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Study of the CD73 role in the immune deficit of dialysis patients

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Studio del ruolo della CD73 nel deficit immunitario dei pazienti in dialisi

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Abstract

Chronic kidney disease (CKD) patients undergoing hemodialysis (HD) face significant clinical complications, including chronic inflammation and immune dysfunction. These pathologies are largely driven by bio-incompatibility and non-selective clearance during extracorporeal circulation. CD73 (ecto-5′-nucleotidase) plays a crucial regulatory role in immune signaling by converting pro-inflammatory extracellular AMP into anti-inflammatory adenosine. While dysregulated purinergic signaling and altered CD73 activity are implicated in renal pathology, CD73 dynamics in chronic hemodialysis remain incompletely understood. We investigated the role of CD73 and purinergic signaling in systemic alterations secondary to chronic hemodialysis. Gene expression study using real-time quantitative PCR (RT-qPCR) and protein levels of CD73 by ELISA were evaluated in peripheral blood mononuclear cells (PBMCs) from hemodialysis patients and compared with healthy control subjects. RT-qPCR analysis revealed a significant upregulation of relative CD73 gene expression in PBMCs from HD patients compared to healthy controls ($p < 0.01$). Conversely, ELISA measurements showed that CD73 protein levels were significantly decreased in the PBMCs of HD patients relative to controls ($p < 0.05$). This inverse relationship points to underlying post-transcriptional or post-translational dysregulation of CD73 in HD patients, which may contribute to the persistent inflammation and immune dysfunction observed during maintenance of hemodialysis. Furthermore, statistical correlations were conducted to analyze associations between NT5E/CD73 levels and clinical/biochemical parameters. The major finding suggests a strong positive correlation of NT5E expression with BMI and an inverse correlation with CRP, whereas the CD73 protein levels were strongly negatively correlated to lymphocytes and CRP in HD patients. On the contrary, in healthy subjects, NT5E gene expression was significantly and positively correlated with CD73 protein levels and both NT5E expression and CD73 protein levels showed significant negative correlations with age, indicating lower CD73/NT5E levels with increasing age.

Sommario della tesi

I pazienti affetti da malattia renale cronica (MRC) sottoposti a emodialisi (HD) vanno incontro a significative complicanze cliniche, tra cui infiammazione cronica e disfunzione immunitaria. Tali patologie sono in gran parte causate dalla bio-incompatibilità e dalla clearance non selettiva durante la circolazione extracorporea. La CD73 (ecto-5′-nucleotidasi) svolge un ruolo regolatorio cruciale nella segnalazione immunitaria, convertendo l'AMP extracellulare pro-infiammatorio in adenosina anti-infiammatoria. Sebbene la segnalazione purinergica deregolata e l'alterata attività della CD73 siano coinvolte nella patologia renale, le dinamiche della CD73 nell'emodialisi cronica non sono ancora del tutto chiarite. Abbiamo studiato il ruolo della CD73 e della segnalazione purinergica nelle alterazioni sistemiche secondarie all'emodialisi cronica. Sono stati valutati l'espressione genica mediante RT-qPCR (real-time quantitative PCR) e i livelli proteici della CD73 tramite ELISA nelle cellule mononucleate del sangue periferico (PBMC) di pazienti in emodialisi, confrontandoli con quelli di soggetti sani di controllo. L'analisi RT-qPCR ha rivelato una significativa sovra-regolazione dell'espressione genica relativa della CD73 nei PBMC dei pazienti in HD rispetto ai controlli sani ($p < 0,01$). Per contro, le misurazioni ELISA hanno mostrato una significativa riduzione dei livelli proteici di CD73 nei PBMC dei pazienti in HD rispetto ai controlli ($p < 0,05$). Questa relazione inversa suggerisce una sottostante deregolazione post-trascrizionale o post-traduzionale della CD73 nei pazienti in HD, che potrebbe contribuire all'infiammazione persistente e alla disfunzione immunitaria osservate durante il trattamento emodialitico di mantenimento. Inoltre, sono state effettuate analisi statistiche per valutare le associazioni tra i livelli di NT5E/CD73 e i parametri clinico-biochimici. Il risultato principale indica una forte correlazione positiva tra l'espressione di NT5E e l'IMC (BMI) e una correlazione inversa con la PCR, mentre i livelli proteici di CD73 sono risultati fortemente correlati in modo negativo con la conta linfocitaria e la PCR nei pazienti in HD. Al contrario, nei soggetti sani, l'espressione genica di NT5E è risultata significativamente e positivamente correlata ai livelli proteici di CD73; inoltre, sia l'espressione di NT5E che i livelli proteici di CD73 hanno mostrato significative correlazioni negative con l'età, indicando una riduzione dei livelli di CD73/NT5E all'aumentare dell'età.

Thesis Summary

Chronic renal failure (also known as chronic kidney diseases, CKD) is a serious, progressive disease that leads to gradual loss of kidney functions and impairs the body's ability to filter metabolic waste, balance fluids, and regulate critical hormones and electrolytes. Dialysis serves as renal replacement therapy to facilitate the clearance of metabolic waste products and excess fluid from the body. However, studies indicate that kidney failure and hemodialysis expose patients to an increased risk of clinical pathologies including chronic inflammation, anemia, and immune dysfunction. The major factors contributing to the dialysis-related diseases include oxidative stress and microinflammation, developed due to foreign-surface bio incompatibility of HD treatment and non-selective clearance by dialysis methods. Bio incompatibility refers to the contact between blood and dialyzer biomaterials during extracorporeal circulation and activates the alternative complement pathway, generating the anaphylatoxins C3a and C5a. The synergistic effect of bio incompatibility and oxidative stress stimulates monocytes to release inflammatory cytokines including TNF-α, IL-1 and IL-6 and trigger neutrophil aggregation, ultimately causing a drop in blood neutrophil levels (neutropenia). Moreover, immunodeficiency in HD patients is caused due to imbalance in Th1/Th2 polarization through suppressing Th1 response and enhancing Th2 differentiation.

CD73 (cluster of differentiation 73) also known as ecto-5′- nucleotidase (5′- NT) is involved in purinergic signaling and immune system regulation. It is a cell surface ectonucleotidase that takes extracellular adenosine-5-monophosphate (AMP) and converts it to adenosine. The adenosine produced acts as a signaling molecule and adenosine signaling through P1 receptor mediates anti-inflammatory response. In this way, CD73 converts pro-inflammatory ATP into anti-inflammatory adenosine molecules and plays an important role in immunological processes. Under normal conditions, purinergic signaling regulates key renal processes, including water and sodium balance, vascular and tubular function, renal blood flow, and autoregulation. However, dysregulated purinergic signaling and altered expression or function of the CD73 are key drivers of pathophysiological conditions such as kidney inflammation, hypertension, and polycystic kidney disease (PKD).

In this study, we investigated the role of CD73 and purinergic signaling in the systemic alterations associated with chronic hemodialysis. For this purpose, NT5E gene expression and protein levels of CD73 were analyzed in peripheral blood mononuclear cells (PBMCs) from HD patients and compared with those from healthy subjects. Furthermore, a correlation study was performed to find the potential association between gene expression and CD73 protein levels with various clinical and biochemical parameters of patients with chronic kidney disease. Real-time quantitative PCR (RT-qPCR) for CD73 expression performed on patients (HD, n=7) and healthy controls (CTRL, n=7) samples, revealed higher relative CD73 gene expression in hemodialysis patients with a significant difference between the two groups ($p < 0.01$). On contrary, CD73 protein levels determined by ELISA were significantly lower in PBMCs from HD patients than in healthy controls ($p < 0.05$). Statistical correlation analysis revealed that in HD patients, NT5E expression showed a non-significant negative correlation with age, while significant positive correlations were observed with BMI, ALT, transferrin, potassium, albumin, and total protein, with BMI showing the strongest association. Conversely, NT5E expression was significantly and negatively correlated with calcium and CRP levels. Whereas CD73 protein levels showed significant positive correlations with dialysis vintage, hemoglobin, and ferritin.
In contrast, CD73 levels were inversely correlated with lymphocyte count, CRP, monocyte count, and serum urea, with the strongest negative association observed for lymphocytes. On the other hand, in healthy subjects, NT5E gene expression was significantly and positively correlated with CD73 protein levels. Also, both NT5E expression and CD73 protein levels showed significant negative correlations with age, indicating lower CD73/NT5E levels with increasing age.

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Introduction

1.1 Introduction to dialysis

Chronic renal failure is a progressive renal insufficiency. Kidneys are involved in maintaining the hydro electrolytic and metabolic balance by regulating the body's extracellular fluid, blood pressure, osmolarity, pH, production of hormones, and excretion of waste products. The inability of kidneys to maintain this balance leads to a condition called uremic state; an accumulation of excessive urea and waste products in blood (Chisholm and Ogg, 1970).

The basic structural and functional unit of kidney is nephron, which is involved in three main renal processes:

a) **Renal filtration** which involves the passage of water, ions (sodium, potassium), amino acids, glucose and nitrogenous wastes from glomerular capillaries through a selectively permeable membrane into Bowman's capsule, Whereas, the large molecules including the plasma proteins and red and white blood cells are unable to pass the infiltration membrane and are retained in the blood.

b) **Tubular reabsorption**, through which renal tubes recover water and solutes from glomerular filtrate back into the blood capillaries. This process aims to prevent the loss of nutrients, solute, and water from extracellular fluids.

c) **Secretion** by which the substances that needs to be eliminated, including metabolic waste products, drugs, H^+ ions and toxins are transported from peritubular capillaries (the blood) into the filtrate.

The final step is excretion, which is physiological elimination of all the waste in the form of urine into the renal pelvis (Ogobuiro & Tuma, 2023).

In chronic renal deterioration, kidneys are unable to excrete the waste compounds through urine and accumulate the catabolic protein products in blood, leading to uremia that has adverse effects on all body systems. The three stages of chronic kidney disease include:

1) **Impaired renal reserve:** This is characterized by the huge loss of nephrons and decreased renal reserve. It can cause loss of up to 40-75% of kidney function.

2) **Renal failure or renal insufficiency:** Nephron function drops and causes 75-90% loss of kidney functions. At this stage, waste products start accumulating in blood.
3) **End stage renal disease (ESRD):** The final life-threatening phase where kidney failure exceeds 90%. Kidneys are unable to perform core functions of filtration, reabsorption and secretion and results in massive accumulation of toxins and waste in blood.

Chronic renal failure affects almost all organ systems of the body. The most serious complication is cardiovascular diseases including hypertension, caused by the retention of salts and water, and inactivation of renin-angiotensin-aldosterone system, congestive heart failure, hypervolemia, and edema. This is caused by the leaking of excess fluid in the surrounding tissues and swelling and pericarditis, where the accumulation of uremic products irritates and inflames the pericardium. Renal insufficiency can also lead to metabolic and electrolyte imbalance disorders including hyperkalemia, characterized by reduced excretion of potassium ions from the body. Their accumulation can in fact disrupt electrical signals causing cardiac arrhythmias. Chronic renal failure is also responsible for metabolic acidosis, in which the kidney is unable to excrete hydrogen ions and ammonia ions. In addition, the inability to reabsorb bicarbonate ions can lead to dangerously acidic blood and in turn affect muscles and bones (Chisholm and Ogg, 1970).

The treatment used for kidney failure is dialysis, that removes excess fluid and waste products from the body. It can be performed in emergency situations or chronic procedures (also called maintenance dialysis for ESRD). The two methods of dialysis include hemodialysis and peritoneal dialysis. In hemodialysis, the blood is diverted to an artificial machine or dialyzer through an arteriovenous fistula. This machine consists of a series of tiny tubes that are made up of semipermeable synthetic membranes and allow waste products and fluid to pass through them. These tubes act as filters and replace damaged kidneys. Within the dialyzer, diffusion, osmosis, and ultrafiltration occurs. Clear fluid called ‘dialysate’ is passed outside the tubes in the opposite direction to that of blood. Diffusion and osmosis allow the removal of waste and water by moving them from higher concentration (blood) to lower concentration (dialysates). The dialysate solution contains essential electrolytes in appropriate concentrations and in this way can help to restore the electrolyte balance in blood. The dialysate containing bicarbonate ions can help maintain the body’s buffer system whereas addition of heparin can help prevent blood clotting in the circuit.

Ultrafiltration is the process that helps to remove excess water, and it works by applying a negative pressure (suction) to the dialysis membrane. This creates a pressure gradient, and water is moved along the gradient, from a region of higher pressure to the region of lower pressure. Dialysis sessions normally last around three to four hours and take place three times a week.

Peritoneal dialysis, on the other hand, uses the inner lining of abdomen called peritoneum to filter the blood. The peritoneum is filled with a dialysate solution that can help blood vessels in the area filter the blood. Continuous ambulatory peritoneal dialysis usually takes about 40 minutes, and three to five sessions are needed each day. Automated peritoneal dialysis needs to be done every day and can take eight to 12 hours (Kidney Research UK, 2024; Cleveland Clinic, 2021).

However, numerous studies indicate that kidney failure and hemodialysis expose patients to an increased risk of clinical conditions such as malnutrition-inflammation complex syndrome that includes chronic inflammation, anemia, immune dysfunction and malnutrition (Kendrick & Chonchol, 2008; Libetta *et al.,* 2011; Choi *et al.,* 2012).

1.2 Dialysis-related damage

During respiration, inside mitochondria an electron transport chain takes place to generate energy. Oxygen is present at the very end of this chain that accepts electrons to undergo reduction. One oxygen molecule (O_2) accepts four electrons and $4H^+$ to convert into water and release plenty of energy. In this process, there is the possibility of intermediate reduction stage resulting in the formation of free radicals (FRs). Free radicals possess one or more unpaired electrons and are able to exist independently. These species have heterogenous reactivity; some of them like hydroxyl radicals are very reactive with short half-life in the range of nanoseconds, whereas others such as the tocopheroxyl free radicals have less reactivity and are more stable having half-life in the range of seconds. The FRs can be derived from oxygen (Reactive Oxygen Species, ROS) or can also be derivatives of nitrogen (Reactive Nitrogen Species, RNS) (Libetta *et al.,* 2011).

However, an imbalance in the production of these free radicals and their removal leads to a condition called oxidative stress. This can occur either due to overproduction of the ROS or RNS species, impaired clearance of these radicals, or due to lack of the activity of antioxidants (Diplock *et al.,* 1998). Above all, the massive production of ROS is linked to changes in electron transport chain. Other than this, white blood cells and neutrophils are also involved in increasing the levels of free radicals as they use oxidative FRs (superoxide, hydrogen peroxide, hydroxyl) to fight against the antigens (Romano *et al.,* 2010; Robinson, 2009). The enhanced ROS in a cell leads to the production of proteolytic enzymes (elastase) and myeloperoxidase (MPO). MPO is specifically involved in generating oxidative stress through the formation of a powerful oxidant called hypochlorous acid, HClO (Libetta *et al.,* 2011).

Studies have reported that in case of renal failure, high levels of free radicals found in these patients are involved in disease pathogenesis. CKD (Chronic Kidney Disease) patients accumulate high levels of low molecular weight (< 3 kDa) protoxins (Hasselwander & Young, 1998). It has been found that the effect of these protoxins is dominant on the activity of antioxidants like Vitamin C, E, and glutathione sulfhydryl (GSH). GSH, a powerful antioxidant, is specifically found to be decreasing with the progression of disease in CKD patients (Ceballos, *et al.*, 1996; Libetta *et al.,* 2011). The reason for this decrease is the alterations in Nuclear Factor Erythroid 2 p-45 related factors 2 (Nrf2) pathway. Nrf2 pathway is involved in transcription of antioxidant genes and phase

II detoxifying enzymes. This pathway is impaired in CKD patients, resulting in decreased production of various endogenous antioxidant and cytoprotective factors (Kim & Vaziri, 2010). The free radicals can have several harmful effects. Especially in case of the renal failure patients, it can lead to a condition called 'carbonyl stress', characterized by the derangements in biochemistry of carbohydrates and the formation of advanced glycated products (AGEs), that can cause atherosclerosis in uremia patients (Miyata *et al.*, 1999). In addition, oxidative stress is responsible for several renal diseases including glomerulonephritis, acute or chronic renal failure and tubule interstitial nephritis (Klahr, 1997; Vaziri, 2004). Oxidants can induce damages in glomerular permselectivity, altered growth responses, changes in cellular phenotype, apoptosis, endothelial cell damage, LDL oxidation and severe inflammatory responses (Lee & Song, 2008; Cochrane & Ricardo, 2003).

Renal failure patients undergoing hemodialysis are at the increased risk of several diseases including cardiovascular diseases, beta-2microglobulin-amyloidosis and protein malnutrition, and various infections. The two crucial processes responsible for dialysis-related diseases include oxidative stress and microinflammation. Enhanced oxidative stress in HD patients can occur due to two main reasons. Firstly, bio incompatibility of HD treatments can lead to increased production of FRs, causing oxidative stress. Secondly, hemodialysis is the non-selective process, and it removes solutes and waste based on the sieving properties of the membrane, on the size and molecular weight of the solutes. Therefore, essential antioxidant molecules, such as water-soluble vitamin C, may be lost (Libetta *et al.,* 2011).

Bio incompatibility refers to the complex series of reactions triggered when blood comes into contact with the dialyzer's artificial biomaterials during extracorporeal circulation. This, in turn, leads to neutropenia, a decline in the number of neutrophils in blood (Kaplow & Goffinet, 1968). Previous studies demonstrated that this contact activates the complement system via the alternative pathway, generating the anaphylatoxins C3a and C5a. These toxins stimulate cytokine synthesis and their release from monocytes. This complement activation is responsible for neutrophil aggregation and causes drop in neutrophils (Craddock *et al.,* 1977; Chenoweth *et al.,* 1983).
The processes that cause interleukin release include complement activation (Haeffner-Cavaillon *et al.,* 1987), back filtration of endotoxin fragments (Laude-Sharp *et al.,* 1990), and cellular contact with dialysis membrane through L-fucose dependent pathways (Betz *et al.,* 1988). However, the

increased release of cytokinin is found to be linked to diseases including hemodialysis-related amyloidosis, cardiovascular diseases and infections as explained by interleukin hypothesis (Gejyo *et al.,* 1988; Henderson *et al.,* 2008; Libetta *et al.,* 2011). This hypothesis is shown in the scheme of Figure 1.

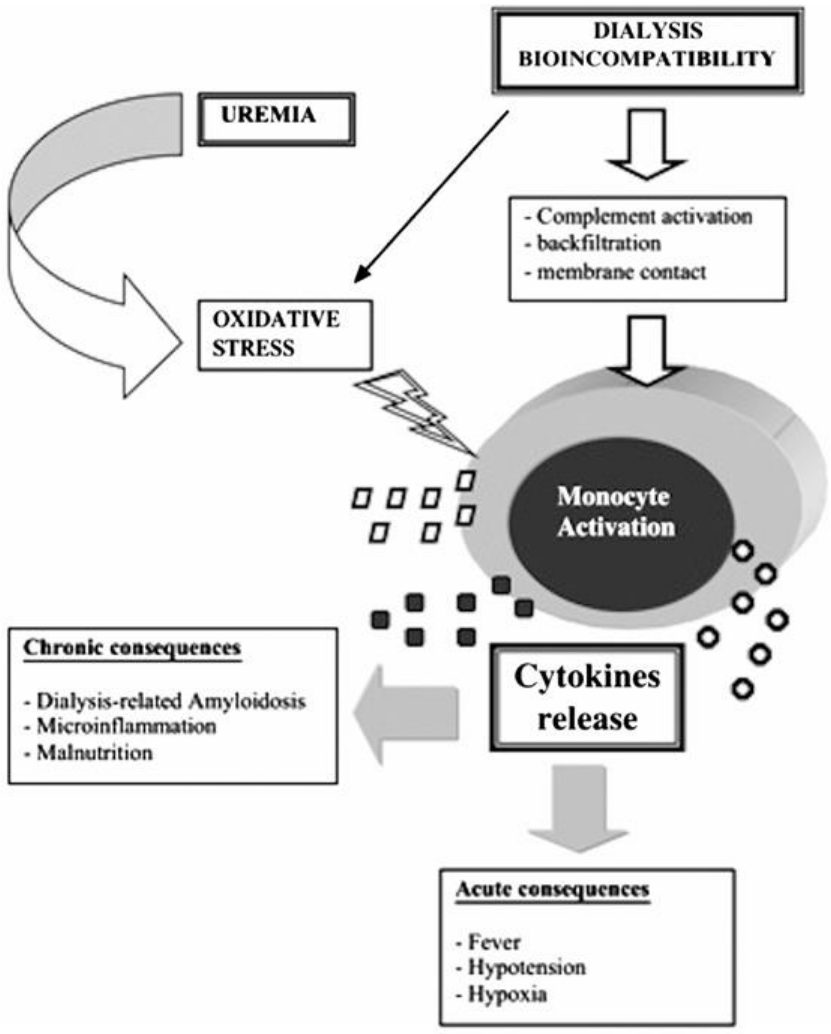


Figure 1: Interleukin hypothesis in hemodialysis patients (Libetta et al., 2011)

Dialysis leads to an increased inflammatory response by releasing inflammatory cytokines, such as TNF-α, IL-1 and IL-6 in blood (Memoli *et al.,* 1992; Tarakçioğlu *et al.,* 2003; Sanchez-Niño *et al.,* 2010). Most importantly, IL-6 exerts a pro inflammatory response and is primarily produced by monocytes. It is involved in acute phase response (response to tissue damage, tissue injury, and infections) and is a main player in systemic inflammation, leading to fever and changes in biosynthetic profile of liver proteins. During inflammatory response, two main acute phase

response proteins CRP and SerumamyloidA (SAA) increase many folds (Steel & Whitehead, 1994). It has been demonstrated that peripheral blood mononuclear cells (PBMC) of patients undergoing hemodialysis show enhanced levels of IL-6 as compared to uremic patients not undergoing HD (Memoli *et al.,* 1992). This suggests that when blood interacts with curophan membrane during dialysis, it leads to chronic activation of monocytes, that in turn release high IL-6 cytokines. However, dialysis performed with polymethyl methacrylate (PMMA) membranes did not activate complement pathways or monocytes, maintaining IL-6 levels comparable to those in non-uremic controls (Memoli *et al.,* 1992). These results overall suggest that bio incompatibility is a major cause of the high production of cytokines via monocyte activation and leads to microinflammatory response in dialysis patients. IL-6 binds to its receptor IL-6R and the signal transduction process takes place through two transmembrane subunits of this receptor; the IL-6 binding glycoprotein gp80 (IL-6R) and the signal-transducing component (gp 130) (Yawata *et al.,* 1993). IL-6 first binds to IL-6R, and this binary complex subsequently binds to two molecules of gp130. However, IL-6R is also present in soluble form in cytoplasm and possesses the same binding affinity for its ligand as the membrane bound. During dialysis, the interaction with curophan membrane leads to high levels and production of this sIL-6R, that can bind IL-6 ligands and elicit strong inflammatory response in HD patients (Memoli *et al.,* 2000; Libetta *et al.,* 2011).

Studies have revealed that in HD patients, synergistic relationship exists between the oxidative stress and the systemic inflammatory response. These two processes act to positively regulate each other. Oxidative metabolites, specifically ROS, including hydrogen peroxide, stimulate NF- κB pathway. NF κB is a transcription factor and leads to upregulation of various proinflammatory cytokines, specifically IL-6 (Interleukin-6) and TNF-α (Tumor Necrosis Factor-alpha). In this way, oxidative stress plays a role in increasing the inflammatory response (Witko-Sarsat *et al.,* 1998; Schwedler *et al.,* 2001). Similarly, acute-phase protein CRP is involved in stimulating phagocytes to produce more intracellular ROS (Zeller & Sullivan, 1992).

This positive correlation was also observed in hemodialysis patient cohorts, which exhibited upregulation of inflammatory and oxidative stress biomarkers, including CRP and lipid peroxidation (Borazan *et al.,* 2004). Moreover, it is observed that patients who have both high inflammation and hypoalbuminemia (low levels of the protein albumin) experience more oxidative stress, as compared to patients that have normal levels of albumin. This is attributed to the antioxidant properties of albumin, as reduced levels lead to elevated oxidative stress and damage

(Danielski *et al.,* 2003). These studies explain that a vicious cycle exists between inflammation and oxidative stress and tends to amplify each other in uremic patients undergoing hemodialysis (Libetta *et al.,* 2011).

The low levels of high-density lipoprotein (HDL) cholesterol and its protective enzymes including paraoxonase, glutathione peroxidase, platelet activating factor acetyl hydrolase, lecithin cholesterol acyl transferase and apolipoprotein A-I (primary structural protein of HDL involved in clearing cholesterol) in plasma of hemodialysis patients (Moradi *et al.,* 2009) is another factor that can play a role in increasing oxidative stress and high risk for cardiovascular disease and atherosclerosis (clogged arteries). HDL is an antioxidant involved in reverse cholesterol transport. It removes cholesterol from the arterial walls and transports it back to the liver, thereby protecting against cardiovascular diseases. However, HD patients present reduced levels of this anti-inflammatory HDL and are at high risk of atherosclerosis and thrombosis (Feig *et al.,* 2008; Vaziri *et al.*, 2009). To study this correlation, Carotid Intima–Media Thickness (CIMT) ultrasound analyses have been performed on patients to observe the thickness of cell wall i.e. atherosclerosis. It was observed that high atherosclerosis was linked with high levels of oxidative stress biomarkers including TBARS (thiobarbituric acid reactive substances) and nitrites/nitrates (related to nitric oxide stress) and was inversely correlated to the levels of antioxidants including SOD (superoxide dismutase), CAT (catalase), and P-SH (plasma sulfhydryl groups) (Dursun *et al.,* 2008).

1.3 T-helper cell imbalance in dialysis

Patients with uremia and renal failure demonstrate strong immunodeficiency and reduced response to vaccination (Stevens *et al.,* 1984). The reason for this immunodeficiency is linked to impaired T cell proliferation and activation. Immune cells like T or B-lymphocytes and monocytes membranes are rich in long-chain unsaturated fatty acids such as arachidonic acid. The double bonds of these unsaturated fatty acid chains are prone to be targeted by the FRs oxidation. This makes these immune cells an easy target of oxidative stress and consequent damage as compared to other cells of the body. Moreover, renal failure patients possess reduced levels of the powerful antioxidant GSH, required to survive in an oxidative stress environment. In the absence of GSH, T cells are unable to proliferate and activate to fight against the infection. This in turn leads to an unnatural shift toward a T helper 2 (Th2) response as compared to T helper 1 (Th1) response (Peterson *et al.,* 1998; Libetta *et al.,* 2011).

Upon antigen interaction, helper T-cells can differentiate into two subsets, T helper 1 (Th1) and T helper 2 (Th2). The process of differentiation starts with T helper precursor cells (Thp) that are naive T cells and have not encountered any infection. Upon antigen exposure, naive helper T cells, Th_p differentiate into an uncommitted intermediate stage known as Th_0. They are a mixture of cell populations and can secrete various chemical signals called cytokines. Finally, they differentiate into specialized cells called Th1 or Th2. Some of them become immediate fighters called effectors, while others become long-term memory cells to remember the infection for next time (Mosmann & Coffman, 1989).

Th1 is involved in cell-mediated immunity. It is responsible for secreting interferon-gamma (IFN-γ), which has multiple biological functions. Firstly, it is involved in stimulating phagocytic defense by activating phagocytes (like macrophages) to kill the pathogen. Secondly, it induces immune cells to secrete specific antibodies called IgG_{2a} required for opsonization of bacteria on the phagocytic cells. Moreover, it promotes expression of FcγRI receptors on phagocytic cells, so they can bind more IgG_{2a} antibodies and enhance opsonization. Also, it leads to expression of adhesin molecules and secretion of the chemokines on endothelial cells to attract monocytes and T cells to

the site of infection and thus enhances the immune response against the infection (Boehm *et al.,* 1997; Maggi *et al.,* 1992; Libetta *et al.,* 2010).

On the other hand, Th2 is involved in phagocyte-independent defense. They work by secreting multiple cytokines to fight parasites like helminthic worms and drive allergic reactions. Cytokines released by Th2 includes IL-4 that in turn leads to production of IgE (antibody responsible for allergic reaction), IL-5 (activating factor for eosinophils), IL-10 and IL-13. Among them, IL-4 and IL-13 play a critical role by suppressing Th1 pathway and thus switching off cell-mediated immune response (Gajewski *et al.,* 1989).

The primary immune defect in uremia and HD patients is impairment in cell-mediated immune response (Kaul *et al.,* 2000). In these patients, 'nodal dysregulation'' characterized by problems in immune communication network (particularly cytokines) takes place, that eventually results in an Th1/Th2 imbalance through suppressing Th1 response and enhancing Th2 differentiation (Libetta *et al.,* 2001). It has also been confirmed by the presence of hyper eosinophilia in RTD patients due to increased release of Th2 cytokines (Cledes *et al.,* 1988).

In healthy individuals, the Th1/Th2 polarization profiles remain balanced. However, in uremia and RDT patients, the polarization is shifted more towards Th2. A previous study showed that stimulated PBMC and CD4+ cells from these patients produce more IL-4 and IL-10 as compared to healthy controls (Peleman *et al.,* 1989) (Fig. 2). Moreover, as Th1 and Th2 possess reciprocal inhibition, it was observed that these activated cells secreted less IFN-γ. In this way, IL-4 and IL-10 (Th2 cytokines) suppress IFN-γ (Th1 cytokines). This was further proved by using neutralizing antibodies against IL-4 and IL-10 and observed that T-cells successfully recovered their ability to release IFN-γ (Libetta *et al.,* 2001). These studies were carried out on anergic tuberculosis patients, that exhibit impaired cell-mediated immunity, and their T-cells produce IL-10 instead of IFN-γ. In this way, they mimic uremic patients (Boussiotis *et al.,* 2000; Libetta *et al.,* 2010). A representation of cytokines influencing the development of naive CD4+ cells into Th1 and Th2 lymphocytes in hemodialysis patients is shown in Figure 2.

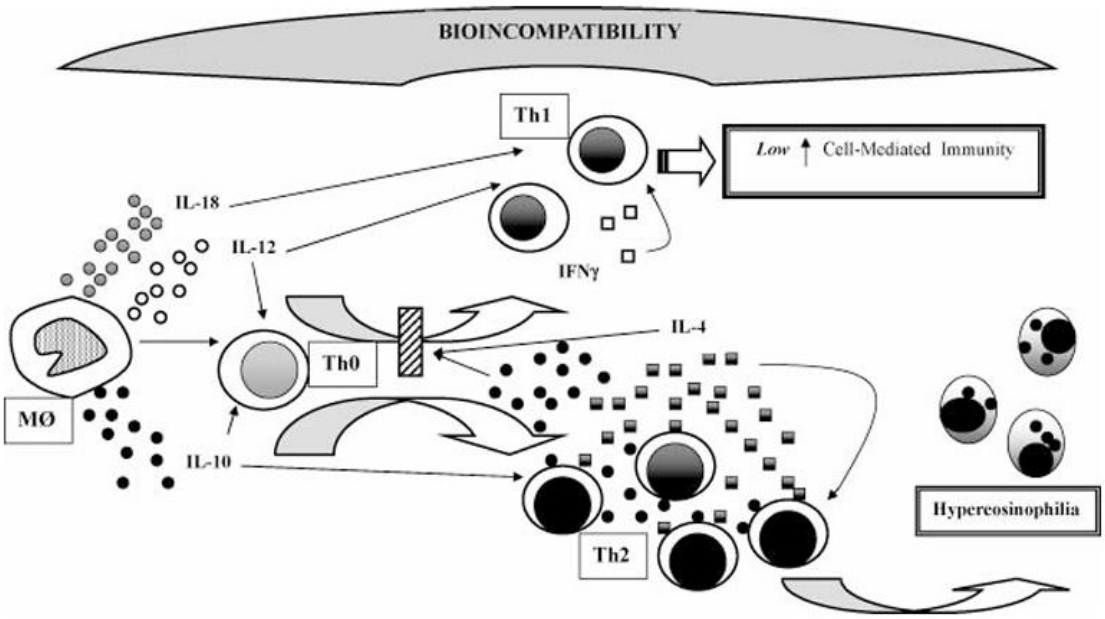


Figure 2: Schematic representation of cytokines influencing the development of naive CD4+ cells into Th1 and Th2 lymphocytes in hemodialysis patients (Libetta et al., 2010)

These findings explain the phenomenon of immunodeficiency in uremia and RDT patients. Impairment in Th1 polarization and suppression in IFN-γ release can lead to defects in immunological processes including loss of CD8+ (killer T-cells) and NK (Natural Killer) cells cytotoxicity**,** cell-mediated functions and inability to properly present antigens using Major Histocompatibility Complex (MHC) class I and II molecules. Moreover, inhibition of IFN-γ suppresses the expression of adhesion molecules on endothelial cells and can affect leukocyte/leukocyte and leukocyte/endothelial interaction. Also, the drop in IgG_{2a} antibodies impairs opsonization (Gajewski *et al.,* 1989). On the other hand, Th2 polarization does not only suppress Th1 pathway but also causes independent damage such as IL-4 and IL-10 down-regulate LPS-induced inflammatory cytokine production by macrophages, inhibit macrophage cytotoxic activity, and block rescue of monocytes from apoptosis (de Waal Malefyt *et al.,* 1991; Libetta *et al.,* 2010).

1.4 CD73 and immunity

CD73 (cluster of differentiation 73) also known as ecto-5′-nucleotidase (5′- NT) plays an important role in purinergic signaling and regulation of immune system homeostasis. It is an ectonucleotidase that converts extracellular adenosine-5-monophosphate (AMP) to adenosine. ATP provides energy to cells and is heavily abundant inside the cell in high concentrations of about 3 to 5 mM, whereas the extracellular environment of a cell consists of relatively lower concentrations of ATP (μM). This difference in concentrations is due to the strong negative charge of ATP, that makes it unable to cross the hydrophobic membrane and keeps it locked inside the cell. However, intracellular ATP can be released into the extracellular space via pannexin hemichannels in apoptotic cells, plasma membrane disruption in necrotic cells, or connexin hemichannels in activated inflammatory cells (Burnstock, 2012; Lohman *et al.,* 2012). ATP, ADP and its degradation product adenosine act as signaling molecules in purinergic signaling pathways. Under normal conditions, purinergic signaling is involved in various physiological processes in kidneys including regulation of water and sodium homeostasis, tubular, glomerular and vascular functions, renal blood flow, and autoregulation. However, in pathophysiological conditions, the uncontrolled ATP release and activation of purinergic signaling are linked to various diseases such as renal inflammation, hypertension, lithium- induced nephrogenic diabetes insipidus and polycystic kidney disease (PKD) (Dwyer *et al.,* 2020).

Purinergic receptors are of two types; P1 and P2. Extracellular ATP and ADP bind to P2 receptors, and this binding acts as a proinflammatory signal to promote inflammation. P2 receptors are present in all parts of the neurons. Out of 15 cell surface P2 receptors, eight P2Y receptors belong to the G-protein-coupled receptor (GPCR) family and can be activated by ATP, ADP, UTP and UDP, whereas other seven are ligand gated ion channels and are capable to bind only ATP as signaling molecule (Burnstock, 2007). P2X is an important P2 receptor and is present in all immune cells, especially inflammatory cells and is involved in causing inflammation responses. Once bound by ATP, it is involved in activation of NLRP3 (NOD-, LRR- and pyrin domain-containing 3) inflammasomes and causes release of cytokines and chemokines from fibroblasts, mast cells, dendritic cells and peripheral- blood mononuclear cells (Virgilio *et al.,* 2017).

On the other hand, adenosine signaling through P1 receptor mediates anti-inflammatory response. P1 receptors are classified as G protein coupled receptors and are called A_1, A_{2A}, A_{2B} and A_3 receptors. A_1 and A_3 receptors are bound to G-inhibitory subunit and are involved in decreasing adenylyl cyclase, thereby reducing intracellular cAMP. Whereas A_{2A} and A_{2B} receptors are coupled to the G- stimulatory subunit and their activation leads to an increase intracellular cAMP (Kukulski *et al.,* 2011; Blackburn *et al.,* 2009). Two key enzymes drive adenosine signalling: they are ectonucleoside triphosphate diphosphohydrolases (NTPDases). Furthermore, CD39 hydrolyses extracellular ATP to ADP and AMP, and CD73 subsequently converts AMP into adenosine. This adenosine can be further degraded into inosine by adenosine deaminase. Therefore, CD73 is a cell surface ectonucleotidase and is responsible for stopping DAMP by converting pro-inflammatory ATP into anti-inflammatory adenosine molecule (Figure 3). CD73 is predominantly present in the glomeruli and on peritubular fibroblasts. However, within the kidney, CD39 works much faster and more efficiently than CD73 (Lazarowski *et al.,* 2000; Dwyer *et al.,* 2020).

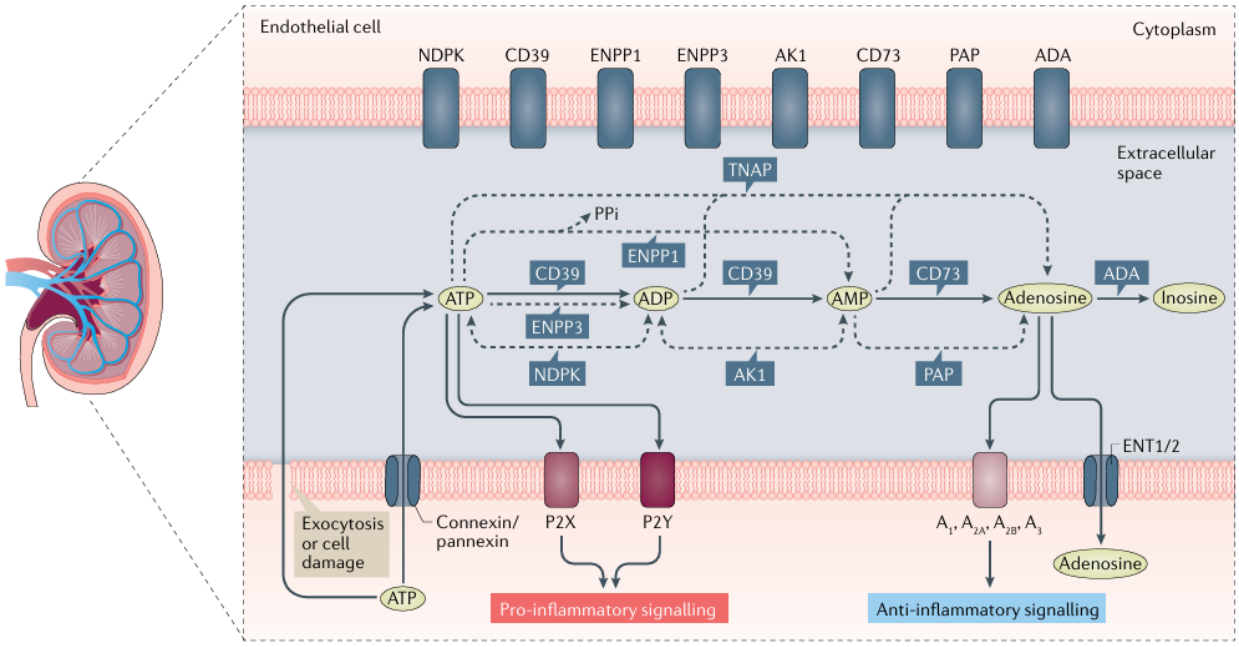


Figure 3: ATP and adenosine signaling via purinergic receptors in the kidney (Dwyer et al., 2020)

Studies have revealed that CD73 plays a crucial role in immunological processes. NLRP3 is the assembly of multiple proteins and is activated during inflammation in response to infection or tissue damage. Activated inflammasomes lead to production of pro-inflammatory cytokines like IL-1β and IL-18 and are involved in pyroptosis (Lamkanfi & Dixit, 2014). NLRP3 inflammasome is a main driver in major kidney pathologies including AKI, CKD, diabetic nephropathy, and crystal-related nephropathy (Hutton *et al.,* 2016). The renal macrophages release intracellular ATP by exocytosis through connexin/pannexin hemichannels. This ATP binds back to the P2X7 receptors on these macrophages and activates NLRP3 inflammasome, which in turn releases IL-1β to promote inflammation (Baron *et al.,* 2015; Dosch *et al.,* 2019). Once a macrophage's P2X7 receptor is activated, it causes cell to release more ATP into the surrounding space (Riteau *et al.,* 2012; Brandao-Burch *et al.,* 2012). The high levels of extracellular ATP are sequentially processed by CD39 and CD73 to generate adenosine. Although the conversion of ATP to adenosine is involved in reducing inflammation, sustained and long-term exposure to adenosine acts in an opposite way and promotes NLRP3 inflammasome by binding to A_{2A} receptor. This in turn can augment inflammation by increasing the secretion of intracellular IL-1β, leading to cytokine storms (Ouyang *et al.,* 2013; Dwyer *et al.,* 2020) (Figure 4).

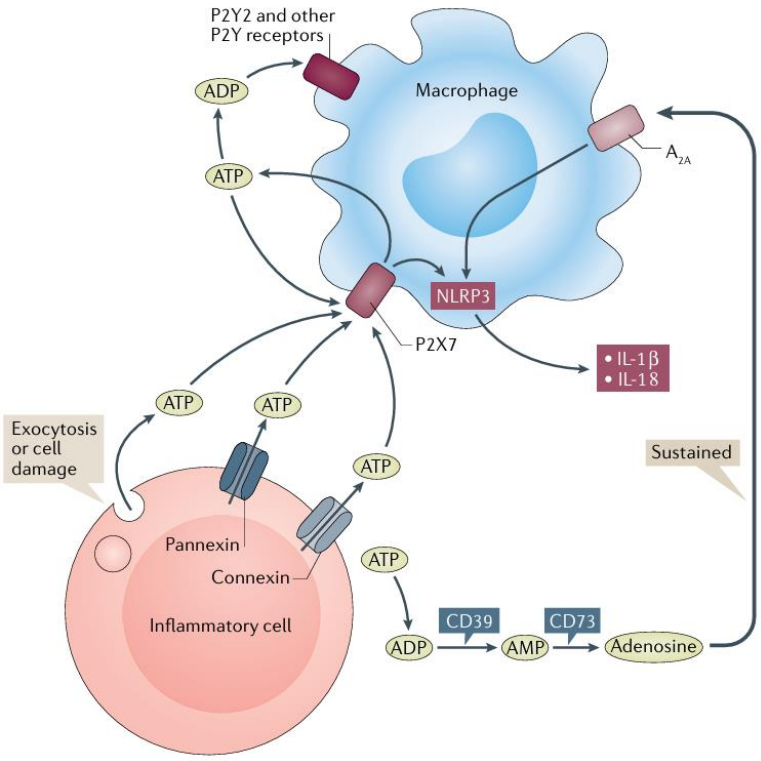


Figure 4: CD73 and adenosine in the activation of the NLRP3 inflammasome (Dwyer et al., 2020)

Regulatory T cells (T_{reg}) play an important role in kidney diseases and transplantation. They are involved in allograft tolerance and prevent immune cells from rejecting the transplanted kidney. The mechanism by which they accomplish this is by converting extracellular ATP to adenosine. Mice Treg cells contain both CD39 and CD73, and they work in tandem to take the excess ATP and convert them into AMP, which is further degraded by CD73 to adenosine. This adenosine binds to A_{2A} receptors on neighboring T cells and thus suppresses immune activation and inflammation (Deaglio *et al.,* 2007). Human cells on the other hand lack CD73 and contain only CD39. They take the ATP, degrade it into AMP, which is then handed over to the neighboring T-cells or target cells that express CD73 and are converted into adenosine (Dwyer *et al.,* 2010). Human memory Treg (mTreg) cells are an important class of $CD4^+$ Treg cells and are vital for transplant rejection. On these cells, combined action of both CD39 and CD73 generates adenosine. This adenosine acts in a positive way and transactivates the Entpd1 promoter to synthesize more CD39 (Borsellino *et al.,* 2007; Liao *et al.,* 2010).

T helper cells can mature into two types of T_H17 cells; aggressive T_H17 and suppressive T_H17 cells. When naive T helper cells mature in the presence of high ATP concentration, they differentiate into pathogenic T_H17 that leads to severe inflammation (Schenk *et al.,* 2011). Whereas if these T cells mature in cells expressing high levels of CD39 and CD73, they differentiate into suppressive T_H17 cells that convert ATP to adenosine, mitigating inflammation and thus in this way acts like Treg cells (Figure 5). Inflammatory Bowel Disease (IBD) are severely lacking in these protective, adenosine-generating T_H17 cells, that leads to chronic inflammation (Longhi *et al.,* 2014).

Macrophages are effector cells of the immune system and are derived from monocytes. They play an important role in various kidney pathologies including obstructive uropathy, ischemia–reperfusion injury (IRI), glomerulonephritis, and diabetic nephropathy. Macrophages can have pro-inflammatory or anti-inflammatory effects in various diseases, depending on the polarization of the macrophages (Anders & Ryu, 2011; Cao *et al.,* 2013; Tian & Chen, 2015). Macrophages are classified into 'classically activated' (M1), 'alternatively activated' (M2) or macrophages with intermediate activation status. M1 mimics Th1 T-cell response and is involved in acute inflammation. In these macrophages, NLRP3 inflammasome expression is very high (Lopez-Castejón *et al.,* 2011; Biswas & Mantovani, 2010). Extracellular ATP binds to P2X7 receptors and acts as a signal for M1 macrophages polarization (Lévesque *et al.,* 2010). On the other hand, M2 macrophages mimic Th2 T-cell response and have an immunoregulatory role. They act to quiet down the immune system and rebuild damaged tissue. M2 macrophages have lower expression of NLRP3 inflammasome. When a macrophage is polarized into the aggressive M1 state by ATP, it expresses high levels of CD39 to convert ATP to ADP and AMP but possesses reduced expression of CD73 (Lévesque *et al.,* 2010; Roberts *et al.,* 2014) As anti-inflammatory adenosine is not produced, this leads to tissue damage. However, once the inflammation stops and cells manage to polarize into the M2 state. These M2 cells rebuild high CD73 expression, allowing it to finally process AMP into adenosine, that acts as a brake to macrophage-mediated inflammation (Roberts *et al.,* 2014; Dwyer *et al.,* 2020). A scheme that illustrates the role of Th17 cells and macrophages in tissue injury and repair is shown in Figure 5.

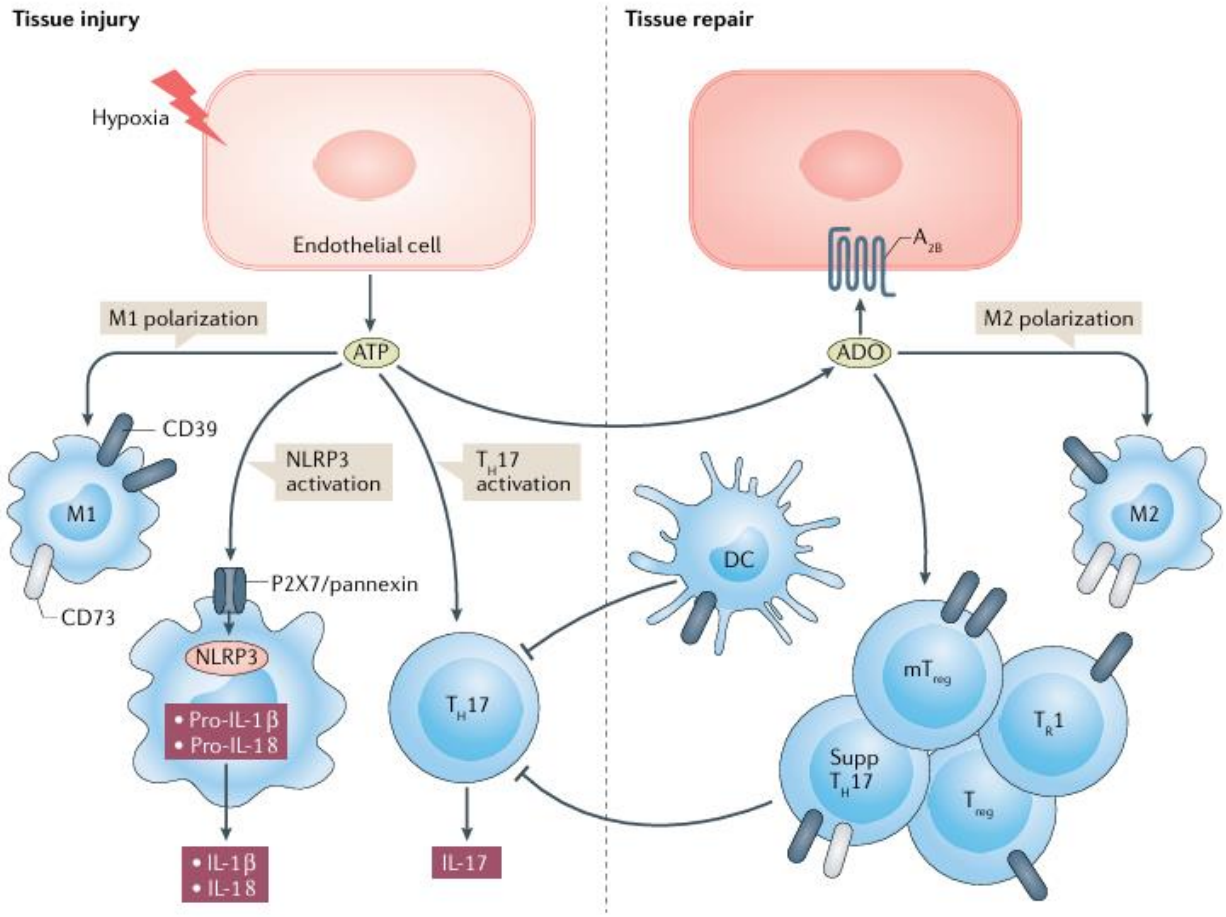


Figure 5: T_H17 cells and macrophages role in tissue injury and repair (Dwyer et al., 2020)

CD73 also plays an important role in maintaining the endothelial barrier function. When tissues are stressed, CD73 produces adenosine from AMP, that binds to the A_{2B} receptors and prevents the leaking of fluid, proteins, and aggressive immune cells out of the bloodstream into surrounding tissues. In this way, it effectively prevents severe tissue swelling (edema) and propagating localized organ damage (Thompson *et al.,* 2004; Eckle *et al.,* 2008).

Platelets are key mediators of thrombosis, and during inflammatory responses, they release large quantities of ADP and ATP from their intracellular dense granules. This ATP and ADP can interact with three different purinergic receptors. ADP interacts with the P2Y1 and P2Y12 receptors whereas ATP acts through P2X1 receptors, and it leads to thrombosis (Hechler & Gachet, 2015). To prevent inappropriate intravascular coagulation, healthy endothelial cells express CD39, which

hydrolyzes excess extracellular ATP and ADP to AMP, and CD73, which subsequently converts AMP into adenosine. This adenosine then binds to the A_{2A} receptor, which raises internal cAMP levels and stops platelet activation and hence prevents blood clotting (Johnston-Cox *et al.,* 2012; Dwyer *et al.,* 2020).

In conclusion, the ectonucleotidase activity of CD73 plays a vital role in modulating physiological functions of the kidney and maintaining immune system homeostasis. However, the dysregulation in purinergic signaling is linked to many renal diseases, and the alterations in expression and function of ecto-5′-nucleotidase (CD73) are linked to many kidney pathologies and disease progression.

Aim of the work

The aim of this study was to evaluate CD73 gene and protein expression levels in peripheral blood mononuclear cells (PBMCs) of maintenance hemodialysis patients relative to healthy controls. Furthermore, CD73 gene and protein expression levels were correlated with key clinical and biochemical parameters in the hemodialysis cohort to assess potential associations with end-stage kidney disease (ESKD) phenotypes. This approach aimed to elucidate the potential role of CD73 and purinergic signaling in the systemic alterations observed during chronic hemodialysis.

Materials and Methods

2.1 Study Population

We analyzed clinically stable uremic patients undergoing maintenance HD for at least six months. Patients with acute infections, active immunological diseases, immunosuppressive therapy, previous transplantation, a history of malignancy, or known blood diseases were excluded. All patients underwent standard bicarbonate HD treatment at the time of observation using polysulfone membranes (F10-HPS, Fresenius Medical Care, Bad Homburg, Germany). We evaluated clinical and biochemical data, including age, dialysis modality, dialysis vintage, intradialytic heparin dose, home therapy, and pre-dialysis serum levels of calcium, phosphate, parathyroid hormone (PTH), C-reactive protein (CRP), and albumin.

The study included two cohorts, each comprising seven maintenance HD patients and seven healthy subjects. Healthy subjects served as the control group and were age- and sex-matched to the HD patients.

The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of the Fondazione IRCCS Policlinico San Matteo of Pavia, Italy (protocol code: 00025750/2024). Written informed consent was obtained from all participants.

2.2 PBMC Isolation

Blood samples were collected from healthy subjects and HD patients before a single hemodialysis session into 7.0 mL heparin-containing tubes. Peripheral blood mononuclear cells (PBMCs) were isolated using density gradient centrifugation (Ficoll-Paque). For this purpose, the blood sample was diluted with the same volume of physiological PBS buffer. Ficoll-Paque corresponding to the equal quantity of diluted blood was added in a separate layer and the diluted blood was carefully and slowly layered over Ficoll-Paque layer. It was centrifuged at 1800 rpm for 30 min. After centrifugation, blood components separate according to density and these layers were visible:

- PBS-diluted plasma

- PBMC layer (interphase) containing lymphocytes and monocytes
- Density medium
- Granulocytes and red blood cells at the bottom

The PBMC layer was successfully isolated in a separate falcon and washed to remove residual density medium, plasma proteins, and platelets. For this purpose, PBS was added and centrifuged at 1600 rpm for 10 min. Supernatant was discarded, and pellet was recovered. To count the cells using Burker chamber, PBS was added in the pellet. Separately, trypan blue solution was prepared by taking 450µL of PBS in eppendorf and adding 50µL of 0.1% of trypan blue. 50µL of the cells were added in trypan blue solution. 20µL of this mixture was loaded on burker chip and cells were counted and calculated. PBMCs isolated were stored at –80ºC.

2.3 RNA Extraction

RNA was extracted from PBMC using QIAGEN miRNeasy Mini Kit. For this purpose, 700µL of QIAzol lysis reagent was added to the cells to disrupt the cell membrane. The homogenate was incubated at room temperature (15-25ºC) for 5 min. 140µL of chloroform was added and was shaken vigorously. After adding chloroform, it was incubated at room temperature for 2-3 min and then centrifuged for 15 min at 12000xg and at 4ºC.

Centrifugation resulted in two different layers separated by an interphase. The upper aqueous layer is RNA. This layer of RNA was transferred to a new collection tube without disrupting the interphase (DNA layer). 1.5 volumes of 100% ethanol were added in RNA and mixed thoroughly. 700µL of this sample was loaded on RNeasy Mini column in a 2ml collection tube and was centrifuged at 8000xg for 15 s at room temperature. The flow-through was discarded, and this step was repeated using the remaining volume. 700µL of buffer RWT was added in Mini columns and was centrifuged at 8000xg for 15 s at room temperature. The flow through was discarded. This was followed by adding 500µL of buffer RPE and was centrifuged at 8000xg for 15 s at room temperature. The flow-through was discarded and 500µL of buffer RPE was added and centrifuged at 8000xg for 2 min at room temperature. After discarding the flow through, RNeasy Mini column was transferred to a new 2ml collection tube and 40µL of RNAse free water was added and

centrifuged for 1 min at 8000xg and room temperature to elute the RNA attached to the Mini column membrane. The eluted sample is RNA and was stored at -80ºC.

After the extraction, a spectrophotometer was used to assess the quantity and quality of the RNA extracted. The A_{260}/A_{280} absorbance ratio was used to evaluate the purity of the isolated RNA.

2.4 Protein Extraction

After cells homogenization and separation of RNA layer, the pink bottom layer of proteins was separated. To extract the proteins, 150µL of ethanol (Et-OH 100%) was added, mixed by inversion (5 times) and incubated for 3 min at room temperature. It was centrifuged at 2000xg for 5 min and at 8ºC. The supernatant was transferred to another tube, and the pellet was discarded. Then 750µL of isopropanol were added, mixed by inversion (4 times) and incubated for 10 min at room temperature. This was followed by centrifugation at 12000xg for 10 min at 8ºC. The supernatant was discarded and 1mL of 0.3M guanidine-HCl in Et-OH 95% was added. This was incubated for 20 min at room temperature. After centrifugation at 7500xg for 5 min at 8ºC, the supernatant was discarded. This step (addition of guanidine, incubation, and centrifugation) was repeated 3 times. Next, 1mL of Et-OH 100% was added, vortexed to break the pellet and incubated at room temperature for 20 min. After centrifugation for 5 min at 7500xg and at 8ºC, supernatant was discarded. The sample was dried in the hood for 30 min. 100µL of 1% sodium dodecyl sulphate (SDS) was added and the pellet dissolved using a pipette or a pestle. This was followed by incubation in a thermocycler at 50ºC for 1 h with sample resuspension after every 15 min. The final step included centrifugation at 10,000xg for 10 min at 8ºC. The supernatant was collected in a separate tube and was stored at -80ºC.

2.5 Reverse Transcription

255ng of the extracted RNA was retro-transcribed using iScript^{TM} cDNA synthesis kit (BIO-RAD). For this purpose, the reaction mixture for each sample was prepared by adding the reagents shown in Table 1.

Table 1: Reaction mixture prepared per reaction for retro-transcription

Reagent	Volume
5x iScript Reaction Mix	4µL
iScript Reverse Transcriptase	1µL
Nuclease-free water	Variable
RNA template	Variable
Total volume	20µL

The RNA template for each sample was calculated as the volume corresponding to 255ng of the concentration and the nuclease free water quantity was calculated to make the final volume up to 20µL. The reaction mix was incubated in a thermocycler using the protocol shown in Table 2.

Table 2: Thermal cycler protocol for retro-transcription

Step	Time and Temperature
Priming	5 min at 25ºC
Reverse transcription	20 min at 46ºC
RT inactivation	1 min at 95ºC
Optional step	Hold at 4ºC

The cDNA obtained was stored at -80ºC.

2.6 RT-PCR: Expression of NT5E/CD73

Real-time polymerase chain reaction was performed using BIO-RAD PrimePCR™ assays to evaluate NT5E gene expression across 14 samples, (7 healthy controls and 7 HD patients). RPLP0 was used as a housekeeping gene. To achieve this, 20 µL of reaction mixture were added per well across three 48-well plates. To prepare 20µL of qPCR reaction mixture, both for the housekeeping and NT5E gene expression, 10µL of 2x SsoAdvanced™ Universal SYBR® Green Supermix, 1µL of respective primer mix, 7µL of Nuclease-free water and 2µL of diluted cDNA (1:3 cDNA to nuclease-free water) was added. Each sample was done in triplicate, and the negative control

containing no cDNA was also used. These 20µL of master mix were transferred on a 48-well PCR plate, The plate was sealed and was loaded on the real time PCR instrument. The PrimerPCR cycling protocol followed is described in Table 3.

Table 3: Thermal cycling protocol for RT-qPCR

Step	Temperature	Time	Number of Cycles
Activation	95ºC	2 min	1
Denaturation	95ºC	5 sec	45
Annealing/extension	60ºC	30 sec	
Melt Curve (for SYBR Green analysis only)	65-95ºC (0.5ºC increment)	5 sec/step	1

2.7 BCA Protein Quantification

Total proteins present in the samples were quantified using a Thermo Scientific Micro BCA Protein Assay Kit. Initial step included standard diluted Albumin samples (BSA) prepared by adding the reagents listed in Table 4.

Table 4: Preparation of diluted Albumin (BSA) standards

Standard	Volume and Source of BSA	Volume of Diluent (SDS 1%)
A	12.5µL of stock BSA	112.5µL
B	5.6µL of stock BSA	69.4µL
C	30µL of A	30µL
D	22.5µL of A	52.5µL
E	30µL of D	30µL
F	30µL of E	30µL

For each protein sample, dilutions were prepared in 1% SDS (1:5 or 1:10) depending on the initial number of PBMC cells used for extraction of the proteins. 20µL of SDS and 5µL of standard or the diluted protein samples were pipetted in the respective microplate wells in duplicates. Micro BCA working reagent (WR) was prepared by mixing 25 parts of Micro BCA Reagent MA, 24 parts of Reagent MB and 1 part of Reagent MC (25:24:1). 25µL of this WR mix were added to all

the wells and was mixed thoroughly on a plate shaker for 30 s. The plate was covered and incubated at 37ºC for 2 h.

The plate was cooled at room temperature, and absorbance was measured on a plate reader at, or near 562nm. A calibration curve was constructed by plotting the mean blank-corrected absorbance at 562 nm for each BSA standard against its concentration (mg/mL). The calibration curve was used to extrapolate the protein concentration of the samples.

2.8 Quantification of CD73 by means of Enzyme-Linked Immunosorbent Assay (ELISA)

The Ab213761–Human CD73 ELISA Kit (NT5E) was used to determine the quantitative measurement of CD73. After preparation of reagents, standards were prepared following the protocol described in Table 5.

Table 5: Standard preparation for ELISA

Standard	Volume to dilute	Volume of diluent	Concentration (pg/mL)
S1	100µL of 10,000 pg/mL stock solution	0µL	10,000
S2	300µL of S1	300µL	5,000
S3	300µL of S2	300µL	25001
S4	300µL of S3	300µL	1250
S5	300µL of S4	300µL	625
S6	300µL of S5	300µL	3121
S7	300µL of S6	300µL	156
S8	0µL	300µL	0

Standards (100 µL) were loaded in duplicate into the wells of a 96-well plate. Similarly, 100 µL of each diluted protein sample (1:2 ratio) were added in duplicate. The wells used as negative control contained only sample diluent buffers. The plate was incubated at 37ºC for 90 min. After incubation, the plate's content was discarded and 100µL of biotinylated anti-Human CD73 antibody working solution were added to each well and incubated at 37ºC for 60 min. Biotinylated anti-Human CD73 antibody working solution was discarded and plate was washed 3 times with 300µL of 1X Wash Buffer (PBS 0.01M). Washing was carried out by incubating the wash buffer in the microplate wells for 1–2 min per cycle.

100µL of prepared ABC working solution were added to each well and incubated at 37ºC for 30 min. The plate was then washed 5 times with 1X Wash Buffer, and each step included a 1–2 min incubation period time. Then, the washing buffer was discarded and 90µL of prepared TMB color developing agent were added to each well and incubated at 37°C in the dark for 15-20 min. The final step included an addition of 100µL of prepared TMB Stop Solution into each well which induced an immediate color shift to yellow.

Absorbance was measured within 30 min after adding the TMB Stop Solution at 450 nm in a microplate reader.

2.9 Statistical Analysis

Quantitative variables are presented as mean ± standard deviation (SD) or as median and interquartile range (IQR) when not normally distributed.

Relative gene expression of NT5E (CD73) was calculated using the 2^ -ΔCT method, where 2^ -ΔCT was determined by subtracting the threshold cycle (Ct) of the RPLP0 housekeeping reference gene from the Ct of the target gene (Ct NT5E – Ct RPLP0).

For ELISA quantification, CD73 protein concentrations were calculated and normalized against total protein input (pg/100pg of proteins).

Correlations between CD73 protein or gene expression and clinical or laboratory variables were evaluated using Pearson's correlation coefficient (*r*).

All statistical tests were two-sided, and a p value < 0.05 was considered statistically significant. Statistical analyses were performed using Stata statistical software (version 11; StataCorp LP, College Station, TX, USA).

Results

3.1 Patient Characteristics

The clinical characteristics of the patient's cohort and control group are shown in Table 6. Twelve patients (8 males and 4 females) were included in this study. Mean age was 61.9±18.2 years, whereas median dialysis vintage was 56.1±24.7 months. Mean BMI was 24.3±5.6 kg/m^2; 1 patient was diabetic. All HD patients were treated with standard bicarbonate hemodialysis. Mean predialysis albumin was 3494± 618.4 mg/dL. According to CRP levels, 5 patients resulted inflamed with median CRP levels of 0.83 (0.55-3.39) mg/dL, and 3 were noninflamed with median CRP of 0.15 (0.07-0.28) mg/dL. Their biochemical evaluations were performed during predialysis.

The mean eGFR predialysis was 5.5 ± 2.6 mL/min and a mean urea level of 166.5 ± 43.7 mg/dL was measured in these patients during predialysis period. The mean Creatinine concentration was 9.7 ± 3.3 mg/dL. The control group (CTRL) consisted of ten healthy subjects (aged 60.7 ± 6.5 years; 4 males).

Table 6: Clinical characteristics of HD patients and Control group

Parameter	Dialysis group (HD)	Healthy subjects (CTRL)
N	12	10
Gender (M/F)	8/12	4/10
Age (y)	61.9 ± 18.2	60.7 ± 6.5
BMI (kg/m^2)	24.3 ± 5.6	-
Systolic BP (mmHg)	132.7 ± 30.2	-
Diastolic BP (mmHg)	70.9 ± 19.2	-
F.C. (bpm)	70.3 ± 12	-
Dialysis age (months)	56.1 ±24.7	-
Blood Pressure, n	3	-
Diabetes Mellitus, n	1	-
Dyslipidemia, n	3	-
Blood flow (Qb) (mL/min)	275 ± 37.8	-

Hemoglobin (g/dL)	11.4 ± 1.5	13.4 ± 2.1
Hematocrit (%)	35.1 ± 4.8	40.1 ± 5.3
MCV (fl)	95.8 ± 8.0	88.7 ± 5
Neutrophils (%)	71.1 ± 7.8	60 ± 7.1
Lymphocytes (%)	16.7 ± 7.2	30.8 ± 6.7
Monocytes (%)	6.34 ± 2.7	6.1 ± 1.6
Eosinophils (%)	3.5 ± 2.1	2.4 ± 1.2
Basophils (%)	0.4 ± 0.1	0.5 ± 0.1
MCH (pg)	31.2 ± 2.6	29.4 ± 1.7
Creatinine (mg/dL)	9.7 ± 3.3	0.9 ± 0.1
Transferrin (mg/dL)	171.4 ± 61.8	-
Albumin (mg/dL)	3494 ± 618.4	3277.1 ± 2199.6
25-OH (Vitamin D)	16.2 ± 9.2	39.3 ± 3.9
Alkaline phosphatase (UI/L)	61 ± 29.3	93.2 ± 43
Sodium (mEq/L)	138.6 ± 2.8	-
Potassium (mEq/L)	5.2 ± 0.7	4.7 ± 0.6
Chloride (mEq/L)	102.8 ± 4.1	105.3 ± 3.5
Total Bilirubin (mg/dL)	0.36 ± 0.14	0.8 ± 0.4
Gamma-GT (MU/mL)	35.4 ± 31.7	28.8 ± 9.5
Creatine kinase (MU/mL)	52.8 ± 37.8	142 ± 72.4
B12 Vitamin (pg/mL)	4222 ± 217.5	418.3 ± 84.3
C-reactive protein (mg/dL)	0.7 ± 0.8	0.5 ± 0.5
Ferritin (ng/mL)	367.3 ± 317	63.5 ± 63.8
Folic Acid (ng/mL)	13.5 ± 11.1	11.3 ± 11.8
ALT (mU/mL)	16.2 ± 8	21.1 ± 6.8
AST (mU/mL)	14.3 ± 4.9	21.8 ± 7.2
Prealbumin (mg/dL)	27.8 ± 11.2	-
eGFR (mL/min)	5.5 ± 2.6	85.1 ± 16.3
Glucose (mg/dL)	124.5 ± 39.5	90.75 ± 5.5
Urea (mg/dL)	166.5 ± 43.7	40.2 ± 7.1

Calcium (mg/dL)	9 ± 0.7	9.7 ± 0.2
LDL (mg/dL)	59.3 ± 37.8	-
HDL (mg/dL)	34.1 ± 7.8	62.1 ± 10.7
Triglycerides (mg/dL)	163.5 ± 100.7	138.1 ± 55.8
Magnesium (mg/dL)	3.6 ± 5.7	-
Total Cholesterol (mg/dL)	119.6 ± 57.2	208.6 ± 21.9
Omocysteine (µmol/L)	42.4 ± 31.4	-
Total protein (g/dL)	6.25 ± 0.5	-
α-1 Globulin (%)	5.5 ± 1.1	-
α-2 Globulin (%)	10 ± 2.3	-
β1- globulin (%)	5.1 ± 0.8	-
β2- globulin (%)	39.4 ± 113.5	-
ɣ- globulin (%)	16.6 ± 3.4	-

3.2 Quantification of Proteins and RNA extracted from PBMCs

Peripheral blood mononuclear cells (PBMCs) collected from study participants were processed to obtain total protein lysates and total RNA, as described in the Materials and Methods section. The number of PBMCs isolated from each participant, along with the corresponding protein yields (BCA assay) and RNA concentrations (NanoDrop spectrophotometry), are summarized in Table 7.

Table 7: Data of number of PBMCs isolated, protein quantification and RNA concentration determined in HD patients and control participants

Participants Code	Number of PBMCs isolated	Total protein quantification (pg/100µL)	RNA concentration (ng/µL)
HD1	3x10^6	3.6x10^8	34.7
HD2	9x10^6	2.53x10^8	71
HD3	2.3x10^6	2.7x10^8	37.4
HD7	4x10^6	0.4x10^8	18
HD8	1.57x10^6	0.51x10^8	22
HD9	1.3x10^6	0.1325x10^8	46.6
HD11	2x10^6	0.17x10^8	72.1
HD12	5.4x10^6	0.465x10^8	22.6
CTRL1	1x10^6	0.365x10^8	16.9
CTRL2	1x10^6	0.1675x10^8	43.7
CTRL3	1x10^6	0.2325x10^8	22.6
CTRL4	1x10^6	0.37x10^8	31.3
CTRL5	1x10^6	0.2775x10^8	34.4
CTRL6	1x10^6	0.36x10^8	43.9
CTRL7	36.7x10^6	1.46×10^8	69.3
CTRL8	33.2x10^6	4.07×10^8	99

3.3 CD73 profile in HD patients

To investigate whether NT5E transcriptional activity differs between healthy controls and clinical HD patients, real-time quantitative PCR (RT-qPCR) was performed on samples from patients (HD, n=7) and healthy controls (CTRL, n=7).

Analysis of ΔCt values revealed a significant difference between the two groups ($p < 0.01$). The HD group showed lower ΔCt values (mean ± SD: 9.84 ± 1.05; median: 10.11; IQR: 9.93–10.43) compared with healthy controls (mean ± SD: 12.16 ± 1.06; median: 12.22; IQR: 11.56–13.14), corresponding to a higher relative CD73 gene expression when expressed as 2^−ΔCt (Figure 6).

Data are presented as box-and-whisker plots representing the median, interquartile range, and minimum-to-maximum values.

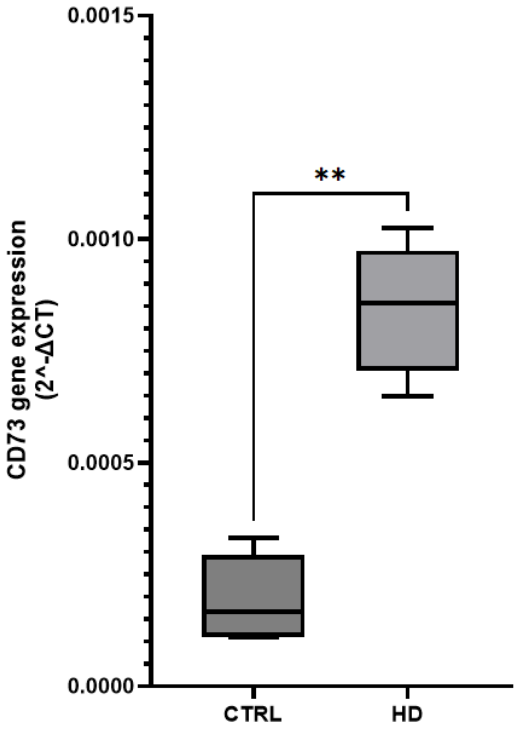


Figure 6: Relative NT5E (CD73) gene expression normalized to RPLP0 in healthy controls and patients, showing significant upregulation in the patient group (Mann–Whitney U test, p< 0.01).

To determine whether upregulation in gene expression corresponds to an increase in protein abundance, CD73 protein levels were subsequently quantified in PBMC lysates by ELISA. The calibration curve used for protein quantification is shown in Figure 7.

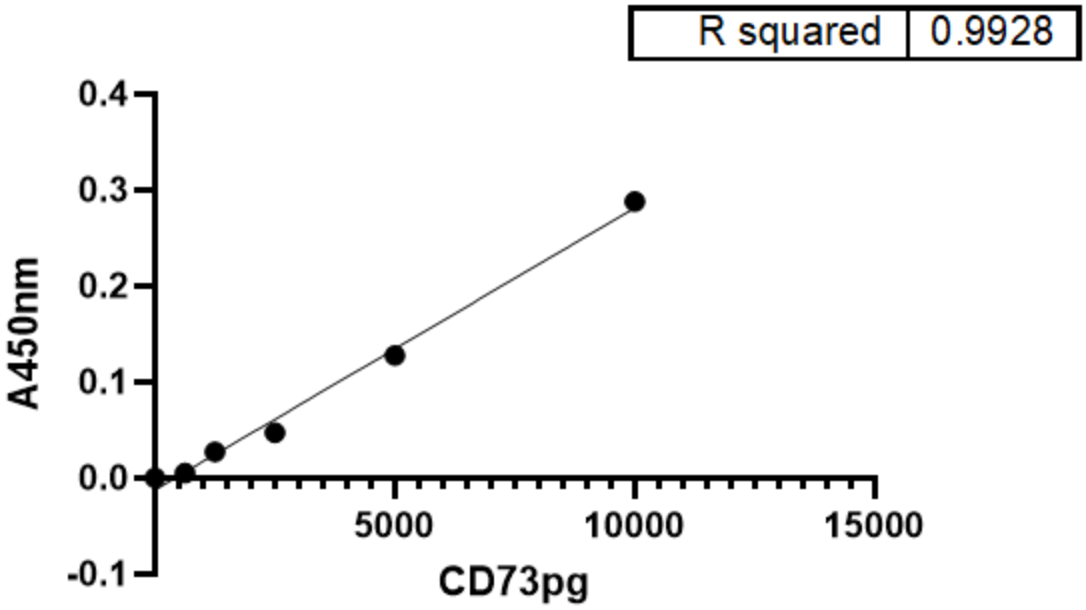


Figure 7: Calibration curve for protein quantification by ELISA

In contrast to the gene expression results, CD73 protein levels were significantly lower in PBMCs from HD patients than in healthy controls ($p < 0.05$). Specifically, the median CD73 protein concentration was 0.0042pg (IQR: 0.0009–0.0112) in the HD group and 0.0153pg (IQR: 0.0082–0.0404) in the control group, as illustrated in Figure 8.

To account for differences in the total protein yield among PBMC lysates, CD73 protein levels were normalized to the total protein content and expressed as picograms (pg) of CD73 per 100 pg of total protein.

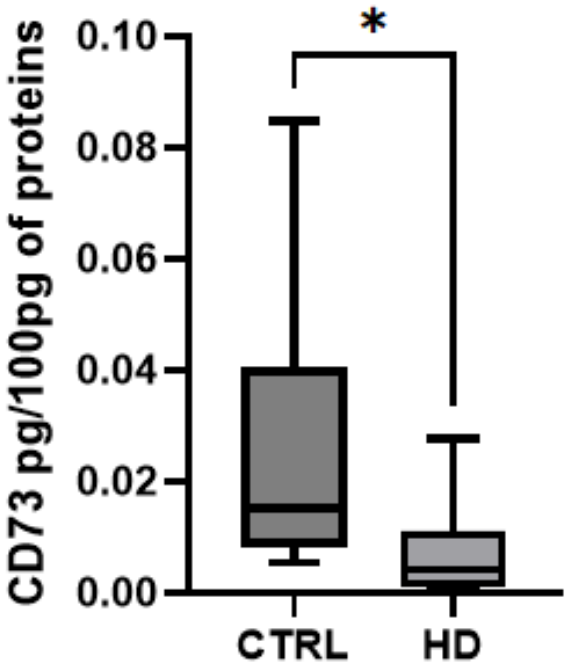


Figure 8: Quantification of CD73 protein levels by ELISA in healthy controls and patients (HD)

3.4 Correlation Analysis

3.4.1 Correlation Analysis of NT5E Gene Expression in HD cohort

No significant correlations were observed between NT5E gene expression and CD73 protein levels, blood pressure, diabetes status, dialysis vintage, hematological parameters, markers of renal function, or most of the biochemical variables analyzed (all $p > 0.05$). A negative, although non-significant, correlation was observed between NT5E gene expression and age ($r = -0.682$, $p = 0.146$; $R^2 = 0.465$).

Among the significant associations, BMI showed the strongest positive correlation with NT5E gene expression ($r = 0.947$, $p < 0.0001$; $R^2 = 0.898$). Moderate positive correlations were also observed with ALT ($r = 0.610$, $p = 0.0121$; $R^2 = 0.372$), transferrin ($r = 0.717$, $p = 0.0086$; $R^2 = 0.515$), potassium ($r = 0.693$, $p = 0.0029$; $R^2 = 0.481$), albumin ($r = 0.603$, $p = 0.0381$; $R^2 = 0.363$), and total protein concentration ($r = 0.593$, $p = 0.0155$; $R^2 = 0.352$).

In contrast, NT5E gene expression was inversely correlated with calcium levels ($r = -0.526$, $p = 0.0362$; $R^2 = 0.277$) and C-reactive protein (CRP) levels ($r = -0.572$, $p = 0.0206$; $R^2 = 0.327$), indicating that these associations accounted for approximately 28% and 33% of the variance in NT5E expression, respectively. The complete set of correlations is reported in Table 8.

Table 8: Correlation among NT5E gene expression, biochemical, and inflammatory parameters in HD patients, represented by Pearson's correlation coefficient (r) ($p < 0.05$, **$p < 0.01$)*

	NT5E expression
NT5E expression	1
Age	−0.682
BMI	0.947**
ALT	0.610*
Transferrin	0.717**
Potassium	0.693**
Albumin	0.603*
Total protein	0.593*
Calcium	−0.526*
CRP	−0.572*

3.4.2 Correlation Analysis of CD73 protein levels in HD cohort

CD73 protein levels did not show significant correlations with NT5E gene expression, age, blood pressure, body weight, BMI, heart rate, diabetes status, or most hematological and biochemical variables analyzed (all $p > 0.05$).

Among the significant associations, moderate positive correlations were observed between CD73 protein levels and dialysis vintage ($r = 0.659$, $p = 0.0055$; $R^2 = 0.435$), hemoglobin ($r = 0.542$, $p = 0.0300$; $R^2 = 0.294$), and ferritin ($r = 0.626$, $p = 0.0295$; $R^2 = 0.392$).

Conversely, CD73 protein levels showed a strong inverse correlation with lymphocyte count ($r = -0.944$, $p < 0.0001$; $R^2 = 0.891$) and CRP levels ($r = -0.727$, $p = 0.0074$; $R^2 = 0.528$), while moderate inverse correlations were observed with monocyte count ($r = -0.616$, $p = 0.0110$; $R^2 = 0.380$) and serum urea levels ($r = -0.704$, $p = 0.0023$; $R^2 = 0.496$). The complete set of correlations is reported in Table 9.

*Table 9: Correlation among CD73 protein levels and biochemical parameters in HD patients, represented by Pearson's correlation coefficient (r) (*p < 0.05, **p < 0.01)*

	CD73 protein
CD73 protein	1
Dialysis Vintage	0.659**
Hemoglobin	0.542*
Ferritin	0.626*
Lymphocyte count	−0.944**
CRP	-0.727**
Monocyte Count	−0.616*
Serum Urea	−0.704**

3.4.3 Correlation Analysis in CTRL cohort

In healthy subjects, a significant positive correlation was observed between NT5E gene expression and CD73 protein levels (r = 0.8852, $R^2 = 0.7835$, $p = 0.0035$). CD73 protein levels showed a

strong negative correlation with age ($r = -0.8393$, $R^2 = 0.7045$, $p = 0.0092$). Similarly, NT5E gene expression was significantly and negatively correlated with age ($r = -0.7540$, $R^2 = 0.5685$, $p = 0.0307$), as illustrated in Table 10.

Table 10: Correlation among NT5E gene expression, CD73 protein levels, and biochemical parameters in controls, represented by Pearson's correlation coefficient (r) ($p < 0.05$, **$p < 0.01$)*

	NT5E gene expression	**CD73 protein**
NT5E gene expression	1	0.8852**
CD73 protein	0.8852**	1
Age	−0.7540*	−0.8393**

Discussion

The present study investigated CD73 (NT5E) at both the transcriptional and protein levels in PBMCs from patients undergoing maintenance hemodialysis (HD) and healthy controls and explored the associations between CD73 expression and the clinical and biochemical characteristics of the dialysis population.

The most notable finding was a marked discordance between NT5E transcription and CD73 protein abundance. Although NT5E mRNA expression was significantly increased in PBMCs from HD patients, CD73 protein abundance was significantly reduced. To the best of our knowledge, this is the first study to simultaneously evaluate both levels of CD73 regulation in PBMCs from patients undergoing maintenance HD and healthy controls. This finding suggests that CD73 regulation in the setting of end-stage kidney disease may involve mechanisms acting beyond transcriptional control and highlights the importance of considering both transcript and protein abundance when investigating the CD73/adenosine pathway.

The available literature on CD73 in HD remains limited, and previous studies have primarily evaluated CD73 at the cellular level using flow cytometry or enzymatic activity assays rather than assessing NT5E transcription and CD73 protein abundance in parallel.

For example, Dalagnol et al. (2026) reported a higher proportion of CD73-positive lymphocytes in patients with chronic kidney disease undergoing HD than in healthy controls, together with an increased ecto-5′-nucleotidase activity in platelets. The authors interpreted these findings as evidence of an overall enhancement of the CD73-dependent purinergic pathway in chronic kidney disease. However, CD73 expression in lymphocytes was quantified by flow cytometry as the proportion of CD73-positive cells rather than as NT5E transcript abundance or CD73 protein content in the overall PBMC population. While their findings indicate an increased representation of CD73-expressing lymphocytes, they cannot be directly equated with increased CD73 protein abundance per PBMC or increased NT5E transcription.

The apparent difference between these observations and our findings may therefore reflect the distinct biological levels assessed rather than a genuine discrepancy. Flow cytometry primarily measures the proportion of cells expressing CD73 and, depending on the experimental design, it may also be influenced by changes in the relative distribution of PBMC subsets. In contrast, quantitative analysis of NT5E mRNA and CD73 protein in bulk PBMCs provides information on transcriptional activity and overall protein abundance within the heterogeneous PBMC compartment. Accordingly, an increased proportion of CD73-positive lymphocytes may coexist with reduced overall CD73 protein abundance in PBMCs if the relative distribution of immune-cell subsets changes or if CD73 expression differs substantially among individual cell populations. Further studies incorporating subset-specific analyses will therefore be required to determine whether the apparently divergent findings reflect differences in cellular composition, cell-specific CD73 regulation, or both.

The significant increase in NT5E transcription observed in PBMCs from HD patients may represent an adaptive response to the altered extracellular environment associated with end-stage kidney disease and chronic dialysis. CD73 is a key component of the purinergic signaling pathway and catalyzes the conversion of extracellular AMP into adenosine, an important immunomodulatory and anti-inflammatory mediator (Lazarowski *et al.,* 2000). Together with CD39, CD73 contributes to the regulation of extracellular nucleotide concentrations and to the control of inflammatory responses (Colgan *et al.,* 2006).

Previous studies have shown that CD73 expression and activity can be modulated in activated peripheral immune cells. Schuler et al. demonstrated that activation of PBMCs/Tregs with anti-CD3/CD28 increased both the proportion of CD73-positive cells and CD73 mean fluorescence intensity, accompanied by increased adenosine production (Schuler *et al.,* 2014). Similarly, TLR7 stimulation of blood-derived plasmacytoid dendritic cells has been shown to increase surface CD73 expression and thereby contribute to ATP-derived adenosine generation (Sosa-Luis *et al.,* 2024). Hypoxia and pseudo-hypoxia have also been implicated in the induction of the CD39/CD73 pathway and subsequent adenosine generation (Serra *et al.,* 2016). Although these observations were not obtained specifically in PBMCs from patients undergoing HD, they support the concept that CD73 expression may be dynamically regulated in response to changes in the extracellular and inflammatory environment.

However, the concomitant reduction in CD73 protein abundance observed in our study indicates that increased transcription does not necessarily translate into increased protein availability. This interpretation is further supported by the absence of a significant correlation between NT5E transcript and CD73 protein abundance in the HD group, in contrast to the strong positive association observed in healthy individuals. This apparent uncoupling between transcriptional and protein levels may reflect regulation at multiple post-transcriptional or post-translational steps, including altered mRNA translation, impaired intracellular trafficking or membrane localization, increased protein turnover or degradation, and changes in protein stability.

The uremic milieu provides a biologically plausible context in which such mechanisms could occur. Uremia is characterized by heightened oxidative stress and the accumulation of uremic toxins. Consequently, circulating and cellular proteins undergo oxidative modifications, carbonylation, glycation, and other post-translational alterations that can compromise their structure, stability, and function (Piroddi *et al.,* 2007; Antonelou *et al.,* 2011). Although direct evidence that these processes specifically promote CD73 degradation in PBMCs from patients undergoing HD is currently lacking, they could contribute to the reduced CD73 protein abundance observed despite increased NT5E transcription.

Furthermore, CD73 is a glycosylphosphatidylinositol (GPI)-anchored ectoenzyme whose biological activity depends not only on its total cellular abundance but also on its appropriate trafficking and localization at the plasma membrane (Alcedo *et al.,* 2021). Consequently, measurement of total CD73 protein in PBMC lysates does not necessarily reflect the fraction of CD73 effectively expressed at the cell surface or its functional capacity to generate extracellular adenosine. This distinction may be particularly relevant in the uremic and dialysis setting, in which alterations in membrane protein homeostasis and protein turnover may occur.

In a previous study (Esposito *et al.,* 2012), we have demonstrated that patients with uremia and those undergoing HD exhibited reduced CD40 gene expression and membrane CD40 expression, together with increased circulating soluble CD40 (sCD40). These alterations were shown to be closely related to impaired renal clearance of the soluble form of CD40. Although CD40 and CD73 have distinct biological functions and regulatory mechanisms, these findings provide an example of how the uremic environment may affect membrane-associated proteins and their distribution

between cellular and soluble compartments. This previous observation therefore provides a useful biological context for considering whether similar alterations in protein homeostasis, trafficking or shedding could contribute to the discrepancy between NT5E transcription and CD73 protein abundance observed in the present study.

A similar mechanism could be hypothesized for CD73, particularly in light of the strong inverse association observed between CD73 protein abundance and serum urea concentration. Urea is a major marker of the uremic milieu and accumulates as renal function declines. The observed association therefore raises the possibility that the degree of uremic disturbance may be related to CD73 protein abundance or turnover. Nevertheless, serum urea is also influenced by dialysis efficiency, dietary and protein intake, residual kidney function, catabolic status, and the timing of blood sampling relative to dialysis. Therefore, the inverse association observed in our cohort should be regarded as exploratory and cannot be interpreted as evidence of a direct effect of urea on CD73 expression, degradation, or stability. Direct mechanistic studies will be required to determine whether uremic toxins or other components of the uremic environment contribute to the observed reduction in CD73 protein abundance.

Interestingly, CD73 protein abundance was positively associated with dialysis vintage. At first sight, this finding might appear inconsistent with the hypothesis that the uremic environment contributes to reduced CD73 protein availability. However, dialysis vintage should not necessarily be interpreted as a direct measure of cumulative uremic exposure. In our cohort, patients with a longer dialysis vintage were predominantly younger individuals (<50 years), and dialysis vintage may therefore be influenced by survivor and selection effects, as well as by differences in access to kidney transplantation. Thus, the positive association between CD73 protein abundance and dialysis vintage may reflect the demographic structure of the cohort rather than a direct effect of prolonged dialysis exposure on CD73 expression.

In this regard, age may represent a key determinant. Across both healthy controls and dialysis patients, CD73 expression tended to decline with advancing age.

This observation is consistent with previous evidence showing an age-related decline in CD73-expressing memory T-cell populations. Fang et al. (2021) demonstrated that CD73 identifies a distinct functional memory T-cell subset and that the proportion of CD73-positive CD4+ and

CD8+ memory T cells declines with age. These findings support the possibility that age-related changes in immune-cell composition and/or CD73 regulation may contribute to the relationships observed in our cohort. Accordingly, the positive association between CD73 protein abundance and dialysis vintage should be interpreted with caution, particularly in light of our small sample size and the age distribution of the study population. (Fang *et al.,* 2021).

The correlation analyses further revealed a distinct pattern of associations between NT5E/CD73 expression and clinical and biochemical parameters in the HD population. Overall, these findings suggest that NT5E/CD73 expression may reflect the broader nutritional, metabolic, inflammatory, hematological, and dialysis-related milieu rather than being driven by any single clinical parameter. Two partially overlapping biological themes may be identified: the potential interplay between the CD73 pathway and the systemic metabolic and nutritional environment, and the relationship between CD73 and the effects of uremic exposure, dialysis adaptation, cellular composition, and protein homeostasis. These associations should be interpreted cautiously, as they are exploratory and do not establish causal relationships.

In particular, the strong positive associations between NT5E expression and BMI, as well as albumin, total protein, transferrin, potassium, calcium, and liver-related parameters, may collectively point to an indirect relationship between NT5E expression and the broader nutritional, inflammatory, and immunometabolic status of patients undergoing hemodialysis. The associations with hematological parameters should also be interpreted within this broader context. For instance, ferritin cannot be regarded as a specific marker of iron stores in patients undergoing hemodialysis, as its levels are also substantially influenced by inflammation. Accordingly, the observed associations of NT5E expression with ferritin and hemoglobin may reflect the complex interplay among erythropoiesis, iron metabolism, inflammation, and dialysis-related factors, rather than a direct relationship between these parameters and NT5E/CD73 expression. Similarly, the associations observed with liver enzymes may reflect systemic rather than organ-specific processes. In particular, the positive association between NT5E expression and ALT may suggest a link between CD73 transcription and the metabolic or hepatic milieu, although the biological mechanisms underlying this relationship remain unclear.

However, these parameters are influenced by multiple, often interrelated biological processes, including nutritional status, inflammation, hydration, and, in the case of transferrin, iron metabolism. Thus, the observed associations should be interpreted cautiously, as they may reflect the complex systemic milieu of patients undergoing hemodialysis rather than direct causal relationships.

Conversely, the inverse association observed between CRP and both NT5E expression and CD73 protein abundance suggests a potential relationship between the CD73/adenosine pathway and systemic inflammatory burden. This interpretation is consistent with previous evidence linking reduced ectonucleotidase expression with inflammatory states. Pietrobon et al. reported reduced expression of ectonucleotidase genes, including NT5E, in the blood of patients with COVID-19, together with an inverse relationship between NT5E expression and circulating CRP levels; CD73-expressing T-cell populations were also reduced in patients with more severe disease (Pietrobon *et al.,* 2022). Although these findings were obtained in the distinct context of acute viral inflammation, they provide independent evidence of an inverse relationship between NT5E/CD73 expression and systemic inflammatory burden. Importantly, this observation is also consistent with findings in HD patients. Xiang et al. reported a reduced proportion of CD4+CD73+ T cells in patients undergoing HD and showed that reduced CD73-positive T-cell fractions were associated with systemic inflammation and terminal T-cell differentiation (Xiang *et al.,* 2024). These findings support the possibility that chronic inflammatory conditions may be associated with alterations of CD73 expression within circulating immune-cell populations.

Finally, the strong inverse association between CD73 protein abundance and lymphocyte count, together with its negative association with monocyte count, should be interpreted with particular caution, as CD73 was quantified in bulk PBMC lysates. These associations may therefore reflect, at least in part, differences in the cellular composition of the PBMC fraction and/or variation in CD73 abundance across individual leukocyte subsets. This is particularly relevant given that CD73 is differentially expressed among circulating immune-cell populations, being predominantly expressed on specific lymphocyte subsets, whereas its expression on circulating monocytes appears to be more limited (Ehlers *et al.,* 2021). Thus, changes in the relative abundance of these cell populations could contribute to the overall CD73 signal measured in bulk PBMCs, independently of changes in CD73 expression at the single-cell level. In patients undergoing

hemodialysis, reduced proportions of CD73-expressing T cells have also been associated with systemic inflammation and immune dysfunction, further suggesting that CD73 expression may be influenced by the broader inflammatory and immunological milieu rather than simply reflecting the absolute number of circulating immune cells (Xiang *et al.,* 2024).

Interestingly, the pattern observed in healthy individuals differed from that identified in the HD population. In healthy subjects, CD73 protein abundance showed a strong positive correlation with NT5E transcript levels, consistent with the expected relationship between transcription and protein expression under physiological conditions. Moreover, CD73 protein abundance was positively associated with eGFR, although the proportion of variance explained by this association was modest. To our knowledge, a direct association between CD73 protein abundance in PBMCs and eGFR has not previously been described in healthy individuals. Although CD73 is a key enzyme in extracellular adenosine generation and CD73-dependent purinergic signaling contributes to the regulation of renal hemodynamics and glomerular filtration, these mechanisms have mainly been demonstrated in renal tissues and experimental models. Therefore, the association observed in PBMCs should not be interpreted as evidence of a direct effect of circulating immune-cell CD73 on glomerular filtration. Rather, it may reflect a broader relationship between CD73 expression in circulating immune cells and systemic physiological or metabolic status, the biological basis of which remains to be elucidated.

CD73 protein abundance and NT5E expression were also positively associated with circulating glucose levels, while NT5E transcript levels were positively associated with total cholesterol. Although direct evidence linking CD73 protein abundance or NT5E transcription in PBMCs with these circulating metabolic parameters in healthy individuals remains limited, these findings may be biologically plausible in light of the established involvement of CD73/adenosine signaling in metabolic regulation. Nevertheless, given the limited sample size and the observational nature of these analyses, these associations should be considered exploratory and hypothesis-generating.

The fact that these renal and metabolic associations were observed in healthy individuals but were not apparent in the HD population does not necessarily indicate that these relationships are absent in patients undergoing haemodialysis. Rather, they may be modified, attenuated, or obscured by the multiple and overlapping biological processes characterizing this population. The complex

biological environment of haemodialysis, together with the previously discussed alterations in transcriptional and protein regulation, inflammatory status, cellular composition, and metabolic homeostasis, may therefore make physiological associations between CD73/NT5E expression and systemic parameters less readily detectable.

A statistical explanation should also be considered. The relatively small sample size, particularly in the healthy control group, limits the precision of correlation estimates and reduces the statistical power to detect moderate associations. In addition, the greater biological heterogeneity of the HD population may further increase variability and make subtle physiological relationships more difficult to identify. Therefore, the absence of significant correlations in HD should not necessarily be interpreted as evidence that the corresponding biological relationships are lost. Conversely, the significant associations observed in healthy individuals should not be considered definitive evidence of physiological mechanisms, particularly given the exploratory nature of these analyses.

Overall, the different correlation patterns observed between healthy and HD individuals suggest that the relationship between CD73/NT5E expression and systemic renal or metabolic parameters may be context dependent. In healthy individuals, CD73 expression may more closely reflect physiological systemic conditions, whereas in the complex setting of haemodialysis, multiple overlapping biological processes may modify or obscure these relationships. These findings should therefore be considered hypothesis-generating, establishing a rationale for future studies to confirm whether these associations persist in larger cohorts and how they are altered by chronic kidney disease and dialysis.

Limitations of the study and Future perspectives

Several limitations should be considered when interpreting these findings. First, the relatively small sample size, particularly the limited number of healthy controls and HD patients, reduces statistical power and limits the precision of correlation estimates. Further patient recruitment and validation in larger, clinically well-characterized cohorts will therefore be necessary to confirm the observed associations and allow more robust multivariable analyses. Second, the cross-sectional design does not allow temporal or causal relationships to be established; longitudinal studies, including assessment before and after dialysis initiation and during long-term treatment, would help clarify the dynamics of CD73 regulation. Third, the use of bulk PBMCs may mask cell-specific alterations and the contribution of changes in PBMC subset composition. Future studies should therefore characterize CD73 expression in individual immune-cell populations, particularly by flow cytometry, and distinguish total from cell-surface CD73. Finally, the study assessed NT5E transcription and CD73 protein abundance but not CD73 enzymatic activity, extracellular adenosine production, or other components of the purinergic pathway, such as CD39 and adenosine receptors. Functional studies integrating these parameters, together with investigation of post-transcriptional and post-translational mechanisms, will be important to determine whether the molecular alterations observed translate into functional changes in purinergic signaling.

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NOTES

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