SCHOOL OF MEDICINE

Department of Health Sciences

MASTER DEGREE IN MEDICAL BIOTECHNOLOGIES

Final Relation

ROLE OF EXTRACELLULAR VESICLES AND UREMIC TOXINS ON VASCULAR AGING AND COGNITIVE DECLINE OF END-STAGE RENAL DISEASE PATIENTS

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ABSTRACT

Title: Role of extracellular vesicles and uremic toxins on vascular aging and cognitive decline of end-stage renal disease patients.

Objective: The project aims to explore pathophysiology, complications, and emerging biomarkers associated with neurovascular impairment in chronic kidney disease (CKD). It focuses particularly on the role of extracellular vesicles (EVs) and their complex interactions with uremic toxins in causing neurocognitive decline and cardiovascular alterations. The study also evaluated plasma concentrations of brain-derived neurotrophic factor (BDNF) in relation to cognitive impairment and assessed the protective effects of kidney transplantation on EVs and cognitive performance.

Background: Chronic kidney disease (CKD) is characterized by a progressive and irreversible decline in renal function, eventually leading to end-stage renal disease (ESRD). CKD is closely associated with significant morbidity and mortality, mainly due to its complications, particularly cardiovascular changes and neurocognitive impairments. Kidney transplantation represent the best therapeutic option for ESRD patients.

Methods: the research project was divided in different phases:

-Screening of hemodialysis population for neurocognitive impairments, cardiovascular alterations, and analysis of EVs and BDNF levels.

-In vitro investigation of the effects of EVs, Indoxyl sulphate and Ahr antagonist on Human Umbilical Vein Endothelial Cells (HUVEC) and astrocytes.

-Evaluation of early vascular aging (EVA) – induced phenotype in isolated artery vessels.

-Assessment of biomarkers and protective role of kidney transplantation in pre- and post-transplantation patients.

Results:

-Correlations between EVs, cognitive impairment, and cardiovascular markers in hemodialysis patients were proved. Specifically, endothelial-derived EVs appear to correlate with pulse wave velocity (PWV); in addition, platelet-derived EVs exhibited a correlation with the cognitive impairment test and carotid intima media thickness (CCA IMT). BDNF levels, diminished in CKD patients, were found to be correlated with cognitive performance.

-Effect of EVs in vascular injury was highlighted by observing elevated expression of markers associated with senescence and calcification, namely p21, p16, and RUNX2.

-The toxic effect of EVs in BBB functionality was proved. The exposure of cells to potential therapeutics, such as BDNF, significantly attenuated the harmful effects of EVs.

-Kidney transplantation was found to reduce EVs levels and improve BDNF levels, thus indicating a protective effect against CKD-induced complications.

Conclusion: EVs have been shown to have a pivotal role in the pathogenesis of CKD-related neurocognitive and cardiovascular complications. Decreased levels of BDNF, on the other hand, are predictive of cognitive impairment. Kidney transplantation appears to ameliorate these effects, heralding promising therapeutic prospects.

Keywords: Chronic Kidney Disease, Extracellular Vesicles, Neurocognitive Impairment, Cardiovascular Alterations, Early Vascular Aging, Blood-Brain Barrier, Kidney Transplantation, Brain-Derived Neurotrophic Factor.

INTRODUCTION

1.1 Characteristics of chronic kidney disease

1.1.1 Definition and classification

Chronic Kidney Disease (CKD) is a condition defined by the presence of kidney damage or an estimated glomerular filtration rate (eGFR) less than 60 ml/min/1.73 m^2, persisting for at least 3 months, regardless of the cause[1].It is a progressive and irreversible clinical entity characterized by loss of renal function leading to the development, in the final stages, of uremic syndrome or end-stage kidney disease (ESKD). Renal damage can manifest as abnormalities identified on imaging or renal biopsy, isolated urinary abnormalities, or an increase in proteinuria. It represents a gradually advancing and irreversible clinical condition marked by the decline in renal functions both exocrine and endocrine, culminating in the onset of uremic syndrome or end-stage kidney disease (ESKD) in its advanced stages. Renal impairment can be identified through imaging or renal biopsy findings, isolated urinary irregularities, or a rise in proteinuria.
The classification of CKD is based on the KDIGO 2012 guidelines, which, according to the eGFR, define 6 stages (Figure 1): G1: > GFR 90 ml/min/1.73 m2, G2: GFR 60-89 ml/min/1.73 m2, G3a: GFR 45-59 ml/min/1.73 m2, G3b: GFR 30-44 ml/min/1.73 m2, G4: GFR 15-29 ml/min/1.73 m2, G5: GFR < 15 ml/min/1.73 m2 or dialysis[2].

GFR categories in CKD

GFR category	GFR (ml/min per 1.73 m^2)	Terms
G1	⩾90	Normal or high
G2	60–89	Mildly decreased*
G3a	45–59	Mildly to moderately decreased
G3b	30–44	Moderately to severely decreased
G4	15–29	Severely decreased
G5	<15	Kidney failure

Abbreviations: CKD, chronic kidney disease; GFR, glomerular filtration rate.
*Relative to young adult level.
In the absence of evidence of kidney damage, neither GFR category G1 nor G2 fulfill the criteria for CKD.

Figure 1 Classification of chronic kidney disease (CKD) based on the KDIGO 2012 guidelines.

The Causes of CKD vary and mostly are[3]:

- Diabetes Mellitus: is the most common cause of CKD in most developed and developing countries.
- Glomerulonephritis.

- Genetic diseases: Adult polycystic kidney disease (APKD) stands as the most prevalent monogenetic disorder leading to chronic kidney disease (CKD).
- Drugs: Several drugs such as penicillins, non-steroidal anti-inflammatory drugs (NSAIDs), proton pump inhibitors, diuretics and anti-retrovirals can cause CKD, usually by provoking interstitial nephritis.
- Urological condition: urinary tract obstruction is a potentially reversible cause of AKI/CKD commonly caused by renal calculi, prostatic hypertrophy, or pelvic malignancies.
- Infections: HIV, hepatitis B and C, tuberculosis and malaria are significant causes of chronic kidney disease.

1.1.2 Epidemiology

Chronic kidney disease (CKD) has appeared as one of the most remarkable causes of death in the 21st century. Due to the increment of risk factors, such as obesity and diabetes mellitus, the prevalence of patients affected by CKD has surged[4].

On a world scale the total number of individuals with chronic kidney disease (CKD), acute kidney injury (AKI), and those on renal replacement therapy (RRT) exceeds 850 million (table 1). Thus, kidney diseases are one of the most common diseases worldwide[5].

The global dimension of kidney disease in 2017	
CKD stages 1-5	843.6 million
Individuals with AKI	13.3 million
Individuals on RRT	3.9 million
Total	860.8 million
AKI, acute kidney injury; CKD chronic kidney disease; RRT, renal replacement therapy	

Table 1: Individuals with kidney disease in 2017.

A more recent study performed a comprehensive systematic review and meta-analysis of 100 studies comprising 6,908,440 patients, and reported a global prevalence of 13.4% for CKD stages 1–5 and 10.6% for CKD stages 3–5.8 The prevalence of the individual CKD stages was 3.5% (stage 1), 3.9% (stage 2), 7.6% (stage 3), 0.4% (stage 4), and 0.1% (stage 5).

1.1.3 Pathophysiology

As a progressive disease, chronic kidney disease has two kinds of risk factors: non-modifiable and modifiable risk factors that, as previously mentioned, are mostly hypertension, diabetes mellitus and other metabolic features. The non-modifiable risk factor are the age, male gender and non-whit ethnicity which includes African Americans, Afro-Caribbean individuals, Hispanics, and Asians (South Asians and Pacific Asians) [6].

Differently from acute kidney injury (AKI), CKD is an irreversible process and contributes to progressive kidney fibrosis that involves the destruction of the normal structure of the kidney. Renal fibrosis is characterized by glomerulosclerosis, tubulointerstitial fibrosis, loss of renal parenchyma, and inflammatory cell infiltration[7].

Transforming growth factor beta (TGF-β) has been identified as the key mediator for the fibrosis in CKD, it can be both produced by resident kidney cells and infiltrating leukocytes. It stimulates the renal cells to change their phenotype and become matrix-producing fibrogenic cells. As a downstream mediator of TGF-β activity, connective tissue growth factor (CTGF) also contributes to renal fibrosis and tubuloepithelial transidifferentation[8].

In renal endothelial cells there is a vasoconstrictor peptide which exerts profibrotic and pro-inflammatory effects and it is Endothelin-1. The overexpression of this substances induces structural and functional changes because it causes hypoxic injury due to the constriction of peritubular capillaries. The main hallmark of deteriorating renal function is inflammation, so there are some proinflammatory cytokines that can induce fibrosis and these are tumor necrosis factor alpha (TNF-α) and interleukin-1 (IL-1) (Figure 2).

Renal senescence plays a crucial role in the pathogenesis and progression of CKD: compared with the general population, the renal ageing and senescence process is greatly accelerated and advanced in this disease. Important senescent biomarkers such as p16 and p21 were found expressed in CKD animal models.

Moreover, chronic stimulation of various stressors in CKD leads to the continuous and excessive induction of chronic senescent cells and release of senescence associated secretory phenotype (SASP), which contributes to their accumulation and persistence and so renal fibrosis and abnormal renal repair.

Various pathways are involved in the promotion of senescence in CKD, they include factor-erythroid 2-related factor 2 (NRF2), Wnt/β-catenin, NF-κB, and mTOR[9] (Figure 3).

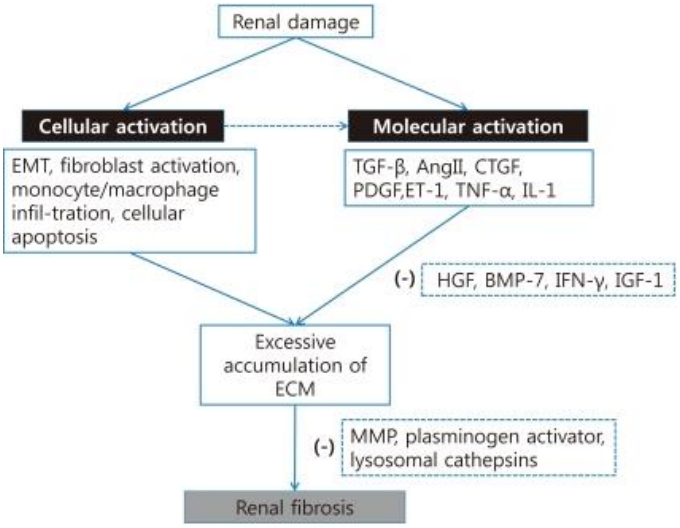


Figure 2: Pathogenetic mechanism underlying renal fibrosis.

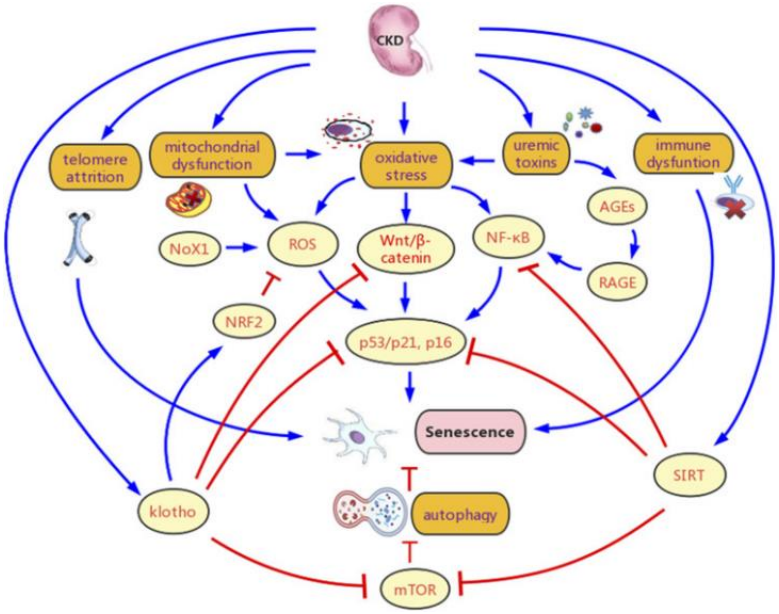


Figure 3: Pathways involved in the promotion of senescence in CKD.

1.2 Uremic toxins

Due to the injuring of the kidney, the nitrogenous substances that would normally be excreted in the urine accumulate in the blood. At increasing concentrations, these substances exert toxic effects and are responsible for the uremic syndrome.
These retention compounds can be defined as uremic toxins and can cause damage in the organism but particularly in the cardiovascular, central nervous and muscular system. There are some criteria that can define a solute as a true uremic toxin[10]:

- The compound must be identified and quantified through analysis in biological fluids.
- The compound's level must be higher in uremic than in nonuremic subjects.
- The lower the concentration of the compound, the lower are the specific uremic dysfunction and/or symptoms related to it.
- Biological activity should be proven in vivo, ex vivo, or in vitro studies.

In 2003, EUTox defined a classification based on the physicochemical properties of uremic toxins that influence their clearance during conventional hemodialysis[11]:

- Small Hydrophilic Toxins: these include compounds that have a molecular weight lower than 500 Dalton, such as urea (60 Da) and uric acid. Conventional hemodialysis removes them using diffusion as the primary transport mechanism.
- Medium-Sized Toxins: are all the compounds that have a molecular weight higher or equal to 500 Da such as, for example, β2 microglobulin (11.8 kDa) and parathyroid hormone (9.5 kDa). Convective transport dialysis exploits movement of molecules through a semipermeable membrane associated with the fluid being removed during ultrafiltration. (solute molecule is swept through a membrane by a moving stream of ultrafiltrate), can remove some of these toxins but their molecular weight interferes with their elimination.
- Protein-Bound Toxins (PBUTs): this category encloses molecules with low molecular weight, such as indoxyl sulfate which displays, both with p-cresyl sulfate, more than 80% plasma protein binding. Regardless of their low molecular weight, clearance is affected due to the lower concentration of unbound toxin at the dialysate side surface (Dialysate solution or dialyzing fluid is a nonsterile aqueous electrolyte solution that is similar to the normal levels of electrolytes) side surface of the membrane.

Protein-Bound Toxins (PBUTs) are a distinct class of toxins characterized by a strong affinity to plasma proteins, particularly albumin. This feature of them makes complex their elimination with traditional dialysis techniques.

Their genesis occurs in the intestine, where dietary proteins undergo metabolism by the intestinal microbiota, producing precursors that later become toxins. Their molecular weight is typically lower than 500 Da; however, their protein binding confers them a larger molecular size.

These toxins have an impactful role in chronic kidney disease (CKD) and can lead to a range of systemic effects. Several studies have underlined the role of PBUTs in initiating renal fibrosis, vascular calcification, and immune system deficiency.

Among these toxins, Indoxyl Sulfate has shown some concerning effects for the kidney.

Its production starts from the indole that is a metabolic product of tryptophan decomposition by bacterial tryptophanase. Once produced, Indole enters the liver where is hydroxylated by cytochrome P450 2E1 (CYP2E1) and afterwards sulfated to form indoxyl sulfate.

This toxin has shown nephrotoxic effects through generations of reactive oxygen species (ROS), depletion of anti-oxidative systems and induction of fibrosis and inflammation.

In human proximal tubule cells (HK-2) the activation of NF-κB by IS suppressed cellular proliferation, induces, and accelerates senescence through activation of p53, and promotes fibrosis by inducing TGF-β1 and PAI-1 expression[12].

Furthermore, in IS was shown to induce a depletion of the expression of Klotho, an anti-aging gene with renoprotective properties by hypermethylation of its gene (Figure 3).

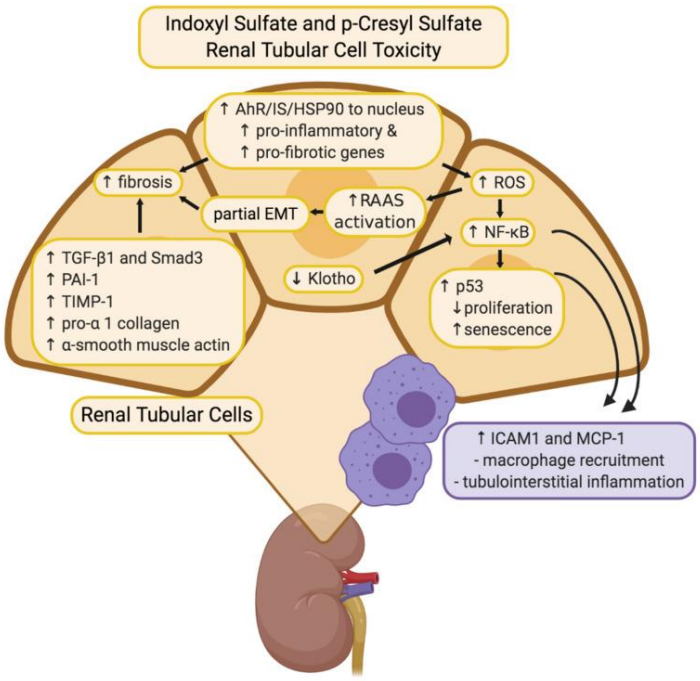


Figure 4: Role of Indoxyl Sulphate on Renal Tubular Cells

1.3 Extracellular Vesicles (EVs)

For tissues to function properly, communication between cells is required. The secretion of soluble factors like growth factors, cytokines, or chemokines; the release of mediators such as nucleotides or bioactive lipids; intercellular adhesion contact, which occur when specific adhesion molecules connect and set off a series of intracellular signals; and the material exchange via the formation of nanotubes or small circulating vehicles called extracellular vesicles are some examples of intercellular communication[13].

Extracellular vesicles (EVs) were seen to have a big impact because they work as transporters for long distances, they carry biomolecules that, if free, they would be easily degraded. They can be classified in two types:

- exosomes: they are extracellular cystic vesicles with a diameter of about 30-100 nm. Their origine initiates when early endosomes evolve into late endosomes leading to the formation of the multivesicular bodies (MVB). MVBs can fuse with lysosomes or with the plasma membrane, releasing their content as exosomes[14].
- microvesicles: they are particles with bigger dimensions (100-1000 nm), generated by a process of shedding from the plasmacellular membrane, that is a process that depends on the intracellular flux of calcium that modifies the distribution of membrane phospholipids and the reorganization of the cytoskeleton. This subsequently brings a redistribution of the membrane components (Figure 5).

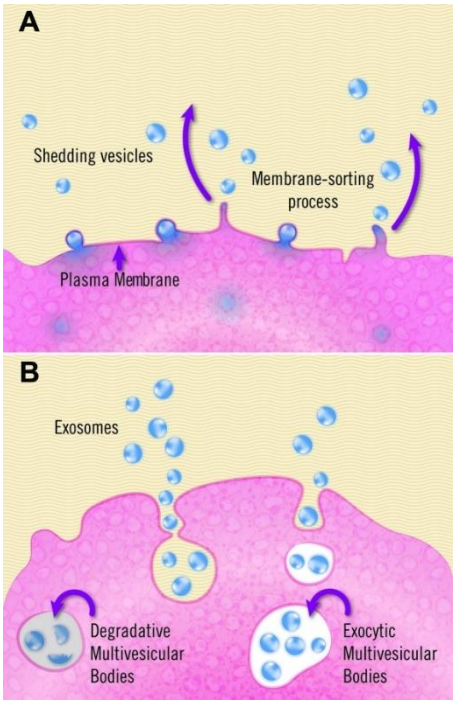


Figure 5: Production and release of shedding vesicles and exosome. (A) Schematic representation of production and release from the cell surface of the shedding vesicles. (B) Schematic representation of exosome release.

The release of EVs is physiological phenomena that is associated with cellular activation and growth. It has been demonstrated that EVs are secreted in greater quantities by quickly expanding cell lines than by slower-growing cell lines. In general, the number of EVs released by cells increases in cases of cellular activation, hypoxia, irradiation, oxidative damage, exposure to complement cascade proteins, and exposure to tangential forces[15]. EVs are composed of proteins and lipids that are similar to those found on the cell membranes from which they originate. In addition, during budding, they have the ability to incorporate a portion of the cellular cytoplasm and therefore they may contain cytoplasmic proteins, mRNA[16], infectious particles (such as HIV or prions) or entire organelles like mitochondria[17]. Indeed, EVs have been found to contain proteins, lipids, mRNA, microRNA, and non-coding RNA sequences[18].

EVs are released by several cell types, and their composition varies depending on the cell of origin (e.g., EVs released by platelets are called microparticles, while EVs released by activated PMNs are defined as ectosomes). They can remain near the cell from which they originated or reach distant sites via body fluids, indeed EVs have been identified in plasma, urine, milk and cerebrospinal fluid.

As previously mentioned, the content of these extracellular vesicles is variable and complex. An analysis aimed at identifying their composition found a total of 1,354 proteins of cytoplasmic origin, often associated with lysosomes and endosomal organelles, and 1,992 mRNAs[19].

Circulating EVs can stimulate distant cells either directly or through the transfer of material. They interact only with specific target cells through receptor interactions that can trigger intracellular signaling cascades or the formation of multimolecular complexes[20]. Another type of interaction involves the direct internalization of the material contained in the EVs through endocytosis. At this point, the EVs can fuse with the endosomes of the target cell, leading to a horizontal transfer of their content into the cell cytosol, or they can remain within the endosomes and be transferred to lysosomes, or even be released again by the cells trough the mechanism of transcytosis[21].

Extracellular vesicles can modify cellular functions of the target cells trough:

-mechanism of direct stimulation: for example, EVs released by platelets in the circulatory system have a fundamental role in the coagulation process because they have a membrane rich in phosphatidylserine that constitutes a good surface for the assemblage of the coagulation factors.

-transfer of membrane receptors: some membrane antigens were seen to be transferred from B activated lymphocytes to B non-activated lymphocytes, this process leads to a more

rapid amplification of the signal and a more effective antigen presentation to the T lymphocytes CD4+[22].

-release of proteins: it has been demonstrated that monocytes stimulated by endotoxins are capable of releasing EVs containing caspase-1, which, when released at the level of smooth muscle cells, induces their apoptosis[23].

-horizontal transfer of genetic material determining epigenetic modifications: EVs from EPCs are capable of transferring their RNA content to endothelial cells, thereby activating their neoangiogenic activity[24].

1.3.1 EVs as RNA and microRNA transporters

MicroRNAs are small non-coding RNA sequences around 22 nucleotides that have been identified in various species (animals, plants, viruses). Most microRNAs are transcribed by RNA polymerase II, forming complexes that are cleaved to produce pre-microRNAs. These are then transferred to the cytoplasm, where they are further cleaved to produce mature microRNAs.

They have an important role in the genic regulation, working during the process of translation of mRNA in proteins. They can induce the formation of complexes of RNA that are able to cleave mature mRNA and inhibit their translation[25].

It is known that a single microRNA can inhibit the translation of hundreds of mRNA.

Most microRNAs are located intracellularly; however, their presence has also been detected in the extracellular space, in various body fluids (plasma, saliva, urine, milk, tears), even in healthy individuals[26]. Changes in the concentration and composition of extracellular microRNAs have been linked to various physiological and pathological conditions, suggesting their potential as valuable disease biomarkers. It is now established that microRNAs play a significant role in the progression of several diseases, particularly cancers, where they can function as both oncogenes and tumor suppressor genes[27].

These circulating microRNAs are remarkably stable, even in the presence of extracellular RNAase enzymes. This stability likely results from their ability to protect themselves by associating with other elements, primarily by forming protein-microRNA complexes or being encapsulated within microvesicles[28]. Numerous studies have shown that exosomes, microvesicles, apoptotic bodies, and protein/RNA complexes released from various cell types can transfer proteins, RNA, and microRNAs to both nearby and distant cells. This transfer has

the potential to influence angiogenesis, cell proliferation, cell death, tumor progression, and other forms of intercellular communication[29].

1.3.2 Pathogenic role of EVs in cardiovascular alterations

Studies have shown that the plasma concentration of EVs released from the endothelium is higher in patients with cardiovascular diseases compared to healthy individuals[30]. The release of EVs is triggered by various pro-inflammatory elements, including cytokines, activated platelets, and oxidized LDL. Furthermore, EVs appear to play a role in modulating inflammation by acting on different targets, functioning as both pro-inflammatory and anti-inflammatory agents[31].

EVs also play a role in thrombus formation, displaying multiple platelet activation mediators on their surface, such as a tissue factor (TF). They increase TF concentration within plaques, triggering the coagulation cascade. Additionally, EVs release pro-inflammatory cytokines (IL-1, IL-6, IL-8, TGF β), which boost the expression of adhesion molecules[32]. This endothelial activation leads to the adhesion and diapedesis of leukocytes and neutrophils, a critical step in the development of atherosclerotic plaques[33].
Also, EVs are involved in the inhibition of the release of NO synthetase leading to an endothelial dysfunction[33]. In patients with cardiac diseases, after the stimulation of acetylcholine it was seen that there was an increase in the endothelial circulating EVs and this phenomenon is correlated with a greater arterial wall stiffness and a decreased capacity of vasodilation. This happens in response to an increase in blood flow together with a decreased capacity of the endothelium to respond to an acetylcholine stimulus[31].
In the same way, in chronic kidney disease, high levels of endothelial EVs are correlated with the development of atherosclerosis and with an increase in the cardiovascular disease incidence. This seems to be a consequence of the inflammatory environment caused by uremia (uremic toxins, hyperphosphatemia, hypercalcemia, high levels of FGF23, decrease in levels of KLOTHO) [32].

High levels of phosphate, together with hypercalcemia and presence of cytokines such as TNFalphas stimulate the secretion of EVs that release miRNA (miRNA133b, -155, -204, -211, -223,) and this favors the accumulation of calcium[32](Figure 5).

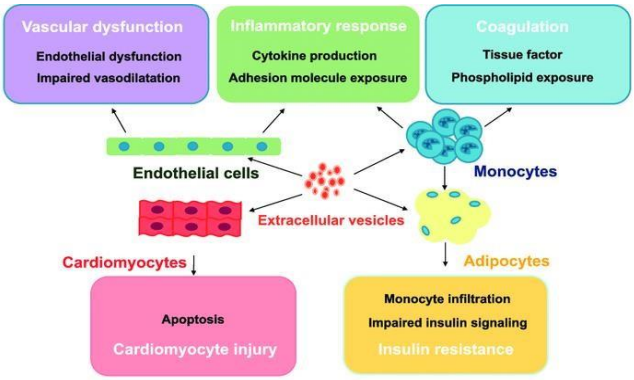


Figure 5: Mode of action of EVs.

1.4 Complications of chronic kidney disease (CKD)

Generally, the renal deterioration proceeds in the first phases without any symptoms. However, as chronic damage advances, characteristic clinical changes emerge, including metabolic acidosis, anemia, hypertension, mineral and bone disorders, and electrolyte imbalances. The progression towards chronic kidney disease is marked by several signs and symptoms known as uremia. Among the numerous complications associated with chronic kidney disease, cardiovascular and neurocognitive alterations play a crucial role in influencing patients' quality of life and survival.

1.4.1 Cardiovascular alterations

Cardiovascular disease (CVD) is a major cause of death in patients with chronic kidney disease. Accumulation of uremic toxins, chronic inflammation, and oxidative stress have been identified to act as CKD-specific alterations that increase cardiovascular risk[34].
Vascular calcification happens because of an impaired renal function and is the main cause of atherosclerosis. Dysregulation in mineral metabolism and the elevated levels of circulating calcium (Ca) and phosphate (Pi) can cause a phenotypic change in the smooth muscle cells (VSMCs) that are sensitive to these factors. VSMCs are the major resident cells in the media layer of the blood vessels and can undergo trans-differentiation to osteoblast-like cells and extrude matrix that contains proteins similar to osteoblastic vesicles[35].

Vascular smooth muscle cells (VSMCs) to osteogenic trans-differentiation (VOT) in a high-phosphate environment is involved in arterial in CKD. WNT/β-catenin signaling is indicated to play a crucial role in osteogenesis via promoting Runx2 expression in osteoprogenitor cells[36]. Previous research have shown that oxidative stressors and cellular senescence can not only reduce Klotho expression (known as an aging suppressor gene) but can also increase the phosphorylation of RUNX2 and actively recruit more calcium and phosphate intracellularly.

Chronic renal failure is associated with an early process of vascular aging EVA, which describes a premature aging of the vessels that occurs with structural and functional changes in the vascular wall. The early vascular ageing has some typical features such as increase arterial stiffness, impaired endothelial function, arterial calcification, and oxidative stress[37].

As renal function declines, the normal regulatory mechanisms for maintaining the balance in phosphate (Pi) and calcium (Ca) levels, such as parathyroid hormone (PTH), fibroblast growth factor 23 (FGF-23), and klotho, become overload. This disruption affects the endocrine system, including the renin-angiotensin-aldosterone system (RAAS). With the loss of nephrons and increased FGF-23 levels there is a reduction in the production of 1,25-dihydroxyvitamin D (calcitriol) and subsequently an activation of RAAS that leads to vasoconstriction.

Furthermore, uremic toxins, such as Indoxyl Sulphate (IS) induce smooth muscle cells (VMSC) to transform into osteoblast-like cells. Consequently, macrophages increase apoptosis and accumulation of apoptotic bodies, transitioning into foams cells to create a pro-inflammatory milieu.

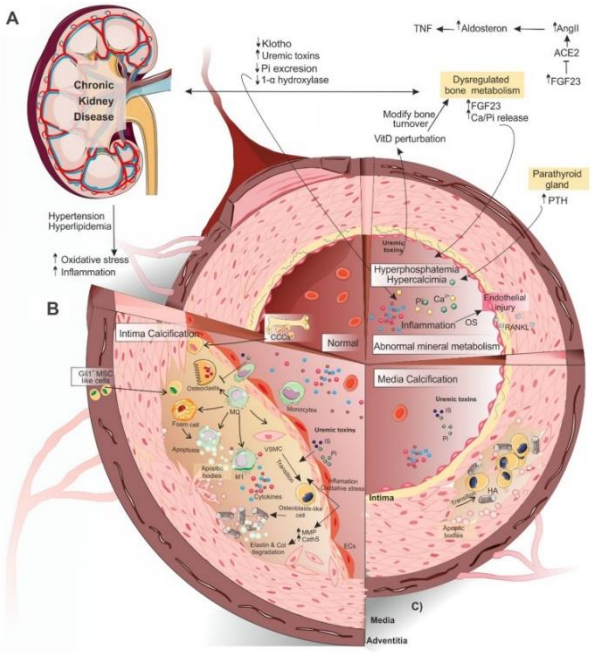


Figure 6: Dysregulated metabolism in chronic kidney disease.

1.4.2 Neurocognitive alterations

The uremic patient may exhibit a range of central and peripheral nervous system alterations, ranging from mild disturbances to a state of dementia.
Compared to the general population, patients with chronic kidney disease (CKD) are significantly more likely to experience cognitive impairment. Both poorer cognitive function and cognitive impairment are linked to lower glomerular filtration rate and the presence of albuminuria[38].
In hemodialysis patients, depression represent the primary psychiatric disorder with a prevalence ranging from 20% to 40%, having a significant impact on the quality of life of patients and being associate with increase hospitalization and higher mortality[39].
Other manifestations include sleep disorders; indeed, several studies have demonstrated a correlation between chronic sleep deprivation and the development of diseases, including hypertension, an important risk factor for the development of CKD[40] and the restless leg syndrome (RLS) that is associate with periodic leg movements that interfere with sleep and lead to frequent awakenings.

1.5 The blood brain barrier

The blood brain barrier (BBB) plays a critical role in controlling the flux of biological substances, so its integrity is fundamental for a correct functioning of the brain metabolic activity[41] (Figure 7). All the mechanisms previously described can also affect it, compromising its integrity with a consequent onset of neurological and cognitive manifestations.
It is composed of endothelial cells (ECs), pericytes (PCs), capillary basement membrane, and astrocytes end-feet, all of which aim to protect the brain from toxic substances[42].
The uremic milieu typical of the patient affected by chronic kidney disease is potentially damaging the BBB, through the disruption of the proteins that constitute the tight junction (TJ), with a consequent alteration of the permeability of the barrier.
Claudin, Occludin and JAM-1 are some “cytoplasmatic adaptors” particularly involved in the BBB damage. Claudins represents a class of more than 25 members of tetraspanins characterized by a W-GLW-C-C domain in the first extracellular loop. Evidence in vitro suggests that claudin are essential for the paracellular barrier formation[43].
Occludin is a tetraspanin expressed by epithelial cells and central nervous system epithelial cells and it was seen that it is important for the resistance of the barrier and it specifically regulates calcium flux across the BBB[43].

JAM-1 is part of the immunoglobulin superfamily, it does some homotypic bound between endothelial cells of the SNC and the epithelial cells. These molecules are fundamental for regulating the passage of leukocytes in the central nervous system[43].

In recent years, there has been growing interest in understanding the mechanisms behind EV transport across the BBB. This interest is driven not only by the need to understand the pathophysiology of neurological disorders but also by the potential to use EVs as disease biomarkers and as carriers for brain-targeting drugs.

The exact mechanism by which uremia-derived EVs penetrate the BBB is still not clear.

In a study investigating exosomes derived from both malignant and non-malignant cell types, researchers observed their ability to cross the BBB[44]. The authors proposed two potential outcomes for exosomes interacting with the BBB: they may either fully traverse the endothelial cell barrier to enter the brain, or they may become sequestered within the brain endothelial cells. This notion was further supported by a study conducted by Jakubec's group, which demonstrated that exosomes derived from human plasma exhibited BBB permeability comparable to that of liposomes[45]. However, unlike liposomes, the exosomes were found to accumulate within endothelial cells[46]. This sequestration could potentially enable exosomes to influence endothelial functions, while complete passage might facilitate their effects on the brain. Recent studies focused on EVs as carriers of biomarkers, facilitating transfer of molecular signals from brain to peripheral circulation. Chronic inflammation within the CNS activates astrocytes, inducing the release of EVs containing molecular cargo, which may hold diagnostic potential for CNS injury. Furthermore, research by Morad's team has demonstrated that tumor-derived EVs can break the intact BBB via transcytosis, highlighting the potential for EV-mediated transport of bioactive molecules across the barrier. This mechanism provides a pathway through which systemic factors associated with CKD can affect the BBB. EV-mediated signaling may exacerbate the progression of neurological complications commonly seen in CKD patients, contributing to increased neuroinflammation and compromised brain health.

Additionally, studies in mice have shown that EVs from older mice induce glial cell activation, evidenced by altered expression of glial fibrillary acidic protein, a brain-specific marker[47].

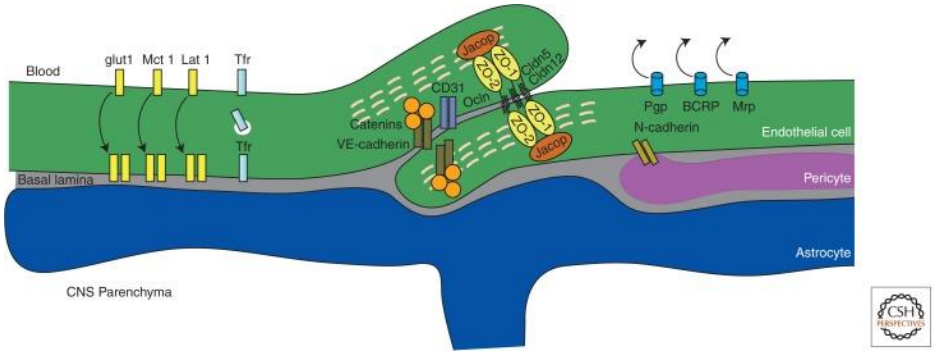


Figure 7: Representation of the blood brain barrier.

1.6 Brain-kidney crosstalk in CKD

The cerebral and renal vasculature, which are both marked by high blood flow and low resistance, are anatomically similar. Both organs have arterioles that are capable of autoregulating tissue perfusion, and both are susceptible to traditional risk factors for atherosclerosis such as advanced age, hypertension, diabetes, dyslipidemia, and smoking[48]. It was seen that CKD itself is a causal risk factor for cerebrovascular disease beyond the traditional risk factors[49]. It is considered that the causes of neurocognitive disorders in CKD patients are multifactorial and, in particular, there are two main hypotheses: the vascular and the neurodegenerative one (Figure 8).

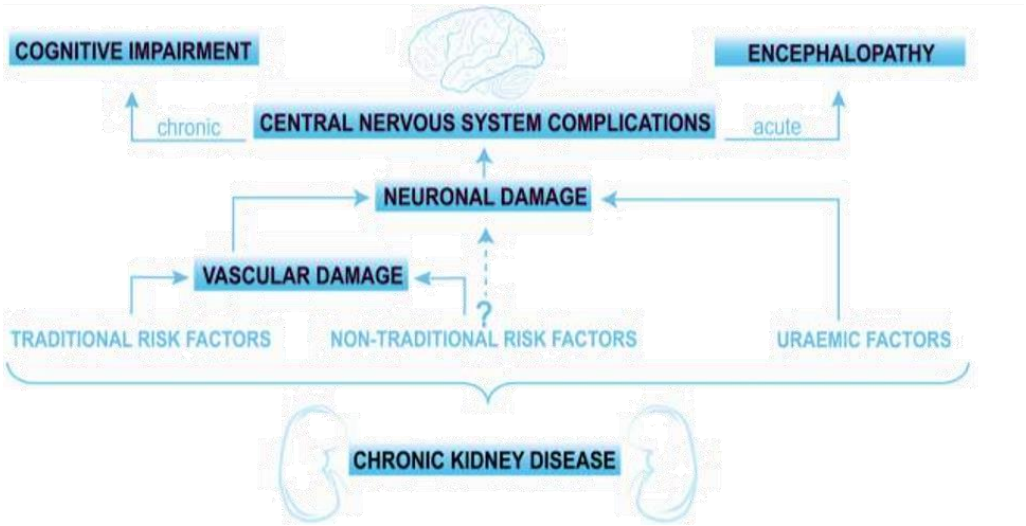


Figure 8: Diagram of traditional and non-traditional risk factors involved in neurocognitive impairment in patients with CKD.

The first hypothesis assumes that the vascular damage is influenced by both traditional risk factors such as hypertension, diabetes, increased age and smoking status and non-traditional risk factors common in chronic kidney disease such as metabolic disorders, related to calcium, phosphate, inflammation and oxidative stress. This leads to a process of accelerated atherosclerosis and endothelial dysfunction. Even if the vascular damage is fundamental for the pathophysiology of the cognitive deficit in CKD patients, it partially explains the high presence of these disorders.

The neurodegenerative hypothesis explains how neurocognitive alterations are influenced, either directly or indirectly, by uremic toxins, which contribute to the persistence of chronic inflammation, endothelial dysfunction, and atherosclerosis[50]. Several toxins can be directly

connected to neurotoxic damage such as Indoxyl sulphate, p-cresol, TNF – α and some interlukines[51]. As previously stated, the uremic environment negatively impacts the blood-brain barrier (BBB), leading to changes in serum levels of some specific brain markers such as brain-derived neurotrophic factor (BDNF) [52].

Consequently, altered BBB permeability caused by tight junction (TJ) destruction allows uremic toxins to penetrate and accumulate in the brain, resulting in damage to brain structures, neurons and glia. This structural damage to neuronal cells would therefore lead to development of the neurocognitive alterations described so far.

1.7 Brain-Derived Neurotrophic Factor (BDNF)

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophins family found ubiquitously in the brain and is expressed by glutaminergic neurons and glial cells such as astrocytes isolated from the cortex and hippocampus[53]. During embryogenesis, BDNF promotes the differentiation of cortical progenitor cells and subsequently their differentiation into neurons; moreover, it can influence synaptic connectivity and neuronal plasticity[54].
BDNF expression is regulated by numerous genetic and epigenetic mechanisms, as well as a wide range of exogenous stimuli (such as stress, physical activity, brain injuries, diet). It exerts its action by binding to two different receptors thereby activating various signaling cascades that induce an increase in CA2+ influx, phosphorylation of transcription factors, and de novo expression of the BDNF gene[55].
Altered levels of this neurotrophic factor were seen in several cerebral pathologies, therefore it is a possible biomarker for a wide range of neuropsychiatric disorders (Figure 9).
Some study have analyzed how in chronic kidney disease patients there is an inverse correlation between plasmatic levels of BDNF and depression levels at the Beck Depression Inventory (BDI test). [56]

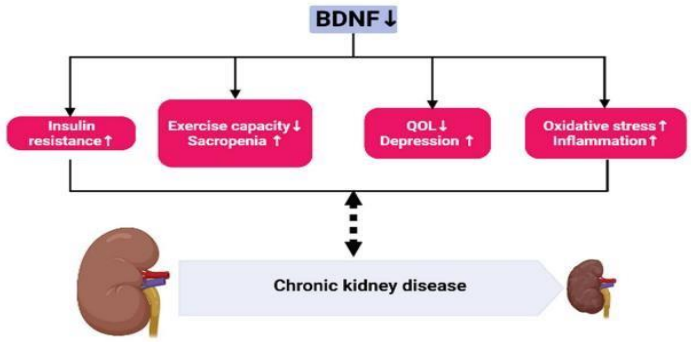


Figure 9: Reduced levels of BDNF have been associated with the development of insulin resistance, sarcopenia, depression, worsening of quality of life (QOL), increased oxidative stress, and inflammatory state, which in turn facilitate the progression of chronic.

1.8 Management of the patient with chronic kidney disease (CKD)

Patients with chronic kidney disease require regular nephrological monitoring to mitigate the advancement of damage and effectively address the various complications associated with kidney failure.
The periodic assessment of renal function and proteinuria helps to determine the progression of the disease and the potential response to the established therapies.
Hypertension is one of the manifestations of the CKD patient: the rate of decline in renal function increases with raising blood pressure.
Two important factors contribute to the increase of blood pressure: sodium retention and activation of the renin-angiotensin-aldosterone system. For these reasons, the first initial step of therapy should be ACE inhibitors or angiotensin II receptor blockers and the second one is a reduction of sodium intake[57].
Dietary education is essential for preventing and managing CKD.
Low-protein diets (LPDs) have been shown to improve hyperfiltration, reduce nitrogenous waste and ease the renal workload by decreasing glomerular pressure[58].
For the end stage kidney disease (ESKD) patient is essential to set up the best therapy between:

- hemodialysis
- peritoneal dialysis
- kidney transplantation

Peritoneal dialysis (PD) is a type of kidney replacement therapy where a sterile solution is introduced into the peritoneal cavity through a catheter. This solution helps eliminate waste products and excess water by using the peritoneal membrane as the exchange surface[59].
Kidney transplantation (KT) can be defined as the first successful modality of renal replacement therapy (RRT) for irreversible chronic kidney disease[60].
It is the natural substitution of the chronic loss of renal function and can be done by two types of donors: deceased or living.
KT can enhance the neurological status of chronic kidney disease (CKD) patients. Indeed, in a study conducted in patients before kidney transplantation and at two-year follow-up, some circulating BBB-specific biomarkers were measured[61]. In particular, it was seen that after transplantation the level of BDNF was increased, emphasizing the importance of this treatment for the patient.

AIM OF THE STUDY

Firstly, an assessment of cognitive impairment and depression levels was conducted in a group of 44 patients with Chronic Kidney Disease (CKD) undergoing chronic hemodialysis treatment. This was achieved by administering the Mini Mental State Examination (MMSE) and Beck Depression Inventory (BDI).
Secondly, the role of Extracellular Vesicles (EVs) in the development of cardiovascular and neurocognitive alterations was evaluated. This assessment involved investigating their impact on the pathogenetic mechanisms of early vascular aging (EVA) through both in vitro and ex vivo experiments. EVs were correlated with key clinical markers of cardiovascular and neurocognitive alterations. These investigations were conducted in collaboration with the Karolinska Institutet (Stockholm, Sweden).
Thirdly, plasma levels of Brain-Derived Neurotrophic Factor (BDNF), a neurotrophin implicated in neuronal plasticity and potentially protective effects on the blood-brain barrier (BBB), were assessed. Low levels of BDNF have been associated with the development of neurocognitive disorders and depression. This analysis was performed in a group of chronic hemodialysis patients, correlating BDNF levels with Mini Mental State Examination (MMSE) and Beck Depression Inventory (BDI) results, used respectively for the evaluation of cognitive impairment and depression levels.
Lastly, to evaluate the protective effect of renal transplantation on cardiovascular and neurocognitive alterations, BDNF levels and EVs were analyzed in a cohort of 74 hemodialysis patients eligible for living donor renal transplantation. These investigations, conducted in collaboration with the Karolinska Institutet (Stockholm, Sweden), assessed the study biomarkers at baseline (pre-renal transplantation) and two years post-transplantation.

MATERIAL AN METHODS

3.1 Population design

For the evaluation of the role of EVs in the development of EVA and neurocognitive alterations, and for the analysis of BDNF levels, we enrolled 44 patients with CKD undergoing chronic hemodialysis from the Nephrology and Dialysis Department of SS Annunziata Hospital in Savigliano (CN), compared with a healthy population (n=20) matched for sex and age. In the same patients, we assessed cardiovascular and neurocognitive alterations using clinical parameters.

3.1.1 Collection and conservation of biologic samples

Blood samples from patients at the Dialysis Center in Savigliano were collected via peripheral venous sampling: to separate the serum, whole blood samples were centrifuged at 3500 g for 15 minutes at room temperature; to separate the plasma, whole blood samples in EDTA anticoagulant were centrifuged at 1500 g for 15 minutes at room temperature. The plasma and serum samples obtained in this way were stored at -80°C until use.
Blood samples from patients who were candidates for living donor kidney transplantation at Karolinska University Hospital were obtained at baseline and two years post-kidney transplantation. After isolating the plasma, the samples were stored at -80°C until use. The laboratory parameters considered (complete lipid profile, calcium, phosphorus, creatinine, blood glucose, IL-6) were obtained from routine analysis of the patients at the Department of Laboratory Medicine at Karolinska University Hospital.

3.2 Analysis of clinical parameters of cardiovascular and neurocognitive alterations

3.2.1 Clinical parameters of cardiovascular alterations

For the evaluation of cardiovascular alterations, the following ultrasound parameters were used as clinical markers of damage:
-arterial stiffness investigated through carotid-femoral pulse-wave velocity
-thickness of the intima-media layer of the right common carotid artery (IMT)
The ultrasound examination was performed using an ESAOTE Crysta Line Mylab ultrasound machine with a linear probe.

3.2.2 Clinical parameters of neurocognitive alterations

The assessment of depressive status was conducted using the Beck Depression Inventory (BDI) test. The 48 enrolled patients were asked 21 questions, and each response was assigned a score. At the end of the questionnaire, these scores were summed up. A higher total score indicated more severe depression in the patient.
For the evaluation of cognitive status, the Mini Mental State Examination (MMSE or MMT) was administered to the 48 patients enrolled in the study.

3.3 Analysis of BDNF

The determination of plasma levels of BDNF was performed using the ELISA technique, employing the DBD00 kit (R&D Systems, Abingdon, UK). Briefly, after reconstituting the reagents as per the manufacturer's instructions, we added 100 µL of RD1S assay diluent and 50 µL of patient serum to each well, which contained a monoclonal antibody specific to BDNF. The plate was then incubated for 2 hours at room temperature. Subsequently, 100 µL of conjugated antibody specific to BDNF was added to each well, followed by covering the plate and incubating for 1 hour at room temperature. After incubation, each well was washed three times with wash solution (400 µL). Once dry, 200 µL of substrate solution was added to the wells and incubated for 30 minutes at room temperature in the dark. Then, 50 µL of blocking solution was added; the color of the liquid in the wells changed from blue to yellow. The plate was read at a wavelength of 450 nm with a reference wavelength set at 540 nm.

3.4 Immunohistochemical analysis on sections of isolated vessels

Immunohistochemical investigations for the evaluation of the phenotype related to EVA were conducted on isolated vessel samples from patients with advanced CKD compared to healthy subjects. Vessel samples from CKD patients were obtained during kidney transplantation, while healthy controls were obtained from individuals undergoing bariatric surgery, cholecystectomy, or kidney donation at Karolinska University Hospital (Stockholm, Sweden), after signing informed consent. The study protocol was approved by the ethics committee.
Isolated vessel samples were frozen with dry ice and stored at -80°C until use. Before immunohistochemical analysis, the samples were fixed in 4% formaldehyde. Subsequently, the sections obtained were fixed with 5% goat serum (Sigma-Aldrich, United States). The primary antibodies used were diluted with PBS as follows: anti-p21CIP1 1:100 (Sigma-Aldrich), anti-p16INK4a 1:200 (Abcam, UK), anti-RUNX2 1:100, anti-Sirtuin-1 1:100, anti-Nrf2 1:100 (Sigma-Aldrich). The diluted antibodies were added to the sections and incubated overnight at 4°C. After incubation, the secondary goat anti-rabbit Alexa Fluor 594 antibody was added at a 1:600 dilution (Thermo Fisher Scientific) for 1 hour in the dark at room temperature. Subsequently, the sections were immersed in DAPI (1:5000) for 1 minute. The sections were then washed twice in PBS and left overnight in 200 µL of SA-β-gal solution (pH=6) at 37°C and 0% CO2. Finally, the sections were examined and photographed with a fluorescence microscope (Olympus, Japan).

The fluorescence intensity of RUNX2 and SIRT1 was evaluated by three different blind observers using a scoring scale from 0 to 3 (0=no fluorescence, 3=maximum fluorescence intensity). The expression of Nrf2 was assessed using Image J software, with the area of positivity measured and expressed as a percentage of the total area. For the cellular expression of p21CIP1 and p16INK4a, Image J software was used to count the positive cells, providing a value expressed as a percentage of the total cells.

3.5 Analysis on EVs

3.5.1 Isolation, quantification and characterization of EVs

All samples were centrifuged at 3000 x g for 10 minutes at 4°C to remove cellular debris or any other potential contaminants and were then diluted (1:1000) with phosphate-buffered saline (PBS) filtered through 0.1 μm filters. The resulting sample was analyzed using NanoSight LM10 (Malvern Instruments, Malvern, UK) to determine the concentration and size of the EVs using NTA 1.4 software and Nanoparticle Analysis Systems: the Brownian motion of the EVs in the sample is visualized by a laser light, recorded by a camera, and then converted into the cited parameters by the software using the Stokes-Einstein equation.
The characterization of the EVs was performed on 44 patients with CKD undergoing chronic hemodialysis and compared with an equal number of healthy subjects to study the role of EVs in the development of EVA and neurocognitive alteration.
The isolation of EVs for subsequent characterization was obtained by ultracentrifugation at 11000 x g for 2 hours at 4°C; the sample contained 1 ml of plasma diluted with 3 ml of phosphate-buffered saline (PBS). At the end of the 2 hours, the supernatant was removed, and the pellet was resuspended in 1 ml of RPMI culture medium to which 10 μl of 1% DMSO was added for longer storage at -80°C.
The characterization of the EVs from the 44 patients on chronic hemodialysis was performed by flow cytometry to identify their cellular origin. For this purpose, platelet markers (CD42b, CD62P, CD41), monocyte markers (CD14, CD15), T lymphocyte markers (CD3), B lymphocyte markers (CD19, CD5, CD40), and endothelial markers (CD144, CD31, CD105, CD146) were used. Briefly, 1 μL of EV-containing suspension was diluted in 100 μL of phosphate-buffered saline (PBS) filtered through 0.1-micron filters. The suspension was then placed in a 96-well plate to which 1 μL of labeled antibody was added. Before analysis, the plate was incubated at 4°C for 1 hour in the dark.

The characterization of EVs from kidney transplant candidate patients was performed by flow cytometry (MACSPlex Exosome Kit, human, Miltenyi Biotech, Auburn, CA, USA) on samples collected both pre-transplant and two years post-transplant. This kit allows the detection of 39 surface antigens using sized beads. The samples analyzed were diluted with MACSPlex buffer to a total volume of 120 µL; subsequently, 15 µL of a solution containing the MACSPlex beads was added to the suspension.
To identify the bead-bound EVs, anti-CD9, CD63, and CD81 antibodies were added and incubated for 1 hour under oscillatory conditions at 450 rpm away from light. After incubation, unbound beads were washed at 3000 x g for 5 minutes 3 times using the MACSPlex buffer provided in the kit; the supernatant was then removed, leaving 150 µL for each sample.
The flow cytometry analysis was performed with the CytoFLEX flow cytometer (Beckman Coulter, Indianapolis, IN, USA).

3.5.2 MiRNA analysis

To analyze the expression profile of EVs' miRNAs secreted in the plasma of the 40 patients undergoing dialysis, RNA was extracted from macrovesicles and used Agilent SurePrint G3 Human miRNA r21 microarrays to detect which miRNAs were expressed. For each sample, 2560 miRNAs were screened. We further analyzed them prioritizing miRNA-target interactions to assess an over-representation of miRNA-target interactions based on the statistical significance associated with each miRNA-target interaction.
RNA extraction from EVs was performed using exoRNeasy Midi Kit (Qiagen, Hilden, Germany; No. 77144), according to the protocol described by the manufacturer. The EVs' total RNA quantification was assessed using NanoDrop One (Thermo Fisher Scientific, Madison, WI, USA).
RNA samples were processed using the Agilent's miRNA complete labelling and hybridization kit and hybridized on SurePrint G3 Human miRNA r21 microarrays (G4870-60530) (Agilent Technologies, Inc., Santa Clara, CA, USA). Briefly, 100ng of total RNA were dephosphorylated, denaturated with DMSO at 100°C and labelled with Cyanine 3-pCp at 16°C for 2 hours. The samples were completely dried in a vacuum concentrator at 45°C, resuspended in hybridization mix and incubated at 100°C for 5 minutes. The hybridization sample mixture was loaded into the Agilent microarray hybridization chamber and hybridized into an oven rotator rack at 55°C for 20 hours. Microarray slides were washed and scanned using an Agilent SureScan microarray scanner or Agilent C scanner device according to the manufacturer's instructions. Microarray data were extracted using Agilent feature extraction software. The Agilent GeneSpring Data Analysis software was used to normalise and filter gene expression data.

The web tool MIENTURNET (MicroRNA ENrichment TURned NETwork) (http://userver.bio.uniroma1.it/apps/mienturnet/) was used to identify and generate the miRNA-target interactions network, by choosing the reference database MiRTarBase.
For statistical significance, a cut-off of p-value < 0.05 was used.

3.5.3 Biologic effect of EVs on TJ and BBB

Sections of vessels isolated from adipose tissue were used as surrogates for BEE vessels given the impossibility of performing such biopsies.
These sections were transferred to a plate consisting of 24 wells containing 0.3 nL of DMEM solution with penicillin and streptomycin added; each section was placed in a single well. EVs-CKD (1 mL containing a particle number of approximately 1.5 x 10^10/ml) was added to one half of the sections contained in the wells. The remaining sections were not incubated with EVs and used as controls.
Internalization of EVs into ECs was assessed by immunofluorescence technique using the PKH26 Red Fluorescent Cell Linker Kit. The protocol used was as follows: after fixation with 4% formaldehyde for 10 min at room temperature, cell nuclei were stained by Hoechst (dilution 1:100). We stained the EVs of CKD patients with a red fluorescent dye (PKH26 Sigma Aldrich), so the internalization of EVs into endothelial cells could be observed. After incubation, fluorescence was detected by spectrophotometer with appropriate wavelength analysis.
The presence of JAM-1, Claudin-5 and Occludin proteins was then assessed on each sample by immunohistochemical technique. The following primary antibodies were used: JAM-1 (1:400) - CSB-PA897579LA01HU, Cusabio Technology USA, Claudin5(1:200) - CSB-PA005507LA01HU, Cusabio Technology USA, Occludin (1:200) - CSB-PA016263LA01HU, Cusabio Technology USA, CD31/PECAM-1(1:100) - 53370, BD Biosciences Pharmigen, USA). Antibody diluted with 5% goat serum anti-rabbit IgG CF594 (1:600) was used as the secondary antibody - SAB4600407, Sigma-Aldrich) for tight junctions; while the secondary antibody used for the detection of CD31/PECAM-1 was Alexa Fluor 488 rabbit anti-rabbit IgG (1:600) A-11059, Fisher Scientific, Sweden.
The protocol used was as follows: each section was fixed with 4% paraformaldehyde for 5 min. Then each section was washed twice with PBS-1X for 5 minutes each. Blocking antibody with Glycine (100 mM) was added for 10 minutes. Two washes with Triton were performed for 5 min each.
Blocking antibody was added with Glycine (100 mM) for 10 min. Two washes with Triton were performed for 5 min each. They were incubated with 10% goat serum at room temperature for 1 hour. They were washed twice with Triton for 5 min each. Primary antibody for Claudin-5

(1:200), Occludin (1:200), JAM-1 (1:400) was added and each section was incubated overnight at 4°C. A 5-minute wash with Triton was performed twice. Fluorochrome-conjugated secondary antibody for tight junctions was added and incubated for 45 minutes at room temperature. A 5-minute wash 3 times with Triton was performed. The endothelial cell marker CD31/PECAM-1 (1:100) was added and incubated for 1 hour at room temperature. A double wash with Triton was performed twice for 5 minutes. The secondary antibody for fluorochrome-conjugated CD31 was incubated. A 2-fold wash with Triton was performed for 5 minutes. DAPI counterstain was added for 5 minutes for visualization of cell nuclei. Three washes with PBS-1X were performed 3 times, for 5 minutes each. Each section was allowed to air dry and fixed.

3.5.4 Cell culture

HUVEC were purchased from ATCC (Manassas, VA, USA) (catalog no. CRL-1730™) and were maintained in Dulbecco's Modified Eagle's Medium DMEM Medium, containing 2 mM L-glutamine (Euroclone S.p.A, Milan, Italy), 1500 mg/L sodium bicarbonate (Euroclone), and supplemented with 0.1 mg/mL heparin (Merck KGaA, Darmstadt, Germany), 1% penicillin, 1% streptomycin, and 10% FBS (Euroclone).

Mice immortalized astrocytes (kindly provided by prof. Dmitry Lim) [33], were cultured in DMEM, Sigma-Aldrich, Milan, Italy with 10% fetal bovine serum (FBS; Euroclone, S.p.A.; Pero, Milan, Italy), 2 mM L-glutamine (Euroclone) and 1% penicillin/streptomycin (Sigma-Aldrich).

In order to detach the adhering-cells from the flask, the media was removed, the cells were washed with 5mL of sterile phosphate saline buffer (PBS) solution 1X. Then the cells monolayer was incubated with 1 mL of trypsin / ethylenediaminetetraacetic acid (EDTA) at 37°C for 5 minutes. In this way, trypsin degrades the proteins of the cellular matrix that keep the cells adherent to each other, while EDTA chelates magnesium (Mg2+) and calcium (Ca2+), essential elements for cell adhesion to the flask. After the incubation, cell detachment was observed using the optical microscope and subsequently, 5 mL of culture medium was added in order to inhibit the action of trypsin / EDTA. The cells were collected and centrifuged at 800 rpm, for 5 minutes, at room temperature. The supernatant was aspirated, and the cell-pellet resuspended in an appropriate volume of new medium. Both HUVEC and astrocytes were then plated in a sterile 96-well plate adding 10 000 cells per well to conduct the experiments.

We examined the effects of EVs (50,000 EVs/cell) on HUVEC and astrocytes cell viability, the mitochondrial membrane potential (i.e., JC1 assay) and mitoROS release (i.e., mitoROS assay). The experiments were performed in triplicate and repeated at least three times by using different pools of HUVEC and astrocytes.

3.5.5 MTT Assay

Cell viability of HUVEC and astrocytes was investigated by using the 1% 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT assay; Cayman Chemical, Ann Arbor, MI, USA), as previously performed in similar or other cellular models [62].
The 10% of the MTT solution was prepared by dissolving 50 mg of the MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) in 10 mL of PBS (pH 7.4) and kept stored at 4 °C protected from the light. Following the EVs stimulation of HUVEC and astrocytes (10,000 cells/well in 96 well plates) for 24 h at the selected concentration, the media was removed and 100 µL of the MTT solution diluted in DMEM high glucose w/o phenol red, supplemented with 2 mM L-glutamine, and 1% penicillin-streptomycin (P/S) were added to each well. Thereafter, the plate was incubated at 37 °C for 2 h.
Once the reaction had occurred, the supernatant was removed and the formazan crystals formed in each well were dissolved with 100 µL of dimethyl sulfoxide (DMSO; Sigma, Milan, Italy). Cell viability was finally determined by measuring the absorbance through a spectrophotometer (VICTOR™ X Multilabel Plate Reader; PerkinElmer; Waltham, MA, USA) with a wavelength of 570 nm. Cell viability was calculated by setting control cells (untreated cells) as 100%.

3.5.6 JC-1 Assay

Mitochondrial membrane potential (ΔψM) is an important parameter of the mitochondrial function used as an indicator of cell health, as previously tested in similar cellular models [63].
In order to examine the mitochondrial membrane potential, the medium of HUVEC and astrocytes stimulated with EVs (as performed for MTT assay) was removed and cells were incubated with 5,51,6,61-tetrachloro-1,11,3,31tetraethylbenzimidazolyl carbocyanine iodide (JC-1) staining solution diluted in Assay Buffer 1X (Cayman Chemical; Ann Arbor, MI, USA) for 20 min at 37 °C, following the manufacturer's instructions. After incubation, HUVEC and astrocyte were washed twice with the Assay buffer 1X and then 100 µL/well of it was added for the final reading. The mitochondrial membrane potential was determined by measuring the red (excitation 535 nm/emission 595 nm) and green (excitation 485 nm/emission 535 nm)

fluorescence through a spectrophotometer (VICTOR™ X Multilabel Plate Reader; PerkinElmer). Normalization of the data was executed versus control cells (untreated cells).

3.5.7 MitoROS release assay

The MitoROS release was examined using the Cayman's Mitochondrial ROS Detection Assay Kit (Cayman Chemical) [64]. In particular, 10,000 HUVEC/astrocytes/well were positioned in 96 WELL plates in complete medium and they were stimulated with EVS, as was the case for cell viability and mitochondrial membrane potential quantification. After stimulation, we blocked the reactions by replacing the culture media with 120 μL of Cell-Based Assay Buffer. Then, we aspirated the Buffer and added 100 μL of Mitochondrial ROS Detection Reagent Staining Solution to each well. After incubation at 37 °C, with protection from light for 20 min, we removed the Staining Solution and washed each well three times with 120 μL of PBS. In each sample, the absorbance was read at excitation/emission wavelengths of 480 nm and 560 nm, respectively, through a spectrophotometer (VICTOR™ X Multilabel Plate Reader). Data were normalized in relation to non-treated cells taken as control.

3.6 Statistical Analysis

Clinical and laboratory characteristics and biomarker levels in the study are expressed as median with interquartile range (IQR). Statistical analysis was performed using GraphPad Prism (v9, GraphPad, San Diego, CA, USA) and SPSS (v28, IBM, New York, NY, USA). For the analysis of changes in BDNF and EVs within the same patient, the Wilcoxon paired test was used to assess differences between baseline and data at two years post renal transplant. The Fisher's exact test was used for categorical variables. In both in vitro and ex vivo experiments, the Mann-Whitney U test was used to compare differences between groups, while concentration-dependent experiments utilized two-way ANOVA. To evaluate correlations between biomarkers in the study and patient-dependent factors (such as demographic and clinical characteristics), Spearman's rank correlation test was used. Results from in vitro experiments represent the mean 3 independent experiments. Statistical significance was set at $p < 0.05$.

RESULTS

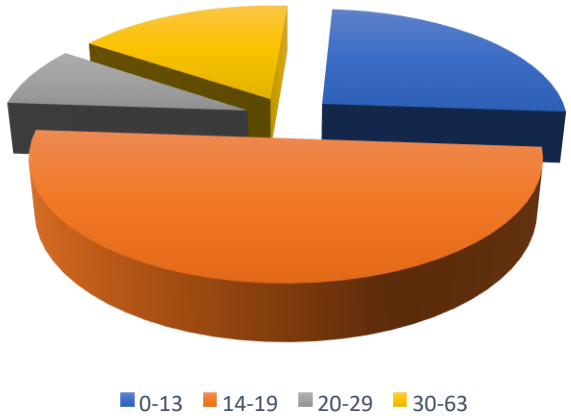
4.1 Analysis of the study population

For the evaluation of the role of EVs and BDNF in the genesis of cardiovascular and neurocognitive alterations, 44 patients undergoing chronic hemodialysis were enrolled, whose demographic and clinical characteristics are reported in Table 2.

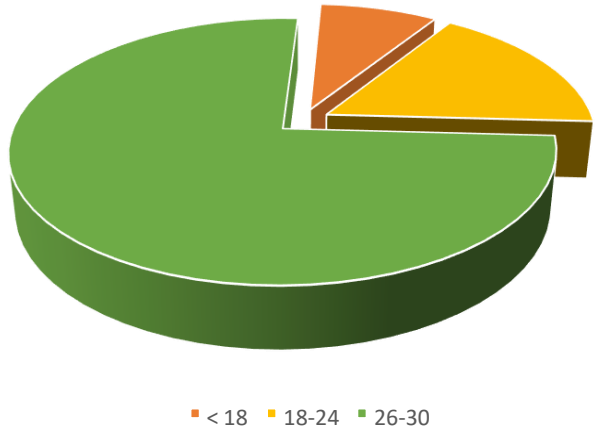
Demographic characteristics	
Age (years)	50 (± 16.3)
Sex (man, %)	42.9
Weight (Kg)	69.3 (± 13.6)
Dialytic age (months)	47.7 (± 20)
Comorbidities	
Pathologies CV (%)	35.7
Diabetes Mellitus	42.9
Arterial Hypertension	82.1

Table 1: Characteristics of the study population. Variable expressed in median ± DS or in percentage.

To evaluate the incidence of depression and neurocognitive alterations, 44 patients were administered the Beck Depression Inventory (BDI) and the Mini-Mental Test (MMT), respectively for the assessment of depression and neurocognitive deficits.
From the analysis of the BDI scores, it emerged that 50% of the population has a mild to moderate level of depression (Graph 1). From the MMT scores, it emerged that about 75% of the study population does not have a cognitive deficit (Graph 2).



Graph 10: division of the scores obtained at Beck Depression Inventory.



Graph 11: Division of the scores obtained at Mini Mental test.

4.2 Analysis of BDNF levels and neurocognitive alterations

Firstly, the levels of BDNF in a group of 44 patients with chronic kidney disease undergoing hemodialysis were compared with those of a healthy population matched for sex and age. BDNF levels were found to be significantly lower compared to those observed in the control population (Figure 13).

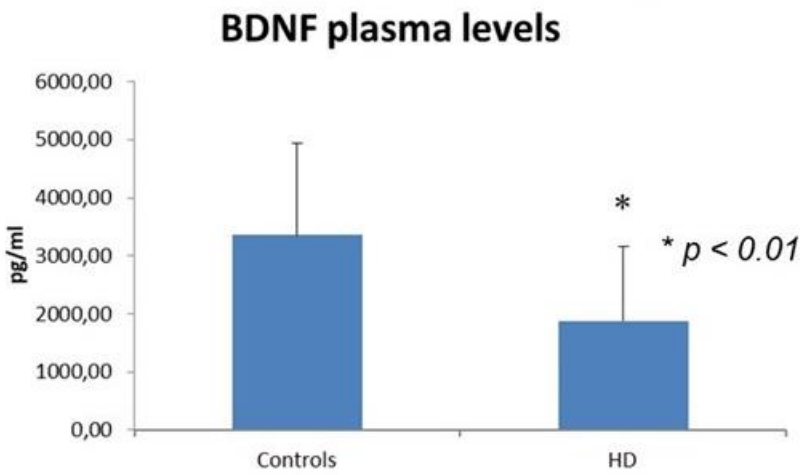


Figure 12: Comparison of BDNF levels of hemodialysis population and control population.

Subsequently, patients underwent tests to assess cognitive status (Mini-Mental Test, MMT) and degree of depression (Beck Depression Inventory, BDI), with results correlated with BDNF

levels. From this preliminary analysis, an association between reduced BDNF levels and the test outcomes emerged (Figure 26 and 27).

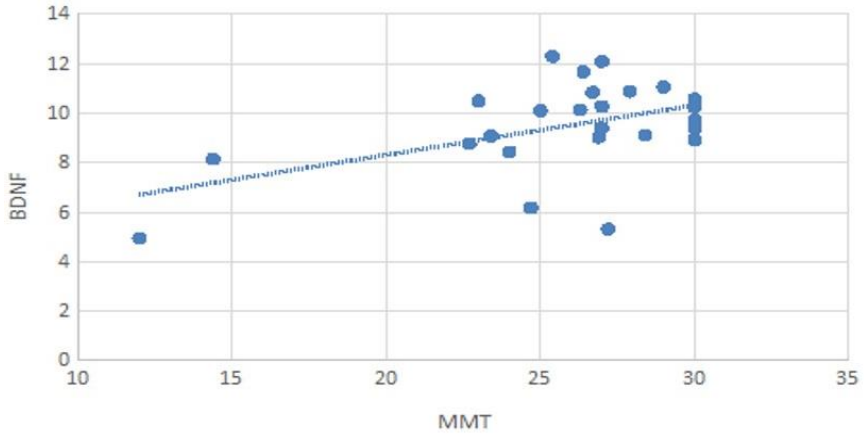


Figure 13: Correlation between BDNF levels and the Mini-Mental test (MMT) results.

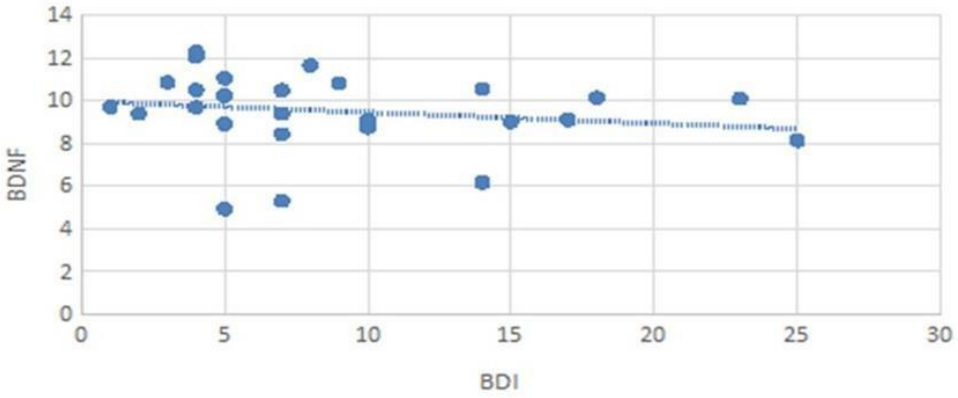


Figure 14: Correlation between BDNF levels and Beck Inventory Scale (BDI) results.

4.3 EVA as an indicator of senescence in patients with CKD

Early vascular ageing (EVA) commonly occurs in CKD patients, and it is characterized by structural and functional changes in microcirculatory vessels during uremia. Key features of EVA include calcifications and impaired vasodilation processes, alongside heightened expression of senescence factors by endothelial cells.

To study the phenotypic traits of EVA, we conducted immunohistochemical analysis on isolated vessels using specific markers: RUNX2 (calcification marker), Nrf2 and SIRT1 (antioxidant markers), and p21CIP1 and p16INK4a (senescence markers).
In uremic patients compared to healthy controls, we observed a significant decrease in the expression of oxidative stress protective markers (Nrf2 and SIRT1). This difference was statistically significant for both markers (Figure 16).

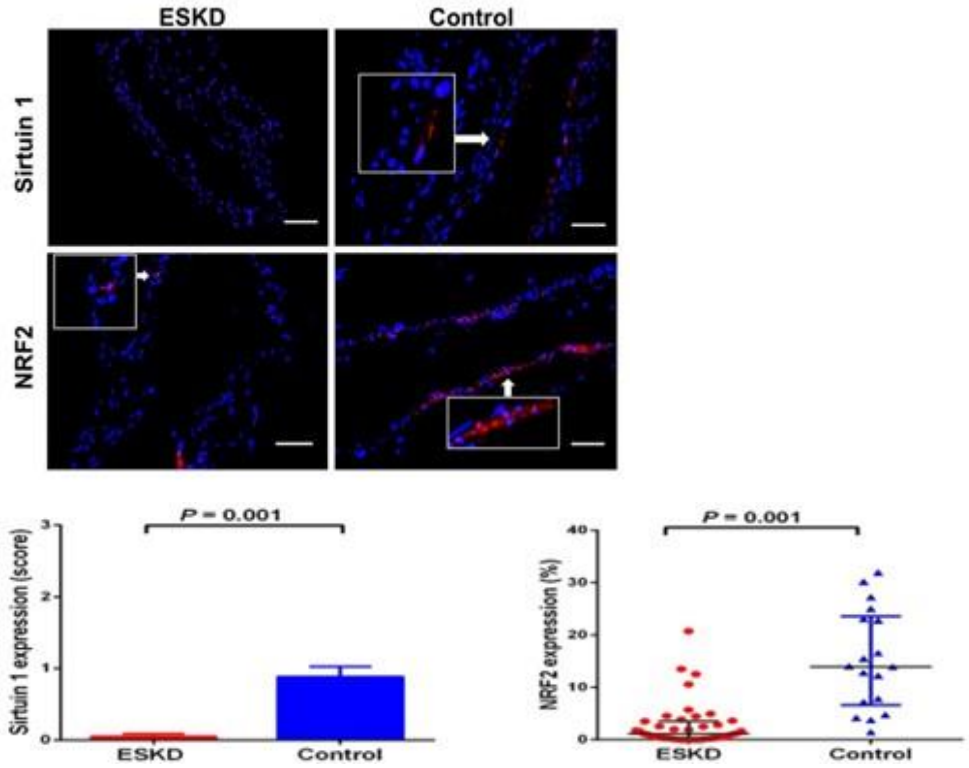


Figure 16: Expression of Nrf2 and SIRT1 in samples of isolated vessels (a) and in vitro (B) incubated with EVs from CKD patients vs healthy controls.

Conversely, senescence markers (p21CIP1 and p16INK4a) and calcification marker (RUNX2) are found to be more highly expressed in the CKD population compared to healthy controls (Figure 17).

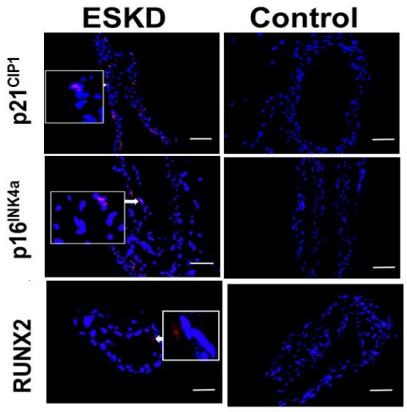


Figure 17: Senescence (p21CIP1 and p16INK4a) and calcification (RUNX2) markers in CKD patients vs healthy controls.

4.4 Role of the Extracellular vesicles (EVs)

For the quantitative analysis and characterization of EVs, 44 patients undergoing chronic hemodialysis were enrolled, selected from the initial study population. Plasma-derived EVs from these patients were compared with those obtained from a group of healthy subjects matched for sex and age. Plasma EVs from hemodialysis patients (EVs-CKD) were found to be significantly higher compared to the control population (1.3x10^12 ± 1.25x10^11 particles/ml vs 4.8x10^11 ± 1.0x10^11 particles/ml). However, there were no statistically significant differences in the sizes of EVs between the two populations (Figure 18).

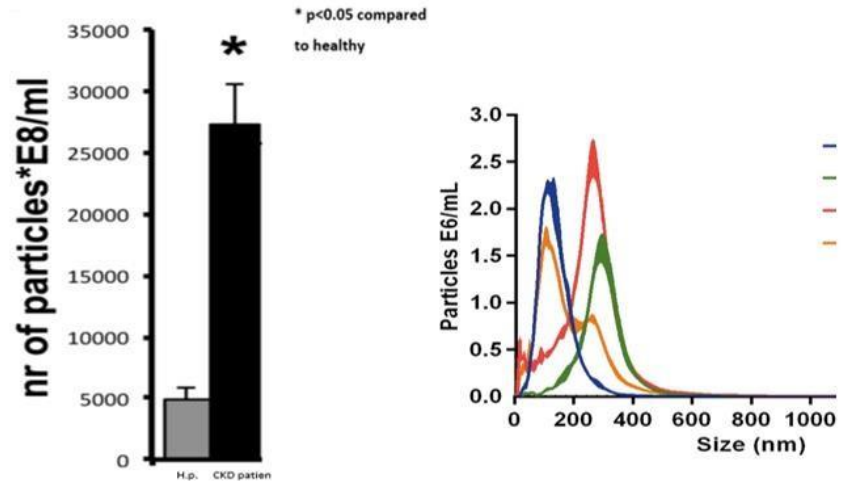


Figure 18: Concentration of EVs in plasma samples from CKD patients measured using Nanotrack Analysis (Nanosight). On the right, a graph showing the sizes and relative concentrations of EVs (Ex = 10x).

Subsequently, using MACSPLEX analysis, an initial characterization was performed to assess the cellular origin of EVs: statistically significant differences were not observed between the

hemodialysis and control populations, except for endothelial-derived EVs ($p < 0.05$) (Figure 19).

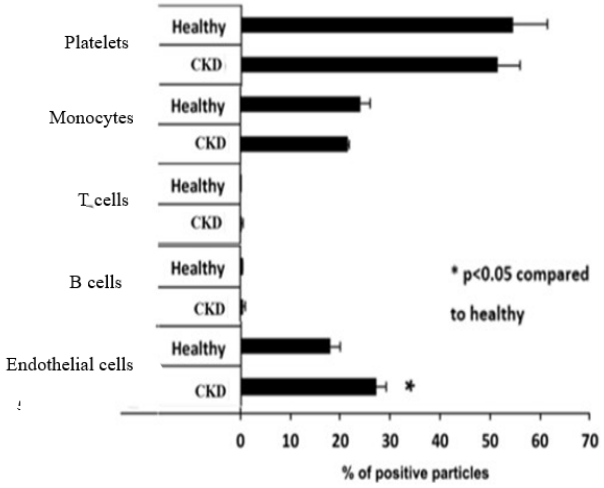


Figure 19: Characterization of EVs with MACSPLEX

Cellular origin (specific markers) % of positivity	**CKD-EVs**	**Controls-EVS**
Platelets (CD42b, CD62P, CD41)	50.30 ± 2.50	53.60 ± 5.05
Monocytes (CD14, CD15)	21.30 ± 0.05	24 ± 3.80
T lymphocytes (CD3)	0.13 ± 0	0
B lymphocytes (CD19, CD5, CD40)	1.43 ± 1.03	0.1 ±0.2
Endothelial cells (CD144, CD31, CD105, CD146)	27. 34 ± 2.75	17.04± 3

Table 2: Characterization of the cellular origin of EVs: average percentages of positivity for specific markers.

Subsequently, we evaluated the membrane expression of specific markers involved in apoptotic, inflammatory, and pro-coagulatory processes. EVs from CKD patients exhibited molecules such as Tissue Factor, C5b-9, CD40L, ICOS, FAS-ligand, NGAL, Class I HLA, and various types of proteins in the selectin and integrin families on their surface (Figure 19 and Table 2). The expression of these markers was higher on EVs derived from CKD patients compared to the control population, showing a statistically significant difference (Table 3 and Figure 20).

Surface cell markers % of positivity	CKD-EVs	Controls-EVs
CD40L	7.50 ± 2.40	2.23 ± 0.01
Tissue Factor	25.44 ± 4.05	5.00 ± 2.08
C5b-9	10.01 ± 3.00	3.34 ± 3.00
ICOS	11.39 ± 0.03	1.39 ± 1.03
FASL	13.37 ± 4.75	2.73 ± 1.75

Table 3: Average percentages of positivity for specific markers expressed on the surface of EVs obtained from CKD patients.

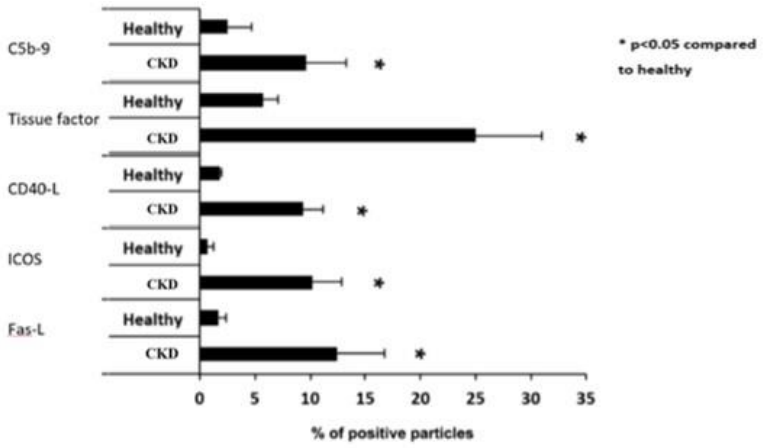


*Figure 20: Characterization of EVs based on specific markers involved in apoptotic, inflammatory, and pro-coagulatory processes: comparison between CKD population and controls. *p<0.05.*

4.4.1 miRNA analysis

For each sample, 2560 miRNAs were screened. Of these, thirty-five miRNAs were found to be expressed in EVs isolated from 50% of the cohort, 16 miRNAs were expressed in 75%, and 5 miRNAs were expressed in 100% of the samples (Table 4). We then focused on these 5 miRNAs that were found to be expressed in EVs of all patients (hsa-miR-33b-3p, hsa-miR-4515, hsa-miR-4665-3p, hsa-miR-6737-3p, hsa-miR-940). Using enrichment analysis, a network of miRNA-target interactions was built (Figure 21, $p<0.05$), identifying 10 target genes regulated by these 5 miRNAs. In the bioinformatic analysis, we only considered each miRNA showing an interaction with at least 2 genes. The central node of this network was miR-940, which has been implicated in the regulation of inflammatory, apoptotic, and angiogenic processes. It interacted with all ten genes in the network: THY1, NRGN, CASP16P, NUCB1, DHODH, SNRPD1, CACNG8, TPM3, NUFIP2, and GDE1. (Table 5)

	p-value	*Number of interactions*	*microRNA 1*	*microRNA 2*
Gene				
THY1	**0,001**	2	hsa-miR-33b-3p	hsa-miR-940
NRGN	**0,003**	2	hsa-miR-4665-3p	hsa-miR-940
CASP16P	**0,01**	2	hsa-miR-4665-3p	hsa-miR-940
NUCB1	**0,01**	2	hsa-miR-4515	hsa-miR-940
DHODH	**0,02**	2	hsa-miR-940	hsa-miR-33b-3p
SNRPD1	**0,03**	2	hsa-miR-4665-3p	hsa-miR-940
CACNG8	**0,04**	2	hsa-miR-4665-3p	hsa-miR-940
TPM3	**0,04**	2	hsa-miR-4515	hsa-miR-940
GDE1	0,07	2	hsa-miR-940	hsa-miR-6737-3p
NUFIP2	0,12	2	hsa-miR-6737-3p	hsa-miR-940

Table 4: List of genes regulated by the five miRNAs expressed in the extracellular vesicles found in 100% of the samples. Each gene was found to interact with two miRNAs (p<0.05).

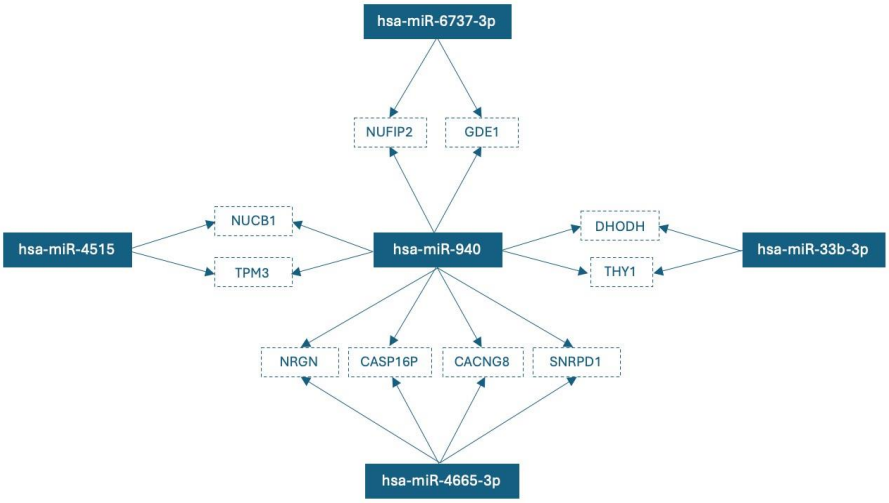


Figure 21: Network of miRNA-target interactions identified by the enrichment analysis of the five miRNAs expressed in EVs of all dialysis patients.

4.4.2 Analysis of the effect of EVs on the Blood Brain Barrier (BBB)

Because of the significant correlation between EVs derived from hemodialysis CKD patients and endothelial dysfunction, and the correlation between EVs and neurocognitive alterations, the effect of EVs on the molecules constituting the tight junctions (TJ) of endothelial cells was evaluated using vessels isolated from adipose tissue to recreate a model of the BBB. The expression of these molecules, particularly Occludin, Claudin, and JAM-1, was assessed through immunohistochemical analysis on sections of isolated vessels previously incubated

with EVs from CKD patients versus healthy controls. Before evaluating the expression of the molecules, we confirmed the internalization of CKD-EVs in endothelial cells using immunofluorescence (Figure 23).

A statistically significant reduction in the expression of Occludin (Figures 22a and 23a and b) and JAM-1 (Figure 22b) was observed in vessels stimulated with plasma EVs from CKD patients compared to healthy controls. Regarding Claudin, a trend towards down-regulation was noted, although it was not statistically significant (Figure 22c).

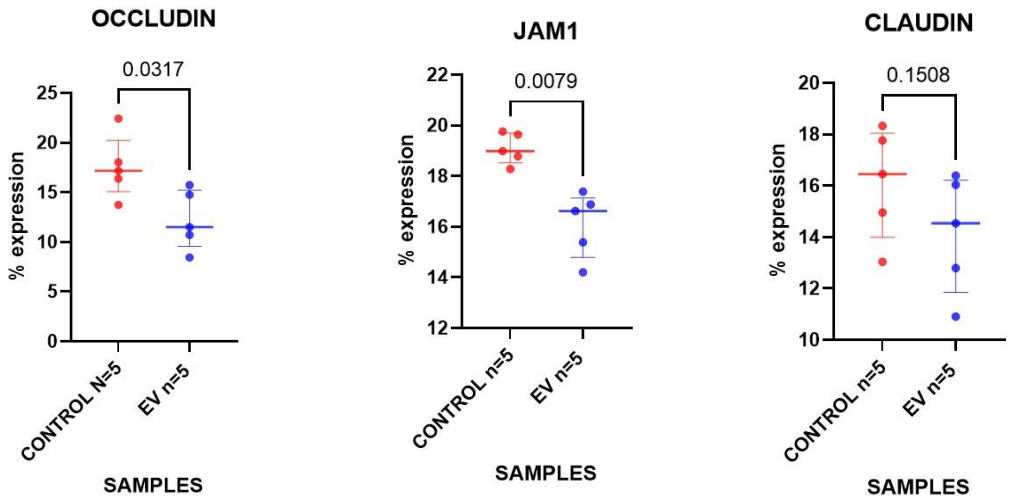


Figure 22: Expression of Occludin (a), JAM-1 (b), and Claudin (c) in endothelial cells after incubation with EVs from CKD patients versus healthy controls. (d) Reduction in Occludin expression in isolated vessels after incubation with EVs from CKD patients.

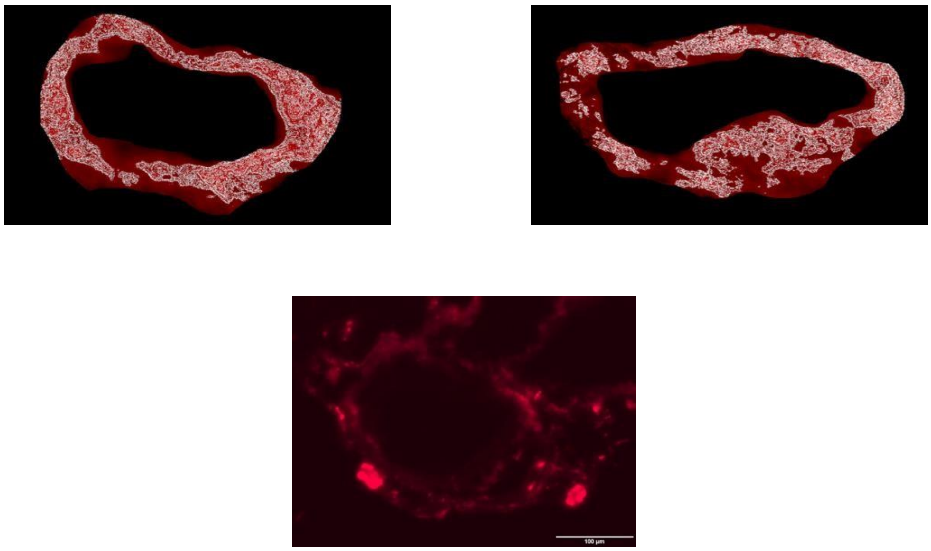


Figure 23: Occludin expression in isolated vessel (a) and after EVS-CKD incubation; experiments were conducted after the confirmation of the internalization of red-labelled EVs in endothelial cells (c).

4.4.3 Correlation of EVs with established markers of vascular damage and cognitive tests

In order to assess the potential harmful effects of EVs on the vascular system, and consequently on the microcirculation and BBB in the uremic population, we conducted a correlation analysis between previously quantified and characterized EVs and several instrumental tests known as indicators of cardiovascular damage and cognitive function. Specifically: arterial stiffness measured by pulse wave velocity (PWV) and carotid intima-media thickness (IMT) using ecoColorDoppler, while Mini-Mental test (MMT) was used for cognitive evaluation and Beck Depression Inventory Scale (BDI) for depression assessment.
The concentration of platelet-derived EVs (P-selectin+) from CKD patients showed a statistically significant correlation with IMT and Mini-Mental test (MMT) scores, whereas endothelial-derived EVs (CD31+) exhibited a significant correlation with arterial stiffness (measured by PWV).

Test	**EVs CD31+**	**EVs CD62P+**
IMT CCA	r=0.543	**r=0.886**
	p value=0.266	**p value=0.019***
PWV	**r=0.829**	r=0.486
	p value=0.042*	p value=0.329
FE	r=0.058	r=0.058
	p value=0.913	p value=0.913
MMT	r=0.706	**r=0.794**
	p value=0.117	**p value=0.050***
BDI	r=0.429	r=0.086
	p value=0.397	p value=0.872

*Table 5: Correlation between EVs from uremic patients and key parameters of vascular and cognitive damage. *p <0.05.*

4.4.4 Analysis of the effects of EVs on Human Umbilical Vein Endothelial Cells (HUVEC)

In vitro experiments were then conducted to study the cytotoxic effect of EVs of CKD patients compared with EVs of healthy controls on endothelial cells (ECs), using the MTT assay. The cell viability was found to decrease after incubation for 24 hours with the EVs of CKD patients. This decrease was more pronounced compared to cell viability of unstimulated cultures (controls) and cultures stimulated with EVs of healthy controls (Figure 24).

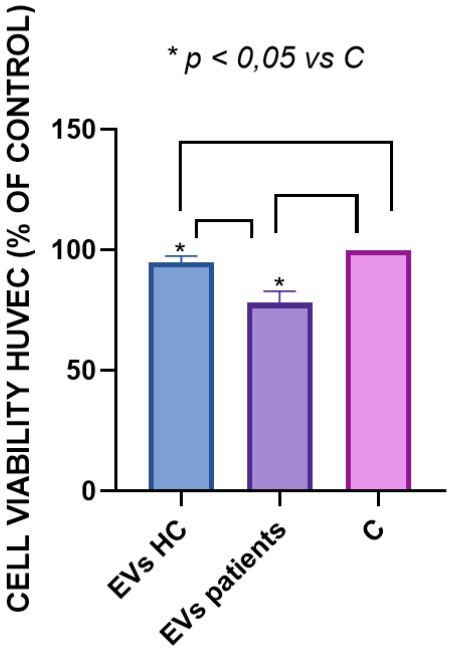


*Figure 24: Effect on cell viability after incubation of EVs-CKD and EVs of healthy controls for 24 h. Data are normalized to the control value. Data are reported as mean ± SD of three independent experiments * p<0.05. Abbreviations: HC: healthy controls; C: controls.*

4.4.5 Analysis of the effects of EVs, Indoxyl Sulphate (IS) and AHR antagonist on Human Umbilical Vein Endothelial Cells (HUVEC)

As previously mentioned, Indoxyl sulphate is classified as a protein bound uremic toxin that is implicated in the progression of CKD, playing a role in endothelial dysfunction. In vitro studies have shown evidence that IS decreases cell viability in HUVEC and increases the production of ROS.

It was decided to perform dose-response and time course study on cell viability about Indoxyl Sulphate (IS)(SANTA CRUZ -sc- 255218). We analysed 4 dosages (10, 100, 200, 400 micromolar) and 3 different timings (3, 12, 24 hours) and we decided to select 200 micromolar and 3 hours stimulation (Figura 25).

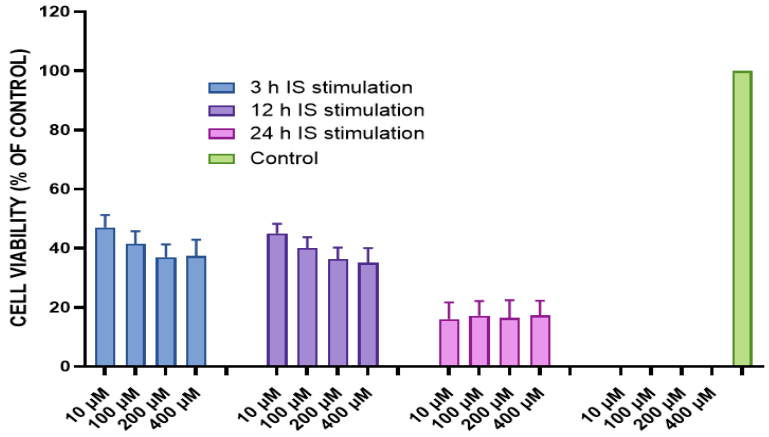


Figure 25: Dose-response and time course study on cell viability with IS. The data are normalized with respect to the control value. The data are reported as means ± SD of three independent experiments.

By triggering the CYP1A1 pathway, IS activates the aryl hydrocarbon receptor (AhR), which in turn mediates oxidative stress and endothelial dysfunction.

So, we decided to use an Ahr antagonist (Ahr Antagonist III, GNF351- SIGMA ALDRICH), in the concentration of 1 and 10 micromolar because of the published data. We selected 10 micromolar for the Ahr antagonist in co-stimulation with indoxyl sulphate.

In vitro experiments where then conducted to observe the effect of EVs, together with IS and the Ahr antagonist (Figure 26). HUVEC were stimulated with CKD-EVs and with healthy controls EVs under three conditions:

- EVs for 24 hours.
- EVs + Ahr antagonist

- EVs + Ahr antagonist + Indoxyl sulphate

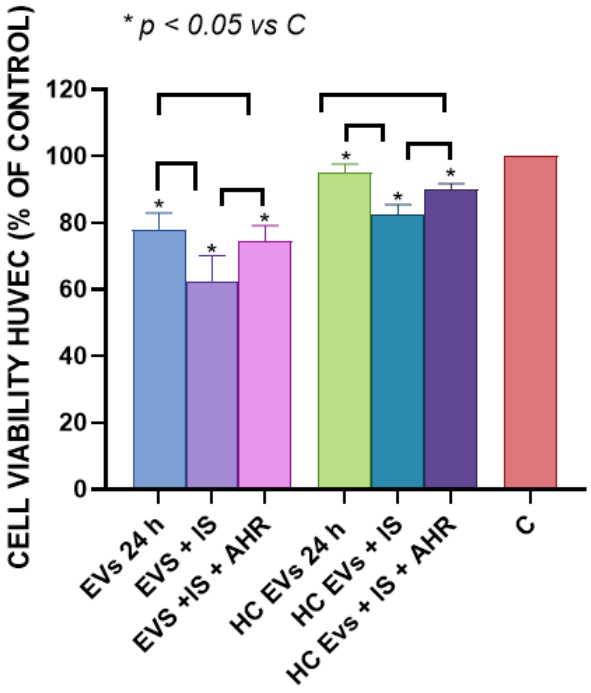


Figure 26: Effect on the viability of HUVEC after incubation with EVs, EVs + IS and EVS + IS + AHR. The data are normalized with respect to the control value. The data are reported as means ± SD of 3 independent experiments. The difference in viability was significant ($p < 0.05$). Abbreviations: HC: healthy controls; AHR: Ahr antagonist.

As expected, cell viability is higher in cells stimulated with EVs of healthy controls. Ahr antagonist exerts a protective effect against IS and EVs together with IS decreases cell viability more than EVs alone.

4.4.6 Analysis of mitoROS production in HUVEC after EVs incubation

To assess the endothelial dysfunction following the damage caused by EVs, it was analysed the production of superoxide in mitochondria. The conditions were, as in the MTT assay, three (Figure 27). Their increase is proportional to the stress that is added to the cell colture; the highest column represents HUVEC stimulated with CKD-EVS and IS. Also in this assay, Ahr antagonist is seen exerting a protective effect, decreasing mitoROS levels.

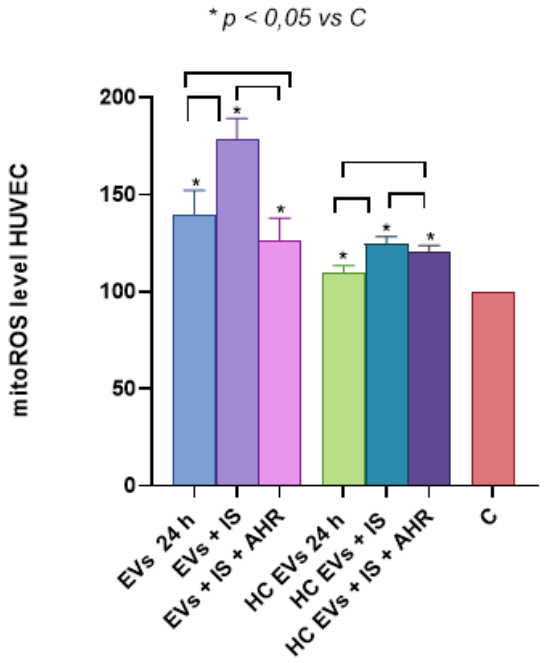


Figure 27: Effect on HUVEC and mitoROS production after incubation with EVs, EVs + IS and EVs + IS + Ahr antagonist. The data are normalized with respect to the control value. The data are reported as means ± SD of three independent experiments. The difference in ROS release was significant (p < 0,05). Abbreviations: HC: healthy controls; AHR: Ahr antagonist.

4.4.7 Analysis of the mitochondrial membrane potential in HUVEC after EVs incubation

In order to confirm the results found in the previous experiments, it was evaluated the variation of the mitochondrial membrane potential of HUVEC after the incubation of the cells with the CKD EVs and healthy controls EVs. As expected, the mitochondrial membrane potential is higher in healthy controls. Ahr antagonist exerts a protective effect, and the combination of EVs and IS decreases the mitochondrial potential more than EVs alone (Figure 28).

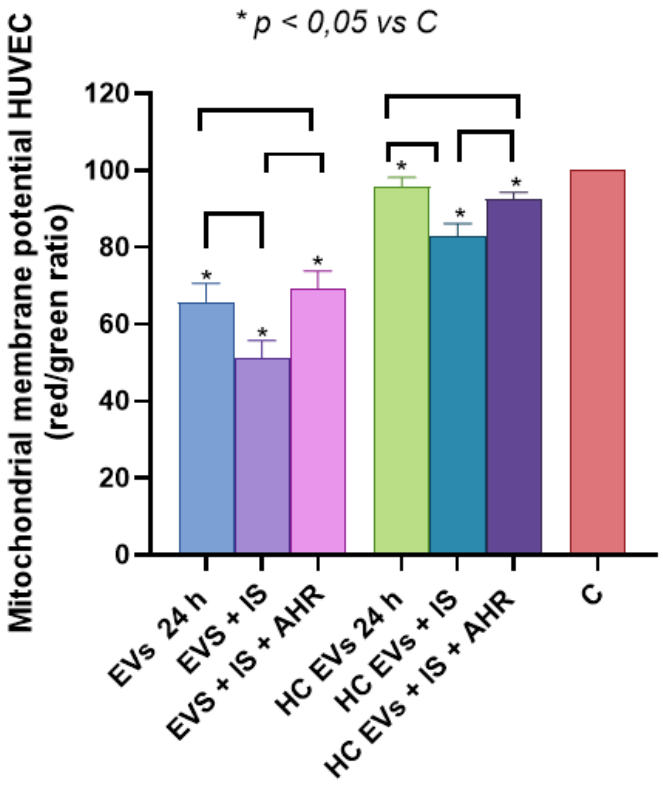


Figure 28: Effect on the change in HUVEC membrane potential after incubation with EVs, EVs + IS, and EVs + IS + Ahr antagonist. The data are normalized with respect to the control value. The data are reported as means ± SD of three independent experiments. The difference in viability was significant (p < 0,05). Abbreviations: HC: healthy controls; AHR: Ahr antagonist.

4.4.8 Analysis of the effects of EVs on astrocytes

We performed the same in vitro experiments that were made in HUVEC, also in astrocytes to analyze the effect of EVs in cells of the central nervous system. The MTT assay was used to study the cytotoxic effect of EVs of CKD patients compared with EVs of healthy controls on astrocytes. The cell viability was found to decrease after incubation for 24 hours with the EVs of CKD patients. This decrease was more pronounced compared to cell viability of unstimulated cultures (controls) and cultures stimulated with EVs of healthy controls (Figure 29).

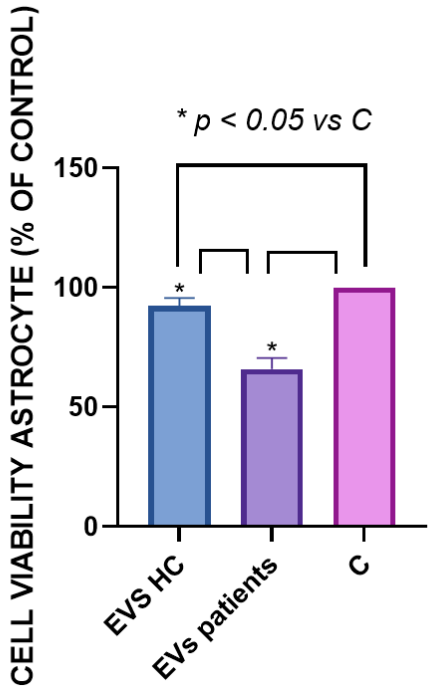


*Figure 29: Effect on cell viability after incubation of EVs-CKD and EVs of healthy controls for 24 h. Data are normalized to the control value. Data are reported as mean ± SD of three independent experiments * p<0.05. Abbreviations: HC: healthy controls; C: controls.*

4.4.9 Analysis of the effects of EVs, Indoxyl Sulphate (IS) and AHR antagonist on astrocytes

Astrocytes were stimulated with CKD-EVs and with healthy controls EVs under three conditions:

- EVs for 24 hours.

- EVs + Ahr antagonist
- EVs + Ahr antagonist + Indoxyl sulphate

As expected, cell viability is higher in cells stimulated with EVs of healthy controls. Ahr antagonist exerts a protective effect against IS and EVs together with IS decreases cell viability more than EVs alone (Figure 30).

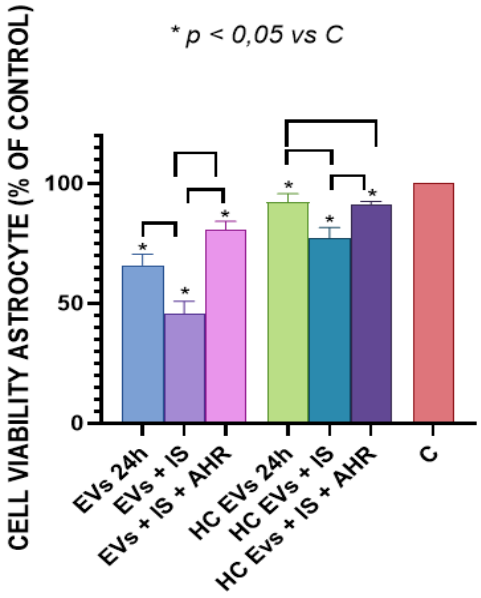


Figure 30: Effect on the viability of HUVEC after incubation with EVs, EVs + IS and EVS + IS + AHR. The data are normalized with respect to the control value. The data are reported as means ± SD of 3 independent experiments. The difference in viability was significant (p < 0.05). Abbreviations: HC: healthy controls; AHR: Ahr antagonist.

4.4.10 Analysis of mitoROS production in astrocytes after EVs incubation

In vitro experiments were made to evaluate the production of mitochondrial reactive species in astrocytes. The conditions were, as in the MTT assay, three (Figure 31). The highest increase is seen when astrocytes are stimulated with CKD EVs together with IS. The presence of Ahr

antagonist decreases the production of mitoROS because of its protective effect against Indoxyl sulphate (Figure 31).

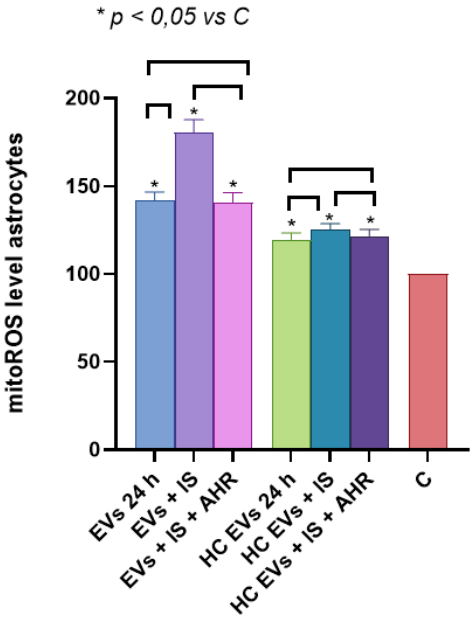


Figure 31: Effect on HUVEC and mitoROS production after incubation with EVs, EVs + IS and EVs + IS + Ahr antagonist. The data are normalized with respect to the control value. The data are reported as means ± SD of 3 independent experiments. The difference in viability was significant (p < 0.05). Abbreviations: HC: healthy controls; AHR: Ahr antagonist.

4.4.11 Analysis of the mitochondrial membrane potential in astrocytes after EVs incubation

In vitro experiments were made to evaluate the variation of the mitochondrial membrane potential of astrocytes after the incubation of the cells with the CKD EVs and healthy controls EVs. As expected, the mitochondrial membrane potential is higher in healthy controls. Ahr antagonist exerts a protective effect, and the combination of EVs and IS decreases the mitochondrial potential more than EVs alone (Figure 32).

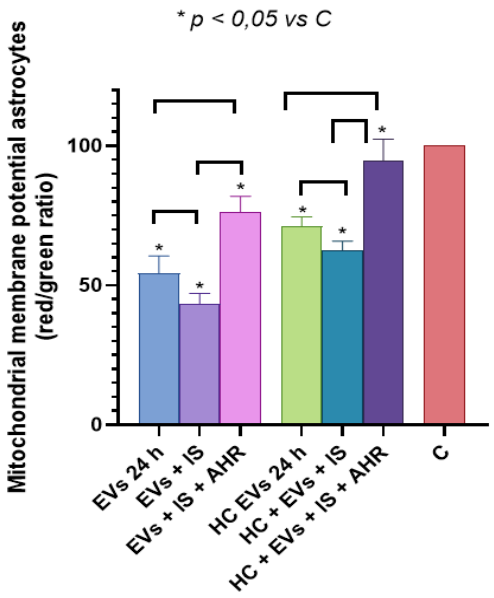


Figure 32: Effect on the change in HUVEC membrane potential after incubation with EVs, EVs + IS, and EVs + IS + Ahr antagonist. The data are normalized with respect to the control value. The data are reported as means ± SD of three independent experiments. The difference in viability was significant (p < 0.05). Abbreviations: HC: healthy controls; AHR: Ahr antagonist.

4.4.12 Quantitative and qualitative analysis of EVs before and after kidney transplantation

EVs were then isolated and characterized in a subgroup of 74 patients both at baseline and two years post-transplant. Nanosight analysis showed a clear reduction in the concentration of circulating EVs post-transplant, without indicating any difference in their size (160-180 nm at both time points considered; Figure 33a). Flow cytometry analysis was then performed for the characterization of EVs: two years after kidney transplantation, there was a significant reduction in the expression of the endothelial antigen CD31, CD40 (a costimulatory molecule), and integrins alpha 2 and 5, and beta 1 (Figure 33b).

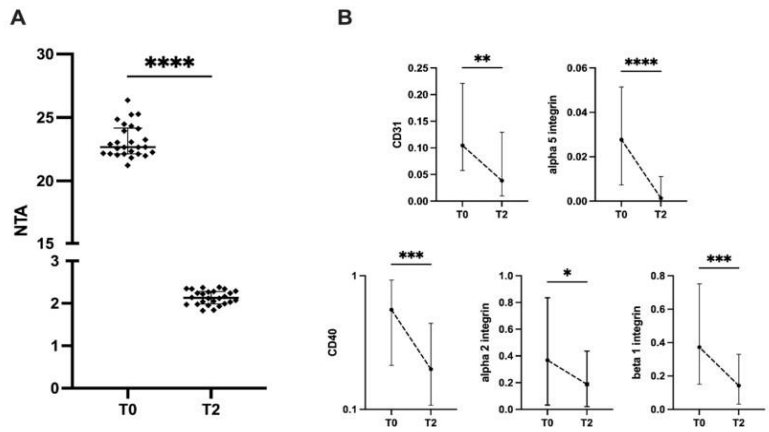


*Figure 33: (A) Nanosight analysis of plasma EVs before (T0) and after kidney transplantation (T2). (B) Flow cytometry of surface protein expression CD31, integrin alpha 5, CD40, integrin alpha 2, and beta 1. Variables expressed as median and IQR. *p < 0.05, **p <0.01, ***p < 0.001, ****p < 0.0001. Abbreviations: NTA: nanoparticle tracking analysis; CD: cluster of differentiation.*

4.5 BDNF levels pre and post kidney transplantation

For the evaluation of the protective effect of kidney transplantation on cardiovascular and neurocognitive alterations, 74 patients undergoing chronic hemodialysis who were candidates for kidney transplantation from a living donor were enrolled. The mean age at baseline was 46 years, and 70% of the study population was male. The analysis of laboratory data shows a significant improvement in systolic blood pressure, BMI, HDL cholesterol, and Apo-A1 values two years after the transplant (although with an increased use of statis).
The remaining characteristics are reported in Table 6.

Demographic characteristics	**Baseline (n = 74)**	**Two years (n = 74)**	***p* Value**
Age	46 (32-53)	48 (34-55)	
Sex (male)	52 (70)		
BMI	24.6(22.6-28), n = 31	25.3 (23.3-29.3), n = 31	0.0086*
PAS (mmHg)	138 (127–159), n = 34	132 (123–142), n = 34	0.0380*
PAD (mmHg)	86 (77–97), n = 34	80 (77–83), n = 34	0.0794
Dialysis seniority (years)	0.8 (0.6		
Comorbidities	-3.0), n = 39 62 (84)		
Pathologies CV		-	-
Diabetes mellitus	6 (8)	-	-
Current therapy			
Ca-antagonist	40 (54)	31 (37)	0.0358*
B blockers	48 (65)	45 (54)	0.1753
ACE-i/AT1 antagonist	42 (57)	62 (75)	0.0176*
Statins	20 (27)	53 (64)	<0.0001*
Laboratory exams			
Creatinine (mg/dl)	7.66 (6.53-9.65), n = 73	1.25 (1.09-1.53), n = 73	<0.0001*

Albumin (g/L)	35.5(32.8–38.0), n = 70	38 (36–39), n = 70	<0.0001*
Calcium (mmol/L)	2. 3 (2.1–2. 4), n = 68	2.4 (2.3–2.5), n = 68	<0.0001*
Phosphorus (mmol/L)	1.7 (1.3–2.0), n = 68	1.0 (0.8–1.1), n = 68	<0.0001*
Troponin T (µg/L)	21 (13-38), n = 62	8 (6–12), n = 62	<0.0001*
Triglycerides (mmol/L)	1.3 (1.0–1.8), n = 66	1.4 (1.0–1.7), n = 66	0.9272
Total cholesterol (mmol/L)	4. 5 (3.7–5.1), n = 66	4.4 (3.9–5.1), n = 66	0.4836
HDL cholesterol (mmol/L)	1.4 (1.0–1.7), n = 67	1.4 (1.2–1.9), n = 67	0.0069*
Apo-A1 (g/L)	1.40 (1.18–1.61), n = 65	1.49 (1.31–1.77), n = 6	0.0003*
Apo-B (g/L)	0.89 (0.71–1.01), n = 64	0.77 (0.65–0.97), n = 64	0.3044
Lp(a), mg/L	51 (14–122), n = 45	12 (10–32), n = 45	<0.0001*
HBA1c mmol/mol	35 (32–39), n = 63	37 (35–42), n = 6	<0.0001*

*Table 6: Characteristics of the study population. Variables expresses as median with interquartile range (Q1-Q3). Differences between baseline and two years post-transplant were analyzed using the Wilcoxon test; statistical significance at *p<0.05. Abbreviations: BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; CV: cardiovascular; Ca: calcium; ACE-i: angiotensin-converting enzyme inhibitors; AT1: Angiotensin 1; HDL: high-density lipoprotein; Apo-A1: apolipoprotein A1; Apo-B: apolipoprotein B; Lp(a): lipoprotein (a); HbA1c: glycated hemoglobin.*

At two years post-transplantation, there is an increase in BDNF values compared to baseline (1.3 ng/mL, IQR 0.8–2.0 vs. 1.1 ng/mL, IQR 0.8–1.5). The interassay coefficient of variation was 9% (Figure 34).

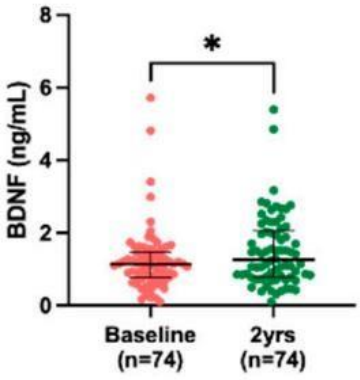


*Figure 34: Comparison of BDNF levels at baseline and 2 years post-kidney transplantation in the study population. Differences between baseline and two years post-transplant values were analyzed using the Wilcoxon paired test. Variables expressed as median with interquartile range (Q1-Q3). Differences between baseline and two years post-transplant were analyzed using the Wilcoxon test; statistical significance at *p<0.05.*

DISCUSSION

Cardiovascular and neurocognitive alterations are significant comorbidities in patients with advanced chronic kidney disease (CKD) and major contributors to mortality. Cardiovascular diseases are the leading cause of death in CKD patients, and CKD is recognized as an independent risk factor for these alterations. Conversely, neurocognitive alterations and depression are often underdiagnosed and pharmacologically undertreated, particularly in the dialysis population, despite their significant impact on quality of life, therapeutic compliance, and consequently, the rates of hospitalization and mortality. An important factor in the pathogenesis of these physiological alterations is the development of early vascular aging (EVA), a process of premature aging that is strongly correlated with chronic kidney disease (CKD). EVA manifests through vascular calcifications, impaired vasodilation, and endothelial dysfunction. Our research linked this phenotype to specific markers of senescence, calcification, and the expression of Nrf2, a key antioxidant involved in aging processes. Notably, CKD patients showed higher levels of the senescence markers p21CIP1 and p16INK4a, as well as the calcification marker RUNX2, and lower Nrf2 levels compared to healthy controls. The reduced Nrf2 expression in uremic patients may be due to oxidative stress caused by circulating uremic toxins such as Indoxyl Sulphate (IS), which has been shown to suppress Nrf2 expression in the kidneys. This suggests that the pro-inflammatory and pro-oxidant toxic environment is at least partly responsible for the diminished Nrf2 levels [65].
Our study investigated the pathogenic role of CKD-EVs in the development of EVA and neurocognitive impairment, along with the protective function of the neurotrophin brain-derived neurotrophic factor (BDNF) in maintaining neuronal plasticity and blood-brain barrier integrity. Additionally, we examined the positive impact of renal transplantation on vascular and neurocognitive outcomes by assessing changes in these biomarkers before and after the transplant.

5.1 Role of EVs in EVA and neurocognitive impairment

EVs are nanoscale particles enclosed in lipid bilayers, released by cells into body fluids and the extracellular matrix either under basal conditions or because of cellular activation or distress. They facilitate intercellular communication that contributes to the maintenance of cellular function and tissue integrity. EVs are implicated in numerous physiological and pathophysiological processes, including inflammation and immune response[66].
Increased production of extracellular vesicles (EVs) is a feature of several diseases, including antiphospholipid antibody syndrome, preeclampsia, acute coronary syndrome, and chronic

kidney disease (CKD). These conditions share common characteristics such as endothelial dysfunction and inflammation. This suggests that EVs could serve as a new marker of endothelial dysfunction in the uremic state and as mediators of these alterations. Consequently, EVs are hypothesized to play a significant role in early vascular aging (EVA) and the development of cognitive dysfunction, which are major comorbidities in CKD patients [67].

As already described in the literature, our data showed that in samples from CKD patients there is a higher concentration of EVs (1.3x10^12 ± 1.25x10^11 particles/ml) than in healthy controls (4.8x10^11 ± 1.0x10^11 particles/ml: uremic patients in chronic hemodialysis have a higher concentration of EVs than healthy controls and that this increase is linked to numerous factors related to CKD itself, including oxidative stress, chronic inflammatory status and alterations in mineral metabolism, but also to individual factors such as comorbidities and therapies.

Regarding the characterization of EVs, our results show a trend, albeit not statistically significant, towards an increase in the production of EVs by platelets and monocyte cells in CKD patients compared to controls, while there is a significant increase in endothelial derived EVs in CKD patients compared to healthy population.

These findings align with existing literature. Multiple studies, including the one by De Laval et al., have demonstrated that uremic patients on chronic dialysis exhibit an increase in EVs not only of endothelial origin but also derived from platelets (CD41; p-value = 0.039) and monocytes (CD14; p-value = 0.001) derivation[68].

The same study demonstrated that platelet- and monocyte-derived EVs from chronic dialysis patients showed increased membrane surface expression of TF (p-value = 0.001), Klotho (p-value = 0.003), and RAGE (p-value = 0.009), as well as p-selectin and sCD40-L (p-value = 0.045) on platelet-derived EVs, compared to those from healthy controls. Our data also revealed that EVs from CKD patients exhibit significantly higher levels of markers associated with apoptotic, inflammatory, and pro-coagulant mechanisms, such as Tissue Factor, C5b-9, CD40-L, ICOS, Fas-ligand, Class I HLA, and various proteins from the selectin and integrin families, compared to healthy individuals.

Another notable finding of this study is the consistent presence of five miRNAs in EVs from all enrolled CKD patients. Additionally, we employed a holistic network theory approach to infer potential miRNA regulation, capturing emergent properties of the miRNA-target regulatory network that might not be apparent through a pairwise analysis of individual components. We prioritized experimentally validated miRNA-target interactions based on statistical significance from a miRNA-target enrichment analysis, generating a network that illustrates the number of interactions for each miRNA.

MiR-940 emerged as a key regulator, binding all the 10 gene targets identified from the enrichment analysis. It is involved in various biological processes, including cell proliferation, apoptosis, cell cycle, migration and epithelial-to mesenchymal transition[69]. Furthermore, it has been shown to inhibit angiogenesis by reducing VEGF expression[70].

miRNAs miR-940 and miR-33b-3p, which were found to be expressed in 100% of the samples regulate Thymocyte antigen-1 (THY-1), also known as CD90. THY-1 is a glycosylphosphatidylinositol (GPI)-anchored glycoprotein expressed on activated endothelial cells, neuronal cells, fibroblasts, and in a subpopulation of hematopoietic stem cells in humans[71]. THY1 can have a role in lung fibrosis. It controls the apoptosis in lung and dermal fibroblasts via Fas-, Bcl-, and caspase-dependent pathways and Thy-1-deficient mice displayed increased fibroblast proliferation, decreased apoptosis, and downregulation of FasL[72]. THY-1 plays a crucial role also in immunological processes, as it is necessary for the recruitment and transendothelial cell migration of human leukocytes to sites of infection, inflammation, and tissue injury[73].

Through our transcriptomic analysis performed on miRNAs isolated from the EVs of 100% dialysis patients, we found that two of these miRNAs in particular, miR-940 and miR-33b-3p, target the dihydroorotate dehydrogenase (DHODH). This enzyme, located on the outer face of the inner mitochondrial membrane, plays a crucial role in inhibiting ferroptosis by detoxifying peroxidized mitochondrial lipids and in the de novo synthesis of pyrimidines[74].

Ferroptosis is a form of programmed cell death triggered by high levels of lipid peroxidation due to iron overload. Excess Fe2+ leads to the production of reactive oxygen species, which oxidize polyunsaturated fatty acids (PUFAs) in cell membranes[75]. This mechanism has been observed to be activated in macrophages residing within atherosclerotic plaques. Iron overload within macrophages destabilises atherosclerotic plaques and promotes the progression of atherosclerosis. Therefore, DHODH plays a role in inhibiting mitochondrial ROS production and lipid peroxidation, thereby preventing ferroptosis.

Two other genes, tropomyosin 3 (TPM3) and neurogranin (NRGN), which were found linked to miRNAs expressed in EV, have a function in vascular smooth muscle cells (VSMCs). TPM3 has been found to be modulated by the miRNAs miR-4515 and miR940.

Altogether the 5 miRNAs may constitute a cluster of molecules that can function as biomarkers of inflammation and fibrosis in hemodialysis patients.

Given the pathogenic role of EVs in endothelial dysfunction we hypothesised that the damaging effect could also affect the structures of the BBB leading to an increase in its permeability, a fundamental mechanism for triggering vascular senescence and cognitive impairment.

We incubated Human Umbilical Vein Endothelial cells (HUVEC) and astrocytes with CKD-EVs and healthy control EVs and we confronted them. Our findings revealed a significant decrease in cell viability in HUVEC and astrocytes stimulated with CKD EVs, which is associated with mitochondrial dysfunction leading to increased ROS production resulting in a worsened inflammatory state and oxidative stress. We then analyzed cell viability, mitochondrial membrane potential and mitoROS production in both astrocytes and HUVEC with 3 different conditions: stimulation with EVs alone, stimulation with EVs and Indoxyl Sulphate and then stimulation with EVs , Ahr antagonist and Indoxyl Sulphate. In both cell lines, the stimulation with EVs and IS decreased the cell viability more than the one with just EVs. Whereas Ahr antagonist exerted a protective effect against the toxicity of IS and EVs.

Incubating isolated vessel samples (used as a surrogate model for BBB studies) from healthy subjects with CKD-EVs, there is a statistically significant decrease in Occludin and JAM-1, and a non-significant declining trend in Claudin compared to controls. This disruption of tight junctions (TJs) may increase blood-brain barrier (BBB) permeability, potentially allowing uremic toxins like IS to cross into the brain and accumulate, thereby damaging neuronal and glial cells. EVs could facilitate this passage; previous studies have highlighted bidirectional EV exchange between blood and the central nervous system. Specifically, in neurodegenerative diseases, EVs from damaged CNS cells can cross the BBB to the blood, serving as disease biomarkers. Conversely, in chronic systemic inflammatory conditions like CKD, circulating EVs may cross the BBB due to increased permeability, reaching neurons, astrocytes, and glial cells, contributing to neuroinflammation and neurodegeneration typical of CKD and other chronic diseases.

This hypothesis suggests that EVs play a dual role in neurocognitive alterations: first, by promoting altered BBB permeability and endothelial dysfunction in brain vessels; and second, by entering the brain through this altered permeability to cause damage to neuronal and glial cells.

We then correlated the EVs with some of the main clinical parameters of vascular damage and with the results of neurocognitive and depression tests. These analyses revealed a significant correlation with the degree of arterial stiffness and IMT, confirming what is already present in the literature: in particular, EVs derived from endothelial cells (CD31+) and platelets (P-selectin+) showed the best correlation with arterial stiffness found in CKD patients.

5.2 BDNF levels analysis

BDNF is a neurotrophin recognized for its beneficial impact on neuronal plasticity and integrity. Reduced levels of this molecule have been associated with conditions including depression[76], neurodegenerative diseases such as Alzheimer's and Parkinson's, inflammatory conditions, sarcopenia, and the onset of insulin resistance[77]. Our analysis observed significantly reduced BDNF levels in chronic renal failure patients on hemodialysis compared to healthy controls. As mentioned previously, the uremic environment sustains an inflammatory state and heightened oxidative stress, significant risk factors for cardiovascular diseases and microvascular damage. This pervasive vascular impairment extends to the blood-brain barrier (BBB), where compromised permeability facilitates the entry of numerous uremic toxins (as previously noted, including EVs). This process contributes to the development of neurocognitive alterations frequently observed in hemodialysis patients.

Additionally, BDNF acts at the endothelial level, where its receptor TrkB is expressed, providing protection against vascular damage. Reduction in BDNF levels could therefore not only deprive neuronal cells of a protective factor but also leave the BBB more vulnerable to the harmful effects of toxic substances and EVs. In this context, our data highlight a significant correlation between low levels of BDNF and certain neurocognitive tests. Specifically, a correlation emerged with the presence of depression (assessed using the BDI scale) and cognitive impairment (assessed using the Mini-Mental test, MMT). These findings support existing literature: numerous studies have linked low levels of BDNF with poorer cognitive and depressive states.

The study by Sun et al. [78] demonstrated that in nephrectomies mice, depressive behaviors were associated with high levels of IS, a uremic toxin capable of inhibiting the expression of BDNF. In the same animal model, increased levels of p-cresol, another uremic toxin that inhibits the expression of the BDNF receptor, correlated with reduced BDNF levels. These changes were associated with depressive and anxious behaviors as well as worsening cognitive deficits.

5.3 Protective role of kidney transplantation

It is well known that kidney transplantation represents the best therapy for chronic kidney failure. Improved renal function allows for the elimination or reduction of the uremic environment and consequently mitigates the mechanisms of damage described so far. To

investigate the positive effects of kidney transplantation, we examined the levels of EVs and BDNF in a cohort of CKD patients treated at the Karolinska University Hospital before and after kidney transplantation. Of interest, at two years post-transplantation, we observed not only a significant reduction in the concentration of circulating EVs but also a marked decrease in the expression of pro-apoptotic, inflammatory, and pro-coagulant markers on their surface. Specifically, there was a notable reduction in the expression of CD31, CD40L, integrins alpha 2 and 5, and integrin beta1. These changes may be induced by the improvement in renal function, which limits inflammation, oxidative stress, and uremic toxin accumulation, thereby reducing the release of EVs and their harmful effects on microcirculation, particularly at the BBB, resulting in reduced cardiovascular risk and cognitive deficits. In this study, the positive effect of transplantation on the BBB and its integrity is also highlighted by favorable changes in BDNF levels. Our analysis reveals that two years after kidney transplantation, there is a significant increase in BDNF levels, supporting the hypothesis that improved renal function, and thus the elimination of the uremic environment, allows for higher BDNF levels. This increase, accompanied by a reduction in inflammation and oxidative stress due to improved renal function, may serve as a marker of enhanced BBB integrity. These data are supported by previous studies present in literature and they demonstrate the protective effect of the transplantation on cognitive functionalities: Harciarek et al. [79] demonstrated that one year after transplantation, hemodialysis patients experienced a significant improvement in cognitive functions and memory. Therefore, to improve patient outcomes, it is essential to facilitate kidney transplantation as much as possible, in order to eliminate the uremic environment early and limit its harmful effects.

STRENGHTS AND LIMITATIONS OF THE STUDY

A significant strength of this study is the correlation, in CKD patients, between the two biomarkers examined and the development of EVA and neurocognitive alterations. This aspect is often greatly underestimated in the clinical setting but has a profound impact on quality of life, therapeutic compliance, and patient mortality.

Another strength of our study is its longitudinal design in evaluating the population before and after transplantation, allowing each patient to serve as their own control, thereby excluding unknown confounding factors.

One limitation of the study is the small sample size, which may have impacted the statistical significance of some results. Therefore, these findings need to be confirmed through larger studies.

Another limitation is the lack of solid data in the literature regarding the actual correlation between BDNF and BBB integrity. This hypothesis is based on the known expression of the BDNF receptor on endothelial cells, but the complexity of replicating the BBB structure limits the possibilities for ex-vivo studies. Thus, confirmation of this correlation is necessary to study the BBB more thoroughly, even indirectly, through its markers of integrity and function.

FUTURE PERSPECTIVES

The traditional dialysis techniques do not allow the filtration of EVs and PBUTs because of their large size and protein binding. For this reason, we could hypothesize new therapeutic targets to counteract the effects of the uremic environment and EVs. For example, the CONVINCE [80] study has demonstrated that the use of specific dialysis techniques, particularly OL-HDF, can reduce cardiovascular risk through better clearance of medium-sized uremic toxins. Similarly, a comparable benefit could be obtained from the use of MCO membranes, utilizing internal hemodiafiltration, or adsorption techniques with membranes such as PMMA or the use of sorbents in series with the classic dialysis circuit. These methods are capable of removing PBUTs, which are among the main culprits of BBB and brain damage.

Our results showed an increase in BDNF concentration in patients who have undergone transplantation. A possible therapeutic approach can be administering BDNF with a minimally invasive approach such as trans-nasal delivery in pateints in maintenance chronic hemodialysis. Literature has demonstrated that the olfactory epithelium expresses the trkB receptor for BDNF, and it may be that receptor binding slows movement of drug into brain tissues. Another approach can be administering BDNF intravenously, so that it can pass by the systemic circulation.

Another interesting point can be to focus on the pathways in which EVs and PBUTs are involved. In our study, five miRNAs present in 100 % of CKD-EVs were highlighted: miR-33b-3p, miR-4515, miR-4665-3p, miR-6737-3p and miR-940; analysis of the interactions between these miRNAs and target genes shows involvement of pathways involved in apoptosis, extracellular matrix (ECM) production and renal fibrosis. One could speculate to design specific Antagomirs, chemically engineered oligonucleotides able to silence microRNAs and consequently to block their pathways.

The final aspect regards the EVs localization in the brain: to this end, we are conducting an ongoing study in collaboration with the Neuroscience group at the CNR in Pisa using an animal

model. Preliminary findings indicate that EVs from CKD patients, when injected into the choroid plexus of experimental animals, can cross the blood-brain barrier and localize to various areas of the central nervous system. Furthermore, direct intracerebral injection of EVs from CKD patients into the hippocampal regions appears to affect the animals' short-term memory processes.

CONCLUSIONS

In this study, we demonstrated that patients with advanced CKD exhibit increased production of EVs, which show elevated expression on their surface of molecules known to be involved in inflammatory, pro-apoptotic, and pro-coagulant processes (C5b-9, TF, ICOSL, FAS-L). Our ex vivo and in vitro studies confirm the toxicity of EVs towards endothelial and neuronal cells. This suggests that EVs in CKD patients may contribute to and promote the onset of EVA, thereby leading to cardiovascular and neurocognitive complications. These conditions are very common in our patients, often underdiagnosed, and have a significant impact on quality of life and mortality.

Conversely, we found that levels of BDNF, a neurotrophin known to be a protective factor against cognitive impairment and depression, and potentially also for BBB integrity, are reduced in the population of CKD patients undergoing hemodialysis. These levels are correlated with the development of neurocognitive impairment and depression. Therefore, BDNF could become an early marker of cognitive disorders in patients with advanced CKD.

Post-kidney transplantation changes in the biomarkers studied further confirm the protective role of kidney transplantation on cardiovascular and neurocognitive alterations. Removal of the uremic state results in a reduction in the concentration of EVs and the inflammatory and apoptotic markers expressed on their surface. This leads to a significant reduction in their harmful effects on BBB, endothelial, neuronal and glial cells, thereby reducing the risk of developing cardiovascular and neurocognitive disorders.

Additionally, the increase in BDNF levels allows for the restoration of the protective effect of this neurotrophin on neuronal and endothelial cells, particularly on the BBB, making it a potential predictor of improved cardiovascular and cognitive damage post-transplantation. These findings confirm how kidney transplantation enhances patient survival and improves quality of life, leading not only to a lower cardiovascular risk but also to an improvement in cognitive function. They also lay the groundwork for evaluating new therapeutic targets to counteract the effects of uremia and EVs.

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