**UNIVERSITA' DEGLI STUDI DI MILANO**

Graduate School in Pharmacological Sciences

Department of Pharmacological and Biomolecular Sciences

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# INFLUENCE OF CHRONIC KIDNEY DISEASE ON THE HAEMOSTATIC PROPERTIES, THE PLATELET TRANSCRIPTOMIC AND PLASMA PROTEOMIC PROFILES OF CORONARY ARTERY DISEASE PATIENTS

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**Paola CANZANO**

Matricola R10166

TUTOR: Chiar.ma Prof.ssa Marina CAMERA

COORDINATORE DEL DOTTORATO: Chiar.mo Prof. Alberto CORSINI

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# INTRODUCTION

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## CHRONIC KIDNEY DISEASE

### Definition and classification

In February 2002, the Kidney Disease Outcomes Quality Initiative (K/DOQI) of the National Kidney Foundation (NKF) published 15 clinical practice guidelines on chronic kidney disease. The goals of the guidelines were to define chronic kidney disease and classify its stages, regardless of underlying cause, evaluate laboratory measurements for the clinical assessment of kidney disease, associate the level of kidney function with complications of chronic kidney disease, and stratify the risk for loss of kidney function and development of cardiovascular disease [1]. In 2012, Levey and colleagues presented the definition and five-stage classification system of chronic kidney disease and summarized the major recommendations on early detection in adults: this paper represented an important shift towards the chronic kidney disease recognition as a worldwide public health problem that should be managed in its early stages by general internists [2]. Improving outcomes for people with chronic kidney disease requires a coordinated world-wide approach to prevention of adverse outcomes through defining the disease and its outcomes, estimating disease prevalence, identifying earlier stages of disease and antecedent risk factors, and detection and treatment for populations at increased risk for adverse outcomes (Figure 1). The definition of chronic kidney disease is based on the presence of kidney damage (i.e., albuminuria) or decreased kidney function (i.e., glomerular filtration rate [GFR] <60 mL/min per 1.73 $m^2$) for 3 months or more.

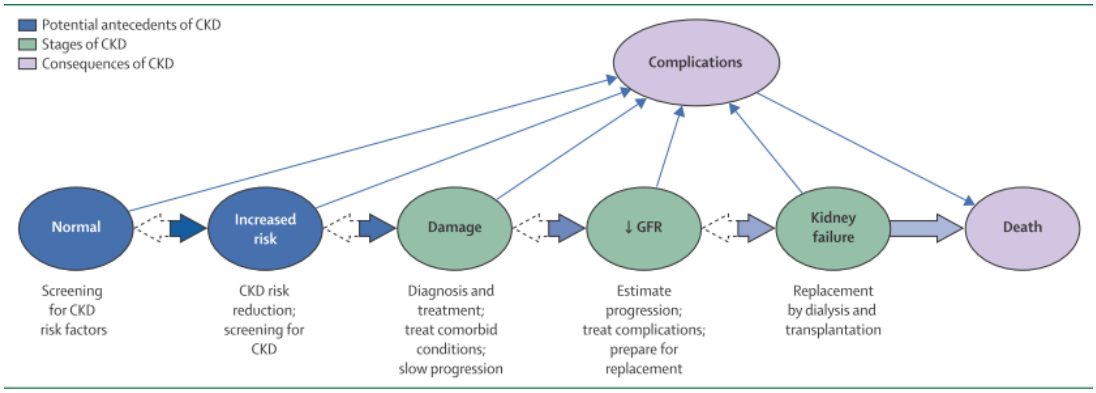



***Figure 1.*** **Conceptual model for chronic kidney disease.** Continuum of development, progression and complications of chronic kidney disease (CKD) and strategies to improve outcomes. Thick arrows circles represent development, progression and remission of CKD. Complication refer to all complication of CKD, including complications of decreased glomerular filtration rate (GFR) and cardiovascular disease. Complications might also arise from adverse effects of interventions to prevent or treat the disease. Horizontal arrows pointing from left to right represent the progressive nature of CKD. Dashed arrowheads mean that remission is less frequent than progression. *from Lancet 2012; 379: 165-80*

CKD is classified based on cause, GFR category and albuminuria category (CGA): chronic renal disease is divided into five stages on the basis of renal function (Table 1).

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

| GFR categories (ml/min/ 1.73 m²) Description and range | | | | 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 |
| | 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 1.*** **Classification of CKD**

**Epidemiology**

Chronic kidney disease is a worldwide public health problem [3]. In 2015, Mills et al [4] estimated the global prevalence and absolute burden of CKD by pooling data from population-based studies. The Global Burden of Disease Study 2013 estimated 956,200 deaths worldwide were directly attributable to CKD in 2013, representing a 134.6% increase from 1990 [5]. In addition, CKD was ranked as the 19$^{th}$ highest cause of years of life lost in 2013 [5]. The global incidence and prevalence of CKD, however, may be underestimated by these data; in fact, first of all, in the World Health Report 2002, the item "Disease of Genitourinary System" is articulated in only 2 specific cause groups: "Nephrosis and nephritis" and "Benign prostatic hypertrophy", a classification that does not provide any significant insight into the contribution of specific kidney diseases to the global burden of disease. Second, patients with CKD are at high risk of cardiovascular diseases (CVD) and cerebrovascular diseases, and they are more likely to die of CVD than to develop terminal renal failure. Moreover, patients with CVD often develop CKD during the course of their disease [6, 7]. Therefore, an unknown proportion of people whose death and disability are attributed to CVD have kidney disease as well. The worldwide rise in the number of patients with CKD is reflected in the increasing number of people with end-stage renal disease (ESRD), treated by renal replacement therapy, dialysis or transplantation [8]. Two factors are important. The first is the ageing of the population: the incidence of ESRD is higher in elderly people than in the general population. The second factor is the global epidemic of type 2 diabetes mellitus: diabetes mellitus is responsible for 895.000 deaths every year, according to the GBD project. There are currently 170 million patients with diabetes: approximately 30% of them have diabetic nephropathy and this proportion is even higher in some ethnic groups. The presence of CKD increases the risk of death of CVD in patients with diabetes [3].

**Diagnosis**

Chronic kidney disease is most frequently diagnosed through blood and urine tests.

The criteria described for the diagnosis of chronic kidney disease are any of the following [2]:

- *Duration >3 months on the basis of documentation:* duration is necessary to distinguish chronic from acute kidney disease
- *GFR <60 mL/min per 1·73 m²:* GFR is the best overall index of kidney function in health and disease
- *Kidney damage as defined by structural abnormalities or functional abnormalities other than decreased GFR:*
    - pathological abnormalities:

      - glomerular disease (diabetes, autoimmune diseases, systemic infections, drugs, neoplasia)

      - vascular diseases (atherosclerosis, hypertension, ischaemia, vasculitis, thrombotic microangiopathy)

      - tubulointerstitial diseases (urinary-tract infections, stones, obstruction, toxic effects of drugs)

      - cystic disease (polycystic kidney disease)
    - history of kidney transplantation - in addition to pathological abnormalities in native kidneys, common pathological abnormalities include:

      - chronic allograft nephropathy (non-specific findings of tubular atrophy, interstitial fibrosis, vascular and glomerular sclerosis)

      - rejection

      - drug toxic effects (calcineurin inhibitors)

      - BK virus nephropathy

      - recurrent disease (glomerular disease, oxalosis, Fabry's disease)
    - albuminuria as marker of kidney damage - increased glomerular permeability , urine albumin/creatinine ratio (ACR) >30 mg/g:

      - the normal urinary ACR in young adults is < 10 mg/g. Urine ACR categories 10-29, 30-300 and > 300 mg are high normal, high and vey high, respectively. Urine ACR > 2000 mg/g is

accompanied by signs and symptoms of nephrotic syndrome (low serum albumin, oedema and high serum cholesterol)

- high urinary ACR can be confirmed by urine albumin excretion in a timed urine collection

- abnormalities in urinary sediment as marker of kidney damage:

  - red-blood-cell casts in proliferative glomerulonephritis

  - white-blood-cell casts in pyelonephritis or interstitial nephritis

  - oval fat bodies or fatty casts in diseases with proteinuria

  - granular casts and renal tubular epithelial cells in many parenchymal diseases (non-specific)

- imaging abnormalities as markers of kidney damage (ultrasound, CT and MRI with or without contrast, isotope scans angiography):

  - polycystic kidneys

  - hydronephrosis due to obstruction

  - cortical scarring due to infarcts, pyelonephritis or vesicoureteral reflux

  - renal masses or enlarged kidneys due to infiltrative diseases

  - renal artery stenosis

  - small and echogenic kidneys (common in late stages of CKD because of many parenchymal diseases)

- Renal tubular acidosis:

  - nephrogenic diabetic insipidus

  - Barrter and Gittelman syndromes

  - Fanconi's syndrome

  - cystinuria

  - familiar hypomagnesaemia with hypercalciuria and nephrocalcinosis

Excretion of urinary creatinine varies with age, sex, race, diet and nutritional status, and generally exceeds 1.0 g per day in healthy adults; therefore, the numeric value for urinary ACR (mg/g) is usually less than the rate of urinary albumin excretion (mg/day). Rates of 30–300 mg per day and >300 mg per day correspond

to microalbuminuria and macroalbuminuria, respectively. Normal urine contains small amounts of albumin, low-molecular-weight serum proteins and proteins that are from renal tubules and the lower urinary tract. In most kidney diseases, albumin is the main urine protein, comprising about 60–90% of total urinary protein when total protein is very high. Values corresponding to normal, high-normal, high, very high, and nephrotic-range total protein are about <50, 50–150, 150–500, >500, and >3500 mg/g, respectively.

**Symptoms**

Symptoms are usually due to complications of decreased kidney function and when severe, they can be treated only by dialysis or transplantation [9]. Signs and symptoms of chronic kidney disease develop over time if kidney damage progresses slowly. The early symptoms of chronic kidney disease are the same as for many other illnesses. These symptoms may be the only sign of a problem in the early stages.

Symptoms may include:

- Appetite loss
- General ill feeling and fatigue
- Headaches
- Itching (pruritus) and dry skin
- Nausea
- Weight loss without trying to lose weight

Symptoms that may occur when kidney function has gotten worse include:

- Abnormally dark or light skin
- Bone pain
- Drowsiness or problems concentrating or thinking
- Numbness or swelling in the hands and feet

- Muscle twitching or cramps
- Breath odor
- Easy bruising, or blood in the stool
- Excessive thirst
- Frequent hiccups
- Problems with sexual function
- Menstrual periods stop (amenorrhea)
- Shortness of breath
- Sleep problems
- Vomiting, often in the morning

**Causes and risk factors**

Much epidemiological and clinical evidence has shown a link between several factors and the initiation and the progression of CKD. These can be classified into two

distinct categories: those proven to be causal (risk factors) and those that are associated with CKD in the absence of established causal relations (risk markers) [10].

*Susceptibility factors*

CKD commonly clusters within families, which implies genetic or familial predisposition [11]. Genetic studies have suggested links between CKD and various alterations or polymorphisms of candidate genes encoding putative mediators. Low birthweight and infant malnutrition in some ethnic minorities might be associated with a reduction in the number of nephrons, predisposing to hypertension and renal disease in later life [12]. Male and elderly people might also be more susceptible to CKD, which would explain the high proportions of these population groups in renal-replacement-therapy programmes [13].

*Initiation factors*

Many cohort studies have identified hypertension, diabetes, hyperlipidaemia, obesity and smoking as risk factors or markers in the general population for the development of CKD. Common risk factors and markers seem to be linked to both renal and cardiovascular diseases in more developed countries.

Also, albuminuria itself is a predictor not only of CKD but also of cardiovascular morbidity and mortality [14]. Impaired kidney function is also a major risk factor for patients with cardiovascular disease [6, 15]. Consequently, early detection and prevention could influence both renal and cardiovascular morbidity and mortality. Evidence is lacking on the etiology of ESRD in many less developed countries owing to poor data collection and the absence of renal registries. In addition, these countries continue to suffer from the burden of infectious diseases with infection-related glomerulonephritis and consequent renal insufficiency. The infections include HIV (40 million infected worldwide), hepatitis C virus (170 million), malaria (300 million cases per year), schistosomiasis (200 million) and tuberculosis (200 million) [WHO, 2003]. The growth in the number of cases of CKD attributable to these infections is likely to parallel the rising number of infected individuals.

*Progression factors*

The progression of established CKD is variable and depends on several risk factors or markers. Non-modifiable factors include genetics, race, age, and sex. For instance, there is much evidence that the rate of progression of CKD is faster among patients who are elderly [16], male [17] or African-American [18]. Most important, among the modifiable progression factors is systemic hypertension [19, 20]. Proteinuria is a reliable marker of the severity of CKD and a powerful and independent predictor of its progression [21, 22]. Controversy prevails as to whether proteinuria is a risk factor for the progression of clinical nephropathies.

Patients with persistently high rates of urinary protein excretion (>3–5 g in 24 h) in general have a much faster rate of progression than those with mild or moderate proteinuria (<1–3 g in 24 h)[22]. Metabolic factors have been implicated in the progression of CKD. The Diabetes Control and Complications Trial[23]

and the UK Prospective Diabetes Study [24] established that poor diabetes control accelerates the progression of diabetic nephropathy in both type 1 and type 2 diabetes. Experimental evidence has also shown a link between hyperlipidaemia and the progression of diabetic and non-diabetic nephropathies [25]. A link between hyperuricaemia and the development of systemic hypertension, cardiovascular disease, and renal disease has been postulated [26]. The worldwide pandemic of obesity could also affect the progression of CKD: obesity has been associated with the initiation and progression of glomerulonephritides [27, 28]; the incidence of focal and segmental glomerulosclerosis is higher in obese than in lean individuals [29] and the progression of IgA nephropathy is thought to be faster in overweight patients. Whether these links are causal or simply associated with CKD remains unclear; obesity is associated with hypertension, albuminuria and dyslipidaemia, all of which are potential modifiers of the progression of CKD [30].

Cigarette smoking has been implicated in the initiation and progression of CKD. A graded increased risk of ESRD was noted in non-diabetic nephropathies with increasing cigarette smoking; the incidence of ESRD was increased by 5,9 times among heavy smokers. And heavy smokers had also a risk of developing albuminuria three times that of non-smokers [31]. Also the consumption of analgesics, especially paracetamol and non-steroidal anti-inflammatory agents, could be linked with a higher risk of developing CKD [32-34].

**Pathophysiology**

Approximately 1 million glomerular capillary units are present in each kidney, each contributing to the total GFR [35]. In the face of renal injury (regardless of the etiology), the kidney has an innate ability to maintain GFR, despite progressive destruction of nephrons, as the remaining healthy nephrons manifest hyperfiltration and compensatory hypertrophy. This nephron adaptability allows for continued normal clearance of plasma solutes. Plasma levels of substances, such as urea and creatinine start to show measurable increases only after total GFR has decreased to 50%. The plasma creatinine value approximately double with a 50% reduction in GFR (Krishna Murthy et al., 2012 A Study of Estimated Glomerular Filtration Rate In Chronic Kidney Disease).

The hyperfiltration and hypertrophy of residual nephrons, although beneficial for the reasons noted, has been hypothesize to represent a major cause of progressive renal dysfunction. The progression of chronic kidney disease tends to follow a stereotypical course in many cases; the increased glomerular capillary pressure may damage the capillaries: a common histologic finding in these cases is focal segmental glomerulosclerosis (FSGS) with tubulointerstitial fibrosis [36].

Decreased renal function interferes with the kidneys' ability to maintain fluid and electrolyte homeostasis (Thompson et al., 2009 Fluid and electrolyte problems in renal disfunction). The ability to concentrate urine declines early and it is followed by decreases in ability to excrete phosphate, acid and potassium. When renal failure is advanced (GFR ≤ 10 mL/min/1.73 $m^2$), the ability to dilute urine is lost; thus urine osmolality is usually fixed close to that of plasma (300 to 320 mOsm/kg) and urinary volume does not respond readily to variations in water intake.

<u>Aging and renal function</u>

The biologic process of aging initiates various structural and functional changes within the kidney [37, 38]. Renal mass progressively declines with advancing age and glomerulosclerosis leads to a decrease in renal weight. Histologic examinations show a decrease in glomerular number of as much as 30-50% by age 70 years. The GFR peaks during the third decade of life at approximately 120 mL/min/1.73 $m^2$; it then

undergoes an annual mean decline of approximately 1 mL/min/1.73 m², reaching a mean value of 70 mL/min/1.73 m² at age 70 years. Ischemic obsolescence of cortical glomeruli is predominant, with relative sparing of the renal medulla. Juxtamedullary glomeruli see a shunting of blood from afferent to efferent arterioles, resulting in redistribution of blood flow favoring the renal medulla. These anatomic and functional changes in renal vasculature appear to contribute to an age-related decrease in renal blood flow. Given the histologic evidence for nephronal senescence with age, a decline in the GFR is expected. However, a wide variation in the rate of GFR decline is reported because of measurement methods, race, gender, genetic variance and other risk factors for renal dysfunction.

Calcium and phosphate abnormalities

Abnormalities of Ca++, phosphate, parathyroid hormone (PTH), vitamin D metabolism and renal osteodystrophy can occur. A study by Schwarz et al suggested that lower serum calcium was associated with higher risk of CKD progression [39]. Decreased renal production of calcitriol contributes to hypocalcemia and it develops primarily from decreased intestinal calcium absorption because of low plasma calcitriol levels. It also possibly results from increased calcium-phosphate binding, caused by elevated serum phosphate levels [40]. Decreased renal excretion of phosphate results in hyperphosphatemia. Secondary hyperparathyroidism is common and can develop in renal failure before abnormalities in Ca++ or phosphate concentrations occur. For this reason, monitoring PTH in patients with moderate CKD, even before hyperphosphatemia occurs, has been recommended. Phosphate retention begins in early CKD [41]; when the GFR falls, less phosphate is filtered and excreted, but because of increased PTH secretion, which increases renal excretion, serum levels do not rise initially. As the GFR falls toward CKD stages 4-5, hyperphosphatemia develops from the inability of the kidneys to excrete the excess dietary intake [42]. Hyperphosphatemia suppresses the renal hydroxylation of inactive 25-hydroxyvitamin D to calcitriol, so serum calcitriol levels are low when the GFR is less than 30 mL/min/1.73 m². Increased phosphate concentration also effects PTH concentration by its direct effect on the parathyroid glands (post-transcriptional effect).

Hyperkalemia and hypokaliemia

Hyperkalaemia is common in patients with chronic kidney disease (CKD), in part because of the effects of kidney dysfunction on potassium homeostasis and in part because of the cluster of comorbidities (and their associated treatments) that occur in patients with CKD [43]; nevertheless, the ability to maintain potassium excretion at near-normal levels is generally maintained in CKD, as long as aldosterone secretion and distal flow are maintained. Another defense against potassium retention in patients with CKD is increased potassium excretion in the gastrointestinal tract, which also is under control of aldosterone. Hyperkalemia usually does not develop until the GFR falls to less than 20-25 mL/min/1.73 m², at which point the kidneys have decreased ability to excrete potassium. Hyperkalemia can be observed sooner in patients who ingest a potassium-rich diet or have low serum aldosterone levels. Common sources of low aldosterone levels are diabetes mellitus and the use of ACE inhibitors, NSAIDs or beta-blockers. Hyperkalemia in CKD can be aggravated by an extracellular shift of potassium, such as occurs in the setting of acidemia or from lack of insulin.

Hypokalemia (K < 3.5) is uncommon but can develop in patients with very poor intake of potassium, gastrointestinal or urinary loss of potassium, or diarrhea or in patients who use diuretics (Thompson et al., 2009 Fluid and electrolyte problems in renal disfunction).

Metabolic acidosis

Anions accumulate during the course of CKD [44-46] and unmeasured anions, such as indoxyl sulfate and p-cresyl sulfate, probably accelerate CKD progression [47] and complications [48, 49], including bone disease, muscle protein catabolism and progressive glomerular filtration rate loss. The KDOQI guidelines, based on "evidence and opinion," call for maintenance of the serum bicarbonate level at ≥22 mEq/L to lessen these complications [9]. In stage 5 CKD, accumulation of phosphates, sulfates and other organic anions are the cause of the increase in anion gap. Metabolic acidosis has been shown to have deleterious effects on protein balance, leading to negative nitrogen balance, increased protein degradation, increased essential aminoacid oxidation and reduced albumin synthesis [50]. In addition, metabolic acidosis is a factor in the

development of renal osteodystrophy [51], because bone acts as a buffer for excess acid, with resultant loss of mineral [52]. Acidosis may interfere with vitamin D metabolism and patients who are persistently more acidotic are more likely to have osteomalacia or low-turnover bone disease.

Salt- and water-handling abnormalities

Salt and water handling by the kidney is altered in CKD. Extracellular volume expansion and total-body volume overload results from failure of sodium and free-water excretion [53]. This generally becomes clinically manifested when the GFR falls to less than 10-15 mL/min/1.73 $m^2$, when compensatory mechanisms have become exhausted. As kidney function declines further, sodium retention and extracellular volume expansion lead to peripheral edema and, not uncommonly, pulmonary edema and hypertension.

Anemia

Normochromic normocytic anemia principally develops from decreased renal synthesis of erythropoietin, the hormone responsible for bone marrow stimulation for red blood cell (RBC) production. The anemia starts early in the course of the disease and becomes more severe as, with the shrinking availability of viable renal mass, the GFR progressively decreases: the prevalence of anemia increased with stage of CKD, from 8.4% at stage 1 to 53.4% at stage 5 [54]. No reticulocyte response occurs. RBC survival is decreased and bleeding tendency is increased from the uremia-induced platelet dysfunction.

Bone disease

Chronic kidney disease-related mineral and bone disease (CKD-MBD) is a syndrome defined as a systemic mineral metabolic disorder associated with CKD which can result in disorders of the bone metabolism and/or the cardiovascular system [55]. Cortical bone thinning, abnormalities in bone turnover and primary/secondary mineralization, elevated levels of circulating sclerostin, increased apoptosis in osteoblasts and osteocytes, disturbance of the coupling phenomenon, iatrogenic factors, accumulated micro-crackles, crystal/collagen disorientation and chemical modification of collagen crosslinks are all possible candidates found in CKD that could promote osteopenia and/or bone fragility [56]. This disease

consists of three components: abnormalities observed in laboratory examinations, including parathyroid gland dysfunction; abnormality in the bone metabolism; and abnormality in the soft tissue calcification including vascular calcification. CKD-MBD may result from alteration in levels of serum phosphorus, PTH, vitamin D and alkaline phosphatase.

Skeletal manifestations

If serum levels of PTH remain elevated, a high–bone turnover lesion, known as osteitis fibrosa, develops [57]. This is one of several bone lesions and develop in patients with severe CKD. Osteitis fibrosa is common in patients with ESRD. The pathogenesis of another bone disease, the adynamic bone disease [58], is not well defined, but several factors may contribute, including high calcium load, use of vitamin D sterols, increasing age, previous corticosteroid therapy, peritoneal dialysis and increased level of N-terminally truncated PTH fragments. Low-turnover osteomalacia in the setting of CKD is associated with aluminum accumulation [59]. It is markedly less common than high-turnover bone disease. Another form of bone disease is dialysis-related amyloidosis, which is now uncommon in the era of improved dialysis membranes. This condition occurs from beta-2-microglobulin accumulation in patients who have required chronic dialysis for at least 8-10 years; it manifests with cysts at the ends of long bones.

**Prognosis**

Patients with CKD generally experience progressive loss of kidney function and are at risk for ESRD. The rate of progression depends on age, the underlying diagnosis, the success of implementation of secondary preventive measures and the individual patient. Timely initiation of chronic renal replacement therapy is imperative to prevent the uremic complications of CKD that can lead to significant morbidity and death. Lower estimated glomerular filtration rate, higher albuminuria and male sex pointed to a faster progression of kidney failure [60]. Also, a lower serum albumin, calcium and bicarbonate level and a higher serum phosphate level were found to predict an elevated risk of kidney failure [60].

Hospitalization

Unadjusted rates of hospitalization in the CKD population, reflecting its total disease burden, are 3-5 times higher than those of patients without CKD [61]. After adjustment for gender, prior hospitalizations and comorbidity, rates for patients with CKD are 1.4 times higher. Rates of hospitalization of these patients for cardiovascular disease and bacterial infection are particularly elevated CKD [61].

Dialysis and Mortality

The mortality rates associated with CKD are striking. After adjustment for age, gender, race, comorbidity and prior hospitalizations, mortality in patients with CKD in 2009 was 56% greater than that in patients without CKD [61]. For patients with stages 4-5 CKD, the adjusted mortality rate is 76% greater. Mortality rates are consistently higher for men than for women and for black than for white individuals and patients of other races. The highest mortality rate is within the first 6 months after the beginning of dialysis. Mortality then tends to improve over the next 6 months, before increasing gradually over the next 4 years. The 5-year survival rate for a patient undergoing long-term dialysis is approximately 35% and approximately 25% in patients with diabetes. At every age, patients with ESRD on dialysis have significantly increased mortality when compared with non-dialysis patients and individuals without kidney disease. At 60 years old, a healthy person can expect to live for more than 20 years, whereas the life expectancy of a patient aged 60 years who is starting hemodialysis is closer to 4 years. Among patients aged 65 years or older who have ESRD, mortality rates are 6 times higher than in the general population [61].

**Prognostic relevance of CKD in cardiovascular disease**

In coronary artery disease (CAD), CKD represents a potent and independent risk factor for adverse outcome [62]. Patients with chronic kidney disease have a high burden of cardiovascular morbidity and mortality. The vast majority of patients with chronic kidney disease do not progress to end-stage renal failure, but do have a significantly higher incidence of all cardiovascular co-morbidities. Traditional cardiovascular risk factors (such as hypertension, dyslipidemia, diabetes and obesity) only partially account for this increased incidence of cardiovascular disease [63]. There are many other cardiovascular risk factors that are either "uremia specific", or at least much more common in patients with CKD than in the general population. These factors include anemia, hyperparathyroidism, carnitine deficiency, hyperhomocysteinemia, low vitamin C, high lipoprotein A levels and small apolipoprotein A size [64]. In patients with kidney disease the basic biology underlying cardiovascular disease may be similar to that in patients without kidney disease, but it would seem many more risk factors are involved as a consequence of renal dysfunction. Although emphasis is placed on delaying the progression of chronic kidney disease, it must be appreciated that for many patients it is vital to address their cardiovascular risk factors at an early stage to prevent premature cardiovascular death [65]. Antithrombotic agents and percutaneous coronary interventions (PCI) are clearly emerging as the cornerstones of treatment patterns in patients presenting with ACS [66]. However, despite the increasing number of CKD patients with a broad range of CAD at presentation, evidence-based data with established or newer drugs and interventional strategies are still lacking in this population because CKD patients have typically been excluded from randomized trials. Ideally, these are the patients to whom recent therapeutic advances should be aggressively applied, in order to minimize their increased risk. However, application of strategies for reducing cardiovascular morbidity and mortality seem to be limited in CKD patients, when compared to patients with normal renal function.

<u>NSTEMI and CKD</u>

Chronic kidney disease is present in a substantial proportion of patients with ACS; indeed, large registries report that almost 40% of patients with NSTEMI and 30% of those with STEMI have CKD [67, 68]. Several observational studies have found that, in the setting of NSTEMI, in-hospital outcomes and mid- to long-

term mortality are worse among patients with CKD [69-71]. The GRACE (Global Registry of Acute Coronary Events) study, a large prospective multinational registry, including the full spectrum of patients with ACS, evaluated the prognostic impact of serum creatinine (sCr) levels on hospital mortality and adverse outcomes in 11.774 NSTEMI patients [69]. Patients were divided into three groups according to their creatinine clearance values: >60 ml/min or normal renal function (including patients with minimally impaired renal function), 30-60 ml/min or moderate renal insufficiency, and <30 ml/min or severe renal insufficiency. In comparison with patients with normal renal function, patients with moderate and severe renal insufficiency were at a significantly increased risk of hospital mortality and major bleeding episodes. The significant contribution of renal function evaluation to risk stratification was demonstrated by Gibson et al. [72] who pooled data from five international multicenter trials (TIMI 11A and B, TIMI 12, OPUS-TIMI 16, and TACTICS-TIMI 18) and analyzed 13.307 patients with NSTEMI. Notably, these trials excluded patients with a sCr level above 2.0 mg/dl. As a consequence, moderate to severe renal insufficiency (GFR <60 ml/min/1.73 $m^2$) was present in 16% of patients, while only less than 1% of the patients had a GFR<30 ml/min/1.73 $m^2$. In this study, a significant and graded association was observed between reduced GFR and short-term (30 days) and mid-term (6 months) mortality: 1.3% and 2.5% for patients with normal renal function, 2.1% and 3.8% for patients with mild renal insufficiency and 5% and 9.5% for patients with moderate to severe renal insufficiency, respectively (P for trend <0.001). In parallel, there was a stepwise increase in the incidence of stroke, major bleeding, recurrent myocardial infarction and recurrent ischemia with worsening GFR. Interestingly, within each GFR category, high TIMI (Thrombolysis in Myocardial Infarction) risk scores and augmented levels of biomarkers (C-reactive protein, B-type natriuretic peptide and troponin I) were associated with a significant increase in mortality as compared with low TIMI scores and normal levels of biomarkers, demonstrating the prognostic value of GFR in addition to the traditional clinical risk stratification of patients with NSTEMI.

SA and CKD

Chronic kidney disease is strongly associated with a higher risk of cardiovascular disease [6, 73-75], and studies have shown that at least 25% of the patients in dialysis had CAD [76, 77]; even earlier stages of CKD have been associated with worse outcomes in patients with CAD [78, 79]. In the MASS II [80], a randomized trial that evaluated the long-term relative efficacies of three possible therapeutic strategies for patients with multivessel CAD, stable angina, and preserved left ventricular function, 611 patients were randomly assigned to medical treatment MT (n=203), PCI (n=205), or CABG (n=203). All patients had angiographicaly documented proximal multivessel coronary stenosis of more than 70% by visual assessment and documented ischemia. Exclusion criteria included unstable angina or acute MI necessitating emergency revascularization, ventricular aneurysm necessitating surgical repair, left ventricular ejection fraction of less than 40%, a history of PCI or CABG, single-vessel disease, or previous cardiac surgery. Patients were categorized in normal renal function (RF) and mild or moderate CKD groups depending on their GFR based on the National Kidney Foundation (NFK) classification. The results of this trial confirm that CAD accompanied by CKD has a worse prognosis, regardless of the therapeutic strategy for CAD. The lowest GFR group had a 4.5-fold increased risk of death within 5 years compared with the normal GFR group. Additionally, these data suggest that the different treatment strategies available for stable CAD may have differential beneficial effects according to the range of GFR strata. Authors showed that patients with stable multivessel CAD and CKD, mainly in early stages, had more events when kept in MT over 5 years of follow-up. Of note, CABG patients compared with the PCI or MT patients had a better free-event survival curve and less overall mortality rates only in patients with mild CKD (or stage 2 CKD). However, among patients with moderate CKD, CABG surgery did not convey a survival benefit over PCI or MT. Prior studies in high-risk populations, such as those who already have CAD or who have many risk factors for cardiovascular disease, have suggested that the level of kidney function is a risk factor for cardiovascular disease outcomes [74, 81-84].

## CHRONIC KIDNEY DISEASE AND CORONARY ARTERY DISEASE

### Acute coronary syndrome

Definition

The term *acute coronary syndrome* (ACS) refers to any group of clinical symptoms compatible with acute myocardial ischemia and covers the spectrum of clinical conditions ranging from unstable angina (UA) to non-ST-segment elevation myocardial infarction (NSTEMI) to ST-segment elevation myocardial infarction (STEMI) [85]. Unstable angina and NSTEMI are closely related conditions: their pathophysiologic origins and clinical presentation are similar, but they differ in severity. A diagnosis of NSTEMI can be made when the ischemia is sufficiently severe to cause myocardial damage that results in the release of a biomarker of myocardial necrosis into the circulation (cardiac-specific troponins T or I or muscle and brain fraction of creatine kinase [CK-MB]). In contrast, the patient is considered to have experienced UA if no such biomarker can be detect in the bloodstream hours after the initials onset of ischemic chest pain. Unstable angina exhibits one or more of three principle presentations: rest angina (usually lasting major 20 minutes), new-onset (< 2 months previously) severe angina and an increased pattern of occurrence (increasing in intensity, duration, frequency or any combination of these factors) [86].

Etiology

Acute coronary syndrome (ACS) is caused primarily by atherosclerosis. Most cases of ACS occur from disruption of a previously non-severe lesion (an atherosclerotic lesion that was previously hemodynamically insignificant yet vulnerable to rupture). The vulnerable plaque is characterized by a large lipid pool, numerous inflammatory cells, and a thin fibrous cap [87]. The major trigger for coronary thrombosis is considered to be plaque rupture [88, 89] caused by the dissolution of the fibrous cap and the dissolution is the result of the release of metalloproteinases (collagenases) from activated inflammatory cells [90]. This event is followed by platelet activation and aggregation, activation of the coagulation pathway and vasoconstriction. This process culminates in coronary intraluminal thrombosis and variable degrees of

vascular occlusion [91]. Distal embolization may occur. The severity and duration of coronary arterial obstruction, the volume of myocardium affected, the level of demand on the heart and the ability of the rest of the heart to compensate are major determinants of a patient's clinical presentation and outcome [92]. Baseline blood glucose levels appear to be an independent risk factor for a major adverse cardiac event (MACE) in patients with suspected ACS [93]. Other significant predictors of MACE include male sex, older age, family history, hypertension, dyslipidemia, ischemic findings on ECG and positive troponin-tests [94].

Prognosis

The prognosis of patients with acute coronary syndromes generally depends on the occurrence and extent of myocardial damage [95]. Patients presenting without persistent ST-segment elevation or a typical enzyme rise have the lowest incidence of mortality and morbidity [96]. Intermediate complication rates are seen in those presenting without ST-segment elevation but with a rise in cardiac enzymes [96], whereas prognosis is worst in patients with ST-segment elevation and substantial myocardial damage. Apart from this simple classification, analyses of the GUSTO-1 (Global Utilization of Streptokinase and Tissue plasminogen activator for Occluded coronary arteries) population have demonstrated that age, heart rate, blood pressure and signs of heart failure are the key factors in predicting outcome in patients with STEMI [97, 98]. The Global Registry of Acute Coronary Events (GRACE) reports that the six-month mortality rate is 13% for patients with NSTEMI ACS and 8% for those with unstable angina [98].

**Stable coronary artery disease**

Definition

Stable coronary artery disease (or stable angina, SA) is generally characterized by episodes of reversible myocardial demand/supply mismatch, related to ischaemia or hypoxia, which are usually inducible by exercise, emotion or other stress and reproducible, but which may also be occurring spontaneously [99]. Such episodes of ischaemia/hypoxia are commonly associated with transient chest discomfort (angina pectoris). SA also includes the stabilized, often asymptomatic, phases that follow an ACS. The various clinical presentations of SA are associated with different underlying mechanisms that mainly include: 1) plaque-related obstruction of epicardial arteries; 2) focal or diffuse spasm of normal or plaque-diseased arteries; 3) microvascular dysfunction; and 4) left ventricular dysfunction caused by prior acute myocardial necrosis and/or hibernation (ischaemic cardiomyopathy). These mechanisms may act singly or in combination. However, stable coronary plaques with and without previous revascularization may also be completely clinically silent [99].

Etiology

Myocardial ischaemia and hypoxia in stable coronary artery disease are caused by a transient imbalance between blood supply and metabolic demand. The consequences of ischaemia occur in a predictable temporal sequence that involves:

a) increased H+ and K+ concentration in the venous blood that drains the ischaemic territory [100];

b) signs of ventricular diastolic and subsequently systolic dysfunction with regional wall motion abnormalities [101];

c) development of ST–T changes [99];

d) cardiac ischaemic pain (angina)

Angina is ultimately caused by the release of ischaemic metabolite, such as adenosine, that stimulate sensitive nerve endings, although angina may be absent even with severe ischaemia owing, for instance, to impaired transmission of painful stimuli to the cortex and other as-yet-undefined potential mechanisms [102].

Prognosis

Within the population with stable CAD, an individual's prognosis can vary considerably, depending on baseline clinical, functional and anatomical characteristics. Prognostic assessment is an important part of the management of patients with stable coronary artery disease. On the one hand, it is important to reliably identify those patients with more severe forms of disease, who may have an improvement in outcome with more aggressive investigation and, potentially, intervention, including revascularization [99]. On the other hand, it is also important to identify those patients with a less severe form of disease and a good prognosis, thereby avoiding unnecessary invasive and non-invasive tests and revascularization procedures. Conventional risk factors for the development of CAD [103-105], such as hypertension [106], hypercholesterolaemia [107], diabetes [108],sedentary lifestyle [109], obesity [109],smoking [106, 110] and a family history [111] have an adverse influence on prognosis in those with established disease, presumably through their effect on the progression of atherosclerotic disease processes.

**Treatment of patients with CAD and CKD**

Treatment of CAD in patients with CKD is particularly problematic. Traditionally, patients with advanced CKD and ESRD receiving dialysis have not been included in randomized CAD trials evaluating either medical or interventional therapies. Thus, only scarce data deriving from limited observational studies are available and, to date, no optimal treatment strategy has been defined for this subgroup of patients.

Coronary reperfusion strategies

Doubts still exist on how CKD patients should be treated in the early phase of CAD. In particular, there are concerns about the use of aggressive reperfusion strategy (fibrinolytic therapy and primary PCI). Undoubtedly, landmark megatrials, such as the GISSI (Gruppo Italiano per lo Studio della Streptochinasi nell'Infarto Miocardico), the ISIS (International Study of Infarct Survival) and the GUSTO (Global Utilization of Streptokinase and Tissue Plasminogen for Occluded Coronary Arteries) trials, have shown the benefit of thrombolytic agents in reducing mortality in patients with ACS [112-114]. However, in all these trials, no subgroup analysis was performed in patients with CKD, and scarce data have been published on the use of thrombolytics in these patients. Reperfusion therapy was used less frequently in patients with any degree of CKD than in patients without renal insufficiency. Renal insufficiency should not preclude the success rate of percutaneous or pharmacological reperfusion therapies, but it may be associated with increased incidence of major adverse events (MACE). The influence of renal insufficiency in patients with STEMI receiving fibrinolytic therapy was investigated by Gibson et al [114]. Despite appropriate treatment with thrombolytics and adjunctive therapies for acute myocardial infarction (including early PCI in many patients) and even though the epicardial and myocardial reperfusion rates were equivalent, there was a stepwise decrease in survival going from normal to mildly and severely impaired renal function that continued through up to two years of follow-up. The incidence of intracranial hemorrhage was also increased in patients with CKD, suggesting that primary PCI may represent a favorable alternative therapy. Nevertheless, the outcomes of primary PCI in patients with STEMI and CKD have not been well characterized, because such patients are typically excluded from clinical trials. According to previous

studies and guidelines, an invasive strategy is superior to an initial conservative strategy (invasive management only after failed medical therapy or for objective evidence of ischemia) and an early invasive strategy is superior to a delayed invasive strategy. In the KAMIR study, an invasive (within 24 hours after admission) strategy decreased mortality compared to a conservative strategy except for severe CKD. In the timing of an invasive strategy, there were trends showing that an early invasive strategy was superior to a delayed strategy in patients with mild CKD, but this tendency decreased as renal function decreased [115].

Standard medical therapy

Berger et al. [116] compared the patterns of care and the effect of standard STEMI therapy on 30-day mortality between ESRD patients on chronic dialysis (either peritoneal dialysis or haemodialysis) and non-ESRD patients. They confirmed that aspirin, beta-blockers, and ACE-inhibitors are less likely to be used in ACS patients on dialysis, even among those considered "ideal candidates" for these medications, than in patients not receiving dialysis. Nevertheless, the authors observed a similar absolute reduction in short-term mortality with aspirin, beta-blocker and ACE-inhibitor therapy when comparing the dialysis and non-dialysis groups. Aspirin was associated with a 21% absolute reduction in mortality in dialysis patients and a 23% reduction in non-dialysis patients. Beta-blocker therapy was associated with a 14% absolute reduction in mortality in both the dialysis and non-dialysis patients. The ACE-inhibitor use was associated with a 16% absolute reduction in 30-day mortality in dialysis patients and a 5% reduction in non-dialysis patients. Recently, data from the ACTION registry documented, lower use of short-term therapies, in-hospital procedures, cardioprotective medications and higher rates of medication overdosing among patients with CKD [68]. Finally, despite these high rates of adverse outcomes, patients with CKD were less likely to receive discharge counseling related to cardiovascular disease risk reduction. Thus, all these studies confirm less aggressive care in CKD patients with CAD that parallel the degree of renal dysfunction and demonstrate that beneficial therapies are underutilized in patients with CKD despite increased prevalence of hypertension, congestive heart failure and coronary artery disease, and despite the fact that these medications are associated with a substantial survival benefit in patients with normal renal function.

Antithrombotic therapy

Refinement of the antithrombotic strategies among CKD patients in CAD setting is still a major and unmet need. The challenge is daunting because, on the one hand, renal insufficiency is associated with prolongation of bleeding time and abnormal platelet aggregation and adhesion [117] and, on the other hand, a state of hypercoagulation has been demonstrated with high levels of von Willebrand factor [118], fibrinogen, factors VII, VIII, and XIII and enhanced thrombin generation [117]. The combination of these alterations puts the patient with CKD at risk, simultaneously, for thrombosis and hemorrhage. Thus, use of well-established antiplatelet drugs, such as aspirin and clopidogrel, should be weighed against bleeding risk in renal patients. Current practice guidelines recommend the addition to aspirin of another antiplatelet agent (ticlopidine, clopidogrel, prasugrel, ticagrelor) for treatment of high-risk CAD patients. Despite never specifically investigated in CKD patients, some differences, in terms of bleeding risk, possibly exist among these drugs. In the CREDO (Clopidogrel for the Reduction of Events During Observation) trial the effectiveness of clopidogrel in reducing adverse cardiovascular events seemed to be greatest in patients with normal renal function, whereas patients with moderate CKD appeared to have experienced less benefit [119]. In the PLATO (Platelet Inhibition and Patient Outcomes) study clinical evidence was provided that ticagrelor is a more effective antiplatelet agent than clopidogrel in patients with CAD, regardless of renal function and without any need for dose reduction to prevent major bleeding [120]. In addition to antiplatelet agents, heparin has become the standard of care in patients with ACS: the two preparations generally available are unfractionated heparin (UFH) and low-molecular weight heparin (LMWH). A major difference between these two therapeutic agents is their mechanism of clearance: at low doses, UHF is cleared primarily by macrophages and endothelial cell binding, whereas LMWH is cleared primarily by renal mechanisms [121]. Clinical studies on enoxaparin, the most widely used LMWH in NSTEMI, excluded patients with CKD, so that the optimal dosing for renal patients has not been established [122, 123]. Until conclusive results are available regarding optimal dosing, it may be safer to use UFH in CKD patients presenting with ACS. It should be highlighted, however, that bleeding risk increases in parallel with the increasing severity of baseline renal insufficiency also in patients receiving UHF alone [124]. Impaired renal clearance of many

pharmacological agents may increase the probability of overdosing in patients with reduced renal function, further increasing their bleeding risk [125]. This is particularly true for renally excreted antithrombotic agents, such as enoxaparin, tirofiban and eptifibatide that are recommended at a lower dose in CKD patients. Use of newer antithrombotic agents, such as platelet glycoprotein (GP) IIb/IIIa receptor inhibitors, has become the standard of care for the higher-risk CAD patients, mainly for those undergoing PCI. However, patients with CKD were also excluded from entry into most randomized trials investigating GPIIb/IIIa antagonists. Thus, it is not clear if they may derive the same therapeutic benefit with equivalent safety from these pharmacological agents, as do patients with normal renal function. Moreover, because agents such as tirofiban and eptifibatide are largely cleared through the kidneys, moderate to severe renal insufficiency would be expected to increase the mean plasma concentration of these drugs, producing a greater inhibition of platelet aggregation. Since platelet-bound abcximab is eliminated by the reticuloendothelial system [126], its use in patients with renal insufficiency should not be associated with greater impairment of platelet function. Thus, unlike tirofiban and eptifibatide, abciximab does not require dosing adjustment in CKD patients.

Statins

Coronary artery disease patients with CKD are less likely to be treated with statins, despite the evidence of reduced mortality with statins use in patients with or at risk for coronary events [69, 83, 127, 128]. In addition to the long-term benefit of statin therapy in ACS patients, data from National Registry of Myocardial Infarction 4 suggest that administration of statins within the first 24 hour of hospitalization for acute myocardial infarction significantly lowers the rate of early complications and in-hospital mortality, possibly due to their pleiotropic effects [129]. Indeed, acute myocardial infarction is associated with a number of abnormalities, including inflammation, endothelial dysfunction and coagulation disorders, all of which appear to be dampened by statins [130]. Patients with CKD have more co-morbidities and, as a consequence, more contraindications to these medications. Statins are primarily eliminated by the liver, while the renal route is usually a minor elimination pathway. However, reluctance to prescribe statins in patients with CKD is likely

due to remaining uncertainty regarding their clinical effects in patients with CKD. In particular, no clear evidence of a positive relationship between blood cholesterol and cardiovascular events has been found in these patients. As they approach end-stage renal disease, there appears to be increased oxidation of low-density lipoprotein, with progressive lowering of total cholesterol levels. The influence of dyslipidemia upon cardiovascular outcomes shows an increasing cardiovascular event rate among patients with severe CKD having low cholesterol levels. This "low-cholesterol paradox" has been attributed to the effects of chronic malnutrition and inflammation, which become increasingly important in severe CKD [131]. This paradox has raised the question of the utility of lipid-lowering therapy, although previous evidence than statin therapy may delay progression of renal dysfunction [132]. Moreover, some concerns about drug toxicity related to high statin doses exist in CKD patients. Indeed, high statin doses have been associated with an increased risk of myopathy in CKD patients [133].

## PLATELETS

### Platelet biology

Platelets were discovered by the pathologist Giulio Bizzozero in 1882. He observed them microscopically in the circulating blood of living animals and in the blood removed from the blood vessels. Platelets, also called thrombocytes, are anucleated discoid cell fragments with a diameter of 1-4 μm and a thickness of 1 μm. Platelets derived from the cytoplasmatic fragmentation of megakaryocytes, their precursor cells, in the bone marrow. Megakaryocytes are rare myeloid cells that reside within the bone marrow and represent less than 0,1% of the myeloid cells [134]; they could be found also in the lungs and in the peripheric bloodstream. Megakaryocytes tailor their cytoplasm and membrane systems for platelet biogenesis. For the release of platelets, megakaryocytes have to enlarge considerably their diameter (100 μm) and they fill with high concentrations of ribosomes which allow the production of platelet-specific proteins. Cellular enlargement is mediated by repeated cycles of endomitosis, a process that amplifies the DNA by as much as 64-fold [135]. Platelet release by megakaryocytes involves the development of cytoplasmic ramifications,

the so called “pro-platelets”, of 100-500 µm of length. This process starts from a single site on megakaryocyte with the emission of pseudopodal extensions, that stretch, in 4-10 hours, into pro-platelets. In the final portion of the pro-platelet, the bundles of microtubules form loops that come out and fall in pro-platelet causing the formation of bulbous tips of 3-5 µm in diameter [136]. Then the single platelets are released into the bloodstream (Figure 2).

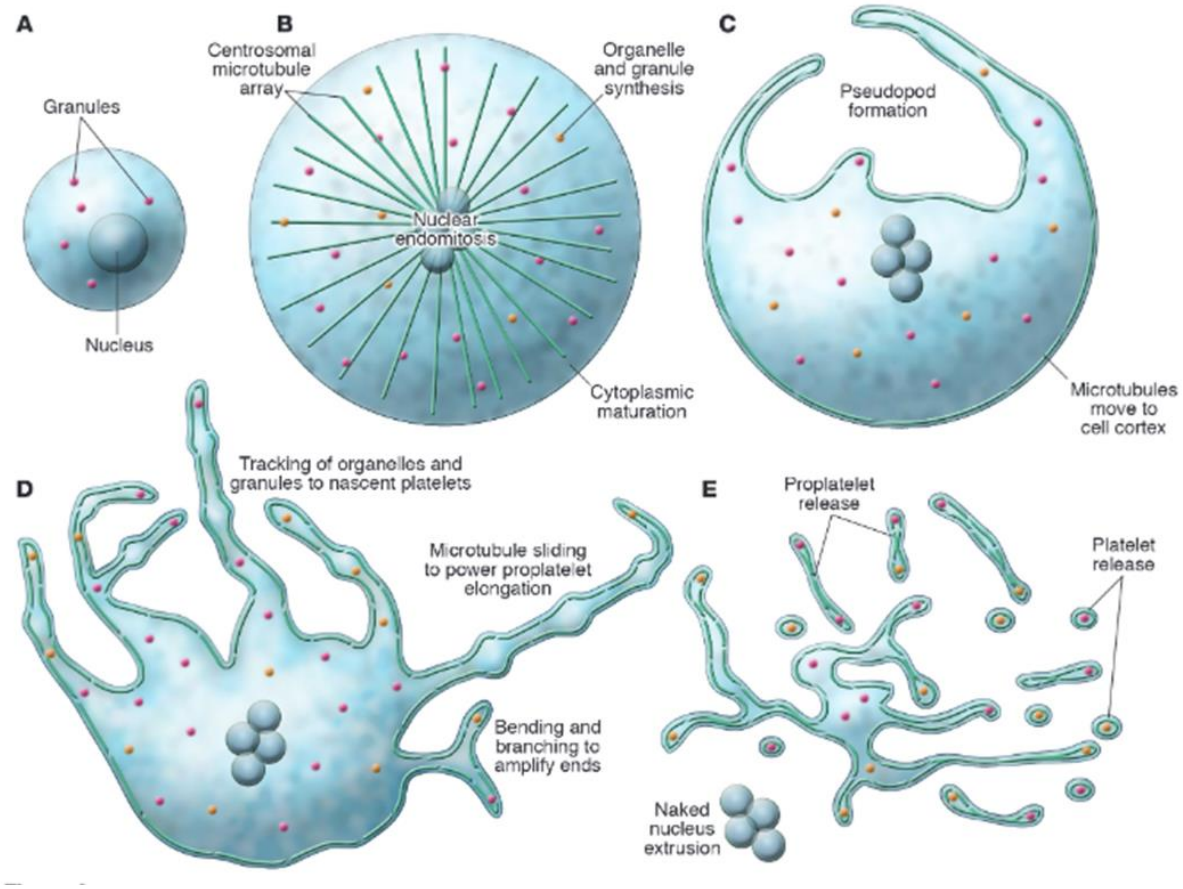



***Figure 2.*** **Schematic overview of platelets release by megakaryocytes.** (B) Megakaryocytes maturation is characterized by repeated cycles of endomitosis and by a significant maturation of the cytoplasm with the formation of internal membranes, granules and organelles while the microtubules extend (C) Shortly before the formation of pro-platelets, microtubules are consolidated into a mass beneath the plasma membrane and blend in linear beams thicker branches that fill pro-platelets determining the elongation (D) In the terminal portion of the pro-platelets, the bundles of microtubules form loops that come out and fall in pro-platelets causing the formation of bulbous tips. (E) The entire cytoplasm of megakaryocytes is converted into pro-platelets that are released from the cell. The individual platelets are then released from the ends of pro-platelet. *From J Clin Invest 2005; 115 (2): 3348-54*

Besides the prevention of bleeding, that is their physiological role, platelets have other important functions, for example in the inflammatory process [137-139]: in fact into their granules, platelets store chemokines, cytokines and growth factors. Every day $10^{11}$ platelets are produced and their average lifespan is about 8-10 days, after which time they reach senescence and they are removed from the bloodstream by the spleen and liver, and this mechanism also works in the case of morphological or functional abnormalities of platelets. In physiological conditions, platelet count in peripheral blood is approximately 250.000/µl, whereas the rate of platelet production may change in pathologic conditions: a platelet count lower than 100.000 platelets/µl is defined as thrombocytopenia and could lead to an increased risk of bleeding; an higher platelet count, more than 600.000 platelets/µl, is defined as thrombocytosis and could lead to an increased risk of thrombotic events.

Structurally, platelets consist of:

- A plasma membrane rich in phospholipids including phosphatidylserine and phosphatidylinositol, negatively charged and abundant in the cytoplasmic side of the membrane where they act as a substrate for the phospholipase. The glycocalyx is rich in glycoproteins, some of which perform receptor functions in response to stimuli and agonists, giving to the platelets the ability to convey information and to give rise to a cellular response [140].

- Internal membranes formed by the open canalicular system and by the tubular dense on which are anchoring the enzymes involved in the synthesis of prostanoids.

- A cytoskeleton composed of microtubules and microfilaments, which are important in the process of platelet activation and secretion.

- Mitochondria, lysosomes and peroxisomes.

- Secretory granules: lysosomes, dense granules (also called delta granules) and alpha granules. Lysosomes contain hydrolytic enzymes such as elastase, which may affect the vessel wall and thus promote atherosclerosis; dense granules are rich in nucleotides (ADP and ATP), serotonin, histamine, pyrophosphate

and calcium; alpha granules contain proteins important for the haemostatic function such as von Willebrand factor (VWF), fibrinogen, P-selectin, glycoprotein IIb/IIIa, PECAM-1, CD40 ligand, platelet factor-4 PF-4), thromboglobulin, beta thrombomodulin, thrombospondin, platelet derived growth factor (PDGF), Factor V, Factor X, interleukin 1 and PAI-1. The content of these granules is secreted during platelet activation (Table 2) .

| α-Granules | Dense bodies |
|---|---|
| Platelet-specific proteins | ADP |
| Platelet factor 4 | ATP |
| β-Thromboglobulin family* | Calcium |
| Multimerin | Serotonin |
| Adhesive glycoproteins | Pyrophosphate |
| Fibrinogen | GDP |
| von Willebrand factor | Magnesium |
| von Willebrand factor propeptide | Other secreted or released proteins |
| Fibronectin | Protease nexin I |
| Thrombospondin-1 | Gas6 |
| Vitronectin | Amyloid β-protein precursor (protease nexin II) |
| Coagulation factors | Tissue factor pathway inhibitor |
| Factor V | Factor XIII |
| Protein S | $\alpha_1$-Protease inhibitor |
| Factor XI | Complement 1 inhibitor |
| Mitogenic factors | High molecular weight kininogen |
| Platelet-derived growth factor | $\alpha_2$-Macroglobulin |
| Transforming growth factor-β | Vascular permeability factor |
| Endothelial cell growth factor | Interleukin-1β |
| Epidermal growth factor | Histidine-rich glycoprotein |
| Insulin-like growth factor I | Chemokines |
| Angiogenic factors | MIP-Iα (CCL3) |
| Vascular endothelial growth factor | RANTES (CCL5) |
| Platelet factor 4 (inhibitor) | MCP-3 (CCL7) |
| Fibrinolytic inhibitors | Gro-α (CXCL1) |
| $\alpha_2$-Plasmin inhibitor | Platelet factor 4 (CXCL4) |
| Plasminogen activator inhibitor-1 | ENA-78 (CXCL5) |
| Albumin | NAP-2 (CXCL7) |
| Immunoglobulins | Interleukin-8 (CXCL8) |
| Granule membrane-specific proteins | TARC (CCL17) |
| P-selectin (CD62P) | |
| CD63 (LAMP-3) | |
| GMP 33 | |

***Table 2.*** **Platelet granular and secreted molecules** *(adapted from Parise et al, 2005)*

**Platelet functions**

Platelets play a key role in haemostasis and in blood coagulation. Vascular and tissue injury involves removal of the anti-thrombotic endothelial cell layer and exposure of matrix molecules to different degrees according to the depth of the injury. Major matrix molecules include several types of collagen and laminin. Von Willebrand factor (VWF) has a major role in haemostasis by binding to collagen in the first instance and by binding to activated platelets at a later stage. Platelets, carried by the bloodstream, are swept up against exposed matrix, adhere, become activated, undergo a number of changes and, in turn, bind further platelets to form a thrombus preventing further bleeding but limited in size. Each of these phases is thought to involve specific or specific sets of platelet receptors [141].

Platelets play important roles in several diverse processes beyond haemostasis and thrombosis, including promoting inflammatory and immune responses, maintaining vascular integrity and contributing to wound healing. Platelets can recruit leukocytes and progenitor cells to sites of vascular injury and thrombosis; they spur thrombin generation; they store, produce and release pro-inflammatory and anti-inflammatory and angiogenic factors and microparticles into the circulation [142]. Platelets are important not only in thrombus formation occurring upon plaque rupture, but they play a pivotal role also in early stage of plaque development by releasing numerous cytokines (such as IL-1, CD40L, beta-thromboglobulin), chemokines (such as RANTES, PF4), growth factors (such as PDGF, TGF, EGF) and adhesion proteins (such as fibrinogen, fibronectin, von Willebrand factor).

*Platelet secretion* - The ability of platelets to store and release bioactive mediators allows them to play an important role in modulating the function of other cells. As described before, platelets contain three types of storage whose contents are released into the circulation or translocated to the platelet surface upon platelet activation.

*Platelet production of bioactive mediators* - Platelets are not only storage houses for bioactive molecules: in fact they also generate lipid-derived mediators such as thromboxane A2 and participate in transcellular metabolism, which results in the production of both pro-inflammatory and anti-inflammatory molecules. In

addition, platelets have several unique, extranuclear mechanisms for translating mRNA into protein in a signal-dependent manner and can produce, among other proteins, interleukin-1b and tissue factor, which may link haemostasis and inflammation.

*Platelet–leukocyte interactions* - Analogously to the interactions of leukocytes with inflamed endothelium, leukocytes can roll on a template of adherent platelets, firmly adhere and then transmigrate through the adherent platelets. Although some of the receptor–ligand pairs and signaling molecules that mediate platelet–leukocyte interactions may differ from those involved in endothelial cell–leukocyte interactions, many of the fundamental aspects are similar. Rolling and adhesion of leukocytes on platelets or endothelial cells are regulated by adhesive receptors, cellular geometry, and, perhaps of greatest overall relevance, shear forces generated within flowing blood.

The selectin family of adhesive receptors mediates the initial stage of cellular rolling. P-selectin plays an essential role in platelet–leukocyte contacts, whereas both P-selectin and E-selectin are present on endothelial cells and contribute to endothelial cell–leukocyte interactions. The third selectin, L-selectin, is present on leukocytes. The best-characterized leukocyte ligand for P-selectin is P-selectin glycoprotein ligand (PSGL)-1, which can interact with all selectin subtypes under inflammatory conditions. Ligation of PSGL-1 transmits signals within the leukocyte that are necessary for adhesion mediated by leukocyte integrins. Although unnecessary for leukocyte rolling on P-selectin, the cytoplasmic domain of PSGL-1 is essential for activation of leukocyte beta2 integrins.

Immobilized and released chemokines are also required for firm leukocyte adhesion and arrest. Prominent among the chemokines released by platelets that influence leukocyte function and platelet–leukocyte interactions are PF4/CXCL4, RANTES (regulated on activation, normal T-cell expressed and secreted; CCL5) and growth-related oncogene-alpha [142].

**Platelet transcriptome**

Platelets, although they do not have a nucleus, contain between 2000 and 7000 transcripts derived from megakaryocytes [143]. They have the translational capacity necessary for protein synthesis [144-146] and they are to do *de novo* protein synthesis through translation of megakaryocyte-derived mRNA; their transcriptome is derived from megakaryocytes through a very well controlled mechanism since not all mRNAs are transferred to platelets, depending on physiological and pathological conditions [147]. Platelet mRNAs from healthy subjects encode for surface receptors and glycoproteins, for proteins involved in metabolism, signaling, inflammation and immunity [143, 148]. When activated, platelets can use their transcriptome to do new protein synthesis, thus modifying their proteome and, as a consequence, their functions [149]. In pathological conditions, specific mRNAs could vary and, recently, it has been shown that platelets harbour an abundant array of microRNAs, which are known as key regulators of mRNA translation [150], and some of them coordinate with reactivity to specific agonists and to pathological states [151]. Thus, platelets may make use of post-transcriptional gene regulation for their morphology and physiologic functions and microRNAs may play a significant role by binding to their target mRNAs.

**Platelets and atherothrombosis**

Atherothrombosis

Atherothrombosis, defined as atherosclerotic plaque disruption with superimposed thrombosis, is the leading cause of mortality in the Western world [152]. Atherosclerosis is a diffuse process that starts early in childhood and progresses asymptomatically through adult life. Later in life, it is clinically manifested as coronary artery disease (CAD), stroke, transient ischaemic attack (TIA) and peripheral arterial disease (PAD). From the clinical point of view, it should envision this disease as a single pathologic entity that affects different vascular territories. The most important factors in atherothrombosis include activated endothelial cells, inflammatory leukocytes (which are a source of thrombogenic stimuli), smooth muscle cells (which act as a source of procoagulants and an amplifier of inflammatory responses during thrombosis) and

platelets (which act as an important source of further inflammatory mediators and are involved in thrombus formation by aggregating). Endothelial dysfunction is a systemic, reversible disorder considered the earliest pathologic process of atherothrombosis [153, 154]. It is involved in the recruitment of inflammatory cells into the vessel wall and in the initiation of atherosclerosis. Endothelial cells produce cytokines, express adhesion molecules such as ICAM-1 (intercellular adhesion molecule-1), VCAM (vascular cell adhesion molecule-1) and selectins, such as E- or P-selectin, which bind monocytes loosely to the endothelial surface, and assist leukocytes and other blood-derived cells in atheroma infiltration. Secondary changes may occur in the underlying media and adventitia, particularly in advanced disease stages. Lesions progress to fibroatheroma by developing a cap of smooth muscle cells and collagen. Atherosclerotic lesions can progress without compromising the lumen because of compensatory vascular enlargement (positive remodelling) [155]. Importantly, lipid-rich lesions leading to acute coronary syndromes (ACS) are often mildly stenotic, due to significant positive remodelling and therefore are not detectable by angiography. Plaque disruption and subsequent thrombus formation is responsible for the onset of most ACSs and strokes. The magnitude of the thrombotic process triggered upon plaque disruption is modulated by different elements that determine plaque and blood thrombogenicity: local shear rate, tissue factor (TF), apoptotic microparticles, circulating monocytes and others. The atherosclerotic and thrombotic processes appear to be interdependent and could therefore be integrated under the term “atherothromhrombosis”, a term that includes both atherosclerosis and its thrombotic complications [153-156]. In spite of a common pathophysiologic pathway, atherosclerotic lesions are very heterogeneous and the “high-risk plaque” of each vascular bed has unique characteristics. Insights into the disease have advanced beyond the notion of progressive occlusion of the coronary artery into the recognition that plaque disruption and superimposed thrombus formation are the leading causes of acute coronary syndromes and cardiovascular death. Consequently, plaque composition (as a determinant of risk of disruption), rather than luminal stenosis, has become the major determinant of this disease [157]. Histologically, these rupture-prone (also called vulnerable or high-risk) lesions consist of a large core of extracellular lipid, a dense accumulation of macrophages, reduced numbers of vascular smooth muscle cells and a thin fibrous cap. So these plaques

are less stable and have a greater propensity to rupture than the fibrous, collagen-rich plaques. Plaque disruption usually occurs at the weakest point ("shoulder"), where the cap is often thinnest and most heavily infiltrated with inflammatory cells [158]. Once the plaque is disrupted, the highly thrombogenic, lipid-rich core, with abundant tissue factor, is exposed to the bloodstream, triggering the formation of a superimposed thrombus that leads to vessel occlusion and subsequent ischaemic symptoms distal to it [159].

Role of platelets in atherothrombosis

The primary role of platelets is to arrest bleeding and promote vessel repair. In addition to preventing excess bleeding, platelets also play an important role in surveying and maintaining the integrity of the endothelium, in part through the release of pro-angiogenic cytokines and growth factors. Dysregulated platelet-endothelial interactions have increasingly been recognized as an important pathogenic mechanism in the development of atherosclerosis. In response to specific pro-inflammatory signals, endothelial cells become more adhesive toward platelets, stimulating the production of various platelet-derived inflammatory molecules that provide a positive feedback loop for further endothelial cell activation. Endothelial-bound platelets are highly effective at recruiting leukocytes from flowing blood and also enhance leukocyte adhesion and transmigration to the site of the pro-inflammatory stimulus. Therefore, pathological derangement of these key interactions among platelets, endothelial cells and leukocytes facilitates the inflammatory process that underlies the development of atherosclerosis [137]. Under normal physiological conditions, platelets circulate in close proximity to the endothelium without forming stable adhesion contacts due to the anti-adhesive properties of quiescent endothelial cells. However, after vascular injury, platelets rapidly adhere to sites of endothelial disruption to establish a haemostatic plug that prevents excess blood loss. This process is critically dependent on the efficiency of platelet adhesion to the subendothelial matrix, as well as on the ability of the platelets to undergo rapid biochemical and morphological changes that support aggregation and the localized activation of the coagulation cascade. After damage to the vessel wall, subendothelial matrix proteins that are highly reactive to platelets,

including collagen, von Willebrand factor (VWF), fibronectin and laminin, become exposed to the blood and immediately engage specific receptors on the platelet surface. The contribution of specific ligands and receptors mediating platelet adhesion is critically dependent on the prevailing blood-flow conditions. Under conditions of high shear stress, as encountered in arterioles and stenotic arteries, VWF plays a critical role in recruiting platelets from blood flow [140]. This tethering function of subendothelial VWF is dependent on the interaction of its A1 domain with the glycoprotein Ibα (GPIbα) component of the platelet GPIb-V-IX complex [160]. Circulating plasma VWF has limited binding potential for GPIbα, however, once immobilized onto subendothelial collagen (type I, III, and VI), the unfolded VWF macromolecule provides a linear array of A1 domains that facilitate binding to multiple GPIbα receptors [161, 162]. The bond between GPIbα and VWF has a rapid dissociation rate that is unable to support stable adhesion, requiring the contribution of other ligand-receptor interactions to promote stable adhesion [163]. Once tethered to the site of vascular injury, platelets form stable adhesion contacts with collagen and other matrix macromolecules. Platelet binding to collagen is mediated by GPVI [164, 165] and integrin α2β1 [166], whereas fibronectin and laminin engage integrin α5β1 and α6β1, respectively [167]. Once adherent, platelets undergo a remarkably complex series of morphological and biochemical changes that lead to the release of platelet granule contents and up-regulation of the adhesive function of integrin αIIbβ3. Activated αIIbβ3 binds multiple ligands, including VWF [168], fibrinogen [169], fibrin and fibronectin [170] and is indispensable for the formation of stable platelet aggregates [171]. Central to platelet activation is the generation and release of soluble agonists at sites of vascular injury. These include thromboxane A2 (TxA2) which is synthesized from arachidonic acid via cyclooxygenase (COX) and thromboxane synthase, and ADP released from platelet dense granules. These endogenous agonists can act in an autocrine or paracrine manner to enhance platelet activation by engaging specific G protein coupled receptors: P2Y1 [172] and P2Y12 [173] (ADP) and the thromboxane receptors TPα and TPβ [174]; ADP and TxA2 act in a cooperative manner to enhance platelet activation and thrombus formation. In addition to the synthesis and release of soluble agonists, platelets provide a catalytic surface for the assembly of coagulation complexes necessary for thrombin generation. Thrombin is among the most potent stimulators of platelets through proteolytic cleavage and activation of platelet protease-

activated receptors (PARs), specifically PAR1 and PAR4, on human platelets [175]. Also central to the haemostatic function of thrombin is its ability to generate fibrin polymers, key step stabilizing formed thrombi.

## TISSUE FACTOR

### TF biology

Structure

Tissue Factor (TF), or Thromboplastin or CD142, the main activator of blood coagulation, is a 47 kDa transmembrane glycoprotein containing three domains: an extracellular domain (residues 1-219), a transmembrane domain (residues 220-242) and an intracellular domain (residues 243-263). The extracellular domain, representing the NH2-terminal part of the molecule, is composed of two fibronectin type III domains; it is involved in complex formation with factor VIIa and increases, in a membrane dependent fashion, the activity of the protease toward its natural substrates factor IX, factor X and factor VII by several orders of magnitude [176, 177]. The transmembrane domain anchors TF to the membrane and the cytoplasmic COOH-terminal domain is involved in signal transduction (Figure 3)

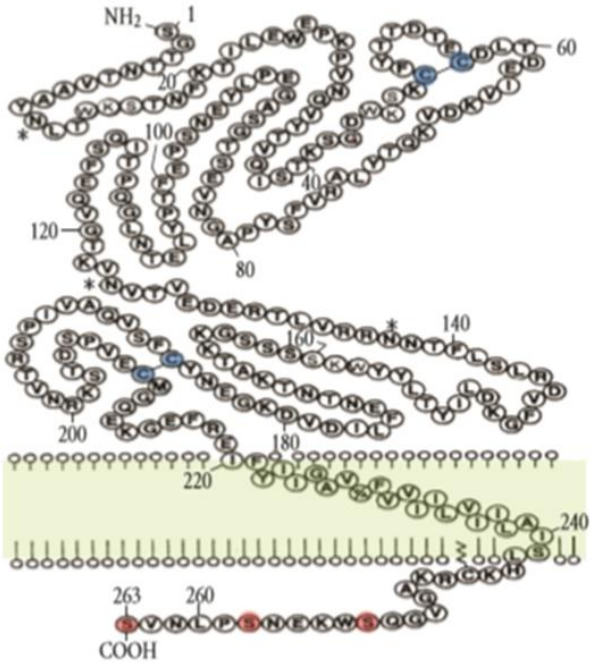



***Figure 3.*** **Tissue Factor structure.** The 47-kDa membrane bound single polypeptide chain consists of extracellular, transmembrane and cytoplasmic domains. There are two intrachain disulfide bridges, shown in blue, in the extracellular region which also contains factor VII/factor VIIa binding domain. There are three serine residues, shown in red, in the cytoplasmic domain for undergoing phosphorylation.

Role

Tissue Factor is considered the major player in blood coagulation and thrombotic complications of atherosclerosis [178]. After vascular injury, clotting is initiated by the binding of plasma FVII/FVIIa to tissue factor (TF). The TF:FVIIa complex of the extrinsic pathway initiates blood coagulation by activating both FX and FIX. The FVIIIa:FIXa complex of the intrinsic pathway provides an alternative route to generate FXa, which participates in the prothrombinase complex (FVa:FXa). This complex activates prothrombin to thrombin, which plays a central role in the coagulation protease cascade. It activates FXI, which is an alternative way to generate FIXa [179, 180]. Thrombin also activates FXIII, as well as various cofactors, cleaves fibrinogen and stimulates platelets via cleavage of protease activated receptors (PARs). Platelets accelerate the activation of the coagulation cascade by binding FXI via the receptor glycoprotein Ib-IX-V and by providing a thrombogenic surface for the assembly of the prothrombinase complex (FVa:FXa) [181] (Figure 4).

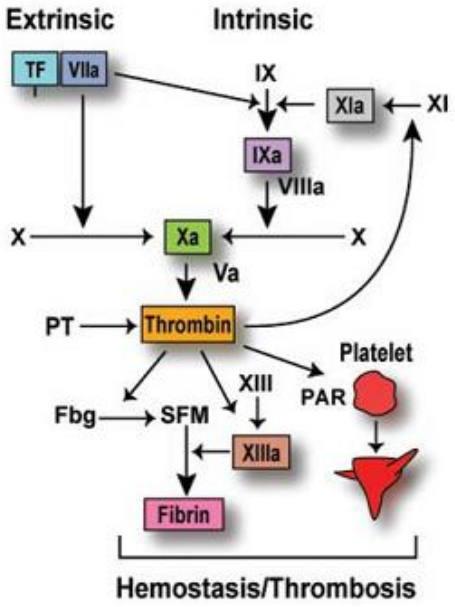



***Figure 4.*** **The coagulation cascade.** TF:FVIIa complex initiates clotting by activating FX and FIX. Alternatively, FXI can activate FIXa. The prothrombinase complex (FVa:FXa) activates prothrombin (PT). Thrombin activates various proteases and cofactors. Thrombin cleavage of fibrinogen to soluble monomers (SFM), which are cross-linked by FXIIIa, and activation of protease-activated receptors (PARs) on platelets leads to the formation of a clot. *from Arterioscler Thromb Vasc Biol. 2004;24:1015-1022*

TF is expressed in subendothelial cells, such as in smooth muscle cells and fibroblasts; endothelial cells do not express TF under physiological conditions but it may be stimulated by various cytokines. The only physiologic inhibitor of the TF-FVIIa complex is the tissue factor pathway inhibitor (TFPI), a serine protease synthesized mainly by the vascular endothelium. A small percentage of TFPI is found also in platelets or in plasma, free or associated with lipoproteins.

Although the main physiologic function of TF is the regulation of the processes of haemostasis and thrombosis, emerging evidence showed a broad spectrum of biological function for TF. In fact, Tissue Factor is involved in many pathological conditions: cardiovascular complications [182], angiogenesis [183], tumor metastasis [184], wound repair, embryonic development, cell adhesion and migration, autoimmune disorders. Cardiovascular complications are closely associated with either inflammation or thrombosis and TF plays a major role in their pathogenesis. TF has been shown to be present in atherosclerotic plaques [185]; in atherosclerosis, TF is expressed by macrophage-derived foam cells within atherosclerotic plaques [186].

Moreover, TF levels are higher in atheroma from patients with unstable angina compared with stable angina [187]. These results strongly suggest that high levels of TF exposed upon plaque rupture trigger thrombosis and myocardial infarction (Figure 5). Thus, the classical view of TF is that it is expressed locally within an atherosclerotic lesion. Inhibition of TF would be expected to reduce thrombosis associated with a variety of diseases [181]. Vessel-wall associated TF, however, does not entirely explain the thrombogenic potential of vascular lesions when they are exposed to flowing blood. It has been proposed that thrombus growth might be promoted by circulating (i.e., microparticles or platelet-associated) TF [188].

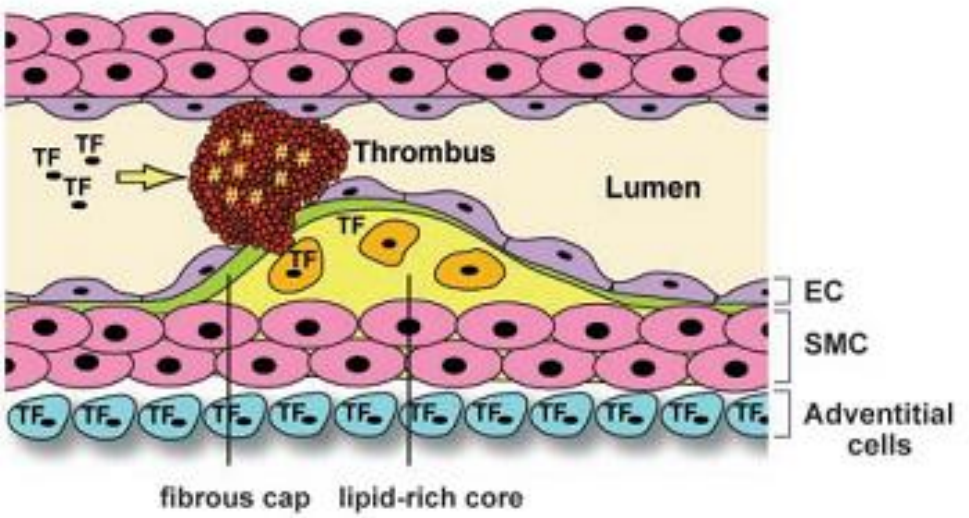



***Figure 5.*** **Role of TF in thrombus formation after rupture of an atherosclerotic plaque.** TF expressed by foam cells (orange) and in the necrotic core (yellow) of the plaque would be exposed to clotting factors in the blood and initiate clotting after plaque rupture. In addition, blood-borne TF may contribute to thrombus propagation. TF is constitutively expressed by adventitial cells (blue). EC, endothelial cells; SMC, smooth muscle cells. *from Arterioscler Thromb Vasc Biol. 2004;24:1015-1022*

Moreover TF hypercoagulability leads to cardiovascular complications and vascular disease such as arterial hypertension [189], hypertrophy [190] and acute coronary syndrome [182, 191]. In condition of hyperglycaemia, such as T2DM, the increased circulating advanced glycation end products (AGEs) lead to an increased expression of Tissue Factor [192]; TF can also assume a pathogenic role in diabetic progression in a close relation with the inflammatory process: in fact, TF could be responsible for insulin resistance [193, 194].

**Platelet-associated Tissue Factor**

At the beginning of 2000, Rauch et al. proposed that thrombosis occurring upon plaque rupture does not necessarily require the exposure of vessel wall-TF [195]. In fact, platelets can be a source of active TF, the so called "blood-borne" TF or "circulating" TF, which can sustain the activation of the blood coagulation on the edge of a growing thrombus. Circulating TF is associated with increased blood thrombogenicity [196] and procoagulant microparticles (MPs) was found in human blood under normal and pathological conditions [188, 197]. In the 2000, Rauch and Nemerson published the finding that TF-positive microparticles, released by activated monocytes, were up taken by activated platelets through a CD15-P-selectin transfer mechanism: platelet-associated TF, resulting from TF-positive MPs transfer, is an alternative source of procoagulant activity [198]. In 2003 Camera et al [199] and Muller et al [200] showed the evidence that human platelets from healthy subjects express TF antigen, using different approaches (western blotting, confocal and immune electron microscopy). Moreover, Camera and colleagues reported, by using flow cytometry, that platelet activation resulted in the expression of TF besides other platelet activation markers (such as P-selectin), on platelet surface. The presence of TF protein and its functional activity was further confirmed showing the bindings of this protein to its physiologic ligand FVIIa and the capacity to generate FXa [199]. Moreover, TF mRNA was found in a sample of resting platelets depleted from leukocytes contamination, suggesting that platelets could perform *de novo* protein synthesis [33]. Three years later, two independent groups provided the evidence that platelet activation results in the translation of TF mRNA, increasing the membrane-bound TF protein [201, 202]. At present, published data suggest that three are the possible mechanisms responsible for the presence of TF in human platelets:

1) the transfer from TF-positive MPs released by activated endothelial cells or leukocytes (as proposed by Rauch and Nemerson in 2000 [195]);

2) the storage of TF protein within the alpha granules [203];

3) the *de novo* protein synthesis from specific TF mRNA [201, 202].

All these mechanisms are not mutually esclusive and one mechanism may dominate over the others depending on the pathophysiologic conditions (Figure 6).

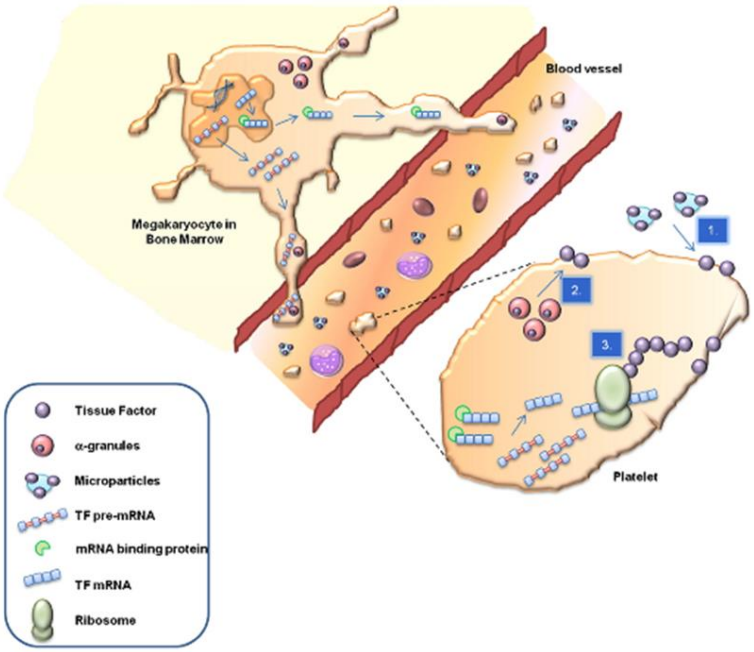



***Figure 6.*** **Schematic view of the three possible mechanisms responsible for the presence of TF in human platelets**

As shown above, previous studies carried out in our laboratory showed that in vitro platelet activation by classical agonists results in expression of functionally active TF on the cell membrane [199]. Furthermore, in 2008, our group showed that NSTEMI patients have a greater number of TF positive platelets and platelet-monocyte aggregates compared to stable CAD patients or healthy subjects, providing additional insight into the prothrombotic potential of CAD platelets [204]. The finding that both TF mRNA and protein are significantly higher in platelets from NSTEMI patients than in stable CAD patients supports the idea that platelets from stable CAD and ACS patients are potentially preconditioned to a different degree of reactivity on the transcriptional level [204, 205].

In conclusion, platelet activation not only results in thrombus formation, but also leads to *de novo* protein synthesis through rapid and highly regulated translation of pre-existing megakaryocyte-derived mRNAs [206].

## PLATELET AND PLASMA PROFILE IN CHRONIC KIDNEY DISEASE

### Platelet activation profile and CKD

Patients with CKD suffer from complex haemostatic disorders. Uremic patients show a bleeding diathesis that is mainly due to abnormalities of primary haemostasis. However these patients present also a high prevalence of cardiovascular and thrombotic complications.

Indeed, platelet activation in CKD patients is a controversial issue: some authors have described a decreased platelet activation in these patients [207-212], while others a higher platelet activation [213-216].

In 2004, Boccardo et al [207] described that ESRD patients present a decreased platelet function, although these patients have a high prevalence of cardiovascular and thrombotic complications. Patients with CKD show a bleeding diathesis, mainly due to abnormalities of primary haemostasis, although the pathogenesis of uremic bleeding is multifactorial; the interactions between platelet-platelet and platelet-vessel wall appear to be of crucial importance. Platelet dysfunction in uremic patients is partially due to uremic toxins present in circulating blood; platelet dysfunction arises by diverse mechanisms: for example, abnormalities of platelet alpha-granules consisting of storage pool defect denoted by reduction in ADP and serotonin and elevated cAMP levels in patients with terminal renal insufficiency, that may contribute to defective platelet aggregation and adhesion to injured vessels [208]. Interaction of platelets with vessel wall play a crucial role in promoting haemostasis at the site of the vessel lesion: decreased binding of both vWF and fibrinogen to stimulated uremic platelets may account for the defective function of the α2β3 complex in these patients. In particular the impaired binding activity is caused by dyalizable toxic substances [209, 210] or it is due to α2β3 receptor occupancy by fibrinogen fragments present in uremic plasma, that prevent the cross-linking between adjacent platelets to form aggregates [211]. On this regard, in 1999, Kozek-Langenecker and collegues [212] reported that fibrinogen fragments, usually absent in human blood, but present in uremic plasma, may play a role in uremia platelet dysfunction. They described that adding fibrinogen fragments to normal PRP or isolated platelets, platelet aggregation decreases both in PRP as well as in isolated platelets. Moreover, they confirmed that fibrinogen fragments diminish the expression of activated GP IIb/IIIa. So

these data provide further support to the role of fibrinogen fragments in causing uremic platelet dysfunction.

In literature it is also described that blood platelet activation and platelet interaction with circulating cells may increase the cardiovascular disease risk of patients with chronic kidney disease [213, 216]. Atherosclerotic cardiovascular disease is a major cause of morbidity and mortality in patients with renal disease [216]. Although "traditional" atherothrombotic risk factors, such as hypertension, dyslipidemia and diabetes mellitus, may account for a large proportion of the excessive burden of cardiovascular disease in CKD patients, recent studies suggest that "non-traditional" risk factors, such as inflammation, oxidative stress and vascular calcification, may also contribute [64].

Furthermore, CKD is also associated with a worse prognosis in patients undergoing PCI [68, 217-219], which is possibly attributable to inadequate platelet inhibition by standard antiplatelet therapy [220, 221]. The successful treatment of coronary artery disease and cerebrovascular disease is frustrated by aspirin resistance (AR) [222-228]. Cardiovascular disease and thrombosis are common and often fatal complications of CKD, possibly due to excessive platelet activity [229-233]. In 2012, Blann and collegues [234] reported an association between AR and CKD. Patients who are AR (defined by both arachidonic acid response by light transmission aggregometry) have lower GFR and have higher soluble P-selectin than patients who are sensitive to aspirin. As the platelet expression of CD62P (P-selectin) and increased soluble P-selectin both mark platelet activation [214, 231, 235-238], thus patients with the worse GFR have the greatest degree of platelet activation. Despite being on aspirin, authors reported that patients still have increased platelet activity (soluble P-selectin, surface expression of CD62P) compared to the healthy controls, reflecting continuing alpha granule degranulation [239]. Indeed, this alone may partially explain the continuing thrombosis risk despite the use of this drug and supports the hypothesis that aspirin does not inhibit the degranulation of alpha granules or the appearance of P-selectin at the surface of the platelet [214, 240-242]. Also Thijs et al [213] described blood platelet activation, evaluated as platelet surface expression of P-selectin and GP IIb/IIIa (PAC1), in patients with renal impairment: the expression of P-selectin and PAC1 is statistically significantly inversely related to the glomerular filtration rate in these patients. CKD patients not only may present

aspirin resistance, but recent findings from placebo-controlled trials suggest that patients with decreased renal function might not accrue equal therapeutic benefit of clopidogrel as compared to patients with a normal renal function [119, 243]. In 2014, Breet et al [215] showed that patients with CKD have significantly higher platelet reactivity, determined in parallel by ADP- and AA-induced light transmittance aggregometry (LTA) and the VerifyNow System (P2Y12 and Aspirin). Patients with CKD more frequently have high on-clopidogrel platelet reactivity (HCPR) and high on-aspirin platelet reactivity (HAPR) regardless of the platelet function used: high on-treatment platelet reactivity strongly influences the occurrence of atherothrombotic events in these patients.

Uraemia and dialysis induce a pro-inflammatory state, with widespread microvascular and circulatory changes [244]. Long recognized as having a role in inflammation, besides platelets, also platelet–leukocyte aggregates are known to contribute to ongoing injury at atheromatous sites and in plaque disruption [245]. In 2002, Ashman et al [216] investigated circulating platelet–leukocyte interactions as a potential cardiovascular risk factor in CKD (continuous ambulatory peritoneal dialyzed (CAPD) and haemodialyzed) patients with no evidence of cardiovascular disease. Circulating platelet–monocyte aggregates are significantly increased in parallel with a significant reduction in PSGL-1 expression on monocytes in CKD patient compared with normal controls. The presence of higher platelet–monocyte aggregates in dialysis patients was associated with increased cardiovascular events. After at least 1 year's follow-up, eight patients of 11 within the group with higher platelet–monocyte aggregates have evidence of cardiovascular disease or have died for a cardiac death. In 2001, Furman et al [246] showed that when patients had 15.3 ± 3.0% platelet–monocyte aggregates, they were proven subsequently to have a myocardial infarction: they assessed that circulating platelet–monocyte aggregates are an early marker of myocardial infarction.

Platelet–monocyte aggregates found in CKD patients are associated with cardiovascular events and they may contribute to local thrombotic changes in acute plaque erosion and in the evolution of atherosclerosis, but may yet prove an epiphenomenon reflecting widespread inflammatory changes in the microcirculation associated with uraemia.

**Transcriptome and CKD**

There is now extensive evidence from a wide variety of approaches that suggests renal disease in the general population has a genetic component. Multiple techniques have been employed in an effort to identify genes that contribute to this genetic susceptibility [247]. Many studies have now been carried out assessing the contribution of specific "candidate genes" that are involved in renal pathogenesis. Many efforts have been made to link specific genes or small numbers of related genes to various forms of renal disease in the general population. Recent genome-wide association studies (GWASs) using estimated glomerular filtration rate (eGFR) as the phenotype of interest have identified several loci that may affect renal function [248, 249]: 13 new loci affecting renal function and CKD (in or near LASS2, GCKR, ALMS1, TFDP2, DAB2, SLC34A1, VEGFA, PRKAG2, PIP5K1B, ATXN2, DACH1, UBE2Q2 and SLC7A9) and 7 loci suspected to affect creatinine production and secretion (CPS1, SLC22A2, TMEM60, WDR37, SLC6A13, WDR72 and BCAS3). These loci potentially influence nephrogenesis, podocyte function, angiogenesis, solute transport and metabolic functions of the kidney [250]. In 2012 Plé and colleagues [251] reported that platelet abnormalities and changes in platelet proteome, that occur in circulating platelets exposed to uraemic toxins [252], could be subsequent to changes in the composition and/or regulation of the platelet transcriptome. They observed that platelet mRNA and microRNA transcriptome is altered in CKD patients and could be restored partially upon dialysis. In particular, the level of metallothionein-encoding genes, such as MT1X, is increased in platelets of both uraemic and dialysis patients, in which plasma zinc deficiency is common [253]. Particularly abundant in platelets and involved in platelet reactivity and haemostasis [254], zinc imbalances are involved in oxidative stress [255], a state that induces expression of metallothionein genes [256]. Elevated levels of platelet metallothionein genes may thus result from the altered zinc homeostasis and oxidative stress conditions that prevail in uraemic and dialysis patients and that may contribute to the relatively high prevalence of CVD in these patients [257, 258]. Additional genes deregulated in platelets of CKD patients could be linked to platelet-related disorders observed in uraemia. For instance, the mRNA encoding for LPAR4, which is suspected to inhibit platelet reactivity to lysophophatidic acid [259], is reduced by ~ 50% in CKD patients. Lysophosphatidic acid accumulation in

atherosclerotic lesions represents an important risk factor for the development of atherosclerosis and thrombosis [260] and the observed decrease in LPAR4 expression in CKD patients may explain the increased risk of cardiovascular events observed in these clinical cases. The authors also identified genes involved in lipid metabolism, such as glycine N-acyltransferase-like 2 (GLYATL2), phospholipase C eta 1 (PLCH1), phosphatidylcholine transfer protein (PCTP), OSBP2 and SERINC1, whose levels are deregulated in uraemic conditions. This may partially explain the altered synthesis of bioactive lipids [261, 262] and membrane lipid composition [263, 264] observed in platelets of uraemic patients. The conditions associated with CKD did not affect the ability of platelets to synthesize or mediate the function of microRNAs. However, they observed an important alteration of the platelet microRNA profile in uraemic patients, as a total of 21 (such as miR-33a and -33b, miR-142-5p and -3p, miR-340 and -340*), out of 247 microRNAs expressed in platelets, displayed more than two-fold changes, as compared to healthy subjects.

As described before, CKD patients have a markedly increased morbidity and mortality, especially caused by cardiovascular disease (CVD) and specific mRNAs may vary in clinical conditions, such as ST-elevation myocardial infarction (STEMI) [265]. In 2011, our group [205] tested the hypothesis that patients with acute coronary syndrome (ACS) have different platelet mRNA composition compared to patients with stable angina (SA), assuming that platelets may be more thrombogenic on the transcriptional level. Heterogeneity of mRNA expression, particularly in the ACS population, is evident from the heat map of the gene expression profiles. Unsupervised hierarchical analysis show that differential expression profiles allowed clustering of SA patients in a distinct homogeneous group. On the contrary, non-ST-elevation myocardial infarction (NSTEMI) patients present a higher degree of inter-individual variation, which is confirmed by qRT-PCR analysis on individual patients. This heterogeneity further supports the concept that differential gene expression does exist in CAD platelets and may be responsible for different degrees of activation and thrombogenicity in these patients. This is consistent with our previous observations on platelet-associated TF expression in CAD patients and on the prothrombotic potential of NSTEMI platelets [204]: indeed, TF mRNA and protein are significantly higher in NSTEMI than in SA resting platelets. Transcriptomic analysis provide consistent evidence that three genes (BAIAP2, CLTA and GP1BB) have a higher expression in NSTEMI in

comparison with SA platelets, at both mRNA and protein level. A fourth transcript (PKIG) shows a higher expression in NSTEMI samples. BAIAP2 and GP1BB belong to one of the modulated functional gene categories (cell surface receptor linked signal transduction). Perturbation of this biological process and, likely, of cell adhesion function may be involved in the platelet increased reactivity and prothrombotic potential found in NSTEMI. GP1BB up-regulation in NSTEMI highlights the role for the von Willebrand factor (VWF) pathway, as it is part of the GPIb-IX-V complex that forms VWF receptor. Binding of the GPIb-IX-V complex to VWF facilitates platelet adhesion to vascular endothelium at sites of injury and initiates a signalling cascade that leads to platelet activation, aggregation, haemostasis, and thrombosis. Consistent with this observation, authors found that PKIG, a potent inhibitor of cAMP-dependent protein kinase (PKA) [266], is up-regulated in NSTEMI: an up-regulation of PKIG could mediate a higher degree of platelet reactivity and also potentiate the effect of GP1BB over-expression. BAIAP2 is involved in structural changes of the actin cytoskeleton downstream of the Rho GTPases Cdc42 and Rac [267], which are involved in the formation of membrane protrusions, such as lamellipodia and mediate platelet shape change [268]. CLTA increased expression in NSTEMI platelets may affect intracellular trafficking and GPCR signaling [269]. Platelet gene expression profiling reveals quantitative differences between ACS and stable CAD. The study provided evidence that NSTEMI platelets are potentially preconditioned on the transcriptional level to a higher degree of reactivity, which eventually lead to the thrombotic event.

**Plasma and platelet proteome and CKD**

Besides the traditional risk factors, such as hypertension, dyslipidemia and diabetes mellitus, also non-traditional risk factors, such as inflammation, oxidative stress and vascular calcification, may contribute to CVD events in CKD patients [270]. The prevalence and extent of vascular and valvular calcification and arterial stiffness are strong predictors of CVD and all-cause mortality in CKD patients. The chronic inflammation present in these patients may promote vascular calcification and, in particular, mediators and inhibitors, such as leptin [271], matrix-gla protein [272], tumor necrosis factor [273], bone morphogenetic protein [274], osteoproteogerin [275] and fetuin A [276] may be related to a process of accelerated vascular calcification. In

particular, it has been demonstrated that CKD patients present low levels of fetuin A (also called alpha 2-Heremans Schmid gly-coprotein [AHSG]), a plasma protein that inhibits ectopic calcium-phosphate production, precipitation and vascular calcification [277]. Low fetuin A levels correlates with inflammation, accelerated atherosclerosis and CVD in uremic patients [270] and could, at least in part, explain the high prevalence of vascular calcification in this population. Since it has been demonstrated that at least four polymorphisms exist in the AHSG gene (at amino acid positions Thr248Met [C→T], Thr256Ser [C→G], Asp276Asn [G→A] and Arg317Cys [C→T]), genetic polymorphisms may have an effect on circulating levels of this protein [278]. Indeed, a recent study [270] showed that ESRD patients with the AHSG 256Ser allele have lower serum fetuin-A levels and higher all-cause and cardiovascular mortality rates. Thus these observations suggest a significant gene–environment interaction in which the involvement of inflammatory processes may further enhance the adverse effects of low circulating fetuin-A levels.

In 2011, Luczak et al [279] carried out a comparative proteomic analysis of blood plasma proteins from patients in various stages of renal dysfunction (CKD group) and healthy volunteers (HV group). In this study they wanted to assess whether and how the progression of CKD influences the composition of blood plasma proteins; the analysis between CKD and HV plasma proteome reveals that there are 4 proteins at different levels: alpha-1-microglobulin (α-1-m), apolipoprotein A-IV (apo-A-IV), γ-fibrinogen (Fb) and beta-haptoglobin (Hp). As far as the plasma levels of α-1-m is concerned, there is a linear correlation between protein concentration and CKD progression; also for the plasma levels of apo-A-IV, authors found a significant increase of protein accumulation during the initial phase of the disease. These data suggest that α-1-m and apo-A-IV may find practical application as early markers of nephropathy.

Also platelet proteome could be affected by chronic kidney disease: in fact, in 2010 Marques and collegues [252] showed that platelet protein expression profile is altered in CKD patients. They analyzed the differences in the expression of proteins associated with the cytoskeleton and platelet-cell interaction energetic metabolism and oxidative stress in normal and dysfunctional platelets from uremic patients. The main finding is that the platelet expression of actin-interacting protein-1 isotype 1, a cytoskeleton-related protein, is down-regulated in the dysfunctional uremic platelets. Moreover, integrin IIb, a protein

associated with platelet-cell interaction and the oxidative stress-related proteins, glutathione-S-transferase isotypes 1 and 2 and peroxiredoxin VI, are up-regulated in dysfunctional uremic platelets when they were compared with the uremic platelets with normal functionality. These observations may be in accordance with previous published results in which abnormalities have been reported in the organization of cytoskeleton in platelets from uremic patients, including a decreased association of actin-interacting protein with the cytoskeleton [280].

Several studies have unequivocally demonstrated that oxidative stress is increased in uremic patients [281, 282]. Since in platelets, the main focus of oxidative stress is platelet activation [283], in the dysfunctional uremic platelets, the up-regulation of antioxidant-related enzymes, such as peroxiredoxin VI and the glutathione-S transferase isotypes 1 and 2, may mitigate the prostimulating platelet effect of circulating oxidative molecules, such as reactive aldehydes, oxidized thiols and lipid peroxidation products, reducing platelet functionality.

# AIMS OF THE STUDY

# AIMS OF THE STUDY

Chronic kidney disease (CKD) is emerging as a major health problem [3, 284, 285]. Patients with CKD are at high risk of cardiovascular diseases and cerebrovascular diseases and they are more likely to die of CVD than to develop terminal renal failure [15]. 'Traditional' atherosclerotic risk factors, such as diabetes, hypertension, dyslipidemia and older age, have been found to be independent predictors of CVD in CKD [15, 286, 287]. In addition, haemodynamic and metabolic factors, such as volume overload, anemia, calcium and phosphorus imbalance, chronic inflammation and a hypercoagulable milieu are unique features of chronic kidney disease, that may contribute to the risk and pathogenesis of CVD [288].

CKD increases bleeding risk on one hand and thrombosis risk on the other [289]. The bleeding tendency of patients with uraemia is characterized by haemorrhagic symptoms and by abnormal prolongation of bleeding time. This bleeding tendency has been attributed classically to abnormalities of platelet function that include impaired adhesion [290] and decreased aggregation [290, 291] and to alteration in the balance of pro- and anti-thrombotic factors in blood [292, 293]. On the other hand, blood platelet activation and platelet interaction with circulating cells increase the risk of thrombosis and, consequently, of cardiovascular events in CKD patients [213, 216].

CKD may also influence the response to antiplatelet therapy [119, 243]. Patients with CKD might have reduced Clopidogrel-induced platelet inhibition due to a specific impairment of the P2Y12 pathway [220, 294, 295]. Moreover, despite the use of Aspirin, in some patients (~10%) thrombosis still occurs and this has lead to the concept of a 'suboptimal response to aspirin', also described as 'high on-treatment platelet reactivity' or 'aspirin resistance' (AR) [222, 223]. This suboptimal response in patients undergoing percutaneous coronary intervention is associated with increased risk of thrombosis and increased major adverse clinical events (MACE) in long term follow-up [224-226, 296].

Activated platelets express a great variety of molecules relevant for the atherothrombotic processes, including membrane glycoproteins involved in adhesion, aggregation and coagulation. In 2003, our group has shown that in vitro platelet activation by classical agonists results in expression of functionally active

Tissue Factor (TF), a major actor in the coagulation cascade and thrombotic complications of atherosclerosis [199]. In 2008, we have also provided evidence that patients with non-ST elevation myocardial infarction (NSTEMI) have a higher number of TF-positive platelets and platelet-monocyte aggregates than stable CAD patients or healthy subjects, providing additional insight into the prothrombotic potential of CAD platelets [204]. However, no study has been carried out so far to investigate whether the expression of platelet-associated TF may provide further insights into the bleeding or thrombotic risk of CAD patients with CKD.

In 2003, Gnatenko et al [143] has shown for the first time that platelets have a transcriptome and that they contain between 2000 and 7000 transcripts derived from megakaryocytes; platelets can use their transcriptome to perform *de novo* protein synthesis in response to activation, thus modifying their proteome and, as a consequence, their function. Our group has previously shown that specific changes in the expression/distribution or amount of platelet mRNAs and proteins are associated with CAD, suggesting that platelet are potentially preconditioned to a different degree of reactivity on the transcriptional level[205]. However no information is still available on the changes that may occur in platelet transcript profile in CAD patients with CKD.

The plasma proteomic profile may also vary in pathologic conditions: the incorporation of proteomics into cardiovascular research provide a means of exploring the mechanisms of disease onset and progression. Proteomics have the potential to reveal those proteins that are associated with pathogenesis and could be potentially used as predictive or prognostic markers [297]. In end-stage renal disease (ESRD) patients, CKD influences the expression of plasma proteins involved in the development of atherosclerosis [252, 298-300]. No study focused on the plasma protein changes that may occur in CVD patients with mild-to-moderate CKD has been yet carried out.

Based on this rational, the general aim of this study is to provide insights into 1) the platelet phenotype, 2) the platelet transcriptomic profiles, 3) the plasma proteomic profiles in CAD patients with and without CKD.

In particular, the specific aims of the present project are to investigate:

- platelet- and leukocyte-associated TF expression in order to better characterize the platelet haemostatic capacity of these patients and to assess whether platelet TF could contribute to the increased prothrombotic propensity or to the bleeding risk characteristic of CKD patients;
- the global haemostatic function of whole blood, studying parameters that describe clot properties during its formation, in order to correlate blood parameters with the haemorrhagic/thrombotic phenotype present in these patients;
- the platelet transcriptome, in order to gain insights into the molecular pathways associated with CKD and cardiovascular disease, and to identify potential predictive platelet biomarkers for classifying patients;
- the plasma proteome, in order to reveal differentially expressed proteins in patients with CKD and cardiovascular disease and could be potentially used as predictive or prognostic biomarkers.

# METHODS

# METHODS

## STUDY DESIGN

The present project is an observational, case-control, and cross-sectional study. All the enrolled subjects were recruited at Centro Cardiologico Monzino IRCCS and gave their written, informed consent to participate in this study after receiving oral and written information; the investigation conforms to the principles outlined in the Declaration of Helsinki and was approved by our local Ethical Committee. 139 consecutive acute coronary syndrome (ACS) patients, 31 with chronic kidney disease (CKD) and 108 without CKD, and 217 stable angina (SA), 49 with CKD and 168 without CKD were enrolled from December 2012 to April 2014. We also recruited 37 healthy subjects (HS) as control.

*Inclusion criteria:*

- Non-ST-segment elevation myocardial infarction (NSTEMI) (for the group of ACS patients): defined as chest pain at rest with documented transient ST-segment depression or T-wave inversion in at least two contiguous electrocardiographic leads, without pathological Q-waves, with or without enzymatic evidence of myocardial necrosis. The last spontaneous episode of chest pain had to have occurred within the 24 hours preceding study entry. All of the patients underwent coronary angiography in order to confirm the presence of significant coronary artery stenosis, which was defined as the presence of at least one >75% stenosis in any major epicardial coronary vessel;
- Stable CAD (for the group of SA patients): diagnosis was made, according to the AHA criteria, based on typical chest pain on exertion associated with ST segment depression >1.0 mm on an exercise test, and a coronary angiography that confirms the presence of significant coronary artery stenosis (≥75%).
- CKD: diagnosis of chronic kidney disease only in the presence of glomerular filtration rate (GFR) <60 ml/min/1.73m2.

*Exclusion criteria:*

- Patients older than 80 years;
- Valvular heart disease, atrial fibrillation, thyrotoxicosis, a history of haemorrhagic diathesis, platelet disorder or thrombocytopenia, malignancies, inflammatory diseases, major surgery or trauma within the preceding month, or severe liver disease.

**BIOLOGICAL MATERIAL COLLECTION**

At study admission, peripheral venous blood samples were collected without stasis, with a large bore needle (19G), discarding the first 4 mL, from each patient in order to:

- performe fresh whole blood flow cytometry analysis of platelet activation;

- study the global haemostatic function of whole blood by Rotem;

- isolate platelets for transcriptome analysis;

- separate plasma for proteomic studies.

**ASSESSMENT OF PLATELET PHENOTYPE BY FLOW CYTOMETRY**

Platelet activation has been evaluate by whole blood flow cytometry with a fluorescence-activated-cell sorter (FACS) Calibur (Becton Dickinson), using specific fluorochrome-labeled monoclonal antibodies; the immunostaining has been performed as previously described [199, 204], within 15 minutes after blood sampling to avoid artificial platelet activation. The sensitivity of fluorescence detectors has been set and monitored using CaliBRITE beads (Becton Dickinson) according to the manufacturer's instruction.

For this analysis, it was used blood collected into tubes with 0.129 mol/L buffered sodium citrate (3.8% sodium citrate) (vacutainer, Becton Dickinson, BD).

The expression of the following platelet activation markers has been assessed: Tissue Factor, P-selectin (CD62P) and activated glycoprotein IIb/IIIa (GP IIb/IIIa, PAC1). Platelet activation has been also studied as formation of platelet-leukocyte aggregates, by specific antibodies (a leukocyte marker, such as CD45; a platelet marker, such as CD41; and a specific activation marker, Tissue Factor). Saturating concentrations of the following mouse anti-human monoclonal antibodies were used: CD41-PE (Instrumentation Laboratory, IL), also known as integrin α2b or GPIIb and used as a marker of platelet population; PAC1-FITC (Becton Dickinson, BD); CD62-FITC (IL); CD45-PerCP (BD); TF4507CJ-FITC (American Diagnostica, AD). FITC-, PE-, PerCP-conjugated isotype controls were used in all the experiment to quantify the background labeling.

Briefly, for platelet activation staining, 5 µl of whole blood has been added to phosphate buffer saline (PBS 1X, pH 7.4, Life Technologies) to a final volume of 100 µl in presence of saturating concentration of the above described antibodies (a sample was prepared for each marker of platelet activation and co-stained with CD41). All the sample have been prepared to assess surface platelet activation marker expression in resting conditions and upon stimulated condition (prepared following the same labeling of the resting sample except for co-incubating ADP 10 µM, Sigma, together with antibodies, blood and PBS in a final volume of 100 µl). Samples have been incubated at room temperature for 15 minutes (kept away from light). Then, they have been diluted with 600 µl of paraphormaldeid (PFA, final concentration 1%; Sigma) to obtain fixation. Finally, they have been kept in the dark until analysis at FACSCalibur. Platelets positive to activation markers have been determined in 10.000 CD41-positive events per sample.

All the data have been analyzed by CELLQuest software (Becton Dickinson, BD) and the results will be expressed as percentage of positive cell and as mean fluorescence intensity (MFI), calculated for the gated population.

For the assessment of platelet-leukocyte aggregates, 100 µl of whole blood have been stained with saturating concentration of the above described antibodies; platelet-leukocyte formation has been studied in resting conditions and upon stimulated condition (prepared following the same labeling of the resting sample except for co-incubating ADP 10 µM). As previously described for platelet activation marker

assessment, samples have been incubated at room temperature for 15 minutes (kept away from light) and then diluted with 700 µl of BD Lysing Solution (10X diluited 1:10 with room temperature deionized water; final concentration 1X) for lysing red blood cells. Finally, they have been kept in the dark until analysis at FACSCalibur. Platelet-monocyte and -granulocyte aggregates have been determined in 1000 CD45-positive monocytes and 5000 CD45-positive granulocytes per sample.

All the data have been analyzed by CELLQuest software (Becton Dickinson, BD) and the results will be expressed as percentage of platelet-leukocyte aggregates and TF-positive platelet-leukocyte aggregates.

Assessment of platelet intracellular TF has been performed on 50 µl of whole blood previously fixed with PFA 1%; after 2 hours of cell fixation, blood has been washed with PBS and centrifuged at 1500 g for 5 minutes. Then, pellet have been resuspended with 100 µl of PBS-Triton 0,1% (Triton X-100, Carlo Erba) to obtain permeabilization of cell membrane and antibodies access to the antigen in the intracellular compartment. After 10 minutes, blood has been added with saturating concentration of the following mouse anti-human monoclonal antibodies: TF4508CJ-FITC (BD) and CD41-PE (IL). After 15 minutes of incubation in the dark, washing in PBS, centrifugation as before described and resuspension in PBS have been performed. TF-positive platelets have been determined in 10.000 CD41-positive events per sample.

All the data were analyzed by CELLQuest software (Becton Dickinson, BD) and the results will be expressed as percentage of positive cell calculated for the gated population.

**PLATELET ISOLATION FROM WHOLE BLOOD**

Blood collected into three acid citrate dextrose (ACD) -containing vacutainers (BD) has been processed within 15 minutes from sampling and platelets have been isolated by centrifugation and filtration. Particular care has been taken in using an isolation technique that minimizes non-specific platelet activation and leukocyte contamination. Briefly, whole blood has been centrifuged at 100 x g for 10 minutes, at room temperature, without break, in order to obtain platelet rich plasma (PRP). PRP has been

transferred into a new tube, added with 4 µM prostaglandin (PG)E1 and 10 µM EDTA. All PRP obtained has been diluted 1:3 in HEPES-Tyrode's buffer (by adding 2 volumes of HEPES Tyrode's buffer to PRP volume). HEPES-Tyrode's buffer's composition is the following: 10 mmol/L HEPES, 134 mmol/L NaCl, 2,9 mmol/L KCl, 1 mmol/L MgCl2, 12 mmol/L NaHCO3, 0,4 mmol/L Na2HPO4, 0,1% glucose, 5 mmol/L EDTA, 1 µM PGE1); all reagents are purchased from Sigma. Diluted PRP has been filtered through Filter Pall Purecell™, which captures residual contaminating leukocytes, whereas platelets pass through the filter and are collected in a new tube. Filter is washed with 2 volume of HEPES-Tyrode's buffer in order to recover all platelets.

Filtered PRP is then divided, since a volume corresponding to at least 800 million of platelet is necessary for transcriptome studies. Filtered PRP is analyzed by haematologic analyzer Sysmex (Sysmex XS-1000, Dasit) to obtain the platelet count and to assess the eventual presence of contaminating leukocytes. A samples of this PRP is used for the assessment of platelet activation: it has been stained with CD62P-FITC (IL) and CD41-PE (IL) and a comparison of P-selectin value in this sample with the value in fresh whole blood to confirm that the procedure does not lead to non-specific platelet activation has been performed; also leukocyte contamination has been assessed by flow cytometry analysis (staining with CD45 PerCP, BD). Only those preparations with less than 5 leukocytes per $10^5$ platelets, without sign of activation are used for genomic analysis.

The sample volume for transcriptome analysis has been centrifuged at 754 x g for 8 minutes and lysed in 500 µL of QIAzol Lysis Reagent (QIAGEN), immediately frozen till RNA extraction.

**PLASMA PREPARATION FOR SUBQUENT PROTEOMIC STUDIES**

Blood collected into citrate-containing tubes has been centrifuged at 1700 x g for 10 minutes at 4°C.

Plasma from the 0.129 mol/L buffered sodium citrate vacutainer has been placed in tube and quickly aliquotated in small volumes (200 µL) and frozen at -80°C.

**EVALUATION OF WHOLE BLOOD HAEMOSTATIC FUNCTION BY THROMBOELASTOMETRY (ROTEM system)**

Global haemostatic function has been assessed by using Rotem coagulation analyzer (Tem International Gmbh, Munich, Germany); in particular we performed Na-TEM assay, used to recalcify the citrate whole blood, which provides a very sensitive assessment of the equilibrium of coagulation activation or inhibition; and Fib-TEM assay, that contains a platelet inhibitor (Citocalasin D), to avoid platelet contribution to clot formation and to assess and measure the formation of a clot only composed by fibrin.

ROTEM is a system for the study of haemostasis that is based on the measurement of the elasticity of the blood through constant graphic recording of the firmness and the size of the clot during its formation and subsequent fibrinolysis.

ROTEM system sets a reaction curve and kinetic parameters derived from the analysis of the curve, describing the kinetics of clot formation.

Blood collected in a sodium citrate-containing vacutainer (BD) has been put into a cuvette and added with 20 µL of 0.2 mol/L $CaCl_2$ to triggers coagulation, according to the manufacturer's instruction. The cuvette has been placed on a rod applied to the lower end of a vertical axis that rotates from left to right with a defined angle. The rotation has been detected optically. In case of clot formation, the clot adheres to the surface of the rod and cuvette; the movement is hindered and the amplitude of the angle decreases to the increase of clot firmness. All measurements is performed at 37°C.

The following parameters have been recorded:

- Clotting Time (min), CT: is the time required to form a clot of 2 mm of amplitude from the beginning of the assay;

- Clot Formation Time (min), CFT: is the time to form a clot of 20 mm amplitude from a clot of 2 mm;

- Maximum Clot Firmness (mm), MCF: represents the maximum amplitude of the curve before fibrinolysis reduce the size of the clot;

- Maximum Clot Firmness Time (min), MCF-t: is the time necessary to reach MCF starting from the 2 mm amplitude time-point;

- Alpha Angle: is the angle between the tangent to the curve at the 2 mm amplitude time point and the baseline;

- Clot Formation Rate, (CFR): is the linked to alpha angle and it is the angle between the baseline and the tangent at the maximum slope;

- Actual clot firmness (mm), ACF: represents the clot firmness at actual time point after reaction start. ACF is not a parameter in its classical sense, but it is for orientation to judge the clot firmness at the actual point.

**TOTAL RNA EXTRACTION**

RNA extraction was performed using matherials RNasi free and always wearing gloves to avoid the presence of RNasi, enzymes that degrade RNA; reagents were purchased from Sigma Aldrich. Total RNA was isolated from platelet samples lysed in phenol/guanidine-based Qiazol Lysis Reagent (sample preparation was described above).

The combination of organic extraction and chaotropic disruption contributes to efficient lysis and higher yields of total RNA.

Each platelet sample in 500 µL of lysis reagent was added with 50 µL of 2M, pH4, sodium acetate and with 100 µL chloroform. After shaking by vortex, sample were kept in ice for 15 minutes. After this, the homogenate was separated into aqueous and organic phases by centrifugation at 9000 x g, 4°c for 20 minutes. RNA partitions to the upper, aqueous phase while DNA partitions to the interphase and proteins and lipids to the lower, organic phase. Aqueous phase was placed in a new tube and RNA was precipitated from the aqueous phase by adding 1 volume of isopropanol and incubating over night, at 4°C. Sample was centrifuged at 9000 x g, 4°C, for 15 minutes and, after removing isopropanol, the pellet was then washed

with 75% ethanol, 500 µL; sample was kept for ten minutes at room temperature and then centrifuged again at 9000 x g, 4°C, for 5 minutes. After removal of ethanol, pellet was dissolved in 15 µL RNase-free water.

**RNA QUALITY CONTROL**

RNA quantification was performed by Infinite M200 Pro Tecan, an instrument that calculates RNA concentration and purity, measuring absorbance at 230, 260 and 280 nm and giving the ratio of the absorbances 260/280, index of protein contamination if higher than 2, and the ratio of the absorbances 260/230, index of solvent contamination if lower than 2.2.

To evaluate the quality and integrity of extracted RNA the Agilent RNA 6000 Pico kit and reagents and the Bioanalyzer 2100 Agilent Technologies were used.

**GENOMIC ANALYSIS**

The primary objective of this study is the search for genes that are differentially expressed (DE) in distinct phenotype (class comparison), performing genome-wide transcriptional analysis in platelets of ACS patients with and without CKD. To do so, we enrolled 14 ACS with CKD patients and 26 ACS without CKD (controls). DE platelet transcripts were assessed by microarray profiling.

RNA was reverse transcribed, labeled, and linearly amplified using the Total Prep RNA Amplification Kit (Life Technologies, Carlsbad, CA), in order to be hybridized to HumanHT-12 v.4 Expression BeadChip microarrays (Illumina, San Diego, CA), according to manufacturers' instructions. This platform provides genome-wide transcriptional coverage of annotated genes, gene candidates and splice variants (>25000 annotated genes, about 42000 unique transcripts, with >47000 50-mer gene-specific probes derived from the NCBI RefSeq Release 38 and the UniGene Build 99 databases). To estimate technical variability, 10% of the samples were hybridized in duplicate. Arrays were read using the high resolution confocal scanner iScan (Illumina) and

signal quantification and quality control (data mining) were performed with the Genome Studio v. 1.9.0 software (Illumina).

Primary analyses were performed using BRB-ArrayTools v. 4.4.1 developed by Dr. R. Simon and BRB-ArrayTools Development Team (http://linus.nci.nih.gov/BRB-ArrayTools.html). Data variance stabilizing transformation and robust spline normalization were conducted with the lumi R package. Probes were filtered out under any of the following conditions: when the 97.5th percentile of intensities showed a detection P-value greater than 0.01 as calculated by Genome Studio (i.e., the 2.5th percentile of intensities did not significantly differ from the background level) and/or the P-value of the log-ratio variation was greater than 0.01 (i.e., probes that showed minimal variation across the entire set of arrays). Multiple probes were reduced to one per gene symbol by using the most variable probe measured by interquartile range across arrays. The number of genes that passed these filtering criteria was 6013.

Replicate arrays were averaged and genes that were differentially expressed among classes were identified using random-variance univariate two-sample t-test. Genes were considered statistically significant if their parametric P-value was less than 0.001. To correct for multiple comparisons, we set the maximum allowed false discovery rate (FDR) at 0.05 (5% of false discoveries in the list of DE genes) with a confidence level of FDR assessment of 80%. The number of genes significant at 0.001 level of the univariate test was 191: the probability of getting at least 191 genes significant by chance (at the 0.001 level), if there are no real differences between the classes, was virtually equal to 0 at the global test. A fold-difference cut-off of ±1.2 (ACS patients with CKD vs ACS patients without CKD) was applied to this list to focus on most meaningful DE genes: thus the list of DE genes was reduced to 144. Further, we performed a univariate test using a Randomized block design adjusting for age deciles (block variable), to take into account and correct for the influence of age on gene expression differences between the two classes of patients. The number of genes significant at $P < 0.001$ and $FDR < 0.1$ in the univariate test was 60: the probability of getting at least 60 genes significant by chance (at the 0.001 level), if there are no real differences between the classes, was equal to 0.002 at the global test. DE genes with a fold-difference of ±1.2 (ACS patients with CKD vs ACS

patients without CKD ) were 38. The 60 DE genes were clustered using Pearson's correlation (centered) and average linkage method.

Quantitative trait analysis, testing the correlation between the glomerular filtrate rate (GFR) of each patient and platelet gene expression, was performed using the Spearman correlation test, setting the significance level of each univariate test at 0.001.

Gene-annotation enrichment analysis for Gene Ontology (GO) biological processes (BP), molecular functions (MF), and cellular components (CC), and for KEGG pathways was performed on the entire dataset using three tests: the LS and the KS permutation tests, which find gene sets with more genes differentially expressed among the phenotype classes than expected by chance; and the Efron-Tibshirani's test, which uses 'maxmean' statistics to identify gene sets differentially expressed and returns the direction of this difference (+ up-, and – down-regulated gene set in ACS patients with CKD vs ACS patients without CKD comparison). A GO term or a KEGG pathway was considered significantly enriched if its P-value was lower than 0.005 for the ACS patients with CKD vs ACS patients without CKD comparison, with a univariate test for each gene with $P < 0.01$. Redundant GO terms were removed from the results of the above analyses using the web-based tool REVIGO (REduce and VIsualize Gene Ontology).

**PROTEOMIC STUDIES**

Because of human plasma proteome complexity and its enormous dynamic range, in order to overcome the problem of dynamic range, different strategies have been developed to remove the most abundant proteins.

In this study we applied an immunoaffinity purification system using the ProteoPrep Blue Albumin depletion kit, which has been designed to specifically remove albumin and immunoglobulin from human plasma. The ProteoPrep Blue albumin and IgG depletion kit is a mixture of two medias: a blue dye

conjugated to an agarose base matrix and a Protein G Agarose, thus enabling the removal of 95% of albumin and 85% of IgG.

In order to compare the different proteomic profile of plasma from patients with stable angina or ACS with and without chronic kidney disease, we employed a proteomic approach based on two dimensional electrophoresis for protein separation and mass spectrometry for their identification on a selected group of patients, 5 well-matched patients for each group. Immunoenzymatic assays have been performed to confirm the results obtained by two dimensional electrophoresis on a larger cohort of patients.

*Two-dimensional electrophoresis and mass spectrometry*

200 µg of proteins from depleted plasma were diluted with a buffer to yield final concentrations of 7 mol/L urea, 2 mol/L thiourea, 0.2% w/v Sodium Dodecyl Sulfate (SDS), 4% w/v CHAPS, 2% v/v carrier ampholytes, pH 3-10, 20 mmol/L Tris, 55 mmol/L dithiothreitol, and bromophenol blue. IPG ready strips, 7 cm, pH 4-7 linear gradient (Biorad, Italy), were actively rehydrated at 50 V for 24 h and, after focusing, were first equilibrated for 15 min using a solution containing 50 mmol/L Tris-HCl, 6 mol/L urea, 30% v/v glycerol, 2% w/v SDS and 2% w/v dithiothreitol, and then with the same buffer containing 4.5% w/v iodoacetamide instead of dithiothreitol. The focused proteins were then separated according to size on 7-17% polyacrylamide gradient gels and stained with Coomassie Colloidal Blue G-250. Briefly, the gels were fixed with a fixing solution containing 40% v/v methanol and 10% v/v acetic acid, and stained overnight with a solution containing 0.12% w/v Coomassie Blue G-250, 8% w/v (NH4)2SO4, 1.6% v/v phosphoric acid, and 20% v/v methanol. They were then destained with a solution containing 25% v/v methanol. All of the images were scanned using a GS-800 densitomer (Biorad, Italy) before being analyzed by means of Progenesis SameSpot software (Nonlinear Dynamics, v 4.1). Each sample was analyzed by 2-DE in triplicate in order to evaluate gel reproducibility and improve the reliability of the qualitative and quantitative changes in protein expression measured by means of electrophoresis. Progenesis SameSpot software (v 4.1, NonLinear Dynamics) was used for gel alignment, spot detection, spot quantification, and normalization for total spot volume in each gel, and the data were statistically analyzed using the

incorporated statistical package. The cut-off level for a differentially expressed protein was defined as at least a 1.2-fold increase or decrease in spot intensity. Statistically significant between-group differences for each protein were computed using analysis of variance (ANOVA) followed by Tukey's post hoc test to allow both multiple group and individual group-to-group comparison; a p value of <0.05 was considered statistically significant.

The differentially expressed protein spots were manually excised from the gels and underwent in-gel digestion with trypsin. The samples were then analyzed by means of LC-ESI-MS/MS, with the spectra being recorded by a Q-TOF spectrometer (Synapt-MS, Waters corporation, Milford, USA) connected to a nano-UPLC chromatograph. The proteins were identified correlating the uninterpreted spectra with entries in UniProt using ProteinLynx Global Server (Version 2.3, Waters Corporation Milford, USA).

*Immunoenzymatic assays*

Plasma alpha-1 microglobulin concentration was measured using a commercially available ELISA kit (Immunology Consultant Laboratories, Portland, OR, USA) and the minimum detectable dose (MDD) was 2.481 ng/ml. Also Haptoglobin levels were measured by a sandwich ELISA, purchased from Immunology Consultant Laboratories, Portland, OR, USA) and the minimum detectable dose (MDD) was 0.819 ng/ml. Commercially available ELISA kits were also used to measure plasma Retinol binding protein 4 (MDD=0.224 ng/ml) and Fetuin-A concentrations (0.62 ng/ml) (R&D Systems, Minneapolis, MN, USA). All protocols were performed following the manufacturer's recommendations.

For multiple comparisons of the normally distributed variables the two-ways analysis of variance (ANOVA) and the Tukey adjustment were performed. For categorical comparisons chi square test was performed. P values >0.05 were considered statistically significant. All data obtained were subjected to a Kolmogorov-Smirnov normality test to check the normal distribution of the analyzed population.

For obtained results, linear regression and correlation analysis were also performed. All statistical analysis were performed using SAS 9.4 software (Software of Analysis System).

## STATISTICAL ANALYSIS

Baseline categorical and continuous variables were expressed as n° (%) and as mean ± SD, respectively. Platelet activation markers were summarized as mean ± SD and were log-transformed before analysis; the effect of CKD in SA and ACS patients was evaluated by covariance analysis (ANCOVA) after adjustment for age, sex, presence of diabetes and anti-platelet drugs. Differences among groups were analyzed by using chi-square test for categorical variables and Student's t-test for continuous variables. Only $p<0.05$ was regarded as statistically significant.

# RESULTS

# RESULTS

## PATIENT ENROLLMENT

In this study we have enrolled, from December 2012 to April 2014, two cohorts of consecutive ACS patients (onset of symptoms in the previous 24 hours) with (n=31) and without CKD (n=108) and two cohorts of consecutive SA patients with (n=49) and without CKD (n=168): the number of CKD patients enrolled in each group reflects the frequency of the disease in the real world (~30%). We also recruited 37 healthy subjects (HS) as control. All the study subjects were recruited at Centro Cardiologico Monzino IRCCS in Milan, Italy, and gave their written informed consent; the investigation conforms to the principles outlined in the Declaration of Helsinki and was approved by our local Ethical Committee.

Table 3 reports the characteristics of patients enrolled in the study.

Clinical, anamnestic and pharmacological characteristics of the patients included in this study matched the inclusion criteria previously described. CKD patients are older and with a higher prevalence of diabetes that is an important risk factor in CKD patients; in patients with CKD the value of hemoglobin, of hematocrit and the number of RBC, as expected, are significantly lower compared to patients without CKD. Furthermore, patients are comparable in terms of past medical history and drug treatments, except for ipoglycaemic agents.

| CHARACTERISTICS | ACS-no CKD n=108 | ACS-CKD n=31 | *P value* | SA-no CKD n=168 | SA-CKD n=49 | *P value* | HEALTHY SUBJECTS n=37 |
|---|---|---|---|---|---|---|---|
| Age, years (mean±SD) | 64±11 | 74±8 | *<0.01* | 66±8 | 70±7 | *<0.05* | 50±9 |
| Gender, %male | 88(81.5) | 19(61.3) | *<0.05* | 147(87.5) | 40(81.6) | *n.s.* | 18(48) |
| GFR (ml/min/1.73 $m^2$) | 88.1±20.7 | 42.7±12.9 | *<0.01* | 86.5±16.9 | 45.9±11.6 | *<0.01* | n.a. |
| BMI ($kg/m^2$) | 28.2±4.8 | 26.9±6.3 | *n.s.* | 27±3.5 | 29.7±5.1 | *n.s.* | 24±3.5 |
| total cholesterol (mg/dl) | 193±44 | 186±41 | *n.s.* | 191±44 | 192±57 | *n.s.* | 189±50 |
| HDL cholesterol (mg/dl) | 42±11 | 42±14 | *n.s.* | 49±20 | 43±9 | *n.s.* | 61±13 |
| LDL cholesterol (mg/dl) | 123±44 | 114±36 | *n.s.* | 116±38 | 104±46 | *n.s.* | 123±35 |
| triglycerides (mg/dl) | 145±106 | 144±86 | *n.s.* | 137±85 | 147±91 | *n.s.* | 101±38 |
| glycaemia (mg/dl) | 131±54 | 141±45 | *n.s.* | 127±51 | 139±51 | *n.s.* | 84±10 |
| glycosylated hemoglobin (%) | 6.4±1.4 | 6.8±0.9 | *n.s.* | 7.9±2.4 | 7.4±1.5 | *n.s.* | 5.5±0.4 |
| **HAEMOCHROME** | | | | | | | |
| PLT (*$10^3$/ul) | 210±49 | 211±53 | *n.s.* | 200±48 | 197±47 | *n.s.* | 210±51 |
| IPF (%) | 3.1±2.2 | 3.2±2.2 | *n.s.* | 3.1±2.5 | 2.7±1.9 | *n.s.* | 2.4±0.7 |
| MPV (fL) | 10.6±1.1 | 10.1±1.3 | *n.s.* | 11±1.5 | 10.6±0.9 | *n.s.* | 10.9±0.8 |
| WBC (*$10^3$/ul) | 8±2.1 | 7.6±3.2 | *n.s.* | 6.9±1.8 | 7±1.5 | *n.s.* | 5.9±1.2 |
| RBC (*$10^6$/ul) | 4.5±0.5 | 3.8±0.5 | *<0.01* | 4.6±0.6 | 4.4±0.6 | *<0.05* | 4.7±0.5 |
| hemoglobin (g/dl) | 13.4±1.5 | 11.9±1.7 | *<0.01* | 14.1±1.3 | 13.2±1.9 | *<0.01* | 14.1±1.4 |
| hematocrit (%) | 39.7±4.1 | 35.3±5 | *<0.01* | 41.3±3.6 | 39.1±5.1 | *<0.01* | 41.6±3.7 |
| **RISK FACTORS** | | | | | | | |
| Current Smoking (%) | 26(25.2) | 4(13.8) | *n.s.* | 26(16.3) | 4(9.3) | *n.s.* | 5(13.5) |
| Family history of CAD (%) | 23(45.1) | 3(20) | *n.s.* | 74(54.4) | 22(55) | *n.s.* | 6(16) |
| Type-2-diabetes (%) | 27(25) | 16(51.6) | *<0.01* | 48(28.6) | 22(44.9) | *<0.05* | 0(0) |
| Hypercholesterolemia (%) | 66(62.9) | 22(73.3) | *n.s.* | 107(74.8) | 24(63.2) | *n.s.* | 3(8) |
| Hypertriglyceridemia (%) | 18(58.1) | 6(50) | *n.s.* | 26(25.7) | 14(42.4) | *n.s.* | 0(0) |
| Hypertension (%) | 67(64.4) | 23(79.3) | *n.s.* | 122(75.8) | 40(81.6) | *n.s.* | 2(5) |
| **PAST MEDICAL HISTORY** | | | | | | | |
| Prior IMA (%) | 33(32.4) | 6(20) | *n.s.* | 52(31.9) | 16(38.1) | *n.s.* | 0(0) |
| Prior PCI (%) | 25(23.8) | 10(33.3) | *n.s.* | 80(48.5) | 17(40.5) | *n.s.* | 0(0) |
| Prior CABG (%) | 21(20.2) | 8(25.8) | *n.s.* | 20(12.2) | 9(21.4) | *n.s.* | 0(0) |
| **DRUG TREATMENT** | | | | | | | |
| Anti-platelet agents (%) | 94(87) | 29(93.5) | *n.s.* | 159(95.8) | 42(86) | *n.s.* | 0(0) |
| only ASA (%) | 35(32) | 15(48) | *n.s.* | 81(48) | 26(53) | *n.s.* | 0(0) |
| only P2Y12 inhibitors (%) | 11(10) | 2(6) | *n.s.* | 8(5) | 2(4) | *n.s.* | 0(0) |
| DAT (%) | 48(44) | 12(39) | *n.s.* | 67(40) | 14(29) | *n.s.* | 0(0) |
| no anti-platelet drugs (%) | 14(13) | 2(6.5) | *n.s.* | 7(4) | 6(12) | *n.s.* | 37(100) |
| Ipoglycaemic medication (%) | 19(17.6) | 12(38.7) | *<0.05* | 39(23.6) | 19(43.2) | *<0.01* | 0(0) |
| Lipid-lowering agents (%) | 44(41.9) | 12(40) | *n.s.* | 101(61.2) | 28(63.6) | *n.s.* | 0(0) |
| Anti-hypertensive (%) | 52(50) | 18(62.1) | *n.s.* | 113(68.5) | 38(77.6) | *n.s.* | 0(0) |

***Table 3.*** Clinical characteristics of enrolled patients and healthy subjects. Differences among groups analyzed by chi-square test for categorical variables and Student's t-test for continuous variables.

## ASSESSMENT OF PLATELET ACTIVATION MARKERS BY FLOW CYTOMETRY

### Comparison between SA and ACS patients and healthy subjects (HS)

Surface expression of platelet activation markers has been assessed by whole blood flow cytometry in SA and ACS patients and in a group of healthy subjects (HS).

Classic platelet activation markers, such as activated glycoprotein IIb/IIIa (GP IIb/IIIa) and P-selectin, have been evaluated. Furthermore, a novel marker of platelet activation, Tissue Factor (TF), has been assessed.

The expression of GP IIb/IIIa is not different among the three groups of subjects, both as mean amount of antigen expressed on each cell (MFI) (HS: 1.18±0.09; SA: 1.22±0,14; ACS: 1.18±0,12; p>0.05) and as percentage of GP IIb/IIIa-positive platelets (HS: 0.44%±0,09; SA: 0.42%±0,06; ACS: 0.44%±0,1; p>0.05) (Figure 7).

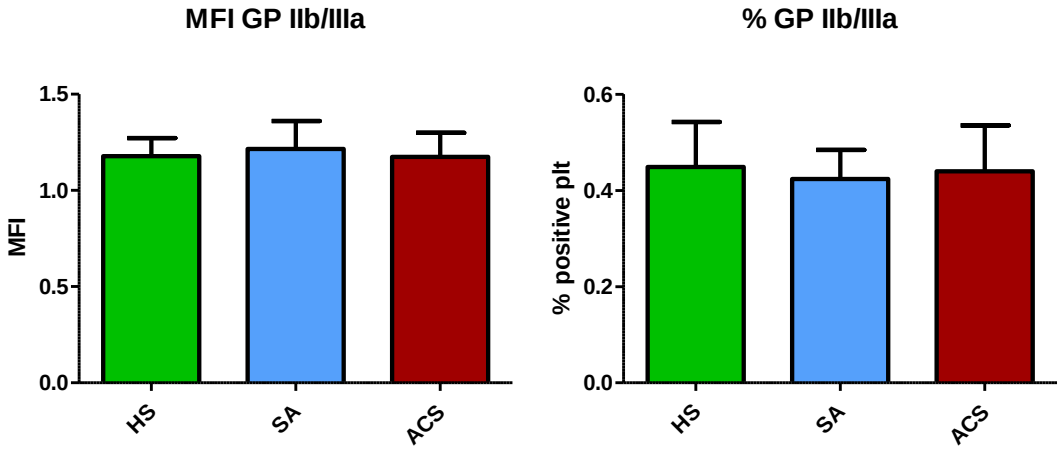



***Figure 7.*** GP IIb/IIIa expression on platelet surface of HS, SA and ACS patients assessed by flow cytometry in resting conditions. Values are reported as mean fluorescence intensity (MFI) as well as % of positive platelets.

As far GP IIb/IIIa platelet expression, also the expression of the other classical platelet activation marker P-selectin is comparable among CAD patients (SA and ACS) and healthy subjects both in terms of mean fluorescence intensity (MFI) (HS: 1.29±0,12; SA: 1.55±0,21; ACS: 1,43±0,25; p>0.05) and in terms of percentage of P-selectin-positive platelets, although a trend toward a higher number of P-selectin-positive platelets is observed in CAD patients compared to healthy subjects (HS: 0.54%±0,04; SA: 0.79%±0,07; ACS: 0.86%±0,17; p>0.05) (Figure 8).

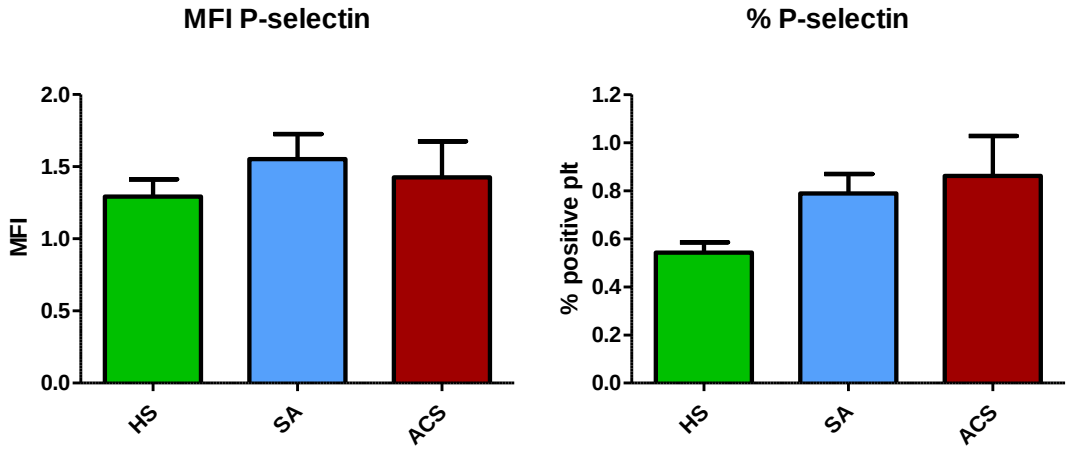



***Figure 8.*** P-selectin expression on platelet surface of HS, SA and ACS patients assessed by flow cytometry in resting conditions. Values are reported as mean fluorescence intensity (MFI) as well as % of positive platelets.

Therefore, these data suggest that the expression of classical markers of platelet activation is not different among CAD patients (that are pharmacologically treated) and HS.

On the contrary, as far as the expression of the novel marker TF is concerned, we have observed that it is significantly higher in SA and ACS patients compared to healthy subjects in terms of mean fluorescence intensity (HS: 1.56±0.48; SA: 2.12±0.68; ACS: 2.08±0.91; p<0.001). Moreover, the percentage of TF-positive platelets significantly increases with the gravity of the coronary artery disease, being highest in ACS patients (HS: 1.49%±0.3; SA: 2.72%±0.41; ACS: 5.23%±0.82; p<0.001). These results fully confirm previously data, published in 2008 by our group, that provided for the first time the evidence that platelet-associated

TF expression is higher in ACS patients compared to SA patients, it is functionally active and therefore it may account for the higher prothrombotic potential of ACS patients [204] (Figure 9).

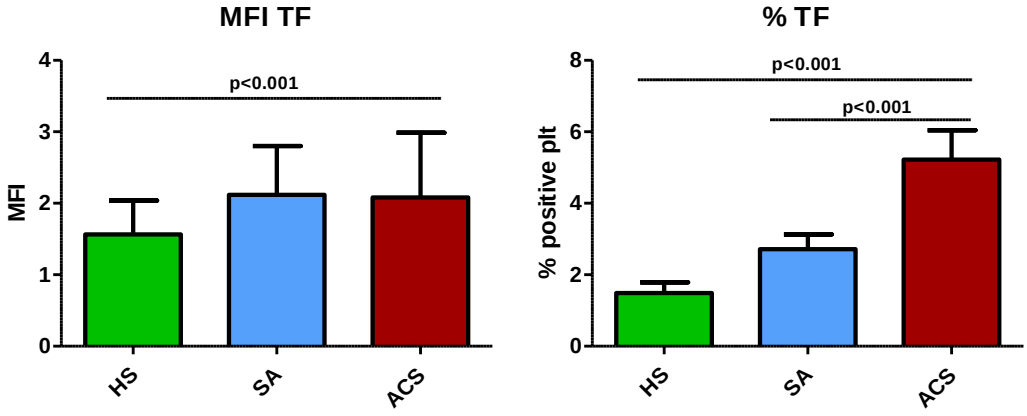



***Figure 9.*** Tissue Factor expression on platelet surface of HS, SA and ACS patients assessed by flow cytometry in resting conditions. Values are reported as mean fluorescence intensity (MFI) as well as % of positive platelets

**Comparison between SA and ACS patients with and without CKD**

In order to evaluate the effect of CKD on the platelet activation profile, whole blood analysis of platelet activation markers has been performed in SA and ACS patients with CKD in comparison to patients without CKD. The presence of platelet-leukocyte aggregates, that are another important marker of platelet activation, has been also assessed.

The percentage of GP IIb/IIIa-positive platelets is not different in ACS patients with and without CKD (resting condition: 0.45%±0.14 in CKD patients *versus* 0.44%±0.10 in patients without CKD, p>0.05; after ADP stimulation: 68.32%±3.93 *versus* 56.11%±2.66, respectively, p>0.05). The same trend is also observed as mean fluorescence intensity (MFI) (resting condition: 1.20±0.11 in CKD patients *versus* 1.18±0.12 in patients without CKD, p>0,05; after ADP stimulation: 10.55±0.90 *versus* 8.63±0.58, respectively, p>0.05). In SA patients with CKD, no statistically significant differences are present in terms of percentage of GP IIb/IIIa-positive platelets in resting conditions and after ADP stimulation (0.26%±0.04 *versus* 0.42%±0.06,

respectively, p>0,05 in resting conditions; 73.18%±2.5 *versus* 66.57%±1.65, respectively, p>0.05, after ADP stimulation); also the expression of GP IIb/IIIa in terms of mean amount of antigen expressed on each cells (MFI) is comparable among patients with and without CKD both in resting conditions and after ADP stimulation (resting condition: 1.17±0.3 *versus* 1.22±0.14, respectively, p>0,05; after ADP stimulation: 12.47±1.22 *versus* 12.01±0.63, respectively, p>0.05) (Figure 10).

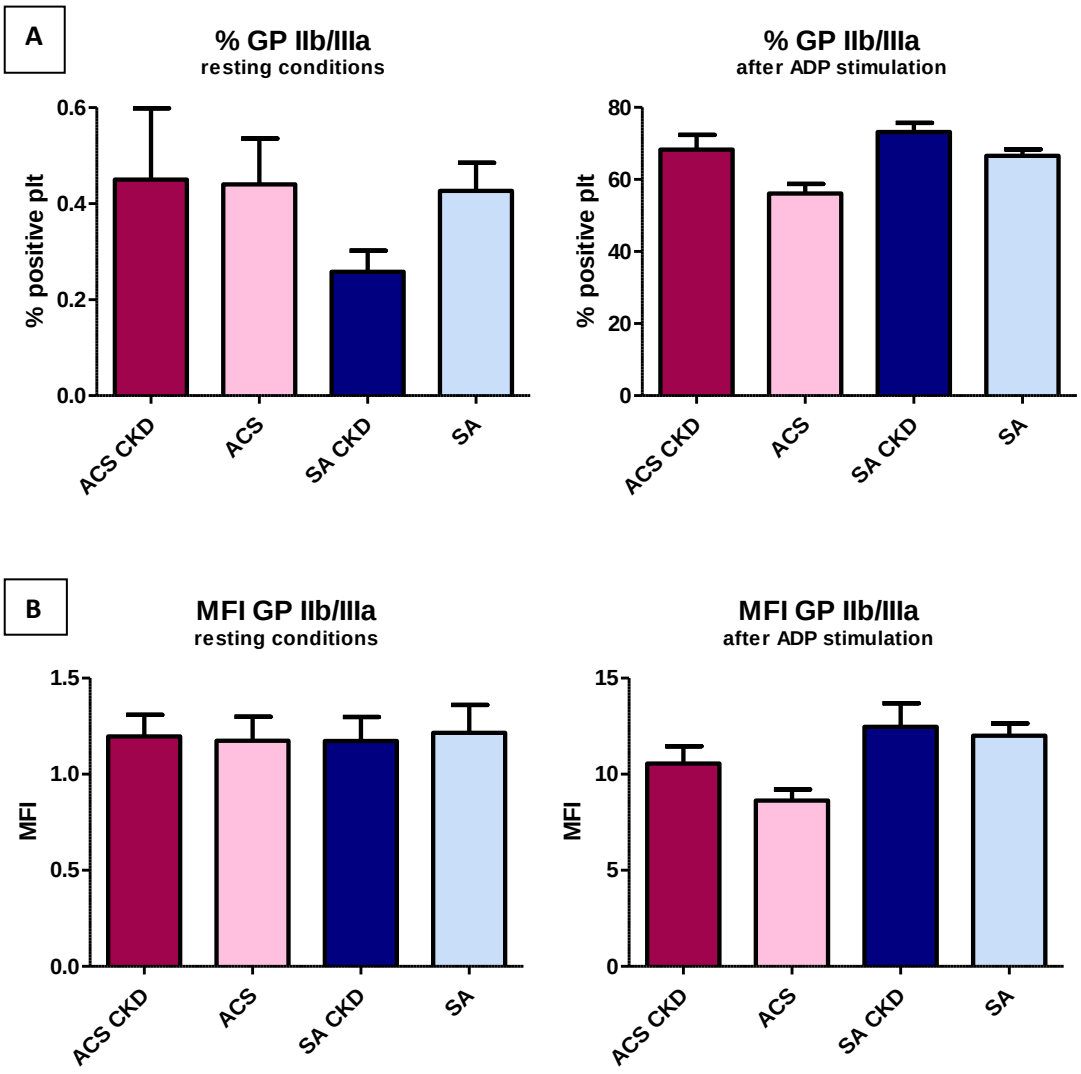
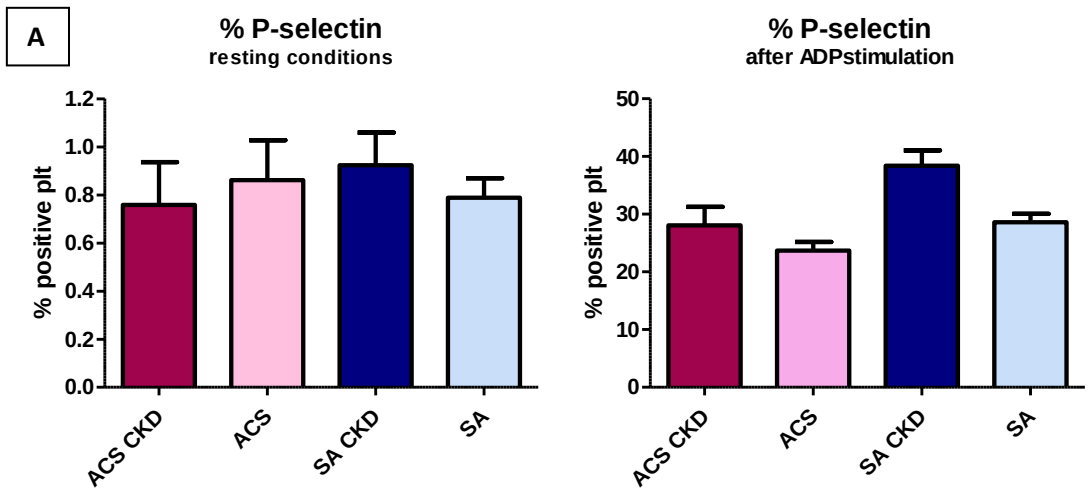



***Figure 10.*** GP IIb/IIIa expression on platelet surface of ACS and SA patients assessed by flow cytometry in resting conditions and after ADP stimulation. Values are reported as % of positive platelets (panel A) and as mean fluorescence intensity (MFI) (panel B).

In ACS patients, the percentage of P-selectin-positive platelets is comparable among those with and without CKD (resting condition: 0.76%±0.18 *versus* 0.86%±0.17, respectively, p>0,05; after ADP stimulation: 28.07%±3.21 *versus* 23.71%±1.49, respectively, p>0.05), as well as the mean fluorescence intensity (MFI) of P-selectin expression (resting condition: 1.42±0.18 *versus* 1.43±0.25, respectively, p>0,05; after ADP stimulation: 3.95±1.74 *versus* 3.54±1.62, respectively, p>0.05). Also in SA patients, the expression of P-selectin is comparable among patients with and without CKD both in terms of percentage of positive platelet (resting conditions: 0.92%±0.14 *versus* 0.79%±0.07, respectively, p>0,05; after ADP stimulation: 38.40%±2.65 *versus* 28.62%±1.43, respectively, p>0.05) and mean fluorescence intensity (resting condition: 1.43±0.22 *versus* 1.55±0.21, respectively, p>0,05; after ADP stimulation: 4.92±1.96 *versus* 4.37±2.10, respectively, p>0.05) (Figure 11).



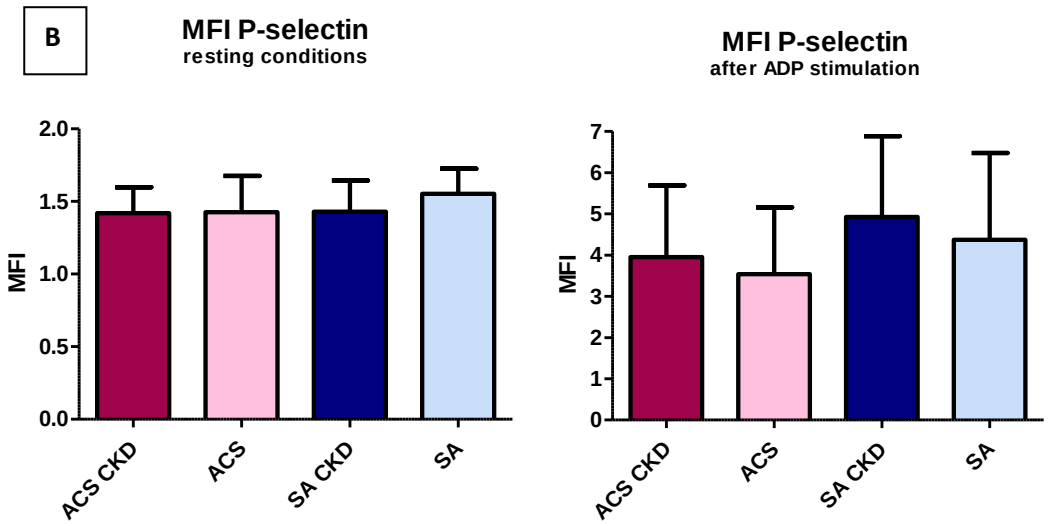



***Figure 11.*** P-selectin expression on platelet surface of ACS and SA patients assessed by flow cytometry in resting conditions and after ADP stimulation. Values are reported as % of positive platelets (panel A) and as mean fluorescence intensity (MFI) (panel B).

These data indicate that the expression of these classical markers of platelet activation, GP IIb/IIIa and P-selectin, is not different among patients with CKD and without CKD both in ACS and in SA patients.

Conversely, the expression on platelet surface in resting condition of TF is significantly lower in ACS patients with CKD compared to patients without CKD (4.07%±1.05 and 5.23%±0.82 respectively, p<0,05), whereas there is not a significant difference after ADP stimulation (20.32%±2.58 and 21.71%±1.52 respectively, p>0,05). Furthermore, the mean fluorescence intensity of TF expression is similar among patients with and without CKD both under resting conditions and after ADP stimulation (resting condition: 1.91±0.56 versus 2.08±0.91, respectively, p>0,05; after ADP stimulation: 3.13±1.03 versus 3.32±1.49, respectively, p>0.05). This means that CKD patients have a lower number of TF-positive platelets compared to patients without CKD, but the mean expression of this marker on each platelet is not different among the two groups of patients. The same trend in TF surface expression is also observed in SA patients with CKD compared to those without CKD in resting condition (1.96%±0.8 *versus* 2.72%±0.41, respectively, p<0,05) and, although not statistically significant, after ADP stimulation (12.93%±2.73 and 16.54%±1.52 respectively, p>0.05). The mean fluorescence intensity of TF expression is lower in SA patients with CKD patients, although not

statistically different compared to patients without CKD (resting condition: 1.80±0.54 *versus* 2.12±0.68, respectively, p>0,05; after ADP stimulation: 3±0.88 *versus* 3.37±1.19, respectively, p>0.05) (Figure 12).

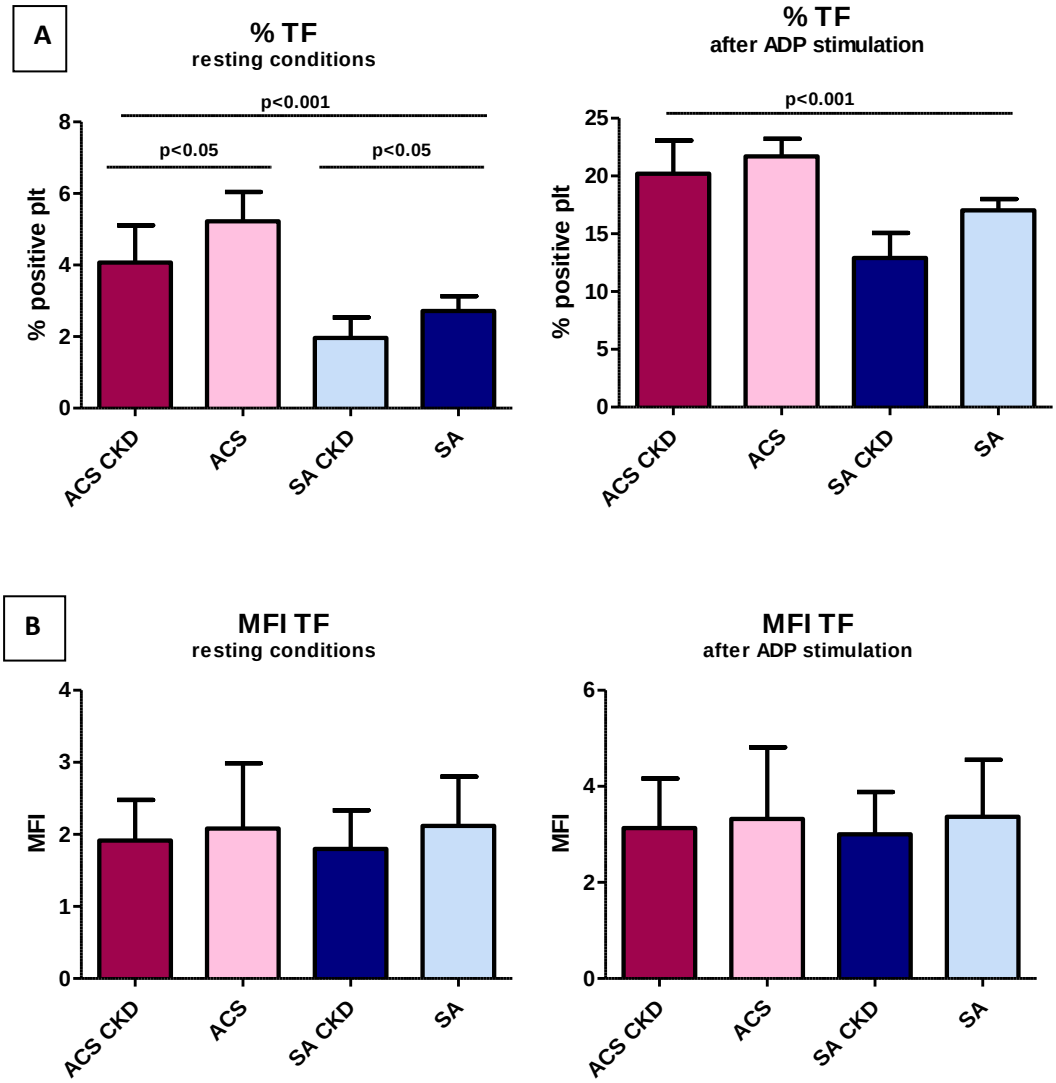



***Figure 12.*** TF expression on platelet surface of ACS and SA patients assessed by flow cytometry in resting conditions and after ADP stimulation. Values are reported as % of positive platelets (panel A) and as mean fluorescence intensity (MFI) (panel B).

To further characterize the lower number of circulating TF-positive platelets found in CKD patients, intracellular staining of TF in resting, fixed and permeabilized platelets has been performed in a subgroup of ACS patients (n=15 with CKD and n=10 without CKD). This approach confirms that the percentage of TF-positive platelets is significantly lower in patients with CKD compared to patients without CKD (19.49%±4.04 and 27.53%±5.16 respectively, p<0,001) (Figure 13).

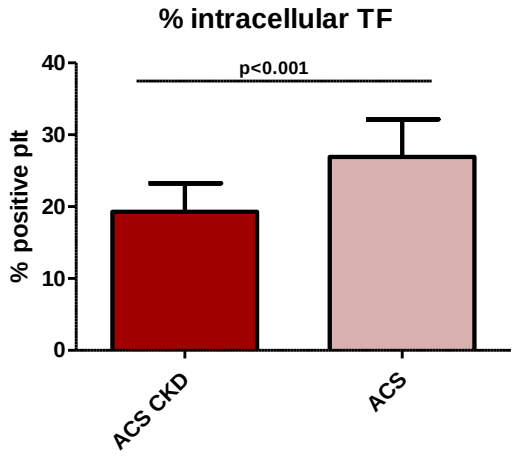



***Figure 13.*** Platelet intracellular Tissue Factor expression of ACS patients assessed by flow cytometry. Values are reported as % of positive platelets.

Of interest, when the number of TF-positive platelets has been correlated with the kidney function, a statistically significant positive correlation has been found between the glomerular filtration rate (GFR) and the percentage of TF-positive platelets (Figure 14).

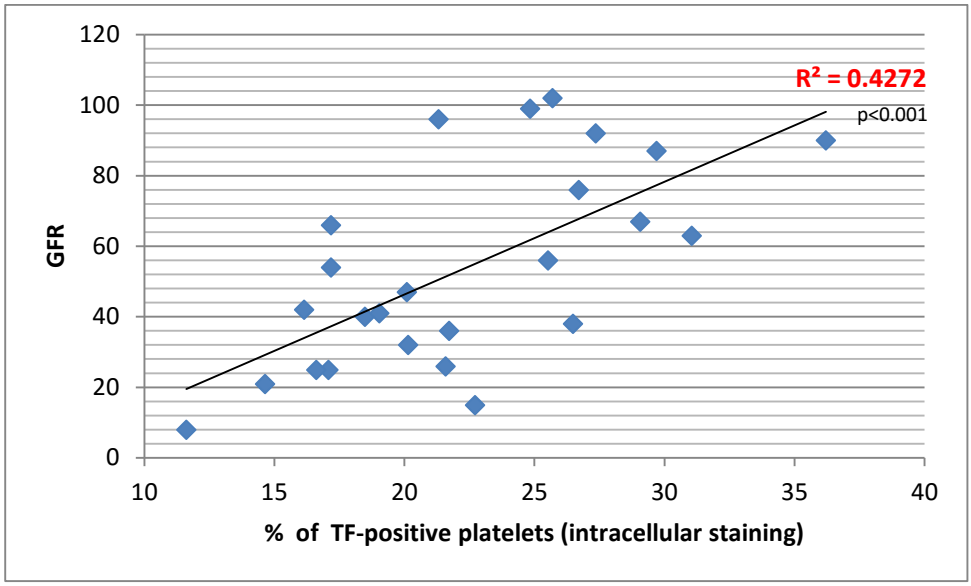



***Figure 14.*** Correlation between platelet intracellular Tissue Factor expression and the Glomerular Filtration Rate (GFR) of a subgroup of ACS patients (n=15 with CKD and n=10 without CKD).

All together these data indicate that, also in presence of CKD, ACS patients have twice the amount of circulating TF-positive platelets compared to patients with SA.

Of interest, CKD significantly affects the expression of TF resulting in a significantly lower number of TF-positive platelets both in ACS and in SA patients: the worst is the kidney function, the lower is the number of circulating TF-positive platelets.

Leukocyte-platelet aggregates (monocyte-platelet and granulocyte-platelet aggregates) that, as mentioned before, are another important marker of platelet activation, is comparable among ACS patients with CKD and without CKD (monocyte-platelet aggregates: 17.16%±2.24 and 15.93%±1.14 respectively $p>0,05$; granulocyte-platelet aggregates: 5.21%±0.45 and 4.62%±0.27, respectively $p>0,05$).

A similar behaviour has been observed in SA patients with no difference among patients with and without CKD (monocyte-platelet aggregates: 14.62%±1.30 and 15.89%±1.05, respectively $p>0,05$; granulocyte-

platelet aggregates: 4.43%±0.41 and 5.11%±0.39, respectively p>0,05). Also the comparison among the four groups of patients has shown no difference (Figure 15).

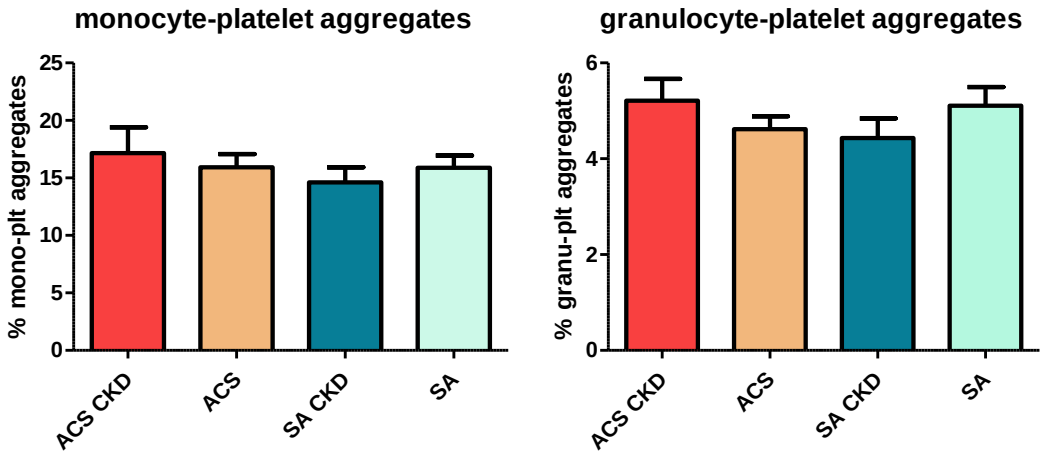



***Figure 15*** Formation of leukocyte-platelet aggregates (monocyte-platelet aggregates in the panel on the left; granulocyte-platelet aggregates in the panel on the right) of ACS and SA patients assessed by flow cytometry. Values are reported as % of leukocyte-platelet aggregates.

On the contrary, the analysis of TF-positive aggregates, TF-positive platelet-monocyte and TF-positive platelet-granulocyte aggregates, has shown a trend towards a lower number in CKD patients, both ACS and SA (in ACS patients: TF positive platelet-monocyte aggregates: 1,58%±0.23 and 2,28%±0.39 respectively p>0,05; TF positive platelet-granulocyte aggregates: 0,09%±0.03 and 0,19%±0.08, respectively p>0,05; in SA patients: TF-positive platelet-monocyte aggregates: 1.98%±0.43 and 2.54%±0.37 respectively p>0.05; TF-positive platelet-granulocyte aggregates: 0.11%±0.017 and 0.24%±0.07, respectively p>0,05) (Figure 16).

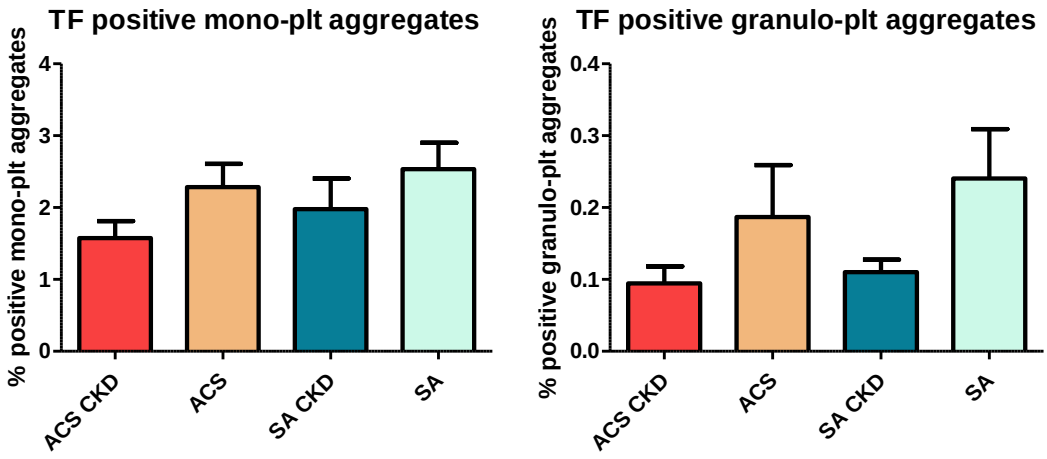



***Figure 16.*** Tissue Factor expression on leukocyte-platelet aggregates (TF-positive monocyte-platelet aggregates in the panel on the left; TF-positive granulocyte-platelet aggregates in the panel on the right) of ACS and SA patients assessed by flow cytometry. Values are reported as % of TF-positive leukocyte-platelet aggregates.

Overall, CKD patients (both ACS and SA patients) show a reduction in the expression of TF-associated to platelet and to leukocyte-platelet aggregates. In view of the haemorrhagic tendency of CKD patients, these data could account for the bleeding profile characteristic of these patients.

## GLOBAL HAEMOSTATIC FUNCTION OF WHOLE BLOOD

The global haemostatic function of whole blood has been assessed by thromboelastometry (ROTEM system), that measures the haemostasis and the interaction of coagulation factors, inhibitors and cellular components (in particular platelets) during the phases of clotting and subsequent fibrinolysis.

The Na-TEM assay, used to recalcify citrate blood, has shown that the haemostatic function is significantly different in SA patients with CKD compared with SA patients without CKD. Indeed in CKD patients there is a significant shorter clot formation time, suggesting a higher procoagulant potential (CT=396±151 in patients with CKD, CT=457±113 in patients without CKD, p<0,01). However, a reduced clot firmness (MCF) which correlates with clot instability has been observed in SA patients with CKD (MCF=55±18 in patients with CKD, MCF=60±5 in patients without CKD, p<0,05) (Figure 17).

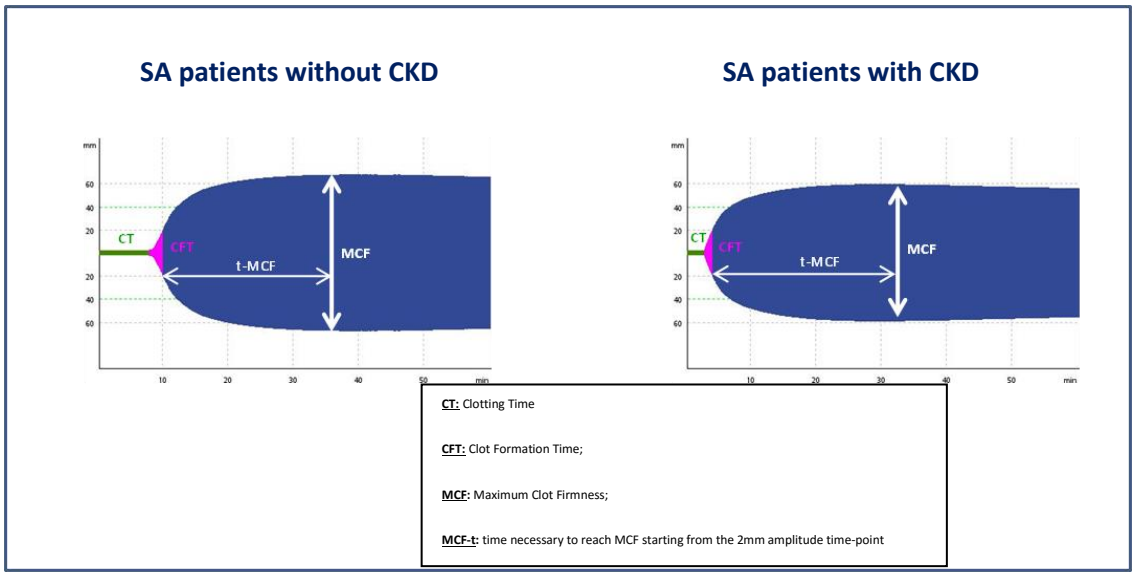



***Figure 17.*** Representative Na-TEM thromboelastometric graphs from whole blood of *SA* patients without CKD, on the left, and of patients with CKD, on the right.

In contrast to what observed in SA patients, ACS patients with CKD have a hypocoagulant phenotype: clot formation time is significantly prolonged (CT=677±399 in patients with CKD, CT=466±189 in patients without CKD, p<0,01,) while no differences has been observed in clot firmness (Figure 18).

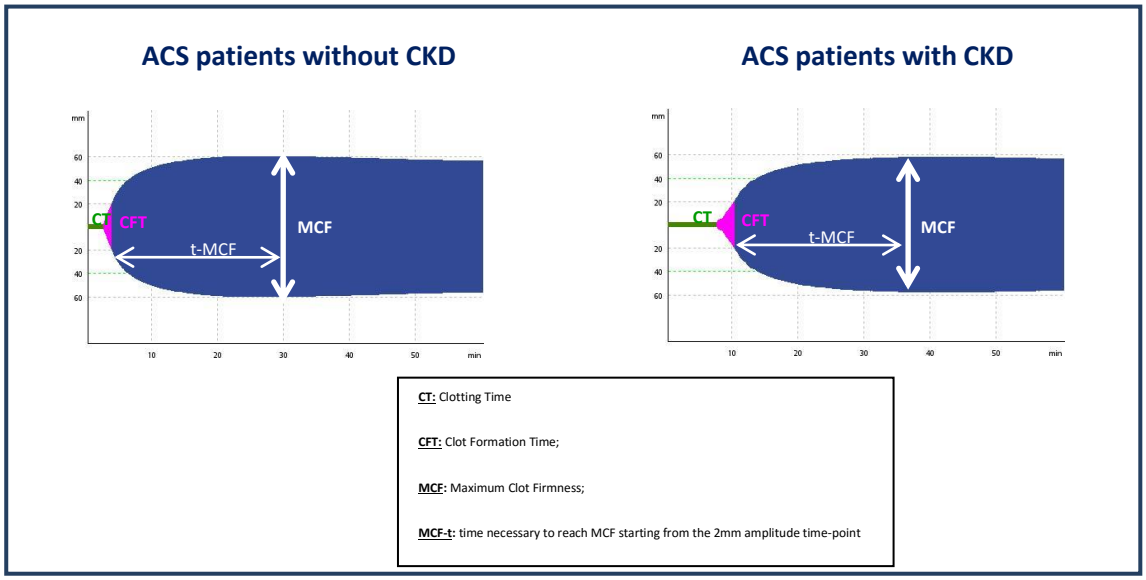



***Figure 18.*** Representative Na-TEM thromboelastometric graphs from whole blood of *ACS* patients without CKD, on the left, and of patients with CKD, on the right.

In order to define the relative contribution of platelet and of the coagulation pathway to the global haemostatic potential, thromboelastometry has been performed in the presence of a platelet inhibitor (Citocalasin D; Fib-TEM assay) thus allowing the formation and measurement of a pure fibrin clot. Under these conditions, the rate of fibrin formation as well as the firmness of the fibrin clot are both significantly increased in ACS and SA patients with CKD compared to patients without CKD. In particular, in ACS patients: maximum clot firmness, MCF=25±17 in patients with CKD, MCF=16±5 in patients without CKD, p<0,01; actual clot firmness, ACF=25±17 in patients with CKD, ACF=17±5 in patients without CKD, p<0,01; rate of clot formation, alpha angle=75±16 in patients with CKD, alpha angle=71±6 in patients without CKD, p<0,05 (Figure 19).

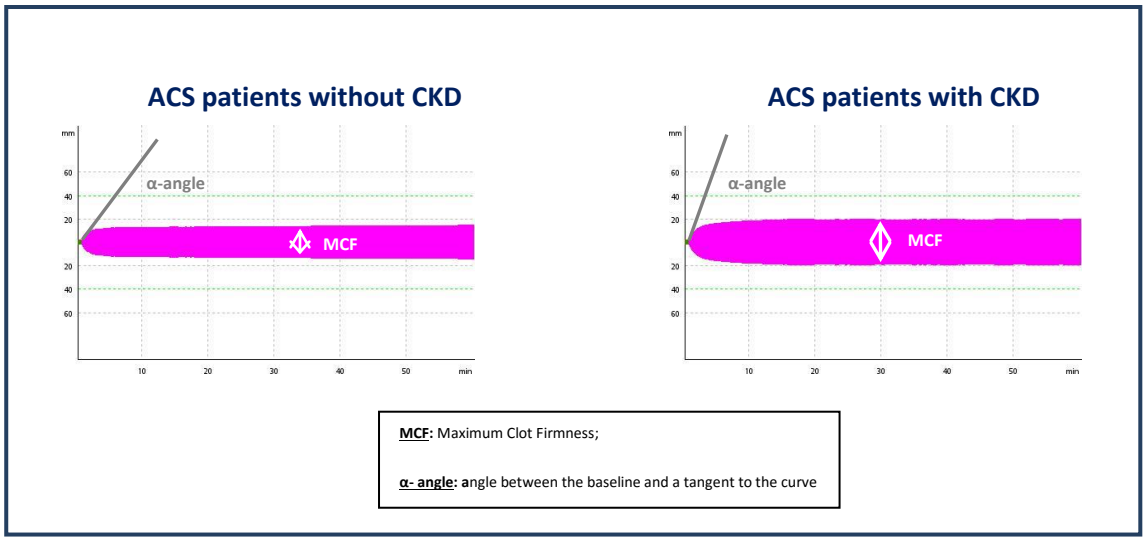



***Figure 19.*** Representative Fib-TEM thromboelastometric graphs from whole blood of *ACS* patients without CKD, on the left, and of patients with CKD, on the right.

A similar trend has been observed in SA patients: clot formation rate, CFR=74±5 in patients with CKD, CFR=73±4 in patients without CKD, p<0,05; clot firmness, ACF=21±9 in patients with CKD, ACF=18±2 in patients without CKD, p<0,05 (Figure 20).

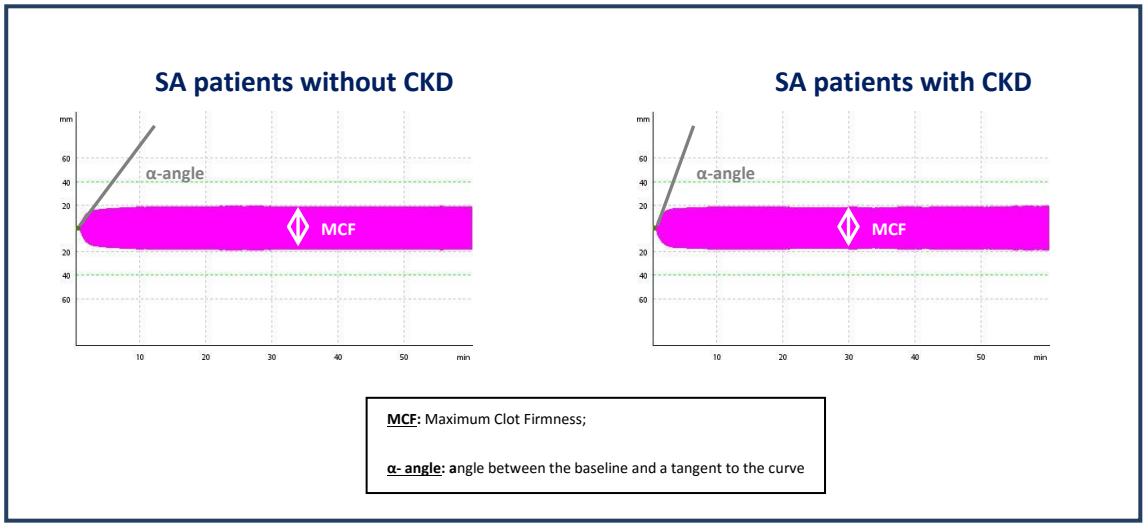



***Figure 20.*** Representative Fib-TEM thromboelastometric graphs from whole blood of *SA* patients without CKD, on the left, and of patients with CKD, on the right.

## QUALITY CONTROLS FOR PLATELET ISOLATION AND RNA EXTRACTION

To check the absence of platelet activation in the samples of isolated platelets for the RNA extraction, the expression of platelet P-selectin has been detected by flow cytometry and it has been compared with platelet P-selectin expression in whole blood. The developed technique and the care in handling the samples allow to have all the isolated platelet preparations not activated by the procedure of separation.

Moreover, to study platelet-derived RNAs, it's necessary that the transcriptome analysis is performed only on platelet RNAs, not contaminated with leukocyte-derived RNAs; to check leukocyte contamination, all preparations has been analyzed for absence of leukocytes both by flow cytometry (Figure 21) and by haematologic analyzer: both the instruments have given the same results. Only platelet samples with less than 50 leukocytes per $10^6$ platelets were considered suitable for RNA analysis.

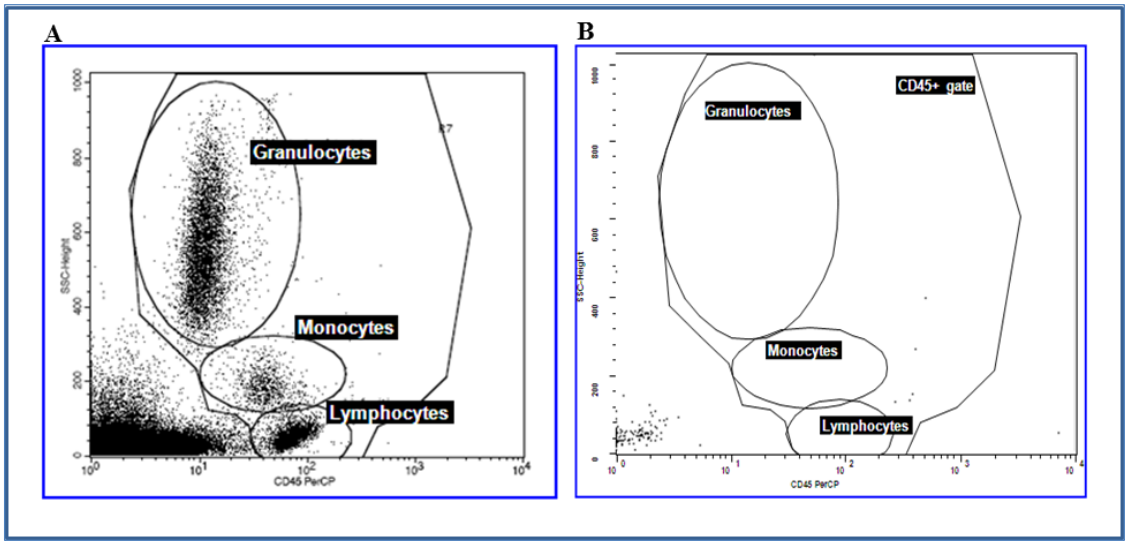



***Figure 21.*** Control of leukocyte contamination in isolated platelets by flow cytometry. **A**, leukocytes detection in whole blood on the basis of side scatter characteristics and positivity to CD45; **B**, detection of leukocyte contamination in isolated platelet samples applying the same setting used for leukocytes acquisition in whole blood and the same strategy of gating and analysis.

RNA was extracted from pure platelet preparations and quantified by a highly sensitive absorbance reader. RNA quality control and quantitation is essential before any downstream application. A positive correlation was found between the platelet number in each sample and the amount of recovered RNA suggestive of the good optimization of the method applied (r=0.78) (Figure 22). Generally, we obtained 1 µg of RNA from 1 billion of platelets. We checked the quality of these RNAs by using Agilent RNA 6000 Pico bioanalyzer and the obtained profiles showed the typical platelet RNA pattern, with the 18S ribosomal RNA more abundant than the 28S ribosomal RNA. The 260/280 ratio indicated absence of contaminant proteins (Figure 23).

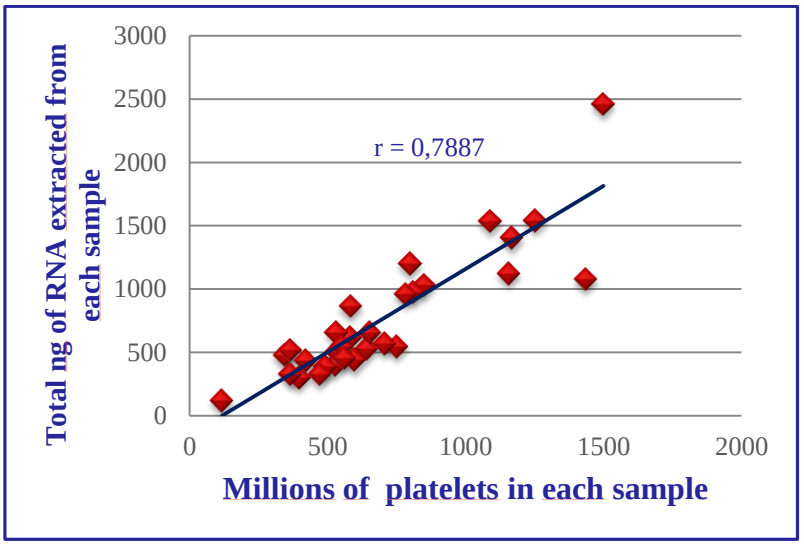
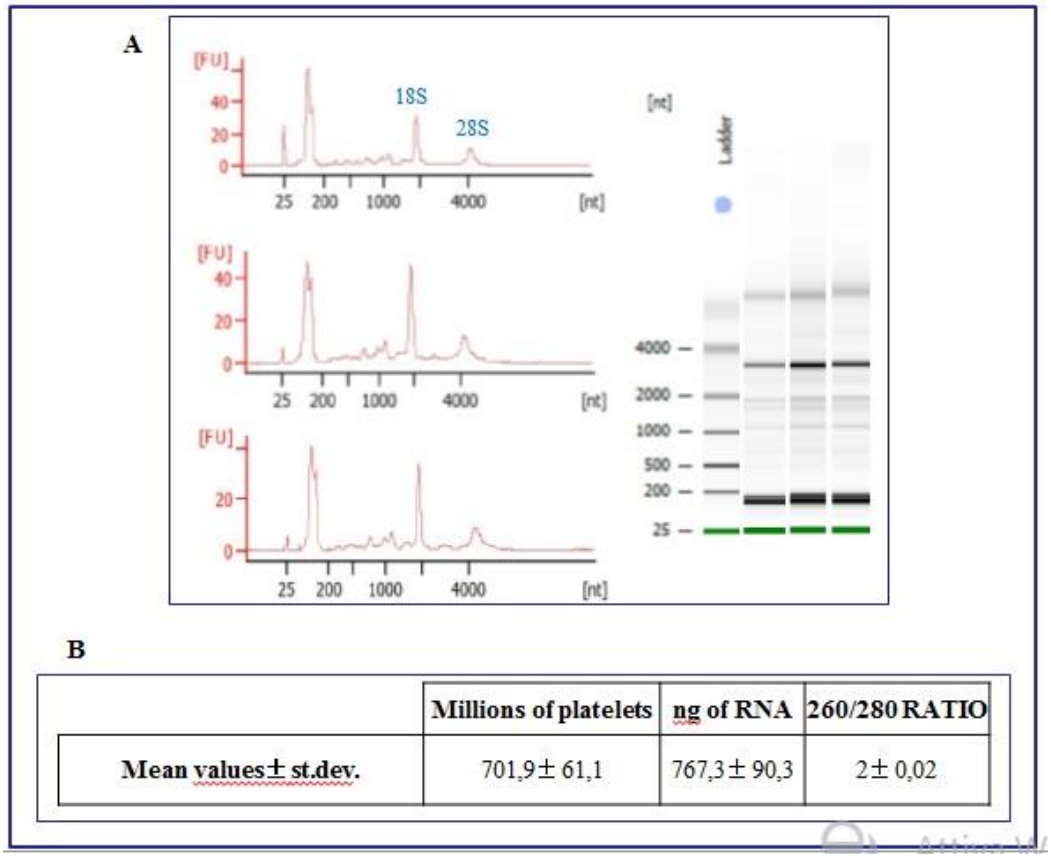



***Figure 22.*** Correlation between the number of platelets isolated from a patient and the amount of recovered RNA in a representative subset of samples.




**B**

| | Millions of platelets | ng of RNA | 260/280 RATIO |
|---|---|---|---|
| Mean values± st.dev. | 701,9± 61,1 | 767,3± 90,3 | 2± 0,02 |

***Figure 23.*** panel A: RNA elettropherograms by Agilent bioanalyzer to assess quality of recovered platelet RNAs; panel B: quantification and quality control by Infinite M200Pro.

**GENOMIC DATA: DIFFERENTIAL PLATELET GENE EXPRESSION PROFILES BETWEEN PATIENTS WITH AND WITHOUT CKD**

Platelet genome-wide transcriptome profiles have been performed on a subgroup of ACS patients (n=14 with CKD, n=26 without CKD) well-matched for age, sex and risk factors, by microarray analysis of RNA samples, in order to study expression signatures that may differentially modulate platelet reactivity in patients with chronic kidney disease compared to patients without CKD. Gene profiling has detected more than 6000 distinct transcripts as being present in ACS with and without CKD.

Differential expression analysis, analyzed by two-sample T-test, followed by correction for multiple testing to obtain false discovery rate (FDR) <0.05, has identified several changes in gene expression profile between patients with and without CKD. In particular, 144 unique mRNAs with a significant ± 1.2-fold or greater difference in expression between ACS patients with and without CKD has been identified: 104 of these genes were over-expressed, whereas 40 had a lower expression in platelets of CKD patients compared to those of patients without CKD. (Figure 24).

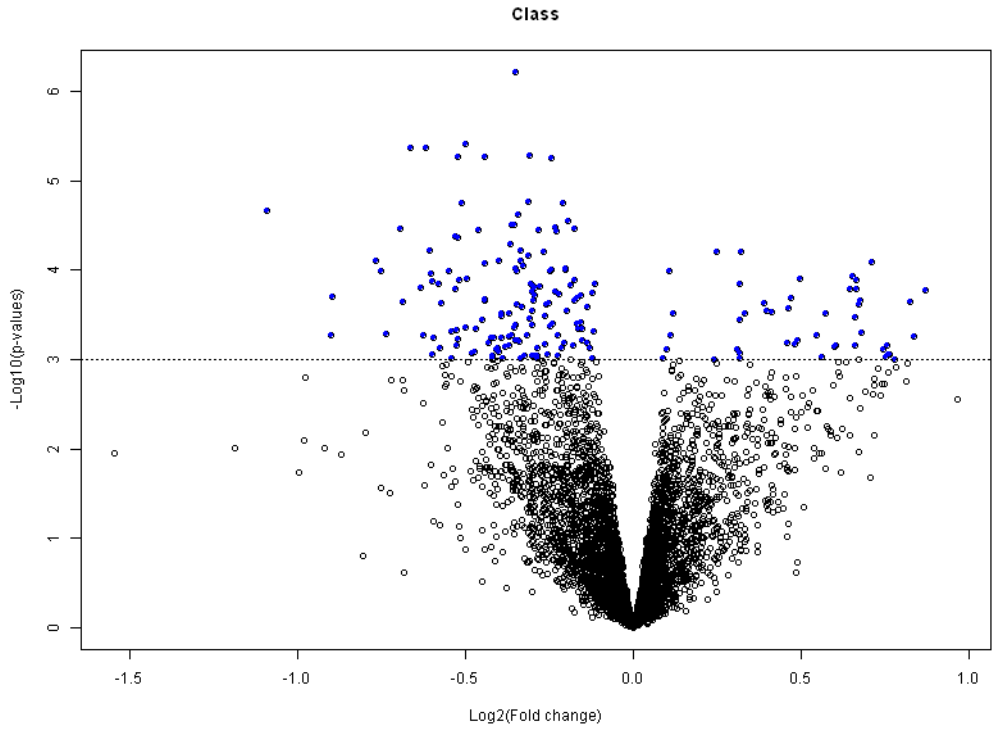



***Figure 24.*** Volcano plot of differential gene expression in platelets of ACS patients with and without CKD

After adjustment for age, 60 mRNAs with a significant difference in expression between ACS patients with and without CKD have been identified ($p<0.001$, FDR<0.1): 44 of them were over-expressed, whereas 16 showed a decreased expression in platelets of ACS patients with CKD compared to platelets of ACS patients without CKD. On a global test analysis, the probability of getting at least 60 genes significant by chance (at the 0.001 level) if there are no real differences between the classes has been 0.002. Hierarchical clustering has shown that differential gene expression profiles correctly discriminate between platelets of patients with and without CKD and there are at least seven different clusters of co-regulated gene (Figure 25 and Table 4).

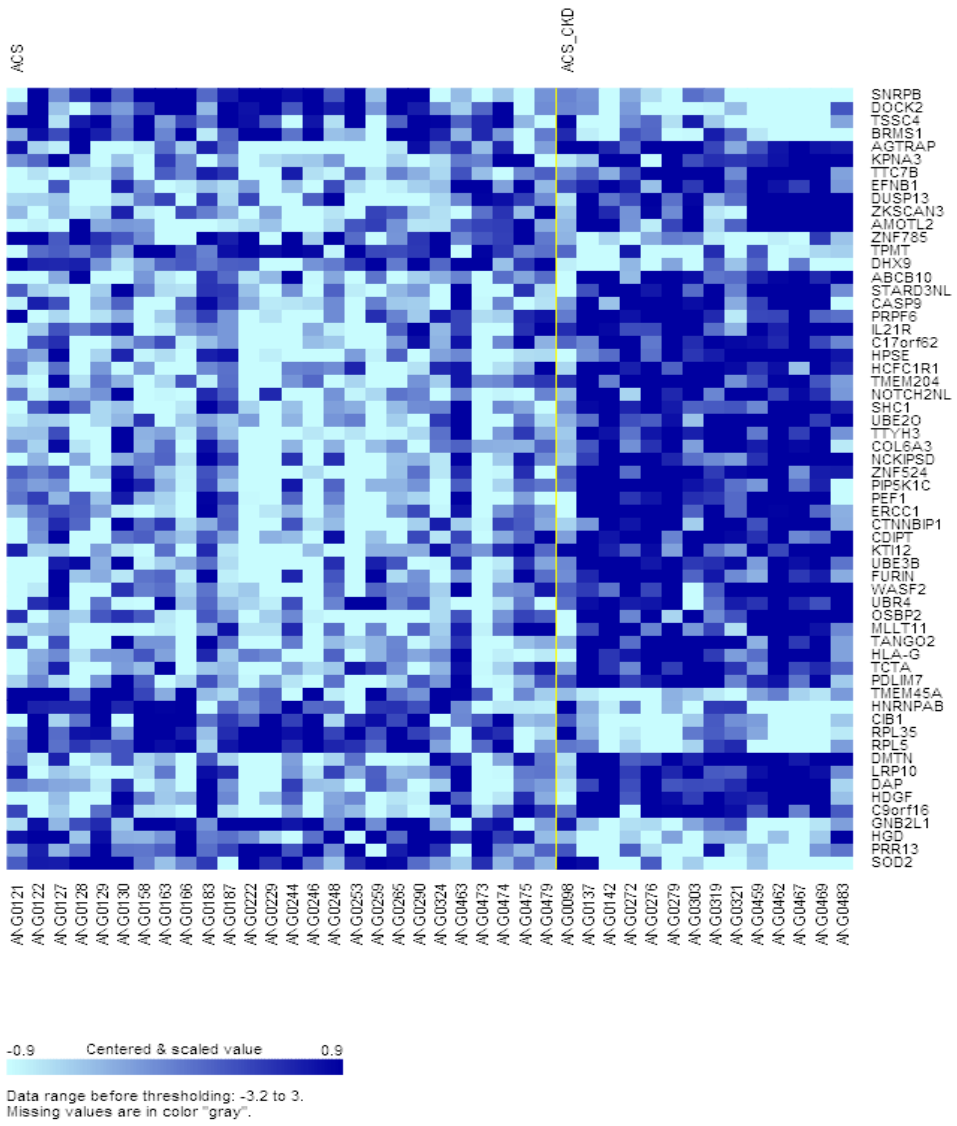



***Figure 25.*** Heat map of clustered differential gene profiles in platelets of ACS patients with and without CKD

| Parametric p-value | FDR | Geom mean ACS | Geom mean ACS-CKD | FC CKD vs. ACS | Symbol | Name |
|---|---|---|---|---|---|---|
| 1.31E-05 | 0.0788 | 116.26 | 134.36 | **1.15** | TTC7B | tetratricopeptide repeat domain 7B |
| 6.85E-05 | 0.0927 | 151.16 | 192.86 | **1.28** | ZNF524 | zinc finger protein 524 |
| 7.23E-05 | 0.0927 | 182.1 | 225.96 | **1.23** | TMEM204 | transmembrane protein 204 |
| 9.20E-05 | 0.0927 | 114.1 | 128.72 | **1.12** | ZKSCAN3 | zinc finger with KRAB and SCAN domains 3 |
| 9.88E-05 | 0.0927 | 501.61 | 794.28 | **1.59** | DMTN | dematin actin binding protein |
| 0.000118 | 0.0927 | 158.21 | 224.07 | **1.41** | TTYH3 | tweety family member 3 |
| 0.0001282 | 0.0927 | 141.48 | 128.24 | **-1.10** | TSSC4 | tumor suppressing subtransferable candidate 4 |
| 0.0001327 | 0.0927 | 115.29 | 124.75 | **1.09** | AMOTL2 | angiomotin like 2 |
| 0.0001418 | 0.0927 | 115.03 | 108.11 | **-1.06** | ZNF785 | zinc finger protein 785 |
| 0.0001744 | 0.0927 | 268.62 | 386.01 | **1.43** | TANGO2 | transport and golgi organization 2 homolog (Drosophila) |
| 0.0002089 | 0.0927 | 116.07 | 137.59 | **1.19** | AGTRAP | angiotensin II receptor-associated protein |
| 0.000261 | 0.0927 | 133.59 | 181.6 | **1.35** | UBE2O | ubiquitin-conjugating enzyme E2O |
| 0.0002714 | 0.0927 | 153.63 | 193.74 | **1.27** | UBE3B | ubiquitin protein ligase E3B |
| 0.0002729 | 0.0927 | 161.38 | 247.69 | **1.54** | HPSE | heparanase |
| 0.0002834 | 0.0927 | 143.82 | 181.41 | **1.27** | PIP5K1C | phosphatidylinositol-4-phosphate 5-kinase, type I, gamma |
| 0.0002925 | 0.0927 | 172.41 | 219.04 | **1.27** | NOTCH2NL | notch 2 N-terminal like |
| 0.0003332 | 0.0927 | 130.94 | 159.35 | **1.22** | STARD3NL | STARD3 N-terminal like |
| 0.0003333 | 0.0927 | 118.98 | 129.45 | **1.09** | DUSP13 | dual specificity phosphatase 13 |
| 0.0003354 | 0.0927 | 389.23 | 593.58 | **1.52** | HDGF | hepatoma-derived growth factor |
| 0.0003452 | 0.0927 | 129.3 | 119.01 | **-1.09** | TPMT | thiopurine S-methyltransferase |
| 0.0003507 | 0.0927 | 125.14 | 142.62 | **1.14** | PRPF6 | pre-mRNA processing factor 6 |
| 0.0003905 | 0.0927 | 183.6 | 262.61 | **1.43** | MLLT11 | myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 11 |
| 0.0003982 | 0.0927 | 1388.03 | 875.38 | **-1.59** | HGD | homogentisate 1,2-dioxygenase |
| 0.0004116 | 0.0927 | 223.69 | 266.48 | **1.19** | TCTA | T-cell leukemia translocation altered |
| 0.0004127 | 0.0927 | 418.46 | 569.39 | **1.37** | C9orf16 | chromosome 9 open reading frame 16 |
| 0.0004127 | 0.0927 | 310.76 | 196.67 | **-1.58** | TMEM45A | transmembrane protein 45A |
| 0.0004245 | 0.0927 | 147.52 | 194.94 | **1.32** | NCKIPSD | NCK interacting protein with SH3 domain |
| 0.0004646 | 0.0927 | 213.81 | 456.42 | **2.13** | OSBP2 | oxysterol binding protein 2 |
| 0.0004778 | 0.0927 | 122.07 | 143.37 | **1.18** | CASP9 | caspase 9, apoptosis-related cysteine peptidase |
| 0.0004811 | 0.0927 | 1224.97 | 888.2 | **-1.38** | PRR13 | proline rich 13 |
| 0.0004992 | 0.0927 | 149.46 | 177.27 | **1.19** | PEF1 | penta-EF-hand domain containing 1 |
| 0.000511 | 0.0927 | 126.68 | 117.3 | **-1.08** | DHX9 | DEAH (Asp-Glu-Ala-His) box helicase 9 |
| 0.0005199 | 0.0927 | 145.09 | 172.48 | **1.19** | CTNNBIP1 | catenin, beta interacting protein 1 |
| 0.0005316 | 0.0927 | 649.89 | 355.42 | **-1.83** | RPL35 | ribosomal protein L35 |
| 0.0005742 | 0.0927 | 125.51 | 143.63 | **1.15** | IL21R | interleukin 21 receptor |
| 0.0005764 | 0.0927 | 517.11 | 746.51 | **1.45** | LRP10 | low density lipoprotein receptor-related protein 10 |
| 0.0005881 | 0.0927 | 148.72 | 132.73 | **-1.12** | BRMS1 | breast cancer metastasis suppressor 1 |
| 0.0006073 | 0.0927 | 117.52 | 134.74 | **1.15** | KPNA3 | karyopherin alpha 3 (importin alpha 4) |
| 0.000608 | 0.0927 | 204.44 | 163.89 | **-1.25** | SNRPB | small nuclear ribonucleoprotein polypeptides B and B1 |
| 0.0006212 | 0.0927 | 292.56 | 220.07 | **-1.33** | HNRNPAB | heterogeneous nuclear ribonucleoprotein A/B |
| 0.0006318 | 0.0927 | 1177.42 | 834.99 | **-1.41** | SOD2 | superoxide dismutase 2, mitochondrial |
| 0.0006688 | 0.0944 | 212.93 | 303.75 | **1.43** | HLA-G | major histocompatibility complex, class I, G |
| 0.0007106 | 0.0944 | 530.96 | 332.58 | **-1.60** | RPL5 | ribosomal protein L5 |
| 0.0007134 | 0.0944 | 167.04 | 207.65 | **1.25** | SHC1 | SHC (Src homology 2 domain containing) transforming protein 1 |
| 0.0007156 | 0.0944 | 154.06 | 196.59 | **1.28** | COL6A3 | collagen, type VI, alpha 3 |
| 0.0007222 | 0.0944 | 363.53 | 618.87 | **1.69** | DAP | death-associated protein |
| 0.0007972 | 0.0978 | 156.99 | 198.58 | **1.27** | FURIN | furin (paired basic amino acid cleaving enzyme) |
| 0.0008165 | 0.0978 | 129.3 | 151.86 | **1.18** | C17orf62 | chromosome 17 open reading frame 62 |
| 0.0008249 | 0.0978 | 125.8 | 154.84 | **1.23** | ABCB10 | ATP-binding cassette, sub-family B (MDR/TAP), member 10 |
| 0.0008308 | 0.0978 | 2953 | 1868.11 | **-1.58** | GNB2L1 | guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1 |
| 0.0008492 | 0.0978 | 140.46 | 161.52 | **1.15** | CDIPT | CDP-diacylglycerol--inositol 3-phosphatidyltransferase |
| 0.0008604 | 0.0978 | 161.29 | 205.3 | **1.27** | WASF2 | WAS protein family, member 2 |
| 0.0008947 | 0.0978 | 173.74 | 239.54 | **1.37** | HCFC1R1 | host cell factor C1 regulator 1 (XPO1 dependent) |
| 0.0009134 | 0.0978 | 140.65 | 158.73 | **1.12** | KTI12 | KTI12 homolog, chromatin associated (S. cerevisiae) |
| 0.0009145 | 0.0978 | 160.57 | 177.73 | **1.11** | UBR4 | ubiquitin protein ligase E3 component n-recognin 4 |
| 0.0009209 | 0.0978 | 189.16 | 151.61 | **-1.25** | DOCK2 | dedicator of cytokinesis 2 |
| 0.0009324 | 0.0978 | 216.87 | 278.72 | **1.28** | PDLIM7 | PDZ and LIM domain 7 (enigma) |
| 0.0009752 | 0.0978 | 110.52 | 123.25 | **1.11** | EFNB1 | ephrin-B1 |
| 0.0009776 | 0.0978 | 346.94 | 264.86 | **-1.31** | CIB1 | calcium and integrin binding 1 (calmyrin) |
| 0.0009916 | 0.0978 | 140.7 | 175.03 | **1.25** | ERCC1 | excision repair cross-complementation group 1 |

***Table 4.*** Genes that are differentially expressed between patients with and without CKD

Spearman analysis has revealed that 98 unique genes significantly correlate ($p<0.001$; FDR<0.05) with the decrease of glomerular filtration rate (GFR), i.e. with the severity of the renal failure: the expression levels of 79 genes have shown an inverse correlation with GFR and 19 a positive correlation. The probability of getting at least 98 genes significant by chance (at the 0.001 level) if there are no real differences between the classes has been 0.007. (Figure 26).

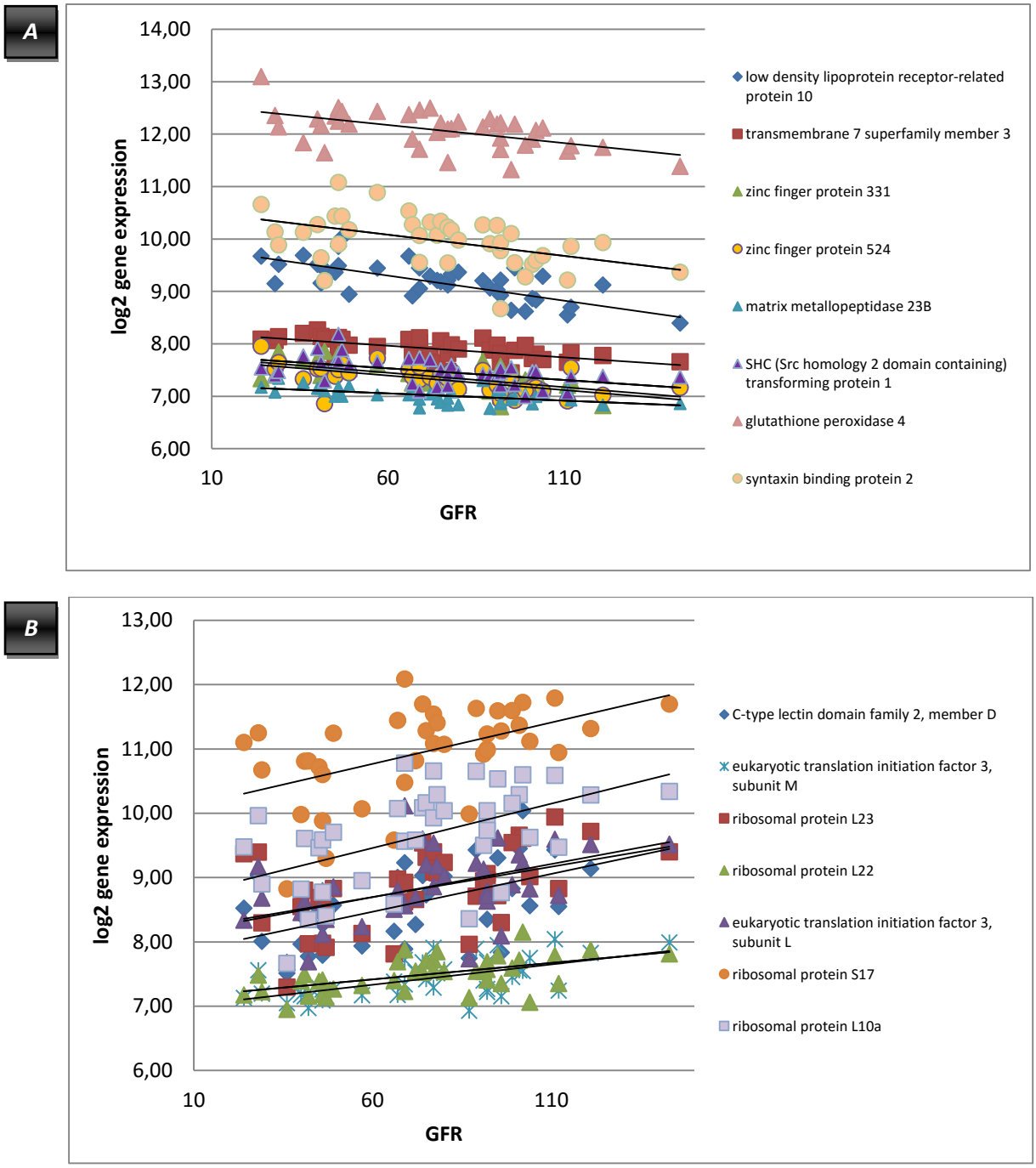



***Figure 26***. Many genes significantly correlated with the glomerular filtration rate (GFR): in panel A, gene expression levels that have an inverse correaltion with GFR; in panel B, gene expression levels that are positive correlated with GFR.

Enrichment analysis of Gene Ontology (GO) categories has allowed the identification of biological processes (BP), cellular components (CC) and molecular functions (MF) whose expression was significantly altered in platelets of ACS with CKD in comparison with platelets of ACS without CKD (Table 5).

| GO ont. | GO term | Number of genes | LS permutation p-value | KS permutation p-value | Efron-Tibshirani's GSA test p-value |
|---|---|---|---|---|---|
| BP | nuclear-transcribed mRNA catabolic process, nonsense-mediated decay | 99 | 0.00001 | 0.00001 | 0.017 (-) |
| BP | SRP-dependent cotranslational protein targeting to membrane | 92 | 0.00001 | 0.00001 | 0.017 (-) |
| BP | protein targeting to ER | 94 | 0.00001 | 0.00001 | 0.017 (-) |
| BP | translational elongation | 91 | 0.00001 | 0.00001 | 0.015 (-) |
| BP | translational termination | 82 | 0.00001 | 0.00001 | 0.018 (-) |
| BP | ribonucleoprotein complex assembly | 74 | 0.00001 | 0.00178 | 0.003 (-) |
| BP | ribonucleoprotein complex subunit organization | 77 | 0.00001 | 0.00531 | 0.004 (-) |
| BP | negative regulation of autophagy | 11 | 0.00012 | 0.00435 | < 0.001 (+) |
| BP | platelet aggregation | 40 | 0.00016 | 0.01574 | 0.009 (+) |
| BP | G2 DNA damage checkpoint | 14 | 0.00022 | 0.01363 | 0.001 (+) |
| BP | platelet degranulation | 56 | 0.0003 | 0.00049 | 0.022 (+) |
| BP | ribosomal small subunit biogenesis | 15 | 0.00046 | 0.0005 | 0.027 (-) |
| BP | pinocytosis | 9 | 0.00073 | 0.00364 | 0.031 (+) |
| BP | regulation of endocytosis | 62 | 0.00073 | 0.00025 | 0.088 (+) |
| BP | mitotic spindle organization | 14 | 0.0008 | 0.03588 | 0.005 (+) |
| BP | ameboidal cell migration | 88 | 0.00087 | 0.02858 | 0.001 (+) |
| BP | calcium-mediated signaling | 33 | 0.00095 | 0.0206 | 0.012 (+) |
| BP | synaptic transmission, dopaminergic | 6 | 0.00152 | 0.00199 | 0.007 (+) |
| BP | homeostasis of number of cells | 92 | 0.00168 | 0.02418 | 0.051 (-) |
| BP | mRNA stabilization | 19 | 0.00174 | 0.03477 | 0.041 (+) |
| BP | cellular response to nerve growth factor stimulus | 8 | 0.00238 | 0.00054 | 0.002 (-) |
| BP | cellular response to starvation | 44 | 0.00241 | 0.02605 | 0.028 (+) |
| BP | phagocytosis | 88 | 0.00251 | 0.00651 | 0.172 (+) |
| BP | response to hydrogen peroxide | 50 | 0.00264 | 0.03034 | 0.079 (-) |
| BP | regulation of mitochondrial membrane potential | 27 | 0.00267 | 0.00594 | 0.007 (-) |
| BP | platelet morphogenesis | 13 | 0.00296 | 0.05584 | 0.074 (+) |
| BP | modulation by symbiont of host cellular process | 5 | 0.00325 | 0.00039 | 0.027 (+) |
| BP | regulation of lamellipodium organization | 14 | 0.00356 | 0.29628 | 0.002 (+) |
| BP | transmembrane receptor protein serine/threonine kinase signaling pathway | 95 | 0.00376 | 0.02467 | 0.017 (+) |
| BP | regulation of membrane protein ectodomain proteolysis | 5 | 0.00456 | 0.01854 | 0.013 (+) |
| BP | mitotic recombination | 17 | 0.00473 | 0.01646 | 0.006 (-) |
| BP | serotonin transport | 8 | 0.00486 | 0.02122 | 0.008 (+) |
| BP | regulation of dopamine metabolic process | 6 | 0.00537 | 0.01314 | 0.004 (+) |
| BP | 'de novo' protein folding | 34 | 0.0061 | 0.00084 | 0.131 (+) |
| BP | protein homooligomerization | 76 | 0.00806 | 0.00353 | 0.053 (+) |
| BP | protein folding | 93 | 0.00844 | 0.00097 | 0.092 (+) |
| BP | cellular response to topologically incorrect protein | 44 | 0.00908 | 0.07441 | 0.003 (+) |
| BP | Rho protein signal transduction | 30 | 0.00969 | 0.00168 | 0.005 (+) |
| BP | rRNA metabolic process | 55 | 0.01254 | 0.00349 | 0.032 (-) |
| BP | regulation of metalloenzyme activity | 5 | 0.01613 | 0.00285 | 0.024 (-) |
| BP | 'de novo' posttranslational protein folding | 31 | 0.01732 | 0.00152 | 0.168 (+) |
| BP | regulation of DNA-templated transcription in response to stress | 22 | 0.01774 | 0.00113 | 0.163 (+) |
| BP | respiratory electron transport chain | 70 | 0.02651 | 0.00258 | 0.108 (-) |
| BP | positive regulation of cell division | 21 | 0.02679 | 0.09165 | 0.003 (+) |
| BP | regulation of transcription from RNA polymerase II promoter in response to stress | 20 | 0.03315 | 0.00403 | 0.176 (+) |
| BP | neutral lipid metabolic process | 39 | 0.05738 | 0.00281 | 0.029 (+) |
| BP | acylglycerol metabolic process | 38 | 0.06976 | 0.00426 | 0.037 (+) |
| BP | regulation of protein stability | 64 | 0.07554 | 0.0033 | 0.047 (+) |
| BP | negative regulation of RNA splicing | 13 | 0.17594 | 0.15046 | < 0.001 (-) |
| BP | DNA-dependent DNA replication | 44 | 0.20998 | 0.47116 | < 0.001 (-) |

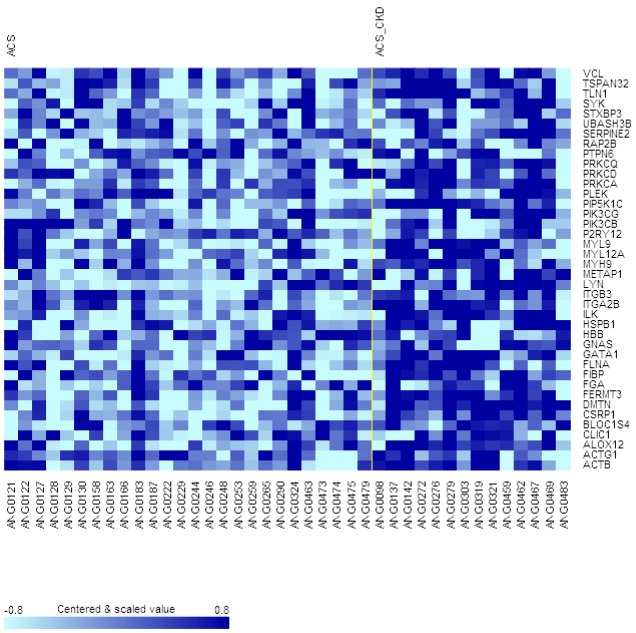
| | | | | | |
|---|---|---|---|---|---|
| CC | small ribosomal subunit | 46 | 0.00001 | 0.00001 | 0.008 (-) |
| CC | large ribosomal subunit | 56 | 0.00001 | 0.00001 | 0.02 (-) |
| CC | cytosolic ribosome | 82 | 0.00001 | 0.00001 | 0.018 (-) |
| CC | cortical cytoskeleton | 36 | 0.00018 | 0.00554 | 0.005 (+) |
| CC | actin filament bundle | 33 | 0.00021 | 0.03187 | 0.017 (+) |
| CC | actomyosin | 37 | 0.00027 | 0.02331 | 0.018 (+) |
| CC | small nuclear ribonucleoprotein complex | 20 | 0.00067 | 0.02257 | 0.021 (-) |
| CC | nuclear replisome | 9 | 0.0009 | 0.00672 | < 0.001 (-) |
| CC | sarcoplasm | 21 | 0.00098 | 0.03499 | 0.004 (+) |
| CC | sarcoplasmic reticulum | 21 | 0.00098 | 0.03499 | 0.004 (+) |
| CC | translation preinitiation complex | 12 | 0.00104 | 0.00025 | 0.016 (-) |
| CC | polysome | 20 | 0.00124 | 0.00118 | 0.022 (-) |
| CC | lamellipodium | 63 | 0.00126 | 0.0564 | 0.084 (+) |
| CC | replication fork | 21 | 0.00148 | 0.00252 | < 0.001 (-) |
| CC | platelet dense tubular network | 7 | 0.00267 | 0.14285 | 0.016 (+) |
| CC | eukaryotic translation initiation factor 3 complex | 13 | 0.00276 | 0.00826 | 0.019 (-) |
| CC | cell trailing edge | 7 | 0.00497 | 0.06893 | 0.059 (+) |
| CC | uropod | 7 | 0.00497 | 0.06893 | 0.059 (+) |
| CC | rough endoplasmic reticulum membrane | 11 | 0.01946 | 0.00479 | 0.026 (+) |
| CC | MHC protein complex | 15 | 0.0757 | 0.00443 | 0.271 (-) |

| | | | | | |
|---|---|---|---|---|---|
| MF | mRNA binding | 54 | 0.00029 | 0.03093 | 0.013 (-) |
| MF | rRNA binding | 24 | 0.00091 | 0.00111 | 0.011 (-) |
| MF | immunoglobulin binding | 10 | 0.00116 | 0.00777 | 0.006 (+) |
| MF | ubiquitin binding | 35 | 0.00151 | 0.00454 | 0.057 (+) |
| MF | small conjugating protein binding | 38 | 0.00185 | 0.00245 | 0.037 (+) |
| MF | ion channel inhibitor activity | 10 | 0.00199 | 0.0184 | 0.001 (+) |
| MF | translation factor activity, nucleic acid binding | 52 | 0.00231 | 0.00147 | 0.021 (-) |
| MF | small GTPase regulator activity | 70 | 0.00492 | 0.01221 | 0.088 (+) |
| MF | pre-mRNA binding | 10 | 0.04631 | 0.0013 | 0.002 (-) |
| MF | hydrolase activity, acting on ether bonds | 5 | 0.0624 | 0.00444 | 0.057 (+) |
| MF | peroxiredoxin activity | 6 | 0.0645 | 0.00424 | 0.143 (-) |
| MF | cargo receptor activity | 11 | 0.52337 | 0.00392 | 0.479 (+) |

***Table 5.*** Gene Ontology (GO) analysis of biological processes (BP), cellular components (CC) and molecular functions (MF) significantly different among patients with and without CKD

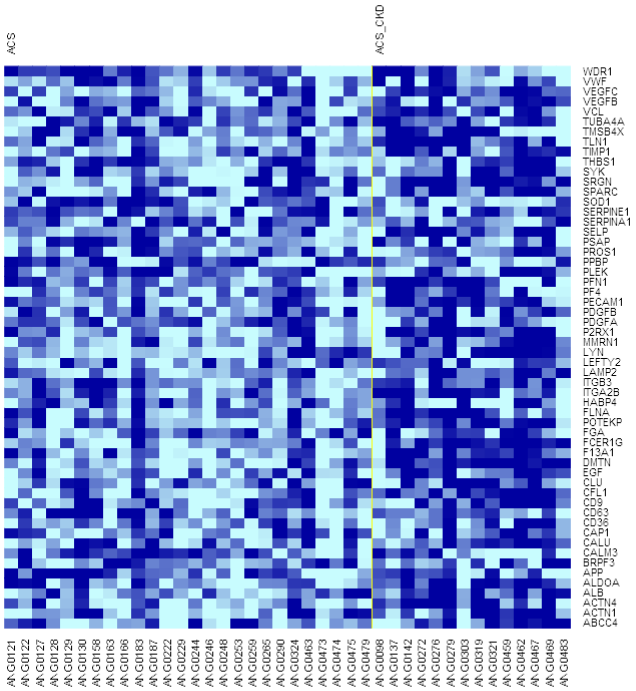
Gene set enrichment analysis of expressed genes has shown that many biological processes and/or molecular pathways are altered in CKD platelets. In particular, gene expression changes in a number of functions/processes were up-regulated in ACS patients with CKD: platelet aggregation (MYL9, FERMT3, ITGB3, STXBP3...), platelet degranulation (PDGFB, PDGFA, ITGA2B, LAMP2…), platelet morphogenesis (CLEC1B, CASP9, CASP3…), regulation of actin cytoskeleton (CAPZB, GSN…), regulation of transcription from RNA polymerase II promoter in response to stress (UBB, SIRT2, NOTCH1…) (Figure 27).

GeneSet:GO:0070527 ***PLATELET AGGREGATION***



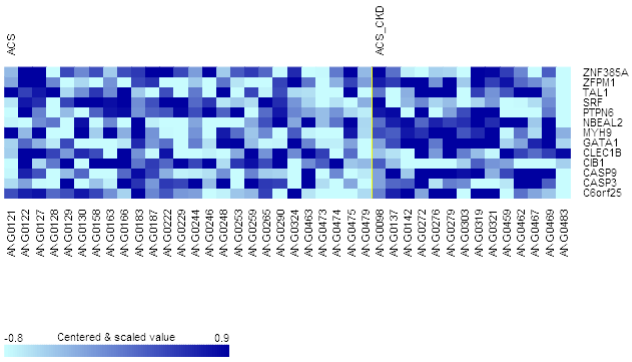

Data range before thresholding: -3.6 to 4.1.
Missing values are in color "gray".

GeneSet:GO:0002576 ***PLATELET DEGRANULATION***

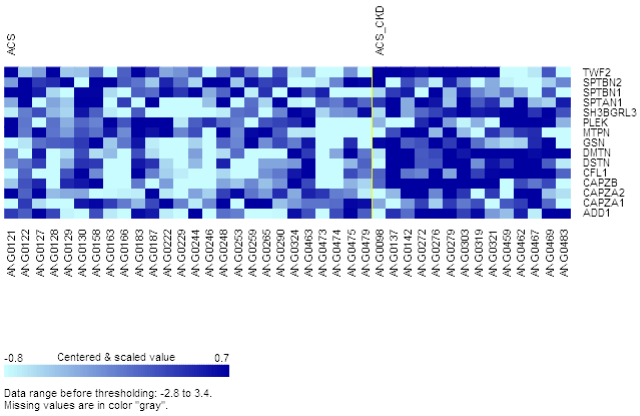



Data range before thresholding: -3.4 to 3.4.
Missing values are in color "gray".

GeneSet:GO:0036344 ***PLATELET MORPHOGENESIS***




GeneSet:GO:0030834 ***REGULATION OF ACTIN CYTOSKELETON***




GeneSet:GO:0043618 ***REGULATION OF TRANSCRIPTION IN RESPONSE TO STRESS***

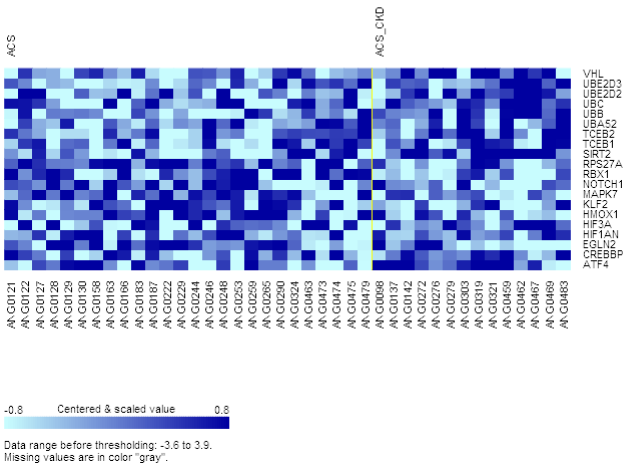
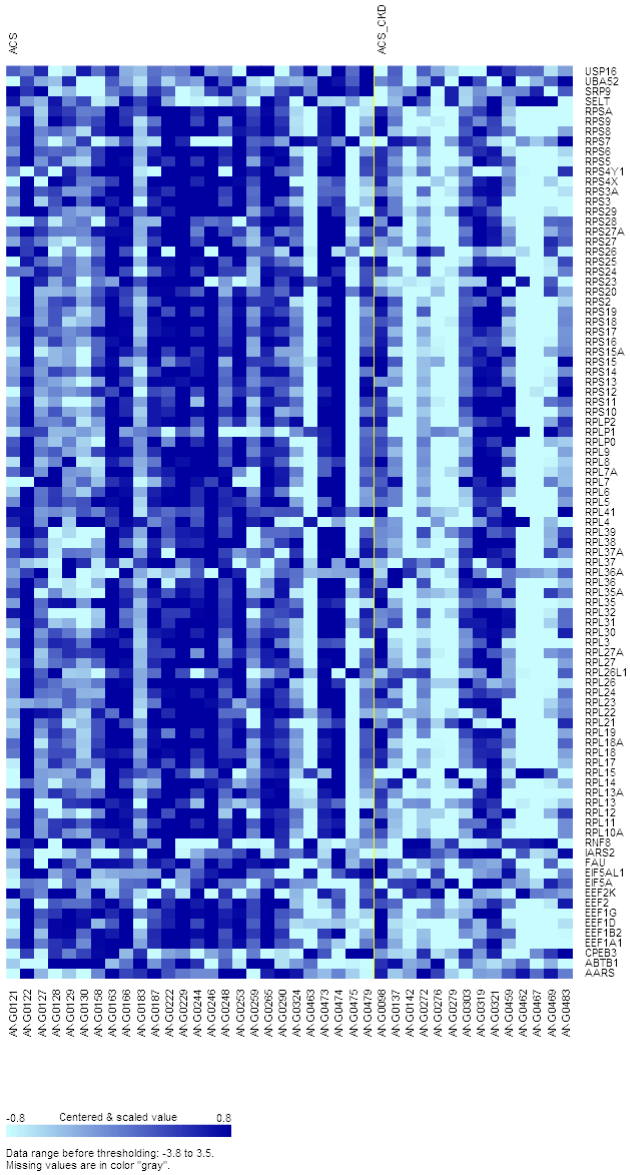


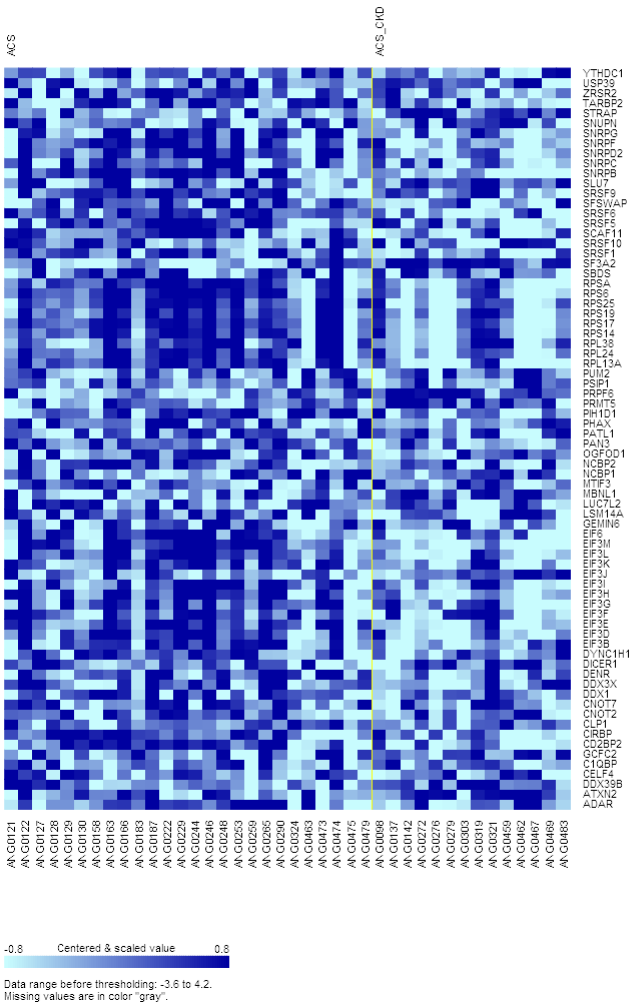

***Figure 27.*** Up-regulated gene expression in biological functions/processes of ACS patients with CKD

Conversely, some GO categories were down-regulated in platelets of ACS patients with CKD, such as translational elongation (EEF1D, EEF1A1, RPS17, RPL10A, RPL33, RPL9…), ribonucleoprotein complex assembly and subunit organization (RPL38, RPS19, EIF3M, EIF3L), negative regulation of RNA splicing (RPS26, RPS13, PTBP3…) (Figure 28).

GeneSet:GO:0006414 ***TRANSLATIONAL ELONGATION***



GeneSet:GO:0022618 ***RIBONUCLEOPROTEIN COMPLEX ASSEMBLY AND ORGANIZATION***




GeneSet:GO:0033119 ***NEGATIVE REGULATION OF RNA SPLICING***

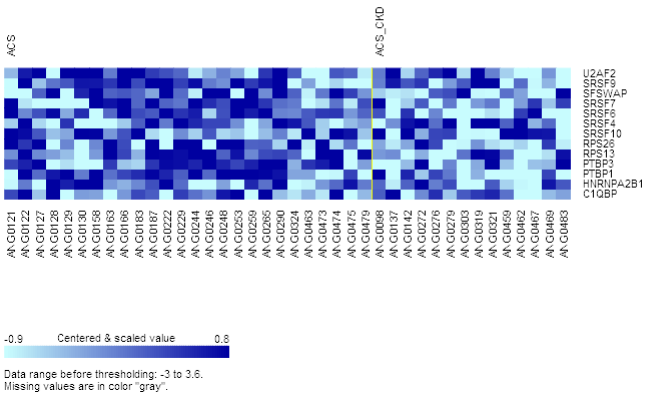
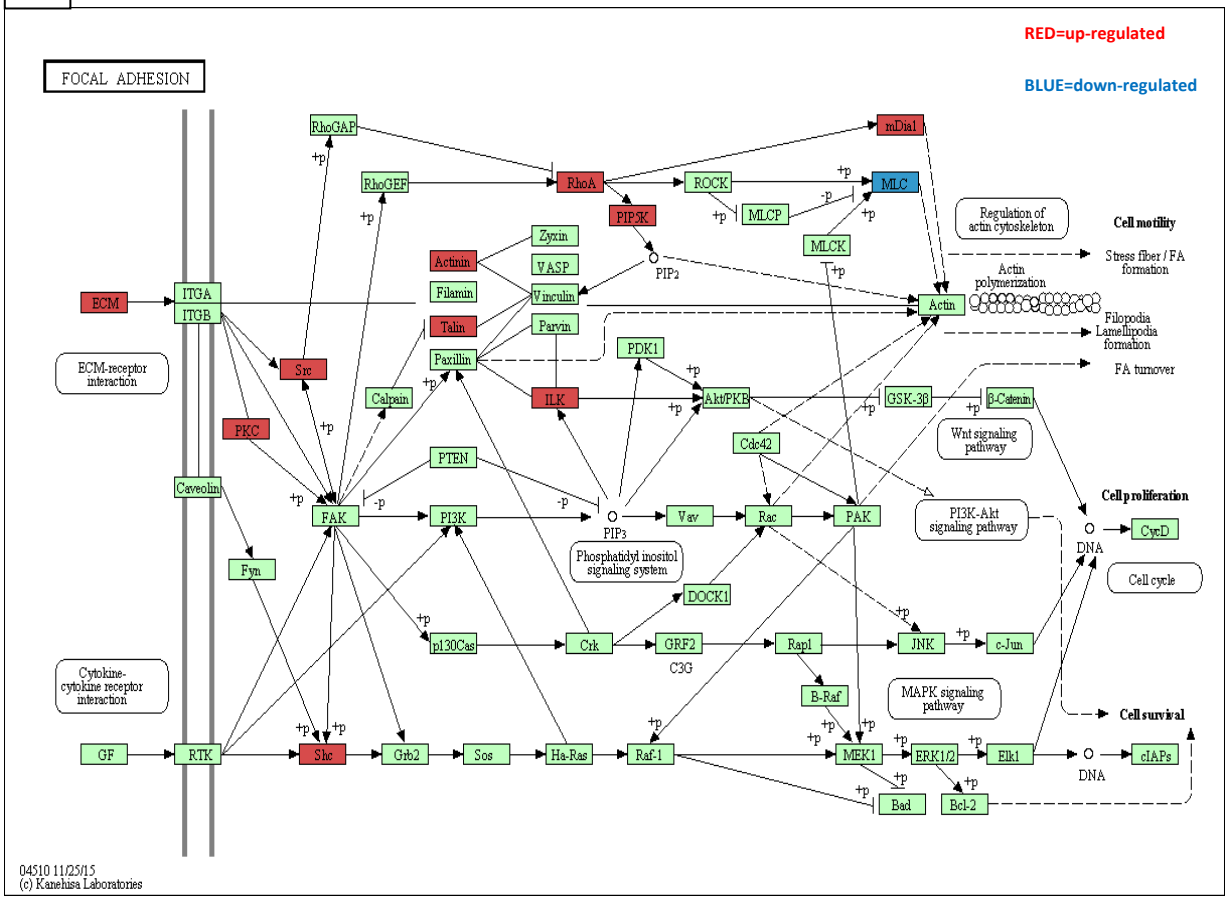



***Figure 28.*** Down-regulated gene expression in biological functions/processes of ACS patients with CKD

Gene expression enrichment analysis performed using the KEGG database, for understanding high-level functions and utilities of the biological system from molecular-level information, has confirmed that some processes (for example those involved in focal adhesion) have transcripts mostly up-regulated in CKD platelets, as well as others are down-regulated in CKD platelets (such as those involving ribosomes and their functions) (Figure 29).

**A**



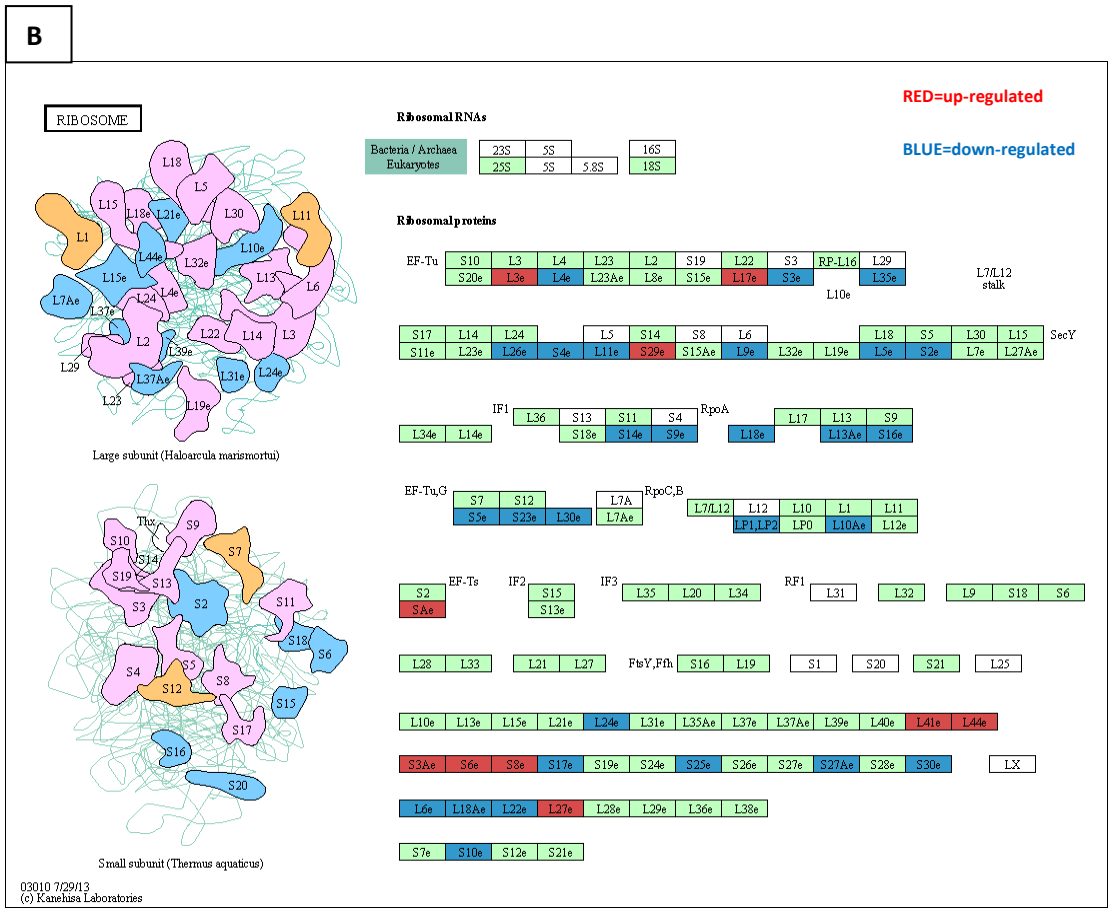



***Figure 29.*** Focal adhesion (panel A) pathway and ribosome (panel B)

Class prediction by Lasso Logistic Regression analysis and cross-validation has shown that the percent of patients correctly classified during cross-validation in a gene expression and covariates combined model is 88%: the analysis has allowed to identify 20 differentially expressed genes that cluster the group of patients with or without CKD with an error of 12%.

**PROTEOMIC DATA: CKD PATIENTS PRESENT A DIFFERENT PLASMA PROTEOME PATTERN COMPARED TO PATIENTS WITHOUT CKD**

In order to compare the proteomic profile of plasma of ACS and SA patients with and without chronic kidney disease, a proteomic approach, based on two dimensional electrophoresis for protein separation and mass spectrometry for their identification, has been employed on a selected group of patients, 5 well-matched patients for each group. Then, immunoenzymatic assays have been performed to confirm the results obtained by two dimensional electrophoresis on a larger cohort of patients.
The proteomic study has revealed that patients with CKD present a different plasma proteome pattern compared to patients without CKD (both SA and ACS): in particular, in ACS patients the image analysis has shown that 8 protein spots, corresponding to different isoforms of 3 proteins (α1-microglobulin, Retinol binding protein 4 and Hemopexin), are significantly up-regulated in patients with CKD, and two proteins, Fetuin A and α1-antichimotrypsin, are down-regulated in CKD patients (Figure 30 and Table 6).

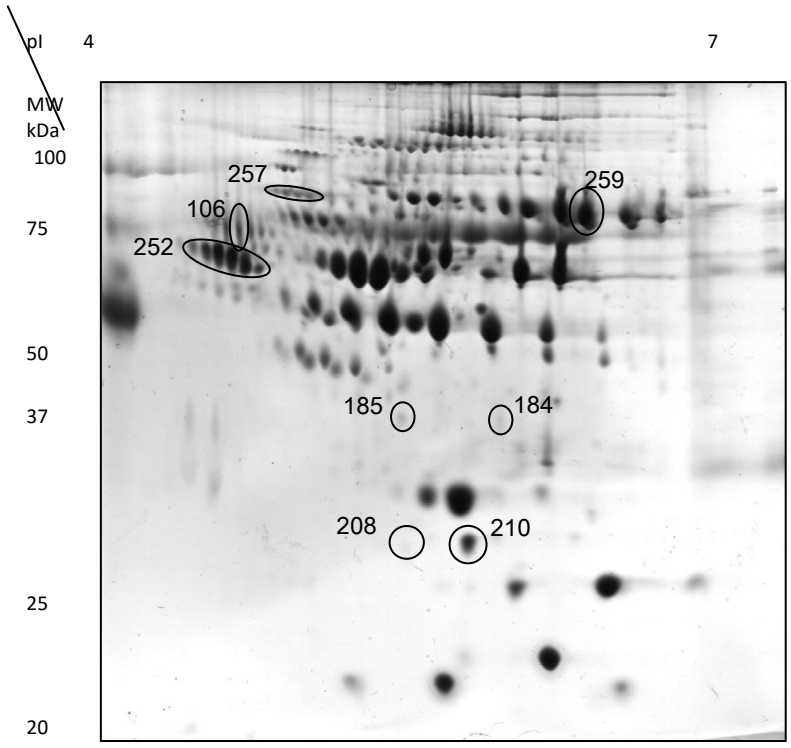



***Figure 30.*** Representative image of 2-DE of plasma proteins from ACS patients. Differentially expressed proteins in patients with or without CKD are circled.

| # | Accession | Description | Fold | Anova (p) | Highest Mean |
|---|---|---|---|---|---|
| 185 | P02760 | AMBP protein precursor | 1.6 | 0.0015 | CKD |
| 210 | P02753 | Retinol-binding protein 4 | 1.5 | 0.0005 | CKD |
| 208 | P02753 | Retinol-binding protein 4 | 2.1 | 0.005 | CKD |
| 259 | P02790 | Hemopexin | 1.3 | 0.0007 | CKD |
| 184 | | Unknown protein | 1.7 | 0.001 | CKD |
| 252 | P02765 | Fetuin A | 1.4 | 0.005 | No CKD |
| 106 | P01011 | Alpha 1 antichymotrypsin precursor | 1.3 | 0.012 | No CKD |
| 257 | | Unknown protein | 1.4 | 0.02 | No CKD |

***Table 6.*** Differentially expressed proteins in ACS patients with or without CKD

In SA patients the image analysis has revealed that 8 protein spots, corresponding to different isoforms of 4 proteins (Haptoglobin, Retinol binding protein 4, α1β-glycoprotein and Ceruloplasmin), are significantly up-regulated in patients with CKD (Figure 31 and Table 7).

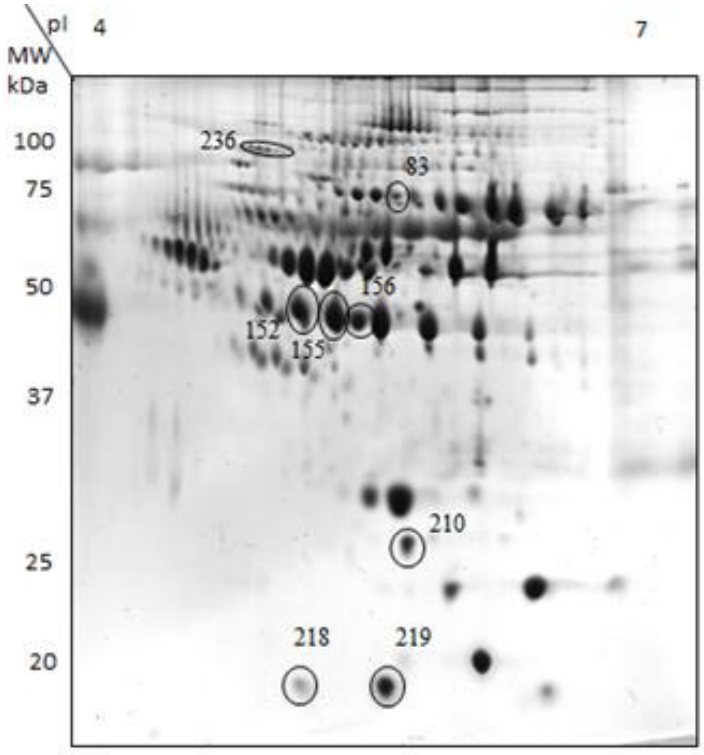



***Figure 31.*** Representative image of 2-DE of plasma proteins from SA patients. Differentially expressed proteins in patients with or without CKD are circled.

| # | Accession | Description | Fold | Anova (p) | Highest Mean |
|---|---|---|---|---|---|
| 83 | P04217 | Alpha 1B glycoprotein precursor | 1.2 | 0.005 | CKD |
| 152 | P00738 | Haptoglobin beta chain | 1.3 | 0.03 | CKD |
| 155 | P00738 | Haptoglobin beta chain | 1.2 | 0.016 | CKD |
| 156 | P00738 | Haptoglobin beta chain | 1.3 | 0.0004 | CKD |
| 210 | P02753 | Retinol-binding protein 4 | 1.3 | 0.004 | CKD |
| 218 | P00738 | Haptoglobin alpha chain | 3.4 | 0.015 | CKD |
| 219 | P00738 | Haptoglobin alpha chain | 4.2 | 0.005 | CKD |
| 236 | P00450 | Ceruloplasmin | 1.6 | 0.05 | CKD |

***Table 7.*** Differentially expressed proteins in SA patients with or without CKD

The immunoenzymatic assays, performed on a larger cohort of ACS patients (n=31 with CKD and n=108 without CKD) and SA patients (n=49 with CKD and n=168 without CKD), have confirmed the up-regulation in CKD patients of α1-Microglobulin (A1M), that is 2-fold higher in ACS CKD patients and 1.5-fold higher in SA CKD patients, Retinol Binding Protein 4 (RBP4), 1.5-fold higher both in ACS and SA CKD patients and Haptoglobin (HPT), 1.2-fold higher both in ACS and SA CKD patients (Figure 32).

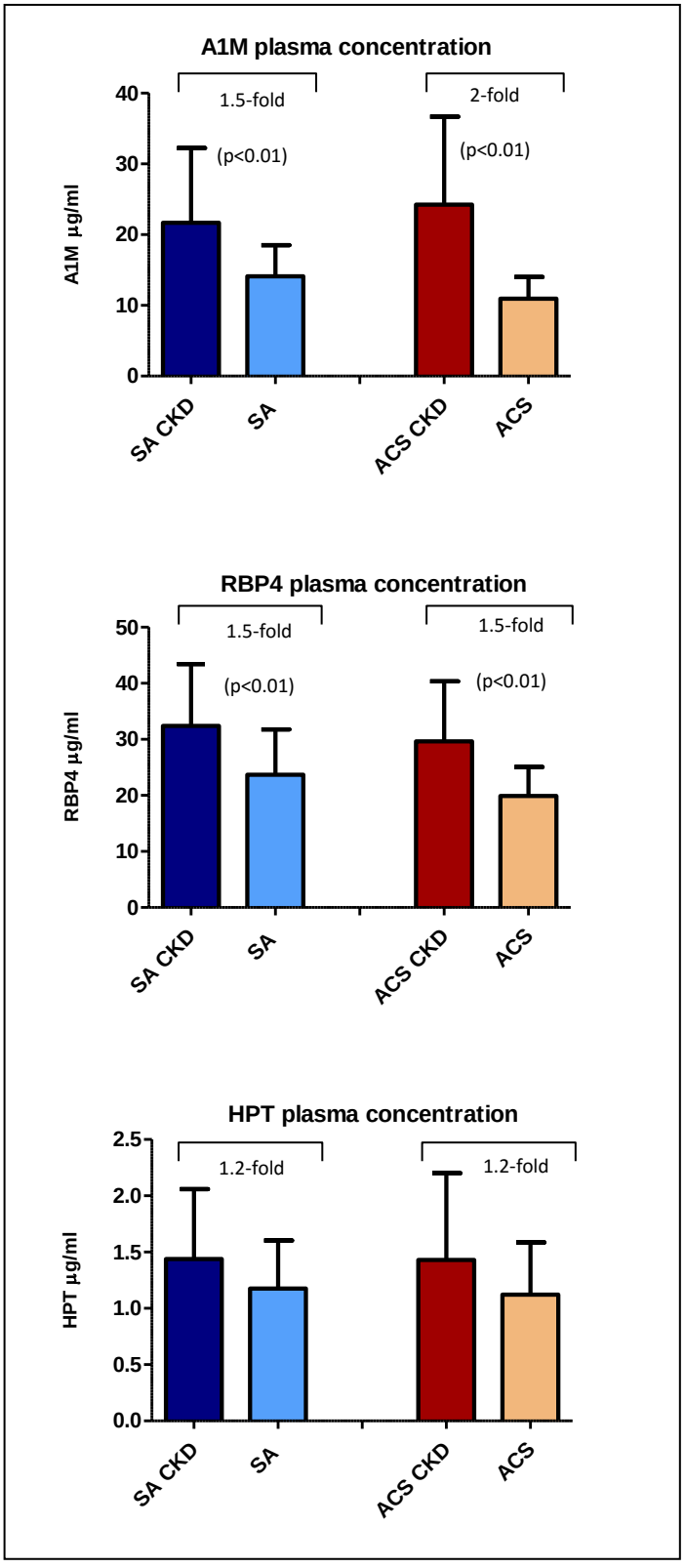
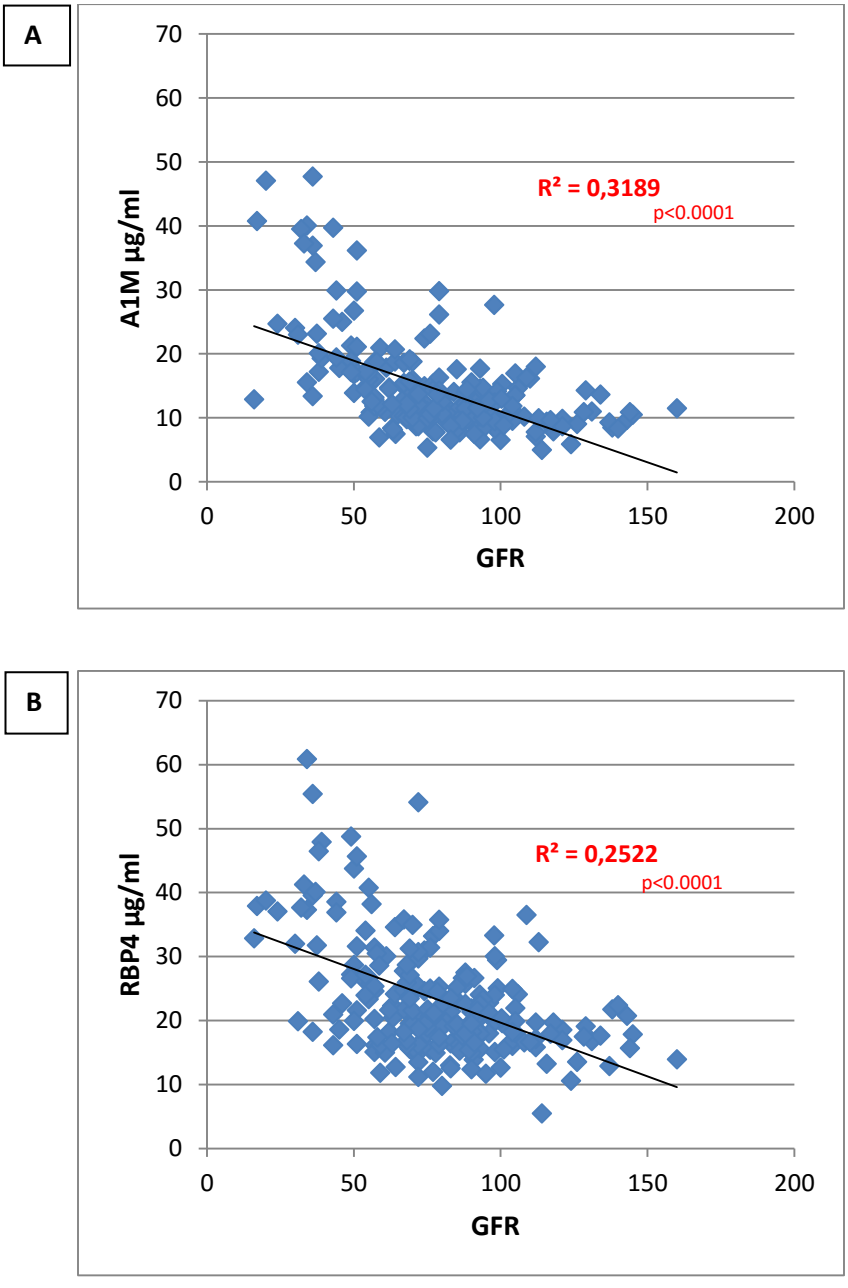



***Figure 32.*** Immunoenzymatic analysis: A1M, RPB4 and HPT plasma concentrations.

The plasma levels of all these proteins have shown a negative correlation with the glomerular filtration rate (GFR), being higher with the increase of kidney disease (Figure 33).



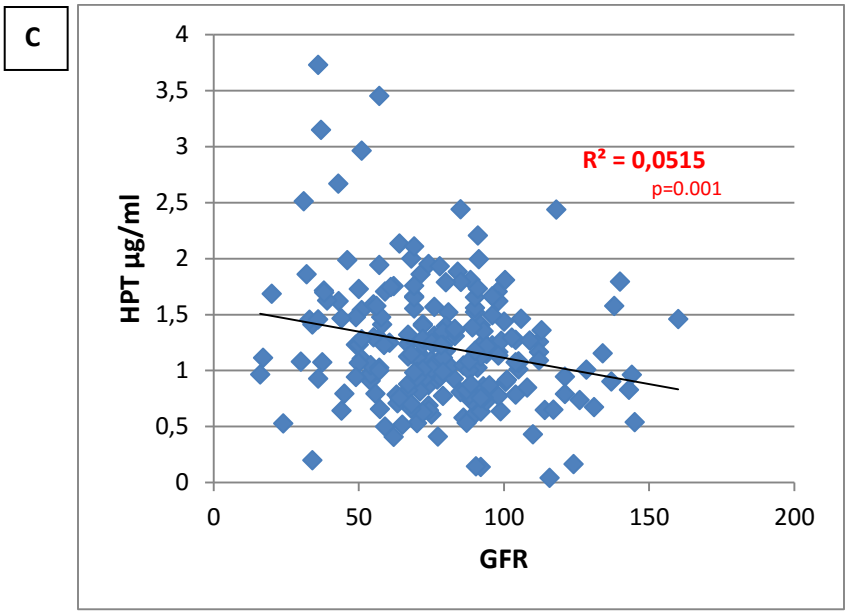



***Figure 33*** Correlation between A1M (panel A), RBP4 (panel B) and HPT (panel C) plasma levels and the Glomerular Filtration Rate (GFR).

Immunoenzymatic analysis has not confirmed the differences observed for Fetuin-A, which is present at similar levels in the plasma of patients with and without CKD (both in ACS and in SA patients) (Figure 34 and 35).

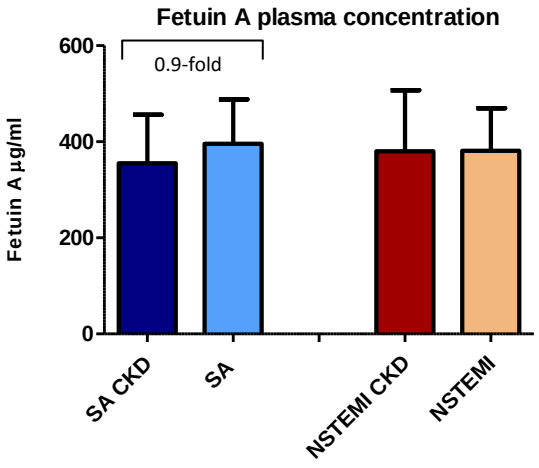



***Figure 34.*** **I**mmunoenzymatic analysis of Fetuin A plasma concentrations.

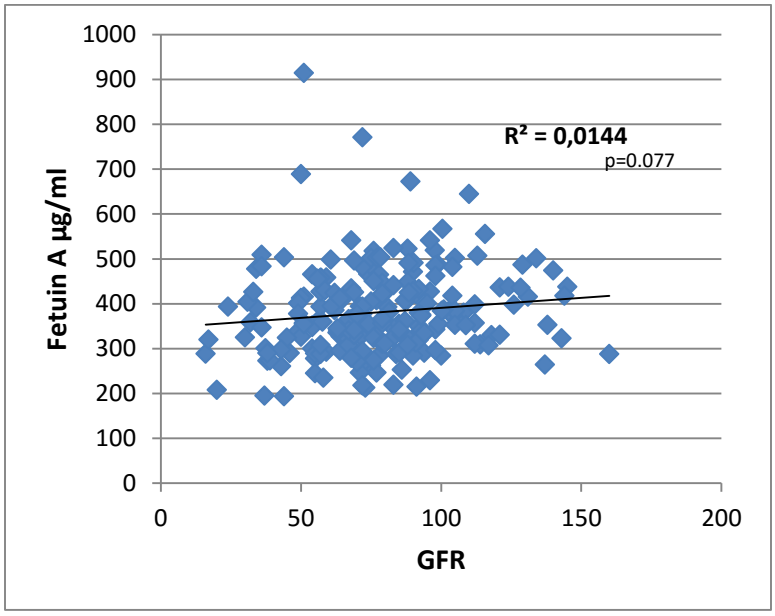



***Figure 35.*** Correlation between Fetuin A plasma levels and the Glomerular Filtration Rate (GFR).

# DISCUSSION

# DISCUSSION

Patients with chronic kidney disease (CKD) have a high burden of cardiovascular morbidity and mortality; indeed, in coronary artery disease (CAD), CKD represents a potent and independent risk factor for adverse outcome [62]. "Traditional" atherosclerotic risk factors such as diabetes, hypertension, dyslipidemia and older age, have been found to be independent predictors of CAD in CKD [15]. Moreover, CKD patients present also "non-traditional" cardiovascular risk factors such as inflammation, oxidative stress, vascular calcification and a uremic milieu, that may contribute to the risk and pathogenesis of CAD [288]. Patients with CKD may experience two opposite haemostatic complications: bleeding diathesis and thrombotic tendencies. The bleeding profile has been attributed classically to abnormalities of platelet function that include impaired adhesion [290] and decreased aggregation [290, 291] and to alteration in the balance of pro- and anti-thrombotic factors in blood [292, 293], while the thrombotic risk seems to be also due to an activated coagulation system of CKD patients [207, 208].

Furthermore, CKD may influence the response to antiplatelet therapy: patients with CKD have less therapeutic benefit of antiplatelet therapy [119, 243]. Recent findings suggest that renal function might affect the clinical efficacy of Clopidogrel. It has been hypothesized that patients with chronic kidney disease might have reduced Clopidogrel-induced platelet inhibition due to a specific impairment of the P2Y12 pathway [243]. Moreover, despite the use of 75 mg/die Aspirin, in some patients (~10%) thrombosis still occurs and this implies that, in these subjects, Aspirin is sub-effective [301]. This suboptimal response in patients undergoing percutaneous coronary intervention is associated with increased risk of thrombosis and increased major adverse clinical events (MACE) in long-term follow-up [224-226, 296].

Some years ago, our group described that ACS patients present a higher expression, compared to SA patients, of platelet-associated TF, a more recently described marker of platelet activation, that may account for the higher prothrombotic potential of ACS patient [204]. In order to further characterize the effect of CKD on the platelet activation profile, in this study we have focused our attention in particular on the expression of Tissue Factor (TF) and of the other classical markers of platelet activation. We have found

that the expression levels of the classical platelet activation markers, P-selectin and GP IIb/IIIa, were comparable between patients with and without CKD, both in stable angina (SA) and in ACS patients. On the contrary, we observed that CKD significantly affects platelet Tissue Factor expression; several approaches have been used to support this finding and the results can be summarized as follows: 1) in resting conditions the percentage of circulating platelets carrying TF on their surface is significantly lower in patients with CKD compared to patients without CKD; a similar feature was observed analyzing the percentage of TF-positive monocyte-platelet and granulocyte-platelet aggregates; 2) after ex vivo ADP stimulation, the percentage of TF-positive platelets is also overall lower in CKD patients compared to that of patients without CKD; 3) the total platelet content of TF, evaluated by performing intracellular staining, confirms that patients with CKD have significantly lower number of circulating TF positive platelets; 4) there is a highly significant inverse correlation between the number of TF positive platelets and the severity of renal failure.

The finding that CAD patients with CKD are characterized by a lower number of circulating TF-positive platelets and leukocyte-platelet aggregates provides further information on the platelet dysfunction state responsible for the haemostatic abnormalities typical of these patients and suggests potential new mechanisms contributing to the higher bleeding rather than the thrombotic risk of CKD patients.

In order to further characterize this peculiar phenotype found in CKD patients and their haemostatic tendencies, we took advantage from the use of thromboelastometry, a technique that provides clinically relevant information on the dynamics of clot development, stabilization and dissolution reflecting *in vivo* haemostasis. While the assay confirmed a higher prothrombotic potential in ACS compared to SA patients (in line with the platelet-associated TF expression), the presence of CKD exerted different effect on the global haemostatic potential. Indeed, CKD resulted in a shorter and slower clotting time in SA and ACS patients, respectively. Since the contribution of the coagulation cascade to the fibrin clot formation is similar between SA and ACS patients with CKD, we can speculate that the differences observed in the global

haemostatic capacity previously described might be related to a platelet function more “compromised” in ACS patients with CKD compared to SA patients.

The presence of TF in platelets has been proposed first in 2000 by Giesen et al. who postulated that thrombus formation and propagation, upon atherosclerotic plaque rupture, could take advantage from blood-born tissue factor: circulating platelets, carrying TF derived from microparticles, may themselves trigger the activation of the coagulation cascade [188]. Since then, several papers have documented the presence of TF in human platelets, suggesting that at least three mechanisms are involved in the presence of TF in platelets: 1) the microparticle-transfer mechanism; 2) the storage within the α-granules and the open canalicular system and 3) the *de novo* protein synthesis from the TF specific messenger RNA (mRNA). In 2003 our group provided the evidence that human CD34+-derived megakaryocytes express the TF mRNA [199]. Thus, although it is commonly believed that the only mechanism responsible for the presence of TF in platelets is through the uptake of TF-positive microparticles released by activated endothelial cells or leukocytes, it can be speculated that the TF mRNA and protein detectable in platelets could be the result of a direct transfer from megakaryocytes. Recently our group, using an *in vitro* cell culture model able to recapitulate megakaryocyte differentiation and platelet biogenesis, has provided consistent evidence that TF is an endogenously synthesized protein that characterizes megakaryocyte maturation. Since the cell system used allowed us to study mRNA and protein expression in the absence of any crosstalk with other cell or microparticle, we have also provided the evidence for the direct transfer of both TF mRNA and protein from megakaryocytes to a subset of platelets where it contributes to their thrombin generation capacity. Of interest, the percentage of TF-positive platelets that we have observed *in vitro* (both with Meg-platelets and with CD34+-derived platelets) was virtually identical to the amount found in blood from healthy individuals. This striking data suggests that a fine-tuned mechanism, which deserves further investigation in order to dissect the molecular pathways involved in its regulation, is responsible for the controlled delivery of TF from megakaryocytes to platelets. All together these data support the concept that, under physiological conditions, a percentage of TF expressing megakaryocytes are committed to

release in the bloodstream a constant number of platelets containing either TF protein or TF pre-mRNA [302]. Under pathological conditions, such as ACS, cancer, autoimmune diseases, alterations in the megakaryocyte transcriptome and proteome as well as in the release of new platelets may occur and the increase in the number of TF-positive platelets may be the result of a (A) mechanism taking place in the bloodstream and resulting in the splicing of the TF pre-mRNA and *de novo* protein synthesis or (B) a mechanism taking place in the bone marrow inducing more megakaryocytes to express TF and to release TF-positive [303]. We have recently reported that in spontaneously hypertensive stroke-prone rats the percentage of circulating TF-positive platelets directly correlated with blood pressure and is the results of an increased number of TF-positive megakaryocytes which release in the bloodstream a higher number of TF-positive platelets (M. Brambilla et al. Captopril downregulates circulating Tissue Factor expression in stroke-prone rats. *Manuscript in preparation*).

Therefore, based on these findings, it could be speculated that the lower number of circulating TF-positive platelets as well as the lower expression of TF-associated to platelets that we have observed in CKD patients, may be the result of: 1) a mechanism acting at the megakaryocyte level affecting both the number and the amount of TF per platelet; 2) a mechanism acting in the bloodstream, where uremic toxins might affect the platelet protein composition.

As previously mentioned, although platelets don't have a nucleus, they contain ~2000-7000 transcripts [143, 148, 304, 305]. Microarray analysis of the platelet transcriptome from healthy subjects identified mRNAs that encode for cell surface receptors and glycoproteins, as well as proteins involved in metabolism, signaling, inflammation, and immunity [143, 306, 307]. The finding that platelets can use their mRNA pool to perform new protein synthesis in response to cellular activation is of great importance, since these mechanisms allow them to modify their protein phenotype and, as a consequence, their functions [149]. It has been reported that specific mRNAs may vary in clinical conditions such as sickle cell disease [308], STEMI [265] and NSTEMI [205]. Identification of disease-associated platelet-specific transcripts is of particular relevance in platelet

pathophysiology, since it may lead to the discovery of novel therapeutic targets. No data are so far available on the platelet transcriptome profiling associated with CKD in CAD patients. The platelet gene expression profile, performed on a subgroup of ACS patients with and without CKD, identified 60 unique differentially expressed genes: 44 genes increased and 16 genes decreased in patients with chronic kidney disease. Functional annotation clustering and gene set enrichment analysis of the differentially expressed genes have shown that many biological processes and/or molecular pathways are altered in CKD platelets. In particular, we have observed the up-regulation of genes involved in platelet aggregation (such as MYL9, FERMT3, ITGB3, STXBP3, etc) and degranulation (such as PDGFB, PDGFA, ITGA2B, LAMP2, etc), regulation of actin cytoskeleton (such as CAPZB, GSN, etc), anti-oxidant genes (such as GPx4, ATOX1, etc), thrombosis-related genes (such as ZNF331, ZNF524, etc). Genes involved in translational elongation (such as EEF1D, EEF1A1, RPS17, RPL10A, RPL33, RPL9, etc), ribonucleoprotein complex assembly and subunit organization (RPL38, RPS19, EIF3M, EIF3L, etc) were among those down-regulated.

The findings that genes involved in platelet activation are up-regulated (~1.3 fold change) in CKD patients seems apparently in contradiction with the well-documented platelet function studies [210, 212, 309, 310]. It is worth mentioning, however, on this regard, that the down-regulation of genes involved in translational elongation and ribonucleoprotein complex assembly and subunit organization, that we have observed, is far higher (~1.8 fold change) compared to the up-regulated genes. This may affect the *de novo* protein synthesis capacity of platelets from CKD patients and globally account for the documented platelet dysfunction.

Furthermore, Spearman correlation analysis between the glomerular filtration rate (GFR) and the expression of platelet genes revealed that the expression of 98 unique genes significantly correlates with the decrease of GFR: the expression levels of 79 genes were negatively and 19 positively correlated. Among the negatively correlated genes, we found glutathione peroxidase 4 (GPx4) and antioxidant 1 copper chaperone (ATOX1), genes which play a prominent role in the defence against oxidative damage to cells [311]. It could be speculated that this might be a mechanism used by platelets to counteract oxidative stress conditions in CKD. In line with this concept is also the expression of ZNF331 and ZNF524, which are two

zinc finger proteins essential for megakaryocyte development, platelet release and, overall, haemostasis [312]. The increased expression of these genes observed together with the reduction of GFR might be again a mechanism acting at the bone marrow level in order to control and maintain haemostasis. Among the genes positively correlated with the GFR, it is worth mentioning C-type lectin domain family 2 (CLEC2) which is a platelet-activating receptor essential for stable aggregate formation [313]. Increased bleeding times and defective arterial thrombus formation in CLEC2–deficient mice has been previously described [313]. Although we have not investigated in the present study whether the lower levels of CLEC2 mRNA are paralleled by a lower amount of CLEC2 protein, this finding may pave the way for further investigation which may help to dissect the mechanisms responsible for the higher haemorrhagic risk of CKD patients.

Similarly to the transcriptome, also the plasma proteomic profile may change in clinical conditions: proteomics has the potential to reveal proteins associated with the etiopathogenesis of diseases and that could be used as diagnostic or prognostic biomarkers [297, 314-317]. In literature there is evidence that patients with end-stage renal disease (ESRD) present changes in the expression of plasma proteins associated to atherosclerosis [252, 298-300], but despite the increasing number of CVD patients with mild-to-moderate CKD, there are no studies focused on the plasma protein changes that may occur in these patients. The plasma proteomic analysis of the patients enrolled in our study (CVD patients with mild-to-moderate CKD) has shown a significant increase in the expression of α 1-Microglobulin (A1M), Retinol Binding Protein 4 (RBP4) and Haptoglobin (HPT) in patients with CKD (both SA and ACS patients).

α1-Microglobulin, member of the lipocalin family, is a tissue housekeeping protein with a central role in cleaning of oxidative waste products (free radicals and heme groups), macromolecular repair, and antioxidation protection; in literature its urinary increase is described in CKD patients and it is considered a diagnostic marker of renal failure [318-320].

The plasma increase of A1M levels in CKD patients enrolled in our study confirms and highlights the importance of this protein as early marker of pathology. It should be considered however that with the worsening of CKD, the protective effects of the protein are weakened by the increased urinary excretion.

In this proteomic study, we have also observed for the first time the plasma over-expression of Retinol binding protein 4 and Haptoglobin, two new CKD-related proteins.

Retinol binding protein4, belonging, as A1M, to the lipocalin family, is an adipokine involved in the early phases of the development of inflammation and insulin resistance [321]. Circulating RBP4 has been found to be associated with some measures of subclinical cardiovascular disease (CVD). Specifically, plasma RBP4 levels have been shown to be positively correlated with the echocardiographically measured left ventricular wall thickness and carotid intima-media thickness (IMT) [322, 323]. The presence of clinical arteriosclerosis is associated with higher circulating RBP4 [324] and it has been associated with any prior cerebrovascular disease and with any prior hospitalization for CVD [325]. Although a well-documented relationship exists between RBP4 and CVD, it remains to be elucidated whether RBP4 is causally involved in the development of CVD [325, 326]. The higher plasma levels of RBP4 in the enrolled CKD patients compared to those without CKD further underline the higher CVD risk.

Haptoglobin is an acute phase protein able to irreversibly bind to haemoglobin, thus preventing iron loss and renal damage. HPT-haemoglobin complexes are endocytosed by the cluster of differentiation receptor 163 (CD163) in mature tissue macrophages present in the spleen, liver, Kupfer cells of the liver and bone marrow [327]. Haptoglobin also acts as an anti-oxidant, has antibacterial activity and plays a role in modulating many aspects of the acute phase response [328, 329].

As mentioned before, these three proteins present a negative correlation with the value of glomerular filtration rate (GFR): the worst is the kidney function, the higher are plasma levels of these proteins. Overall, in CKD patients, there is the over-expression of protective proteins, but also of proteins that could promote the progression of CVD.

In conclusion, all these data shed new light on additional mechanisms involved in CKD-associated haemostatic (thrombotic and haemorrhagic) profile of CAD patients. The peculiar platelet phenotype (lower amount of TF-positive platelets and leukocyte-platelet aggregates), found in CKD patients, doesn't seem to account for the prothrombotic potential that could be ascribed to these patients, but rather it may provide further explanation for their bleeding risk which is related to platelet dysfunction.

Furthermore, the global haemostatic capacity of whole blood assessed in the presence of an inhibitor of platelet contribution to clot formation has revealed that CKD patients have a higher procoagulant potential which may in part account for their thrombotic tendencies.

CKD patients present also a differential gene expression profile compared to patients without CKD. Among the pathways affected by CKD, interestingly, we found a significant down-regulation of genes involved in RNA processing and protein synthesis thus potentially affecting the total RNA expression and overall the platelet function.

Finally this proteomic study has revealed that patients with chronic kidney disease have a different plasma proteome pattern both in ACS and SA patients: the plasma proteomic analysis has identified differentially expressed proteins involved in mechanisms responsible for the progress of the renal and cardiovascular disease (pro-inflammatory proteins), but also in protective mechanisms (anti-oxidant and anti-inflammatory proteins).

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