PONTIFÍCIA UNIVERSIDADE CATÓLICA DO PARANÁ
ESCOLA DE MEDICINA E CIÊNCIAS DA VIDA
PROGRAMA DE PÓS-GRAÇÃO EM CIÊNCIAS DA SAÚDE

The Uremic Toxin CMPF increases the Osmotic Fragility of Red Blood Cells through the mechanoreceptor Piezo1.

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Curitiba, 2023

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A Master's Thesis Submitted to the Post-Graduation Program in Health Sciences (PUC-PR) in Fulfillment of the Requirements for a Master of Science Degree.

Mentor: Professor Andréa Novais Moreno Amaral, PhD

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List of abbreviations

CKD	Chronic Kidney Disease
CMPF	3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid
CNS	Central Nervous System
DMSO	Dimethyl Sulfoxide
EC	Endothelial Cells
EPO	Erythropoietin
ESAs	Erythropoiesis Stimulating Agents
FA	Fatty Acid
GsMTx-4	*Grammostola* mechanotoxin number 4
Hb	Hemoglobin
HD	Hemodialysis
HX	Hereditary Xerocytosis
ICAM-4	Intracellular Adhesion Molecule 4
icCa $^{2+}$	Intracellular calcium
IC50	Median Inhibitory concentration
MA	Mechanic Activity
nm	Nanometers
OFC	Osmotic Fragility Curve
OFI	Osmotic Fragility Index
OF	Osmotic Fragility
OFT	Osmotic Fragility Test
PAF	Platelet Activating factor
PBS	Phosphate Buffered Saline
PS	Phosphatidylserine
RBC	Red Blood Cell
RNA	Ribonucleic Acid
siRNA	Small Interfering Ribonucleic Acid
THU	Transmembrane Helical Units
UT	Uremic Toxins

Abstract

Chronic kidney disease (CKD) patients tend to develop renal anemia, mainly by erythropoietin deficiency. Still, it also can be associated with the retention of uremic toxins (UT), previously related to increased red blood cell (RBC) eryptosis. 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF), an UT, is 5 times higher in CKD patients compared to healthy subjects. This compound shares chemical similarities with the small molecule Jedi1, an activator of the mechanosensitive ion channel Piezo1, which is expressed on several cell types, including on RBC. This study aimed to investigate the ability of the uremic toxin CMPF induce osmotic fragility (OF) in RBC through Piezo1. RBC from healthy donors (n = 5) in phosphate buffered saline (PBS) pre-treated or not with the Piezo1 inhibitor GsMTx-4 were incubated with CMPF or Jedi1 for 30 minutes. Negative control represented cells incubated with buffer. Cells were added to increasing concentrations (from 0 to 9 g/L) of sodium chloride (NaCl), diluted in distilled water. The content was homogenized and centrifuged, the supernatant was added to a 96-well flat bottom plate, and absorbance was obtained at 540 nm. The results showed that CMPF had a significant impact on RBC, affecting osmotic fragility and increasing hemolysis compared to the negative control (5.57 ± 0.39; p = 0.002), but not for cells treated with the channel blocker GsMTx-4 (4.47 ± 0.13; p = 0.546). Jedi1 also increased the OF compared to the negative control (5.02 ± 0.28; p = 0.03), which GsMTx-4 prevented (4.47 ± 0.13; p = 0.94). These findings are relevant to the basic mechanisms behind the establishment of renal anemia, which in the future, could support clinical research aiming to improve the quality of life of patients.

Keywords: Renal anemia, CMPF, uremic toxin, osmotic fragility, Piezo1.

Fact sheet

Renal anemia is a common condition observed in chronic kidney disease (CKD) patients, elevating the burden of the disease and reducing the quality of life of the individuals. Due to insufficient renal clearance, compounds are retained in the organism and are potentially harmful. The compound 3carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) is like the molecule Jedi1, which activates Piezo1, a protein located in the membrane of RBC that is sensitive to mechanical stimuli. This study investigated the effects of the retention solute CMPF on red blood cells (RBC). CMPF or Jedi1 was added or not to isolate RBC. After, cells were submitted to increasing saline solutions. The results showed that CMPF could increase RBC osmotic rupture through Piezo1. These findings are relevant because this might be the first endogenous compound described as interacting with a mechanosensitive channel. This could support future research regarding the processes of establishment of renal anemia during the progression of CKD, helping patients to improve their quality of life.

1. Introduction

Renal anemia is frequently reported in individuals with chronic kidney disease (CKD) and is associated with poor outcomes and reduced quality of life (Babitt & Lin, 2012). The major cause of renal anemia is the insufficient production of the hormone erythropoietin (EPO) by the damaged kidney (Hanna et al., 2021). In addition, other factors were shown to be related to renal anemia, such as functional iron deficiency due to chronic inflammation (Babitt & Lin, 2012), and reduced red blood cell (RBC) lifespan caused by the phenomenon of the erythrocyte suicidal death entitled eryptosis (F. Lang et al., 2006).

In patients undergoing hemodialysis (HD) treatment, it was demonstrated a reduced RBC lifespan to about 73 ± 18 days (range 38 – 116) (Sato et al., 2012). This reduction might be due to exacerbated eryptosis, that was previously associated with elevated levels of uremic toxins (UTs) (Dias et al., 2018; Tozoni et al., 2019), solutes retained due to insufficient renal clearance, causing the uremic syndrome or uremia (Nigam & Bush, 2019). The compound 3-carboxy-4-methyl-5-propyl-2furanpropanoic acid (CMPF), a protein-bound UT, which contains the active moiety 3-carboxylic acid methylfuran, was previously noted for being responsible for inhibiting erythropoiesis (Niwa et al., 1995), participating in the formation of thyroid abnormalities (Everts et al., 1995), impairing neurological functions (Costigan et al., 1996) and causing damage to proximal tubular cells (Miyamoto et al., 2012). There is no evidence of direct interactions of CMPF with erythrocytes and possible effects on its physiology.

Eryptosis is induced by oxidative stress, Cl^- removal, hyperosmotic shock (F. Lang & Qadri, 2012), and ATP depletion (E. Lang & Lang, 2015). These factors culminate in various responses, including red cell shrinkage due to loss of KCl after activation of Ca^{2+}-sensitive K^+ channels and exit of osmotically obliged water; membrane blebbing as result of calpain activation of cytoskeletal protein degradation; membrane scrambling with exposure of phosphatidylserine (PS) at the outer leaflet, signalizing phagocytosis (Lang et al., 2012). All these events are triggered by increased intracellular Ca^{2+} ($icCa^{2+}$) concentration (Pretorius et al., 2016).

The mechanosensitive cation channel Piezo1 is located in the RBC plasma membrane and is critical in cell volume regulation (Danielczok et al., 2017). Shear stress in the microcirculation activates Ca^{2+} influx via Piezo1. The rise of $icCa^{2+}$ subsequently activates calcium-activated potassium KCa3.1 Gardos channels, leading to hyperpolarization and a loss of K^+, Cl^-, and water, resulting in RBC shrinkage (Picard et al., 2019). This process is physiologically important as the RBC traverses narrow anatomical structures such as capillaries and the slits that are formed in the spleen by the spaces between the endothelial cells lining the organ's blood vessels. After the passage, Piezo1 is inactivated, and $icCa^{2+}$ is extruded by a Ca^{2+}ATPase. Consequently, the RBC reverts to its prepassage shape (Aglialoro et al., 2021). Despite mechanical stimuli, the small molecule Jedi1 was identified as a chemical activator of Piezo1, gating the channel possibly by the chemical motif 3-carboxylic-acidmethylfuran (Wang et al., 2018).

Given the structural similarity between the small molecule Jedi1 and the uremic solute CMPF, it was hypothesized that increased levels of CMPF lengthen the Piezo1 activation and thereby increase Ca^{2+} influx via Piezo1 in a Jedi1-like fashion (Kotanko et al., 2022). This could contribute to elevated osmotic fragility of the RBC, reducing its lifespan.

2. Justification

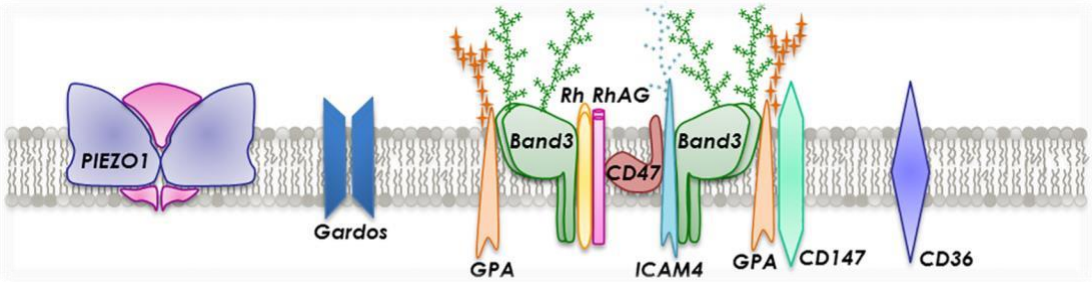
Renal anemia is associated with poor outcomes, elevating the burden of CKD and the cellular mechanisms by which this condition is established constantly need improvement. The structural similarity between the uremic toxin CMPF and the Piezo1 agonist Jedi1 was identified, but there is no experimental evidence regarding if there is also a functional similarity of these molecules on mature RBCs. The possible interaction of an endogenous retained compound with a mechanosensitive channel could instigate new research aiming to describe a new pathway behind renal anemia.

3. Literature Review

Red blood cells

Erythrocytes are the most common cell type in human blood. Although they lack nuclei, ribosomes, mitochondria, and other cell machinery (Pretini et al., 2019), the amount of Hemoglobin (Hb), a protein in charge of the oxygen (O_2) delivery to peripheral tissues, is abundant (Pretini et al., 2019). Red Blood Cell (RBC) production, or erythropoiesis, is a fine-tuned process that starts in the bone marrow and ends at circulation, allowing around 2 million newly formed RBC to enter the blood flow, while about the same number is cleared (Higgins, 2015). During its assembly, erythroid precursors sit in a rich environment containing other immature cells and extracellular matrix proteins, and they are also in constant contact with cell adhesion molecules and cytokines (Dzierzak & Philipsen, 2013). This promotes is maturation and release to the circulation as newly formed RBC. At the bloodstream, throughout their final stages of maturation, reticulocytes enter the peripheral blood and go through a selective sorting process, losing 20% of the plasma membrane and the remaining RNA content (Pretini et al., 2019). After changes in the membrane, such as the loss of several transmembrane proteins (Moras et al., 2017), the mature RBC reaches is the biconcave shape, remarkable for the ability to be deformable because of external forces (Huisjes et al., 2018).

To maintain their shape and deformability properties, RBC are equipped with a complex structure called membrane skeleton (Lux, 2016), composed of a lipid bilayer with embedded transmembrane proteins that form multi-protein complexes (Pretini et al., 2019) (Figure 1). **Figure 1**. Section of the RBC membrane with a focus on the composition of integral membrane proteins incorporated into a phospholipid bilayer.



Pretini et al., 2019.

The recently discovered cation channel Piezo1 is a mechanosensitive channel that plays a notable role in maintaining erythrocyte volume homeostasis (Coste et al., 2010). Its mutations were reported to be related to hereditary Xerocytosis (HX), leading the cell to increased dehydration (Bae et al., 2011). The Gardos channel, or KCNN4/IK-1, is a calcium-dependent potassium channel (Thomas et al., 2011), and interacts with Piezo1 when RBC are undergoing narrow capillaries and interstices (Danielczok et al., 2017). Band 3 is the major anion exchanger protein in RBC membranes, providing integrity to the membrane. Its numerous binding sites connect Band 3 with other membranes, allowing the efficient transduction of signals from the membrane and cytoskeleton, regulating the flexibility, stability, and deformability of the RBC (Pretini et al., 2019). The glycophorin-A is a transmembrane glycoprotein that maintains the net negative surface of the cell membrane (Poole, 2000). It also affects pathogen recognition (Morera & MacKenzie, 2011). Intercellular adhesion molecule 4 (ICAM-4) is a glycoprotein that is responsible for cell-to-cell interaction or adhesion and plays a role when it comes to many physiological processes such as hemostasis and thrombosis (De Oliveira & Saldanha, 2010).

Mature erythrocytes present a life span of around 100-120 days and undergo senescence with subsequent removal of aged RBCs (Lang et al., 2010). In this process, hemichromes bind to the Band 3 complex, complement C3 fragments are deposited, and anti-Band 3 immunoglobulins signalize phagocytosis (Lang et al., 2017). However, removal of RBC before senescence can happen due to various conditions, in a process, called eryptosis (Lang et al., 2010), or suicidal erythrocyte death. Increased eryptosis is found in numerous diseases. Genetic disorders of Hb synthesis such as sickle cell anemia and thalassemia; deficiency of glucose-6-phosphate dehydrogenase (Lang et al., 2010). High levels of eryptotic cells were also found in metabolic diseases, including iron deficiency, diabetes, and renal insufficiency (Lang et al., 2012). In this programmed cell death, a wide range of challenges such as Cl^- removal, oxidative stress, and hyperosmotic shock can stimulate Ca^{2+} entry through permeable non-selective cation channels (Lang et al., 2010). Increased cytosolic Ca^{2+} is followed by activation of Ca^{2+}-sensitive K^+ channels with the consequent exit of K^+, hyperpolarization of the cell membrane, Cl^- loss, and exit of osmotically obliged water, shrinking the cell (Lang et al., 2017). Upon cell shrinkage, platelet-activating factor (PAF) is released and stimulates sphingomyelin degradation,

leading to ceramide formation (Lang et al., 2010). Moreover, the presence of intracellular Ca^{2+} activates calpain, which in turn degrades cytoskeletal proteins and promotes membrane blebbing (Lang et al., 2012). Membrane scrambling with the breakdown of the phospholipid asymmetry after the exposure of PS at the extracellular membrane is also stimulated by elevated intracellular Ca^{2+} (Lang et al., 2012). PS-exposing RBC are rapidly removed from circulation. Macrophages with specific receptors bind to the cells, engulf, and degrade them (Lang & Qadri, 2012). Enhanced eryptosis, if not compensated by the differentiation of mature RBC, leads to anemia. Furthermore, erythrocytes with exposed PS can adhere to the endothelial wall and may obstruct microcirculation (Lang & Qadri, 2012).

RBC Osmotic Fragility (OF) is defined by the propensity to hemolysis under a variation of osmotic conditions (Parpart et al., 1947). The Osmotic Fragility Test (OFT) is broadly used to characterize the hydration state of RBCs (Cueff et al., 2010). It helps to identify hemolytic disorders such as Hereditary Spherocytosis and Hereditary Xerocytosis (Archer et al., 2014). In this type of assay, the resistance of cells to lysis as a function of increased NaCl concentrations is measured (Godal et al., 1981). In hypotonic media (low salt concentrations), RBCs are more prone to uptake water and reach spherocytic shape to the point where the phenomenon of lysis occurs. The normal range of RBC OF usually includes 45 to 50% of hemolysis and is commonly estimated at a salt concentration of 4,5 g/L NaCl (Godal et al., 1981). Different factors were previously reported to influence OF, such as shear stress and mechanical hemolysis (Sowemimo-Coker, 2002), temperature (Pribush et al., 2003), ultrasound effects (Ivanov, 1999), drugs (Sowemimo-Coker, 2002), and irradiation (Ivanov, 1999). Due to low osmotic resistance, intravascular hemolysis takes accountability for reducing the RBC life span. The Osmotic Fragility Curve (OFC) of an individual's RBCs indicates its standard membrane and cytoplasmic properties and is a marker of erythrocyte physiology (Walski et al., 2014).

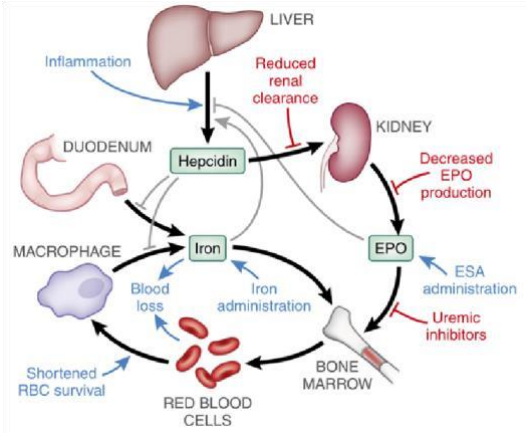
Renal anemia

Anemia is a common issue of chronic kidney disease, elevating the burden on patients and healthcare systems (Mikhail et al., 2017; St Peter et al., 2018). While CKD progresses, the prevalence of anemia is higher, affecting most patients with end-stage kidney disease (Babitt & Lin, 2012). The

clinical practice guidelines kidney disease: Improving Global Outcomes defines anemia of CKD as Hb < 13 g/dL for men and < 12 g/dL for nonpregnant women (KDIGO, 2012). Anemia in CKD is related with diminished quality of life and increased cardiovascular disorders, hospitalizations, cognitive impairment, and mortality (Babitt & Lin, 2012).

Typically normocytic, normochromic, and hypoproliferative, anemia of CKD is attributed mainly to EPO deficiency, as the kidneys decrease production due to the disease condition (Babitt & Lin, 2012). Therefore, a standard method of clinical management is the administration of recombinant human EPO and erythropoiesis-stimulating agents (ESAs) (Babitt & Lin, 2012). However, plenty of adverse effects were reported in patients receiving ESAs, and this treatment does not reduce adverse outcomes associated with renal anemia, including mortality, cardiovascular dysfunctions, hospitalizations, and progression of kidney disease (Babitt & Lin, 2012). Besides EPO deficiency, other factors were previously pointed out as contributors to the anemia of CKD (Figure 2).

Figure 2. Multifactorial mechanisms of anemia in CKD. In healthy conditions, the hepatic hormone hepcidin is cleared by the kidney. In CKD, hepcidin accumulates, and the kidneys can no longer synthesize EPO, leading to reduced erythropoiesis. Newly produced RBC are removed before senescence, releasing insufficient iron to stimulate erythropoiesis.



Babitt & Lin, 2012.

Shortened RBC survival is known to play a role in increased renal anemia (Dias et al., 2020); although the etiology is yet to be elucidated, metabolic and mechanical factors have been proposed (Kuhn et al., 2017); disordered iron homeostasis in which hepcidin (a hormone produced by the liver that controls iron availability) of CKD patients is abnormally elevated, due to the insufficient renal

clearance and induction by inflammation, resulting in iron-limited erythropoiesis (Babitt & Lin, 2012). In addition, many studies observed increased eryptotic events *in vitro* in the presence of circulating UTs (Dias et al., 2018; Tozoni et al., 2019; Ahmed et al., 2013), solutes that would be physiologically removed by healthy kidneys and are found to be retained in CKD patients (Duranton et al., 2012).

The accumulation of metabolic waste products, including UTs, due to diminished kidney function is broadly named uremia (Rosner et al., 2021). Compared to healthy individuals, UTs are detected in CKD patients in abnormally high concentrations (Meert et al., 2007). They can be classified according to their metabolic and biochemical properties, such as origin, affinity to serum proteins, molecular weight, or removal by different dialysis strategies (Duranton et al., 2012). The categories of UTs are 1. Small protein-bound molecules, derived from the gut, with molecular weight < 0.5 kDa; 2. Small water-soluble molecules, also gut-derived, weighing < 0.5kDa; 3. Small-middle proteinbound molecules, hydrophilic, generated by endogenous metabolism, 0.5-15 kDa; 4. Medium-middle water-soluble molecules with a molecular weight > 15-25 kDa; 5. Large-middle molecules, with the same biochemical properties mentioned before, > 25-58 kDa; 6. Large molecules whose molecular weight is > 170 kDa (Rosner et al., 2021).

A furan dicarboxylic acid UT is included in the high molecular weight category, 3-carboxy4methyl-5-propyl-2-furanpropanoic acid, or CMPF, derives from dietary furan fatty acids (Sassa et al., 2000). Furan fatty acids comprise phospholipids and cholesterol esters and are catabolized into dibasic urofuran acids, including CMPF, which are eliminated in the urine (Spiteller, 2005). In healthy individuals, the CMPF concentration in serum was 3.7 mg/L. In uremia, the CMPF levels increased to

61 mg/L (Meert et al., 2007). The origin of elevated circulating CMPF levels remains unknown (Luce et al., 2018). Due to its high affinity to human serum albumin (HSA), CMPF is one of the major contributors to the defective binding of drugs and organic acids in uremic plasma is CMPF since the compounds cannot bind to albumin (Sakai et al., 1995). Erythropoiesis was found to be blocked by CMPF, as this solute has an inhibitory effect on erythroid progenitor cells (Niwa et al., 1990). CMPF also plays a role in irregularities of thyroid function as a consequence of being responsible for low total

T3 levels in CKD patients (Lim et al., 1993). In addition, severe neurologic abnormalities were associated with elevated levels of CMPF in uremic patients (Costigan et al., 1996). Moreover, high CMPF levels were associated with development of gestational diabetes, metabolic syndrome, or type 2 diabetes, due to its ability to cause mitochondrial β-cell dysfunction and induce oxidative stress (Prentice et al., 2014). Lastly, CMPF inhibits active tubular secretion by competitive inhibition of the uptake of p-aminohippurate by rat kidney slices (Henderson & Edward, 1992). Possible interactions between CMPF and its direct effect on RBCs have not yet been investigated.

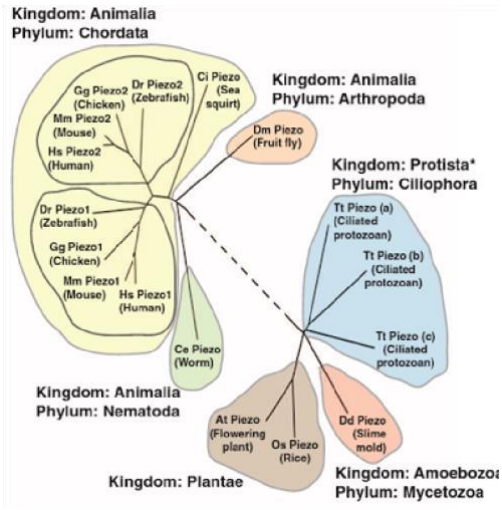
Piezo channels

Mechanotransduction is the process that converts mechanical force into biological signals, and it plays a crucial role in physiology (Coste et al., 2010). Cells of various types physically interact with their surroundings within a broad spectrum of time (Chalfie, 2009), regulating the growth and organization of tissues (Hamill & Martinac, 2001). One group of proteins behind the mechanotransduction pathways was first described in mammals in 2010 and was entitled Piezo channels (Coste et al., 2010).

Using Neuro2A mouse cell lines, scientists applied force to the cell surface with a pressureclamped glass probe attached to a whole-cell patch pipette (Coste et al., 2007) to record mechanically activated (MA) currents (Coste et al., 2010). With this strategy, small interfering RNA (siRNA) was tested in selected proteins with unknown function, until knockdown of Fam38 sequence caused a pronounced decrease of MA currents. Since Fam38 encodes a protein required for the expression of pressure activated ion channels, the gene was named Piezo1 and Piezo2, from the Greek "πίεση" (píesi), meaning pressure (Coste et al., 2010; Parpaite & Coste, 2017).

Many eukaryotic species contain a single Piezo, and vertebrates, early chordate Ciona, and unicellular forms from the Ciliophora kingdom are reported to express both molecules (Figure 3) (Coste et al., 2010; Parpaite & Coste, 2017). The present study focused its experiments on Piezo1, and the literature reviewed was driven to this protein.

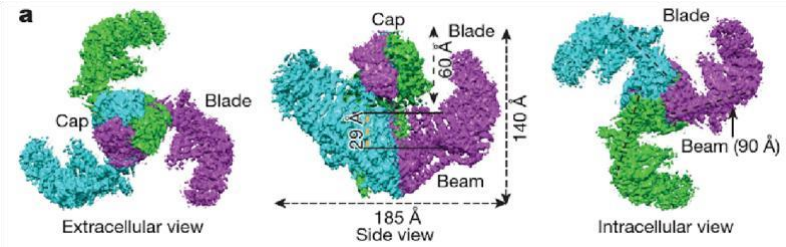
Figure 3. Evolutionary conservation and expression profile in an unrooted phylogenetic tree of mouse Piezo1 and Piezo2. Relationships between groups were generated by using Megalign (DNASTAR, Madison, Winsconsin), and DrawTree programs. Hs, *Homo sapiens*; Mm, *Mouse musculus*; Gg, *Gallus gallus*; Dr, *Danio rerio*; Ci,*Ciona intestinalis*; Dm, *Drosophila melanogaster*; Ce, *Caenorhabditis elegans*; Dd, *Dictyostelium discoideum*; At, *Arabidopsis thaliana*; Os, *Oryza sativa*; and Tt, *Tetrahymena thermophila*.



Coste et al., 2010.

Piezo1 channel has a size of approximately 2500 amino acids (Coste et al., 2010). It exhibits a three-bladed, propeller-shaped homotrimeric structure that comprises a central cap containing an ionconducting pore module, three peripheral blade-like structures on the extracellular side, three long beams on the intracellular side that bridge the blades to the cap and a transmembrane region among these features (Figure 4) (Ge et al., 2015; Zhao et al., 2018; Saotome et al., 2018).

Figure 4. Multiple views of the tridimensional structure of mechanosensitive ion channel Piezo1, obtained through cryoelectron microscopy, with a resolution of 3.97 Å (angstroms). Each subunit is colored, and major domains are labeled.



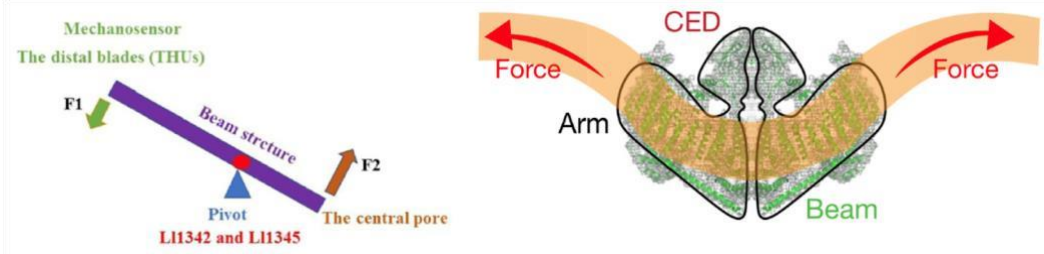
Source: Zhao et al., 2018; Fang et al., 2021.

Given Piezo1's unique structural conformation, its mechanotransduction mechanism was previously proposed as a lever-like model (Ge et al., 2015). Transmembrane Helical Units (THU) blades act as a mechanosensory, while the beam region is a lever-like apparatus, acting as a pivot (Fang et al., 2021). Engaging between the distal blades and the central pore through the beam transforms mechanical stimulus into cation conduction. Since the pivot is closer to the central cap, the lever-like

setup amplifies the input force throughout its extension. Alterations in the structural conformation of the distal blades result in a slight opening of the central pore, allowing cation intracellular flow (Zhao et al., 2018; Fang et al., 2021).

Piezo 1 kinetics involves three states: open, closed, and inactivated. Motion from one state to another determines its mechanosensitivity (Lew & Tiffert, 2017). An experimental study proved a membrane dome mechanism of opening, in which, under membrane tension, the lateral membrane flattens the Piezo dome, increasing the energy of the membrane-channel system in contrast to the expansion of the projected area of the dome (Guo & Mackinnon, 2017). Given the relative energy difference, Piezo1 consequently opens the central pore, allowing the influx of Ca^{2+} at the end of the activation process (Figure 5) (Guo & Mackinnon, 2017). The ensuing $icCa^{2+}$ increase subsequently activates calcium-activated potassium KCa3.1 Gardos channels (Picard et al., 2019), leading to hyperpolarization and a loss of K^+, Cl^-, and water, resulting in RBC shrinkage. Also detected after Ca^{2+} influx, the formation of the calcium-calmodulin complex (Ca-CaM) desestablizes the actinaddusin-Band 4.1 complex, which turns the cross-linked spectrin network more flexible (Danielczok et al., 2017). This process is physiologically important as the RBC traverses narrow anatomical structures such as capillaries and the slits that are formed in the spleen by the spaces between the endothelial cells lining the organ's blood vessels (Aglialoro et al., 2021). After the passage, both Piezo1 and the Gardos-channels are inactivated and return to their respective closed states. Na^+/K^+ and Ca^{2+}ATPase pumps re-establish pre-passage intracellular ion concentrations, allowing RBC to return to its original size (Danielczok et al., 2017).

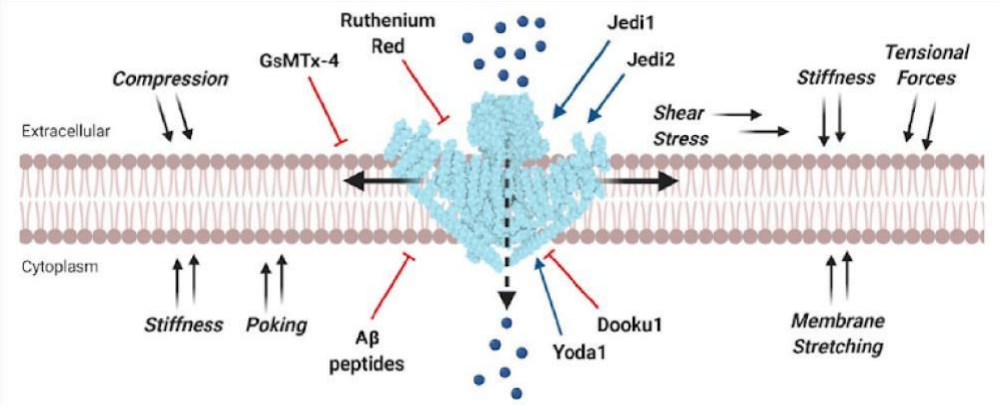
Figure 5. The left image indicates the lever-like mechano-gating model in Piezo1. The blue and red dashed arrows indicate input and output forces, respectively. The right image shows the dome opening membrane mechanism model. Changes in membrane curvature lead to a gating force applied to the Piezo1 channel.



Source: Fang et al., 2021

Activation of Piezo1 can also be made by pharmacological stimulation. Small molecules Jedi 1/2 and Yoda1 were reported to activate Piezo1 without mechanical stimulation (Wang et al., 2018; Botello-Smith et al., 2019). Jedi 1/2 characterizes as a hydrophilic Piezo1 activator, and it acts from the extracellular side through the peripheral blades and uses the lever-like module to gate the ionconducting pore (Wang et al., 2018), while Yoda1 induced conformational changes to happen from the intracellular environment (Figure 6) (Botello-Smith et al., 2019).

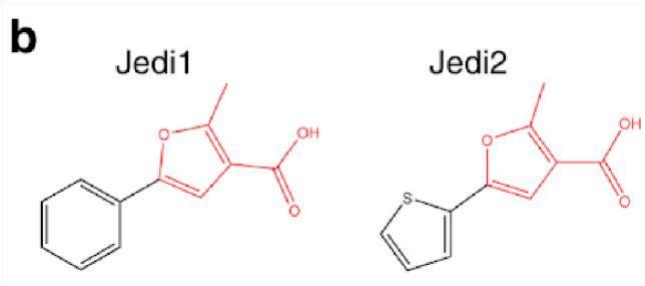
Figure 6. Representational scheme of the pharmacological and mechanical modulation of Piezo1 channel.



Source: De Felice & Alaimo, 2020.

Jedi1 and Jedi2 are identified as small molecules with molecular weights of 202.81 and 208.23 Da, respectively. The chemical structure 3-carboxylic acid methylfuran (Figure 7, highlighted in red), present in both agonists, indicates that this motif might be in charge of their activating properties on Piezo1 (Wang et al., 2018).

Figure 7. The two-dimensional structure of the small molecule Jedi1, at the right, and Jedi2, at the left.



Source: Wang et al., 2018.

Nonspecific inhibitors of Piezo1, such as Ruthenium Red and Dooku1, have been identified to block the ion channel at an inhibitory concentration (IC50) of 5mM (Coste et al., 2012), the broadspectrum mechanical channel inhibitor GsMTx-4 obtained from the tarantula *Grammostola*

spatulata toxin, was also found to inactivate Piezo1, modulating membrane tension near the channel (Suchyna, 2017).

Piezo1 channels are expressed in various cell types and allow Ca^{2+} influx in response to external *stimuli*, such as fluid flow, shear stress, pulling, and ultrasonic forces (Cahalan et al., 2015). Piezo1 can be detected in endothelial cells (ECs) and engages physiological shear stress under endothelium morphogenesis, development (Qiu et al., 2019), and vascular tone (Félétou et al., 2010). The presence of Piezo1 is also found in neurons in the vertebrate central nervous system (CNS), which allows the cells to be sensitive towards mechanical signals that regulate cell division, fluid homeostasis, gene expression, cell migration, morphogenesis, ion channel gating, and vesicular transport (Tyler, 2012). Piezo1 also plays a role in the response to mechanical force in gastrointestinal epithelial enterochromaffin cells, which accounts for the synthesis of most of the body's serotonin (5hydroxytryptamine) (Mawe & Hoffman, 2013).

Red blood cells (RBC), the major blood constituents, are able to undergo morphological deformations during vascular circulation, which is deeply related to the optimal functioning of the RBC and vascular physiopathology (Lew & Tiffert, 2017). A dominantly inherited disorder of RBC volume homeostasis, Hereditary Xerocytosis (HX), has been shown to be associated with mutations in Piezo1 genes, characterizing dehydrated erythrocytes and resulting in hemolytic anemia (Zarychanski et al., 2012). This mutation was further investigated in a study that revealed gain-offunction under plasmodium infection in vitro, where the dehydration of the RBC protected against malaria (Ma et al., 2018).

Once the relation between cell volume regulation and Piezo1 was stated through the machenism mentioned above, another function of Piezo1 in RBCs was brought up in a recent study, which experimentally demonstrated that Piezo1 is related to RBC volume regulation via downstream activation of the KCa3.1 Gardos channel (Cahalan et al., 2015). The activation of Piezo1 on RBC elicits calcium influx and potassium efflux through the Gardos channels, accompanied by water loss and decreased volume (Cahalan et al., 2015). Moreover, ATP release from RBC can be related to calcium influx via shear-induced Piezo1 activation (Cinar et al., 2015).

4. Objectives

General

i) Analyze the effect of CMPF and Jedi1 on RBC through Piezo1 activation under different experimental conditions.

Specifics

i) Determine CMPF concentrations in literature.

ii) Evaluate RBC osmotic fragility in the presence of the uremic concentration of CMPF.

iii) Evaluate RBC osmotic fragility in the presence of Jedi1.

iv) Evaluate RBC osmotic fragility in the presence of the inhibitor GsMTx-4.

5. Methodology

This project was developed at the Anemia & Immunology Research Laboratory (LAB AIRe) - PPGCS, PUCPR in partnership with Renal Research Institute - Fresenius Medical Care and was approved by the Pontifícia Universidade Católica do Paraná Ethics Committee under number 5.697.460.

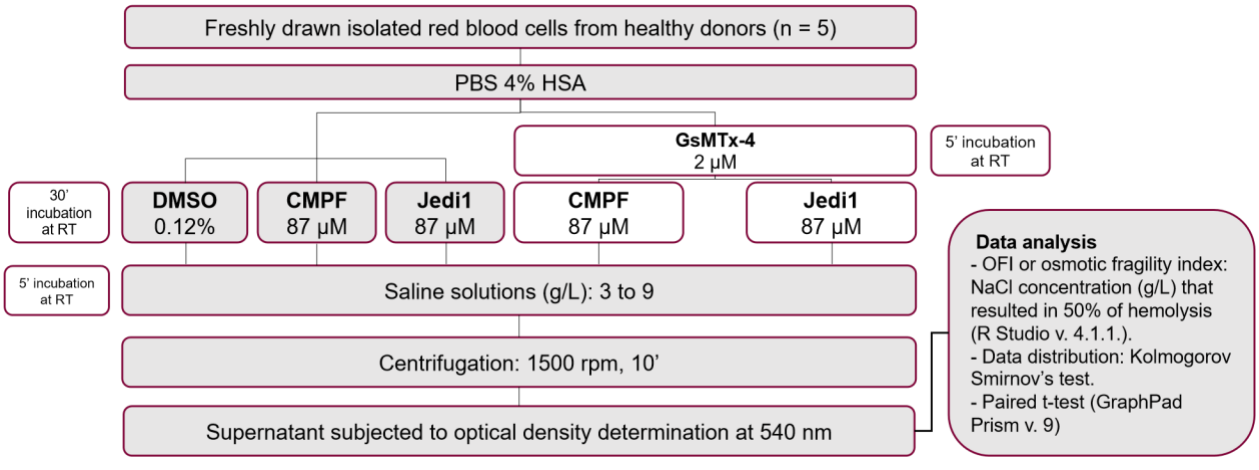
Literature review

To standardize the uremic concentration of CMPF for the assays, a literature review was carried out using the keywords: #CMPF and #3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid. The articles were then selected based on the following criteria: a) articles that measured the concentration of CMPF in the serum of patients on hemodialysis (HD) before the dialysis session, containing or not serum samples from healthy individuals; b) detailed description of extraction method of compounds in the sample; c) method of quantification by high-performance liquid chromatography (HPLC) or liquid chromatography/mass spectrometry (LC/MS). Concentrations of CMPF found in g/dL were converted to the µM range using the molarity formula.

Experimental design

This study is under the basic research cell studies scope. An experimental testing model was used to investigate whether CMPF has a similar effect as Jedi1 on the Piezo1 channel, in which the RBC undergoes volume and shape variation. The experimental conditions tested are represented below.

Figure 8. Representation of the experimental design of the study.



Isolated RBC from healthy volunteers were incubated at room temperature (RT) with CMPF or Jedi1 in the presence (white boxes) or not (gray boxes) of the inhibitor. After treatment, cells underwent osmotic stimulations during 5 minutes by adding an aliquot in increasing tonicity solutions.

Reagents & Solutions

Stock solutions of Jedi1 and CMPF (Sigma-Aldrich, USA) were prepared by dissolving reagent powder in Dimethyl Sulfoxide (DMSO). The final stock concentration was 69.6 mM for both molecules.

Mechanosensitive channel blocker GsMTx-4 was obtained from Cayman Chemicals (Cambridge, UK) and was prepared by dissolving 100 µg in 100 µL of aqueous buffer, according to the manufacturer's instructions.

A Phosphate Buffered Saline (PBS) solution containing 0.136M of NaCl, 0.0027M of KCl, 0.0015M of KH_2PO_4, and 0.008M of $Na_2HPO_4 \cdot 7H_2O$ with 4% of Human Serum Albumin (HSA) (Sigma-Aldrich, USA), was prepared in 1 L of ultrapure distilled MilliQ water and stored at 2-8ºC for up to 3 months.

Saline solutions made of NaCl (sodium chloride) were prepared at a 10X stock concentration in 50 mL of ultrapure distilled MilliQ water and diluted on the day of the experiments.

Blood samples

Whole blood from 5 healthy volunteers was drawn into citrate vacutainers on the day of the experiment after obtaining informed written consent. Criteria for healthy subjects' inclusion were those with age above 18 years, non-smokers, without a history of renal or inflammatory diseases, and who did not receive anti-inflammatory medication prior to the blood draw.

The blood was spun at 3000 rpm for 15 minutes at 4ºC, had its plasma and buffy coat removed, then spun down twice at 1500 rpm for 10 min, and the RBC washed with cold PBS after each spin.

Cell suspensions

An aliquot of 200 µL of isolated cells was mixed with PBS 4% HSA and added 1.25 µL of CMPF, Jedi1, or DMSO (control). These suspensions were kept incubated for 30 minutes at room temperature. DMSO in all experiments did not exceed the concentration of 0.12%.

Osmotic fragility test

The procedure for this step was based on the methodology described by Orbach et al., 2017. Briefly, an aliquot of 10 µL from each cell suspension was submitted to 1 mL of ultrapure distilled MilliQ water (representing the positive control, where all cells were lysed), and 1 mL of the following saline concentrations (in g/L): 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 8 and 9, in the presence of Jedi1 or CMPF or DMSO. The higher saline concentration was considered as the physiological concentration without lysis. The tubes with saline + cells were then spun down at 1500 rpm for 10 min at room temperature. 250 µL of the supernatant was collected and subjected to Hb determination by its optical density at 540 nm (nanometers). The amount of lysis was obtained as below:

$$\%\ hemolysis = 100 \times \frac{OD_s - OD_0}{OD_t - OD_0}$$

where OD_0 = OD of the supernatant obtained from the physiological saline, untreated RBCs, OD_S = OD of the supernatant obtained from RBC subjected to osmotic stimuli (saline solutions from 3 to 9 g/L), OD_t = OD of the supernatant from RBC subjected to total hemolysis. Absorbances were used to determine the Osmotic Fragility Index (OFI), the NaCl concentration (in g/L) where 50% of hemolysis occurred.

Data analysis

Both OFI and individual Osmotic Fragility Curve (OFC) were obtained using the following packages from R version 4.1.1: dplyr, drc, ggplot2, grid, gridExtra e readxl. The OFI distribution was

evaluated through Kolmogorov Smirnov's test, and OFI differences were analyzed through a paired ttest using GraphPad Prism Version 9.

6. Results

CMPF data in the literature

A total of 13 publications met the inclusion criteria. As shown in Table 1, different concentrations of CMPF were reported in the articles. To standardize concentrations for use in osmotic fragility assays, the mean concentration of CMPF was arbitrarily calculated from data obtained from articles on healthy subjects (normal or low concentration) at < 1 mg/dL, mean uremic concentration up to 1 mg /dL and peak or highest uremic concentration above 1 mg/dL. Thus, mean CMPF concentrations of 0.2 mg/dL in healthy individuals, 0.4 mg/dL (mean), and 2.19 mg/dL (high) in patients on HD before dialysis were considered. Still, to adapt the concentrations according to the in vitro toxicity studies using uremic toxins, the concentrations calculated in the present study were converted into µM.

Table 1. CMPF data found in literature.

	Study	Patients	Extraction method	Healthy (mg/dL)	PreHD Total CMPF (mg/dL)	Detection method
1990	Niwa et al.	N = 23	Heat denaturation		4.11±18.3	HPLC—UV detection 270 nm
2000	Lessafer et al.	N = 10	Heat denaturation		1.76±7.7	HPLC—UV detection 254 nm
2002	Fagugli et al.	N = 7	Heat denaturation		0.37±0.25	HPLC—UV detection 254 nm
2007	De Smet et al.	N = 11	Heat denaturation		0.84±5.0	HPLC—UV detection 254 nm
2008	Nishio et al.	N = 14	Acetonitrile		1.88±5.8	HPLC—UV detection 261 nm
2010	Branderburg et al.	N = 41	Heat denaturation		0.4±2.9	RP-HPLC—UV detection 254 nm
2012	Itoh et al.	N = 45	Acetonitrile	0.39±0.10	2.11±1.3	LC/ESI-MS/MS
2013	Eloot et al.	N = 71	Heat denaturation		0.38 [2.0–6.1]	HPLC—UV detection 254 nm
2013	Boelaert et al.	N = 59	Heat denaturation	0.11±1.1	0.54±4.0	UHPLC/MS
2016	Rroji et al.	N = 126	Heat denaturation		0.42 [2.3–7.7]	RP-HPLC-UV detection 254nm
2018	Luce et al.	N = 238	Heat denaturation		0.25 [1.0–5.2]	HPLC—UV detection 215 nm
2020	Suzuki et al.	N = 14	Methanol	0.129±7.83	1.1019 ± 6215	UHPLC-MS/MS
2021	Wang et al.	N = 16	Methanol		0.18±7.5	LC/MS

Data are presented as mean ± SD or median [interquartile range]. Abbreviations: HD, hemodialysis, HPLC, highperformance liquid chromatography, LC/ESI-MS/MS, liquid chromatography/electrospray ionization–mass spectrometry/mass spectrometry, UHPLC, ultra-high performance liquid chromatography

Finally, as the main objective of the present study was to evaluate the interaction of CMPF with PIEZO1 in RBCs, the highest concentration of this uremic toxin (87 µM) was chosen for the osmotic fragility test.

Study population

RBC were obtained from two healthy males and three healthy females. Their mean±SD age was 28.2±6.57 years. Baseline characteristics are presented in Table 2.

Table 2. Demographical and laboratory data from the study population.

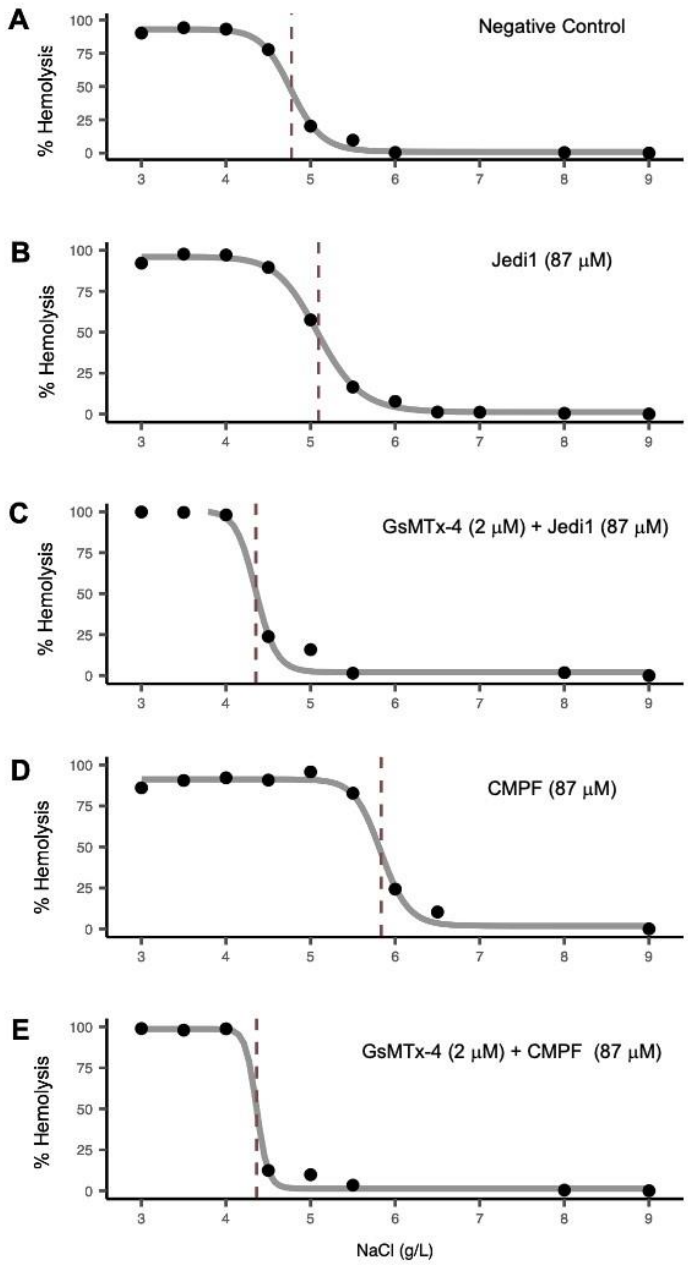
Parameters	Healthy subjects (n = 5)
Age (years)	28.2±6.57
Sex	2 males; 3 females
Caucasians (%)	100
Body mass index (kg/m^2)	22.8±3.07
Hemoglobin (g/dL)	14.2±1.90
Creatinine (mg/dL)	0.94±0.21
Urea (mg/dL)	4.24±0.33

Data expressed as mean ± SD, or binary variables (frequency).

Osmotic fragility test

Effects of CMPF and Jedi1 on the hemolysis of RBCs *in vitro* were determined at 30 minutes of incubation, followed by 5 minutes of saline incubation. The OFC of one donor obtained from the DMSO control (Fig. 9 – A) allowed the visualization of changes in the distribution of osmotic fragility after exposure to stimuli (Fig. 9 – B to E).

Figure 9. Representative RBC osmotic fragility curves. The dashed red lines indicate the osmotic fragility index (OFI; concentration of NaCl that exerted 50% of hemolysis) in g/L. Negative control buffer consisted of phosphate buffered saline, 4% human serum albumin, and DMSO 0.12%. The incubation buffer comprised phosphate buffered saline, 4% Human Serum Albumin, and DMSO 0.12%.



The OFI of the control from one donor was 4.77± 0.31 g/L. In the presence of Jedi1 (87 μM), a slight shift of the curve to the right was observed (Fig 9B), indicating an increased osmotic fragility. This effect of Jedi1 was abolished by the Piezo1 inhibitor GsMTx-4 (2 μM; Figure 9C; OFI 4.35 ± 0.06 g/L). More markedly, the change in the osmotic fragility curve to the right in the presence of CMPF (87 μM) (Fig 9D; OFI 5.83 ± 0.04 g/L) indicates that the RBC becomes more fragile, increasing hemolysis compared to the negative control (Fig 9A). On the other hand, when cells were pretreated

with the Piezo1 inhibitor GsMTX-4 (2 μM) and then challenged with CMPF (87 μM), a slight shift to the left in the curve occurred (Fig 9D; OFI 4.36 ± 0.02 g/L), rapidly reducing hemolysis.

The summarized results from all donors are shown in Table 3.

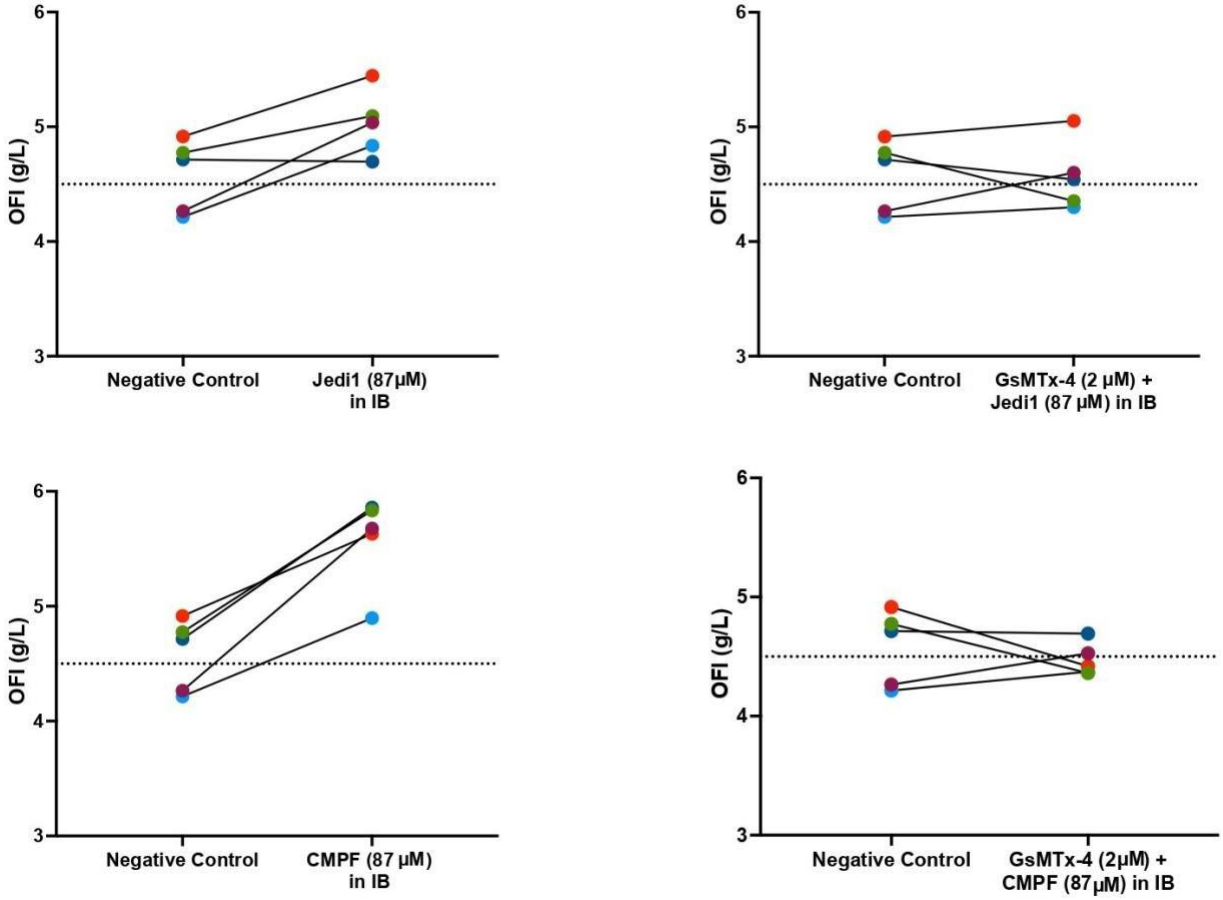
Table 3. Osmotic Fragility Index of RBC under different experimental conditions.

Experimental condition	OFI (95% CI)	Mean difference to control (95%CI)	p-value
Negative control (incubation buffer)	4.57 ± 0.31 (4.18 – 4.97)	n/a	n/a
Jedi1 (87μM)	5.02 ± 0.28 (4.66 – 5.37)	0.45 (0.06 – 0.83)	0.031
Jedi1 (87μM) + GsMTx-4 (2μM)	4.56 ± 0.29 (4.19 – 4.93)	-0.01 (-0.38 – 0.36)	0.944
CMPF (87μM)	5.57 ± 0.39 (5.09 – 6.06)	1.00 (0.61 – 1.38)	0.002
CMPF (87μM) + GsMTx-4 (2μM)	4.47 ± 0.13 (4.30 – 4.64)	-0.10 (-0.52 – 0.32)	0.546

OFI, osmotic fragility index. The OFI is defined as the concentration of NaCl that exerted hemolysis in 50% of the RBC. The p-values were calculated using paired t-test. Data are presented as mean ± SD (95% confidence interval lower and upper limit). Incubation Buffer: Phosphate buffered saline (PBS) containing 0.12% of dimethyl sulfoxide (DMSO) and 4% of human serum albumin (HSA).

The results described next are shown in Table 3, Figure 10. The mean OFI obtained for all 5 donors in the control cell supernatant was 4.57 ± 0.31 g/L. Incubation with Jedi1 (87 μM) increased the OFI to 5.02 ± 0.28 g/L. The mean difference between this condition and the negative control was 0.45 ± 0.31 (95% CI [0.06, 0.83]). Pre-treatment of RBC with the Piezo1 inhibitor GsMTx-4 (2 μM) followed by Jedi1 incubation resulted in an OFI of 4.56 ± 0.29 g/L. The difference between the means of this treatment in contrast with the control was -0.01 ± 0.3 (95% CI [-0.38, 0.36]). OFI increased in RBC incubated with CMPF (5.57 ± 0.39) compared to negative controls, with a mean difference of 1 ± 0.31 (95% CI [0.61, 1.38]). Pre-treatment of RBC with GsMTx-4 prevented the CMPF-induced increase in OFI (4.47 ± 0.13), making it comparable to the control, with a difference of means of -0.01 ± 0.34 (95% CI [-0.52, 0.32]).

Figure 10. Osmotic Fragility Index (OFI) expressed as the concentration of NaCl (in g/L) that exerted 50% hemolysis. The individual donors are color-coded, the experimental conditions are indicated on the x-axis. Solid lines connect OFI results obtained under control and incubation conditions, respectively. The horizontal dotted lines indicate the mean OFI in controls.



Legend: Legend: IB - Incubation buffer. The incubation buffer comprised phosphate buffered saline, 4% human serum albumin, and DMSO 0.12%. Negative control buffer consisted of phosphate buffered saline, 4% human serum albumin, and DMSO 0.12%. CMPF – 3-carboxy-4-methyl-5-propyl-2-furan propionic acid. GsMTx-4 – Grammostola spatulata mechanotoxin 4.

7. Discussion

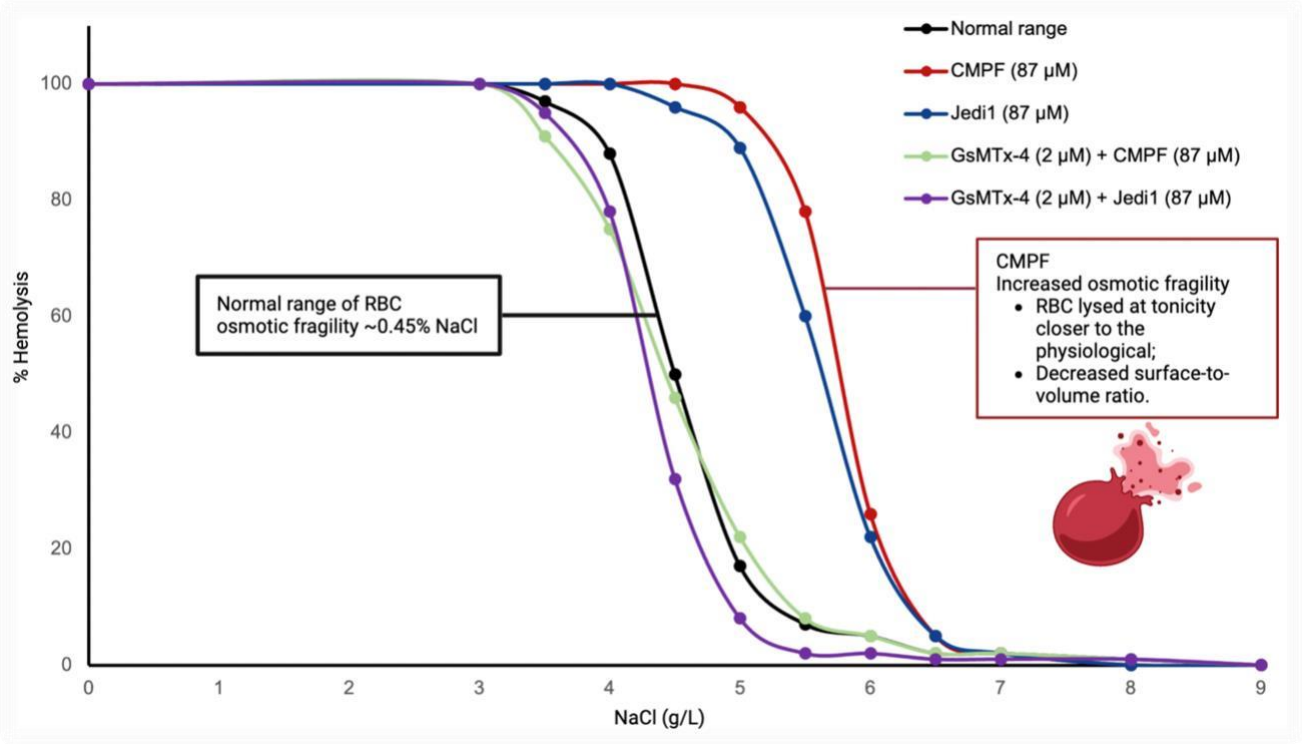
The main finding of this study is that the uremic toxin CMPF increases RBC osmotic fragility. This is the first endogenous compound to have a possible interaction with Piezo1, a phylogenetically old and highly conserved mechanosensitive cation channel on several cell types, including mammalian RBC.

In this study, a model of mechanical stimulation was used: osmotic swelling. When RBC pass across narrow capillaries with a diameter inferior to the cross-section of the red cells, or interendothelial gaps in the spleen, they must be adapted to severe variations in their shape and volume (Svetina et al., 2019). The regulation of RBC volume during these passages was related to Piezo1 activity, in which the $icCa^{2+}$ concentration increased after osmotically stimulated cells (Danielczok et al., 2017). The

presence of high levels of cytosolic Ca^{2+} can elicit various responses, including cell membrane scrambling and blebbing (Lang et al., 2010), activation of Ca^{2+}-sensitive K^+ channels followed by K^+ efflux, alteration of the membrane electrical charges, turning it hyperpolarized, which in turn, leads to Cl^- exit (Lang & Qadri, 2012). The loss of KCl is thenceforward by the efflux of osmotically obliged water, resulting in cell shrinkage (Lang et al., 2010).

The present results suggests that elevated CMPF caused increased hemolysis in osmoticallystimulated RBCs, probably due to Piezo1 opening. OF in RBC can be induced by three general mechanisms: (i) cell hydration with subsequent net solute gains, (ii) membrane area loss due to exo vesiculation of Hb molecules, and (iii) altered membrane properties, turning the RBC prone to prespherical membrane rupture while in an abrupt volume expansion, as it is observed during osmotic fragility experiments (Cueff et al., 2010). The normal OF range in healthy conditions is expected at 0.45% of sodium chloride (Godal et al., 1981). Shifts on the curve to the left indicate decreased OF (or increased resistance) and shifts to the right reveals elevated OF (or decreased resistance) (Figure 11).

Figure 11. Subjective RBC osmotic fragility curves in different experimental conditions.



In the present study, it was observed that CMPF moved the curve to the right, in a greater distance from the control curve when compared even with Jedi1, suggesting that this treatment

increased OF since RBCs lysed at saline concentrations that were closer to the physiological, presenting a decreased surface-to-volume ratio since the cells were osmotically stimulated. Therefore, the reason why RBCs presented increased OF in the presence of CMPF is most likely to be by the loss of membrane area.

Uremia is characterized by abnormal concentrations of retained compounds, which in a healthy physiology, are cleared by the kidneys (Meert et al., 2007). Elevated levels of UTs have a notable biological impact (Vanholder et al., 2008), and this factor was associated with increased RBC death (Dias et al., 2018; Tozoni et al., 2019), that could contribute, in a long term, to the establishment of renal anemia. During the progression of CKD, CMPF values were 40 times higher than in healthy individuals (Meert et al., 2007). In this study, initial insights of a possible interaction between a poorly investigated UT, CMPF, could interact with Piezo1 in RBC were raised. This event might be related to eryptosis due to increased membrane fragility.

These findings are relevant to the pathophysiology of renal anemia in end-stage kidney disease patients. In addition to the physiological mechanical stress when traversing narrow anatomical structures, RBC are exposed to mechanical forces in the extracorporeal dialysis circuit, e.g., exerted by roller pumps or the dialyzer. Moreover, hemodialysis is accompanied by osmotic perturbations with distinct effects on RBC (Schneditz et al., 2015). Depending on the sodium concentration in the dialysate relative to the plasma sodium level, RBC volume changes during hemodialysis. All these events result most likely in the activation of Piezo1. Increased CMPF levels may decelerate the inactivation of Piezo1 and trigger processes that increase membrane fragility resulting in premature RBC death and shortened RBC life span in CKD patients.

8. Limitations

During the conduction of this research, a few limitations were detected.

Since the investigated variable (OFI) gave a macroscopic read-out of the results, quantification of intracellular Ca^{2+} levels would enrich the analysis.

Due to the effort and time-consuming features involved in the performance of an osmotic fragility experiment, only one CMPF concentration was tested. It would be interesting to evaluate RBC response in different uremic scenarios.

The present results provided preliminary inferences. A larger sample of donors could increase the confidence level, especially regarding the statistics.

Besides these limitations, the conduction of this research provided initial inferences about a poorly investigated mechanism involving a uremic toxin and a mechanosensitive channel. If confirmed, this could improve the understanding about eryptosis and link it with renal anemia.

Conclusion

This study described for the first time that an endogenous compound, CMPF, retained during the CKD progression, is probably able to interact with the mechanosensitive ion channel Piezo1 in vitro.

The consequence of this event is increased osmotic fragility, which was observed by increased hemolysis compared to the negative control.

Together the results suggest that Piezo1 prolonged activation by CMPF could contribute to the aggravation of renal anemia probably shortening the RBC lifespan, due to a possible relation between decreased resistance and membrane instability with eryptosis.

Further studies are needed to improve understanding of the mechanisms involved in the CMPF interaction with Piezo1 and their influence on renal anemia.

Final considerations

The development of this study resulted in relevant findings under the basic research field. In the future, these findings can support broad investigations to improve knowledge of renal anemia and new therapeutic target having CKD patients' quality of life as the most gain.

Details regarding the methodological approach in this study could be improved by automated advanced lab machinery. Osmotic fragility tests tend to be time and effort-consuming for the scientist. With automated tools or specialized equipment, differences between independent experiments could be reduced, and the data replicability would be higher.

Application of refined methodologies of analysis is in the Author's perspective for a Ph.D. project regarding the main theme. Also, a better comprehension of the CMPF interaction on Piezo1 and the consequences, like increased eryptosis, is a subject that will be tested in the Ph.D. project.

Ph.D. progression Project

Background and Hypothesis

In Chronic Kidney Disease (CKD), as the loss of kidney function occurs, several complications arise, with anemia being one of the most common in people with kidney failure. This condition can appear early in the diagnosis of CKD and worsen as the kidneys lose their ability to filter and produce erythropoietin (EPO). Although the preponderant cause is related to the decrease in EPO production, the etiology of anemia is multifactorial, manifested by iron, folic acid, and vitamin B12 deficiency, systemic inflammation, as well as the reduction in the life span of red blood cells (RBC) mainly promoted by a uremic environment (Portolés et al., 2021; Dias et al., 2020).

Several groups reported shorter RBC life spans in hemodialysis (HD) patients, with values of 73 ± 18 days (Ma et al., 2017) and 89 ± 28 days (Sato et al., 2012). However, the causes that lead to this reduction are not fully clarified. An increase in RBC death or eryptosis has been documented in CKD patients, in which uremic toxins were described as eryptosis inductors (Ahmed et al., 2013; Lang et al., 2017; Tozoni et al., 2019), as well as stressors such as osmotic disorders and oxidative stress (Lang et al., 2006). Eryptosis is characterized by critical morphological changes in the cell membrane, such as shrinkage and shuffling of the lipid bilayer, with consequent exposure of phosphatidylserine (PS) (Lang & Qadri, 2012). These events are triggered by the influx of Ca2+ through selective channels, increasing cytosolic Ca^{2+} (Segawa & Nagata, 2015; Pretorius et al., 2016).

On the other hand, the molecular mechanisms involved in detecting mechanical forces and their effects on RBC volume still need to be fully understood. The mechanosensitive Ca^{2+} channel Piezo1 expressed on the RBC membrane is an essential mechanical regulator of RBC volume via the influx of Ca^{2+} that allows the passage of these cells through small-diameter capillaries. Of note, the intensity of mechanical force can induce the initial steps of eryptosis since a Piezo1-dependent increase in Ca^{2+} influx stimulates Gardos channels and subsequent erythrocyte shrinkage (Cahalan et al., 2015).

Recently, Kotanko et al. (2022) observed that the active center of Piezo1 chemical activator Jedi1 shows structural similarity with the uremic toxin 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF), which is 5 to 15 times higher in CKD patients (Vanholder et al., 2003).

In this way, the results presented in this master's defense thesis suggest that increased levels of CMPF possibly lengthen the Piezo1 activation, increasing intracellular Ca^{2+} and promoting decreased RBC lifespan. In the Ph.D. project, we aimed to continue this evaluation by observing the effect of CMPF on RBC life span.

Goals

General

Evaluate the effects of different concentrations of the CMPF and Jedi1 in inducing cell membrane change and eryptosis.

Specifics

1) Optimize the osmotic fragility test assay to highlight our previous results.
2) Observed the PS exposure induced by the stimuli using an osmotic fragility test based on flow cytometric analysis (FCM OF).
3) Characterize the RBC morphology change using light and fluorescence microscopy.
4) Quantify the intracellular Ca^{2+} after the RBC challenge with the stimuli.
5) Evaluate the biological events proposed above in the presence of specific inhibitors to Piezo1 (GsMTx-4) and Gardos channel (TRAM-34).

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