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Erythropoietin resistance index and erythropoiesis stimulating agents use in haemodialysis patients with previous SARS-CoV-2 infection.

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Introduction

CKD is defined as kidney damage or a decrease in renal function lasting for three months or longer. Based on the estimated glomerular filtration rate (eGFR), it is divided into five stages. The stages range from Stage 1 (mild renal damage with normal or high eGFR) to Stage 5 (end-stage renal disease with very low eGFR requiring renal replacement therapy).
CKD can have various underlying causes. The most common causes include diabetes, diabetic kidney disease (DKD) and high blood pressure. Glomerulonephritis, polycystic kidney disease, kidney infections, autoimmune diseases, urinary tract obstruction and certain medications or toxins are all possible factors that can trigger CKD.

CKD is a common medical problem affecting millions of people worldwide. According to the Global Burden of Disease Survey data, about 9.1% of the world's population is affected by CKD.
In the last stages of CKD, it is necessary to start to consider Renal replacement therapies (RRT) to replace kidney function in these patients.
People with CKD have an altered immune system and are more susceptible to infections, which are worse compared to the general population.
The SARS-CoV-2 virus, the agent of the infectious disease COVID -19 infected about 600 million people and killed more than 7 million. This virus did not take long to spread among the most susceptible patients, including patients with CKD and on RRT, especially those on haemodialysis. Up to 37% of hemodialysis (HD) patients are at high risk of developing SARS-CoV-2, with need of hospitalization up to 88% and fatal cases of more than 20%[1]. These findings are significantly higher than documented rates in the overall population.

HD patients are highly susceptible to infection because they have a number of risk factors that make them the perfect "target" for the virus: a possibly advanced age, a high degree of comorbidity, possible immunosuppressive therapy, the use of "collective" transport ” to reach dialysis centres, and sharing a close environment with other patients and healthcare workers during haemodialysis treatment are just some of the many factors responsible for their higher susceptibility to infection risk. In addition, several studies show that these patients are more prone to develop a moderately severe form of the disease, which entails a higher need for intensive treatment and a higher mortality rate compared to the general population.

Moreover, patients with a past infection exhibit symptoms such as prolonged asthenia, muscular weakness, broad discomfort, and decline in subjective health status even after remission.

Patients with COVID 19 frequently experience anemia and a compromised iron metabolism[2]. In an observational study of 11,265 COVID 19 patients elevated D-dimer was linked to lower mean Haemoglobin (Hb) and greater serum ferritin. This also applies to patients with AKI and those who are on dialysis[3]. Although the precise processes of COVID 19 are not fully understood, individuals with severe COVID 19 frequently have a prothrombotic state and an extensive inflammatory phase. Erythropoietin-Stimulating drugs (ESA) effectiveness in these situations is matter of debat. Fishbane et al advised continuing ESA treatment at the same posology but with a lower Hb target (Hb 8–9 g/dL) in the case of maintenance dialysis[4]. The possible role of HIF-PHDs as COVID-19 defence mechanisms has been theorised by some other authors. Indeed, stimulation of the HIF1 pathway would inhibit the entry of the COVID 19 membrane spike protein associated with ACE2, thereby reducing the invasiveness of SARS-CoV-2[5].

Systemic inflammatory reactions, as they occur in severe COVID 19, limit iron availability in the case of iron supplementation.

Iron is also necessary for viral replication[6]. Patients with iron excess and viral infections have a poor prognosis. Consequently, limiting iron supplementation may be beneficial for patients with severe COVID 19, although further research is needed to clarify this point[7]. More authors proposed to promote trials to study the administration of EPO in severe cases COVID-19[8].

The aim of this study was to investigate the anaemia parameters and changes in the erythropoietin resistance index (ERI) in HD patients with previous SARS-CoV-2 virus infection.

Chapter 1: Chronic Kidney Disease

1.1 Epidemiology of Chronic Kidney Disease and Renal Replacement Therapy

Chronic kidney disease (CKD) is a progressive disease characterised by the progressive loss of kidney function over time. It is a significant public health problem worldwide and has a major impact on morbidity, mortality and healthcare costs. In fact, in the twenty-first century, CKD has become a leading cause of death and disability. The number of CKD patients has also increased due to the rise in risk factors such as obesity and DM. In 2017, an estimated 843.6 million people worldwide were affected by CKD[9]. Despite a decline in mortality among people with end-stage kidney disease (ESKD)[10], CKD has emerged as a major cause of mortality worldwide, according to global Burden of Disease (GBD) research[11]. It is therefore crucial that CKD is recognised. In 2017, there were 697 cases of CKD worldwide. Almost a third of CKD patients lived in two countries: China (132 million cases) and India (115 million cases). In 2017, 79 of the 195 countries covered by the Global, regional, and national burden of CKD had more than 1 million prevalent CKD patients[12] (Figure 1.).

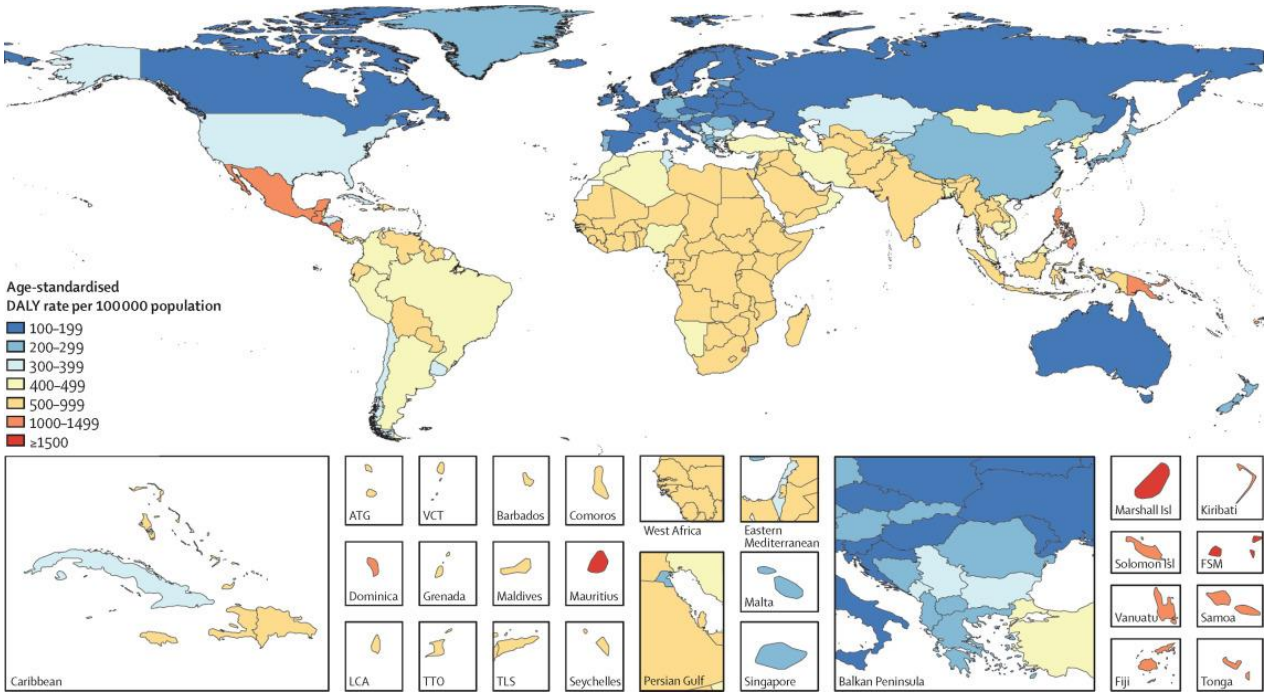
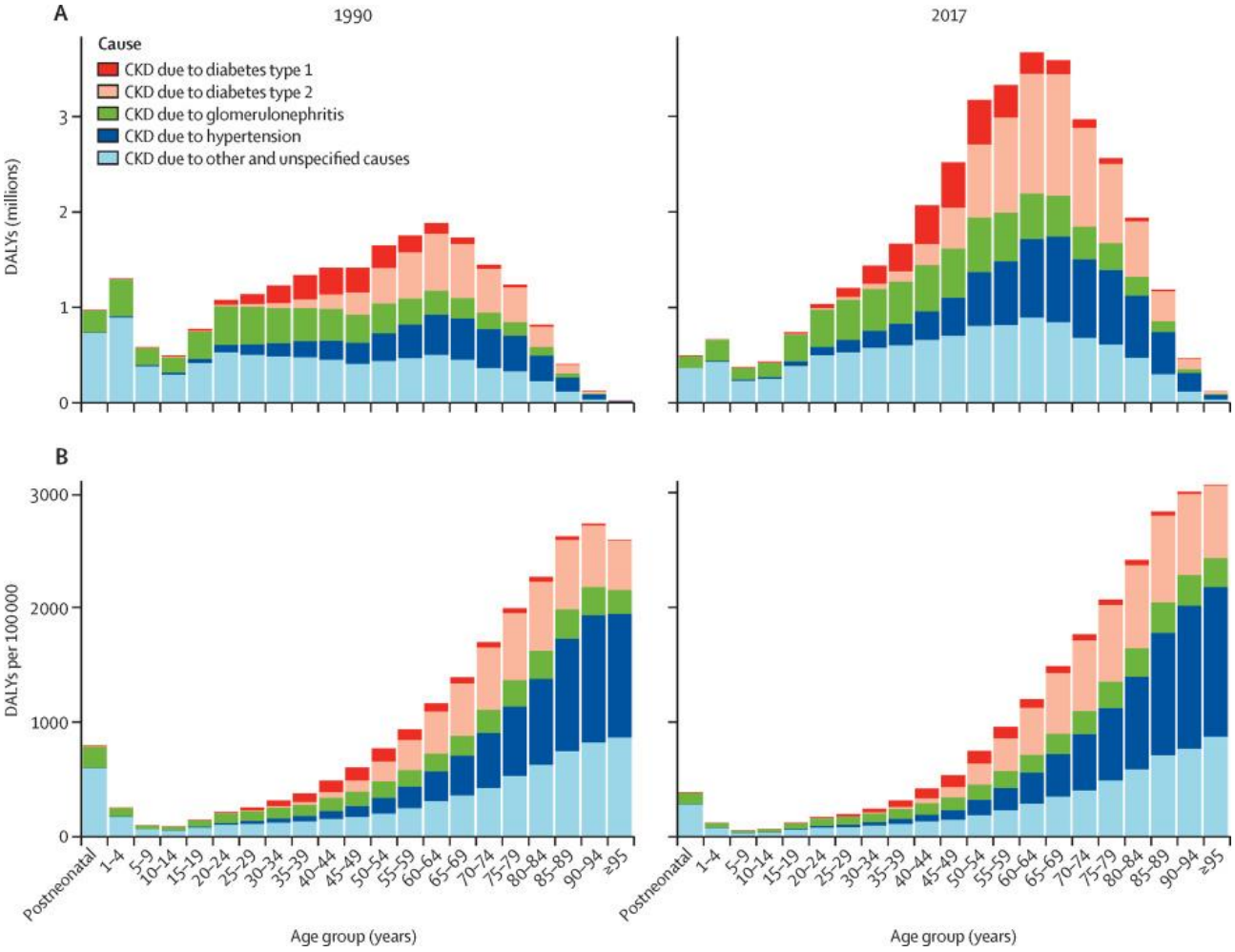


Figure 1 Age-standardised rate of disability-adjusted life-year. for CKD in 2017.

Moreover, CKD and its natural and progressive tendency to CKD are constantly increasing. Recent studies show that the global incidence of the disease has increased by 89% in the last 27 years, mainly due to socio-economic progress and higher life expectancy of the world population. Prevalence has also increased by about 87% worldwide over the same period[13]. These growth trends are mainly related to the progressive ageing of the world's population as a whole, but also, and more importantly, to the increase in the incidence of diseases such as arterial hypertension, diabetes mellitus and obesity, which inevitably lead to significant involvement and consequent impairment of the kidneys over time.

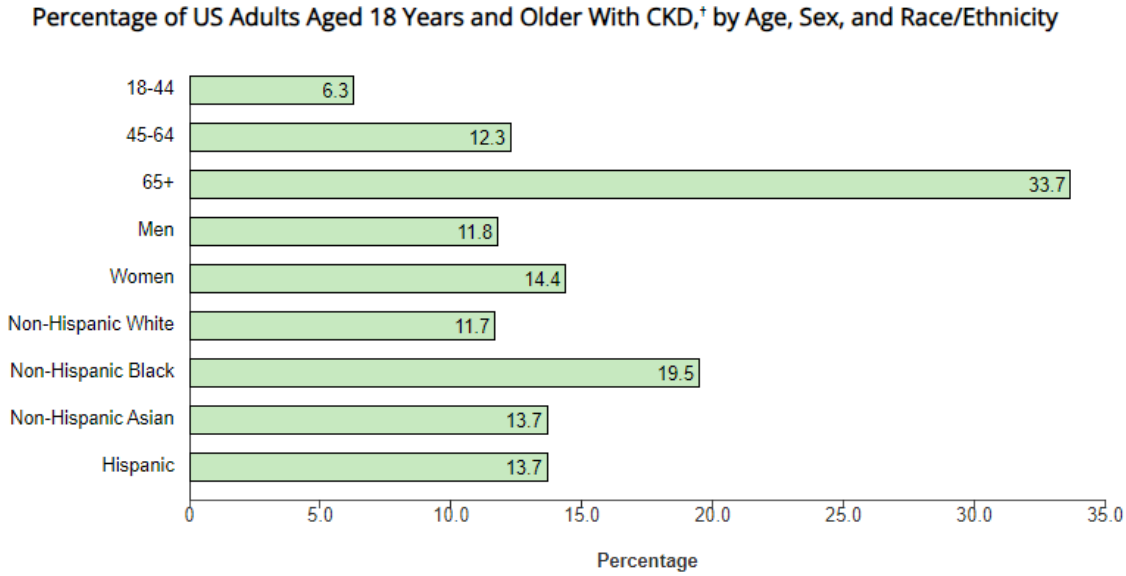
In this large study the main cause in terms of absolute number of disability-adjusted life-year from any cause was CKD secondary to diabetes, which

accounted for 30.7% cases. Type 1 and type 2 diabetes caused 29 million and 81 million (71 to 92) DALYs, respectively (Figure 2)



The mortality rate in CKD patients has decreased over time, due to the decrease in fatal cardiovascular and infectious events, the result of increasingly precise prevention of such episodes.

The Centres for Disease Control and Prevention stated that approximately 35.5 million people in the US, or 14%, will have CKD (Figure 3) [14].



†CKD stages 1–4 using data from the 2017–March 2020 National Health and Nutrition Examination Survey based on 2021 CKD Epidemiology Collaboration GFR estimating equation, including serum creatinine, age, and sex.

In Italy, the absolute prevalence of CKD is 7%, specifically: 4.16% in stages I- II and 2.89% in stages III. Regarding the latter stages, particularly in patients on RRT, according to the report of the Italian Dialysis and Transplantation Registry (RIDT)[15], managed by the Italian Society of Nephrology (SIN), the incidence in Italy is 162 pmp, of which 84.3% in haemodialysis, 14.3% in peritoneal dialysis (PD) and 1.4% in renal transplantation. According to RIDT, the incidence has gradually increased since 2010 before stabilising in recent years. However, this is an increasing trend that can also be observed in most other European registries. In fact, between 1999 and 2009, the incidence in Italy ranged between 130 pmp and 160 pmp. After 2009, however, the incidence increased progressively (in 2010

and 2011 the incidence was 168 pmp) and then remained almost stable for about six years with values between 158 pmp and 162 pmp.

1.2 CKD Classification

CKD is classified into five stages based on the estimated glomerular filtration rate (eGFR) and the presence of kidney damage. Kidney Disease: Improving Global Outcomes (KDIGO) divides CKD into five stages. Stages 1 and 2 call for kidney impairment, such as proteinuria. Glomerular filtration rates less than 60 ml/min/1.73 m2 for at least three months are required to be in stages 3-5. Stages 3 and 4 (GFR 59–15 ml/min/1.73m2) are regarded as the cutoff for clinically severe CKD since they indicate a decrease of 50% of normal renal function. Stages 3 A (from 59 to 45 ml/min/1.73m2) and 3 B (from 44 to 30 ml/min/1.73m2) of CKD are further separated. Stage 5 is for GFR less than 15 ml/min/1.73 m2[16,17] (Figure 3.).

GFR categories (ml/min per 1.73 m²) Description and range				Persistent albuminuria categories Description and range		
				A1	A2	A3
				Normal to mildly increased	Moderately increased	Severely increased
				<30 mg/g <3 mg/mmol	30–300 mg/g 3–30 mg/mmol	>300 mg/g >30 mg/mmol
	G1	Normal or high	⩾90		Monitor	Refer*
	G2	Mildly decreased	60–89		Monitor	Refer*
	G3a	Mildly to moderately decreased	45–59	Monitor	Monitor	Refer
	G3b	Moderately to severely decreased	30–44	Monitor	Monitor	Refer
	G4	Severely decreased	15–29	Refer*	Refer*	Refer
	G5	Kidney failure	<15	Refer	Refer	Refer

Progression to ESRD: Not all people with CKD progress to ESRD. The rate of progression depends on multiple factors, including the underlying cause of CKD, concomitant diseases and adherence to treatment and lifestyle changes. Timely recognition and treatment of CKD can help prevent the development of ESRD.

1.3 Personalized medicine in CKD

Personalized medicine approaches in CKD aim to tailor treatment strategies based on individual patient characteristics, disease progression, underlying causes, genetic factors, and other personalized factors. Personalized medicine focuses on early detection and risk stratification of CKD. Screening tests, such as measuring blood pressure, assessing kidney function through blood and urine tests (e.g., serum creatinine, eGFR, urinary protein), and evaluating risk factors, help identify individuals at high risk of developing CKD. Early detection allows for timely intervention and management to slow disease progression.

Accurate diagnosis and subtyping of CKD are essential for personalized medicine. Diagnostic tests, including kidney imaging (e.g., ultrasound, CT scan), kidney biopsy, and genetic testing, help determine the underlying cause and specific subtype of CKD. This information guides treatment decisions, as different subtypes may require distinct management strategies.

Certain types of CKD are influenced by genetic factors. Personalized medicine may involve genetic testing to identify specific gene mutations or variants associated with CKD. Genetic evaluation helps in understanding disease mechanisms, predicting disease progression, assessing familial risk, and guiding treatment decisions. In some cases, genetic findings may influence medication selection, dosing, or the need for genetic counseling.

Individualized lifestyle modifications are essential for the correct management of CKD. Factors such as diet, exercise, smoking cessation, and blood pressure control are tailored to each patient's specific needs and risk

factors. Dietary adjustments may include sodium and protein restriction, while exercise recommendations are based on the patient's overall health and physical capabilities.

Personalized medicine considers individual patient factors, including kidney function, comorbidities, and medication tolerability, when selecting and dosing medications. Certain medications may require dose adjustments or avoidance in patients with reduced kidney function. Pharmacogenomic approaches can also help identify genetic variants that impact drug metabolism and response, guiding personalized medication selection and dosing.

Diabetes, hypertension, and cardiovascular disease (CVD) are all common co-morbidities of CKD. These conditions and their interactions with CKD are taken into consideration in personalized medication. To improve overall health outcomes, treatment techniques are targeted to address both CKD and the related diseases. Personalized medicine involves risk prediction and monitoring disease progression over time. Various risk prediction models incorporate patient characteristics, biomarkers (e.g., eGFR decline, urinary albumin excretion), and genetic information to estimate future kidney function decline and the likelihood of complications. Longitudinal monitoring, including regular follow-up visits and laboratory tests, allows for timely intervention and adjustment of the treatment plan as needed.

Personalized medicine emphasizes patient education and shared decision-making. Patients actively participate in education about their disease, treatment alternatives and the associated risks and benefits.

Shared decision-making allows patients to align their treatment plans with their personal preferences, values, and goals of care.

It's important to note that personalized medicine in CKD is a rapidly evolving field, and treatment approaches can vary based on individual patient characteristics. Collaborating with healthcare professionals experienced in CKD management and participating in clinical trials or research studies can provide access to cutting-edge personalized treatment approaches.

1.4 Renal Replacement Therapy Modalities

RRT is vital for people with ESRD. The two main modalities of RRT are kidney transplantation and dialysis.

a. Kidney transplantation is regarded as the best therapeutic option for appropriate patients. Patients with ESKD who are placed on a waiting list and finally obtain a kidney transplant outlive those who stay on dialysis. In addition, people who undergo transplantation often have a higher quality of life (QoL) and a 10-year survival advantage over those who remain on dialysis. However, the availability of donor organs and the need for immunosuppressive drugs remain a problem in many nations.
b. Dialysis is a life-sustaining procedure used to equilibrate ions, remove toxins and excess fluid from the body when the kidneys are no longer able to do so. Dialysis is divided into two main types: HD and PD.
   HD is the preferred RRT for individuals in urgent need of dialysis and for many people as maintenance therapy, proving good and rapid

removal of solutes and fluid excess. One of the most important and first transport mechanisms used in dialysis is diffusion, the spontaneous movement of particles along a concentration gradient, stimulated by thermal motion and modified by the interaction of particles with barriers such as the pore walls of the dialysis membrane. When dialysis was used to replace renal function, ultrafiltration (UF), which could be produced by osmotic, oncotic or hydrostatic pressure, was necessary. Due to the effects of UF on solvent resistance, the importance of convective transport and its beneficial ability to accelerate the removal of larger molecular sizes was recognised. As a result, diffusive and convective transport are widely used in modern dialysis, and both processes can be used individually or in combination thanks to sophisticated machines and equipment. The development of novel haemodialysis membranes using nanotechnology, creative manufacturing techniques and recent advances in materials science are just a few examples of the new insights that will shape haemodialysis strategies in the future.

In PD the peritoneal membrane in the abdomen acts as a natural philtre. PD is a successful RRT that provides patients with many benefits, including greater scheduling freedom than inpatient HD. Other advantages of PD include the elimination of salt and water without significantly altering the patient's haemodynamics. Preservation of residual renal function is enhanced by the constant but gentle elimination of solutes and fluid. A more patient-centred strategy emphasises the value of individualised care, especially taking into account progressive PD and other lifestyle-dependent prescriptions. PD

remains a desirable, patient-centred form of renal replacement therapy and a valid choice for a growing number of patients.

Demand for RRT is increasing worldwide due to the increasing prevalence of CKD and ESRD. However, there are significant differences in access to RRT between high-income and low/middle-income nations. Many regions worldwide face challenges in terms of infrastructure, affordability and trained healthcare professionals to provide RRT services.

1.5 Altered immune response and inflammation in CKD

CKD is characterized by a high level of systemic inflammation, which complicates anemia control, QoL, and overall body homeostasis. Patients with CKD, especially in the end stage, have numerous comorbidities. ESRD subjects in particular are linked with a relevant increase in morbidity and mortality due to CVD (50% all cause mortality) and infections (20% all cause mortality).[18]. The increased infectious and, to some extent, cardiovascular risk is nothing more than the result of one of the many and varied homeostatic changes that occur in CKD and uraemia. Indeed, both conditions appear to be associated with dysregulations of the immune system, as CKD and subsequently the uraemic state are characterised by a state of immune dysfunction.

The immune system would thus show typical changes in terms of both innate or non-specific immunity and adaptive or specific immunity (cell-mediated and humoral). Moreover, these abnormalities, which are not yet fully known, have been found in some cases in early stages of CKD and thus not exclusively

in the end stage, have worsened with the progression of uraemia and have even been exacerbated and/or attenuated by dialysis procedures[19].

Uraemia is characterised by the accumulation of a large number of toxins in the plasma of patients, a condition due to the progressive impairment of renal function. These plasma components appear to be critical in the breakdown of both non-specific and specific immunity. The immunological diseases reported are numerous: for example, leukocyte function appears to be changed, with decreased chemotactic and phagocytic capability of polymorphonuclear cells and macrophages, hypoergia or anergy in delayed hypersensitivity reactions, and a decreased response to vaccinations. Such a scenario of immune compromise results in a greater tendency to infections for these patients.

1.6 Changes in innate immunity in patients with ESKD

Innate or non-specific immunity is a primitive mode of organismal defence that involves pathogen recognition, phagocytosis and digestion by monocytes, neutrophils and other leukocytes, induction of inflammation with the production of specific cytokines, and finally antigen presentation, a crucial event for the activation of adaptive or specific immunity.

Recognition is a rather rapid process that is triggered by the connection between the so-called "PAMPs" (Pathogen Associated Molecular Patterns), i.e. specific elements of each pathogen, or the "DAMPs" (Damage Associated Molecular Patterns), i.e. damaged and/or damaged parts of cells in the organism or necrotic cells, and the recognition receptors of these, the “PRR”

(Pattern Recognition Receptor), which is present on the surface of cells of innate immunity.

When PAMP-PRR binding occurs, the cells are activated and can carry out their activities. There are three types of PAMP recognition receptors: circulation receptors, endocytic receptors and signalling or transduction receptors.

- Circulating recognition molecules are mainly represented by the mannose-binding lectin (MBL) family, whose role is to bind to carbohydrates present on the surface of microorganisms and thus initiate the so-called "lectin pathway" of the complement system, leading to the destruction of the pathogen.
- Endocytic receptors are expressed on the surface of phagocytes and are represented by the so-called SR, "scavenger receptors", i.e. scavenger receptors whose task is to carry out the careful elimination of pathogens, but also of potentially harmful substances produced by the organism (think of necrotic cell debris, for example), from the bloodstream.
- Signaling or transduction receptors present on the surface of innate immunity cells recognize PAMPs and trigger the expression of a number of immune response genes, including those for proinflammatory cytokines, by activating signal transduction pathways. This class of molecule includes the "Toll Like Receptor" (TLR) receptor family. Indeed, by activating nuclear factor B (NF-B), the TLR has a significant impact on the initiation of the inflammatory response and serves as a "contact point" between non-specific and specific responses.

All three classes of recognition receptors show alterations in CKD and uraemia.

Regarding circulating molecules in particular, numerous studies have shown that the presence of varying amounts of lectins in plasma may correlate with higher morbidity and mortality in terminal CKD patients. Indeed, very high levels of mannose-binding lectins were found in the serum of patients awaiting transplantation, but not only that, it was also found that these same persistently high levels contributed to a reduction in survival after simultaneous kidney and pancreas transplantation. In contrast, very low lectin levels were found in patients undergoing HD who had recurrent infectious episodes, and in this case, they also correlated with an increase in mortality in these patients[20].

Regarding scavenger receptors, Chmielewski et al. and Ando et al. found that the expression of the two primary macrophage scavenger receptors, SR -A and CD36, is increased in patients with ESKD. This is mainly due to sustained activation as a result of inflammation and/or oxidative stress events, which are particularly pronounced in ESKD[21],[22]. In addition, these receptors perform an essential part in the formation and development of the characteristic atherosclerotic lesions seen in dialysis patients, contributing to increased cardiovascular risk. Namely, through the expression of several SR receptors on the surface, these cells phagocytose large amounts of oxidised LDL molecules and rapidly transform into "foam cells"," which represent the first step of atherosclerotic plaque formation.

Another important aspect is the dysregulation of signalling receptors in CKD. Indeed, uraemia impairs the ability of dendritic cells and macrophages to transmit antigens to T and B lymphocytes via alterations in costimulatory molecules, particularly CD80 and CD86. Since it has been discovered that TLRs control the production of these costimulatory molecules, it seems likely that disruption of TLR expression and/or activity causes dysfunction of

antigen-presenting cell (APC) activities. Indeed, pre-dialysis patients has been demonstrated to have constitutively decreased expression of TLR4, a characteristic molecule of monocytes and dendritic cells[23]. This would mean that due to the decreased expression of TLR4, fewer potentially pathogenic agents are recognised and consequently less intracellular signalling and processing of antigens takes place. This results in less expression of costimulatory molecules, which are essential for proper interaction between APC and lymphocytes, causing the lymphocytes to enter a state of anergy and the immune system to respond inefficiently.

Ando et al. have performed studies demonstrating altered expression and/or activity of TLR4 in innate immune cells, focusing mainly on the functionality of monocytes in the uraemic context. These studies have shown that monocytes from dialysis patients are extremely hyporeactive to LPS, a TLR4 ligand (lipopolysaccharide, component of the outer cell membrane of Gramme - bacteria) compared to healthy subjects, with the levels of IL -1 and TNF- produced by the same stimulated monocytes being significantly lower than those of the healthy control group[24]. Furthermore, monocytes and monocyte-derived dendritic cells have been shown to have not only reduced endocytic capacity, but also reduced maturation and activation when cultured in uraemic serum or when obtained directly from the serum of patients with ESKD[25].

Other immune cells such as neutrophil granulocytes also show changes in CKD and uraemia. Anding et al. reported that the bactericidal capacity of these leukocytes is reduced in HD individuals compared to healthy subjects[26]. Because the HD process restored some of this capability, the authors assumed that some dialysable substances were responsible for these modifications. Indeed, this neutrophilia impairment appears to be the outcome of the effects

of uraemic retention solutions on the balance of apoptosis and survival in these cells[27]. Indeed, some uraemic retention solutions may delay apoptosis, such as Ig light chains, while others favour the process. The latter include advanced glycation end products such as oxidised low-density lipoprotein (ox-LDL) and TNF-α. These results seem to indicate that this uraemic milieu seem to contribute to the changes in neutrophils and other immune cells.

The progressive reduction in renal function also leads to a "hypercytokinaemia" status involving both anti-inflammatory cytokines such as IL -10 and pro-inflammatory cytokines such as TNF-α and IL -6, which contributes to creating a highly inadequate environment for an efficient immune response[28].

1.7 Changes in adaptive immunity in patients with ESKD

Acquired or adaptive immunity is a form of highly specialised body defence that includes the activity of T lymphocytes and B lymphocytes. While innate immunity is a non-specific defence activity, acquired immunity is instead a process defence that recognises some specific parts of the pathogen, the so-called antigens, and is therefore an extremely tailored defence for a specific pathogen. Moreover, in contrast to innate immunity, it has a kind of "infectious information archive" or the so-called "immunological memory" function", which is known to be exercised by the B lymphocytes and their antibody production.

The disorders of acquired immunity in patients with CKD and uraemia undergoing RRT are many and varied, as has been found with the disorders of innate immunity.

Several studies have shown that the proliferation of T lymphocytes is reduced in the uraemic milieu and that there is also a shift in the Th1/Th2 ratio in favour of the former. T helper cells (Th or CD4+) have an important role in controlling the immune response and produce many "important" inflammatory cytokines, including TNF-, IL -12 and IFN-. Th2 cells, on the other hand, primarily produce IL -4, IL -13 and IL -5. They influence the immune response differently by producing different cytokines: Th1 lymphocytes activate macrophages, neutrophil granulocytes, dendritic cells and cytotoxic lymphocytes (CD8+) and enhance cell-mediated immunity, while Th2 cells are involved in activating the second arm of humoral immunity.

With regard to the changes in these cells, it was shown that in patients with PD, the maturation of both subsets of Th cells is reduced not only compared to healthy controls, but also compared to patients with HD, who had lower levels compared to healthy controls. Eleftheriadis et al. discovered that the quantitative alteration of lymphocytes is in the antigen-presenting cells (APCs), which have lower co-stimulatory activity, resulting in poor proliferation and activation of these cells in PD and HD patients[29].

The maturation and number of Th lymphocytes is reduced in HD patients compared to healthy controls, but dialysis patients have a significantly higher proportion of Th1 compared to Th2, resulting in an "imbalance" of the Th1/Th2 ratio in favour of the former.

Since the cytokine environment is fundamental for Th lymphocyte differentiation, a possible explanation for this altered ratio could be a higher production of IL -12, a cytokine produced by both APCs and Th lymphocytes themselves, which acts as a "third signal" (after the presentation of the antigens, resp. of the first signal and the costimulus or second signal) acts on the differentiation of Th lymphocytes by causing an increase in the secretion of INF -γ and a decrease in IL -4 by Th lymphocytes, thus promoting their differentiation into the Th1 type compared to Th2[30]. This thus leads to a stronger activity of cell-mediated immunity compared to humoral immunity with the resulting production of antibodies.

In ESKD, in addition to a weak activation of humoral immunity, it is possible to find a condition of B cell lymphopenia. This situation, according to several studies, in particular the one conducted by Fresnedo et al. it seems to be linked to accelerated cellular apoptosis which has been widely studied, however, in vitro[31]. The study was conducted by collecting B cells from the peripheral blood of both patients already undergoing HD and pre-dialysis and cultivating them in vitro without stimuli for a period of approximately 96 hours. The results demonstrated that B lymphocytes from both patient groups had a higher rate of apoptosis than healthy controls, and that this increased susceptibility to cell death was linked, at least partially, to the patients' reduced expression of an anti-apoptotic protein localized at the mitochondrial level, namely Bcl-2. Despite the importance of this study, a direct effect of apoptosis on B lymphocytes in vivo has yet to be demonstrated. This phenomenon is unfortunately difficult to study, as apoptotic cells and their remaining components are quickly eliminated from circulation by nearby phagocytic cells.

All these data suggest that people with ESKD are more susceptible to infections because of their altered immune response. There is an imbalance between cell-mediated and humoral responses, which leads not only to a qualitative but also to a quantitative change in T and B cells.

CHAPTER 2 Anemia in CKD

2.1 Renal Anemia

Anaemia, which is often normocytic, normochromic and hypoproliferative, is a common consequence of CKD. It is often associated with poor CKD outcomes and higher mortality, in addition to other CKD problems.

Anaemia worsens as kidney disease progresses and affects almost all people with stage 5 CKD. Anaemia caused by CKD often impairs QoL and increases the risk of CVD, cognitive impairment, hospitalisation and death.

The production of the hormone erythropoietin (EPO), which stimulates red blood cell production, is impaired in the course of CKD, and this is the largely recognised aetiology of anaemia in this cohort of patients.

Hypoxia-inducible factor (HIF), a transcription factor that regulates the expression of the erythropoietin gene, has recently been linked to decreased erythropoietin production[32]. Other factors include iron deficiency, folic acid deficiency, bleeding caused by platelet dysfunction, inflammation, shortened red blood cell lifespan, impaired utilization of iron, uraemia and unusual blood loss after dialysis[33]. Hepcidin overproduction impacts the absorption of iron from diet and the release of iron from body storage, which is an important cause of anemia and inadequate iron management in CKD.

Anaemia in CKD can cause symptoms such as fatigue, weakness, shortness of breath, dizziness, pale skin, and difficulty concentrating. This symptomatology can have a significant impact on QoL and daily activities.

Anaemia is diagnosed through blood tests that measure Hb levels and other parameters such as haematocrit, ferritin, transferrin saturation and red blood

cell count. Additional tests may be performed to evaluate, vitamin B12, and folic acid levels.

2.2 Anaemia management for CKD patients

The Kidney Disease Improving Global Outcomes (KDIGO) recommendations are the current standard of care for anaemia in CKD. The KDIGO recommends measuring Hb in people without anaemia at different intervals, depending on the patient's demographic and medical condition. Anaemia is diagnosed at an Hb level of 13.0 g/dL in men and 12.0 g/dL in women.

After confirming a diagnosis of anaemia, the following action is to exclude out any other possible reasons. Anaemia in CKD can be treated with iron supplementation, ESAs, and/or red blood cell (RBC) transfusion. Key factors for commencing and monitoring anaemia therapy in CKD are also discussed. The dangers as well as advantages to patients should be evaluated for all therapies, and the lowest effective dose is normally suggested to correct anaemia.

Anaemia in CKD is treated by increasing Hb levels and relieving symptoms associated with this status.

Patients with CKD typically suffer from chronic inflammation that can be attributed to a variety of causes. CKD patients typically respond poorly to ESA and fluctuating Hb levels, both of which can be caused by persistent inflammation. Understanding how inflammatory cytokines affect erythropoietin formation and hepcidin synthesis can help us better understand the complex web of interactions between the various components involved in

the aetiology of chronic disease-related anaemia. Pharmacological treatments for inflammation-related hyporesensitivity to ESA appear to be moving towards anticytokine and antioxidant therapies[34].

Approaches may include:

a. Iron supplementation: Iron deficiency is common in CKD-related anemia. To balance iron levels and promote red blood cell formation, oral or intravenous iron supplements can be given. Patients with CKD may be iron deficient in both absolute and relative terms. Blood loss can also be caused by blood that remains in the HD circuit.

Furthermore, the "uraemic" condition, as well as other comorbidities causing inflammation to hinder the release of iron from the body's reserves as well as adequate intestinal iron absorption. Proinflammatory cytokines cause functional iron deficiency in many ways. They increase pical divalent metal transporter 1 expression in macrophages, hepcidin synthesis in the liver, ferritin expression and ferroportin inhibition. The kidneys also excrete hepcidin, and when eGFR decreases, the amount excreted also decreases. All these processes favour intracellular iron storage and thus limit iron availability in CKD.

b. ESAs are synthetic erythropoietin analogues that stimulate the bone marrow to create more red blood cells. When iron reserves are sufficient but Hb levels are low, ESAs are used. The dose is carefully controlled to keep Hblevels within therapeutic limits.

c. Blood transfusions: Blood transfusions may be required to rapidly improve Hb levels in severe cases of anaemia or when other therapies are unsuccessful. It is important to avoid unnecessary blood transfusions in prospective kidney

transplant patients because numerous blood donations can lead to alloimmunisation against class I human leukocyte antigens (HLA) on white blood cells. HLA antibodies can interfere with transplanted kidneys, increasing the risk of acute rejection and reducing long-term graft survival. Since the introduction of universal leukocyte depletion of blood components and the use of ESA in CKD, the risk of alloimmunisation has decreased.

Monitoring: Treatment of anaemia in CKD requires regular monitoring of Hb levels and iron status to establish the adequate EPO and iron supplementation dosage. Managing anaemia can improve QoL, relieve symptoms and possibly halt the progression of CKD.

2.3 Erythropoietin treatment for renal anaemia

Since erythropoietin-producing cells can transdifferentiate into myofibroblasts during disease and cause their own fibrosis, they are closely associated with fibrotic processes[35]. In addition, EPO has a number of pleiotropic effects on the kidney, including decreased apoptosis and inflammation, decreased fibrosis and decreased tubular regeneration[36]. In addition, EPO seems to lower oxidative stress in experimental models of glomerulonephritis[37] and DKD[38].

Epoetin was the first EPO analogue to be made available, shortly followed by Epoetin. It is produced in cell cultures using recombinant DNA technology. Methoxy polyethylene glycol epoetin beta and darbepoetin alfa (DA) were produced later and had an extended half-life. Biosimilar versions of the original epoetin have been launched recently.

There are different types of ESAs. For Non-dialysis CKD patients taking long-acting ESAs, their different pharmacokinetic and pharmacodynamic features permit a lower weekly dose and easier administration. Although the conversion factor between short-acting and long-acting ESAs is probably not linear, it should not be forgotten[39]. However, several Cochrane meta-analyses indicate that there is insufficient evidence to support the superiority of any ESA formulation or administration pattern based on efficacy and safety data[40],[41].

As for these results, a number of observational studies have yielded contradictory findings. For example, the Japanese Dialysis Registry study found that patients receiving long-acting ESAs had a 20% higher risk of dying from any cause than patients receiving short-acting ESAs[42]. On the other hand, Non-dialysis CKD people receiving short-acting ESAs at high doses had a higher risk of mortality and progression to ESKD, according to an Italian observational study[43]. A recent RCT comparing monthly administration of continuous erythropoiesis receptor activator with the shorter-acting drugs epoetin alfa/beta and Darbepoetin alfa showed no inferiority in terms of Hb target attainment, serious adverse cardiovascular events or all-cause mortality among study participants.

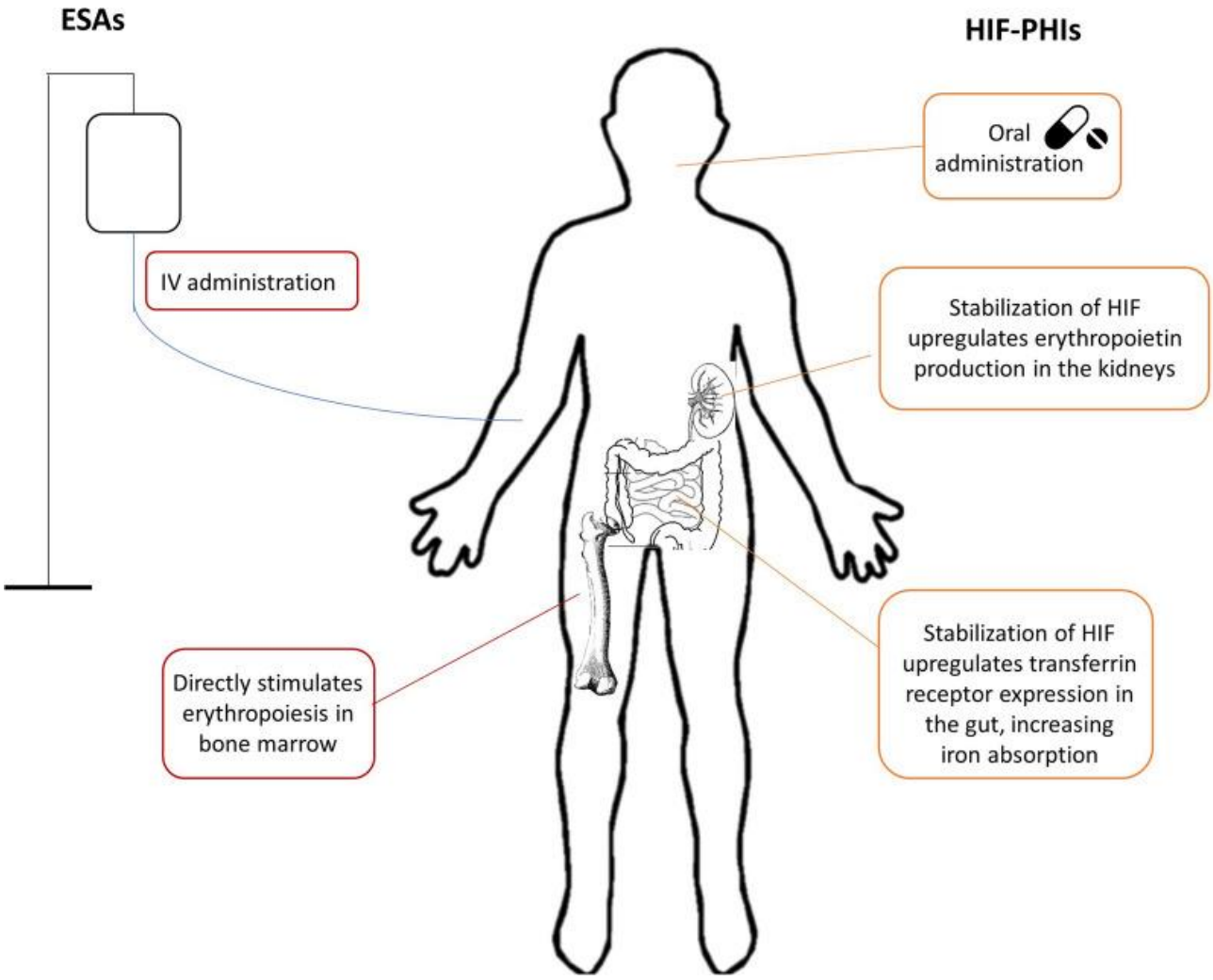
However, it was found that regardless of the medication prescribed, patients who did not reach Hb values >10 g/dL or who received ESA doses in the highest quartile had a higher risk of cardiovascular events or fatal events[44]. Further RCTs are needed to evaluate the different ESA formulations and modes of administration, especially in patients requiring higher ESA doses.

2.4 New Frontiers of anaemia treatment in CKD: HIF

Hypoxia-inducible factor (HIF) plays a significant role in the pathophysiology of CKD. HIF is a transcription factor that regulates the cellular response to low oxygen levels (hypoxia). In the context of CKD, HIF activation is observed due to the impaired oxygen supply and tissue hypoxia that occurs as a result of progressive kidney damage. In response to hypoxia, HIF regulates the expression of numerous genes involved in cellular adaptation and survival. It promotes angiogenesis to improve oxygen delivery, stimulates glycolysis, and modulates cell proliferation and apoptosis. CKD is characterized by the accumulation of extracellular matrix proteins, leading to renal fibrosis. HIF has been found to contribute to this process. It promotes the activation of fibroblasts, which produce excessive amounts of collagen and other components of the extracellular matrix, leading to fibrosis and scarring of the kidney tissue.

Moreover, HIF has been shown to interact with inflammatory pathways and contribute to the inflammatory response in the kidneys. It can modulate the production of pro-inflammatory cytokines and chemokines, as well as the activation of immune cells, such as macrophages.

In addition, HIF activation occurs as a compensatory mechanism to increase EPO production and restore erythropoiesis (Figure 4.).



(Figure 4). Actions of ESAs and HIF-PHIs[45].

Given the role of HIF in CKD, targeting HIF pathways has emerged as a potential therapeutic strategy. HIF stabilizers, such as prolyl hydroxylase inhibitors (PHIs), have been investigated as a means to enhance HIF activation and potentially mitigate the progression of CKD. These agents aim to mimic the hypoxic response and promote cellular adaptation and tissue repair.

The PHD family is made up of three different enzymes: PHD1, PHD2, and PHD3. In normoxia, PHD2 is the main HIF function modulator[46],[47]. HIF-PH activity in normoxia causes HIF to degrade quickly, whereas in hypoxia, this

activity is suppressed by HIF formation and direct EPO stimuli, which increases the expression of transferrin receptors, boosts iron uptake from proerythrocytes, and promotes the growth of erythrocytes with Hb. For the regulation of hematopoietic stem cells, the HIF-1a subunit is essential. They replenish the body's erythrocyte population in response to stress. HIF-PH inhibitors have been demonstrated to induce hematopoiesis in in vivo studies, suggesting a distinct mode of action from typical ESAs[48].

The effects of HIF-PHI on the correction of anaemia have been summarised in a number of systematic reviews and meta-analyses. Thirty phase 2 and 3 trials with a total of 13,146 patients across the spectrum of CKD were included in a recent and comprehensive meta-analysis[49]. The HIF-PHIs utilized were roxadustat, daprodustat, desidustat, enarodustat, molidustat and vadadustat, 21 on placebo and 17 on ESA treatment as the comparator medication. HIF-PHIs significantly increased Hb compared with placebo or ESAs according to the researchers.

HIF-PHIs appeared to be more successful in young people than in older patients and in studies with less than 40% diabetics compared to studies with a high prevalence of diabetes. This also applies to the placebo-controlled trials, which showed the same effect on Hb changes regardless of patient population and treatment duration.

Compared with the ND-CKD group, Hb changes recorded with HIF-PHIs were greater in dialysis patients. Studies longer than 24 weeks showed a significantly greater Hb increase with HIF-PHIs compared with ESA comparators; however, as mentioned earlier, this effect may be related to the drug doses chosen for comparison rather than differential efficacy. Finally, patients younger than 60 years showed a better Hb response than patients between 60 and 65 years and patients older than 65 years.

In their network meta-analysis, Zheng et al. compared the effect of six HIF-PHIs (vadadustat, desidustat, enarodustat, molidustat, roxadustat, daprodustat) with epoetin and darbepoetin in CKD-related anaemia. With the exception of vadadustat, all other HIF-PHIs significantly increased Hb levels compared to placebo. There were no differences in the increase in Hb between these drugs. Furthermore, there was no discernible association between the drugs and all-cause mortality compared to placebo. No differences were found in all-cause mortality between the drugs. HIF-PHIs are effective and well tolerated in the treatment of anaemia patients with CKD who are not undergoing dialysis[50].

CHAPTER 3 Covid-19 and CKD

3.1 The Global Burden of COVID-19

The SARS-CoV-2 virus story begins in December 2019 in Wuhan, China, where the first groups of patients with the clinical picture of bilateral interstitial pneumonia of unknown cause were identified. In January 2020, the WHO (World Health Organisation), having identified the agent responsible for the disease, declares the epidemic of the so-called "Severe Acute Respiratory Syndrome Coronavirus 2" (SARS-CoV-2) a public health emergency of "international concern".

In March 2020, after the virus had spread to large parts of the world, that the WHO COVID -19 declared it a pandemic.

After the emergence of SARS-CoV and MERS-CoV, the pathogens of SARS and Middle East Respiratory Syndrome respectively, SARS-CoV-2 is the third zoonotic human coronavirus that the entire planet has had to deal with and is still learning to live with today.

SARS-CoV-2 is part of the large coronavirus family, specifically it belongs to the order of betacoronaviruses. It is a positive single-stranded RNA virus with an envelope that is mainly, but not exclusively, airborne. In addition, its genome shares 79% of the genome sequence of the SARS-CoV virus and 50% of the genome sequence of the MERS-CoV virus[51].

The genetic material of the virus has gene sequences that code for specific proteins, which can be divided into:

- structural proteins

- non-structural proteins

The former include: the spike protein (S), the pericapsid protein (E, from the envelope), the membrane protein (M) and the nucleocapsid protein (N), which, among other things, is the only protein that can bind to the viral genome. The non-structural proteins (NSP), on the other hand, are a set of enzymes and more that are able to regulate and direct the processes of viral replication and assembly.

The S protein covers the entire surface of the virus and gives it the typical appearance of a "crown". In addition, this protein appears to be covered by countless carbohydrate molecules, especially glycans, which act like a "shield" for the virus, protecting it from possible recognition by the immune system. This protein is a trimer, it consists of 3 completely identical units called "protomers". Each protomer is composed of two functional subunits:

- The S1 subunit, which has an amino acid sequence known as RBD, “Receptor Binding Domain”. This protein part is essential for the correct recognition and subsequent binding between the virus and its receptors on the surface of the cells it will infect.
- The S2 subunit, which contains a number of amino acid sequences that are extremely important for the fusion and entry of the virus into the host cells. These are mainly: the fusion peptide (FP), HR1, HR2, the transmembrane domain and the cytoplasmic domain.

The S protein, in particular the RBD region of subunit 1, has two conformations: the “up” conformation (top) and the “down” conformation (bottom). The difference between the two is quite simple: in the "down" state, i.e. when the virus is not infecting host cells, the receptor-binding domains (RBDs) do not protrude from the viral surface, which is instead appreciated in

the "on" state. The latter state is necessary for the virus to properly fuse with host cell membranes and enter their cytoplasm.

The virus uses the RDB sequence of the S1 subunit to bind to the host cell. It is now known that this part of the protein interacts with a receptor located on the cell membrane of host cells called "ACE2", an enzyme homologous to ACE, the protein responsible for converting angiotensin I into angiotensin II. In the human organism, ACE2 is mainly found on the surface of the plasma membrane of pneumocytes, enterocytes, myocardial cells and kidney cells. This association between the spike protein and ACE2 occurs primarily through the conformational change of the RBD region of subunit 1 of the S protein, which transitions from the "down" state to the "up" state, resulting in the RBD-ACE2 interaction and subsequent fusion between the two membranes and finally, the subsequent entry of the virus into the host cell cytoplasmic environment through subunit S2. All this is made possible by various "protein cuts" of the spike structure mediated by enzymes such as furin and serine protease transmembrane 2.

The spike protein is the main feature of vaccines given to the world's population. This includes the Comirnaty BioNTech/Pfizer vaccine, an mRNA vaccine designed precisely to induce the production of anti-neutralising antibodies in the body. -The spikes are directed against the RBD epitope of subunit 1.

The complex of viral RNA and N-protein forms the so-called “nucleocapsid”. It is interesting to note that the N protein is highly conserved in coronaviruses: the protein of SARS-CoV-2, for example, has an amino acid sequence that is 90% identical to that of SARS-CoV.

The virus is highly contagious and is mainly transmitted via aerosols and "droplets" or particles emitted by infected people when they cough or simply

breathe. Therefore, measures have been taken to limit transmission. Prevention measures that have become an integral part of everyday life in the last two years, namely the use of masks and gloves, keeping a safe distance (at least 1 metre) and proper hand hygiene. To a lesser extent, infection can also occur through surface contamination by droplets.

After an incubation period of about two weeks, the virus presents with a range of signs and symptoms of varying severity. The people most likely to become infected are predominantly male and of advanced age. Another important risk factor for the disease, in addition to age and gender, is the high degree of comorbidity that the patient may have[52].

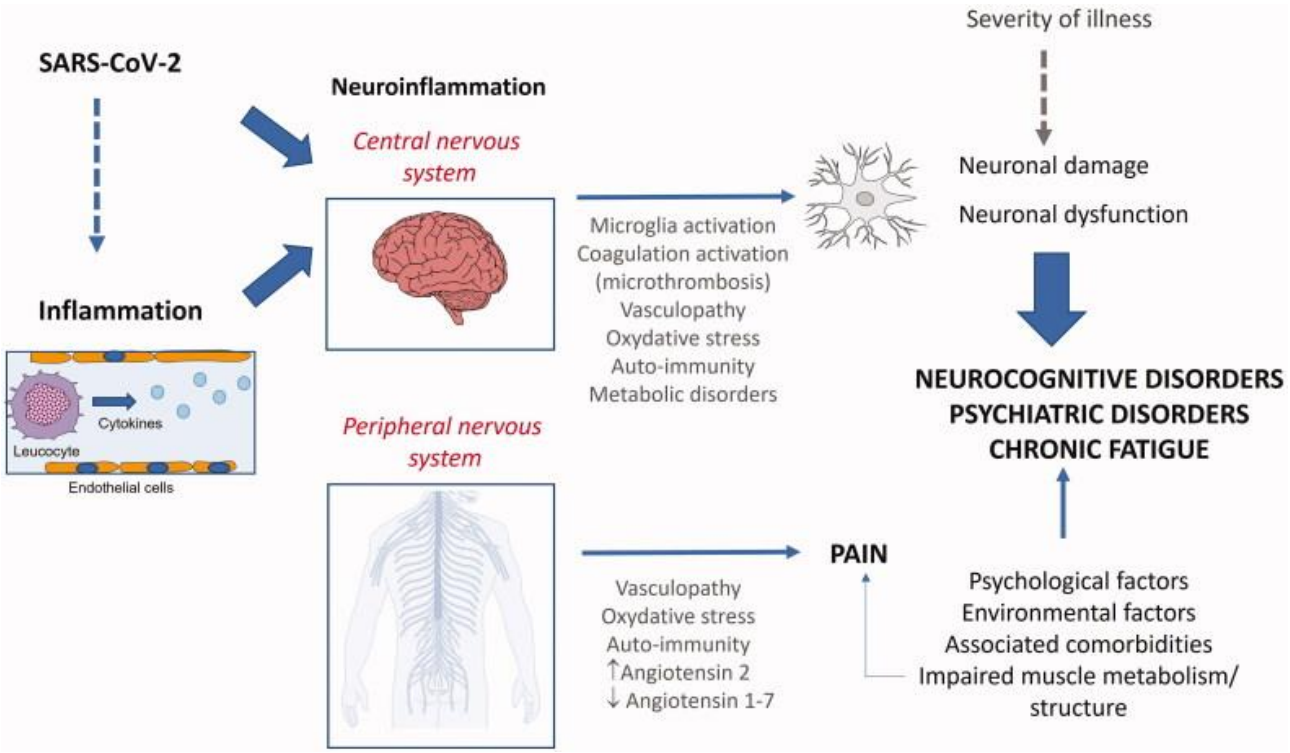
The most common clinical manifestations of COVID -19 in the general population are mainly fever, cough, asthenia, anosmia and ageusia, i.e. flu-like symptoms. Less common are sore throat, headache, osteo-muscular pain, nausea and diarrhoea. In the majority of patients, especially those most at risk, a picture of bilateral interstitial pneumonia with typical faintness opacities is seen on the chest X-ray, but much more clearly on CT.

Based on the severity of the clinical picture, the disease can be divided into three stages: mild, severe and critical. The increasing severity is mainly related to the impressive inflammatory response that occurs in some patients, leading to the production of an impressive amount of cytokines, especially IL -6, the so-called "cytokine storm", which is responsible for the most severe clinical pictures. Moreover, in the most severe cases of COVID -19, the complement system, one of the main players in innate immunity, also seems to be involved. Several studies now state that the N protein of the virus activates the lectin pathway through its interaction with the MASP2 protein. Others suggest that the SARS-CoV-2 spike protein activates the alternative pathway through interaction with C3. However, regardless of the initial

activation, the complement system converges in cleaving C3 and subsequently C5 to form a series of molecules that facilitate thrombosis, chemotaxis and vasodilatation (C3a and C5a). Lina Ma et al. have also shown that circulating markers of complement activation are greatly increased in patients with COVID -19 compared to patients with respiratory illnesses not associated with SARS-CoV-2 virus or with simple seasonal influenza[53]. This would mean that, in addition to elevated levels of cytokines such as IL -6, assessment of complement activation must also be included in key markers of multi-organ failure and death.

In addition to the typical respiratory manifestations, COVID -19 can also affect the functioning of other organs and systems, resulting in a more or less heterogeneous set of symptoms. Indeed, the excessive inflammatory reaction can lead to involvement of the heart, especially the cardiac muscle, which in the most severe cases can lead to arrhythmic complications that can sometimes even be fatal. On the neurological level, on the other hand, symptoms and conditions such as dizziness, impaired consciousness (confusion, lethargy and delirium), intracranial haemorrhages and strokes can occur. Conditions such as Guillain-Barré syndrome, trigeminal neuralgia and necrotising haemorrhagic encephalopathy have also been noted. The kidneys may also be affected, both by the presence of ACE -2 receptors, i.e. a direct effect of SARS-CoV-2 at the renal level, and by the prothrombotic blood changes resulting from the "cytokine storm". Indeed, patients may present with proteinuria and haematuria, which quickly lead to acute renal failure and dialysis. Involvement of the gastrointestinal tract is also possible, particularly at the level of the liver, which has not been well studied but in any case cannot be ruled out, as this has been reported from previous SARS and MERS epidemics.

It should be noted that critically ill patients can rapidly develop ARDS, i.e. acute respiratory distress syndrome, metabolic acidosis and septic shock, until they suffer multiple organ failure, which can inevitably lead to death.
Moreover, patients who had recovered from an acute COVID-19 infection were found to have long-term consequences. In individuals with a recent history of SARS-CoV-2 infection, long COVID is prevalent at a rate of between 10 and 30%[54] (Figure 5).



(Figure 5). Several Mechanisms potentially leading to persisting symptoms after infection[55].

When diagnosing COVID -19 it is therefore useful to consider not only the clinical and instrumental picture, but above all to detect the presence of the

virus in the organism. This can be done by using three main tests, namely molecular, antigenic and finally serological.

The molecular test or molecular smear uses the "real-time reverse polymerase chain reaction" (rRT - PCR), i.e. a molecular biology technique capable of identifying and amplifying the genetic material of the virus on a sample generally represented by the secretions of the respiratory tract obtained by a nasopharyngeal or salivary smear. It is a simple and safe test that gives you a result within 24-48 hours. Even faster are the results of the antigen test or smear test, which looks not for the genetic material but for parts of the viral protein structure, i.e. the viral antigens, within 5-30 minutes in a sample of biological material always obtained by a nasopharyngeal or saliva swab. However, this test is less sensitive than the previous one, as it can give both false-positive and false-negative results. Therefore, the results obtained should generally always be confirmed by molecular tests. Finally, the serological test is based on the search for anti-SARS-CoV-2 IgG and IgM antibodies in the patients' blood, mainly directed against the nucleocapsid protein and the spike protein. Remember that IgM are the first immunoglobulins produced during an infection. They therefore indicate a recent infection event, unlike IgG, which replace previous ones when the infection is no longer ongoing and are therefore reliable indicators of a previous infection[56].

Serological tests can be divided into rapid tests and quantitative tests. The former, for which a small drop of blood is sufficient, are usually qualitative tests, i.e. they only allow you to determine whether there has been contact between the patient and the virus and whether the immune system has produced antibodies against the virus. In contrast, quantitative tests, which require a blood sample, allow accurate and specific dosing of anti-SARS-

CoV-2 antibodies and provide important information about the "quantity" of antibodies produced by the immune system. Despite the quality of the information obtained with the serological test, it cannot be considered as a tool for confirming the positivity of the subject as the molecular test does, because positivity occurs quite late, considering that the incubation period of the virus is about two weeks. Therefore, the serological test could be considered not so much as a diagnostic positive test, but rather as a valid epidemiological tool to accurately estimate the spread of the virus within a population.

3.2 Autoimmunity dysfunctions in course of COVID-19

Autoimmunity dysfunctions are common consequences of COVID-19 infection[57]. Our understanding of the aetiology of inflammation has advanced as a result of information on acute local infections with systemic inflammatory responses generated by persistent low-intensity inflammation triggered by the SARS-CoV-2 virus.. Gusev et al[58] outline four phases of invasion as follows:

1. Blockage of primary innate immunity: during the invasion phase, the spike protein binds to the ACE2 receptors, co-receptors and alternative receptors (as described above). As SARS-CoV-2 grows after uptake into the cell, it also inhibits the innate immune systems.
2. Virus defence against adaptive immunity with activation of T and B cells
3. Acute consequences due to the resulting inflammation
4. Long-term consequences as a result of the acute consequences

The SARS-CoV-2 virus invades target cells through a variety of cell surface receptors, including ACE2 and TMPRSS2. While certain receptors (such as ACE2) and co-receptors/cofactors enable cell entry, others (called pattern recognition receptors) trigger an antiviral immune response. PAMPs or DAMPs are pattern recognition receptors (PRRs) that identify components of the infecting virus or cellular components of damaged/dead host cells.

Patients with long covid have elevated levels of pro-inflammatory cytokines[59], which can persist for up one year post-infection[60],[61]. Autoantibodies have been found in both covid-related and non-covid-related autonomic diseases, suggesting an underlying autoimmune pathology. The lack of specificity in these data limits a clear determination of cause and association, but the frequency is compelling along with the supporting evidence.

Autoantibodies are also increasingly common in Long Covid[62]. Autoantibodies against inflammatory cytokines are present and are associated with anti-SARS CoV2 IgG antibodies[63]. Autoantibodies to antinuclear and extractable nuclear antigens tend to be higher in long covid patients and are related with fatigue and dyspnoea[64],[65]. Finally, the prevalence of GPCR autoantibodies in people with long covid is related to reduced peripapillary vascular density in the eye, which is an indicator of microcirculatory function[66]. Viral clearance is delayed when the immune system is not functioning properly, leading to persistent inflammation and COVID -19 symptoms[67].

This pro-inflammatory milieu can lead to persistent immunological activation or dysfunction by stimulating microbiome-mediated autoantibody production[68]. The frequent and common signs of fatigue, cognitive symptoms and mental exhaustion, gastrointestinal dysfunction, which are not exclusive

to these disorders but are widespread outcomes of any immune-mediated disorder[69], link the above symptoms. This is crucial for treatment approaches, which may focus on the underlying aetiology or the subsequent symptoms.

3.3 Renal Involvement in COVID-19 Infection

ACE2 is highly expressed in renal parenchymal cells, especially proximal tubule cells[70]. These cells also express TMPRSS2 and other proteases that can enhance S protein cleavage and virus entry into the kidney[71], suggesting that the kidney may be susceptible to SARS-CoV-2 infection. Several studies have documented the detection of SARS-CoV-2 RNA and protein in the kidneys of COVID -19 patients[72],[73], as well as the visualisation of SARS-CoV-2 virions by electron microscopy[74],[75].

In a recent study of 62 individuals with COVID -19 , SARS-CoV-2 was found in all kidney samples (RT-PCR), and the nucleocapsid protein was detected in 6 of 6 samples (14). SARS-CoV-2 RNA was found in renal tubular cells and podocytes, and cells carrying viral RNA had higher gene expression associated with injury, inflammation and fibrosis[76]. Although these and other studies suggest that direct viral infection of renal cells contributes to the progression of kidney disease, there is no consensus on the prevalence of COVID -19 kidney infections[77].

3.4 COVID-19 and AKI

AKI is a common side effect of COVID -19, especially in hospitalised patients. Proteinuria and haematuria are common in people with COVID -19 and AKI, in addition to an abrupt decrease in eGFR and/or urine output[78],[79]. At the beginning of the pandemic, when the incidence of severe COVID -19 illness was high, studies from the United States and Europe indicated a pooled incidence of AKI of 28.6%, while it was much lower (5.5%) in studies from China. Older age, male gender, acute respiratory distress syndrome (ARDS) and/or the need for mechanical ventilation, and concurrent conditions such as CKD, hypertension and diabetes mellitus are all risk factors for AKI. Higher levels of CRP, ferritin and D-dimer in the blood are also associated with an increased risk of AKI[80](Figure 6.).

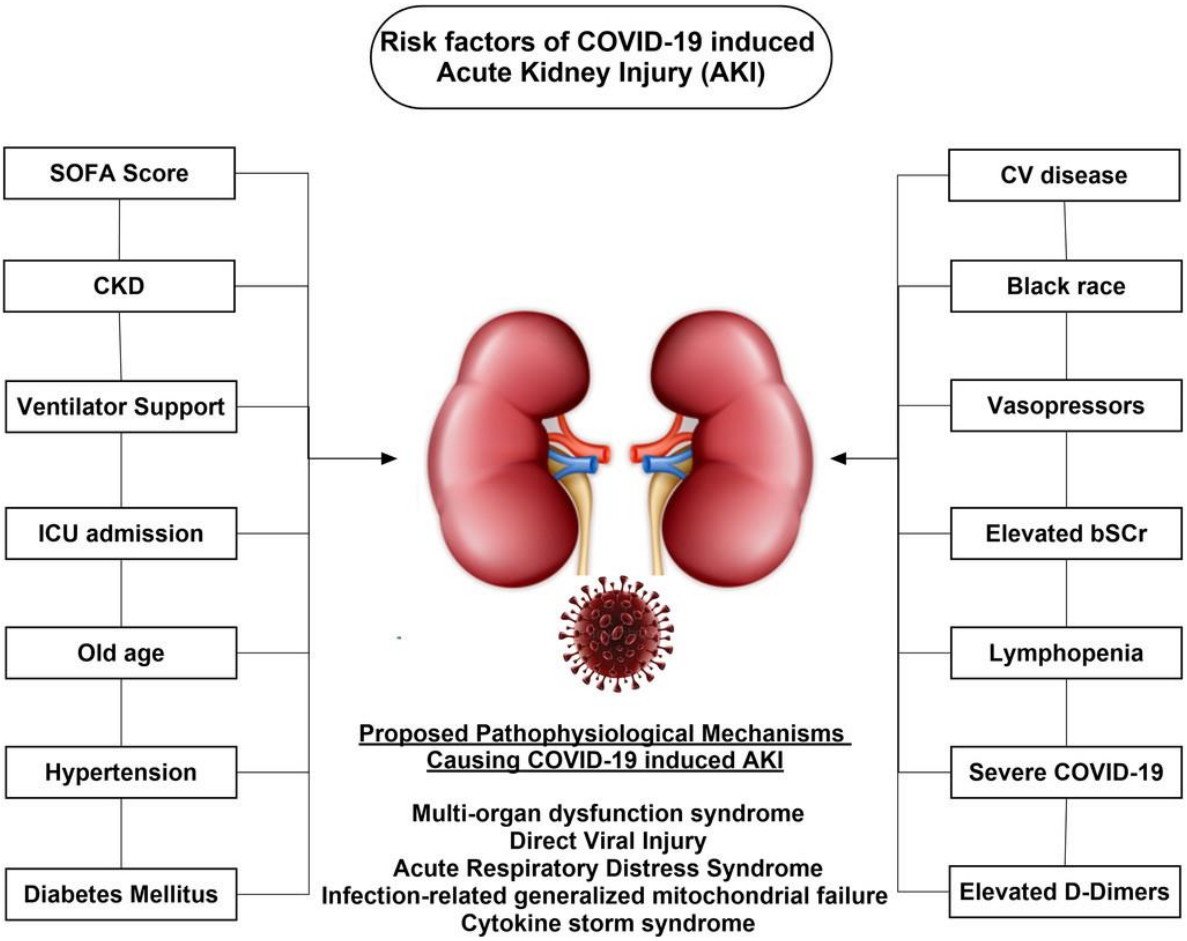


Figure 6. Risk factors for the development of AKI in course of COVID-19 infection[81].

Several causes, including decreased renal blood flow, use of nephrotoxic drugs, increased systemic and local cytokine production and endothelial damage, are common in patients with severe COVID -19[82]. Decreased renal blood flow may be the result of systemic hypotension, volume depletion or cardiac failure. Acute lung injury increases systemic cytokine levels and causes damaged cells to produce DAMPs. Cytokines and DAMPs can bind to cytokine and DAMP receptors in the kidney, including Toll-like receptors, activating innate immune responses and increasing inflammation and renal damage[83]. The pathogenesis of AKI in COVID -19 patients is complex and

includes not only COVID -19-related mechanisms, but also the direct effects of the SARS-CoV-2 virus on the kidney, along with indirect processes caused by the systemic effects of viral infection or the effects of the virus on distant organs such as the lungs (Figure 7.).

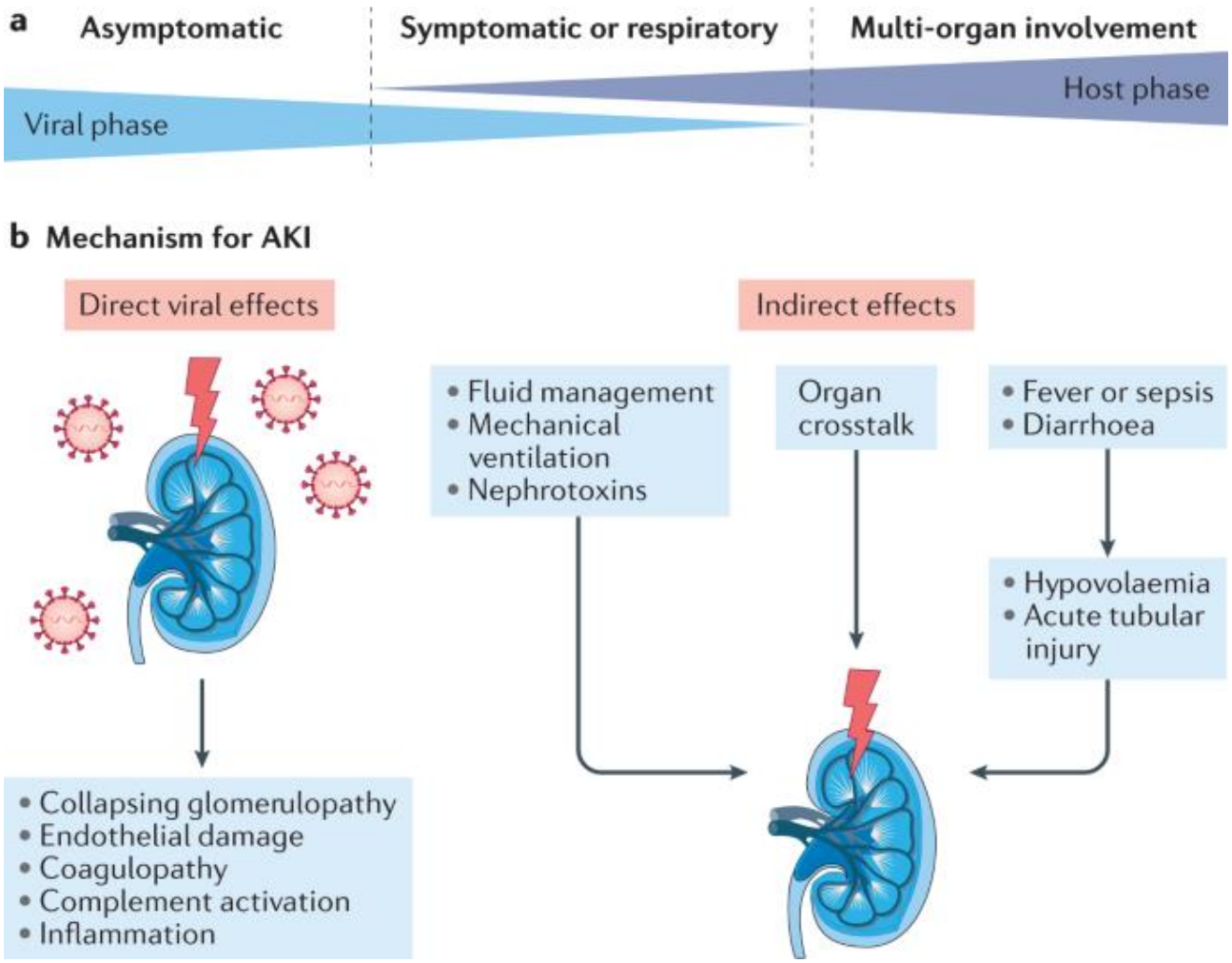


Figure 7. COVID-19-related AKI mechanisms[84].

In a large US cohort study, 20.6% of COVID -19 ICU patients required RRT. The best RRT method is not known and the choice may depend on the experience of the clinician and the availability of drugs[85]. Continuous RRT may be beneficial in haemodynamically unstable patients requiring prone positioning, while PD may be recommended in patients with coagulopathy in the dialysis circuit and/or contraindications to anticoagulants[86]. Fluid management in COVID -19 and AKI patients can be difficult and clinicians need to correct volume depletion while avoiding excessive volume resuscitation, which can worsen oxygenation in ARDS patients[87].

Although renal function improves in the majority of COVID -19 and AKI patients, AKI lasts more than 7 days in 35-40% of patients, and 30% of survivors with AKI who required CRRT continue to require dialysis at the time of hospital discharge[88]. The severity of AKI is associated with the likelihood of developing new and progressive CKD, as well as mortality[89],[90]. As a considerable number of patients with COVID -19 and AKI later develop CKD[91], renal function should be maintained after AKI has resolved to rule out the development of CKD[92].

Even before the vaccine became available, the severity of AKI and the need for RRT in COVID -19 patients declined in the first year of the pandemic, most likely due to breakthrough advances in COVID -19 therapy[93],[94].

3.5 COVID-19 in Dialysis Patients

Because of its high contagiousness, the SARS-CoV-2 virus spread rapidly among the most susceptible patients, including patients undergoing RRT, especially at HD. This group of patients, who have a severely weakened immune system, advanced age, but also various comorbidities such as diabetes mellitus, arterial hypertension and CVD, is an easy and preferred target for the virus. In addition, patients undergoing haemodialysis often have to travel to hospital to undergo the dialysis procedure, and this is mostly done by using "collective" transport systems. Then, when they arrive at the dialysis centre, which from a structural and logistical point of view often does not have rooms and pathways suitable for an epidemic emergency, they have to live in close quarters with other patients and medical staff for at least 3-4 hours per session. All these factors increase the risk of indirect infection and spread of the virus among these patients[95].

HD patients began receiving vaccinations during the COVID-19 beta wave of infections and a first and second vaccination was available before the next wave with the delta variant.

Despite the fact that HD patients are less receptive to vaccination than the general population[96], studies have shown that vaccination reduces clinical severity in HD patients[97]. However, these results should take into account viral variants with lower pathogenicity and partial immunity from previous COVID -19 infections. In September or October 2021, patients were given the option of receiving a third or booster shot. Nevertheless, despite patients receiving

up to three vaccines, several studies found a rise in COVID -19 infections caused by the Omicron variations. According to these data, 39% of HD patients with two vaccines developed COVID-19, and booster immunization had no further impact on lowering infection rates according to the observation of a 38% infection rate[98].

Goodlad et al[99] recorded 795 cases of infection in 710 dialysis patients with 85 of these individuals that have been infected with COVID-19 twice or three times.

COVID -19 is more likely to infect patients receiving RRT. According to a recent study, the total pooled prevalence of COVID-19 in RRT patients from 12 countries was 22 times greater than the general population[100]. This could be partly due to the fact that HD patients are unable to care for themselves and have to travel to and from the dialysis centre three or more times a week, often using public or group transport. Patients receiving home dialysis, on the other hand, have a lower risk for COVID -19 than patients undergoing inpatient home dialysis [101]. Other risk factors for COVID -19 in RRT patients include living in a residential neighbourhood, being black or Hispanic and having diabetes[102],[103].

Due to their impaired innate and adaptive immunity, RRT patients are also at risk of infection[104]. High clinical suspicion COVID -19 is necessary as up to 50% of RRT patients have asymptomatic disease and only 47% have fever compared to 90% in the general population[105].

Approximately 50% of RRT patients identified at COVID -19 required hospitalisation at the onset of the pandemic, and the mortality rate was 20-30%[106]. Individuals receiving inpatient HD were three to four times more probable than those receiving PD to be hospitalized with COVID -19[107]. The risk factors linked with an increased risk of mortality in RRT patients are

comparable to those associated with an enhanced risk of death in the overall population, but they are more elevated in the RRT group.[108].

More emphasis has been paid to COVID-19 data in HD patients than PD and the data are still scarce[109],[110]. A study from the Wuhan region of China found that only 1% of PD patients had COVID -19, but this trial has been performed in the first months of the pandemic emergency[111].

In addition, Yavuz et al[112] found no differences between PD and CAPD patients' rates of SARS-CoV-2 positive, mortality, hospitalization, need for intensive care and ventilator support, and total mortality rates.

As previously indicated, HD patients are more prone to contract an infection from airborne microbes than PD patients. This is due to HD patients swarming in crowded public waiting areas, taking public transportation, and having their dialysis in under-ventilated facilities[113]. The majority of HD facilities have improved infection control practices and expanded the use of personal protective devices to reduce the chance of infection for HD patients.

Along with these measures and the vaccinations given to patients, a lot of governments have put indication to lock-down. Due to improved infection control procedures and governmental constraints, the number of infections in our HD patients may have reduced to levels comparable to those reported in PD patients during the periods when the alpha and beta variants were widespread. The delta and later omicron genotypes were associated with an increase in infections in HD patients. This could be the outcome of a confluence of more contagious virus strains and a relaxation of some of the stricter infection control measures[114].

In the first pandemic wave COVID -19 HD patients had a much higher incidence of infection than PD patients, which was associated with a higher

need for hospitalisation and intensive care, as well as a higher mortality rate. Due to regulatory closures and improved infection control practises, alpha and beta variant infection rates were lower in HD patients. The impact of immunisation, immunity acquired through previous infections, pharmacological therapy and changes in viral variant severity, hospital and intensive care admissions and mortality rates have declined. However, it appears that immunisation and previous infections cannot prevent de novo infections for the new, more infectious forms.

Dialysis patients have a much higher risk of developing a moderate and severe form of the disease due to numerous risk factors and that their clinical course is much more complicated, with significantly higher rates of intensive care requiring treatment and mortality compared to the general population[115].

3.6 COVID-19 and Anemia

The ability of the host immune system, including innate and adaptive immunity, to respond effectively to COVID-19 infection is critical for viral control and eradication. The severity of this illness may be connected to an overabundance of pro-inflammatory cytokines, resulting in a "cytokine storm" and ARDS. Following SARS-CoV-2 infection, the immune system hyperactivity and creation of autoantibodies can result in autoimmune disorders such as autoimmune hemolytic anaemia, autoimmune thrombocytopenia, vasculitis, prothrombotic condition, and widespread coagulopathy[116].

Although rare, COVID -19-associated haemolysis has been reported. Haemolysis can lead to anaemia and other complications[117]. Taneri et al[118]reported in a meta-analysis of 57,563 Covid-19 patients that the degree of disease and outcome of COVID -19 patients may rely on lower Hb levels, as severe cases had considerably lower Hb levels than mild moderately severe cases.

They explained this by saying that lower Hb levels, especially in high-risk groups, may indicate that the patient has a reduced Hbcapacity to maintain the increased oxygen demand of peripheral tissues as a result of the hypermetabolic state during infection.

Anaemia in the course of COVID -19 has been associated with poorer clinical outcomes, including increased mortality rates and longer hospital stays. It may worsen respiratory distress and impair tissue oxygenation in COVID-19 patients[119]. Interestingly, given the significant association between anaemia and COVID-19 severity, it can be said that anaemia can be considered an independent predictive risk factor for COVID-19 and that Hb should be used for risk stratification in patients treated at home or in hospital[120].

Chapter 4 Experimental Part

4.1 Materials and Methods

This single center retrospective cohort study was performed in patients treated with subcutaneous erythropoietin and receiving maintenance HD between October 2020 and January 2023 to the Unit of Nephrology and Dialysis of the Policlinic G. Martino of Messina, Italy. Patients were divided in 2 cohorts: 15 patients with history of COVID-19 and 10 patients without COVID-19. For each patient, a total of 4 visits were retrospectively evaluated; for COVID-19 patients the baseline visit was considered as the most recent visit before the onset of COVID-19. All patients underwent a comprehensive assessment including age, sex, weight, the main diagnoses present at the time of the visit (arterial hypertension, cancer, cerebrovascular disease and diabetes mellitus), and laboratory tests (hb, ferritin, transferrin and transferrin saturation, creatinine, blood urea nitrogen, and electrolytes. Assessment of medications taken by admitted patients was also conducted. The inclusion criteria were as follows: Age over eighteen years, HD therapy longer than three months and in stable condition, use of an arteriovenous fistula and anuria. The following were the exclusion criteria: (1) severe cardiac or cerebral disease, (2) infections that occurred within the last month, (3) active liver disease or cancer, (4) recent surgical procedures or blood transfusions, (5) pure red cell aplasia or active bleeding.

Each patient received HD three times a week for four hours each using heparin anticoagulants. Dialysate flow was 500 mL/min and blood flow was 250–300

mL/min. The dialysate consisted of the following chemicals: calcium 1.50 mmol/L, potassium 2.5 mmol/L and sodium 140 mmol/L.

The study complied with the Declaration of Helsinki and was approved by the local ethics committee.

Covariates

Erythropoietin resistance index (ERI) was calculated at each time point as average weekly erythropoietin (EPO) dose per kg body weight (wt) per average Hb, over a 3-month period (ERI = (EPO/wt)/Hb). All enrolled patients were negative for COVID-19 at the baseline visit; patients underwent 4 different follow-up visits and were divided in two groups according to the presence of COVID-19 infection at the second follow-up visit. All clinical and laboratory data were repeatedly measured during the follow-up time.

4.2 Statistical Analysis

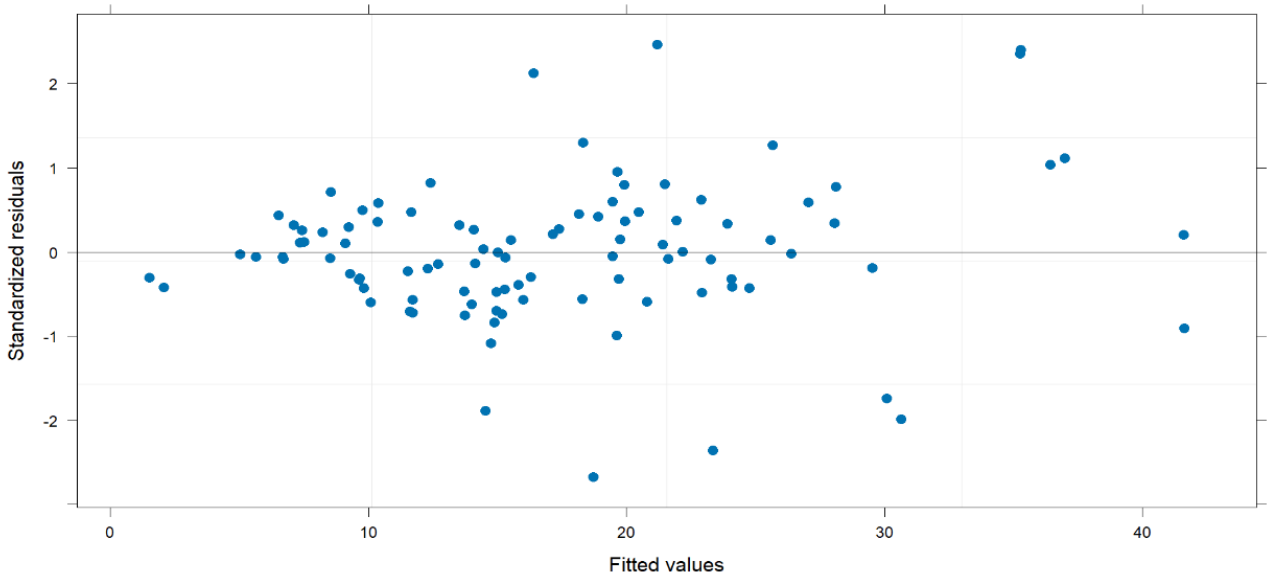
To compare continuous variables between 2 groups, an independent samples *t* test was used in case of normality and a Mann-Whitney U test otherwise. For categorical variables, Fisher's exact test was used because of low numbers. Distribution of study variables was evaluated with Kolmogorov-Smirnov test.

Comparisons between among ERI values across the 4 time points was evaluated by using ANOVA and Mann-Whitney U test. Comparisons between median ERI across groups and time points was graphically displayed. To

compare the evolution over time of ERI between COVID-19 and no-COVID-19 group a family of linear mixed models was fitted using time (as a continuous variable), group (COVID or no-COVID), and the interaction term between time and group as fixed effects and subject as a random effect. In order to improve model's capacity to capture the two distinct directions of continuous ERI change over time (increase vs decrease), we fitted two distinct linear mixed models: one including time points from the baseline visit to the third one; the second including time points from the second visit to the 4th one. Additional models were fitted by using time as a categorical variable.

Assumptions

We checked the independence of the random errors using a dispersion plot of the standardized residuals versus the predicted values. As seen in Supplementary Figure 1, the behavior of residuals was appropriate and only few observation were greater than 2 or lower than -2; we did not observe neither groupings or trends of observations.



As there was a significant interaction between time and group, we looked at the difference in median ERI per measured time point between the COVID-

19 and no-COVID-19 groups. *p* values were adjusted for multiple testing using the Holm correction. Goodness of fit of obtained models was based on evaluation of Bayesian Information Criterion and R2. Statistical analysis was performed using R 3.5.2 (R Foundation for Statistical Computing, 2018). Statistical significance was set at 2-tailed *p* values <0.05.

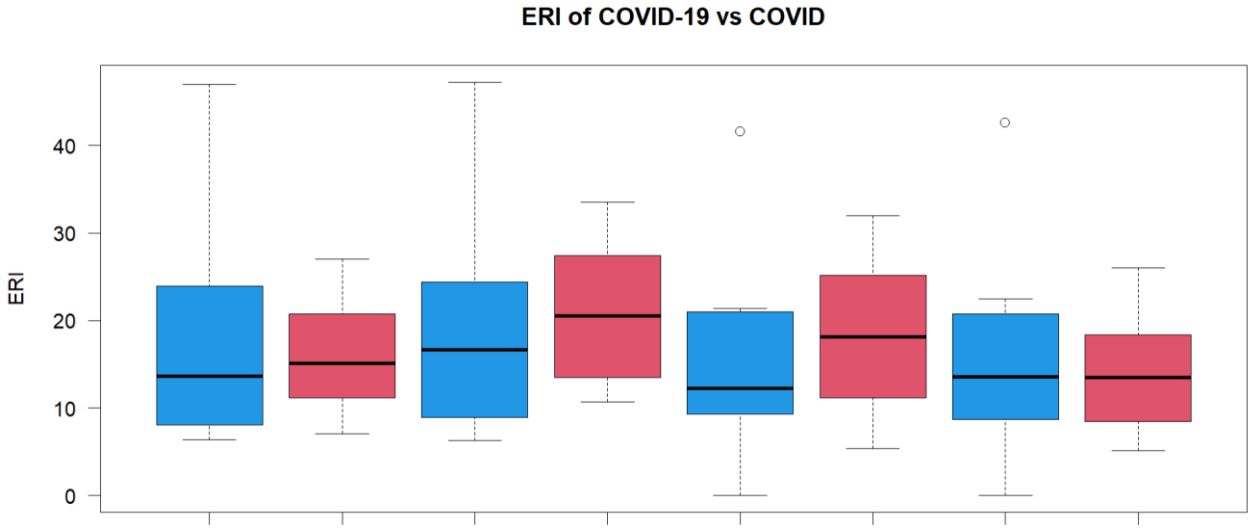
4.3 Results

Clinical and demographic characteristics of the study population and of the 2 groups are reported in table 1. The 25 patients included in the study had a mean age of 66.5 (14.9) years, and were more commonly men (62.5%); the most common comorbidities were hypertension (88%), followed by cerebrovascular disease and diabetes. No significant difference in age, sex, and comorbidities was found between COVID-19 patients and the control group.

	All (n=25)	Control group (n=10)	COVID-19 (n=15)	*P*
Age, mean (SD)	66.5 (14.9)	66.7 (14.4)	66.3 (15.3)	0.913
Male sex, n (%)	15 (62.5)	5 (55.6)	10 (66.7)	0.913
Weight, Kg, median (IQR)	74 (67-87.7)	74 (56-86.1)	79 (68.1-95.2)	0.678
Hypertension, n (%)	22 (88)	8 (80)	14 (93.3)	0.542
Diabetes, n (%)	7 (28)	1 (10)	6 (40)	0.179
Previous Neoplasia, n (%)	4 (16)	2 (20)	2 (13.3)	0.999
Cerebrovascular disease, n (%)	16 (64)	5 (50)	11 (73.3)	0.397
Transferrin, median (IQR)	197 (174-223)	201 (174-220.7)	195 (163-228)	0.218
Haemoglobin, median (IQR)	10.6 (10.0-11.1)	10.4 (9.7-11)	9.9 (9.2-10.9)	0.212
ERI, median (IQR)	15.0 (9.8-22.5)	10.3 (9.3-10.9)	10.3 (9.3-10.9)	0.999

The kinetics of ERI over time and comparative differences in the 2 groups are shown in Figure 1. Despite having almost the same baseline ERI,

COVID-19 and non-COVID-19 patients significantly differed for ERI trends over time. Indeed, patients with COVID-19 exhibited a trend of increased ERI between the T1 and T2, with a median deltaERI being +4.65 (1.80-6.25), which was significantly higher than deltaERI of non-COVID group (-0.27, -1.32-0.21, *p<0.001)*. After the second visit, the ERI slope declined steeper in COVID-19 group (-1.60, -4.35-0.25) than in non-COVI-19 group (-0.1. -8.92-0.89, *p=0.03)* but among COVID-19 patients, it reached baseline-like levels at the fourth visit only. Patients without COVID-19 presented non-significant fluctuations of their ERI across the visits.



Results of univariate linear mixed modelling analyses are reported in Table 2 and table 3.

Table 2. Linear mixed models exploring factors associated with ERI between the first and the 2nd follow-up visit, with time expressed as continuous variable.

Predictor variable	**Estimate**	**Standard Error**	***P value***	**Intercept**
Age	0.035	0.14	0.80	
Time	4.78	1.21	**<0.001**	
Male sex	-9.51	3.76	0.02	
Hypertension	-4.03	6.23	0.52	
Diabetes	-3.38	3.50	0.34	
Cerebrovascular disease	2.47	4.22	0.56	
Haemoglobin	-2.62	0.54	**<0.001**	
Time*Group	4.76	1.21	**0.02**	
Age*Time	-0.014	0.07	0.85	
Sex*Time	2.07	0.96	0.35	
Hb*Time	-0.41	-0.43	0.58	

Table 3. Univariate linear mixed models exploring factors associated with ERI between the the 2nd and the 4th follow-up visit, with time expressed as continuous variable.

Predictor variable	**Estimate**	**Standard Error**	***P value***	**Intercept**
Age	0.075	9.21	0.15	

Time	1.16	1.03	0.27	
Male sex	-10.60	-2.78	**0.01**	
Haemoglobin	.0.69	1.24	0.58	
Time*Group	-3.65	-2.20	**0.03**	**<0.001**
Age*Time	-0.11	0.05	0.05	
Sex*Time	2.23	1.29	0.20	
Hb*Time	-1.39	-1.60	0.11	

Both LMM models using continuous time variables showed the existence of a significant interaction between time and COVID-19 Group (0.02 and 0.03, respectively); according to the models, the slope for ERI was +4.76 between time points 0 and 1, and -3.65 between time points 2 and 3. Models containing categorical time in the overall study population showed a significant interaction between COVID-19 group and time visit 2 (p=0.01)

Bonferroni corrected post-hoc comparisons showed a trend of nonsignificant increase in ERI in patients with COVID-19 at time 1, and a subsequent significant decrease in from Time 1 to Time 3 in COVID-19 only patients.

4.5 Discussion

Several potential mechanisms contribute to the development of anaemia in the course of COVID -19. These include inflammatory cytokine-mediated effects on red blood cell production, impaired iron metabolism, direct viral invasion of bone marrow cells and the formation of blood clots.

Of note, COVID -19 can affect iron metabolism, leading to functional iron deficiency. Inflammation and hepcidin can interfere with iron absorption and utilisation, possibly contributing to anaemia.

Among the several variables that can limit quality of life and increase mortality in patients with CKD, anaemia is one of the most common. An unmet need is the adequate compensation of anaemia in these high-risk patients.

Our study aimed to investigate the role of erythropoietin resistance, a key issue in the treatment of anaemia, in HD patients with previous COVID-19 infection. To the best of our knowledge this is the first research designed to investigate ERI in COVID -19 patients in HD. This study highlights a close relationship between COVID -19 and resistant anaemia, as evidenced by the need to increase the erythropoietin dose in patients with previous infection, even months after diagnosis.

A previous research[121] showed no significant association between EPO use and mortality in HD patients with COVID -19. A major limitation of this study was the possibility of asymptomatic positives, the retrospective nature of the study and the fact that the variables analysed were measured only once. However, several studies have shown the influence of anaemia on the main Covid-19 related outcomes in frail patients[122],[123],[124],[125].

The analysis of our study showed the presence of a significant interaction between time and COVID -19 group. Models that included categorical time variables for the entire study population showed a significant interaction between the ERI in COVID -19 group and the timing of visit 2 (the visit at the time of infection). In fact, patients who tested positive for COVID experienced a significant increase in EPO needs between T1 and T2 compared with patients without infection, with a significant negative ERI delta and statistical significance of the two deltas.

In addition, Bonferroni-corrected post-hoc comparisons showed a trend towards a non-significant increase in ERI in patients with COVID -19 at time 1 and a subsequent significant decrease from time 1 to time 3 only in COVID -19 patients.

Between T2 and T3 the behaviour was different, in patients with earlier infection the need for erythropoietin generally decreases (although some COVID-10 outliers also increase ERI between T2 and T3). There was a significant interaction between time and group, both continuous and categorical. The tendency for persistent anaemia in COVID -19 patients confirms the insidious consequences of long-covid symptoms.

Our study has shown that complete control of anaemia has not yet been achieved in high-risk patients, such as those who have had a previous COVID-19 infection and are suffering the consequences of this disease.

In the context of anaemia treatment, one aspect that emerges is the potential future benefit of HIFs in this type of patient, a possibility that has not yet been addressed or explored in the current literature.

One of the few available studies evaluating drug therapy in haemodialysis patients with COVID -19 is the study by Bao et a[126]. The authors investigated the benefit of using Roxadustat in patients with COVID -19 receiving PD. The researchers concluded that this drug can effectively improve anaemia and is well tolerated in patients undergoing PD who have difficulty taking EPO, and that drug compliance during COVID -19 was better than with EPO.

Which pharmacological strategy is best for the treatment of resistant anaemia in dialysis patients is still debated and the available data are still insufficient.

Our research has several limitations. As it was a single centre study, there was no truly independent external validation cohort. Patients with ESKD have significant multimorbidity and altered physiology that may influence the results. Our patients cohort is still small. We increased the statistical power of the study by using one hundred repeated measures. Long-term follow-up of patients with larger cohorts and global data sharing are needed to support these results and research on this topic.

4.6 Conclusion

COVID -19 can cause or contribute to a variety of renal problems, usually as a result of systemic immunological activation and/or ischaemic injury. Although renal cells contain proteins that may promote SARS-CoV-2 infection, it is unclear how frequently renal cells are infected in COVID -19 patients and whether infection of renal cells leads to renal disease. Patients treated with renal therapy have a greater risk of COVID -19 adverse events compared to the general population, and thus a significantly higher risk of morbidity and death. Anaemia during the course of COVID -19 has also been associated with poorer clinical outcomes, including increased mortality rates and longer hospital stays. Anaemia can worsen respiratory distress and impair tissue oxygenation in COVID -19 patients.

It is important to note that the available scientific evidence on anaemia associated with COVID -19 is not yet mature. Researchers continue to investigate the underlying mechanisms, prevalence rates and clinical impact of anaemia associated with COVID -19. Further studies are needed to determine the best strategy to prevent COVID -19 in people with kidney disease and improve clinical outcomes such as anemia.

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