzani A, Badalamenti S, Dalle-Donne I.
  *Journal:* Free Radic Res. 2019 Dec;53(11-12):1114-1124. doi: 10.1080/10715762.2019.1690651. Epub 2019 Nov 22.

- **Chapter 3:** Iron deficiency is the most common reversible cause of anemia among CKD patients. Iron can be administered via oral or parenteral routes, however both routes show important side effects. In particular, intravenous iron, that is the most commonly used in HD patients, is correlated with an increase in mortality. With this study, we tried to assess if sucrosomial iron (an oral iron preparation containing ferric pyrophosphate covered by phospholipids plus sucrose ester of fatty acid matrix) is safe and able to maintain adequate hemoglobin levels. We assessed also if it has anti-inflammatory capabilities, as previously reported in other studies, measuring inflammation and oxidative stress biomarkers.

  *Title:* Sucrosomial iron in the treatment of anemia of hemodialyzed patients: focus on hemoglobin maintenance and oxidative stress
  *Authors:* Reggiani F, Astori E, Colombo G, Finazzi S, Garavaglia ML, Angelini C, Milzani A, Badalamenti S, Dalle-Donne I.
  *Draft*

- **Chapter 4:** Urea is the uremic toxin with the highest concentration and whose toxicity have been questioned over time. About its effects on the cardiovascular system (CVS), urea is reported as a mortality and atherosclerosis risk factor, able to induce ROS production, pro-inflammatory and pro-apoptotic pathways. Since literature lacks in studies systematically exploring urea effects on protein expression and protein modification potentially involved in CVD, the aim of this study was to assess the effects of urea (at concentrations found in healthy subjects or measurable in CKD patients) on a human endothelial cells line (HMEC-1). We focused on urea effects on proliferation, cytoskeleton structure, endothelial-to-mesenchymal transition (EndoMT) biomarkers and on oxidative stress level. Finally, we performed a proteomic analysis of the cells medium, in order to identify the secreted proteins up or down-regulated by the urea treatment.

  *Title*: Effects of physiological and pathological urea concentrations on Human Microvascular Endothelial Cells (HMEC-1)
  *Authors:* Astori E., Colombo G., Altomare A., Garavaglia M.L., Milzani A., Dalle-Donne I.
  *In preparation*

- **Chapter 5:** Indoxyl sulfate (IS) is a protein-bound uremic toxin. It derives from a microbiota metabolite, indole, than oxidized and sulfated in the liver. Normally cleared through renal tubular secretion, IS accumulates in CKD patients plasma. IS is reported to have adverse effects mainly on kidneys, bones and cardiovascular system. The strongest correlation is with CVD. IS appears to induce ROS production and inflammation, leading to endothelial dysfunction and to an higher risk of chronic heart failure, arrythmia, coronary calcification and atherosclerotic vascular diseases.
  Since most of the studies about IS and CVD tested concentrations much higher than those measured in vivo, with this study we tried to better elucidate some IS side effects when endothelial cells (HMEC-1) are exposed to physiologic concentrations of this toxin. We focused on IS effects on proliferation, cytoskeleton structure, endothelial-to-mesenchymal transition (EndoMT) biomarkers and on oxidative stress level. Finally, we performed a proteomic analysis of the cellular proteins, in order to identify the proteins up or down-regulated by the IS treatment.

  *Title*: Effects of physiological and pathological indoxyl sulphate concentrations on Human Microvascular Endothelial Cells (HMEC-1)
  *Authors:* Astori E., Colombo G., Altomare A., Garavaglia M.L., Milzani A., Dalle-Donne I.
  *In preparation*

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# 4. CHAPTER 1

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*Research Article*

## Plasma Protein Carbonylation in Haemodialysed Patients: Focus on Diabetes and Gender

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Patients with end-stage renal disease (ESRD) undergoing haemodialysis (HD) experience oxidative/carbonyl stress, which is postulated to increase after the HD session. The influence of diabetes mellitus and sex on oxidation of plasma proteins in ESRD has not yet been clarified despite that diabetic nephropathy is the most common cause of ESRD in developed and developing countries and despite the increasingly emerging differences between males and females in epidemiology, pathophysiology, clinical manifestations, and outcomes for several diseases. Therefore, this study aimed to evaluate the possible effect of type 2 diabetes mellitus, gender, and dialysis filter on plasma level of protein carbonyls (PCO) in ESRD patients at the beginning and at the end of a single HD session. Results show that mean post-HD plasma PCO levels are significantly higher than mean pre-HD plasma PCO levels and that the type of dialysis filter and dialysis technique are unrelated to plasma PCO levels. The mean level of plasma PCO after a HD session increases slightly but significantly in nondiabetic ESRD patients compared to diabetic ones, whereas it increases more markedly in women than in men. These novel findings suggest that women with ESRD are more susceptible than men to oxidative/carbonyl stress induced by HD.

### 1. Introduction

Compared to the general population, patients with chronic kidney disease (CKD) are at higher risk for cardiovascular disease (CVD) because of higher prevalence of traditional (such as diabetes mellitus, left ventricular hypertrophy, dyslipidaemia, hypertension, and obesity) and nontraditional cardiovascular risk factors. The latter include uraemia, anaemia, inflammation, and oxidative stress, which all together form part of the malnutrition-inflammation complex (or cachexia) syndrome, which is a strong predictor of morbidity and mortality in these patients. [1–7]. The subgroup of CKD patients that undoubtedly experience the highest degree of oxidative stress is constituted by those patients with end-stage renal disease (ESRD) undergoing haemodialysis (HD). Oxidative stress in ESRD derives from both enhanced oxidative capacity, which is at least partly due to systemic (micro)-inflammation and upregulation of superoxide-producing enzymes [5, 8] and diminished antioxidant defences, the latter including impaired enzyme activities and decreased levels of antioxidant vitamins C and E [5, 8–10]. Depletion of circulating antioxidant vitamins in ESRD may originate from diet restriction, reduced absorption, uraemia-related alterations of metabolic pathways, and intradialytic losses [10].

In haemodialysed patients, oxidative stress, which may act synergistically with inflammation, is involved in the development of long-term complications such as amyloidosis, atherosclerosis, and CVD [1, 3, 11]. Plasma biomarkers

of protein oxidation in ESRD patients on maintenance HD were measured as indicator of oxidative stress in several studies. Plasma protein oxidation is highlighted by decreased protein thiols [12, 13], which might result from the formation of mixed disulphides between protein thiols and low molecular mass aminothiols (*S*-thiolation). *S*-thiolated plasma proteins, measured as protein thiolation index (PTI) [14], are indeed increased in ESRD patients on maintenance HD [12, 13]. Other biomarkers of protein oxidation in haemodialysed patients are plasma protein-bound dityrosines [15, 16] as well as plasma protein carbonyls (PCO), whose levels are elevated compared to healthy subjects [17–19]. Similarly, biomarkers of inflammation are elevated in haemodialysed patients [20–22].

While it appears that oxidative stress in haemodialysed patients may result from uraemia per se [1, 23], the HD procedure itself may contribute to oxidative/carbonyl stress. Indeed, a few studies showed that plasma PCO levels were significantly higher at the end of a single HD session than before it [17–19, 24, 25]. A prospective cohort study demonstrated that initiation of maintenance HD procedure does not have significant influence on serum levels of the inflammation biomarkers, C-reactive protein, interleukin-6, and interleukin-10, as well as plasma PCO [26]. Otherwise, levels of pentraxin-3, an inflammation biomarker belonging to the same protein family of C-reactive protein (pentraxins), which is rapidly produced locally to the site of inflammation by several cell types and released by neutrophils upon stimulation, were significantly increased at the end of the HD session [22]. This finding suggests that the HD procedure is an exacerbating factor for both oxidative/carbonyl stress and inflammation, presumably due to the activation of neutrophils upon contact with the dialysis filter [11]. Hence, it is postulated that oxidative/carbonyl stress increases in ESRD patients after the HD session.

Only a few studies have focused specifically on the impact of a single HD session on plasma PCO levels in ESRD patients [17–19, 24, 25]. Moreover, the relative importance of diabetes mellitus and sex on plasma PCO levels in haemodialysed patients remains poorly defined and with conflicting results [26–28]. This despite that diabetic nephropathy, alone or in combination with hypertensive nephropathy, is the most common cause of ESRD in developed and developing countries and despite the increasingly emerging differences between male and female in epidemiology, pathophysiology, clinical manifestations, and outcomes for several diseases, among which those displaying oxidative stress-mediated inflammation, such as CVD [29–31]. However, whether sex differences exist with respect to biomarkers of oxidative stress before and after a single HD session in ESRD patients is largely unknown. Therefore, we determined the plasma PCO levels in each individual HD patient before and after a single HD session. This was done by dividing the HD population into groups based on the cooccurrence of type 2 (non-insulin-dependent) diabetes mellitus and the gender. Moreover, the HD population was divided into groups also based on the dialysis filter used, in order to ascertain eventual differences attributable to filter characteristics and, therefore, to the different HD techniques. In fact, there is some evidence indicating that techniques that combine diffusion and convection, such as online haemodiafiltration, may reduce oxidative stress improving the haemodynamic tolerance and the clearance of uremic toxins [32, 33]. However, the effect of convective transport on oxidative stress needs a stronger confirmation.

## 2. Materials and Methods

*2.1. Study Design and Participants.* The study was approved by the institutional review board before initiation and carried out according to the Code of Ethics of the World Medical Association (Declaration of Helsinki). All the 69 Caucasian patients enrolled in the study belong to stage 5 of CKD and are referred to as ESRD patients on maintenance HD. In addition to HD, patients are treated with a pharmacological treatment that varies upon the clinical necessities and consists mainly on the treatment of ESRD complications. Most of the patients assume drugs for anaemia (i.e., iron intravenous supplementation and/or erythropoietin) and bone mineral disorder (i.e., calcium supplementation, phosphate binders, vitamin D, paricalcitol, and/or calcimimetics). In addition, patients may also take specific drugs for other comorbidities, for example, hypertension, diabetes mellitus, ischemic cardiopathy, and other vasculopathies. Blood samples were collected, after informed written consent, from ESRD patients undergoing maintenance HD at the Nephrology and Dialysis Unit of the Humanitas Clinical and Research Center (Rozzano, Milan, Italy). The samples were collected at the arterial line at the beginning and at the end of the HD session. The presence of a clinically overt infectious process was the only exclusion criteria. For every patient, an anamnestic record was collected. A deidentification of the samples was performed before any additional data processing. The baseline clinical characteristics of recruited patients are shown in Table 1. Control blood samples were collected from 20 (10 males and 10 females) age-matched voluntary healthy donors at the Analysis Laboratory of the University of Milan (Laboratorio Analisi, Università degli Studi di Milano) after obtaining informed verbal consent. Criteria included no known history of CKD or other diseases that could influence the analysis. In particular, healthy subjects were tested for serum creatinine in order to exclude CKD.

*2.2. Dialysis Filters.* We used different dialysis filters, all characterised by high-flux (defined as a $\beta$2-microglobulin clearance of over 20 ml/min) synthetic biocompatible membranes. In particular, the filters used are the following, classified by the dialysis technique:

(1) Diffusive technique (standard bicarbonate haemodialysis) ($n = 41$)

   (a) Revaclear™ 300, whose membrane is made of polyarylethersulphone (PAES) + polyvinylpyrrolidone (PVP) (surface: 1.4 $m^2$, thickness: 35 $\mu$m), for 23 patients

Table 1: Characteristics of haemodialysed patients with ESRD. Data are expressed as mean ± standard deviation.

| | Haemodialysed patients ($n = 69$) | Reference range |
|---|---|---|
| Age (years) | 69.0 ± 1.5 | — |
| Dialysis vintage (years) | 5.8 ± 0.46 | — |
| Sex | 45 male, 24 female | — |
| Diabetes mellitus | 47 nondiabetic, 22 diabetic | — |
| Creatinine (mg/dl) | 9.24 ± 0.35 | 0.6–1.3 |
| Urea (mg/dl) | 148.98 ± 4.55 | 10.00–50.00 |
| C-reactive protein (mg/dl) | 0.55 ± 0.08 | 0.01–1 |
| Albumin (g/dl) | 3.5 ± 0.04 | 3.5–5 |
| White blood cells (cells/mm$^3$) | 7257.97 ± 271.06 | $4 \cdot 10^3$ |
| Haemoglobin (g/dl) | 11.03 ± 0.12 | 13–18 |
| Sodium (mmol/l) | 137.80 ± 0.38 | 135–145 |
| Potassium (mmol/l) | 5.26 ± 0.09 | 3.5–5.1 |
| Calcium (mmol/l) | 2.22 ± 0.02 | 2.1-2.6 |
| Phosphorus (mmol/l) | 1.63 ± 0.05 | 0.8–1.5 |
| Ferritin (ng/ml) | 201.26 ± 16.85 | 20–250 |

(b) Revaclear™ 400, whose membrane is made of PAES + PVP (surface: 1.8 m$^2$, thickness: 35 $\mu$m), for 14 patients

(c) Filtryzer® 1.6, whose membrane is made of polymethyl metacrylate (PMMA) (surface: 1.6 m$^2$, thickness: 30 $\mu$m), for 4 patients

(2) Diffusive plus convective techniques (online haemodiafiltration (online HDF) and acetate-free biofiltration (AFB)) ($n = 27$)

(a) Polyflux™ 170 H, whose membrane is made of PAES + PVP + polyamide (PA) (surface: 1.7 m$^2$, thickness: 50 $\mu$m), for 14 patients

(b) Polyflux™ 210 H, whose membrane is made of PAES + PVP + PA (surface: 2.1 m$^2$, thickness: 50 $\mu$m), for 11 patients

(c) Nephral™ ST 400, whose membrane is made of acrylonitrile/methallyl sulphonate copolymer, coated with high-molecular-weight polyethyleneimine (PEI) (surface: 1.65 m$^2$, thickness: 42 $\mu$m), for one patient

(d) Nephral™ ST 500, whose membrane is made of acrylonitrile/methallyl sulphonate copolymer, coated with PEI (surface: 2.15 m$^2$, thickness: 42 $\mu$m), for one patient

Filters a, b, and d–g are produced by Gambro®-Baxter, whereas filter c is produced by Toray Industries Inc.

*2.3. Sample Collection.* Venous blood samples of 10 ml were collected from ESRD patients before HD session, and 5 ml were obtained after the same session. All samples were collected on the long interdialytic interval, that is, two days apart from the previous HD session. Blood was taken from the arteriovenous fistula or central venous catheter. $K_3$EDTA was used as anticoagulant in all the blood samples. From healthy donors, 10 ml of venous blood was collected from the antecubital vein. All the samples were processed within the first hour from blood sampling through centrifugation for 10 min at 1000$g$, obtaining pre-HD and post-HD plasma aliquots from ESRD patients and plasma aliquots from healthy subjects. Such aliquots were stored at −80°C until the execution of the assays.

*2.4. Detection of Plasma Protein Carbonylation by SDS-PAGE and Western Blot.* Plasma proteins were fractionated on 12.5% (*w/v*) reducing SDS-PAGE gels and electroblotted onto a polyvinylidene difluoride (PVDF) membrane. Protein carbonylation was detected, after derivatization with DNPH, with anti-DNP antibodies specific for the 2,4-dinitrophenyl hydrazone-carbonyl adduct by Western blot immunoassay as previously reported [34, 35]. Immunoreactive protein bands were visualized by enhanced chemiluminescence (ECL). Protein bands on PVDF membranes were then visualized by washing the blots extensively in PBS and then staining with Ponceau Red.

*2.5. Determination of Plasma Protein Carbonyls by Enzyme-Linked Immunosorbent Assay (ELISA).* Plasma PCO were measured using the ELISA kit manufactured by Enzo Life Sciences (ALX-850-312-KI01). Carbonylated protein standard (40 mg/ml containing 0-0.12-0.22-0.42-0.7-0.9 nmol carbonyls/mg protein) and human plasma samples (60–75 mg/ml) were diluted 1 : 40 in DNPH solution and incubated 45 min to allow PCO derivatization. A 1 : 200 dilution in ELISA buffer was then performed before adding 200 $\mu$L (1-2 $\mu$g of protein) in each ELISA plate well. We incubated ELISA plate overnight at 4°C to allow protein binding. ELISA assay was performed according to the manufacturer's instructions. Absorbance of plate wells was read at 450 nm using the Plate Reader TECAN Infinite® 200 PRO. In all the performed assays, calibration line showed an $R^2$ close to 0.99. We then calculated carbonyl content of samples by using the regression factors (intercept with the $y$-axis and line slope) obtained from standard curve.

*2.6. Determination of Clinical Laboratory Parameters.* Creatinine, C-reactive protein, white blood cell count, albumin, fibrinogen, haemoglobin, ferritin, total iron-binding capacity, urea, sodium, potassium, calcium, and phosphorus were measured *by* standardized methods at the clinical laboratory of the Humanitas Clinical and Research Center [12, 16].

*2.7. Statistical Analysis.* The paired Student's $t$-test was used to test whether differences in plasma PCO level in ESRD patients before (pre-HD) and after (post-HD) a single HD session were significant. The paired Student's $t$-test was also used to test for differences in plasma PCO levels before and after a single HD session by dividing the haemodialysed

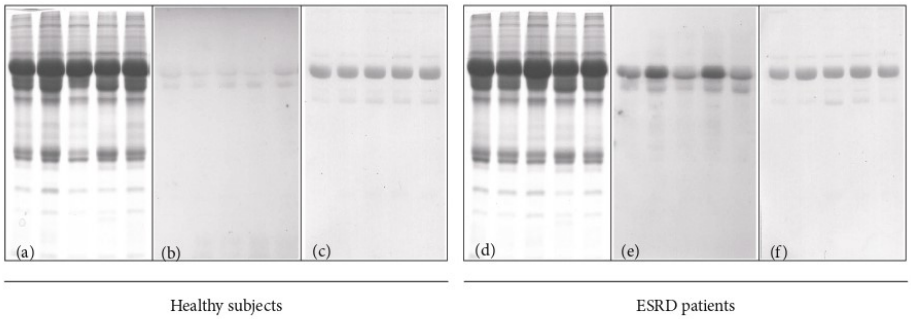


Figure 1: Plasma protein carbonylation. Representative SDS-PAGE (a and d) and Western blot with anti-DNP antibody developed with ECL (b and e) of plasma proteins in age-matched voluntary healthy subjects (a and b) and in five representative ESRD patients on maintenance HD (d and e). Visualization of proteins in PVDF membrane with Ponceau Red staining (c and f).

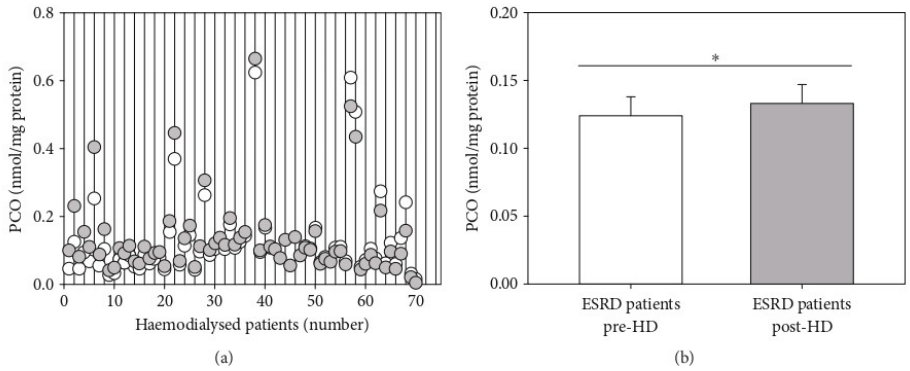


Figure 2: Effect of a single HD session on the level of plasma PCO in ESRD patients on maintenance HD. (a) Scatter diagram showing plasma PCO level in individual haemodialyzed patients immediately before (white circles) and after (grey circles) a single HD session. (b) Histograms showing the mean plasma PCO level in MHD patients immediately before (pre-HD) and after (post-HD) a single HD session. Data are expressed as the mean ± SE. $^{*}p < 0.05$.

patients depending on the dialysis filter used, the cooccurrence of type 2 diabetes mellitus, and the gender. All the values are expressed as mean and standard errors (SE). A $p$ value <0.05 was considered to be significant. The statistical significances are marked as $^{*} = p < 0.05$ and $^{**} = p < 0.01$. The relationship between pre-HD and post-HD PCO levels was investigated by simple linear regression analysis.

## 3. Results

PCO are considered the most general and the most commonly used biomarkers of severe oxidative protein damage. The results of protein carbonylation assessed by Western blotting using anti-DNP antibodies from five ESRD patients and five age-matched voluntary healthy donors are presented in Figure 1. Plasma proteins from healthy subjects showed a very low level of carbonyl content (Figure 1(b)), whereas plasma proteins from ESRD patients clearly exhibited an increase in carbonyl content (Figure 1(e)). We applied reversible Ponceau Red staining to assess equal loading of gels (Figures 1(c) and 1(f)).

We also determined the effect of HD on the plasma PCO levels, measured by a sensitive ELISA method [36, 37], in each individual ESRD patient at the beginning and at the end of the HD session. In this regard, it is important to note that we had previously shown that the total plasma protein concentration in ESRD patients increases significantly after the HD session due to net volume ultrafiltration [12]. Therefore, in this study, plasma PCO are expressed as nmol/mg protein. Scatter diagram of plasma PCO levels in haemodialyzed patients is shown in Figure 2(a). In most ESRD patients, we observed a small increase in the plasma PCO level after the HD procedure compared to the pre-HD value. Differently, some ESRD patients showed the same or a slightly lower plasma PCO level immediately after the HD session compared to the pre-HD value. The result of the paired Student's $t$-test applied to the mean value of plasma PCO level measured in ESRD patients pre-HD (mean $0.1239 \pm 0.0140$ nmol/mg protein) and post-HD (mean

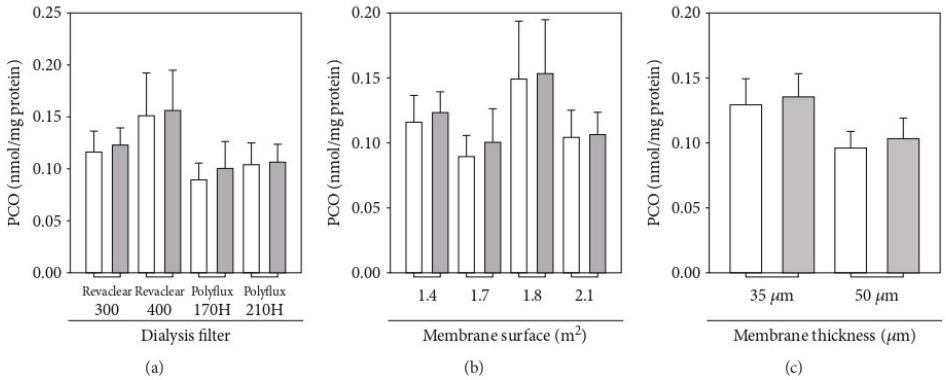


FIGURE 3: Influence of the dialysis filter type on the level of plasma PCO in ESRD patients on maintenance HD. Haemodialyzed patients ($n = 62$) were divided into subgroups based on the characteristics of the dialysis filters used during the HD session: (a) filter type (i.e., Revaclear™ 300, Revaclear 400, Polyflux™ 170 H, and Polyflux 210 H), (b) membrane surface area, and (c) membrane thickness. In all panels, histograms show the plasma PCO level in haemodialysed patients immediately before (pre-HD, white bars) and after (post-HD, grey bars) a single HD session. For filter details, see the Materials and Methods section. The seven remaining ESRD patients, who were dialyzed with Filtryzer 1.6, Nephral™ ST 400, or Nephral ST 500 filters, were not included because their number is too low to make a reliable statistical analysis. Data are expressed as the mean ± SE.

$0.1332 \pm 0.0140$ nmol/mg protein) proved that the means are significantly different ($p < 0.05$) (Figure 2(b)). There were no differences between patients showing an increase as compared to those showing no change or a slight decrease in plasma carbonyl levels after dialysis. In particular, we did not notice any difference in chronological age, HD vintage, body mass index, WBC count and concentration of C-reactive protein, albumin, fibrinogen, haemoglobin, urea, creatinine, sodium, potassium, calcium, phosphorus, and ferritin (not shown).

In HD, patient's blood is allowed to flow through a filter (the haemodialyser), whereby waste products and excess water are removed across a semipermeable membrane separating flowing blood from the dialysate stream. The clean blood is then returned to the haemodialysed patient's body, while wastes are discharged. The main determinant of the quality of HD therapy is represented by the artificial membrane packed into the haemodialyser. The HD therapy per se, in particular the type of dialysis membrane, contributes to the increased production of ROS in ESRD patients [38]. Indeed, typically, an HD patient's blood is in contact with the synthetic HD membrane for a ~3.5 to 4 h/session and three sessions/a week. This prolonged contact of blood with the synthetic polymer surface results in two long-term complications, namely membrane-induced oxidative stress and membrane-induced inflammation, both of which contribute to CVD development [39]. Therefore, the dialysis filter may have a potential relevant impact on plasma PCO levels post-HD. Sixty-two out of 69 ESRD patients recruited in the study were dialyzed with filters made (mainly) of PAES + PVP that differ in extension and thickness (see Materials and Methods). Therefore, we measured the plasma PCO level immediately before and after the HD session by subdividing those 62 ESRD patients based on the characteristics of the filter membrane or the dialysis technique (diffusive versus diffusive plus convective) used during the HD session. As shown in Figures 3 and 4, the comparison of filter type (Figure 3(a)), membrane surface area (Figure 3(b)), and membrane thickness (Figure 3(c)) as well as dialysis technique (Figure 4) did not reveal any statistically significant difference in plasma PCO levels before (pre-HD) and after (post-HD) the HD session.

Considering that diabetes mellitus occurs as an important comorbidity in the ESRD population (often composed predominantly of subjects in advanced age) and that some studies suggest that oxidative stress in diabetic patients leads to increased plasma PCO levels [40, 41], we hypothesized that diabetic ESRD patients could experience a significant increased oxidative stress in comparison with nondiabetic ESRD patients. Therefore, we evaluated the plasma PCO levels immediately before and after a single HD session by subdividing all ESRD patients into diabetics ($n = 22$; mean age: $71.6 \pm 2.1$ yrs; HD vintage: $5.1 \pm 0.7$ yrs) and nondiabetics ($n = 47$; mean age: $67.8 \pm 2.0$ yrs; HD vintage: $6.1 \pm 0.6$ years) (Figure 5). Data were analysed according to a paired sample $t$-test used to compare means of pre- and post-HD plasma PCO level in each of the two groups of haemodialyzed patients. The results proved that the means pre-HD and post-HD are significantly different in nondiabetics ($0.1172 \pm 0.0166$ nmol/mg protein and $0.1268 \pm 0.0172$ nmol/mg protein, resp., $p < 0.05$), whereas they are not significantly different in diabetics ($0.1382 \pm 0.0264$ nmol/mg protein and $0.1467 \pm 0.0244$ nmol/mg protein, resp.) (Figure 5(a)); in addition, differences in both pre-HD and post-HD plasma PCO levels between diabetics and nondiabetics are not statistically significant ($t$-test for independent samples). Pre-HD plasma PCO levels were significantly positively correlated with post-HD plasma PCO concentrations both in nondiabetic ($r = 0.9766$, $p < 0.0001$) (Figure 5(b)) and diabetic ($r = 0.9033$, $p < 0.0001$) (Figure 5(c)) ESRD patients.

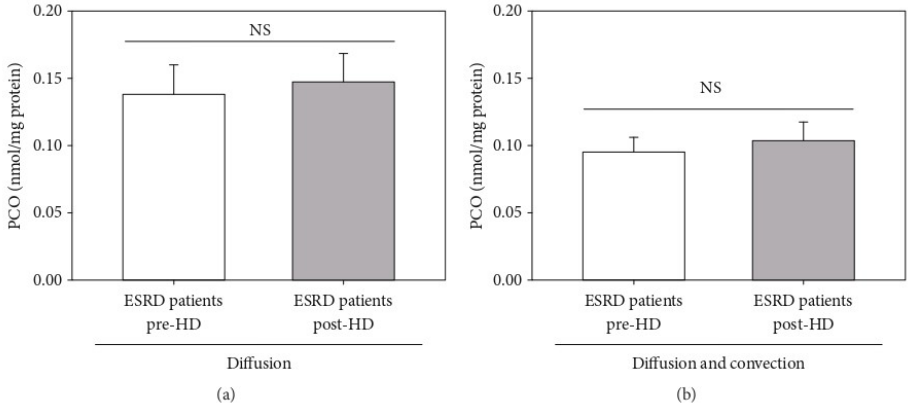


Figure 4: Influence of the dialysis technique (diffusive versus diffusive plus convective) on the level of plasma PCO in ESRD patients on maintenance HD. Haemodialyzed patients were divided into subgroups based on the dialysis technique used during the HD session: (a) diffusive technique (standard bicarbonate haemodialysis) ($n = 41$), (b) diffusive plus convective techniques (online HDF) and acetate-free biofiltration ($n = 28$). In both panels, histograms show the plasma PCO level in haemodialysed patients immediately before (pre-HD, white bars) and after (post-HD, grey bars) a single HD session. Data are expressed as the mean ± SE. In panel (a), $p = 0.0880$; in panel (b), $p = 0.2188$.

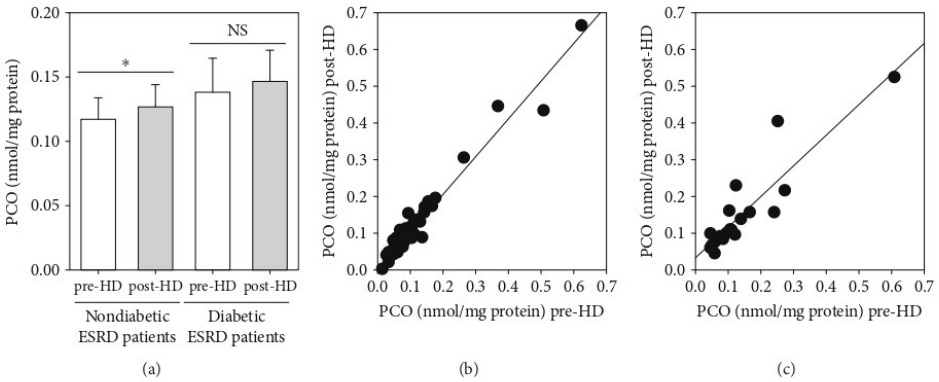


Figure 5: Plasma PCO levels measured in nondiabetic and diabetic ESRD patients on maintenance HD. (a) Histogram showing the plasma PCO level in nondiabetic and diabetic haemodialyzed patients measured immediately before (pre-HD, white bars) and after (post-HD, grey bars) a single HD session. Data are expressed as the mean ± SE. (b) Regression analysis showing the relation between pre-HD and post-HD plasma PCO levels in nondiabetic haemodialyzed patients. Slope of the line is $a = 1.0154$. (c) Regression analysis showing the relation between pre-HD and post-HD plasma PCO levels in diabetic haemodialyzed patients. Slope of the line is $a = 0.8349$. $^*p < 0.05$.

We also evaluated the plasma PCO levels immediately before and after a single HD session by separating ESRD patients on HD by gender (males: $n = 45$, mean age 70.1 ± 2.4 yrs, HD vintage 5.6 ± 0.6 yrs; females: $n = 24$, mean age 66.9 ± 3.1 yrs; HD vintage 6.1 ± 0.8 yrs) (Figure 6). Data were analysed according to a paired sample $t$-test used to compare means of pre- and post-HD plasma PCO level in each of the two groups of haemodialysed patients. The results proved that the means pre-HD and post-HD are not significantly different in males (0.1180 ± 0.0163 nmol/mg protein and 0.1187 ± 0.0134 nmol/mg protein, resp.), whereas they are significantly different in females (0.1348 ± 0.0267 nmol/mg protein and 0.1604 ± 0.0313 nmol/mg protein, resp., $p < 0.01$) (Figure 6(a)); in addition, differences in both pre-HD and post-HD plasma PCO levels between men and women are not statistically significant (unpaired $t$-test). Pre-HD plasma PCO levels were significantly positively correlated with post-HD plasma PCO concentrations both in male ($r = 0.9730$, $p < 0.0001$) (Figure 6(b)) and female ($r = 0.9702$, $p < 0.0001$) (Figure 5(c)) ESRD patients.

## 4. Discussion

Protein carbonylation, which may result from direct oxidation of lysine, arginine, proline, and threonine residues and interaction with reactive carbonyl species produced from

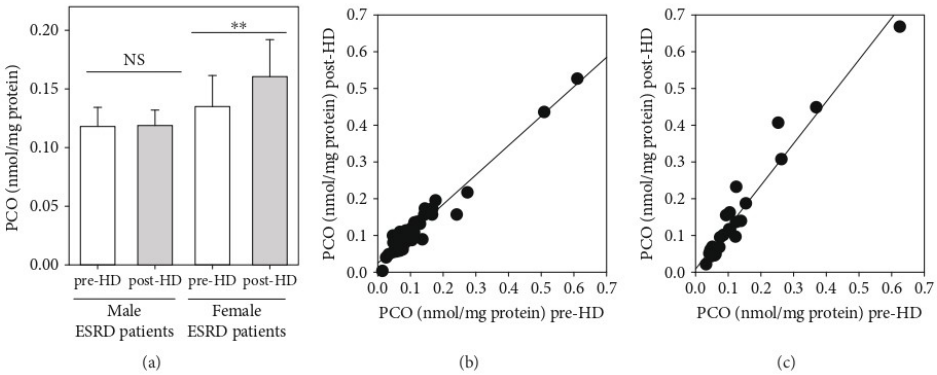


FIGURE 6: Plasma PCO levels evaluated in male and female ESRD patients on maintenance HD. (a) Histogram showing the plasma PCO levels in male and female ESRD patients measured immediately before (pre-HD, white bars) and after (post-HD, grey bars) a single HD session. Data are expressed as the mean ± SE. (b) Regression analysis showing the relation between pre-HD plasma PCO levels and post-HD plasma PCO levels in male ESRD patients. Slope of the line is $a = 0.7993$ (c) Regression analysis showing the relation between pre-HD plasma PCO levels and post-HD plasma PCO levels in female ESRD patients. Slope of the line is $a = 1.1383$. $^{**}p < 0.01$.

carbohydrate and lipid oxidation or non-oxidative reactions with dicarbonyl compounds, is an indicator of oxidative protein damage [42]. Its use as a biomarker of oxidative stress has some advantages because of the stability of PCO in comparison with other oxidation products. We showed that plasma proteins of ESRD patients on HD exhibited an increase in carbonyl content compared to plasma proteins of the controls, which was especially evident in albumin (Figure 1). These results are in agreement with a previous study, which showed that post-HD plasma PCO levels are significantly increased compared to pre-HD levels and that carbonylation affects almost a dozen of plasma proteins, among which albumin is the most susceptible to carbonyl formation [24]. In this regard, it is interesting to note that following carbonylation, albumin vasculoprotective effects in haemodialysed patients are impaired [43] and, therefore, carbonylated albumin may play a role in the early atherogenic events of chronic uraemia by directly damaging the endothelium [24].

The increased levels of PCO measured in haemodialysed patients suggest that protein carbonylation may be an important biomarker of oxidative stress in ESRD patients as well. A number of studies compared biomarkers of oxidative stress between ESRD patients and age-matched healthy subjects [19, 28, 44] or between CKD patients with different CKD stages [45–47]. Here, we compared plasma levels of PCO of each patient before and after a single HD session. We found that mean post-HD levels of plasma PCO are significantly higher than mean plasma PCO levels before the HD session (Figure 2). These results are in agreement with previous ones showing that the levels of plasma PCO were significantly higher at the end of a single HD session than before it [17–19, 24, 25, 48], whereas they differ from those of a unique study that did not report any increase in plasma PCO level after HD [49] (Table 2).

About the possible influence of the dialysis membrane on PCO levels, we have considered that during the HD session, the contact of blood with the dialysis membrane and the loss of antioxidants may promote oxidative stress. In a study conducted on 15 nondiabetic ESRD patients (9 males and 6 females), PCO levels were found to be significantly increased after a HD session with a cuprophane membrane [50] and increased, but not significantly, after a HD session with a polysulfone membrane [50]. This data is intuitive, as hydrophilic cuprophane membranes are known to severely activate complement and leukocytes [51]. In addition, proteomic investigations suggest that dialysis membranes may retain, at least in part, plasma proteins, especially carbonylated ones [52, 53]. This would occur mainly via the protein adsorptive properties of the membrane material [54], since the main mechanisms for solute removal during HD, diffusion, and convection have poor ability to remove high molecular weight solutes, such as proteins. Most of haemodialysed patients recruited into the study were dialyzed using membranes made of PAES and PVP ± PA that differ in surface and thickness (see Materials and Methods). In this regard, neither dialysis membrane composition, surface, and thickness (Figure 3) nor dialysis technique (Figure 4) significantly affected pre-HD and post-HD levels of plasma PCO. Therefore, the membrane surface area and thickness of the dialysis filter as well as the dialysis technique do not significantly affect the observed increase in PCO during the HD session. It is interesting to note that some evidence suggests a progressive significant decrease in concentration of inflammatory biomarkers [55] and advanced oxidation protein products [56] when vitamin E-coated polysulfone membranes are used for the dialysis sessions. This might suggest a protective effect of vitamin E-coated polysulfone membrane against inflammation and oxidative stress in haemodialysed patients.

It is well known that oxidative stress is increased in diabetes mellitus. In particular, some studies have shown that plasma PCO levels are increased in diabetics [40, 41]. Unfortunately, the combination of CKD and diabetes is associated

Table 2: Studies that examined the plasma PCO levels in haemodialysed patients before haemodialysis (pre-HD) and after haemodialysis (post-HD).

| Study | HD group number (age and sex) and dialysis vintage | Control group number (age and sex) | PCO HD group | PCO control group |
|---|---|---|---|---|
| Ward et al. [17] | 22 HD patients (age 51 ± 5 years, 8 M and 4 F). Divided into two groups: 11 patients treated with polysulfone membrane 11 patients treated with cellulose triacetate membrane Dialysis vintage 49 ± 11 months | 17 healthy subjects (age range 23–54 years, both M and F) | Polysulfone membrane pre-HD 0.144 ± 0.037 mmol/mg protein post-HD 0.175 ± 0.029 mmol/mg protein $p < 0.05$ Cellulose triacetate membrane pre-HD 0.145 ± 0.030 mmol/mg protein post-HD 0.178 ± 0.035 mmol/mg protein $p < 0.05$ | 0.041 ± 0.008 mmol/mg protein |
| Dursun et al. [48] | 20 HD patients (age and sex unspecified) Dialysis vintage unspecified | 20 healthy subjects (age and sex unspecified) | Pre-HD 0.889 ± 0.063 nmol/mg protein post-HD 0.997 ± 0.066 nmol/mg protein $p < 0.05$ | 0.417 ± 0.036 nmol/mg protein |
| Pieniazek et al. [18] | 10 HD patients (mean age 58 ± 11 years, sex unspecified) Dialysis vintage unspecified | 9 healthy subjects (age 46 ± 15 years, sex unspecified) | Pre-HD 2.27 ± 0.2 mmol/l post-HD 2.94 ± 0.12 mmol/l $p < 0.0002$ | 0.67 ± 0.07 mmol/l |
| Terawaki et al. [49] | 83 anuric HD patients divided into two groups: patients with CVD ($n = 66$, age 63.5 ± 12.5 years, 32 M and 34 F) Dialysis vintage 85.0 ± 64.6 months patients without CVD ($n = 20$, age 74.3 ± 12.8 years, 11 M and 9 F) Dialysis vintage 58.3 ± 33.3 months | — | patients with CVD pre-HD 0.81 ± 0.16 nmol/mg protein post-HD 0.53 ± 0.13 nmol/mg protein patients without CVD pre-HD 0.82 ± 0.17 nmol/mg protein post-HD 0.58 ± 0.16 nmol/mg protein | — |
| Albarello et al. [25] | 23 HD patients (9 men and 14 women, mean age 50.8 ± 17.3 years) Dialysis vintage unspecified | — | Pre-HD 0.62 ± 0.14 nmol/mg protein post-HD 0.86 ± 0.16 nmol/mg protein $p < 0.001$ | — |
| Caimi et al. [19] | 31 HD patients (61.5 ± 12.8 years, 16 men and 15 women) Dialysis vintage 48.5 ± 35.7 months | 26 healthy subjects (age 43.54 ± 6.92 years, 17 M and 9 F) | Pre-HD 0.62 ± 0.14 nmol/mg protein post-HD 0.86 ± 0.16 nmol/mg protein $p < 0.01$ | 0.440 ± 0.134 nmol/mg protein |

with increased morbidity and mortality, mainly due to increased cardiovascular risk [57]. About one-third (32%) of the haemodialysed patients recruited in our study are diabetics. Hence, we compared plasma PCO levels before and after a single HD session in diabetic and nondiabetic ESRD patients (Figure 5). Unexpectedly, pre-HD PCO levels are not significantly different in nondiabetic and diabetic ESRD patients nor are the post-HD ones. Nevertheless, the mean value of plasma PCO levels increases slightly but significantly in nondiabetic ESRD patients after the HD session. Differently, the mean values of pre-HD and post-HD plasma PCO levels are not significantly different in diabetic ESRD patients. These findings could suggest that the HD session induces a moderate increase in oxidative/carbonyl stress. Therefore, nondiabetic ESRD patients seem to be more susceptible to oxidative stress induced by the HD session. These results are only partially consistent with those described by Dursun and colleagues [58], who determined the levels of several biomarkers of oxidative stress in 20 nondiabetic ESRD patients (9 males and 11 females) and 20 diabetic ESRD patients (9 males and 11 females) before and after HD. They concluded that both diabetes and HD increase

oxidative stress and that their combined effect on oxidative stress is greatest in diabetic ESRD patients. However, a limitation of their study was the very small number of subjects.

In contrast to what is observed in the general population, where females have a longer life expectancy than males [59], female ESRD patients have as poor survival as male ESRD patients [60, 61]. These observations are somewhat surprising considering that haemodialysed women have a lower prevalence of CVD [62] are less likely to develop left-ventricular hypertrophy [63] and are less predisposed to cardiovascular calcification [64]. It has been suggested that noncardiovascular mortality is the main explanation for the loss of the survival advantage in ESRD women on HD [62]. Moreover, the HD procedure too may contribute to cancel out the survival advantage in ESRD women [60]. So, we compared plasma PCO levels before and after a single HD session in male and female ESRD patients (Figure 6). In men, the mean values of pre-HD and post-HD plasma PCO levels are not significantly different, whereas in women, the mean value of post-HD plasma PCO level is significantly higher than that of pre-HD. These novel findings suggest that haemodialysed women seem to be more susceptible to oxidative stress induced by the HD session.

In summary, all these results suggest that (i) the HD session increases plasma protein carbonylation; therefore, although important advances have been done in the field of dialysis biocompatibility, the HD session probably still represents a source of oxidative stress; (ii) plasma PCO level measurement may become an indicator of oxidative/carbonyl stress in ESRD and could be included in the routine monitoring of haemodialysed patients, since standard uraemia and inflammation biomarkers may not be sufficient on their own to describe the inflammatory/oxidative state of ESRD patients on HD; and (iii) haemodialysed women seem to be more susceptible to oxidative/carbonyl stress induced by the HD session than men. On the basis of these findings, it seems appropriate to suggest that the female sex could be considered a fundamental biologic variable (or a "risk factor") associated with HD-induced plasma protein carbonylation in ESRD patients on maintenance HD. Therefore, this study shows that gender differences exist in plasma PCO levels of haemodialysed patients before and after a single HD session and highlights the critical importance of reporting of sex information in study description, data analyses, results, and their interpretation in basic science and medical/clinical research studies concerning ESRD. As a matter of fact, the prevalence of CKD stages 1–5 among US adults aged 18 years or older is higher in women than men (16% versus 13%); however, men are 64% more likely than women to develop ESRD [65], yet most studies of ESRD group together men and women and assume any underlying pathophysiology is the same. So, this study can contribute, in its own small way, to increase our understanding of the gender differences of diseases, in particular in the field of nephrology, where some gender differences have been documented [66]. Indeed, women seem to be somewhat protected from developing ESRD [67]. In addition, the cumulative incidence of ESRD is low during the reproductive ages and begins to rise ten years later in women than in men among participants in community-based screenings. Moreover, the mean age at the start of HD is also higher in women than in men [67].

Finally, our study has some limitations. Firstly, it includes a relatively small number of ESRD male and female patients and has been performed in only one single HD center. Secondly, the majority of the patients studied in this cohort are from Italy and of Caucasian race, and the applicability of the study findings across nationalities and races remains unclear. However, we hope that these results stimulate further research with a larger number of men and women on HD recruited from different dialysis centers, possibly of different nationalities and races, to advance our understanding of the pathophysiology of sex (and possibly of nationality and/or race) differences in CKD and improve clinical care of women with CKD. While some advances have been made in both clinical and basic research, much remains poorly understood, both at the molecular and clinical levels.

## 5. Conclusions

Post-HD plasma PCO level increases in nondiabetic but not in diabetic ESRD patients, more markedly in women than in men. Women with ESRD are more susceptible than men to HD-induced oxidative/carbonyl stress.

## Abbreviations

CKD: Chronic kidney disease
CVD: Cardiovascular disease
DNPH: 2,4-Dinitrophenylhydrazine
ELISA: Enzyme-linked immunosorbent assay
ESRD: End stage renal disease
HD: Haemodialysis
PCO: Protein carbonyls
ROS: Reactive oxygen species.

## Data Availability

The authors are available to share their data.

## Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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# 5. CHAPTER 2

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## Advanced oxidation protein products in nondiabetic end stage renal disease patients on maintenance haemodialysis

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ORIGINAL ARTICLE



# Advanced oxidation protein products in nondiabetic end stage renal disease patients on maintenance haemodialysis

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**ABSTRACT**

In chronic kidney disease (CKD), the impairment of the excretory function leads to elevation in the blood concentrations of urea, creatinine, and various protein metabolic products. Advanced oxidation protein products (AOPP), along with protein carbonyls, protein-bound di-tyrosines and *S*-thiolated proteins, are considered biomarkers of oxidative stress in end-stage renal disease (ESRD) patients on maintenance haemodialysis (HD). In this study, we evaluated the correlations between plasma levels of AOPP (measured by size exclusion/gel filtration high performance liquid chromatography) and those of protein-bound di-tyrosines, protein carbonyls, albumin and fibrinogen in 50 nondiabetic ESRD patients on maintenance HD. Considering that AOPP could represent the bridge between oxidative stress and inflammation, having been identified as proinflammatory mediators, we also evaluated the association between AOPP levels, C-reactive protein concentration and white blood cells count. Finally, we assessed the associations between plasma level of AOPP and serum concentrations of creatinine and urea, both of which showed a strong dependence on the chronological age of haemodialysed patients. Taken together, our results confirm the robust relationship between uraemia and oxidative stress, especially when measured as biomarkers of severe protein oxidative damage (e.g. plasma AOPP).

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Advanced oxidation protein products; albumin; creatinine; fibrinogen; haemodialysis vintage; protein carbonyls

## Introduction

Over the past years, many studies focussed on oxidative stress and its role in end-stage renal disease (ESRD) patients on haemodialysis (HD). Oxidative stress in haemodialysed patients may result from uraemia per se [1], but there are other many potential sources of oxidative stress in these patients. HD procedure is an exacerbating factor for oxidative stress, mainly attributed to the activation of neutrophils, provoking chronic inflammation with release of reactive oxygen species (ROS). Chronic inflammation is not confined to the kidneys, rather it is systemic, and it contributes to oxidative stress in HD patients [2,3]. Also intravenous iron administration, an integral part of anaemia management in ESRD, is among the factors associated with the increase in oxidative stress [4].

Some plasma biomarkers of oxidative stress, such as protein carbonyls [5–8], protein-bound di-tyrosines [9], and *S*-thiolated proteins, are elevated in haemodialysed patients [10,11]. Plasma advanced oxidation protein products (AOPP) are the most used biomarker of oxidative stress in ESRD patients on HD [12–16]. Interestingly, a recent study identified a novel panel of six biomarkers, which include AOPP and can be utilised in predicting overall and cardiovascular survival in ESRD patients [16]. AOPP are generated by both myeloperoxidase (MPO)-dependent and MPO-independent mechanisms during oxidative stress [12,17]. MPO/$H_2O_2$/chloride system of activated neutrophils and the consequent generation of hypochlorous acid (HOCl) represents the major pathway for AOPP production, as suggested by *in vitro* incubation of human plasma with HOCl [12,18,19] and by positive correlation between plasma AOPP levels and plasma MPO activity in haemodialysed patients [20]. The formation of AOPP is irreversible: they cannot be easily hydrolysed by proteases or reduced by antioxidants [21] and are mostly eliminated by the liver and spleen [22].

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Supplemental data for this article can be accessed here.

As oxidative stress and chronic inflammation are common findings in ESRD and they increase over time in patients on maintenance HD [2,3], it has been suggested a possible association between them [23]. It is worth noting that AOPP are not only biomarkers of oxidative stress but could also be a bridge between oxidative stress and inflammation. Indeed, AOPP are proinflammatory mediators because they can trigger oxidative bursts in neutrophils and monocytes and promote the synthesis of inflammatory cytokines, such as IL-1, IL-6, and TNF-α [12,24]. AOPP are also involved in vascular endothelial cell dysfunctions [25] and AOPP levels independently predict endothelial function level in ESRD patients on peritoneal dialysis [26]. Moreover, AOPP directly impair the metabolism of high-density lipoproteins (HDL), being potent HDL receptor antagonists and, therefore, might be directly involved in the development of cardiovascular diseases [27]. A study that compared the serum levels of AOPP in patients on HD and patients on continuous ambulatory peritoneal dialysis concluded that accumulation of AOPP was more significant in the former and that the level of AOPP was independently associated with ischaemic heart disease only in haemodialysed patients [28].

In the present study, we have measured plasma AOPP level by size exclusion-HPLC separation both in healthy subjects and in ESRD patients on maintenance HD. The aim of the study was twofold: on the one hand, we have compared plasma AOPP abundance in the control and HD group, while, on the other hand, we have looked for possible correlations between AOPP and other biomarkers of oxidative stress, such as protein carbonyls or protein-bound di-tyrosines, or between AOPP and clinical parameters.

## Materials and methods

### Reagents

Formic acid (FA), trifluoroacetic acid (TFA), acetonitrile (ACN) were LC-MS grade and purchased from Sigma-Aldrich (Milan, Italy). Threo-1,4-dimercapto-2,3-butanediol (DTT) and iodoacetamide (IAA) were supplied by Bio-Rad Laboratories, Inc (Hercules, CA). Trypsin was purchased from Roche Diagnostics SpA (Monza, Italy).

Digestion buffer was 50 mM ammonium bicarbonate; destaining solution was prepared mixing pure acetonitrile with digestion buffer in a molar ratio 1:1; reducing solution was 10 mM DTT in digestion buffer; alkylating solution was 55 mM iodoacetamide in digestion buffer; extraction solution (3% TFA/30% ACN in $H_2O$ MilliQ, Burlington, MA).

### Study participants

This was an observational study conducted on 50 ESRD patients on HD recruited at the Nephrology Unit of the Humanitas Clinical and Research Centre. The presence of diabetes mellitus (which can be independently related to increased AOPP levels) or a clinically overt infectious process were the only exclusion criteria. For every patient an anamnestic record was collected. A deidentification of the samples was performed before any additional data processing. Control blood samples were collected from 25 age-matched voluntary healthy donors (13 males and 12 females) after obtaining informed verbal consent. Criteria included no known history of CKD or other diseases that could influence the analysis. In particular, healthy subjects were tested for serum creatinine in order to exclude CKD.

### Sample collection

Venous blood samples were collected before (10 mL) and after (5 mL) HD session. All samples were collected on the long interdialytic interval, i.e. 2 days apart from the previous HD session. Blood was withdrawn from the arteriovenous fistula or central venous catheter, using $K_3$EDTA as anticoagulant. All the samples were processed within the first hour from blood withdrawal through centrifugation for 10 min at 1000 *g*. AOPP remain stable during sample storage both at −20 °C and −80 °C for about 6 months [29]; therefore, such aliquots were stored at −80 °C until the execution of the assays.

### Determination of clinical laboratory parameters

Creatinine, urea, C-reactive protein, white blood cells (WBC) count, albumin, fibrinogen, haemoglobin, ferritin, total iron-binding capacity, urea, sodium, potassium, calcium, and phosphorus were measured in plasma by standardised methods at the clinical laboratory of the Humanitas Clinical and Research Centre. It should be noted that urea concentration was directly measured in serum, whereas, in others studies, serum urea concentration indistinctly referred to blood urea nitrogen (BUN) or serum urea nitrogen (SUN) and it is expressed as the mass concentration of nitrogen equivalents. The conversion between different units is the following: BUN (mg/dL)=0.47 × [urea in mg/dL] = 2.8 × [urea in mmol/L] [30]. Body mass index (BMI) was calculated from the formula BMI = weight/height$^2$ (kg/m$^2$).

### Plasma protein fractioning

Plasma proteins from healthy subjects and haemodialysed patients were fractionated by size exclusion/gel filtration high performance liquid chromatography (HPLC) on a BioSep-SEC-S4000 (BioSep-SEC-S4000 Phenomenex, Torrance, CA) column (300 × 7.8 mm) with a guard column (SecurityGuard™ GFC-4000, 4-mm length × 3 mm ID) and UV–vis detector. Plasma samples were diluted 1:15 in 50 mM Tris-HCl, pH 7.4 and 20 μL were loaded into the column for each sample. The mobile phase consisted of Milli-Q water, containing 0.5% (w/v) SDS and was eluted at 1 mL/min.

### Determination of plasma AOPP and protein-bound di-Tyr

Eluates were monitored both at 340 nm for measuring AOPP absorbance and at 215 nm for measuring the absorbance of peptide bonds using HPLC detector 332 from Kontron Instrument SpA (Milan, Italy). The ratio $A_{340}/A_{215}$ was calculated for each plasma sample. For protein-bound di-Tyr determination, eluates were monitored both at 215 nm for measuring absorbance of peptide bonds and at 415 nm emission with 325 nm excitation using Kontron SFM-25 Spectrofluorimeter equipped with 150 W Xenon N-800-LO, ozone-free, lamp. The ratio between fluorescence and absorbance ($IF_{415nm\ em}/A_{215nm}$) was calculated for each sample.

### Determination of plasma protein carbonyls

The covalent reaction of the protein carbonyl group (PCO) with 2,4-dinitrophenylhydrazine (DNPH) leads to the formation of a stable 2,4-dinitrophenyl hydrazone product, which can be detected spectrophotometrically at 370 nm [31] enabling an accurate quantification of PCO. In this study, we used a commercial assay, i.e. the enzyme-linked immunosorbent assay (ELISA) kit by Enzo Life Sciences (Farmingdale, NY) (ALX-850-312-KI01), according to the manufacturer's instructions in order to guarantee less variability in preparation of reagents and standards, generating more accurate and precise results.

### Statistical analysis

Unpaired Student's *t*-test was used to evaluate differences in AOPP values involving age-matched healthy subjects vs. haemodialysed patients. Linear regression analyses, using Pearson's rank correlation coefficients (*r*) and significance, were performed to assess associations between creatinine or urea levels and chronological age or HD vintage. Linear regression analyses were also performed to assess associations between AOPP, protein-bound di-tyrosines and protein carbonyl levels and between AOPP, albumin and fibrinogen levels. Linear regression analyses were also performed to assess associations between AOPP, C-reactive protein and WBC levels and between AOPP, creatinine, and urea levels. In all cases, a $p$ value $<0.05$ was taken to represent a statistically significant difference.

### MS Sample Preparation: in gel tryptic digestion

Target protein bands were cut using a scalpel, transferred in a new Eppendorf and processed by the canonical in-gel digestion experimental protocol starting from the removal of protein stain. The destaining of gels was followed by the reduction and alkylation of the cysteine residues in the proteins. Hereby, the disulphide bonds of the proteins are irreversibly broken up and subsequently irreversible alkylated by iodoacetamide. The reduction was accomplished by adding 150 μL of reducing solution followed by 1 h of incubation at 56 °C in Thermomixer (500 RPM); similarly, the alkylation was achieved by adding 150 μL of alkylating solution and incubating samples for 45 min at room temperature, in the dark. Once discharged the reagent solutions, samples were washed in digestion buffer and 0.5 μg of proteolytic enzyme (trypsin in digestion buffer) was added. Samples were incubated overnight at 37 °C in Thermomixer (500 RPM) and the day after the tryptic mixtures were acidified with formic acid up to a final concentration of 1%. At the end of digestion, the peptides generated during this process were extracted from the gel mashes; this was accomplished by several extraction steps: 100 μL of extraction solution was added twice to the gel portions incubated in the Thermomixer for 10 min at 37 °C at high speed shaking (1400 RPM); this step was followed by two similar steps by adding ACN. The four extracted fractions were then collected, mixed, dried in Speed Vac at 37 °C and finally stored at −20 °C until the MS analysis.

MS analysis was performed as described in Supplementary Materials.

## Results

We studied 50 ESRD nondiabetic patients on maintenance HD (34 males and 16 females). The mean chronological age was 69.94 ± 13.76 years (ranged from 39 to 92 years), the haemodialysis vintage (length of time on dialysis) was 7.60 ± 3.98 (ranged from 1 to 21 years) and the mean BMI was 22.72 ± 2.82 kg/m$^2$. Considering the

criteria established by the World Health Organisation (WHO) for the classification of nutritional status according to the BMI [32], three patients showed malnutrition (BMI $<18.5\,kg/m^2$), one patient was obese (BMI $\geq 30.0\,kg/m^2$) and 11 patients presented overweight (BMI 25.0–29.9 $kg/m^2$). The main clinical and analytical data of the studied cohort of ESRD patients on HD are shown in Table 1. Serum creatinine concentration before HD session ranged from 4.15 to 19.52 mg/dL (mean 9.49 mg/dL), while serum urea concentration before HD session ranged from 74.00 to 243.00 mg/dL (mean 148.42 mg/dL). As expected, creatinine levels in haemodialysed patients were negatively correlated with chronological age ($r = 0.536$, $p < 0.0001$) (Figure 1(A)); also the concentration of urea was negatively correlated with chronological age ($r = 0.484$, $p = 0.0003$) (Figure 1(B)).

In ESRD patients on HD, AOPP are one of the most relevant biomarker of oxidative stress, reflecting severe oxidative protein damage. We have measured AOPP in the blood plasma of healthy subjects and haemodialysed patients by size exclusion-HPLC (Figure 2(A,B)). Eluates were monitored both at 215 nm for measuring the absorbance of peptide bonds (Figure 2(A)) and at 340 nm to detect AOPP absorbance (Figure 2(B)). In the retention time range between 6.5 and 9 min, both the area under the 340 nm absorbance chromatogram ($A_{340}$) and the area under the 215 nm absorbance chromatogram ($A_{215}$) were considered. The ratio $A_{340}/A_{215}$ was calculated for each plasma sample. In agreement with other studies [13–15], AOPP levels were significantly ($p < 0.00000$) higher in the HD group compared to the age-matched healthy group (Figure 2(C)).

AOPP are a mixture of di-tyrosines, pentosidines, and carbonyl-containing protein products [12,17,18]. Therefore, we examined the relationship between plasma AOPP and both protein bound di-Tyr levels and PCO levels in haemodialysed patients (Figure 3(A,B)). Simple linear regression analysis revealed that plasma AOPP levels were significantly positively correlated with

**Table 1.** Main clinical and analytical parameters of the study cohort of nondiabetic ESRD patients on maintenance HD.

| | Haemodialysed patients ($n = 50$) | Reference range |
|---|---|---|
| Creatinine (mg/dL) | 9.49 ± 3.14 | 0.6–1.3 |
| Urea (mg/dL) | 148.43 ± 40.29 | 10.00–50.00 |
| C-reactive protein (mg/dL) | 0.47 ± 0.49 | 0.01–1 |
| White Blood Cells (cells/$mm^3$) | 6982.00 ± 2153.85 | 4000 |
| Albumin (g/dL) | 3.46 ± 0.33 | 3.5–5 |
| Fibrinogen (mg/dL) | 342.63 ± 76.99 | 150–400 |
| Haemoglobin (g/dL) | 11.01 ± 1.04 | 13–18 |
| Sodium (mmol/L) | 138.04 ± 3.12 | 135–145 |
| Potassium (mmol/L) | 5.19 ± 0.83 | 3.5–5.1 |
| Calcium (mmol/L) | 2.22 ± 0.19 | 2.1–2.6 |
| Phosphorus (mmol/L) | 1.67 ± 0.48 | 0.8–1.5 |
| Ferritin (ng/mL) | 221.46 ± 144.02 | 20–250 |

Data are expressed as mean ± SD.

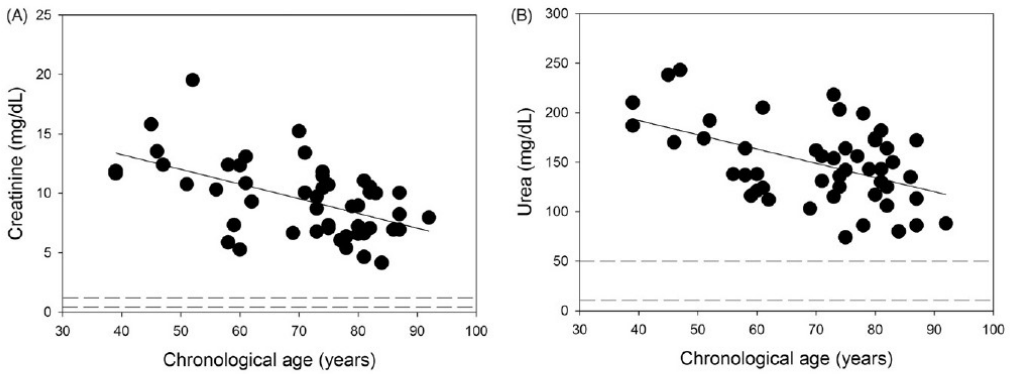



**Figure 1.** Creatinine, urea and chronological age in nondiabetic ESRD patients on maintenance HD. (A) Linear correlation between creatinine concentration and chronological age in haemodialysed patients. (B) Linear correlation between urea concentration and chronological age in haemodialysed patients. In (A) and (B), the horizontal dashed lines represent the reference range of creatinine and urea in healthy subjects.

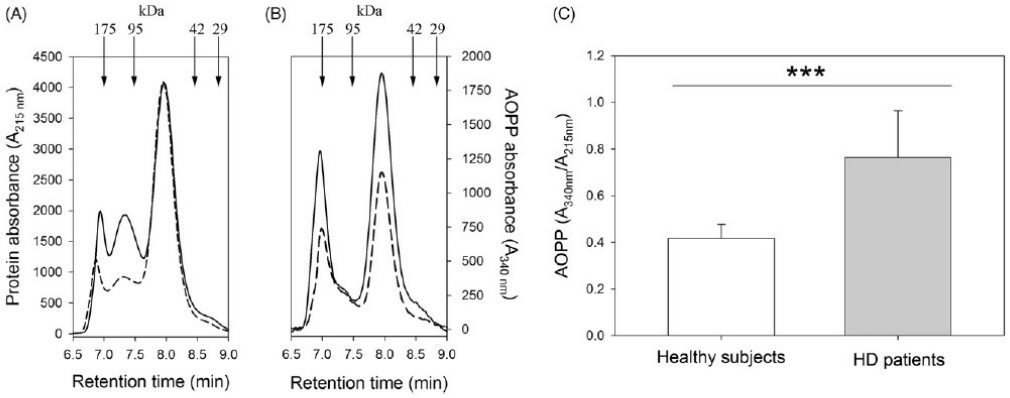



**Figure 2.** AOPP levels in healthy subjects and in nondiabetic ESRD patients on maintenance HD. Plasma proteins from healthy controls and haemodialysed patients were analysed by size exclusion/gel filtration-HPLC. (A) Eluates from control samples (dashed line) and HD samples (solid line) were monitored at $A_{215\,nm}$. (B) Eluates from the same control (dashed line) and HD (solid line) samples were monitored for AOPP absorbance at 340 nm. In all samples, $A_{215\,nm}$ and $A_{340\,nm}$ were calculated for the peak area in the time frame 6.5–9 min. (C) Histograms showing the mean plasma AOPP level in age-matched healthy subjects (control group) and haemodialysed patients immediately before the HD session. Data are expressed as mean ± SE. ***=$p < 0.001$.

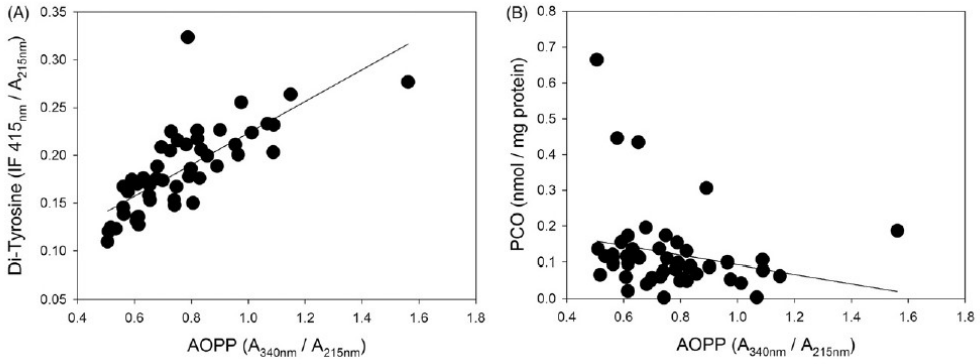



**Figure 3.** Linear regression analysis between AOPP, protein-bound di-tyrosines and PCO in nondiabetic ESRD patients on maintenance HD. (A) Positive linear correlation between plasma AOPP and protein-bound di-tyrosine levels. (B) Statistically not significant correlation between plasma AOPP and PCO levels.

plasma protein-bound di-Tyr levels ($r = 0.747$, $p < 0.0001$) (Figure 3(A)) but not with plasma PCO levels ($r = 0.223$, $p = 0.1212$) (Figure 3(B)).

Although the exact composition of AOPP is not fully defined, it is known that AOPP mainly derive from oxidised albumin, but also from fibrinogen [12,18,33–36]. For this reason, we evaluated the possible correlation between plasma AOPP, albumin and fibrinogen levels in haemodialysed patients (Figure 4(A,B)). Simple linear regression analysis revealed a moderate positive correlation between AOPP levels and albumin levels ($r = 0.294$, $p = 0.0134$) (Figure 4(A)) and no correlation between AOPP and fibrinogen levels ($r = 0.193$, $p = 0.1068$) (Figure 4(B)).

C-reactive protein (CRP) is an easily measured and widely investigated biomarker of inflammation in ESRD patients on HD [37]. Considering the dual role of AOPP, as systemic inflammation promoter and biomarker of oxidative stress, we assessed the possible correlation between plasma AOPP and CRP levels and WBC count in ESRD patients on HD (data not shown). Simple linear regression analysis revealed no correlation between plasma AOPP and CRP levels or WBC count.

Finally, we have evaluated the possible association between plasma levels of AOPP and creatinine and urea concentration. Simple linear regression analysis revealed a significantly positive correlation between

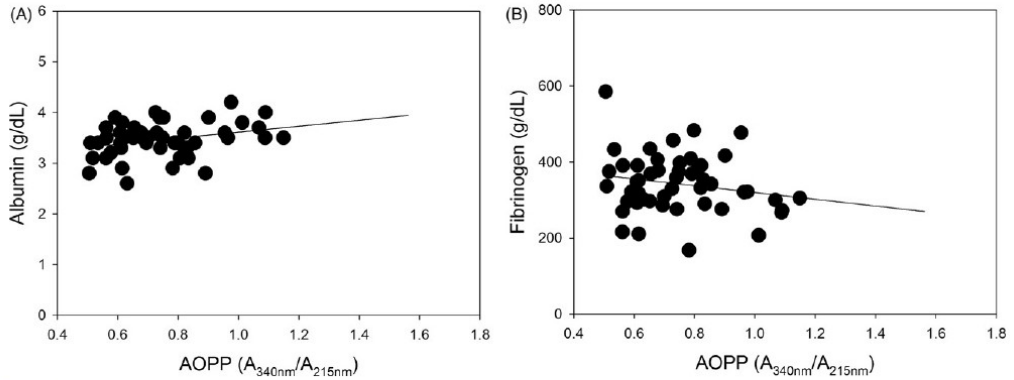



**Figure 4.** Linear regression analysis between AOPP, albumin and fibrinogen in nondiabetic ESRD patients on maintenance HD. (A) Positive linear correlation between plasma AOPP and albumin levels. (B) Statistically not significant correlation between plasma AOPP and fibrinogen levels.

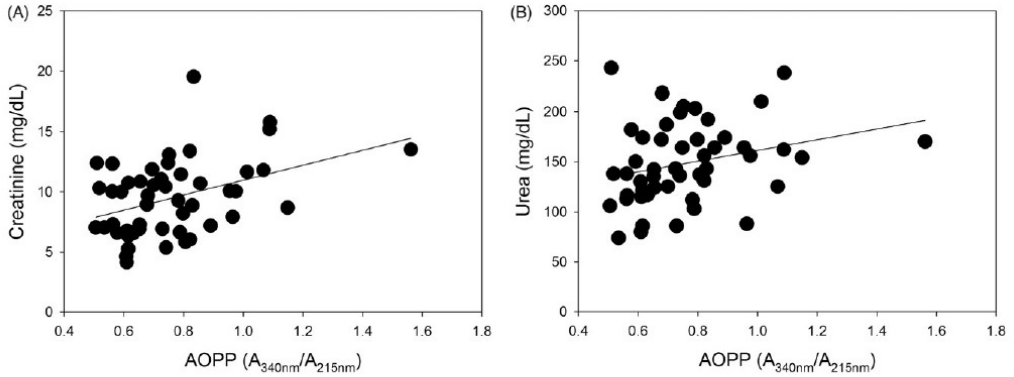



**Figure 5.** Linear regression analysis between AOPP, creatinine and urea in nondiabetic ESRD patients on maintenance HD. (A) Positive linear correlation between plasma AOPP and creatinine levels. (B) Statistically not significant linear correlation between plasma AOPP and urea levels.

AOPP and creatinine levels ($r = 0.400$, $p = 0.0042$) (Figure 5(A)) and no significant correlation between AOPP and urea levels (Figure 5(B)).

## Discussion

In haemodialysed patients, high creatinine level has been associated with greater survival, whereas low creatinine level has been associated with increased mortality [38–40]. We found a significant inverse correlation between creatinine levels and chronological age in ESRD patients on maintenance HD (Figure 1(A)). This finding is in agreement with another study reporting the impact of advancing age on creatinine levels in haemodialysed patients, which showed a significant inverse correlation between chronological age and creatinine ($r = -0.331$, $p = 0.001$) in 126 haemodialysed patients (mean age $70 \pm 13$ years) [41]. Creatinine is almost exclusively a product of the metabolism of creatine and phosphocreatine in skeletal muscle, so it is usually produced at a fairly constant rate by the body depending on muscle mass. It is the most commonly used parameter to assess kidney function, since it is excreted with urine. In ESRD patients on HD, whose residual kidney function is minimal to nonexistent, creatinine is not a reliable surrogate for residual kidney function [42], but it is a surrogate for total skeletal muscle mass and HD efficiency. Skeletal muscle mass and strength decline with advancing chronological age. After the age of 30, muscle mass begins to decline at an average rate of 1–2% per year [43]. Reduction in both skeletal muscle mass and strength (sarcopenia) is a common feature of haemodialysed patients, especially in elderly subjects [44] and is closely associated with significantly lower

creatinine levels [45,46] and systemic inflammation [37,44].

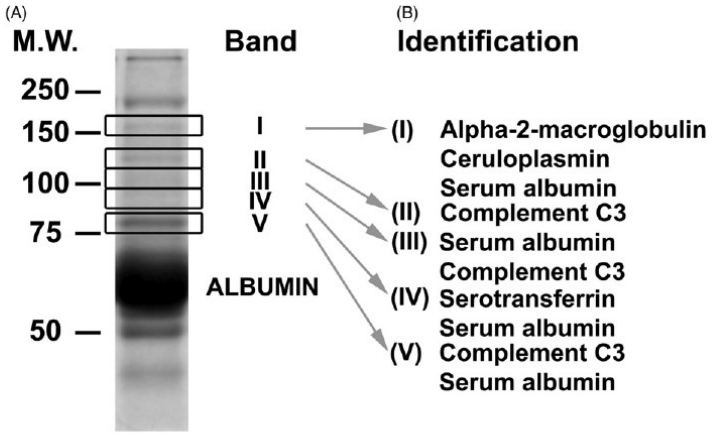
Pre-HD urea concentration and chronological age were also found to be inversely correlated (Figure 1(B)). A study done on haemodialysed patients (subdivided into two age groups: younger $\leq$72 years, vs. older $>$72 years) showed a BUN borderline lower in the older age group and a significant inverse correlation between chronological age and BUN ($r = -0.281$, $p = 0.002$) [41]. The inverse correlation between urea concentration and chronological age could be explained considering that urea is a breakdown product of amino acids resulting from catabolism of proteins, mainly those introduced with the diet. Hence, serum urea concentration may be a surrogate for nutritional status, especially in older haemodialysed patients.

Measurements of plasma levels of AOPP in ESRD patients on HD have generated conflicting data. Numerous studies found higher AOPP levels in haemodialysed patients than in healthy subjects [12–15,47], while another found no difference in AOPP levels between the two groups [48]. It is appropriate to consider that AOPP assays are based on the rationale that AOPP absorb light at 340 nm more than native proteins [12,33,49]. Some protocols do not provide for plasma protein separation: therefore, the $A_{340}$ is measured in diluted whole plasma [47,50,51]. However, measurement of AOPP in diluted whole plasma presents some methodological criticisms [12,13,52], some of which can be overcome [15,53]. Furthermore, plasma components, such as NADH and NADPH, absorb light at 340 nm [54], so they may interfere with the AOPP assay. Moreover, in many protocols chloramine T is used as a standard [50], though a number of plasma constituents contribute to the oxidation of $I^-$ to $I^{3-}$, such as peroxidases [55] and dehydromethionine [56]. As ESRD patients receive many drugs (including anti-inflammatory ones), it is not possible to rule out the potential interference of these drugs on the measurements of AOPP levels in diluted whole plasma. Other protocols provide for the separation of plasma proteins by HPLC [49], size exclusion fast protein liquid chromatography [12], or size exclusion HPLC [18]. In this study, we have measured AOPP in healthy subjects and HD patients by size exclusion-HPLC, detecting, in agreement with other studies [13–15], significantly higher AOPP levels in the HD group compared to the age-matched healthy group (Figure 2(C)).

It is interesting to note that, in the molecular weight range between 66 and 175 kDa (HPLC retention time between 7 and 7.75 min), proteins do not seem to give rise to the formation of AOPP (Figure 2(A)). Mass spectrometry analysis suggests that these proteins could presumably include $\alpha$-2-macroglobulin, ceruloplasmin, complement C3 and transferrin (Figure 6). We can hypothesise that AOPP-resistant proteins do not undergo modifications due to their amino acid composition or based on their 3 D structure. Alternatively, these proteins could be protected from oxidation as a consequence of the high concentration of serum albumin, an abundant protein reported to play an important role of scavenger against oxidising molecules (in this case the molecules able to induce the formation of AOPP) [19].

The positive correlation between AOPP levels and plasma protein-bound di-Tyr levels (Figure 3(A)) suggests that the formation of di-tyrosines strongly contributes to the $A_{340nm}$, whereas the formation of protein carbonyls presumably do not contribute, or contribute slightly, to the $A_{340nm}$ since no association was observed between plasma AOPP and PCO levels in ESRD patients on HD (Figure 3(B)). This involves that, when measuring AOPP as $A_{340nm}$, PCO are not assessed even if they are a component of the AOPP. Therefore, when measuring AOPP as $A_{340nm}$, PCOs should be measured separately in the same samples.

In the plasma of haemodialysed patients, AOPP consist of high molecular weight-AOPP ($\sim$600 kDa), which mainly contain albumin aggregates and low molecular weight-AOPP ($\sim$70 kDa), which mainly contain albumin and globulins [12,33]. Fibrinogen (Fb), too, has been proposed as a key molecule responsible for AOPP formation in human plasma [34,35], as suggested by *in vitro* studies on effects of HOCl on human plasma [18,57]. In particular, HOCl-induced oxidative damage of Fb induces AOPP formation in a concentration-dependent manner [18]. Furthermore, Fb-AOPP formation may promote the change of Fb structure and function increasing its coagulation activity [57]. By contrast, a cross-sectional study including 102 haemodialysed patients suggested that Fb is not a component of AOPP but rather it interferes with their measurement in diluted total plasma at $A_{340nm}$ [14]. In addition, AOPP levels from Fb-free samples showed a significant correlation with MPO activity and levels of IL-6 and TNF-$\alpha$ as compared to AOPP levels obtained from diluted total plasma samples. Therefore, authors suggested that serum AOPP are more appropriate than plasma AOPP as a biomarker of oxidative stress in ESRD patients on HD [14]. Our results confirm that albumin is the main protein contributing to the formation of AOPP in haemodialysed patients (Figure 4(A)). Differently, the lack of correlation between plasma AOPP and fibrinogen levels in haemodialysed patients (Figure 4(B)) suggests that, unlike what's observed *in vitro* [18,57], Fb does not




(C)

| Band | I.D. | Description | Coverage [%] | N° Peptides | N° Unique Peptides | N° PSMs | N° AAs | MW [kDa] |
|---|---|---|---|---|---|---|---|---|
| I | P01023 | Alpha-2-macroglobulin (A2M) | 36 | 38 | 38 | 93 | 1474 | 163.2 |
| | P00450 | Ceruloplasmin (CP) | 32 | 27 | 27 | 68 | 1065 | 122.1 |
| | P02768 | Serum albumin (ALB) | 43 | 27 | 27 | 64 | 609 | 69.3 |
| II | P01024 | Complement C3 (C3) | 33 | 48 | 48 | 140 | 1663 | 187 |
| III | P02768 | Serum albumin (ALB) | 61 | 39 | 39 | 120 | 609 | 69.3 |
| | P01024 | Complement C3 (C3) | 17 | 25 | 25 | 65 | 1663 | 187 |
| IV | P02787 | Serotransferrin (TF) | 52 | 42 | 42 | 193 | 698 | 77 |
| | P02768 | Serum albumin (ALB) | 44 | 30 | 30 | 80 | 609 | 69.3 |
| V | P01024 | Complement C3 (C3) | 30 | 39 | 39 | 106 | 1663 | 187 |
| | P02768 | Serum albumin (ALB) | 45 | 28 | 28 | 79 | 609 | 69.3 |

**Figure 6.** MS identification of presumed AOPP-resistant proteins. (A) Plasma proteins from CKD patients were separated on SDS-PAGE and the five most abundant protein bands (molecular weight between 66 and 175 kDa) were identified by MS analysis. (B) The identification list reports the most relevant identified proteins in the band according to higher peptide spectral match score (PSMs). The complete list of identified proteins is reported in Supplementary Materials. (C) The coverage percentage, the number of peptides and unique peptides, the peptide spectral match score (PSMs), the number of amino acid and the molecular weight of each identified protein are reported in the table.

contribute, or contributes slightly, to the formation of low molecular weight-AOPP in the plasma of ESRD patients on HD. However, we have to consider that plasma proteins from both healthy subjects and haemodialysed patients were fractionated on a size exclusion/gel filtration HPLC column with an exclusion range of 15–500 kDa (in the presence of 0.5% SDS). Therefore, we cannot rule out that Fb may promote high molecular weight-AOPP formation in the plasma of haemodialysed patients.

About the supposed role of AOPP as a bridge between oxidative stress and inflammation, the hypothesis of some correlations between plasma AOPP and CRP levels in haemodialysed patients has generated conflicting data. One study showed a significant correlation between plasma AOPP and CRP levels [14], whereas, two other studies failed to demonstrate any correlations between AOPP and CRP [13,58]. We also could not prove any correlations between AOPP and CRP levels (data not shown). These results are in accordance with our other recent results showing no correlation between plasma protein-bound di-tyrosine levels and CRP [9]. Oxidative stress and inflammation are usually inseparably linked and they reciprocally participate in a self-perpetuating vicious circuit. Taken together, these findings seem to suggest that oxidative stress and inflammation may also be partly independent of each other in ESRD patients on HD. The absence of correlation between AOPP and CRP levels (measured as "pinpoint marker") could be explained by the fact that CRP concentration fluctuates substantially over time in haemodialysed patients: therefore, reliable CRP levels can be obtained only following regular, repeated measurements [37].

It is well established that creatinine is able to deeply affect the oxidative status in CKD patients [59,60].

Furthermore, urea induced oxidative stress in a mouse model of CKD [61]. Urea also induces mitochondrial ROS production and causes endothelial dysfunction and activation of proatherogenic pathways at concentration normally measured in CKD [62]. The positive correlation between AOPP and creatinine that we found in haemodialysed patients (Figure 5(A)) is congruent with recent studies in haemodialysed patients showing a significant positive correlation between creatinine and plasma protein-bound di-tyrosine levels [9]. However, it should be pointed out that, in our haemodialysed patients, creatinine values basically represent dialytic efficiency and nutritional status (i.e. muscle mass). Taken together, these data seem to confirm the robust association between uraemia and oxidative stress, especially if measured as biomarkers of severe oxidative protein damages, e.g. plasma AOPP.

Our study had some limitations that should be noted. First, this study included a relatively small number of ESRD patients on maintenance HD and it has been performed in a single HD centre. Secondly, AOPP levels, also in HD population (composed predominantly of aged subjects in old age), may depend on many factors, such as age, physical condition and cigarette smoking [53,63,64]. Because of these limitations, our results could not be extrapolated to the general HD population. Therefore, additional studies with a larger number of haemodialysed patients enrolled from different HD centres, nationalities, and races are needed to extend these findings.

In conclusion, we would spend some considerations concerning the measurement of AOPP. The successful translation of a biomarker into a clinical diagnostic test requires the availability of an artefact-free and simple assay that is selective, specific, and sensitive. Although AOPP are promising biomarkers of oxidative stress, current methodological problems (discussed above), the poor standardisation of measurement methods and the molecular composition of AOPP not yet fully clarified make AOPP a clinical biomarker still difficult to use. Consequently, before using AOPP as a clinical biomarker, other more in-depth studies including larger number of haemodialysed patients should be carried out.

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## Disclosure statement

The authors confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome. The authors further confirm that the manuscript has been read and approved by all named authors and that the order of authors listed in the manuscript has been approved by all of us.

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# 6. CHAPTER 3

## Sucrosomial iron in the treatment of anemia of hemodialyzed patients: focus on hemoglobin maintenance and oxidative stress

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**Keywords:** iron supplementation, anemia, dialysis, oxidative stress

## Abstract

Iron deficiency is the most common cause of anemia among hemodialyzed patients. In this population the optimal route to administrate iron is still controversial. Nowadays intravenous administration is preferred, since it determines a greater increase in hemoglobin levels and reduces the need for erythropoiesis stimulating agent (ESAs). However, intravenous iron has many side effects, probably mediated by oxidative stress. With this work we evaluated an alternative type of oral iron containing ferric pyrophosphate covered by phospholipids plus sucrose ester of fatty acid matrix, named sucrosomial iron, whose absorption isn't influenced by hepcidin levels. 24 patients undergoing hemodialysis stopped the intravenous iron treatment to start and continue for 3 months the sucrosomial iron supplementation. Over the 3 months, hemoglobin values remained stable, as the values of hematocrit and mean corpuscular volume. In parallel, other anemia parameters dropped: ferritin, transferrin saturation and serum iron decreased significantly at the end of the study, while transferrin level increased. We observed a tendency towards better inflammatory and oxidative stress biomarkers (C-reactive protein, White blood cells, protein carbonyls tend to decrease during the 3 months), but we did not find statistical differences. Other oxidative stress biomarkers (dityrosines, AOPPs and protein thiols) remained stable.
The stability of hemoglobin levels after three months of sucrosomial iron therapy and the oxidative stress and inflammation biomarkers trends suggest that this iron formulation may be an alternative to intravenous iron. Although, further studies with higher dosage, larger sample size and longer duration are needed to confirm if sucrosomial iron could represent an efficacious alternative to administrate iron in hemodialyzed patients.

## Introduction

There are more than 2 million patients on dialysis worldwide and this number is going to increase to more than 5.4 million by 2030[1]. Most of these patients are anemic because of the relative deficiency of erythropoietin, the reduced erythrocytes life span and the chronic blood losses caused by the extracorporeal treatment and the frequent blood analyses[2]. However, the most common reversible cause of anemia among such patients is iron deficiency. It occurs in more than 50% of patients with non-dialysis-dependent CKD and in a greater percentage of patients receiving dialysis. Iron deficiency in dialysis patients is caused by the chronic blood losses and impaired intestinal absorption secondary to elevated hepcidin concentrations[2]. For this reason, virtually all hemodialysis patients will develop iron deficiency if it is not supplemented. In addition to this absolute iron deficiency, dialysis patients may also develop functional iron deficiency. In fact, even if iron stores appear to be adequate by conventional criteria, iron can't be mobilized when erythropoiesis is stimulated by an erythropoiesis stimulating agent (ESA)[3]. Hence, iron replacement is a cornerstone of the treatment of anemia in dialysis patients. Although iron can be administered via oral or parenteral routes, intravenous administration is preferred in hemodialysis patients, since it has been widely demonstrated that intravenous iron determines a greater increase in hemoglobin

levels and reduces the need for ESAs[4–6]. The Kidney Disease: Improving Global Outcomes (KDIGO) guidelines suggests a goal-directed intravenous iron replacement when Transferrin Saturation (TSAT) ≤30% and serum ferritin ≤500 ng/mL[7]. However, Karaboyas *et al.*, analyzing the Dialysis Outcomes and Practice Patterns Study (DOPPS) database, showed that there is a diffuse increase in mean ferritin values, in particular in United States and Europe[8]. One concern about the use of intravenous iron and the risk of iron overload is the possible effect on mortality. In two different studies an increased mortality was observed in patients treated with higher doses of intravenous iron[9,10]. Also a correlation between high ferritin values and mortality has been described in the afore mentioned paper by Karaboyas *et al.*[8]. The possible negative effect of intravenous iron and iron overload on mortality may be mediated by oxidative stress. Iron has a high reactivity with oxygen, as described by the Haber-Weiss and Fenton reactions. Hence, the human body has highly conserved mechanisms, in which hepcidin has a pivotal role, for strict control of iron homeostasis[11,12]. Injection of iron directly into the bloodstream bypasses these protective controls, and this may determine an increase in oxidative stress[13–18].
Sucrosomial iron is an oral iron preparation containing ferric pyrophosphate covered by phospholipids plus sucrose ester of fatty acid matrix[19]. This iron formulation may be a valid alternative to intravenous iron in dialysis patients, since its absorption isn't influenced by hepcidin levels[19]. Moreover, the oral administration may overcome the concern for oxidative stress negative effects. Therefore, the rationale of this study is to verify if sucrosomial iron is able to maintain adequate hemoglobin levels and at the same time reduce the levels of oxidative stress biomarkers.

## Methods

### Study design and partecipants

The study was approved by Istituto Clinico Humanitas review board before initiation and carried out according to the Code of Ethics of the World Medical Association (Declaration of Helsinki). 30 hemodialyzed patients in treatment with sodium ferric gluconate complex have been initially screened. Patients have been enrolled at the Dialysis Center of Istituto Clinico Humanitas, Rozzano, MI. An informed consent has been obtained for each participant. 5 patients have been excluded because of the necessity of more than one sodium ferric gluconate complex administration per week. Patients enrolled interrupted the therapy with sodium ferric gluconate complex and started to take Sucrosomial® iron for three months at a dosage of 90 mg per week. One patient didn't complete the study for causes not related to the aim of the study. 24 patients completed the study and have been included in the per protocol analysis. The baseline characteristics of the participants are shown in *Table 1.*

### Statistical analysis

Statistical analysis has been carried out with the software Stata (StataCorp LLC). Descriptive analysis has been reported as mean ± standard deviation. The statistical test used were Mixed Effect REML Regression and Student's t-test. A p-value less than 0.05 has been considered significative.

### Blood samples

Blood samples have been obtained at the beginning of the study and every month for three months. Blood has been taken from the arteriovenous fistula or from a central venous catheter. Blood samples for biochemical parameters have been obtained and analyzed at the Clinical Analysis Laboratory of Istituto Clinico Humanitas according to standardized procedures.

### Oxidative stress biomarkers

For the measurement of oxidative stress biomarkers 10 mL of venous blood have been taken with ethylenediaminetetraacetic acid (EDTA) as anticoagulant. Within one hour the blood samples have been centrifuged at 3000 RPM for 15 minutes, obtaining plasma aliquots. Aliquots have been stored at -80°C until the execution of the different analyses.

*Detection of Plasma Protein Carbonylation by SDS-PAGE and Western Blot*

Plasma proteins were fractionated on 12.5% (w/v) reducing SDS-PAGE gels and electroblotted onto a polyvinylidene difluoride (PVDF) membrane. Protein carbonylation was detected, after derivatization with DNPH, with anti-DNP antibodies specific for the 2,4-dinitrophenyl hydrazone-carbonyl adduct by Western blot immunoassay as previously reported [35]. Immunoreactive protein bands were visualized by enhanced chemiluminescence (ECL). Protein bands on PVDF membranes were then visualized by washing the blots extensively in PBS and then staining with Ponceau Red.

*Determination of plasma AOPP and protein-bound di-Tyr*

Eluates were monitored both at 340 nm for measuring AOPP absorbance and at 215 nm for measuring the absorbance of peptide bonds using HPLC detector 332 from Kontron Instrument SpA (Milan, Italy). The ratio $A_{340}/A_{215}$ was calculated for each plasma sample. For protein-bound di-Tyr determination, eluates were moni- tored both at 215 nm for measuring absorbance of peptide bonds and at 415 nm emission with 325 nm excitation using Kontron SFM-25 Spectrofluorimeter equipped with 150 W Xenon N-800-LO, ozone-free, lamp. The ratio between fluorescence and absorbance (IF415nm em/A215nm) was calculated for each sample.

*Total plasma thiol determination with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB)*

The free thiol concentration of plasma samples was quantified by the Ellman assay measuring the increase in absorbance at 412 nm caused by the released TNB anion upon reaction of thiols with DTNB and using a molar absorption coefficient of 14.15 $mM^{-1}$ $cm^{-1}$. In detail, 50 ml of plasma was diluted with 900 ml of 50 mM potassium phosphate buffer (PBS), pH 7.4, mixed with 50 µl of 3 mM DTNB prepared in PBS and incubated for 15 min at 25 °C. In order to subtract the intrinsic absorbance of plasma at 412 nm, a parallel sample was assembled mixing 950 ml of PBS with 50 µl of plasma for each sample. All measurements were performed in triplicate and the mean intrinsic absorbance was subtracted from the mean absorbance of TNB release. The molar concentration of thiols was calculated from the molar absorbance of the TNB anion.

*Determination of plasma protein thiols by means of biotin-maleimide and SDS-PAGE*

Biotin-maleimide stock solution was prepared at 40 mM in DMSO and stored at − 20 °C. Plasma protein samples were diluted to a final concentration of 1 mg/mL in 50 mM PBS, pH 7.4, containing 15 µM biotin-maleimide. Protein labelling was performed for 1 h at room temperature. After labelling, protein samples were mixed with an equal volume of 2 × reducing Laemmli sample buffer, boiled for 5 min at 90 °C and analysed by SDS-PAGE using 10% (w/v) Tris–HCl polyacrylamide gels.

After electrophoretic run, proteins were transferred to PVDF membrane and biotin tag revealed with streptavidin-HRP. Briefly, PVDF membranes were washed with PBST [10 mM Na-phosphate, pH 7.2, 0.9% (w/v) NaCl, 0.1% (v/v) Tween-20] and blocked for 1 h in 5% (w/v) non-fat dry milk in PBST. After washing three times with PBST for 5 min each, biotin tag was probed by 2-h incubation with 5% non-fat dry milk/PBST containing streptavidin-HRP (1:5000 dilution). After three washes with PBST, biotinylated proteins were visualized by ECL detection.

## Results

In *Table 2* and *Figure 1* are shown the changes of the main biochemical parameters. Hemoglobin values remained stable during the three months period of treatment with Sucrosomial iron. The mean changed from 11.4 ± 1.0 g/dL at baseline to 11.0 ± 1.0 g/dL at the conclusion of the study ($p = 0.45$). Also, the values of hematocrit and mean corpuscular volume remained stable, passing respectively from 34.7 ± 2.89% to 34.14 ± 3.08% ($p = 0.46$) and from 92.51 ± 8.62 fL to 92.15 ± 8.38 fL ($p = 0.53$). ESA dosage remained stable (9667 ± 7335 UI/week to 10583 ± 7283 UI/week). Ferritin values resulted lower at the end of the study, resulting 97.19 ± 97.25 ng/mL from 225.50 ± 167.00 ng/mL ($p < 0.01$). Ferritin, transferrin saturation and serum iron resulted lower at the end of the study (respectively 97.19 ± 97.25 ng/mL from 225.50 ± 167.00 ng/mL, $p < 0.01$; 16.8 ± 7.3% from 30.0 ± 9.9%, $p < 0.01$; 45.91 ± 20.31 mcg/dL from 70.37 ± 24.91 mcg/dL, $p < 0.01$). Transferrin increased from 1.66 ± 0.32 g/L to 2.05 ± 0.43 g/L ($p < 0.01$). In *Figure 2* are shown

other biochemical parameters measured. C-reactive protein reduced from 1.46 ± 3.59 mg/dL to 0.95 ± 1.51 mg/dL ($p = 0.42$). White blood cells reduced from 6.4 ± 1.82*$10^3$/mmc to 5.84 ± 1.38*$10^3$/mmc ($p < 0.05$).
In *Table 3* and *Figure 3* are shown the results of oxidative stress biomarkers analysis. Protein carbonyl groups reduced from 0.13 ± 0.14 nmol/mg to 0.10 ± 0.10 nmol/mg ($p = 0.088$). Dityrosine and AOPPs remained stable (respectively 0.19 ± 0.04 AU, 0.18 ± 0.04 AU, $p = 0.112$ and 0.91 ± 0.23 AU, 0.88 ± 0.18 AU ($p = 0.165$). SH BNEM didn't change from the beginning to the end of the study, SH DTNB increased from 4.02 ± 0.99 pmol/µg prot to 4.27 ± 1.79 pmol/µg prot ($p = 0.714$). No correlation has been found between ferritin and protein carbonyl groups, dityrosine and AOPPs (*Figure 4*).

## Discussion

The presence of anemia in dialysis patients is frequent and iron deficiency is the most common reversible cause. The chronic blood losses and impaired intestinal absorption secondary to elevated hepcidin concentrations are responsible for the loss of 1-2 grams of iron every year in dialysis patients[20]. Elevated hepcidin levels, caused by the chronic inflammatory status, determines a down-regulation of ferroportin, which is a carrier protein that regulates the transport of iron from enterocytes cytoplasm to the bloodstream. The consequence of this pathway is a reduction of iron absorption in dialysis patients[21,22].
Hence, intravenous iron administration have become the preferred route of administration in this population and its superiority to oral one has been widely demonstrated[4–6]. According to KDIGO guidelines the most used approach is to treat dialysis patients with intravenous iron when transferrin saturation is less than 20% and ferritin less than 200 ng/mL independently from hemoglobin. If hemoglobin is less than 10.5 g/dL or the patient is in treatment with ESAs the suggested cut-off are a transferrin saturation of 30% and a ferritin level of 500 ng/mL[7]. In recent years new sucrosomial iron has emerged as a possible alternative to intravenous iron in dialysis patients, since its peculiar absorption is independent from hepcidin levels and thus not influenced by the micro-inflammatory status[19,23–25]. Different studies on CKD patients not on dialysis have demonstrated that sucrosomial iron is effective in increasing Hb and ferritin values[19,26]. However, few studies investigated the effectiveness of sucrosomial iron in dialysis patients. These studies showed that sucrosomial iron is able to maintain stable hemoglobin levels, but its effect on iron status is controversial[19]. We analyzed in our study the effect on hemoglobin values and iron metabolism parameters shifting from sodium ferric gluconate complex to Sucrosomial® iron. Sucrosomial iron has been administered at a dosage of 90 mg per week. Previously patients were taking sodium ferric gluconate complex at a dosage of 62,5 mg per week. During the study we didn't report any adverse events attributable to sucrosomial iron, which proved to be well tolerated. Hemoglobin levels remained stable during the study (11.4 ± 1.0 g/dL, 11.0 ± 1.0 g/dL, $p = 0.45$) and the required ESA dosage didn't change. However, we observed a significant reduction of ferritin and transferrin saturation. The stability of hemoglobin levels after three months of therapy with sucrosomial iron suggest that this iron formulation may be an alternative to intravenous iron in dialysis patients. However, it's likely that the dosage we used (90 mg/week) is insufficient to maintain iron stores as suggested by the main guidelines. In fact, patients showed a tendency to develop a functional iron deficiency, that uncorrected will finally bring to a reduction of hemoglobin levels or an increase in ESAs dosage. Further studies are required to determine if sucrosomial iron, at a greater dosage, is able to maintain an adequate iron storage in dialysis patients.
Another motivation to evaluate the use of sucrosomial iron in dialysis patients is the possibility of having a beneficial effect on the oxidative status. Oxidative stress, together with the micro-inflammatory status, represents one of the causes of cardiovascular and metabolic complications of patients on hemodialysis[27]. Different studies have demonstrated that intravenous iron administration is able to increase oxidative stress biomarkers in dialysis patients[13–16,18]. This may determine a vicious circle, in which oxidative stress further reduces intestinal iron absorption and determines an increase in intravenous iron demand. Increased oxidative stress seems to be caused by the generation of unbound free iron, a potential trigger of Haber-Weiss and Fenton reactions, after intravenous iron infusion[5]. Moreover, Lim *et al.* observed higher levels of oxidative stress biomarkers in patients with higher ferritin values (> 600 ng/mL). These data suggested that intravenous iron may be a potential target in the perspective of reducing oxidative stress in dialysis patients. However, in a metanalysis by Hougen a correlation between higher dosages of

intravenous iron and mortality, infections and cardiovascular events hasn't been observed[28]. On the contrary in the recent PIVOTAL study, higher dosages of intravenous iron are able to reduce cardiovascular events and mortality rate in dialysis patients[29]. Karaboyas *et al.* performed an anlysis of DOPPS data observing that there is a diffuse increase in mean ferritin values, in particular in United States and Europe[8]. This increase in ferritin values is also associated with an increase in mortality[8]. Therefore, it's unclear if higher dosages of intravenous iron or higher levels of ferritin are truly associated with an increased risk of cardiovascular events and mortality mediated by an increased oxidative stress. The scenario is further complicated by the fact that ferritin may also be a marker of inflammation[30]. We investigated the possible beneficial effect of sucrosomial iron on oxidative stress. In fact, the peculiar intestinal absorption of sucrosomial iron prevents the generation of labile iron available for redox reactions[25]. With this aim we evaluated, before and after the three months of therapy of sucrosomial iron, the levels of protein carbonyl groups, dityrosine, AOPPs and thiol groups, analyzing in this way different oxidative pathways[31–33]. Conversely to Lim et al. study[13], we didn't find a significative correlation between ferritin values and protein carbonyl groups, dityrosine and AOPPs. This may be explained by the fact that in our population just two patients had ferritin values greater than 500 ng/mL. We also didn't observe a statistically significant change of oxidative stress biomarkers values after the switch from sodium ferric gluconate complex to sucrosomial iron. We just observed a not significant reduction of protein carbonyl groups, dityrosine and AOPPs. We also observed a slight reduction of CRP values, which may suggest an improvement in inflammatory status. These results are not sufficient to prove that the shift to sucrosomial iron is able to reduce oxidative stress biomarkers. This is possibly a consequence of the limitations of this study, which are the study design (observational study without a control group), the duration of the study and the limited size. Considering also the contrasting results present in literature[13–16,18], more studies are needed to fill this gap in our knowledge.

## Conclusions

Our study demonstrates that a therapy with sucrosomial iron is able to maintain stable hemoglobin levels at three months. However, the reduction of ferritin and transferrin saturation suggests that a weekly dosage of 90 mg is not sufficient in hemodialysis patients in the long time. This therapy also determined a slight reduction of different oxidative stress biomarkers, however this result is not significant and must be confirmed in studies with a greater size and a longer duration. Although this study doesn't give a definitive answer on the possible advantage of oral iron formulation in dialysis patients, it may represent a starting point for further studies to investigate the complex correlation between iron supplementation, oxidative stress, inflammation, cardiovascular disease and mortality.

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## Tables and figures

***Table 1.*** *Baseline characteristics of study participants. Values are expressed as mean ± standard deviation or frequency*

| | |
|---|---|
| Age | 67.7 ± 16.2 |
| Gender (% men) | 15/25 (60%) |
| Dialysis vintage (months) | 67.3 ± 72.1 |
| BMI | 25.9 ± 6.9 |
| Diabetes (% of patients affected) | 9/25 (36%) |
| Cardiovascular disease (% of patients affected) | 14/25 (56%) |
| Vascular access for dialysis (% of arteriovenous fistula) | 18/25 (72%) |
| Malnutrition-Inflammation Score[34] | 6.44 ± 3.58 |
| ESAs dosage (UI/week) | 9440 ± 7269 |

***Table 2.*** *Hemoglobin, ferritin, TIBC, serum iron and transferrin saturation levels during the three months of therapy with sucrosomial iron. Statistical analysis performed with mixed-Effects REML Regression.*

| | **T0** | **T1** | **T2** | **T3** | **p** | **Δ 0-3 (95% CI)** |
|---|---|---|---|---|---|---|
| Hemoglobin (g/dL) | 11.2 ± 1.0 | 11.1 ± 1.0 | 11.4 ± 1.0 | 11.0 ± 1.0 | 0.681 | -0.175 (-0.652 – 0.302) |
| Ferritin (ng/mL) | 225 ± 166 | 188 ± 147 | 166 ± 155 | 97 ± 97 | < 0.001 | -128 (-176 – -80) |
| Transferrin TIBC (g/L) | 1.66 ± 0.32 | 1.75 ± 0.42 | 1.84 ± 0.51 | 2.05 ± 0.43 | < 0.001 | 0.38 (0.29 – 0.48) |
| Serum iron (mcg/dL) | 70 ± 24 | 58 ± 24 | 51 ± 17 | 46 ± 20 | < 0.001 | -24.5 (-36.2 – -12.7) |
| Transferrin saturation (%) | 30.0 ± 9.9 | 22.7 ± 8.2 | 20.1 ± 8.3 | 16.8 ± 7.3 | < 0.001 | -13.2 (-18.25 – 8.15) |

***Figure 1.*** *Hemoglobin, ferritin and transferrin saturation levels during the three months of therapy with sucrosomial iron.*

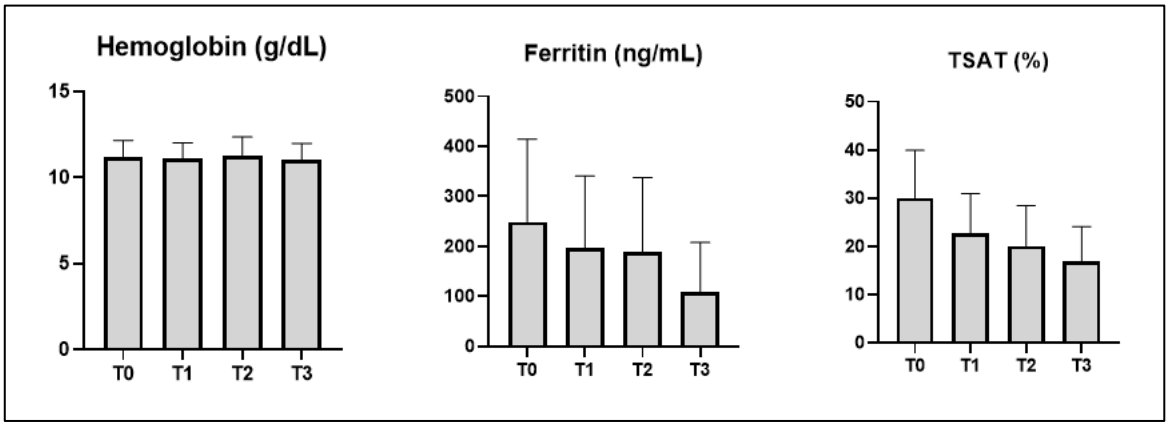



***Figure 2.*** *CRP levels, white blood cells count and total proteins during the three months of therapy with sucrosomial iron*.

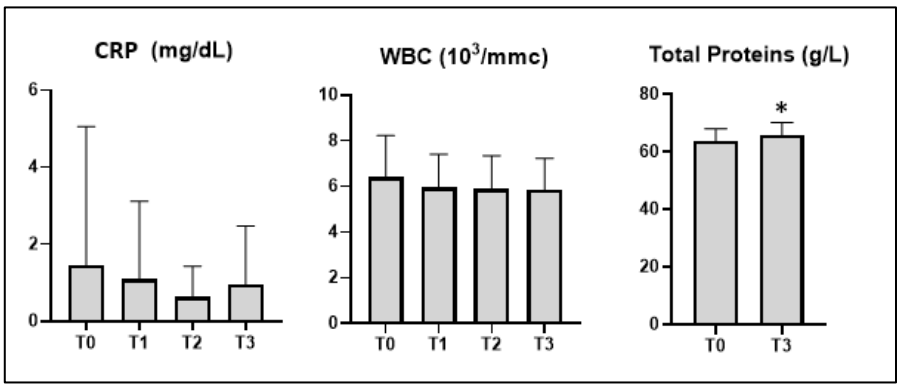



***Table 3.*** *Mixed-Effects REML Regression of oxidative stress biomarkers*

| | T0 | T1 | T2 | T3 | p |
|---|---|---|---|---|---|
| Protein carbonyl groups (nmol/mg) | 0.13 ± 0.14 | 0.13 ± 0.16 | 0.11 ± 0.11 | 0.10 ± 0.10 | 0.088 |
| Dityrosine (AU) | 0.19 ± 0.04 | 0.19 ± 0.05 | 0.18 ± 0.04 | 0.18 ± 0.04 | 0.112 |
| AOPPs (AU) | 0.91 ± 0.23 | 0.95 ± 0.31 | 0.90 ± 0.24 | 0.88 ± 0.18 | 0.165 |
| SH BNEM (AU) | 0.04 ± 0.01 | 0.03 + 0.02 | 0.04 ± 0.02 | 0.04 ± 0.01 | 0.268 |
| SH DTNB (pmol/µg prot) | 4.02 ± 0.99 | 4.53 ± 1.47 | 3.97 ± 0.91 | 4.27 ± 1.79 | 0.714 |

***Figure 3.*** *Oxidative stress biomarkers changes during the three months therapy with sucrosomial iron*

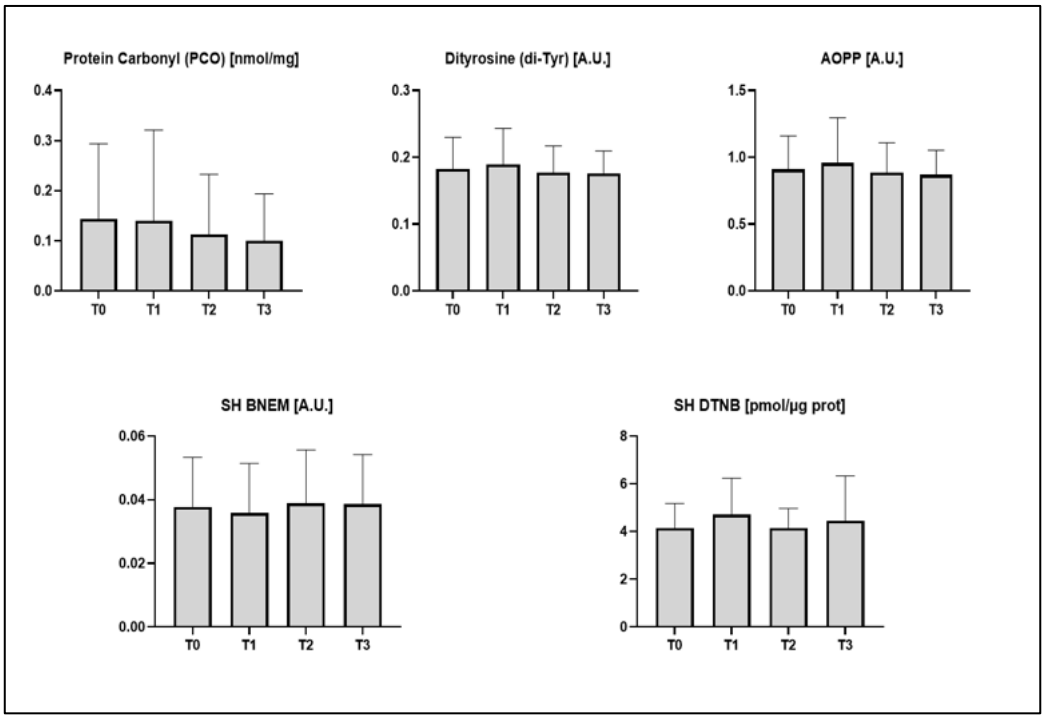
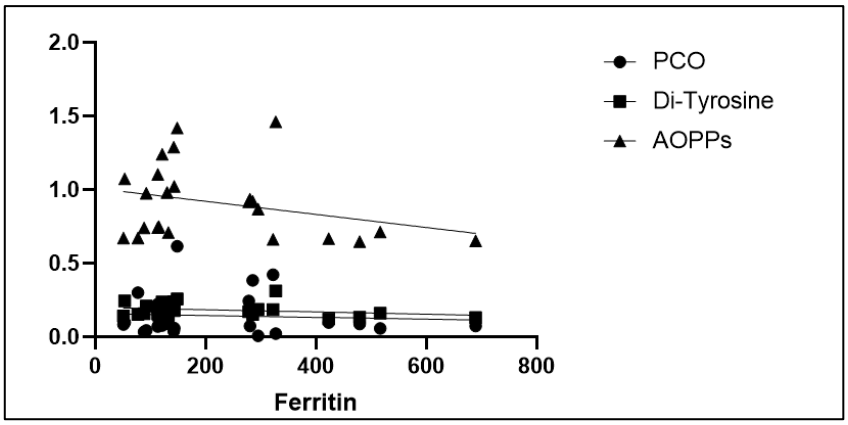



***Figure 4.*** *Correlation between oxidative stress biomarkers and ferritin values*



# 7. CHAPTER 4

## Effects of physiological and pathological urea concentrations on Human Microvascular Endothelial Cells (HMEC-1)

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## Abstract

Urea represents the uremic toxin with the highest concentration. It tends to accumulate in plasma samples of chronic kidney disease (CKD) patients and it is not completely cleared by the dialysis treatment. Urea accumulation is reported to exert direct and indirect side effects on the gastrointestinal tract, on kidneys, adipocytes and on the cardiovascular system (CVS), although its pathogenicity is still questioned, since works evaluating its side effects lacks in homogeneity. With this work we investigated systematically the effects of physiologic and pathologic concentrations of urea on a human endothelial cell line from the microcirculation (Human Microvascular Endothelial Cells-1, HMEC-1), concentrating on protein expression. Urea (5 gr/l) caused a reduction in the proliferation rate after 72 hours of exposure. It seemed not to induce oxidative stress (the level of protein thiols and carbonylated proteins, evaluated as oxidative stress biomarkers, did not change during the treatment). The most interesting results pointed out urea exposition as a potential endothelial-to mesenchymal transition (EndoMT) stimulus. In fact, we found that at 72 hours urea induced an actin rearrangement, a significant increase in matrix metalloproteinases 2 (MMP-2) expression in the medium, and it caused a significant up- or down-regulation of other EndoMT biomarkers (keratin, fibrillin-2 and collagen IV), according to the proteomic analysis.
Looking individually at each protein whose expression resulted to be significantly dysregulated by the urea treatment according to the proteomics, two remarkable supernatants proteins turned out to be significantly down-regulated: dimethylarginine dimethylaminohydrolase (DDAH) and vasorin (VASN). Both these proteins have been directly linked to cardiovascular diseases (CVD) by *in vitro* and *in vivo* studies. It could be interesting to deepen DDAH and VASN pathways, since these proteins could represent a link between CKD and CVD.

## Introduction

Uremic toxins are biologically active molecules with side effects on several physiologic functions. These compounds normally are excreted by the kidneys, while in chronic kidney disease (CKD) patients they accumulate in the blood [Vanholder et al., 2003]. This retention phenomenon is dependent to the reduced renal function observed in CKD and it worsens with the progression of the pathology [Stevinkel et al., 2008]. Urea is a water-soluble molecule, produced during the urea cycle in the hepatocytes. This cycle is necessary to convert toxic ammonia, which originate from protein catabolism, into urea, subsequently excreted through the urine [Barmore et al., 2020].

Urea represents the uremic toxin with the highest concentration [Vanholder et al., 2003], but its biologic activities appeared to be relatively limited when compared to the others [Vanholder et al., 2001]. Some studies questioned urea pathogenicity, while others showed its direct and indirect side effects [Lau et al., 2017].

Given its ubiquity, urea has multiple effects on different organs and tissues. Urea is reported to exert toxic effects on the gastrointestinal tract, contributing to epithelial barrier breakdown and microbiome alteration; on kidneys, indirectly promoting renal fibrosis; on adipocytes, inducing insulin resistance; on blood components, causing erythropoietin carbamylation; and on cardiovascular system (CVS). More in detail, urea has direct and indirect effects on CVS. CKD patients are at higher risk of cardiovascular diseases (CVD) than the normal population [Jha et al., 2013]. In this context, also uremic toxins play a role. In particular about urea, many studies report that high level of blood urea nitrogen (BUN) is associated with increased mortality and hospitalization in patients with CVD [Mok et al., 2017]. CKD patients also show high level of carbamylated LDL (cLDL) [Apostolov et al., 2005]. Urea itself induces the formation of cLDL, that are recognized to be atherogenic both *in vivo* and *in vitro* [Apostolov et al., 2010]. So overall *in vivo* studies seem to indicate urea as a mortality and atherosclerosis risk factor. In clinical trials, results interpretation is complex since CKD patients have multiple comorbidities and urea acts together with the other toxins. It's worth mentioning also some *in vitro* experiments. Urea treatment induced ROS production in human aortic endothelial cells, leading to the activation of pro-inflammatory pathways and the inactivation of the anti-atherosclerosis enzyme PGI2 synthase [D'Apolito et al., 2015]; urea increased ROS production also in human arterial endothelial cells, causing alteration in mitochondrial proteins and in inflammatory markers expression [D'Apolito et al., 2018]. In human aortic smooth muscle cells, urea elevated BAD [B-cell lymphoma 2 (BCL2)-associated death promoter] expression, a pro-apoptotic member of the BCL2 family [Trécherel et al., 2012]. This phenomenon could contribute to the increased apoptosis observed in the arterial wall of CKD patients and it could promote vascular medial calcification [Shroff et al., 2008].

Altogether these papers support the emerging notion that urea might be an important uremic toxin in CKD, even if more studies exploring urea effects on different organ and tissues are needed. Since literature lacks in works systematically exploring urea effects on protein expression and protein modification, with this study we tried to better elucidate some urea side effects potentially involved in CVD, when cells are exposed to physiologic concentrations of this toxin, found in healthy or CKD subjects. We evaluated this effects on a cell line from the microvasculature, often underestimated even if microcirculation is the principal seat of exchanges between circulation and tissues.

# Materials and methods

## Cell line and solutions for cell maintenance

HMEC-1 (Human Microvascular Endothelial Cells-1), an immortalized human cell line, were grown in plates with MCDB 131 Medium (Sigma, Italy), supplemented with 10 % fetal bovine serum (FBS, Euroclone), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 ng/ml Epidermal Growth Factor, 0.1 ug/ml Hydrocortisone (Sigma, Italy). Cell cultures were maintained at 37 °C with 5% $CO_2$ and passaged every 3–4 days.
For experiments, HMEC-1 cells were cultured in the presence or absence of different concentrations of urea (Sigma, Italy) for 24, 48 or 72 h.

## Treatment of HMEC-1 with urea

For all the following experiments, HMEC-1 were seeded at a concentration of 15,000 cells/$cm^2$ and let grown for 24 hours, at 37 °C with 5% $CO_2$. After 24 hours, a half of the medium was removed and replaced with an equal volume of solution with or without urea. This expedient is necessary since HMEC-1 release growth factors in the medium and a complete remove of the medium slows down their growth.

The treatment solutions were prepared dissolving urea powder in Phosphate buffered saline (PBS), obtaining a mother solution with a concentration of 100 gr/l. This solution was diluted in complete medium (prepared as described before) at the following concentration: 0.5 - 4 – 10 g/l. These concentrations are double to the desired ones, since, as mention before, only a half of the medium was changed. The solutions were filtered through a syringe with a 0.22-µm pore-sized filter to remove bacteria and particulate. At this point the solutions were added to the cells supports, obtaining the following final concentrations: 0.25 – 2 – 5 g/l (equal to 4 - 33- 83 mM). For control cells, they had the same treatment, adding a solution made of complete medium and PBS, without the toxin. By this way, in each treatment solution there was the same volume of PBS and they differed only for the presence or absence of the toxin. The treatment lasted 24, 48 or 72 hours, without changing the medium.

### Proliferation Assay

Sulforhodamine B (SRB) assay is a colorimetric test which allows quantifying cellular protein content and it's largely used to indirectly quantify cells proliferation [Orellana et al., 2016]. Briefly, cells were seeded and treated as described before in 24-multiwell plates. At each timepoints, cells were fixed with 50% trichloroacetic acid (Sigma, cod. T6399) for 2 h at 4°C, then washed five times with milliQ water. 0.04% (w/v) SRB protein-bound dye (Sulforhodamine B Sigma, cod. S1402, dissolved in 1% acetic acid) was added to each well and incubated at RT for 30 minutes, then each well was washed four times with 1% (v/v) acetic acid and left to air-dry at room temperature. Finally, 1.2 ml of 10 mM Tris base solution (pH 10.5) was added to each well and the plate was shaken on an orbital shaker for 10 min to solubilize the protein-bound dye. The absorbance at 490 nm was detected using a multimode microplate reader (EnSight Multimode Plate Reader, PerkinElmer).

### Quantification of Proteins Thiols

Cellular proteins extracts were obtained lysing cells with ice-cold lysis buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% TRITON X-100, 0.1% SDS, 0.5% sodium deoxycholate supplemented with protease inhibitors (Sigma P8340)]. Each lysate was incubated on ice for 30 min and centrifuged at 10,000 rpm for 10 min at 4°C to remove cell debris. BCA protein assay was used to assess protein concentration. To detect proteins thiol groups, a biotin-maleimide assay was carried out. Briefly, 40 mM biotin-maleimide stock solution was prepared in DMSO and stored at −20°C. Then, 1 mg/ml of protein was incubated with 75 µM biotin-maleimide solution for 1 h at RT and then mixed to Laemmli sample buffer (2% SDS, 20% glycerol, and 125 mM Tris-HCl, pH 6.8), boiled for 5 min at 90°C and separated on 12% SDS-PAGE Stain-free gel (Biorad) [Hill B.G et al., 2009]. Separated proteins were then electroblotted onto a low-fluorescence polyvinylidene difluoride (LF-PVDF) membrane. Biotin tag was revealed using streptavidin-HRP assay as following. LF-PVDF membrane was washed with PBST [10 mM Na-phosphate, pH 7.2, 0.9% (w/v) NaCl, 0.1% (v/v) Tween-20 (Sigma Aldrich, cod. P9416)] [Hill B.G et al., 2009] and blocked for 1 h in 5% (w/v) non-fat dry milk in PBST. After washing three times with PBST for 5 min, biotin tag was probed by 2 h incubation with 5% non-fat dry milk/PBST containing streptavidin-HRP (1:5000 dilution, GE Healthcare). Biotinylated proteins were visualized by ECL detection (cod.1705061, Biorad) using Chemidoc Touch Imaging System (Biorad). ECL signals were normalized with respect to PVDF stain free [Rivero-Gutiérrez et al., 2014].

### Western blot

Proteins from cell extracts were separated and transferred to PVDF membrane as described previously. After washing three times with TBST for 5 min, membrane was incubated for 2 h with 5%

non-fat dry milk/TBST containing the following primary antibodies: anti-Actin (1:2000, Abcam); anti-Tubulin (1:40000, Abcam). The membrane was washed three times with TBST for 5 min and then incubated for 1h with the following secondary antibodies respectively: anti-mouse (1:10000); anti-rabbit (1:20000). Proteins of interest were visualized by ECL detection (cod.1705061, Biorad) using Chemidoc Touch Imaging System (Biorad). ECL signals were normalized using tubulin as housekeeping.

### Immunofluorescence

HMEC-1 cells were cultured on 12-mm diameter round coverslips, seeded at a concentration of 15,000 cells/cm$^2$ on 24-well culture plates and treated with urea as described before. At each timepoints, cells were washed in PBS, fixed in 4% paraformaldehyde in PBS containing 2% sucrose for 10 min at room temperature, post-fixed in 70% ethanol, and stored at −20°C until use.

For cytoskeleton analysis, cells were washed in PBS three times, incubated 5 minutes at RT with 0.1% Triton X-100/PBS and blocked with 1% bovin serum albumin (BSA, Sigma) in PBS for 1 h. Cells were then incubated with the primary monoclonal anti-Tubulin antibody (1:300, diluted in BSA 0.5%/PBS, Abcam) at 4°C overnight. The next day cells were washed 4 times with PBS, incubated for 1 h with the secondary antibody TRITC anti-rabbit 1:200 in BSA 0.5%/PBS (Abcam) in the dark and washed extensively in PBS. For actin detection, cells were then incubated 1h in the dark with Phalloidin 1:1000 in BSA 1%/PBS (Abcam). After the labeling procedure was completed, the coverslips were incubated for 10 min with DAPI and mounted onto glass slides using mowiol mounting medium. Fixed cells were imaged with a ViCo confocal microscope (Nikon) and TCS NT confocal laser scanning microscope (Leica).

### Zymography

ProMMP-2 protein levels were assessed in the supernatants of cultured HMEC-1 cells by SDS-zymography. HMEC-1 cells were seeded on 24-well culture plates at a concentration of 15,000 cells/cm$^2$; after 24h, they were treated with urea as described before. Supernatants of cells treated for 24 and 72h were collected and concentrated in an AmiconY10 at 6500 ×g for 15 min at 4 °C. The concentrated culture media were mixed 3:1 with sample buffer, containing 10% SDS. Four µg total proteins per sample were run under non-reducing/nondenaturing conditions onto 7.5% polyacrylamide gel (SDSPAGE) co-polymerized with 1 mg/ml type I gelatin. The gels were run at 4 °C. After SDS-PAGE, the gels were washed twice in 2.5% Triton X-100 for 30 min each and incubated overnight in a substrate buffer at 37 °C (Tris–HCl 50 mM, CaCl2 5 mM, NaN3 0.02%, pH 7.5). The MMP gelatinolytic activity was detected after staining the gels with Coomassie brilliant blue R250, as clear bands on a blue background. To confirm the identity of MMP gelatinolytic activity, purified MMP-1 and MMP-2 (100 ng, Calbiochem) were run as controls.

### Proteomic analysis of cellular proteins after 72h treatment: sample preparation

In order to perform a quantitative proteomic analysis of cellular proteins, HMEC-1 cells were seeded and treated as described above. The treatment with urea lasted 72 hours, without changing the medium. After removal of medium and three washes with PBS, cellular proteins extracts were obtained lysing cells with the following lysis buffer: 8M Urea, 100 mM Tris-HCl pH 8.5, protease inhibitors (Sigma P8340). Each lysate was incubated for 30 min at RT and centrifuged at 14,000 g for 30 min at 4°C to remove cell debris. BCA protein assay was used to assess protein concentration. In order to check the quality of proteins, a part of the lysate was mixed to Laemmli sample buffer (2% SDS, 20% glycerol, and 125 mM Tris-HCl, pH 6.8), boiled for 5 min at 90°C and separated on 12% SDS-PAGE Stain-free gel (Biorad) [Hill B.G et al., 2009]. Protein gel was acquired using Chemidoc Touch Imaging System (Biorad). The remaining part of each lysate was used to perform tryptic digestion as described later.

### Proteomic analysis of released proteins in the medium after 72h treatment: sample preparation

Beside evaluating protein expression inside the cells, we also assessed changes in the released proteins in the medium during the treatment. For this experiment, HMEC-1 cells were seeded as described above. After 24 hours, all the medium was removed and replaced with a medium without FBS, added or not with urea. This expedient was necessary since the presence of serum in the medium was not compatible with a correct protein separation using Bio-Gel P6 columns (Biorad). The treatment with urea lasted 72 hours, without changing the medium. After 72h, 30 ml of medium were collected for each condition. Media were freeze-dried over-weekend in a lyophilizer (marca). Each lyophilized sample was resuspended in 1.5 ml MS-grade water allowing a 20X concentration factor. In order to isolate proteins, samples were processed using Bio-Gel P6 columns (Biorad), according to manufacturer's instructions. A further concentration (factor 10X) of the sample was obtained using a Savant SpeedVac Concentrator. The protein content of the concentrated (200X) fraction of control and urea treated medium were assayed with BCA protocol. In order to check the quality of proteins, a small amount of each sample was mixed to Laemmli sample buffer (2% SDS, 20% glycerol, and 125 mM Tris-HCl, pH 6.8), boiled for 5 min at 90°C and separated on 12% SDS-PAGE Stain-free gel (Biorad) [Hill B.G et al., 2009]. Protein gel was acquired using Chemidoc Touch Imaging System (Biorad). The remaining part of each sample was digested as described later.

### Proteomic analysis of cellular proteins and released proteins in the medium after 72h treatment: protein sample digestion and analysis

Both cellular proteins and released proteins in the medium samples underwent the following treatment to allow the protein digestion. 10 µg of proteins were mixed with 36 µl of 50 mM ammonium bicarbonate (AMBIC) dissolved in MS-grade water (Sigma). pH was checked to ensure that it was around pH 8 – 8.5. Then 5mM dithiothreitol (DTT, diluted in AMBIC) was added and the samples were incubated in a Thermomixer at 600 rpm, 52°C for 30 minutes. At this point 15 mM iodoacetamide (IAM, diluted in AMBIC) was added and the samples were incubated in a Thermomixer at 600 rpm, RT for 20 minutes, in the dark. 0.5 µg trypsin in 50 mM acetic acid was added (after activation for 15 minutes at 30°C) respecting a ratio 1:20 trypsin:proteins. Samples were incubated in a Thermomixer at 600 rpm, 37°C overnight. The day after, 2 µl of 50% trifluoroacetic acid (TFA, diluted in MS-grade water) were added and the pH was checked to ensure that it was lower than pH 2. In order to check the quality of proteins and to perform a Western blot for validating the results, a small amount of each sample was mixed to Laemmli sample buffer (2% SDS, 20% glycerol, and 125 mM Tris-HCl, pH 6.8), boiled for 5 min at 90°C and separated on 12% SDS-PAGE Stain-free gel (Biorad) [Hill B.G et al., 2009]. Protein gel was acquired using Chemidoc Touch Imaging System (Biorad). The remaining part of each sample was digested as described later.

### High resolution mass spectrometry analysis (nLC-MSMS)

Tryptic peptides were analyzed at UNITECH OMICs (University of Milano, Italy) using a Dionex Ultimate 3000 nano-LC system (Sunnyvale CA, USA) connected to an Orbitrap Fusion™ Tribrid™ Mass Spectrometer (Thermo Scientific, Bremen, Germany) equipped with a nano-electrospray ion source. Peptide mixtures were pre-concentrated onto an Acclaim PepMap 100 - 100 mm_ 2 cm C18 and separated on EASY-Spray column,15 cm _ 75 mmID packed with Thermo Scientific Acclaim PepMap RSLC C18, 3 mm, 100 Å. The temperature was set to 35 _C and the flow rate was 300 nL min_1.
Mobile phases were the following: 0.1% Formic acid (FA) in water (solvent A); 0.1% FA in water/acetonitrile (solvent B) with 2/8 ratio. Peptides were eluted from the column with the following gradient: 4%-28% of B for 90 min and then 28%-40% of B in 10 min, and to 95% within the following

6 min to rinse the column. Column was reequilibrated for 20 min. Total run time was 130 min. One blank was run between triplicates to prevent sample carryover. MS spectra were collected over an m/z range of 375-1500 Da at 120,000 resolutions, operating in the data dependent mode, cycle time 3 s between master scans. HCD was performed with collision energy set at 35 eV. Each sample was analyzed in three technical triplicates. LTQ raw data was searched against a protein database using SEQUEST algorithm in Proteome Discoverer software version 2.2 (Thermo Scientific) for peptide/protein identification. The searches were performed against Uniprot KnowledgeBase (KB) (taxonomy Homo sapiens). The minimum peptide length was set to six amino acids and enzymatic digestion with trypsin was selected, with maximum 2 missed cleavages. A precursor mass tolerance of 8 ppm and fragment mass tolerance of 0.02 Da were used; acetylation (N-term), oxidation (M) were used as dynamic modifications and carbamidomethylation (C) as static modification. The false discovery rates (FDRs) at the protein and peptide level were set to 0.01 for highly confident peptide-spectrum matches and 0.05 for peptide-spectrum matches with moderate confidence.
We considered only proteins with a score of coverage >2% with at least two identified peptides. Differences in abundance ratio (AR) of proteins between control and treated samples were considered only with at least a 2-fold change and with a standard deviation between replicates less than 20%.

## MS data analysis – Label-free quantitative proteomics

Resulting MS raw files from all the technical and biological replicates were analyzed by using Proteome Discoverer software (Version 2.4.0.305) based on SEQUEST algorithm as database search engine. Database search against the latest Human UniProtKB/SwissProt FASTA files Release (UniProt release 2019_11 - December 18, 2019) was performed according to the following parameters: Trypsin was specified as proteolytic enzyme, cleaving after lysine and arginine except when followed by proline. Up to two missed cleavages were allowed. The precursor ion tolerance was set to 10 ppm and the fragment tolerance was set to 0.6 Da. Carbamidomethylation of cysteine was defined as fixed modification, while oxidation of methionine and acetylation at the protein N-terminus were specified as variable modifications. The false discovery rate (FDR) for peptide identification was calculated using the Percolator algorithm in the Proteome Discoverer workflow based on the search results against a decoy database and was set at 1% FDR. Identified peptides were quantified by a typical Processing workflow for Minora feature detection, based on the quantification of isotopic clusters regardless of whether or not they are associated with a PSM. The RT alignment was performed with a maximum RT shift of 10 min. For quantification all unique and razor peptides were considered, and the normalization of intensity values was performed over precursor (consensus features) against the total peptide amount. Samples were previously categorized by the cell line (cell-line 1 – 21) and by the treatment type (control – urea 5 g/l, urea 0.25 g/l – urea 5 g/l), and for the identification of differentially regulated proteins quantification jobs were alternatively launched using the individual ratios option. Ratio calculation was based on Pairwise Ratio based Approach using summed abundances for single proteins abundance calculations. Proteins were grouped applying strict parsimony principle and filtered at a 1% FDR at the protein level, and further categorized on the basis of annotation aspects (Biological Process, Molecular Function and Cellular Components).

## Validation of proteomic results of released proteins in the medium after 72h treatment: vasorin Western blot

In order to validate proteomic results, a small amount of each sample of proteins released in the medium (after it has been concentrated as described before) was mixed to Laemmli sample buffer (2% SDS, 20% glycerol, and 125 mM Tris-HCl, pH 6.8), boiled for 5 min at 90°C and separated on 12% SDS-PAGE Stain-free gel (Biorad) [Hill B.G et al., 2009]. After washing three times with TBST for 5 min, membrane was incubated overnight with 5% non-fat dry milk/TBST containing the

following primary antibody: anti-Vasorin (1:5000, Abcam). The membrane was washed three times with TBST for 5 min and then incubated for 1h with the following secondary antibody: anti-rabbit (1:5000). Vasorin was visualized by ECL detection (cod.1705061, Biorad) using Chemidoc Touch Imaging System (Biorad).

# Results

## Urea alters growth rate of HMEC-1 cells only after 72 hours of exposure

The growth of cultured HMEC-1 cells were followed up to 72 h. By using SRB assay (*Figure 1*), we observed that only the exposure for 72h to the highest urea concentration tested (5 g/l), measurable in pathologic conditions, caused a significative reduction in the cells proliferation rate ($p < 0.05$).
Control HMEC-1 cells and cells treated with urea 0.25 gr/l and 2 gr/l grew exponentially over three days, whereas cells treated with urea 5 gr/l showed a progressive reduction in growth over time.

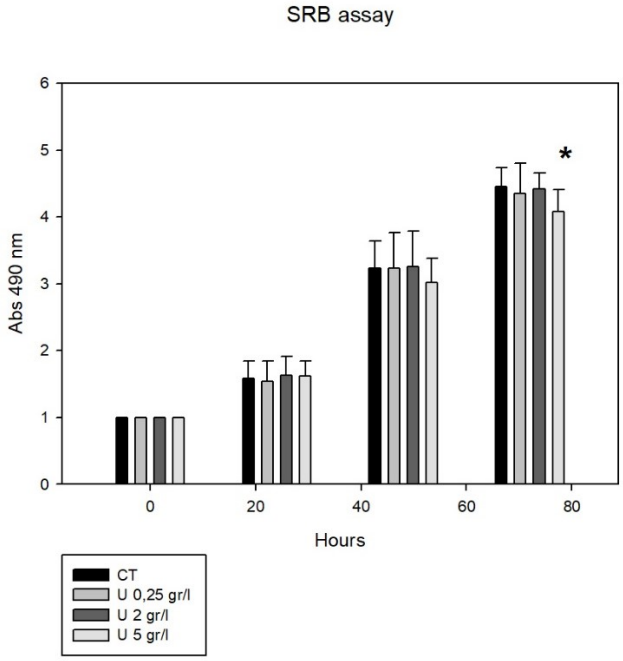


*Figure 1. Effects of urea treatment on cell proliferation. Histogram showing the mean absorbance measured at 490 nm in control cells and cells treated with 0.25 – 2 – 5 gr/l of urea for 0 -24 -48 – 72 hours. Data are expressed as the mean ± SD. *p < 0 05.*

## Urea induces oxidative stress only within the first 24 hours of exposure

Uremia is correlated to oxidative stress [Vaziri et al., 2004]. More in detail, uremic toxins seem to contribute to CVD onset and progression in CKD, exacerbating problems as oxidative stress and inflammation, that are non-traditional risk factors for CVD [Kendrick et al., 2008]. We evaluated urea-induced oxidative stress using protein carbonylation and oxidation of protein thiols as biomarkers. We didn't find differences in protein carbonylation over the treatment (data don't shown). We found a tendency toward reduction in the total amount of protein thiols at 24 hours in cells treated with pathologic concentrations of urea (2 – 5 gr/l), with a significative difference when compared to control cells only in cells treated with urea 2 gr/l (*Figure 2*). It is known that oxidative stress leads to the formation of unwanted disulfide bonds in the cytoplasm, resulting in a lowering in

the total amount of thiols, eventually leading to impaired protein function. Nevertheless, thiols oxidation-reduction is reversible and it seems that urea-induced oxidation at 24h is restored in the following timepoints.

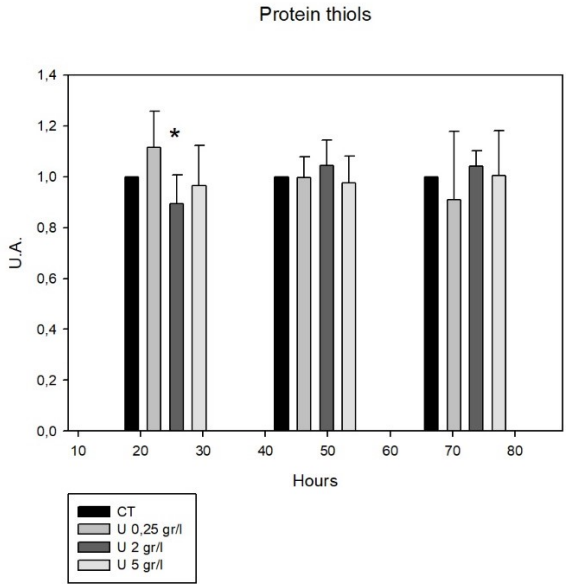


*Figure 2. Effects of urea treatment on the total amount of protein thiols. Histogram showing the protein thiols level measured in control cells and cells treated with 0.25 – 2 – 5 gr/l of urea for 0 -24 - 48 – 72 hours. Data are expressed as the mean ± SD. *p < 0 05.*

## Urea alters actin filaments organization

Since both microtubules and microfilaments rearrangement may contribute to endothelial dysfunctions [Hirase et al., 2012; Sun et al., 2015], we evaluated tubulin and actin expression in HMEC-1 treated with urea. According to WB results, tubulin expression didn't change over the treatment (data don't shown), while actin amount appeared to decrease in a concentration-dependent manner when cells are exposed to urea, even if differences weren't statistically significant (*Figure 3*). This observation was corroborated by immunofluorescence results. In fact, comparing control cells with cells treated with urea 5 gr/l at 72h, actin signal in treated cells is weaker. In addition, in control cells actin is organized in randomized arrays, while in urea 5 gr/l cells it forms peripheral bands and actin filaments appears to be broke apart in the middle (*Figure 4 - 5*).

Seen this alteration, we evaluated also some proteins junction expression. A cytoskeleton modification is often accompanied by a modification in junctional proteins, all contributing to alter endothelial barrier permeability [Rho et al., 2017]. However, we didn't find significant differences nor in VE-cadherin or in beta-cathenin expression (data don't shown).

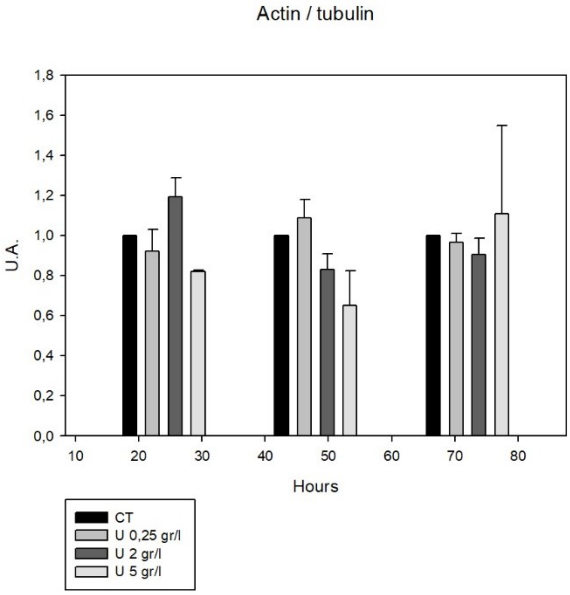


*Figure 3. Effects of urea treatment on the actin amount. Histogram showing actin level measured in control cells and cells treated with 0.25 – 2 – 5 gr/l of urea for 0 -24 -48 – 72 hours. Tubulin was used ad housekeeping. Data are expressed as the mean ± SD. *p < 0 05.*

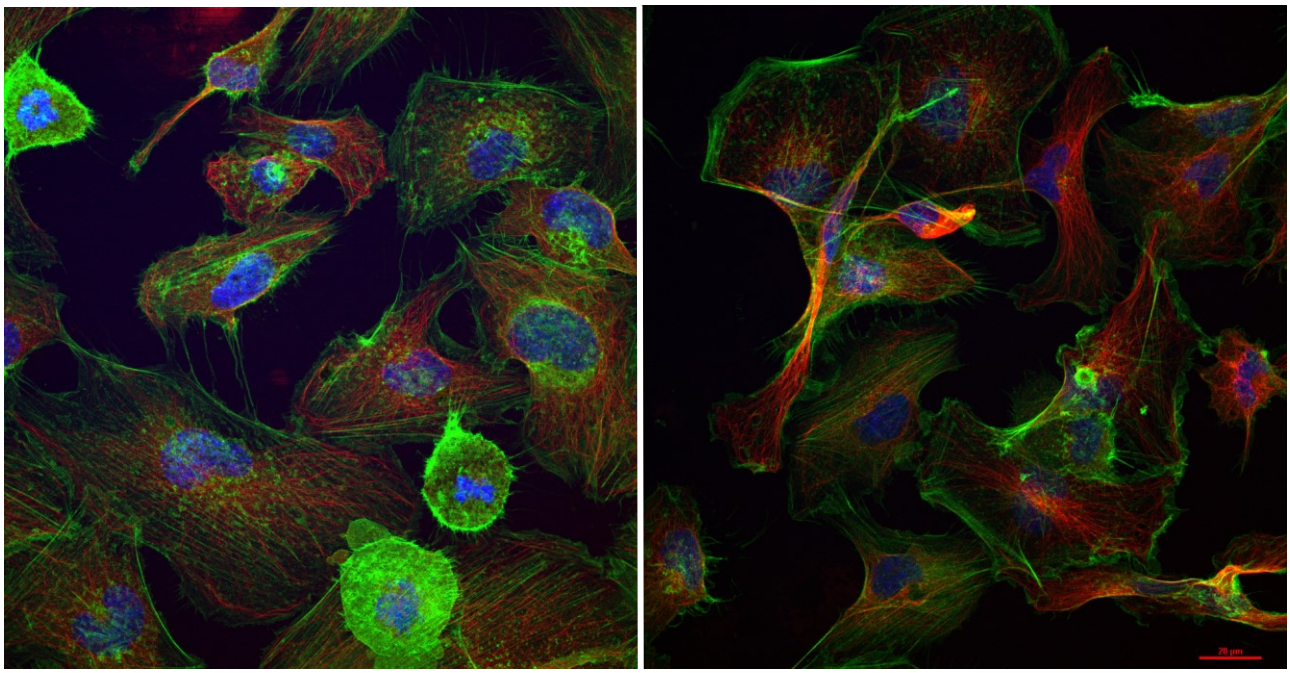

*Figure 4. Effects of urea treatment on the cytoskeleton organization. On the left, control cells at 72h. On the right, cells treated with 5 g/l of urea at 72h. Images acquired with TCS NT confocal laser scanning microscope (Leica).*

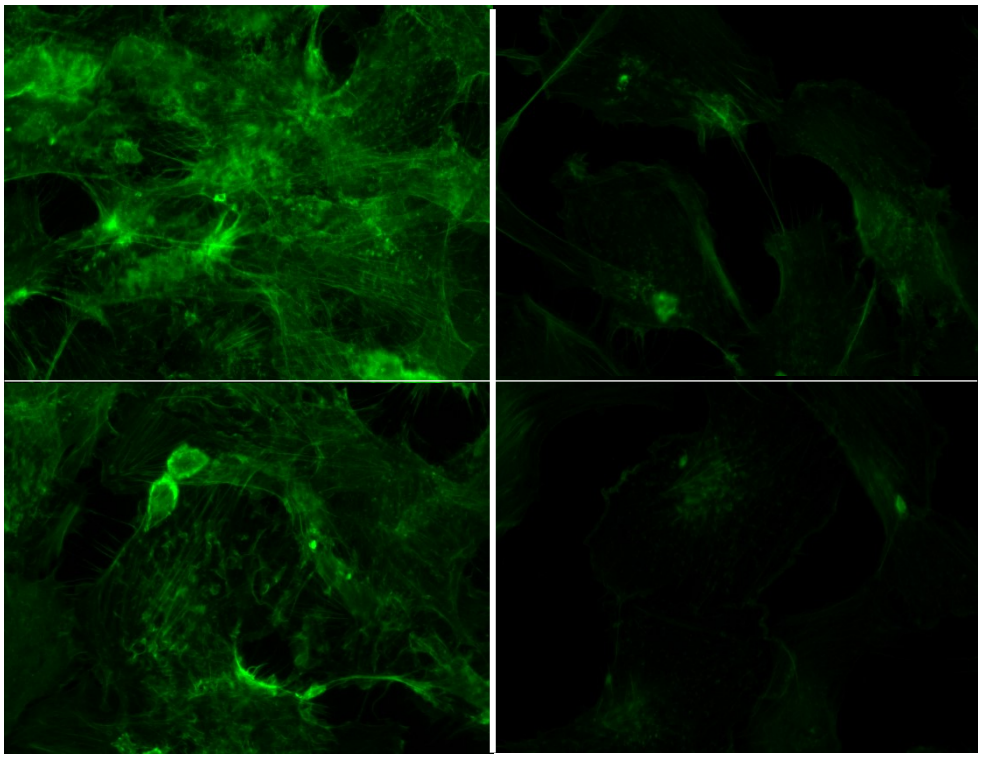

*Figure 5. Effects of urea treatment on the actin organization. On the left, control cells at 72h. On the right, cells treated with 5 gr/l of urea at 72h. Images acquired with ViCo confocal microscope (Nikon).*

## Urea induces endothelial to mesenchymal transition (EndoMT)

Endothelial to mesenchymal transition (EndoMT) is implicated in the pathogenesis of several CVD [Gong et al., 2017]. Remarkable morphological rearrangement of the actin filaments is a feature of EndoMT [Piera-Velazquez et al., 2019], so we decided to evaluate the presence of other biomarkers of this potential transdifferentiation. In particular, we measured matrix metalloproteinase, MMP-2, through zymography [Toth et al., 2012]. After 72h of urea treatment, urea increases MMP-2 release in the medium, in a concentration-dependent way (*Figure 6*). We observed a difference statistically significant comparing control cells and urea 5 gr/l treated cells at 72h ($p < 0,05$), and comparing urea 0,25 gr/l treated cells and urea 5 gr/l treated cells at 72h ($p<0,05$). So urea may represent a stimulus for EndoMT, which in turn could play a role in CVD initiation or progression.

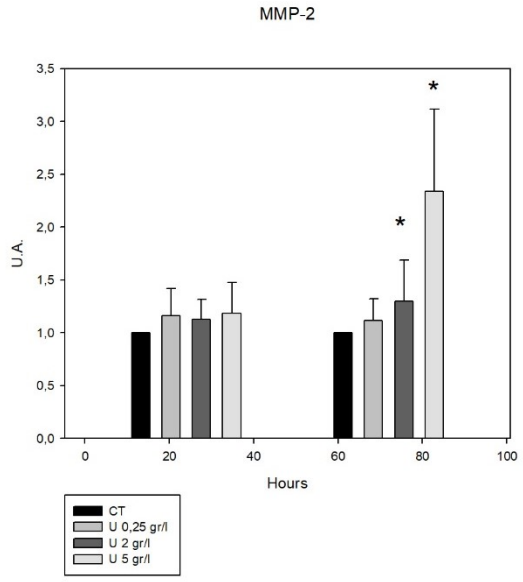


*Figure 6. Effects of urea treatment on the release of MMP-2 in the medium. Histogram showing MMP-2 level measured in control cells and cells treated with 0.25 – 2 – 5 gr/l of urea for 0 -24 -48 – 72 hours. Data are expressed as the mean ± SD. *p < 0 05.*

## Urea weakly affects proteins expression

For the proteomic analysis, we decided to focus on the comparison between cells treated with urea 0.25 g/l, the mean of the physiologic concentration measurable in healthy subjects, and cells treated with urea 5 g/l, a concentration measurable in CKD patients. We did not focus on the comparison between control and treated cells, since the control condition does not exist physiologically. The volcano plot (*Figure 7*) shows that only a few proteins resulted to be up or down-regulated when comparing cells treated with urea 0.25 g/l vs urea 5 g/l. To verify if these proteins were linked according to their functions, we performed an analysis with STRING obtaining the network of up-regulated proteins (*Figure 8*) and the network of down-regulated proteins (*Figure 9*). The networks do not have significantly more interactions than expected, according to STRING lambda calculation. This means that our sets of proteins are composed by an apparently random collection of proteins that are not very well connected, or whose interactions are not still known by STRING upon the available data.

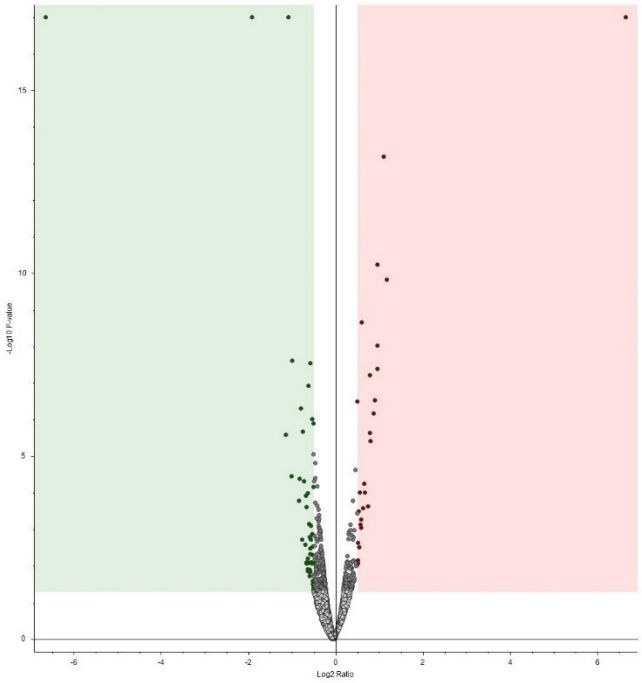


*Figure 7. Volcano plot which compares protein expression of cells treated with 0.25 gr/l of urea vs 5 gr/l of urea for 72h. Down-regulated proteins are in green. Up-regulated proteins are in red.*

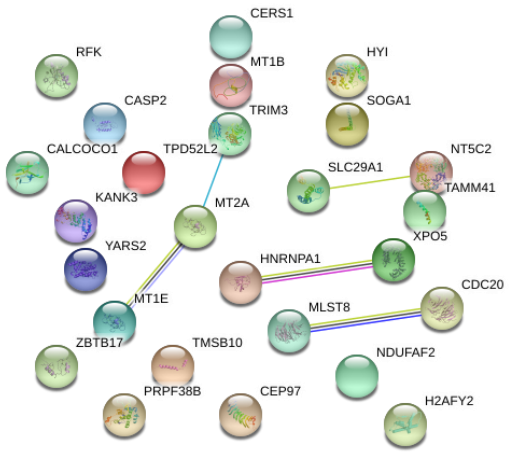


*Figure 8. Network of the up-regulated proteins obtained with the software String. The network comprehends the proteins that emerged as up-regulated in cells treated with 5 gr/l of urea when compared to cells treated with 0.25 gr/l of urea, at 72 h.*

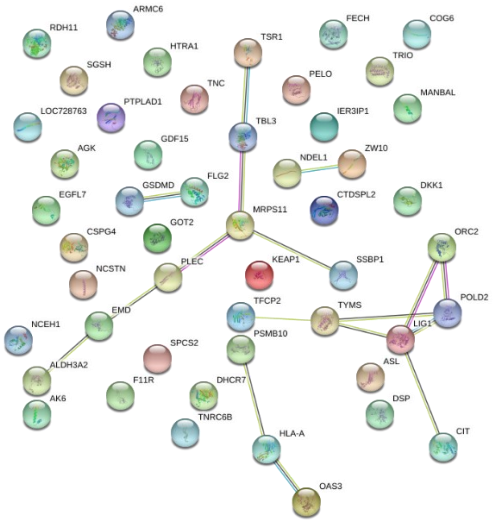


*Figure 9. Network of the down-regulated proteins obtained with the software String. The network comprehends the proteins that emerged as down-regulated in cells treated with 5 gr/l of urea when compared to cells treated with 0.25 gr/l of urea, at 72 h.*

## Urea weakly affects secretoma proteins

For the proteomic analysis of protein released in the medium, we decided to focus on the comparison between control cells and cells treated with urea 5 g/l, a concentration measurable in CKD patients. The volcano plot shows that only a few proteins found in the medium resulted to be up or down-regulated when comparing control cells and cells treated with urea 5 g/l (figure not reported). To verify if these proteins were linked according to their functions, we performed an analysis with STRING obtaining the network of up-regulated proteins (*Figure 10*) and the network of down-regulated proteins (*Figure 11*). The networks do not have significantly more interactions than expected, according to STRING lambda calculation. This means that our sets of proteins are composed by an apparently random collection of proteins that are not very well connected, or whose interactions are not still known by STRING upon the available data.

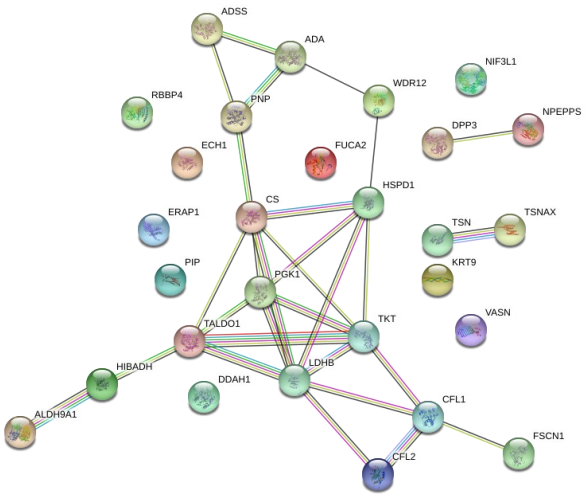


*Figure 10. Network of the up-regulated proteins in the medium obtained with the software String. The network comprehends the supernatant proteins that emerged as up-regulated in cells treated with 5 gr/l of urea when compared to control cells, at 72 h.*

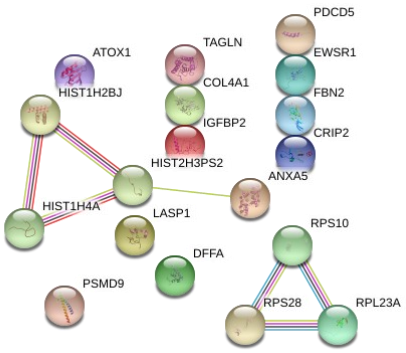


*Figure 11. Network of the down-regulated proteins in the medium obtained with the software String. The network comprehends the supernatant proteins that emerged as down-regulated in cells treated with 5 gr/l of urea when compared to control cells, at 72 h.*

## Urea induces a significative down-regulation of vasorin expression

Vasorin is a transmembrane glycoprotein with a lot of function yet to know. It has recently been proposed as potential biomarker for nephropathies and tumorigenesis [Bonnet et al., 2018]; it is involved in acute arterial injury and in age-associated vascular remodeling [Pintus et al., 2018]; it protects vascular smooth muscle cells against calcification and osteo-/chondrogenic transdifferentiation [Luong et al., 2019]; it plays a role in TGFß-mediated epithelial-to-mesenchymal transition [Malapeira et al., 2011]. So vasorin appears to be linked both with kidneys pathologies and CVD. In our proteomic analysis of released proteins in the medium, comparing control cells and urea 5 gr/l medium, after 72h of treatment, vasorin resulted to be downregulated in urea medium with a fold decrease of 9,03. Therefore we decided to validate its down-regulation also by WB. WB results confirmed a significative decrease in vasorin expression in the medium of cells treated with urea 5 gr/l for 72h, when compared to control cells medium, with around a 188 fold decrease (*Figure 12 – 13*).

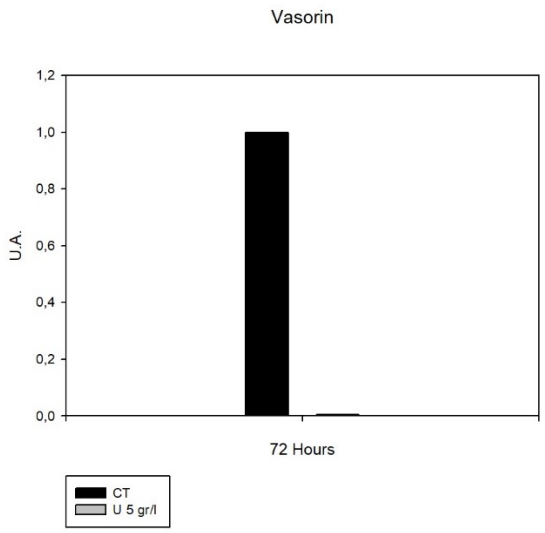


*Figure 12. Effects of urea treatment on vasorin expression. Histogram showing vasorin level measured in control cells and cells treated with 5 gr/l of urea for 72 hours.*

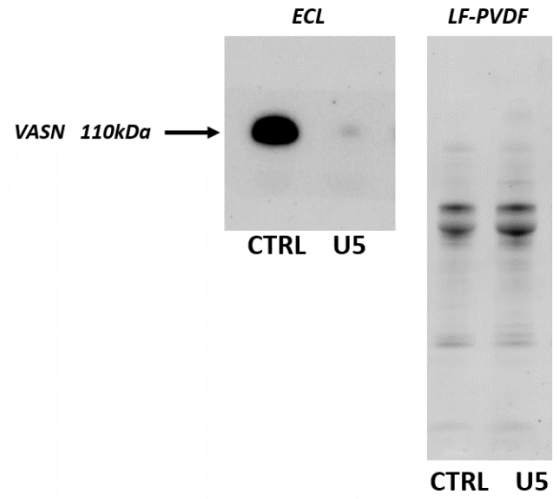


*Figure 13. Effects of urea treatment on vasorin expression. SDS-page and Western blot showing vasorin level assessed in control cells and cells treated with 5 gr/l of urea for 72 hours.*

## Discussion and conclusions

Chronic Kidney Disease is a non-communicable disease with a world prevalence of 8-16% and the WHO declared it as a public health problem which is continually increasing [Jha et al., 2013]. It is diagnosed when there's a decreased kidney function shown by glomerular filtration rate (GFR) less than 60 mL/min (established for a reference man with 1.73 $m^2$ body surface area), or markers of kidney damage, or both, of at least 3 months duration [Webster et al., 2016]. Patients are classified in stages (1-5) according to their GFR values. Complications and morbidities increase in parallel with the GFR decline. Among them there are cardiovascular diseases, acute kidney injury, bones disorder, minerals balance disorder, hospitalization, anemia, oxidative stress, chronic inflammation and dysbiosis [Stevinkel et al., 2008]. The decreased kidney function is responsible for the retention of several molecules, which normally are in part eliminated through faeces and in part cleared by kidneys, so they accumulate in CKD and are called uremic toxins [Evenepoel et al., 2009]. Interest in uremic toxins is continually enhancing since they have been recognized as causes of a lot of side effects [Bouré et al., 2004; Yavuz et al., 2005].

Urea represent the uremic toxin with the highest concentration [Vanholder et al., 2003], whose toxicity is still discussed [Lau et al., 2017]. Nevertheless clinical treatments for CKD patients have focused for years on this molecule and the possibilities to lower its concentration in the plasma, with promising results. In this regard, it's worth remembering that both conservative therapy (for CKD stages 1-4) and renal replacement therapy (for CKD stage 5) aim at preserving or substituting kidney functions not only but also lowering blood urea concentration. In conservative therapy, the nutritional therapy is designed firstly to reduce the total amount of protein intake in order to decrease urea generation. Many studies show that this approach exerts several advantages, as the delay of CKD progression, because it leads to metabolic effects that help preserving kidney functions and controlling uremic symptoms [Di Micco et al., 2019; Ko et al., 2017; Shah et al., 2016]. Renal replacement therapy (hemodialysis or peritoneal dialysis) is prescribed to achieve target values of urea clearance and its adequacy is assessed measuring urea levels [Canaud et al., 2000]. Reaching the established urea values appears to improve patients outcomes [Depner et al., 2001; Meyer et al., 2007], even if it doesn't completely relieve uremic symptoms, since dialysis is not able to remove all uremic toxins, whose retention causes the so called "residual syndrome" [Meyer et al., 2014].

Urea seems to exert toxic effects on gut, kidneys, adipocytes, blood components and on CVS [Lau et al., 2017]. About CVS, high levels of urea have been correlated to a higher rate of mortality and hospitalization in CKD patients with CVD [Mok et al., 2017], and to a higher level of cLDL, that represent a pro-atherogenic factor [Apostolov et al., 2005]. So overall it seems that urea could contribute, at least in part, to the increased risk of CVD observed in CKD patients when compared the normal population [Jha et al., 2013]. Although, results interpretation in clinical trials is complex since CKD patients have multiple comorbidities and urea acts together with the other toxins. So, in these study, we tried to better elucidate urea effects potentially linking urea with CVD, exposing cells to physiologic concentrations of this toxin. We tested them on an endothelial cell line from the microvasculature, since literature lacks of works evaluating urea effects in the microcirculation, even though it is the principal seat of exchanges between circulation and tissues.

We decided to tested the fallowing concentration: 0,25 – 2 – 5 gr/l, after consulting the European Uremic Solutes Database (EUTox-DB), which reports the fallowing concentrations: 0.30 (+/-0.12) (0.30-0.40) g/l grand mean in healthy subjects; 1.57 (+/-0.64) (1.30-4.60) g/l grand mean in uremic patients.

Urea appears to affect cell proliferation. We observed that the exposure for 72h to the highest concentration of urea tested (5 gr/l) caused a significative reduction in the cells proliferation rate ($p < 0.05$). This phenomenon could depend on a pro-apoptotic effect of urea. In a recent study, Trécherel et al. showed an increase in BAD [B-cell lymphoma 2 (BCL2)-associated death promoter] expression, a pro-apoptotic member of the BCL2 family, in human aortic smooth muscle cells exposed to urea 20 mM (equal to 1,2 g/l) [Trécherel et al., 2012]. Apoptosis of the arterial cells is a key event in vascular remodeling and in the progression of atherosclerosis, and CKD patients show an increased apoptosis in their arterial wall [Shroff et al., 2008]. So urea pro-apoptotic capability could participate to vascular medial calcification.

Non-traditional uremic risk factors for vascular dysfunction and calcification include chronic oxidative stress and inflammation [Covic et al., 2010; Lau et al., 2013]. Patients with CKD show high oxidative stress level and uremic toxins contribute to this phenomenon [Chao et al., 2014]. In particular, an incubation with urea 20mM resulted in an increased reactive oxygen species (ROS) production both in human aortic endothelial cells [D'Apolito et al., 2015], in human arterial endothelial cells [D'Apolito et al., 2018] and in 3T3-L1 adipocytes [D'Apolito et al., 2010]. We evaluated two well recognized oxidative stress biomarkers after 24, 48 and 72 hours of urea exposure: protein carbonylation and the total amount of protein thiols [Dalle Donne et al., 2006]. Regarding protein carbonylation, we didn't find significant differences over treatment (data don't shown). Looking at protein thiols, we found a tendency toward reduction in the total amount of protein thiols at 24 h in cells treated with pathologic concentrations of urea (2 – 5 gr/l), with a significative difference when compared to control cells only in cells treated with urea 2 gr/l. Since thiols oxidation-reduction is a reversible modification, we could hypothesize that over time some antioxidant systems start in the cells, explaining the trend observed in the following timepoints.

Another emerging risk factor for CVD is endothelial to mesenchymal transition (EndoMT) phenomenon. EndoMT is a complex biological process during which endothelial cells start to express mesenchymal cell-specific proteins and progressively reduce the expression of endothelial cell-specific proteins [Atkins et al., 2011; Arciniegas et al., 2007]. So they undergo a transition toward a more mesenchymal-like phenotype, mainly induced by the members of the transforming growth factor-β (TGF-β) family. EndoMT is implicated in the pathogenesis of several diseases as malignant diseases, fibrotic diseases, pulmonary arterial hypertension, atherosclerosis, diabetes mellitus, cavernous malformation, fibrodysplasia ossificans progressiva [Piera-Velazquez et al., 2019]. To investigate this potential transition, we evaluated cells morphology and cytoskeleton organization, proteins junction expression and the total amount of MMP-2 in the supernatants.

Concerning the cytoskeleton, we observed a reduction in actin expression both by WB and by immunofluorescence (even if differences by WB were not statistically significative) in cells treated with the pathologic concentrations of urea. In addition, after 72h of incubation with urea 5 gr/l, actin filaments appear to be broke apart in the middle, rather than organize in randomized arrays. So urea appear to induce a morphological rearrangement of the actin filaments, that is a feature of EndoMT [Piera-Velazquez et al., 2019].
Seen this alteration, we evaluated also some proteins junction expression. A cytoskeleton modification is often accompanied by a modification in junctional proteins, all contributing to alter endothelial barrier permeability [Rho et al., 2017]. In addition, junctional proteins, as VE-cadherin, are among those proteins whose expression decrease or disappear during EndoMT, which is characterized by loss of cell-cell junctions and polarity [Sánchez-Duffhues et al., 2018]. However, we didn't find significant differences nor in VE-cadherin or in beta-cathenin expression (data don't shown). This result could depend on a limit of our *in vitro* model: HMEC-1 never reach confluency and when too numerous, they arrange in more than one layer, rather than form a well-organized monolayer.
Other EndoMT features are the acquisition of cellular motility and invasive properties. In this regard, we measured the amount of MMP-2, through zymography. Matrix metalloproteinases are able to degrade extracellular matrix proteins, playing important roles in vascular tissue remodeling processes. They can influence cell migration, proliferation, contraction and calcium signaling [Cui et al., 2018]. MMPs alterations are correlated with CVD as hypertension, atherosclerosis, excessive venous dilation and lower extremity venous disease [Wang et al., 2017]. After 72h of urea treatment, urea increase MMP-2 release in the medium, in a concentration-dependent way.
So urea may represent a stimulus for EndoMT, which in turn could play a role in CVD initiation or progression.

In order to deepen protein expression modifications in our cell model, we performed a proteomic analysis, evaluating proteins both inside and outside the cells (proteins released in the medium). In 2008 Carbo et al. compared protein expression in HUVECs treated with uremic serum, that was serum of patients undergoing hemodialysis, or with normal serum, of healthy subjects. Mainly they found differential expression in proteins link to inflammation, oxidative stress and cytoskeleton [Carbo et al., 2008]. Uremic serum contains all the uremic toxins. With our proteomic job we systematically studied the differential protein expression induced by a single uremic toxin, urea, with the aim to better elucidate the effects linked specifically with this molecule.
The volcano plots show that only a few proteins resulted to be up- or down-regulated by the highest urea concentration (5 gr/l), when compared to the lowest one (urea 0.25 g/l, in the analysis of cellular proteins), or with control cells (in the analysis of released proteins in the medium) after 72h of treatment. Trying to better understand the ratio under the differential protein expression, we analyzed the proteomic data with the software String, gathering proteins according to their functions. The resulting networks did not show pathways markedly influenced by urea 5 gr/l treatment, when looking at protein expression both inside or released by HMEC-1.
Looking individually at each protein whose expression resulted to be significantly up- or down-regulated by urea 5gr/l, we figured out some proteins released in the supernatants particularly interesting.
Annexin A5 and programmed cell death are among those proteins significantly over-expressed in the medium of cells treated with urea 5 gr/l. They may corroborate the hypothesis that urea could have pro-apoptotic effects, as observed by Tréchel et al [Trécherel et al., 2012]. In fact, annexin A5 detection is employed in molecular imaging technology assays in order to reveal apoptotic cells. Annexin A5 binds with high-affinity to negatively charged phospholipids such as phosphatidylserine (PS), that is expressed on the cell membrane of apoptotic cells as an "eat me" signal for phagocytes [Kenis et al., 2007]. Programmed cell death is defined as an apoptosis-accelerating protein. Its overexpression cannot directly induce cell apoptosis, but it can enhance it [Li et al., 2016]. Apoptosis phenomenon plays an important role in the loss of cells during myocardial infarction and heart failure

[Garg et al., 2003] and it is strongly correlated with the development of atherosclerotic plaque vulnerability [Kolodgie et al., 200]. Annexin A5 has been purposed as an imaging biomarker of cardiovascular risk since preclinical and clinical studies evidenced that PS exposure in the cardiovascular system is an attractive biological target in atherosclerosis, heart failure, and cardiac ischemia [Laufer et al., 2008]. It could be interesting deepen annexin A5 over-expression induced by urea and urea pro-apoptotic effects, trying to elucidate a possible mechanism which links urea and CVD.

Keratin, fibrillin-2 and collagen IV are other proteins differentially expressed in the medium of cells treated with urea 5 gr/l; they support the hypothesis that urea may represent a stimulus for EndoMT. Keratin is down-regulated after urea treatment and a loss of keratin is an hallmark of epithelial to mesenchymal transition (EMT) [Roth et al., 2012]. The alteration or the disruption of the keratin cytoskeleton makes epithelia susceptible to tissue damage and various stresses [Roth et al., 2012], and it leads to an increased migration of cancer cells [Kim et al., 2015]. Fibrillin-2 is up-regulated by urea treatment. It is a cysteine-rich glycoprotein which supports structures essential to maintain tissue integrity and which regulates signaling events [Ramirez et al., 2010]; it is employed as a marker of EMT induced by TGFß [Boyer et al., 1999]. Also collagen IV resulted to be up-regulated by urea exposure. It is a principal component of epithelial basement membranes where it organizes in sheet-like network. It is produced by mesenchymal cells and by cancer cells that have undergone EMT [Revert et al., 2018]. It is an EMT biomarker, since it contributes to the microenvironment remodeling typical of cells after EMT [Smith et al., 2016]. So these data corroborate the hypothesis that urea could induce EndoMT, a phenomenon which in turn could link urea and CVD [Gong et al., 2017].

Maybe the two most interesting proteins that we found significantly downregulated in supernatants of cells treated with urea 5 g/l compared to supernatants of control cells are dimethylarginine dimethylaminohydrolase (DDAH) and vasorin (VASN). Both these proteins have been directly linked to CVD by *in vitro* and *in vivo* studies.

DDAH is the enzyme responsible for the degradation of asymmetric dimethylarginine (ADMA). In turn, ADMA is an important regulator of nitric oxide (NO) production. A downregulation of DDAH leads to high level of ADMA, that is considered a CVD risk factor [Anderssohn et al., 2010]. DDAH downregulation or knockdown causes endothelial dysfunction, increases systemic vascular resistance and elevates systemic and pulmonary blood pressure [Leiper et al., 2007]. Interestingly, ADMA is not only a CVD risk factor, but also a CKD progression risk factor, making DDAH an important regulator of renal and vascular function integrity. Recently, enhancing DDAH activity has been purposed as a novel therapeutic strategy to prevent CVD and CKD progression [Palm et al., 2007]. CKD patients show high levels of ADMA [Tripepi et al., 2011], explained by higher levels of protein methylation; increased rate of protein turnover; impaired activity of DDAH; impaired renal excretion [Jacobi et al., 2008]. Today, we can speculate another cause to add to this list that is higher levels of urea, since the treatment with only this molecule seems to be able to induce a strong downregulation of DDAH, that will result in an increase of ADMA level. Other studies are needed to confirm this hypothesis, which could link urea to CVD and CKD progression.

Vasorin is a transmembrane glycoprotein whose functions are not still well elucidated. It directly binds to transforming growth factor beta (TGF-ß) and it attenuates TGF- ß signaling *in vitro* [Ikeda et al., 2004]. In addition, it stabilizes Notch1 protein at the cell membrane, influencing its pathways [Man et al., 2018].

VASN expression appears to be altered in several diseases: it is higher than normal in synovial fluid of patients with osteoarthritis [Liao et al., 2018], in plasma of patients with diabetic nephropathy [Ahn et al., 2010], in serum of subjects with hepatocarcinoma [Li et al., 2015], in urinary exosomes of patients with thin basement membrane nephropathy (TBMN); on the contrary, VASN is lower than normal in subjects with early IgA nephropathy (IgAN) [Moon et al., 2011; Samavat et al., 2015]. Our proteomic analysis of supernatants of cells treated or not with urea showed an important downregulation of vasorin expression in the medium of cells treated with urea 5 g/l. After, we confirmed this difference

also by WB analysis. VASN downregulation was previously observed also *in vivo*, after vascular injury: as consequence the expression of several cytokines, including TGF- ß, was upregulated, leading to neointimal formation, the typical fibroproliferative response to vascular injury [Malapeira et al., 2011]. Reverting vasorin downregulation significantly diminished injury-induced vascular lesion formation. For these reasons, VASN has been purposed as potential therapeutic target for vascular fibroproliferative disorders [Ikeda et al., 2004]. VASN is also linked to EMT. Vasorin protein is effectively cleaved by activated MMP-2 both *in vitro* and *in vivo*. In particular, a metalloprotease containing A Disintegrin And a Metalloprotease domain (ADAM) 17 tightly controls VASN secretion [Malapeira et al., 2011]. Only soluble VASN is able to bind TGF-ß. We observed a significant increase in MMP-2 amount in cells treated with urea, so we could hypothesize that they contributed to increase soluble VASN level in the supernatant. VANS in this form is able to interfere with TGF- ß – mediated EMT, modulating E-cadherin expression and actin organization [Li et al., 2018], phenomenon observed also in HMEC-1. In addition, VASN is able to modulate collagen expression [Ikeda et al., 2004], another EMT marker that we found to characterize urea treated cells through the proteomic approach. So VASN downregulation, promoting TGF- ß pathway, could represent a EndoMT stimulus. Since ADAM17 is the principal metalloproteases controlling VASN cleavage, it could be interesting evaluating if ADAM17 modulation is able to prevent EndoMT phenomenon.

We can conclude that urea in HMEC-1, at the concentration tested, seems to affect cells proliferation and actin organization, together with inducing EndoMT transition. Also the proteomic analysis confirm a dysregulation in proteins expression involved pro-apoptotic pathways and in the EndoMT process, together with the alteration of some proteins directly linked to CVD . It could be useful a further in-depth analysis to better elucidate these proteins pathways and the effects of their dysregulation.
The modest effects of urea observed in our study, sometimes in contrast with literature, could be explained by the choice of urea concentrations belonging only to a range measurable physiologically. In addition, we added the toxin to the cell medium the first day and evaluated the effects after 24, 48 or 72 h, without re-perpetuating the toxin insult. So it is possible that over time cells are able to metabolize this molecule, showing limited effects in long timepoints. However, these limitations could represent also strengths from another point of view: they show that even in these conditions urea is able to compromise cells proliferation, reorganize actin filaments and induce EndoMT.
Further studies are necessary to better understand urea toxic effects. The proteomic results reported in this study could represent a start point, from which choose the up- or down-regulated protein better linked to CVD and confirm their modulation also by other methods, in other cells lines or *in vivo*, evaluating them in plasma sample of healthy and CKD subjects.

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# 8. CHAPTER 5

## Effects of physiological and pathological IS concentrations on Human Microvascular Endothelial Cells (HMEC-1)

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## Abstract

Indoxyl sulfate (IS) is a protein-bound uremic toxin. It tends to accumulate in plasma samples of chronic kidney disease (CKD) patients and it is not cleared by the dialysis treatment. IS accumulation is reported to exert side effects of kidneys, bones and on the cardiovascular system (CVS). Most studies assessed IS effects testing concentrations much higher than those measured *in vivo*, so with this study we focused on the link between IS and cardiovascular diseases (CVD) exposing a human endothelial cell line from the microcirculation (Human Microvascular Endothelial Cells-1, HMEC-1) to IS concentrations measured in plasma of healthy subjects (physiological concentration) or of CKD patients (pathological concentrations). Both the pathologic concentrations tested caused a reduction of the proliferation rate at 72 h. In our conditions, IS did not cause a relevant increase in the oxidative stress level: the total amount of protein thiols (measured as oxidative stress biomarker) decreased only within the first 24 h of exposure, then probably some recovery systems started into the cells (as suggest by the increase of Nrf-2 expression) and the trend disappeared in the following timepoints. The most interesting results pointed out IS exposition as a potential endothelial-to mesenchymal transition (EndoMT) stimulus. In fact, we found that at 72 hours IS induced an actin rearrangement characterized by the formation of stress fiber and supported by the proteomic analysis, thanks to which we identified some proteins linked to actin organization as dysregulated by IS treatment (elongation factor 1-alpha 2, ephrin type-B receptor 6, isoform 2 of low molecular weight phosphotyrosin protein phosphatase and thrombomodulin); in addition, IS caused a significant up-regulation of armadillo repeat-containing protein 8 and a significant down-regulation of ephrin type-B receptor 6, that are two proteins linked to the epithelial-mesenchymal transition (EMT) phenomenon. Looking individually at each protein whose expression resulted to be significantly dysregulated by the IS treatment according to the proteomics, two remarkable proteins turned out to be significantly down-regulated: COP9 signalosome complex subunit 9 (CSN) and thrombomodulin (TM). Both these proteins have been directly linked to cardiovascular diseases (CVD) by *in vitro* and *in vivo* studies. It could be interesting to deepen CSN and TM pathways, since these proteins could represent a link between CKD and CVD.

## Introduction

Chronic kidney disease (CKD) patients are characterized by a reduced renal function, which worsens with the progression of the pathology [Stevinkel et al., 2008]. For this reduced function, they show a pathological retention of various molecules, which normally are excreted by the kidneys [Vanholder et al., 2003]. When these molecules have side effects are called uremic toxins. Indoxyl sulfate (IS) is a protein-bound uremic toxin. It is a tryptophan metabolite produced by the gut microbiota. More in

detail, some microbes in the colon are able to convert dietary tryptophan in indole. Indole then is absorbed into the blood circulation, through which it reaches the liver, where it is oxidized and sulfated to form IS [Leong et al., 2016]. Normally IS is cleared through renal tubular secretion, while in CKD patients it accumulates in blood and tissues, at least 90% bound to plasma proteins [Eloot et al., 2016].

IS have adverse effects mainly on kidneys, bones and cardiovascular system [Leong et al., 2016]. Regarding the nephrotoxicity, it accelerates CKD progression inducing tubulointerstitial fibrosis and glomerular sclerosis [Niwa et al., 1994] other than inflammation [Sun et al., 2012]. For bone toxicity, IS appears to deteriorate bone mechanical proprieties [Iwasaki et al., 2013] and to induce skeletal resistance to parathyroid hormone [Nii-Kono et al., 2007]. Overall most studies are about the correlation between IS and cardiovascular diseases (CVD), in fact this toxin seems to have an important role in the progression of CVD observed in CKD patients [Gao et al., 2017]. CKD patients are at higher risk of CVD than the normal population. To give an emblematic example: End-Stage Renal Disease (ESRD) subjects, who belong to CKD stage five, have a cardiovascular mortality 10-30 fold higher than healthy subjects [Jha et al., 2013]. In this context, also uremic toxins play a role and in particular IS seems to contribute to higher risk of chronic heart failure, arrythmia, coronary calcification and atherosclerotic vascular diseases [Gao et al., 2017]. These correlations may depend mainly on the enhanced oxidative stress induced by IS in myocardium and vasculature [Lekawanvijit et al. 2012; Ito et al., 2014]. Impairment of endothelial function has many consequences which can lead to vascular diseases, as atherosclerosis [Endemann et al., 2004]. IS is considered to induce endothelial dysfunction through several mechanisms, mostly involving an unbalance between pro- and antioxidant mechanisms [Dou et al. 2007; Yu et al., 2010], inflammation pathways [Adelibieke et al., 2014; Watanabe et al., 2013; Pletinck et al. 2013] and dysfunctions in cells proliferation and wound repair capabilities [Dou et al. 2004; Yu et al., 2010], compromising endothelial barrier functions [Peng et al. 2012].

Since most of studies to assess IS effects on endothelium have been performed on Human Umbilical Vein Endothelial Cells (HUVEC), testing concentration much higher than those measured in vivo [Vanholder et al., 2014], with this study we tried to better elucidate some IS side effects when cells are exposed to physiologic concentrations of this toxin. We evaluated this effects on a cell line from the microvasculature, often underestimated even if microcirculation is the principal seat of exchanges between circulation and tissues.

# Materials and methods

### Cell line and solutions for cell maintenance

HMEC-1 (Human Microvascular Endothelial Cells-1), an immortalized human cell line, were grown in plates with MCDB 131 Medium (Sigma, Italy), supplemented with 10 % fetal bovine serum (FBS, Euroclone), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 ng/ml Epidermal Growth Factor, 0.1 ug/ml Hydrocortisone (Sigma, Italy). Cell cultures were maintained at 37 °C with 5% CO2 and passaged every 3–4 days.
For experiments, HMEC-1 cells were cultured in the presence or absence of different concentrations of Indoxyl sulfate potassium salt (IS, Sigma, Italy) for 24, 48 or 72 h.

## Treatment of HMEC-1 with IS

For all the following experiments, HMEC-1 were seeded at a concentration of 15,000 cells/cm$^2$ and let grown for 24 hours, at 37 °C with 5% CO2. After 24 hours, a half of the medium was removed and replaced with an equal volume of solution with or without IS. This expedient is necessary since HMEC-1 release growth factors in the medium and a complete remove of the medium will slow down their growth.

The treatment solutions were prepared dissolving IS in Phosphate buffered saline (PBS), obtaining a mother solution with a concentration of 10 gr/l. This solution was diluted in complete medium (prepared as described before) at the following concentration: 1 - 50 – 100 mg/l. These concentrations are double to the desired ones, since, as mention before, only a half of the medium was changed. The solutions were filtered through a syringe with a 0.22-µm pore-sized filter to remove bacteria and particulate. At this point the solutions were added to the cells supports, obtaining the following final concentrations: 0.5 – 25 – 50 mg/l. For control cells, they had the same treatment, adding a solution made of complete medium and PBS, without the toxin. By this way, in all the treatment solutions there was the same volume of PBS and they differed only for the presence or absence of the toxin. The treatment lasted 24, 48 or 72 hours, without changing the medium.

## Proliferation Assay

Sulforhodamine B (SRB) assay is a colorimetric test which allows quantifying cellular protein content and it's largely used to indirectly quantify cells proliferation [Orellana et al., 2016]. Briefly, cells were seeded and treated as described before in 24-multiwell plates. At each timepoints, cells were fixed with 50% trichloroacetic acid (Sigma, cod. T6399) for 2 h at 4°C, then washed five times with milliQ water. 0.04% (w/v) SRB protein-bound dye (Sulforhodamine B Sigma, cod. S1402, dissolved in 1% acetic acid) was added to each well and incubated at RT for 30 minutes, then each well was washed four times with 1% (v/v) acetic acid and left to air-dry at room temperature. Finally, 1.2 ml of 10 mM Tris base solution (pH 10.5) was added to each well and the plate was shaken on an orbital shaker for 10 min to solubilize the protein-bound dye. The absorbance at 490 nm was detected using a multimode microplate reader (EnSight Multimode Plate Reader, PerkinElmer).

## Quantification of Proteins Thiols

Cellular proteins extracts were obtained lysing cells with ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% TRITON X-100, 0.1% SDS, 0.5% sodium deoxycholate supplemented with protease inhibitors (Sigma P8340). Each lysate was incubated on ice for 30 min and centrifuged at 10,000 rpm for 10 min at 4°C to remove cell debris. BCA protein assay was used to assess protein concentration. To detect proteins thiol groups, a biotin-maleimide assay was carried out. Briefly, 40 mM biotin-maleimide stock solution was prepared in DMSO and stored at −20°C. Then, 1 mg/mL of protein was incubated with 75 µM biotin-maleimide solution for 1 h at RT and then mixed to Laemmli sample buffer (2% SDS, 20% glycerol, and 125 mM Tris-HCl, pH 6.8), boiled for 5 min at 90°C and separated on 12% SDS-PAGE Stain-free gel (Biorad) [Hill B.G et al., 2009]. Separated proteins were then electroblotted onto a low-fluorescence polyvinylidene difluoride (LF-PVDF) membrane. Biotin tag was revealed using streptavidin-HRP assay as following. LF-PVDF membrane was washed with PBST [10 mM Na-phosphate, pH 7.2, 0.9% (w/v) NaCl, 0.1% (v/v) Tween-20 (Sigma Aldrich, cod. P9416)] [Hill B.G et al., 2009] and blocked for 1 h in 5% (w/v) non-fat dry milk in PBST. After washing three times with PBST for 5 min, biotin tag was probed by 2 h incubation with 5% non-fat dry milk/PBST containing streptavidin-HRP (1:5000 dilution, GE Healthcare).

Biotinylated proteins were visualized by ECL detection (cod.1705061, Biorad) using Chemidoc Touch Imaging System (Biorad). ECL signals were normalized with respect to PVDF stain free [Rivero-Gutiérrez et al., 2014].

### Western blot

Proteins from cell extracts were separated and transferred to PVDF membrane as described previously. After washing three times with TBST for 5 min, membrane was incubated for 2 h with 5% non-fat dry milk/TBST containing the following primary antibodies: anti-Nrf2 (1: 1000, Enzo Life Sciences); anti-Actin (1:2000, Abcam); anti-Tubulin (1:40000, Abcam); anti-VE-cadherin (1:1000, Cell Signaling Technology). The membrane was washed three times with TBST for 5 min and then incubated with the following secondary antibodies respectively: anti-rabbit (1:10000); anti-mouse (1:10000); anti-rabbit (1:20000); anti-rabbit (1:20000). Proteins of interest were visualized by ECL detection (cod.1705061, Biorad) using Chemidoc Touch Imaging System (Biorad). ECL signals were normalized with respect to PVDF stain free [Rivero-Gutiérrez et al., 2014] or using tubulin as housekeeping.

### Immunofluorescence

HMEC-1 cells were cultured on 12-mm diameter round coverslips, grown on 24-well culture plates and treated with IS as described before. At each timepoints, cells were washed in PBS, fixed in 4% paraformaldehyde in PBS containing 2% sucrose for 10 min at room temperature, post-fixed in 70% ethanol, and stored at −20°C until use.

For cytoskeleton analysis, cells were washed in PBS three times, incubated 5 minutes at RT with 0.1% Triton X-100/PBS and blocked with 1% bovin serum albumin (BSA, Sigma) in PBS for 1 h. Cells were then incubated with the primary monoclonal anti-Tubulin antibody (1:300, diluted in BSA 0.5%/PBS, Abcam) at 4°C overnight. The next day cells were washed 4 times with PBS, incubated for 1 h with the secondary antibody TRITC anti-rabbit 1:200 in BSA 0.5%/PBS (Abcam) in the dark and washed extensively in PBS. For actin detection, cells were then incubated 1h in the dark with Phalloidin 1:1000 in BSA 1%/PBS (Abcam). After the labeling procedure was completed, the coverslips were incubated for 10 min with DAPI and mounted onto glass slides using mowiol mounting medium. Fixed cells were imaged with a ViCo confocal microscope (Nikon) and TCS NT confocal laser scanning microscope (Leica).

### Proteomic analysis of cellular proteins after 72h treatment: sample preparation

In order to perform a quantitative proteomic analysis of cellular proteins, HMEC-1 cells were seeded and treated as described above. The treatment with IS lasted 72 hours, without changing the medium. After removal of medium and three washes with PBS, cellular proteins extracts were obtained lysing cells with the following lysis buffer: 8M Urea, 100 mM Tris-HCl pH 8.5, protease inhibitors (Sigma P8340). Each lysate was incubated for 30 min at RT and centrifuged at 14,000 g for 30 min at 4°C to remove cell debris. BCA protein assay was used to assess protein concentration. In order to check the quality of proteins, a part of the lysate was mixed to Laemmli sample buffer (2% SDS, 20% glycerol, and 125 mM Tris-HCl, pH 6.8), boiled for 5 min at 90°C and separated on 12% SDS-PAGE Stain-free gel (Biorad) [Hill B.G et al., 2009]. Protein gel was acquired using Chemidoc Touch Imaging System (Biorad). The remaining part of each lysate was used to perform tryptic digestion as described later.

### Proteomic analysis of cellular proteins after 72h treatment: protein sample digestion and analysis

After 72h of treatment, samples underwent the following treatment to allow the protein digestion. 10 µg of proteins were mixed with 36 µl of 50 mM ammonium bicarbonate (AMBIC) dissolved in MS-

grade water (Sigma). pH was checked to ensure that it was around pH 8 – 8.5. Then 5mM dithiothreitol (DTT, diluted in AMBIC) was added and the samples were incubated in a Thermomixer at 600 rpm, 52°C for 30 minutes. At this point 15 mM iodoacetamide (IAM, diluted in AMBIC) was added and the samples were incubated in a Thermomixer at 600 rpm, RT for 20 minutes, in the dark. 0.5 µg trypsin in 50 mM acetic acid was added (after activation for 15 minutes at 30°C) respecting a ratio 1:20 trypsin:proteins. Samples were incubated in a Thermomixer at 600 rpm, 37°C overnight. The day after 2 µl of 50% trifluoroacetic acid (TFA, diluted in MS-grade water) were added and the pH was checked to ensure that it was lower than pH 2.

### High resolution mass spectrometry analysis (nLC-MSMS)

Tryptic peptides were analyzed at UNITECH OMICs (University of Milano, Italy) using a Dionex Ultimate 3000 nano-LC system (Sunnyvale CA, USA) connected to an Orbitrap Fusion™ Tribrid™ Mass Spectrometer (Thermo Scientific, Bremen, Germany) equipped with a nano-electrospray ion source. Peptide mixtures were pre-concentrated onto an Acclaim PepMap 100 - 100 mm_ 2 cm C18 and separated on EASY-Spray column,15 cm _ 75 mmID packed with Thermo Scientific Acclaim PepMap RSLC C18, 3 mm, 100 Å. The temperature was set to 35 _C and the flow rate was 300 nL min_1.
Mobile phases were the following: 0.1% Formic acid (FA) in water (solvent A); 0.1% FA in water/acetonitrile (solvent B) with 2/8 ratio. Peptides were eluted from the column with the following gradient: 4%-28% of B for 90 min and then 28%-40% of B in 10 min, and to 95% within the following 6 min to rinse the column. Column was reequilibrated for 20 min. Total run time was 130 min. One blank was run between triplicates to prevent sample carryover. MS spectra were collected over an m/z range of 375-1500 Da at 120,000 resolutions, operating in the data dependent mode, cycle time 3 s between master scans. HCD was performed with collision energy set at 35 eV. Each sample was analyzed in three technical triplicates. LTQ raw data was searched against a protein database using SEQUEST algorithm in Proteome Discoverer software version 2.2 (Thermo Scientific) for peptide/protein identification. The searches were performed against Uniprot KnowledgeBase (KB) (taxonomy Homo sapiens). The minimum peptide length was set to six amino acids and enzymatic digestion with trypsin was selected, with maximum 2 missed cleavages. A precursor mass tolerance of 8 ppm and fragment mass tolerance of 0.02 Da were used; acetylation (N-term), oxidation (M) were used as dynamic modifications and carbamidomethylation (C) as static modification. The false discovery rates (FDRs) at the protein and peptide level were set to 0.01 for highly confident peptide-spectrum matches and 0.05 for peptide-spectrum matches with moderate confidence.
We considered only proteins with a score of coverage >2% with at least two identified peptides. Differences in abundance ratio (AR) of proteins between control and treated samples were considered only with at least a 2-fold change and with a standard deviation between replicates less than 20%.

## Results

### IS alters growth rate of HMEC-1 cells only after 72 hours of exposure

The growth of cultured HMEC-1 cells were followed up to 72 h. By using SRB assay (*Figure 1*), we observed that only the exposures for 72h to the IS concentrations found in pathologic conditions (25 mg/l and 50 mg/l) caused a significative reduction in the cells proliferation rate ($p < 0.05$).
Control HMEC-1 cells and cells treated with the concentration of IS found in physiological status (0.5 mg/l) grew exponentially over three days, whereas cells treated with IS 25 mg/l and 50 mg/l showed a progressive reduction in growth over time. These results are the mean of four independent experiments.

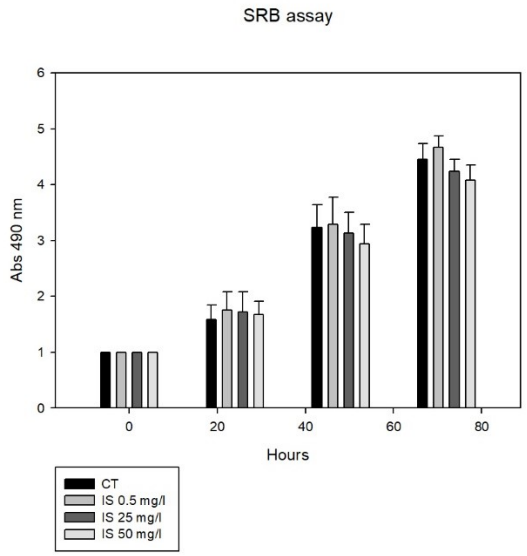


*Figure 1. Effects of IS treatment on cell proliferation. Histogram showing the mean absorbance measured at 490 nm in control cells and cells treated with 0.5 – 25 – 50 mg/l of IS for 0 -24 -48 – 72 hours. Data are expressed as the mean ± SD. *p < 0 05.*

## IS induces oxidative stress and oxidative stress-responses only within the first 24 hours of exposure

It is known that oxidative stress leads to the formation of unwanted disulfide bonds in the cytoplasm, eventually leading to impaired protein function. In literature IS is described to induce oxidative stress in vitro and in animal models [Edamatsu et al., 2018; Yang et al., 2015; Ji et al., 2018] and it is correlated to oxidative stress also in CKD patients [Fujii et al., 2011; Gao et al. 2017]. For these reasons, we evaluated oxidative stress biomarkers as protein carbonylation and oxidation of protein thiols in whole-cell lysates by Western immunoblotting. We didn't find differences in protein carbonylation over the treatment (data don't shown). We found a tendency toward reduction in the total amount of protein thiols at 24 hours, that appears to be concentration-dependent, even if differences were statistically significant only within the 24 h (*Figure 2*). This trend disappeared at 48 and 72 hours. It is known that oxidative stress can lead to the formation of unwanted disulfide bonds in the cytoplasm, resulting in a reduction in the total amount of thiols. This event can lead to impaired protein function. To face this, cells have several mechanisms to increase the intracellular levels of thiols [Deneke et al., 2000]. Notably, intracellular increase of thiol levels are strongly associated with an increased tolerance to an oxidant stress [Deneke et al., 2000] since they act as extraordinarily efficient antioxidants protecting the cells against consequences of damage induced by ROS [Włodek et al., 2002]. Since thiols decreased only at 24 hours in cells treated with IS, trying to assess eventual recovery mechanisms, we evaluated Nrf2 expression with Western blot (*Figure 3*). Nfr2 is a transcription factor whose activation is induced by oxidative stress [Ma Q, 2013]. As shown in *Figure 3*, we found a significative increase in the level of Nrf2 in cells treated with IS 50 gr/l after 24 hours of toxin exposure ($p < 0.01$). Both thiols and Nrf2 Western blot were performed for 3 independent experiments and these results are the mean.

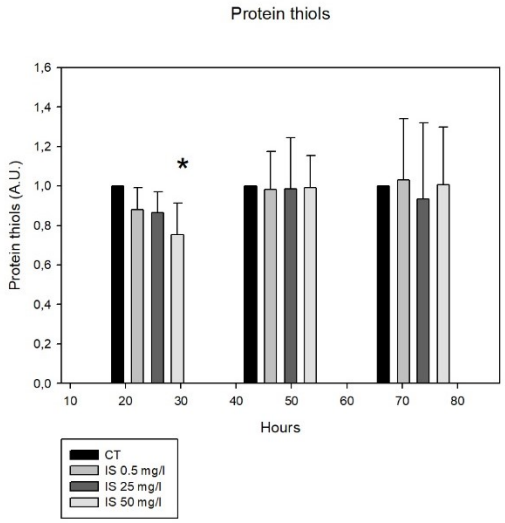


*Figure 2. Effects of IS treatment on the total amount of protein thiols. Histogram showing the protein thiols level measured in control cells and cells treated with 0.5 – 25 – 50 mg/l of IS for 0 -24 -48 – 72 hours. Data are*
*expressed as the mean ± SD. *p < 0 05.*

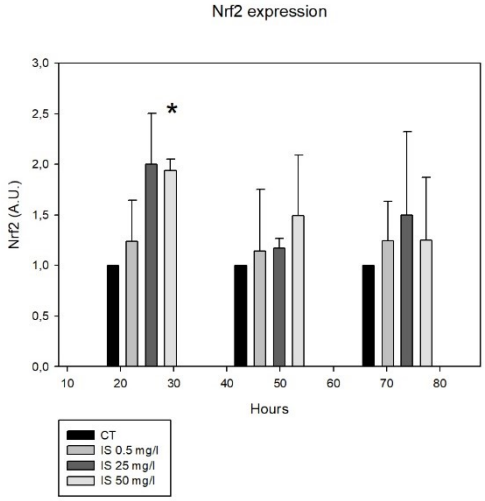


*Figure 3. Effects of IS treatment on Nrf2 expression. Histogram showing the Nrf2 level measured in control cells and cells treated with 0.5 – 25 – 50 mg/l of IS for 0 -24 -48 – 72 hours. Data are expressed as the mean ± SD. *p < 0 05.*

## IS induces stress fiber formation

IS is reported to modify the cytoskeleton organization and to disrupt cell junctions, events that contribute to compromise endothelial barrier function [Peng et al., 2012]. We evaluated cytoskeleton and tight junctions proteins expression in whole-cell lysates by Western immunoblotting. Regarding junctions, we didn't find significant differences nor in VE-cadherin or in beta-cathenin expression (data don't shown). For the cytoskeleton, there were not significant differences nor in actin or tubulin total amount by WB (data don't shown), however we found some differences looking at the cytoskeleton organization by immunofluorescence (*Figure 4*). In particular, control cells showed randomized organization of actin filaments, while after the treatment with IS 50 mg/l for 72 h, actin filaments seem to be organized in prominent parallel-oriented stress fiber (*Figure 5*), as observed

previously by Peng et al. [Peng et al., 2012]. Stress fiber weren't observed in other timepoints or cells treated with lower concentrations of IS (data don't shown).

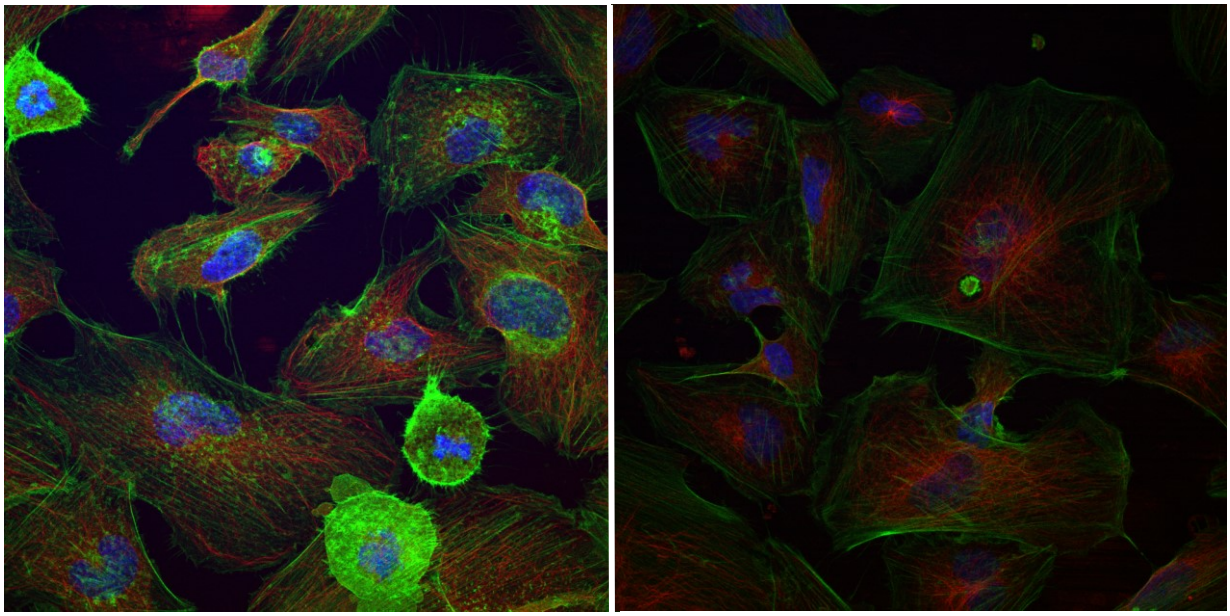

*Figure 4. Effects of IS treatment on the cytoskeleton organization. On the left, control cells at 72h. On the right, cells treated with 50 mg/l of IS at 72h. Images acquired with TCS NT confocal laser scanning microscope (Leica).*

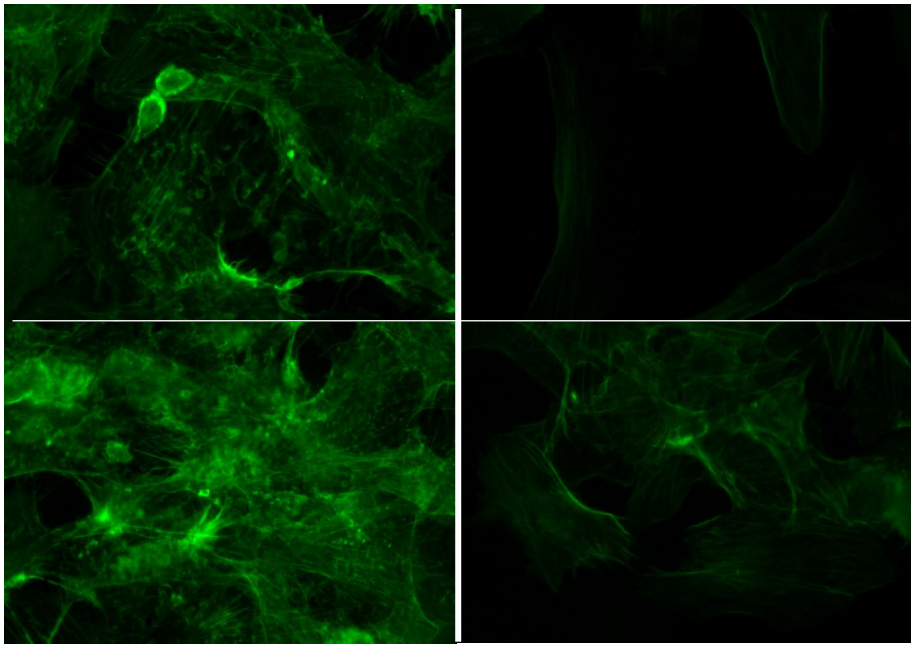

*Figure 5. Effects of IS treatment on the actin organization. On the left, control cells at 72h. On the right, cells treated with 50 mg/l of IS at 72h. Images acquired with ViCo confocal microscope (Nikon).*

### IS weakly affects proteins expression

For the proteomic analysis, we decided to focus on the comparison between cells treated with IS 0.5 mg/l, the mean of the physiologic concentration measurable in healthy subjects, and cells treated with IS 50 mg/l, a concentration measurable in CKD patients. We did not focus on the comparison

between control and treated cells, since the control condition does not exist physiologically. The volcano plot (*Figure 6*) shows that only a few proteins resulted to be up or down-regulated when comparing cells treated with IS 0.5 mg/l vs IS 50 mg/l. To verify if these proteins were linked according to their functions, we performed an analysis with STRING obtaining the network of up-regulated proteins (*Figure 7*) and the network of down-regulated proteins (*Figure 8*). The networks do not have significantly more interactions than expected, according to STRING lambda calculation. This means that our sets of proteins are composed by an apparently random collection of proteins that are not very well connected, or whose interactions are not still known by STRING upon the available data.

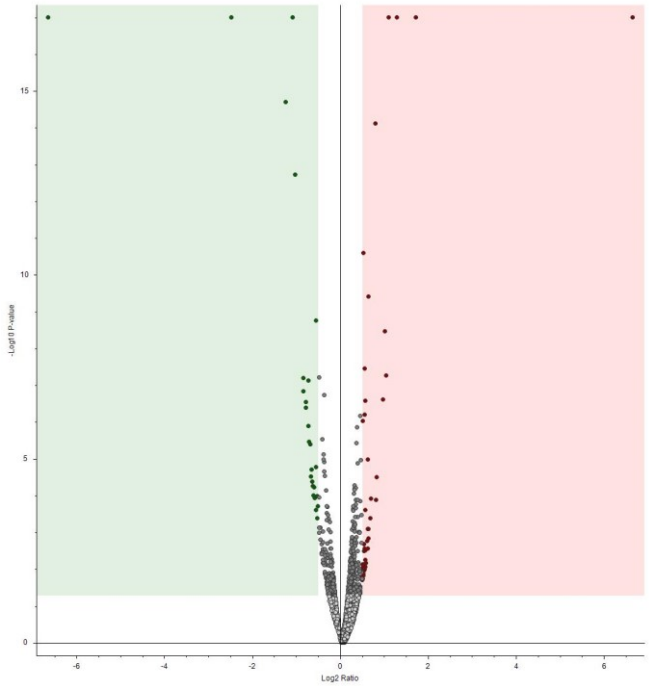


*Figure 6. Volcano plot which compares protein expression of cells treated with 0.5 mg/l of IS vs 50 mg/l of IS for 72h. Down-regulated proteins are in green. Up-regulated proteins are in red.*

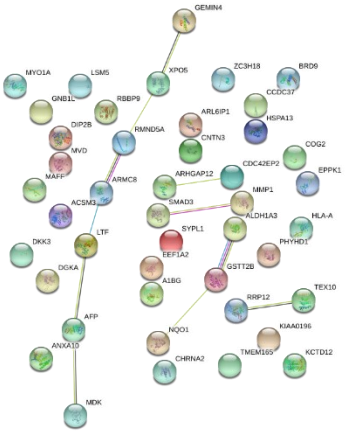


*Figure 7. Network of the up-regulated proteins obtained with the software String. The network comprehends the proteins that emerged as up-regulated in cells treated with 50 mg/l of IS when compared to cells treated with 0.5 mg/l of IS, at 72 h.*

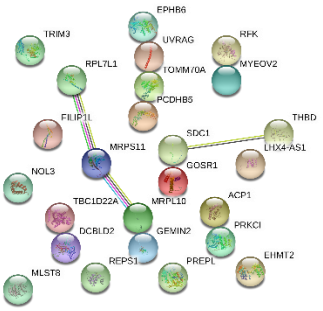


*Figure 8. Network of the down-regulated proteins obtained with the software String. The network comprehends the proteins that emerged as down-regulated in cells treated with 50 mg/l of IS when compared to cells treated with 0.5 mg/l of IS, at 72 h.*

## Discussion and conclusions

Chronic Kidney Disease is a non-communicable disease with a world prevalence of 8-16% and the WHO declared it as a public health problem which is continually increasing [Jha et al., 2013]. It is diagnosed when there's a decreased kidney function shown by glomerular filtration rate (GFR) less than 60 ml/min (established for a reference man with 1.73 $m^2$ body surface area), or markers of kidney damage, or both, of at least 3 months duration [Webster et al., 2016].

The renal impairment predisposes to numerous complications and complications seriousness increases in parallel with the GFR decline. Among them there are cardiovascular diseases, acute kidney injury, bones disorder, minerals balance disorder, hospitalization, anemia, oxidative stress, chronic inflammation and dysbiosis [Stevinkel et al., 2008]. Dysbiosis is a feature of CKD patients. CKD patients show a quantitative and qualitative alteration of intestinal microflora. From the early stages of CKD, there's a change in composition and structure of the microbiota. Uremic patients show a higher number of *Enterobacteria* and *Enterococci* and a lower number of *Lactobacillaceae* and *Prevotellaceae* families [Vaziri et al, 2013]. In particular, hemodialyzed (HD) patients present an overgrowth of aerobic bacteria, with the number of *Enterobacteria* and *Enterococci* species approximately 100 times higher than in healthy subjects. Regarding anaerobic bacteria, HD patients have a significantly lower number of *Bifidobacteria* and higher *Clostridium perfringens* [Hida et al., 1996].
Gut microbiota and CKD have a bidirectional relationship: kidney disease may disrupt microbiota balance and at the same time the unbalanced microbiota affects kidney disease progression and complications. About this last point, many recent studies suggest that toxic products generated by a dysbiotic gut microbiome may contribute to the progression of CKD and CKD-related complications [Ramezani et al., 2014]. In the colon, protein fermentation by intestinal bacteria generates several metabolites including ammonium, amines, thiols, phenols and indoles. Normally, they are in part eliminated through faeces and in part cleared by kidneys, so they accumulate in CKD and are called uremic toxins [Evenepoel et al., 2009]. For the biological and clinical consequences of its accumulation, indoxyl sulfate (IS) is among the most studied metabolites in this context. It is associated with CKD progression, cardiovascular complications, alteration of bone-mineral metabolism, insulin resistance and anemia [Cigarran Guldris et al., 2017].

In this study, we tried to better elucidate some IS side effects when cells are exposed to physiologic concentrations of this toxin. We tested them on an endothelial cell line from the microvasculature, since IS is strongly correlated with CVD [Gao et al., 2017] and the literature lacks of works evaluating IS effects in the microcirculation, even though it is the principal seat of exchanges between circulation and tissues.

IS appears to affect cell proliferation. We observed that the exposures for 72h to IS concentrations found in pathologic conditions (25 mg/l and 50 mg/l) caused a significative reduction in the cells proliferation rate ($p < 0.05$). This result is in accordance with other studies in which comparable IS concentrations were tested on HUVEC [Dou et al., 2004; Yu et al, 2011]. In particular, Yu et al. observed also cell aging, a decreased nitric oxide production, and oxidative stress, so they suggested that IS-induced oxidative stress could cause senescence, resulting in an inhibition of cell proliferation [Yu et al, 2011]. Considering that cell capacity to proliferate, together with cell capacity to migrate, plays an important role in the healing of endothelial injury, Dou et al. evaluated wound repair capability. They found an inhibition of this capability in HUVEC treated with IS [Dou et al., 2004]. We tried to assess if also HMEC-1 showed this alteration, but we were not able to perform a scratch assay, because HMEC-1 never reach confluency (data don't shown).

Patients with CKD show high oxidative stress level, which is strongly correlated with two important risk factors for mortality in these patients: chronic inflammation and cardiovascular diseases [Colombo et al., 2015; Caimi et al., 2013]. Uremic toxins contribute to oxidative stress and IS has been extensively correlated with this problem. IS-induced oxidative stress appears to exert side effects on skeletal system [Liu et al., 2018], on kidneys [Chao et al., 2014] and on cardiovascular system [Gao et al., 2017]. About IS pro-oxidant effects on the cardiovascular system, it seems to enhanced oxidative stress both in myocardium and vasculature [Lekawanvijit et al. 2012; Ito et al., 2014]. An *in vitro* study on HUVEC reports that IS (after an incubation of 5 hours) stimulates ROS production, increases NAD(P)H oxidase activity, and decreases glutathione levels, so it increases oxidative stress both promoting pro-oxidant activities and inhibiting antioxidant activities [Dou et al 2007]. Another study on HUVEC hypothesizes that IS-induced oxidative stress could arise from NADPH oxidase and mitochondrial respiratory chain complex activities and that it could be responsible for endothelial dysfunctions seen after 24 or 48 h of IS exposure [Yu et al 2011]. It's interesting to note that in this last study ROS production was measured only until 12 hours of exposure, during which even the highest concentration of IS tested (125 mg/l, more than 2 fold higher compared to our highest concentration, 50 mg/l) caused an increase in ROS that reaches a plateau within the first hours. Also Masai et al. reported an increase in ROS after exposure to 125 mg/l of IS for 2 minutes, identifying NADPH oxidase and Nox4 (evaluated after 0 – 60 min of IS treatment) as important players in ROS production in HUVEC [Masai et al., 2010].

In our study, we evaluated two well recognized oxidative stress biomarkers after 24, 48 and 72 hours of IS exposure: protein carbonylation (data don't shown) and the total amount of protein thiols [Dalle Donne et al., 2006]. In both cases, we didn't find significant differences. These results appear to be in contrast with the other studies mentioned above, but we can speculate that these differences could depend on different timepoints. In most cases reported in literature, IS effects are evaluated within the first hours of exposure, while we tried to mimic a chronic exposure, assessing IS effects over three days. During this time cells may start some recovery systems. This could explain the oxidative stress biomarkers trend observed. In this regard, interestingly at 24 h we found a tendency toward reduction in the total amount of protein thiols, that appeared to be concentration-dependent; this trend disappeared at 48 and 72 hours. At the same time, we found also a significative increase in the level of Nrf2 in cells treated with IS 50 mg/l after 24 h ($p < 0.01$). Nrf2 activates genes that encode for phase II detoxifying enzymes and antioxidant enzymes, which counteract oxidative stress [Stockler-Pinto et al., 2014]. So we can assume that Nfr2 activation in HMEC-1 treated with IS could be among the recovery mechanisms able to reduce oxidative stress and so to justify the oxidative stress biomarkers levels in medium-long timepoints. This increase in Nrf2 level at 24 h appears to be in contrast with literature, in which is reported a reduced expression of Nrf2 in peripheral blood mononuclear cells

from 20 hemodialyzed patients [Stockler-Pinto et al., 2014] and a downregulation of Nrf2 expression in HK-2 cells treated with IS [Bolati et al., 2013]. However, these studies are not comparable with ours, since the first is *in vivo*, while the second is performed on a kidney cell line, so other work is necessary to better elucidate Nrf2 and IS relationship in vascular cells.

Other events that can compromise endothelial barrier function are cytoskeleton remodeling and disruption of intercellular junctions. A treatment lasted 24 h with 50 – 250 mg/l of IS modified Bovine pulmonary artery endothelial cells (BPAECs) shape: while control cells had cobble stonelike shape, with actin organized in randomized arrays, cells treated with IS were elongated, similar to fibroblast, with actin organized in parallel-oriented stress fiber [Peng et al., 2012]. In HMEC-1, we observed a similar behavior in cells treated with IS 50 mg/l at 72 h, compared with control cells. Actin filaments change their organization in treated cells, resulting in prominent parallel-oriented stress fiber. Dissolution of the dense peripheral band and appearance of stress fibers can arise from stresses exposure, as shear stress or oxidative stress. These events are in turn associated with an increase in endothelium permeability [Ogunrinade et al., 2002]. For this reason, since junctional proteins contribute to regulate permeability between endothelial cells, we evaluated also VE-cadherin and beta-catenin expression by WB, but we did not find significant differences comparing their expression in control or treated cells (data don't shown). It could be interesting evaluating their localization within the cells rather than their total amount. In this regard, Peng et al. found by immunofluorescence continuous linear staining for p120-catenin, VE-cadherin and beta-catenin at cell–cell contacts in control cells and intercellular gap and discontinuous staining in cells treated with IS [Peng et al., 2012]. Nevertheless our *in vitro* model did not allow us to replicate a similar job, since HMEC-1 never reach confluency and when too numerous, they arrange in more than one layer, rather than form a well organized monolayer.

Finally, we performed a proteomic analysis in order to check protein expression modifications in our cell model, both inside and outside the cells (proteins released in the medium). In 2008 Carbo et al. compared protein expression in HUVECs treated with uremic serum, that was serum of patients undergoing hemodialysis, or with normal serum, of healthy subjects. Mainly they found differential expression in proteins link to inflammation, oxidative stress and cytoskeleton [Carbo et al., 2008]. Clearly, uremic serum contains all the uremic toxins. Our proteomic job is the first one to assess the differential protein expression induced by a single uremic toxin, IS, with the aim to better elucidate the effects linked specifically with this molecule. The volcano plots (*Figure 6; Figure 10*) show that only a few proteins resulted to be up- or down-regulated by the highest IS concentration (50 mg/l), when compared to the lowest one (IS 0.5 mg/), after 72h of treatment. Here we report only the comparison between these two concentrations; we decided to exclude the comparison between cells treated with IS 50 mg/l and control cells, since physiologically endothelial cells are always exposed to concentration of IS differently from zero (above 0.5 mg/l). Trying to better understand the ratio under the differential protein expression, we analyzed the proteomic data with the software String, gathering proteins according to their functions. The resulting networks did not show pathways markedly influenced by IS 50 mg/l treatment, when looking at protein expression inside the HMEC-1 (*Figure 8; Figure 9*); while looking at proteins released in the medium, pathways linked to actin organization and organic catabolism emerged, so they appeared to be dysregulated in cells treated with a pathologic concentration of IS (*Figure 11; Figure 12*).
It is worth deepening the functions of the proteins emerged to be the strongest up- or down-regulated inside the cells. Among them, elongation factor 1-alpha 2, ephrin type-B receptor 6, isoform 2 of low molecular weight phosphotyrosin protein phosphatase and thrombomodulin have been linked to actin organization in cell models differently from HMEC-1 [Kurasawa et al, 1996; Murray et al, 1996; Truitt et al, 2011; Chiarugi et al., 2000; Chiarugi et al, 2001; Shimizu et al, 2005; Hsu et al., 2012], confirming the dysregulation of this pathway. So these proteins significantly up- or down-regulated could explain, at least in part, the abnormal organization of actin filaments in cells treated

with IS, which are prone to form stress fiber rather than randomized arrays. Armadillo repeat-containing protein 8 and ephrin type-B receptor 6 are reported to lower alpha-catenin, beta-catenin and cadherin 17 expression (respectively when up- or down-regulated) [Gul et al, 2019; Suzuki et al., 2008; Tewari et al., 2010; Liang et al, 2019; ], supporting the hypothesis mentioned before that IS could modify junctional proteins expression, even if we were not able to confirm this using other methods (Western blots or immunofluorescence). In addition, these two proteins are linked to epithelial-mesenchymal transition (EMT) (armadillo repeat-containing protein 8 acting on TGFbeta pathway [Liang et al, 2017], while ephrin type-B receptor 6 modulating metalloproteases expression [Truitt et al, 2011]), so it could be interesting to assess if cells treated with IS show markers of EMT.
The two most interesting proteins that we found strongly downregulated by IS 50 mg/l treatment vs IS 0.5 mg/l treatment are COP9 signalosome complex subunit 9 (CSN) and thrombomodulin (TM).
CSN appears to have potent protective activities in the vasculature and heart [review cvd e infiam]. It blocks inflammatory signaling in myeloid cells, regulates the cholesterol efflux pathway in foam cells, contributes to control vascular smooth muscle (VSM) cells and T cells proliferation, plays a role in adipocyte differentiation and it inhibits atherogenic signaling pathways in endothelial cells [Milic et al., 2019]. Since the functions described represent all pathways implicated in atherosclerotic pathogenesis, CSN dysregulation is correlated with atherosclerosis. It is implicated also in cardiovascular ischemia [Milic et al., 2019]; interestingly, a previous study on HMEC-1 showed that an over-expression of CSN downregulated TNF-alpha/LPS-induced proinflammatory cytokine levels and avoided the increased endothelial permeability induced by LPS stimulation [Colgan et al., 2010]. In addition, CSN seems to regulate vascular tone at several levels, due to its involvement in VSM and endothelial mechanisms which modulate vascular tone [Martin et al., 2015]. Overall, CSN is emerging as a potential target for several CVD, from atherosclerosis and ischemia, to hypertension, Raynaud's disease or coronary artery spasm.
Thrombomodulin is known to participate in the regulation of coagulation, innate immunity, inflammation and cell trafficking. Seen its multiple functions, TM plays important roles in many diseases as cancer, diabetic nephropathy, pulmonary disease, preeclampsia and CVD [Loghmani et al., 2018]. Regarding CVD, TM dysregulation is linked to atherosclerosis. For example, it is downregulated in endothelial cells that overly atherosclerotic lesions [Laszik et al., 2001] and it seems to inhibit endothelial-mesenchymal transition, so its down-regulation could exacerbate this phenomenon, which in turn contributes to vascular calcification, hypertension, system sclerosis and organ fibrosis [Sanchez-Duffhues et al., 2018]. Reduced TM function causes thrombosis, as observed in several animal studies [Weiler et al., 2003]. Plasma TM level appear to have an inverse relationship with hemorrhagic stroke [Johansson et al., 2002]. TM seems to exert its anti-inflammatory activities attenuating NF-KB/NLRP3 pathway, reducing IL-1beta and HMGB1 release and enhancing Nrf2 antioxidant activity [Yang et al., 2014]. These activities could explain, at least in part, some results obtained in pre-clinical studies, in which administration of soluble TM showed to have benefits in transplantation-associated vasculopathies, to protect heart, lung and kidney from ischemia-reperfusion injury [Loghmani et al., 2018] and to improve outcome in spinal injury [Taoka et al., 2000]. Altogether these evidences make TM another potential target for CVD treatments.
So both COP9 signalosome complex subunit 9 and thrombomodulin could represent a link between IS and CVD.

We can conclude that in HMEC-1 IS, at the concentration tested, seems to affect cells proliferation and actin organization, together with increasing oxidative stress level (measured as protein thiols oxidation), soon restored by the activation of antioxidant pathways (as Nrf2). Also the proteomic analysis confirm a dysregulation in proteins expression involved in cytoskeletal organization (and in particular in actin organization), explaining, at least in part, the actin filaments arranged in stress fibers observed by immunofluorescence in cells treated with IS 50 mg/l. A further in-depth analysis could be useful to better elucidate the link both between IS and COP9 signalosome complex subunit 9 and between IS and thrombomodulin. In fact, this two proteins resulted to be significantly down-regulated

by IS treatment and they are strongly correlated with CVD and inflammation. Maybe they could represent a key link between IS and CVD.
The modest effects of IS observed in our study, often in contrast with literature, could be explained by the choice of IS concentrations belonging only to a range measurable physiologically. In addition, we added the toxin to the cell medium the first day and evaluated the effects after 24, 48 or 72 h, without re-perpetuating the toxin insult. So it is possible that over time cells are able to metabolize this molecule, showing limited effects in long timepoints.
Further studies are necessary to better understand IS toxic effects. The proteomic results reported in this study could represent a start point, from which choose the up- or down-regulated protein better linked to CVD and confirm their modulation also by other methods, in other cells lines or *in vivo*, evaluating them in plasma sample of healthy and CKD subjects.

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# 9. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Overall, the mainly studies worked out during my PhD course can be summarized as follows:

- **Chapter 1:** This study has confirmed that the hemodialysis treatment itself is responsible for an increase in oxidative stress in ESRD patients, in fact the results obtained showed that post-HD plasma PCO levels were significantly higher than pre-HD plasma PCO levels. At the same time, the type of dialysis filter or the dialysis technique did not show any correlations with PCOs concentration. Dividing the population in diabetic and nondiabetic, it emerged that the PCO level increased slightly but significantly in nondiabetic ESRD patients when compared to diabetic ones. The most interesting data is the difference between men and women: plasma PCOs increased more markedly in women than in men after the HD session. According to these results, we could speculate that female sex may represent a risk factor for protein carbonylation induced by the HD treatment. So, this study contributed to point out a gender difference in CKD, often underestimated.

- **Chapter 2:** This work confirmed the robust relationship between uremia and oxidative stress, measured using plasma AOPP as oxidative stress biomarker. In particular, AOPP levels showed a positive correlation with protein-bound di-Tyr levels, another oxidative stress biomarker; with albumin, confirming that albumin is the main protein contributing to the formation of AOPP in hemodialyzed patients; and with creatinine, which basically represents dialytic efficiency and nutritional status (i.e. muscle mass) in HD patients. Differently, the lack of correlation between plasma AOPP and CRP levels could suggest that oxidative stress and inflammation may be two CKD comorbidities partly independent of each other in ESRD patients on HD. Overall our results confirmed the robust relationship between uraemia and oxidative stress, contributing to pave the way to other studies assessing if AOPP could be a useful clinical biomarker.

- **Chapter 3:** Switching HD patients from intravenous iron (62,5 mg/week) to sucrosomial iron (90 mg/week) treatment proved to be safe after a period of 3 months, since hemoglobin values appeared stable. Sucrosomial iron was well tolerated and did not cause noticeable side effects. However, others iron parameters dropped: a significative decrease in ferritin and Transferrin Saturation (TSAT) was observed. These results suggest that sucrosomial iron could represent an alternative route for iron administration, even if higher dosage should be tested. Concerning iron administration and oxidative stress, a modest but not significative decrease in protein carbonyls (PCO), di-tyrosines and advanced protein products (AOPPs) levels was observed. Also C-

reactive protein (CRP) level decreased during the sucrosomial iron treatment, but not in a significative way. Maybe the too low dosage, the short period of treatment and the small population size could explain, at least in part, the results observed. It could be interesting to repeat the study improving these factors in order to confirm or deny the positive impact of sucrosomal iron on oxidative stress and inflammation biomarkers. Although this study did not give a definitive answer on the possible advantage of sucrosomial iron formulation in HD patients, it may represent a starting point for further studies to investigate the complex correlation between iron supplementation, oxidative stress, inflammation, cardiovascular disease and mortality.

- **Chapter 4:** Pathologic concentrations of urea (compared to concentrations measurable in healthy subjects) reduced cells proliferation, modified action organization and induced EndoMT in HMEC-1, after 72 h of exposure. These effects have been confirmed also by the proteomic analysis, which showed a significative dysregulation in proteins expression involved in pro-apoptotic pathways and in the EndoMT process. In addition, further proteomic in-depth analysis brought out two interesting proteins significantly downregulated in supernatants of cells treated with urea 5 g/l: dimethylarginine dimethylaminohydrolase (DDAH) and vasorin (VASN). Both these proteins have been directly linked to CVD by *in vitro* and *in vivo* studies, so it could be interesting to deepen their altered expression in CKD, because they could represent potential biomarkers for CVD in CKD or site of targeting interventions. In this regard, we started confirming the VASN downregulation induced by urea also by SDS-page and Western blot. Since VASN functions are not still well elucidated, this protein could represent a potential link between CVD and CKD to explore more fully.

- **Chapter 5:** Pathologic concentrations of IS (compared to concentrations measurable in healthy subjects) affected cells proliferation and actin organization. In HMEC-1 treated with IS 50 mg/l for 72h, actin seemed to arrange in stress fibers; also the proteomic analysis confirmed a dysregulation in proteins expression involved in actin organization (elongation factor 1-alpha 2, ephrin type-B receptor 6, isoform 2 of low molecular weight phosphotyrosin protein phosphatase and thrombomodulin). IS appeared to increase the oxidative stress level (measured as protein thiols oxidation), soon restored by the activation of antioxidant pathways (as Nrf2). From the proteomic analysis, COP9 signalosome complex subunit 9 (CSN) and thrombomodulin (TM) emerged as the two most interesting proteins strongly downregulated by IS treatment. Since they are both connected with CVD and inflammation, they may represent a

key link between IS and CVD to explore. Overall our study demonstrated that even modest concentrations of IS (belonging to the range measured in healthy subjects or CKD patients) are able to modulate protein expression, suggesting to focus on the proteins dysregulated by the treatment to better understand why an accumulation of IS is correlated with CVD.

Overall, the experiments and the resulted publications realized during my PhD studies have given a contribute, albeit a small one, in CKD comorbidities knowledge, necessary starting point for therapeutic strategies more and more specific and effective. In the future, we would like to use more frequently proteomic approaches to study CKD and CKD-related complications, in order to identify proteins and molecular pathways responsible for the effects observed. Nowadays, we are following this route, deepening the most interesting proteins that resulted to be up or down-regulated by the uremic toxins treatments and validating their trends not only *in vitro*, using other quantitative approaches, but also *in vivo*, verifying if they maintain the same trends in plasma samples of ESRD patients and healthy subjects. They could represent new sensible and easily measurable biomarkers in this context.

# 10. OTHER PUBLICATIONS

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