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Biomarkers and Bioanalysis Overview

Edited by Ane Claudia Fernandes Nunes

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Published in London, United Kingdom







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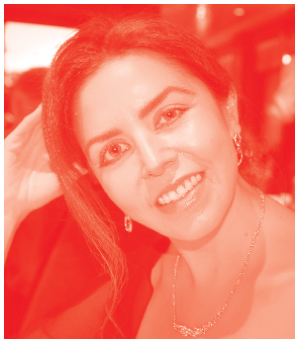

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Biomarkers and Bioanalysis Overview
http://dx.doi.org/10.5772/intechopen.88772
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First published in London, United Kingdom, 2021 by IntechOpen
IntechOpen is the global imprint of INTECHOPEN LIMITED, registered in England and Wales, registration number: 11086078, 5 Princes Gate Court, London, SW7 2QJ, United Kingdom
Printed in Croatia

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library

Additional hard and PDF copies can be obtained from orders@intechopen.com

Biomarkers and Bioanalysis Overview
Edited by Ane Claudia Fernandes Nunes
p. cm.
Print ISBN 978-1-83968-324-4
Online ISBN 978-1-83968-325-1
eBook (PDF) ISBN 978-1-83968-326-8

Meet the editor

Dr. Ane C.F. Nunes is a geneticist with a master's degree and Ph.D. in Medical Sciences and Nephrology from UFRGS/Brazil. She has postdoctoral experience in renal physiology (UFRJ/Brazil), clinical medicine and nephrology (USP/Brazil), and nephrology and hypertension (UCI/USA). She is a Professor of Medical Genetics, Human Genetics, and Molecular Biology. Her research fields are human genetics diseases, cellular and molecular biology applied to nephrology, biochemistry, and microbiology with projects in the following subjects: inflammatory markers, molecular diagnosis, DNA polymorphisms, chronic kidney disease (CKD), polycystic kidney disease (PKD), Fabry disease, rare diseases, cellular and murine models for CKD, RNA processing, fluorescent image analysis, nanoparticles development, and nanomedicine.

Contents

Preface

Diagnosis is one of the milestones of medical assistance and an important research area in biological and medical sciences. Even considered as a subdiscipline of analytical chemistry, bioanalysis is a complex and translational field in charge of the investigation of biotics and xenobiotics structures in biological contexts.

In this book, readers will find different multidisciplinary studies in three sections.

Section 1, "Pharmacology and Drugs Biomarkers," includes interesting reviews on pharmacokinetics markers and multidrug resistance. Chapter 1 by Dr. Ashok Palaniappan, et al. brings us an understanding of computational studies, describing homology modeling, differential ligand affinity, and receptor-ligand docking, among other subjects. Chapter 2 by Dr. Raman Sureshkumar, et al. reports the mechanism of natural inhibitors using P-glycoprotein transporters as a representation to describe a mechanism of action model. Chapter 3 by Dr. Wai-Kit Ma, et al. presents a specific and well-done study about the urological effects of ketamine abuse. This subject provides understanding of the use of biomarkers as monitoring tools for clinical aims.

Section 2, "Nephrology and Renal Physiology," includes three chapters that link basic and clinical backgrounds using renal failure to demonstrate the diversity of bioanalysis applications and its uses in the medical environment. Chapter 4 by Professor Silvia De Rosa, et al. discusses nutritional status, muscle mass, and muscle wasting through serum creatinine analysis. Chapter 5 by Professor Jiang Liu is a comprehensive review of the impact of Na+/K+-ATPase signaling in the renal proximal tubule and its outcome on natriuresis, cardiotonic steroids, and reactive oxygen species (ROS). Chapter 6 by Dr. Henrique Muela, et al. discusses hypertension, salt intake, and the effect of salt dietary restriction in vascular scenarios.

Section 3, "Biochemistry, Cellular and Molecular Biology," presents studies that merge different aspects of basic sciences. In Chapter 7, Dr. David Mole, et al. presents his study on hypoxia, through the analysis of the hypoxia-inducible factor (HIF) pathway, and its effects over clear cell renal cancer. Chapter 8 by Dr. Ernest Anyabolu describes urine excretion, using creatinine as a reference, and the differences on its mechanism comparing HIV and non-HIV subjects. Chapter 9 by Dr. Tatyanny Fucci, et al. focuses on the DNA polymorphisms related to thromboembolism and the significance of molecular screening as a strategy for following up patients with COVID-19.

This project was launched in early 2019. During this period, the world faced the most important public health issue of the century, the COVID-19 pandemic. As an academic editor, I had the pleasure of reviewing high-level proposals and, at the same time, taking part in a tremendous effort to control the pandemic in different countries through collaborations and voluntary work.

For that reason, I wish to dedicate this book to all scientists and health workers who played an important role in this historical moment. Beyond them, I also wish to

dedicate this book to all patients and their families. Each life, saved or lost, makes a difference and contributes to the improvement of health sciences.

I am grateful to all authors, contributors, and experts who took considerable effort to ensure that each chapter provides updated and innovative data. I wish to thank the team at IntechOpen for their support, especially Sara Debeuc and Lucija Tomicic-Dromgool.

The scope of this work goes beyond this preface. I wish you all an excellent reading and learning from *Biomarkers and Bioanalysis Overview*.

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Section 1

Pharmacology and Drugs Biomarkers

Chapter 1

Computational Studies of Drug Repurposing Targeting P-Glycoprotein-Mediated Multidrug Resistance Phenotypes in Priority Infectious Agents

Arvindh Kumar, Sangeetha Muthamilselvan and Ashok Palaniappan

Abstract

ABCB1 P-glycoprotein (P-gp) is an ATP-dependent efflux pump with broad substrate specificity associated with cellular drug resistance. Homologous to role in mammalian biology, P-glycoproteins of bacterial and fungal pathogens mediate the emergence of multidrug resistance phenotypes, with widespread clinical/socioeconomic implications. This work aims to characterize P-gp homologues in certain WHO-prioritized infectious agents, namely (1) bacteria: *Acinetobacter baumannii* and *Staphylococcus aureus* and (2) fungi: *Aspergillus fumigatus*, *Candida albicans*, and *Cryptococcus neoformans*. PSI-BLAST searches against the genome of each of these organisms confirmed the presence of P-gp homologues. Each homologue was aligned against five known P-gp structures, for structural modeling. FDA-approved antibiotics used in the current line of therapy were retrieved from PubChem, and potential antibiotics were identified based on similarity and repurposing of the existing drugs. The most tenable target-ligand conformations from docking studies of the respective modeled P-gp structures and the antibiotic ligands were assessed for interacting residues within 4.5 Å of the ligand, probable binding pockets and relative efficacies of the new drugs. Our studies could lay the foundation for the development of effective synergistic or new therapies against these pathogens.

Keywords: P-glycoprotein, priority pathogen list, nosocomial infection, multidrug resistance, homology modeling, receptor-ligand docking, differential ligand affinity, synergistic effects

1. Introduction

1.1 Multidrug resistance (MDR)

Bacterial evolution tends to respond to the selection constraint of reckless antibiotic use, which has led to the emergence of drug-resistant strains mediated by

varied defense mechanisms. The main mechanisms whereby infectious agents develop resistance to antimicrobial chemotherapy include enzymatic inactivation, modification of the drug target(s), and reduction of intracellular drug concentration by changes in membrane permeability or by the overexpression of efflux pumps [1]. Multidrug resistance efflux pumps are recognized as an important component of resistance in both Gram-positive and Gram-negative bacteria [2]. Some bacterial efflux pumps may be selective for one substrate or transport antibiotics of different classes, conferring a multidrug resistance phenotype. With respect to efflux pumps, they provide a self-defense mechanism whereby antibiotics are extruded from the cell interior to the external environment. This results in sublethal drug concentrations at the active site that in turn may predispose the organism to the development of high-level target-based resistance [3]. Therefore, efflux pumps are viable antibacterial targets and the development of potent efflux pump inhibitors is a promising and valid strategy to rejuvenate the activity of antibiotics that are no longer effective against bacterial pathogens. The world is searching for new tools to combat multidrug resistance.

1.2 P-glycoprotein (P-gp)

ATP-binding cassette (ABC) transporters are found in all phyla and constitute one of the largest protein superfamilies. ABC transporters such as ABCB1 (P-glycoprotein/P-gp), ABCG2, and ABCC1 are well known for their association with multidrug resistance, effluxing structurally diverse compounds, powered by the hydrolysis of ATP [4]. P-gp also plays an important role in the pharmacokinetics of many drugs, altering their absorption, distribution, and excretion. P-gp has been extensively studied since 1976, when it was identified as the multidrug efflux pump in Chinese hamster ovary cells that had been selected for resistance to colchicine [3].

In eukaryotes, it takes the form of a single polypeptide chain consisting of two transmembrane domains (TMDs) that are usually arranged into six transmembrane-spanning α-helices that form the pathway through which substrate crosses the membrane. These domains also form the substrate-binding site (or sites) which contribute to transport specificity. The two nucleotide-binding domains (NBDs) couple the energy of ATP catalysis to transport [5]. In some prokaryotes, however, the P-gp structure comprises a monomeric assembly, namely, a single TMD and a single NTD. The various domains can comprise one, two, or four polypeptide chains, encoded by the same or different genes, which assemble into monomers, homo- or heterodimers, or tetramers.

Prokaryotes harbor both importers for nutrient uptake (including amino acids, sugars, and metal ions) and exporters (drugs, toxins, polysaccharides, lipids, and proteins), whereas eukaryotes harbor only exporters [6]. It is believed that this transporter functions through an alternate access mechanism involving two different conformations. Drug binding occurs to the inward-facing from the cytoplasm or the inner leaflet of the bilayer. After binding two molecules of MgATP, the nucleotide-binding domains (NBDs) dimerize and switch the transmembrane domain (TMDs) from the inward- to the outward-facing conformation, followed by the release of the drug to the extracellular milieu. ATP hydrolysis, ADP/Pi release, and NBD dissociation reset the transporter to the inward-facing conformation. The switch from inward to outward form certainly requires a highly flexible structure [4, 7, 8].

Substrate “promiscuity” or polyspecificity is a well-known characteristic of P-gp and the subject of much research. Attempts have been made to understand the ability of P-gp to recognize various chemically and structurally diverse substrates

through biochemical investigations and structural studies. Despite all these studies, the molecular basis of this unusual property still remains poorly understood and is a matter of intense debate [9].

2. Prioritizing pathogenic agents

Opportunistic pathogens with a response profile of drug resistance to antibiotic treatment are good candidates for study. The organisms chosen here included bacteria and fungi identified by the WHO as priority pathogens [10] as well as other nosocomial pathogens that pose an elevated threat level due to acquisition of MDR over the recent years. Nosocomial pathogens are subject to the evolutionary pressure exerted by constant exposure to antibiotics in hospitals that could accelerate the emergence of pathogenicity-related mutations.

2.1 *Acinetobacter baumannii*

Multidrug-resistant *Acinetobacter baumannii* strains are opportunistic bacterial pathogens primarily associated with nosocomial infections worldwide [11]. Due to the remarkable ability of *A. baumannii* to gain resistance to antibiotics, this bacterium is now considered to be a "superbug." *Acinetobacter baumannii* strains resistant to all clinically relevant antibiotics known have also been isolated. Although MDR *A. baumannii* (MDR-Ab) continues to disseminate globally, very little is known about its pathogenesis mechanisms. Once detected within specific areas of the hospital, various levels of intervention have been attempted to reduce the incidence and prevalence of infection due to MDR-Ab [12].

Acinetobacter baumannii and its close relatives belonging to genomic species 3 (*Acinetobacter pittii*) and 13TU (*Acinetobacter nosocomialis*) are important nosocomial pathogens, often associated with epidemic outbreaks of infection, that are only rarely found outside of a clinical setting. These organisms are frequently pandrug-resistant and are capable of causing substantial morbidity and mortality in patients with severe underlying disease, both in the hospital and in the community [13]. Several epidemic clonal lineages of *A. baumannii* have disseminated worldwide and seem to have a selective advantage over non-epidemic strains. Physicians are also facing challenging therapeutic quandaries when treating patients infected with MDR-Ab, because the increasing prevalence of resistance continues to restrict their treatment options [14].

Urban et al. [12] gave us a look into the MDR in *Acinetobacter baumannii*, discussing its medical relevance and treatment options. They sought to control infection due to MDR-Ab by identifying isolates as clonally related, leading to enhanced infection-control measures, including cohorting, surveillance, contact precaution, initial therapy with ampicillin/sulbactam and local polymyxin B, and, more recently, therapy with synergistic antibiotic combinations. Gupta et al. [15] demonstrated the existence of MDR-Ab and its significance. Park et al. [16] determined the complete genome sequence of *A. baumannii* strain 1656-2 to study biofilm formation. This strain is significant to the project due to its use in target selection.

2.2 *Staphylococcus aureus*

Staphylococcus aureus is a major human pathogen that causes a wide range of clinical infections. Approximately 30% of the human population is colonized with

S. aureus; however, it is a leading cause of bacteremia and infective endocarditis as well as osteoarticular, skin and soft tissue, pleuropulmonary, and device-related infections [17]. The WHO has categorized *Staphylococcus aureus* as a high-priority pathogen that possesses MDR, as a consequence of its acquisition of methicillin and vancomycin resistance.

Hiramatsu et al. [18] described the genetic basis for the remarkable ability of *S. aureus* to acquire multi-antibiotic resistance and proposed a novel paradigm for future chemotherapy against the multiresistant pathogens. The evolution of *Staphylococcus* or for that matter any bacterium does not halt. Lemaire et al. [19] examined the effect of P-gp on the modulation of the intracellular accumulation and activity of daptomycin towards phagocytosed *Staphylococcus aureus* in human THP-1 macrophages, in comparison with MDCK epithelial cells. Handzlik et al. [2] delineated recent achievements in the search for new chemical compounds able to inhibit multidrug resistance mechanisms in Gram-positive pathogens.

2.3 *Aspergillus fumigatus*

Aspergillus fumigatus is a saprophytic fungus that plays an essential role in recycling environmental carbon and nitrogen. Its natural ecological niche is the soil, wherein it survives and grows on organic debris. *Aspergillus fumigatus* is of the more prevalent opportunistic pathogens involved in human aspergillosis in which, though a minor disease, because of the increase in the number of immunosuppressed patients and the degree of severity of modern immunosuppressive therapies, the situation has changed dramatically in recent years. The diversity of patients and risk factors complicates diagnostic and therapeutic decision-making [20]. Invasive procedures are often precluded by host status; noninvasive diagnostic tests vary in their sensitivity and specificity. The ability of *Aspergillus* species to withstand antifungal treatment may be due in part to the presence of the MDR mechanism of drug efflux.

Latge [20] reviewed taxonomy of aspergillosis, its symptoms, diagnosis, virulence factors, defense mechanisms, epidemiology, and treatment. Little is known of the cellular and humoral defense mechanisms which are essential for the killing of *A. fumigatus* conidia and hyphae in the immunocompetent host. Tobin et al. [21] identified genes encoding proteins of the ATP-binding cassette superfamily in *Aspergillus fumigatus* and *Aspergillus flavus*. In *A. fumigatus*, two genes (AfuMDR1 and AfuMDR2) encoding proteins of the ATP-binding cassette superfamily were identified, which are the probable homologue of human P-gp.

2.4 *Candida albicans*

Candida species have emerged among the top three causes of microbial nosocomial infectious diseases in humans, resulting in 46–75% mortality. The incidence of candidiasis has increased sharply over the past few decades, primarily due to hospital interventions such as cancer chemotherapy, surgery, organ/bone marrow transplantation, and indwelling devices [22]. Of note, recently, the incidences of *albicans* and non-*albicans* species of *Candida* acquiring resistance to antifungals (particularly to azoles) have increased considerably which poses problems towards its successful chemotherapy [23]. Drug transporters, such as the ATP-binding cassette transporters encoded by *CDR1* and *CDR2* (*Candida* drug resistance), and a major facilitator superfamily (MFS) transporter encoded by*MDR1*, play key roles in azole resistance as deduced by their high level of expression in the majority of azole-resistant clinical *Candida albicans* isolates [22].

Schubert et al. [24] stated that constitutive overexpression of the Mdr1 efflux pump was an important mechanism of acquired drug resistance *C. albicans*. The Mdr1 efflux pump is a P-gp homologue and is hence significant to this project. Sun et al. [22] highlighted an extensive upregulation of *MDR1* as well as polyamine transporter genes in a fluconazole-resistant strain, going further to correlate the presence of MDR1 in *C. albicans* and its role in fluconazole resistance.

2.5 *Cryptococcus neoformans*

Cryptococcus neoformans is an encapsulated fungal pathogen that is remarkable for its tendency to cause meningoencephalitis, especially in patients with AIDS. While the disease is less common in children than adults, it remains an important cause of morbidity and mortality among HIV-infected children without access to antiretroviral therapy [25]. *Cryptococcus neoformans* is a basidiomycetous yeast ubiquitous in the environment and a model for fungal pathogenesis. *CneMDR1*, a gene encoding a protein related to several eukaryotic multidrug resistance proteins, was identified, cloned, and characterized from a clinical isolate of *Cryptococcus neoformans* [26].

Kao and Goldman [25] reviewed recent insights into both the biology and treatment of cryptococcosis with a special emphasis on the pediatric literature. Thornewell et al. [26] characterized the *CneMDR1* gene. Protein structure predictions suggested the presence of two putative 6-transmembrane (TM) domains as well as two ATP-binding domains, structural characteristics typical of ATP-binding cassette (ABC) proteins, including P-glycoprotein.

3. Sequence and structure analyses

3.1 Bacterial P-glycoprotein efflux pumps

Bacterial P-glycoproteins were identified based on homology to the mammalian P-gp in the following manner. The position-specific iterated BLAST (PSI-BLAST) was performed against a search set of nonredundant protein sequences in the organism of interest, using hP-GP as the query (hP-gp; UniProt P08183). Through a PSI-BLAST search, a large set of related proteins are compiled. It is used to identify distant evolutionary relationships between protein sequences. The algorithm parameters were set with an E-value of 0.001, and the scoring matrix BLOSUM62 was used. This step was performed on all four organisms of interest (*Aspergillus fumigatus*, *Acinetobacter baumannii*, *Staphylococcus aureus*, *Candida albicans*, *Cryptococcus neoformans*). Hundreds of hits were obtained for P-glycoprotein, and these results were prioritized according to predetermined parameters such as medical relevance, annotation status, and the presence of conserved regions. The results were analyzed, and the P-glycoprotein sequence of each organism was finalized and recorded as in Appendix A. The results were filtered for the organisms of interest and shown in **Table 1**.

Hundreds of hits are obtained for P-glycoprotein, and these results were prioritized according to medical relevance and sequence identity. The significance of the sequence identity is that, with a higher sequence identity, there is a higher similarity between the query sequence and the aligned sequence. This project will focus on nosocomial bacterial and fungal strains. The chosen sequences would have conserved regions determined through multiple sequence alignment with the ClustalX2

Organism	Max score	Total score	Query cover (%)	Ident (%)	Length
Aspergillus fumigatus	966	1389	97.00	42.00	1349
Acinetobacter baumannii	256	498	82.00	32.00	555
Staphylococcus aureus	268	504	58.00	34.00	578
Candida albicans	183	352	88.00	23.00	1606
Cryptococcus neoformans	931	931	97.00	42.00	1408

Table 1.
Summary of BLAST results.

software, the most widely used multiple alignment programs. The guide trees in Clustal were calculated using the neighbor-joining (NJ) method [27].

3.2 Homology modeling

The target sequences and the suitable templates were chosen and aligned using ClustalX2. Multiple sequence alignment was performed between the targets and the templates so that the homology and evolutionary relationship between the sequences of the biological data set can be inferred [27]. This information was considered in the structure validation. The templates chosen are:

- 4M1M—*Mus musculus*
- 2HYD—*Staphylococcus aureus*
- 3B5Z—*Salmonella enterica*
- 3WME—*Cyanidioschyzon merolae*
- 4F4C—*Caenorhabditis elegans*

The p-glycoprotein sequences would be used as target sequences for structure modeling with SWISS-MODEL [28]. SWISS-MODEL is an open-source, structural bioinformatics tool used for the automated comparative modeling of three-dimensional protein structures. Several P-glycoprotein structures were modeled for each organism, using multiple templates. The templates having high sequence similarity with the target sequences were given preference. The objective of homology modeling is to identify the best template and build the PDB model of the macromolecule to be used in docking. Modeling of the predetermined templates was accepted if they resulted in high modeling (GMQE) scores. Each modeled structure was saved as a PDB file. The results are summarized in **Table 3**.

The validity was checked using the Ramachandran plot with tools such as Procheck. The structures were refined using energy minimization protocols, and the least energetic structure corresponding to each efflux pump protein was chosen for docking studies.

In summary, the FASTA sequences of the BLAST results were obtained and fed into the SWISS-MODEL to build homology models with the above set of templates. The SWISS-MODEL provided us with the top 100 templates that can be used to generate a homology model. To generate the best possible homology model, the templates were aligned with the target organisms using the multiple alignment tool Clustalx2, and a phylogenetic analysis is subsequently conducted.

Templates	Phylogenetic distance				
	A. fumigatus	***A. baumannii***	***C. albicans***	***S. aureus***	***C. neoformans***
3wme	0.635	0.701	0.844	0.707	0.632
4f4c	0.641	0.703	0.831	0.716	0.637
4m1m	**0.584**	0.707	0.842	0.711	**0.577**
2hyd	0.728	0.623	**0.81**	**0.602**	0.711
3b5z	0.678	**0.592**	0.827	0.661	0.694

Bold values indicate the phylogenetically nearest structure.

Table 2.
Phylogenetic distance between templates and the target sequence of each organism.

From **Table 2**, it could be inferred that in the cases of *Aspergillus fumigatus* and *Cryptococcus neoformans*, 4m1m was the most phylogenetically favored templates. *Candida albicans* and *Staphylococcus aureus* are phylogenetically favored to the 2hyd template, and *Acinetobacter baumannii* is phylogenetically closer to 3b5z.

The validity of the homology models was further checked with Phi-Psi graphs and Chi1-Chi2 plots for each residue type. The template comparison is done based on:

- Taxonomy of the target organism with respect to the templates
- Distance analysis

Subsequent to the Ramachandran plot validation, from **Table 3**, we can infer that 4m1m is preferred in *Aspergillus fumigatus, Aspergillus nidulans, Acinetobacter*

	Total residues	Query cover	(%) Sequence identity
Organism: *Aspergillus fumigatus*			
3wme.1.a	565	0.43	37.8
4f4c.1.a	1241	0.91	37.48
4m1m.2.a	**1251**	**0.91**	**42.15**
Organism: *Acinetobacter baumannii*			
3wme.1.a	**550**	**0.99**	30.29
4f4c.1.a	537	**0.99**	28.88
4m1m.2.a	545	0.97	**32.22**
Organism: *Candida albicans*			
4f4c.1.a	912	0.52	17
4m1m.2.a	**1272**	**0.7**	**18.83**
Organism: *Staphylococcus aureus*			
3wme	**575**	**0.98**	**29.75**
Organism: *Cryptococcus neoformans*			
3wme.1.a	608	0.41	**37.89**
4f4c.1.a	**1259**	**0.88**	37.74
4m1m.1.a	582	0.41	38.5

Bold values indicate optimal parameter values for each organism.

Table 3.
Results of template parameter comparison—homology results.

Antibiotic	PubChemID	SMILES format
Amikacin	37768	C1C(C(C(C(C1NC(=O)C(CCN)O)OC2C(C(C(C(O2)CO)O)N)O)O)OC3C(C(C(C(O3)CN)O)O)O)N
Colistin	5311054	CCC(C)CCCC(=O)NC(CCN)C(=O)NC(C(C)O)C(=O)NC(CCN)C(=O)NC1CCNC(=O)C(NC(=O)C(NC(=O)C(NC(=O)C(NC(=O)C(NC(=O)C(NC1=O)CCN)CC(C)C)CC(C)C)CCN)CCN)C(C)O
Kanamycin	6032	C1C(C(C(C(C1N)OC2C(C(C(C(O2)CN)O)O)O)O)OC3C(C(C(C(O3)CO)O)N)O)N
Netilmicin	90658113	CCNC1CC(C(C(C1OC2C(C(C(CO2)O)NC)(C)O)O)OC3C(CC=C(O3)CN)N)N
Sulbactam	130313	CC1(C(N2C(S1(=O)=O)CC2=O)C(=O)O)C
Amphotericin B	5280965	CC1C=CC=CC=CC=CC=CC=CC=CC(CC2C(C(CC(O2)(CC(CC(C(CCC(CC(CC(=O)OC(C(C1O)C)C)O)O)O)O)O)O)O)O)C(=O)O)OC3C(C(C(C(O3)C)O)N)O
Anidulafungin	166548	CCCCCOC1=CC=C(C=C1)C2=CC=C(C=C2)C3=CC=C(C=C3)C(=O)NC4CC(C(NC(=O)C5C(C(CN5C(=O)C(NC(=O)C(NC(=O)C6CC(CN6C(=O)C(NC4=O)C(C)O)O)C(C(C7=CC=C(C=C7)O)O)O)C(C)O)C)O)O)O
Isavuconazonium	6918606	CC(C1=NC(=CS1)C2=CC=C(C=C2)C#N)C(CN3C=[N+](C=N3)C(C)OC(=O)N(C)C4=C(C=CC=N4)COC(=O)CNC)(C5=C(C=CC(=C5)F)F)O
Itraconazole	55283	CCC(C)N1C(=O)N(C=N1)C2=CC=C(C=C2)N3CCN(CC3)C4=CC=C(C=C4)OC[C@H]5CO[C@](O5)(CN6C=NC=N6)C7=C(C=C(C=C7)Cl)Cl
Micafungin	477468	CCCCCOC1=CC=C(C=C1)C2=CC(=NO2)C3=CC=C(C=C3)C(=O)NC4CC(C(NC(=O)C5C(C(CN5C(=O)C(NC(=O)C(NC(=O)C6CC(CN6C(=O)C(NC4=O)C(C)O)O)C(C(C7=CC(=C(C=C7)O)OS(=O)(=O)O)O)O)C(CC(=O)N)O)C)O)O)O
Porfimer	57166	CC1=C(C2=CC3=NC(=CC4=NC(=CC5=C(C(=C(N5)C=C1N2)C(C)OC(C)C6=C(C7=CC8=C(C(=C(N8)C=C9C(=C(C(=N9)C=C1C(=C(C(=N1)C=C6N7)C)CCC(=O)O)CCC(=O)O)C)C(C)O)C)C)C(=C4CCC(=O)O)C)C(=C3C)CCC(=O)O)C(C)O
Voriconazole	71616	CC(C1=NC=NC=C1F)C(CN2C=NC=N2)(C3=C(C=C(C=C3)F)F)O
Fluconazole	3365	C1=CC(=C(C=C1F)F)C(CN2C=NC=N2)(CN3C=NC=N3)O
Clotrimazole	2812	C1=CC=C(C=C1)C(C2=CC=CC=C2)(C3=CC=CC=C3Cl)N4C=CN=C4
Nystop	11953884	CC1C=CC=CCCC=CC=CC=CC=CC(CC(C(C(CC(=O)CC(C(CCC(CC(CC(CC(=O)OC(C(C1O)C)C)O)O)O)O)O)O)O)C(=O)O)O)OC2C(C(C(C(O2)C)O)N)O
Clindamycin	29029	CCCC1CC(N(C1)C)C(=O)NC(C2C(C(C(C(O2)SC)O)O)O)C(C)Cl
Finafloxacin	11567473	C1CC1N2C=C(C(=O)C3=CC(=C(C(=C32)C#N)N4CC5C(C4)OCCN5)F)C(=O)O
Vancomycin	14969	CC1C(C(CC(O1)OC2C(C(C(OC2OC3=C4C=C5C=C3OC6=C(C=C(C=C6)C(C(C(=O)NC(C(=O)NC5C(=O)NC7C8=CC(=C(C=C8)O)C9=C(C=C(C=C9C(NC(=O)C(C(C1=CC(=C(O4)C=C1)Cl)O)NC7=O)C(=O)O)O)O)CC(=O)N)NC(=O)C(CC(C)C)NC)O)Cl)CO)O)O)(C)N)O

Table 4.
List of known FDA approved antibiotics.

baumannii, and *Candida albicans*; 4m1m is the best template. In *Cryptococcus neoformans, 4*f4c is preferred, and 3wme is preferred in *Staphylococcus aureus.*

4. Antibiotics of interest

A set of antibiotics were identified for the purposes of investigation and included known FDA-approved antibiotics (**Table 4**) against each of the target organisms as well as promising antibiotics that ranged from repurposed to investigational (**Table 5**).

For each structure, we surveyed the literature to determine the known antibiotics that are effective against it and against which the pathogenic strain might have

Compound	PubChemID	SMILES format
Levofloxacin	149096	CC1COC2=C3N1C=C(C(=O)C3=CC(=C2N4CCN(CC4)C)F)C(=O)O
Moxifloxacin	152946	COC1=C2C(=CC(=C1N3CC4CCCNC4C3)F)C(=O)C(=CN2C5CC5)C(=O)O
Tigecycline	54686904	CC(C)(C)NCC(=O)NC1=C(C2=C(CC3CC4C(C(=O)C(=C(C4(C(=O)C3=C2O)O)O)C(=O)N)N(C)C)C(=C1)N(C)C)O
Trovafloxacin	62959	C1C2C(C2N)CN1C3=C(C=C4C(=O)C(=CN(C4=N3)C5=C(C=C(C=C5)F)F)C(=O)O)F
Echinocandin	71723607	CC1CN2C(C1O)C(=O)NCC(CC(C(=O)NC(C(=O)N3CC(CC3C(=O)NC(C(=O)NC(C2=O)C(CCNC(CO)CO)O)C(CC4=CC(=C(C=C4)O)OC)O)O)C(C)O)NCC5CCC(CC5)C(=N)SC(=N)C6=CC=C(C=C6)N7CCC(CC7)(C8CCCCC8)OC)O
Terbinafine	1549008	CC(C)(C)C#CC=CCN(C)CC1=CC=CC2=CC=CC=C21
VL-2397	77843968	CC(C)CC1C(=O)NC(C(=O)NC(C(=O)NC(C(=O)NC(C(=O)NC(C(=O)N1)CC(=O)N)CCCN(C(=O)C)[O-])CCCN(C(=O)C)[O-])CCCN(C(=O)C)[O-])CC2=CC=CC=C2.[Al+3]
Bithionol	2406	C1=C(C=C(C(=C1Cl)O)SC2=CC(=CC(=C2O)Cl)Cl)Cl
Carvacrol	10364	CC1=C(C=C(C=C1)C(C)C)O
VT-1129	91886002	C1=CC(=CC=C1C2=CN=C(C=C2)C(C(CN3C=NN=N3)(C4=C(C=C(C=C4)F)F)O)(F)F)OC(F)(F)F
Aminocandin	16072305	CCCCCCCCOC1=CC=C(C=C1)C2=CC=C(C=C2)C(=O)NC3CC(CNC(=O)C4C(C(CN4C(=O)C(NC(=O)C(NC(=O)C5CC(CN5C(=O)C(NC3=O)C(C)O)O)O)C(CC6=CC=CC=C6)O)CO)C)O)NCCN
Caspofungin	16119814	C1CC(C(C1)N)C(=O)O
E1210	16719049	C1=CC=NC(=C1)OCC2=CC=C(C=C2)CC3=NOC(=C3)C4=C(N=CC=C4)N
Ceftobiprole	6918430	Nc1nc(ns1)\C(=N\O)\C(=O)N[C@H]2[C@H]3SCC(=C(N3C2=O)C(=O)O)\C=C\4/CCN([C@@H]5CCNC5)C4=O
Brilacidin	25023695	C1CNCC1OC2=C(C=C(C=C2NC(=O)CCCCN=C(N)N)C(F)(F)F)NC(=O)C3=CC(=NC=N3)C(=O)NC4=C(C(=CC(=C4)C(F)(F)F)NC(=O)CCCCN=C(N)N)OC5CCNC5
Radezolid	11224409	CC(=O)NCC1CN(C(=O)O1)C2=CC(=C(C=C2)C3=CC=C(C=C3)CNCC4=NNN=C4)F

Table 5.
List of repurposed and investigational antibiotics.

developed resistance via efflux pump activity. A set of ligands is created for each efflux pump, comprising of known and potential antibiotics. The PDB model of each antibiotic is generated using MarvinView by converting the canonical SMILES. This PDB model will act as the ligand during the docking process.

Open Babel is a file conversion software that provides a wide variety of options [29]. We use it to convert the canonical SMILES of the ligand set into a .pdb file in order to perform docking. However, we use this software again during visualization to convert the docked complex from .pdb to .pdbqt format in order for it to be recognized by RasMol.

5. Docking studies of pump-drug combinations

5.1 Docking of the bacterial efflux pumps with known and potential antibiotics

Computational docking is widely used for the study of protein-ligand interactions and for drug discovery and development. The methods are fast enough to allow virtual screening of ligand libraries containing tens of thousands of compounds. Typically, the process starts with a target of known structure, such as a crystallographic structure of an enzyme of medicinal interest. Docking is then used to predict the bound conformation and binding free energy of small molecules to the target. Single docking experiments are useful for exploring the function of the target, and virtual screening, in which a large library of compounds are docked and ranked, may be used to identify new inhibitors for drug development. With AutoDock, it is possible to accomplish the following: basic docking of a drug molecule with an anticancer target, a virtual screen of this target with a small ligand library, docking with selective receptor flexibility, active site prediction, and docking with explicit hydration.

The molecular docking was carried out using the AutoDock suite of tools [30]. The search algorithm used was the Lamarckian genetic algorithm (LGA), which could handle ligands with more degrees of freedom than the simulated annealing method used in earlier versions of AUTODOCK. LGA is the most efficient, reliable, and successful search algorithm and mimics a heuristic Lamarckian evolution, a controversial hypothesis proposed by Jean Batiste de Lamarck that phenotypic characteristics acquired during an individual's lifetime could become heritable traits. The affinity maps were used to compute for each ligand-target pair. The docking parameters were set to 10 runs per receptor-ligand complex yielding 10 poses per each docked complex. Based on the interaction energies, the pose with the smallest free energy of binding was identified as the best pose of the drug and the target.

Each drug is docked with each subsequent target using AutoDock 4.2. The results are analyzed to verify whether the pathogenic strain could develop resistance to known antibiotics using efflux pump activity and if the novel antibiotics could be effective against the development of such resistance.

5.2 Best pose analysis

The ligand pose with the least binding energy is defined as the best pose which was validated by clustering at 2.0 Å r.m.s. The clusterings signify the extent of difference between the various poses. Extremely similar poses will be clustered together, increasing the validity of that respective pose. Thus the best pose is selected based on the combination of the binding energy released and the clusterings of the pose. The output contains the docked structure between the

Organism	Known drug	ΔG kcal/mol
Aspergillus fumigatus	Amphotericin	−6.99
	Itraconazole	−3.71
	Anidulafungin	−3.06
	Micafungin	−2.97
	Voriconazole	−2.6
	Isavuconazonium	−1.1
	Porfimer	−0.31
Candida albicans	Amphotericin	−7
	Nystatin	−6.22
	Clotrimazole	−5.43
	Caspofungin	−3.57
Acinetobacter baumannii	Sulbactam	−4.86
	Kanamycin	−4.11
	Amikacin	−3.46
	Netilmicin	−1.75
	Colistin	4.54
Staphylococcus aureus	Finafloxacin	−5.9
	Cephalexin	−3.19
	Vancomycin	−3.18
Cryptococcus neoformans	Amphotericin	−6.7
	Fluconazole	−3.55
	Voriconazole	−2.3

Table 6.
Summary of the docking results of known antibiotics.

macromolecule and the best pose ligand. The output is a PDBQT file which is then converted to PDB format using Open Babel. **Tables 6** and **7** depict the best pose for every organism in a hierarchy, in the case of both known and investigational drugs, respectively.

5.3 Differential ligand binding affinity

The differential binding affinities of the repurposed ligands will be determined using the conventionally used drugs as a baseline. The differential binding affinity of a potential antibiotic with respect to a known antibiotic can be calculated by subtracting the binding energy value generated by the known antibiotic from that of the unknown antibiotic. A lower differential energy value is indicative of a more stable complex.

$$\Delta\Delta G_{potential} = \Delta G_{bind,potential} - \Delta G_{bind,known} \quad (1)$$

In the above formula, $\Delta\Delta G_{potential}$ is the differential binding affinity of the potential ligand, and ΔG_{bind} is the free energy released during docking. From **Table 8** it is evident that bithionol is the best investigational drug for the *Aspergillus*

Organism	Investigational drug	ΔG kcal/mol
Aspergillus fumigatus	Bithionol	−5.9
	Moxifloxacin	−4.76
	e1210	−4.12
	Terbinafine	−3.76
	Cresemba	−0.45
	Echinocandin	0.03
Candida albicans	e1210	−5.22
	Moxifloxacin	−4.76
	Levofloxacin	−4.66
	Aminocandin	−2.71
	Bithionol	−4.6
Acinetobacter baumannii	Levofloxacin	−6.34
	Gepotidacin	−5.58
	Tigecycline	−4.85
	Ceftobiprole	−4.72
	Moxifloxacin	−4.68
	Trovafloxacin	−4.03
	Tigecycline	−5.75
Staphylococcus aureus	Gepotidacin	−5.12
	Moxifloxacin	−4.92
	Ceftobiprole	−4.18
	934628_27_0	−3.89
	Radezolid	−3.63
	Brilacidin	0.82
Cryptococcus neoformans	Bithionol	−4.98
	e1210	−4.89
	Carvacrol	−4.23
	Moxifloxacin	−4.04
	vt-1129	−3.85

Table 7.
Summary of the docking results of investigational drugs.

fumigatus compared with other repurposed ligand used. From **Table 9** we can infer E1210 as a potential repurposed ligand for *Candida albicans*. **Table 10** depicts the differential binding abilities of repurposed ligand for *Acinetobacter baumannii* of which moxifloxacin is the best investigational drug. In **Tables 11** and **12**, tigecycline and bithionol were the most efficient potential antibiotics for the organisms *Staphylococcus aureus* and *Cryptococcus neoformans*, respectively.

5.4 Identification of interacting residues in each docked complex

The best pose of each docked complex is viewed using RasMol and Pymol v.1.3. All interacting residues within a radius of 4.5 Å of the ligand are restricted using

Aspergillus fumigatus	$\Delta\Delta G_{amphotericin}$	$\Delta\Delta G_{itraconazole}$	$\Delta\Delta G_{anidulafungin}$	$\Delta\Delta G_{micafungin}$	$\Delta\Delta G_{voriconazole}$	$\Delta\Delta G_{isavuconazonium}$	$\Delta\Delta G_{porfimer}$
Bithionol	1.09	**−2.19**	**−2.84**	**−2.93**	**−3.3**	**−4.8**	**−5.59**
Moxifloxacin	2.23	**−1.05**	**−1.7**	**−1.79**	**−2.16**	**−3.66**	**−4.45**
E1210	2.87	**−0.41**	**−1.06**	**−1.15**	**−1.52**	**−3.02**	**−3.81**
Terbinafine	3.23	**−0.05**	**−0.7**	**−0.79**	**−1.16**	**−2.66**	**−3.45**
Cresemba	6.54	3.26	2.61	2.52	2.15	0.65	**−0.14**
Echinocandin	7.02	3.74	3.09	3	2.63	1.13	0.34

Bold values indicate that the differential free energy of binding of the potential antibiotic is negative (i.e., stronger binding).

Table 8.
Differential binding affinities of the repurposed ligands for Aspergillus fumigatus.

Candida albicans	$\Delta\Delta G_{amphotericin}$	$\Delta\Delta G_{nystatin}$	$\Delta\Delta G_{clotrimazole}$	$\Delta\Delta G_{caspofungin}$
E1210	1.78	1	0.21	**−1.65**
Moxifloxacin	2.24	1.46	0.67	**−1.19**
Levofloxacin	2.34	1.56	0.77	**−1.09**
Bithionol	2.4	1.62	0.83	**−1.03**
Aminocandin	4.29	3.51	2.72	0.86

Bold values indicate that the differential free energy of binding of the potential antibiotic is negative (i.e., stronger binding).

Table 9.
Differential binding affinities of the repurposed ligands for Candida albicans.

Acinetobacter baumannii	$\Delta\Delta G_{sulbactam}$	$\Delta\Delta G_{kanamycin}$	$\Delta\Delta G_{amikacin}$	$\Delta\Delta G_{netilmicin}$
Moxifloxacin	**−1.48**	**−2.23**	**−2.88**	**−4.59**
Tigecycline	0.01	**−0.74**	**−1.39**	**−3.1**
Ceftobiprole	0.14	**−0.61**	**−1.26**	**−2.97**
Levofloxacin	0.18	**−0.57**	**−1.22**	**−2.93**

Bold values indicate that the differential free energy of binding of the potential antibiotic is negative (i.e., stronger binding).

Table 10.
Differential binding affinities of the repurposed ligands for Acinetobacter baumannii.

Staphylococcus aureus	$\Delta\Delta G_{finafloxacin}$	$\Delta\Delta G_{cephalexin}$	$\Delta\Delta G_{vancomycin}$
Tigecycline	0.15	**−2.56**	**−2.57**
Gepotidacin	0.78	**−1.93**	**−1.94**
Moxifloxacin	0.98	**−1.73**	**−1.74**
Ceftobiprole	1.72	**−0.99**	**−1**
934628_27_0	2.01	**−0.7**	**−0.71**
Radezolid	2.27	**−0.44**	**−0.45**
Brilacidin	6.72	4.01	4

Bold values indicate that the differential free energy of binding of the potential antibiotic is negative (i.e., stronger binding).

Table 11.
Differential binding affinities of the repurposed ligands for Staphylococcus aureus.

Cryptococcus neoformans	$\Delta\Delta G_{amphotericin}$	$\Delta\Delta G_{fluconazole}$	$\Delta\Delta G_{voriconazole}$
Bithionol	1.72	**−1.43**	**−2.68**
E1210	1.81	**−1.34**	**−2.59**
Carvocrol	2.47	**−0.68**	**−1.93**
Moxifloxacin	2.66	**−0.49**	**−1.74**
VT-1129	2.85	**−0.3**	**−1.55**

Bold values indicate that the differential free energy of binding of the potential antibiotic is negative (i.e., stronger binding).

Table 12.
Differential binding affinities of the repurposed ligands for Cryptococcus neoformans.

Antibiotics	Interacting residues
Amikacin	Ala64, Ser67, Arg68, Arg75, Met103, Tyr104, Glu107, Thr110, Glu115
Colistin	Ala87, Tyr90, Leu91, Ser94, Ser95
Kanamycin	His290, Glu294, Leu295, Asp297, Leu298, Pro299
Netilmicin	Asp277, Val278, Asn279, Glu280, Lys281
Sulbactam	Ser160, Lys161, Arg164, Lys165, Met168, Gln283
Ceftobiprole	Ile120, Asp123, Gly153, Val156, Arg157, Ser160, Met163, Leu268, Lys273, Thr276
Gepotidacin	Gly153, Val156, Arg157, Ser160, Leu268, Lys273, Thr276, Asn279, Leu282, Gln283, Leu286
Levofloxacin	Val78, Tyr79, Ala80, Lys81, Leu82, Leu83, Arg84, Leu85, Tyr90, Asn93, Lys101, Ser291, Val292, Glu294, Leu295, Leu296
Moxifloxacin	Leu384, Ser385, Arg388, Met393, Tyr411, Gly412, Leu414, Arg465, Ala466, Lys469
Tigecycline	Tyr79, Leu83, Tyr90, Ser95, Ile98, Met393, Asn395, Gln397, Val398, Val399, Phe401, Tyr411, Arg465
Trovafloxacin	Ile391, Ala392, Met393, Asn395, Phe401, Tyr411, Gly412, Leu414, Arg465, Ala466, Lys469

Table 13.
Analysis of interacting residues for Acinetobacter baumannii.

Antibiotics	Interacting residues
Amphotericin	Thr221, Asn225, Phe756, Tyr759, Ser760, Gln953, Lys956, Ser957, Glu960, Ala963
Anidulafungin	Trp762, Thr763, Leu764, Phe942, Gly1058, Thr1059, Phe1061, Ser1062, Asp1066, Met1067, Gly1068, Lys1071, Asn1072
Isavuconazonium	Asn392, Leu938, Gly941, Phe942, Arg944, Phe945, Gln1055, Ala1057, Gly1058, Thr1059, Phe1061, Ser1062
Itraconazole	Phe388, Gly391, Asn392, Leu938, Gly941, Phe942, Phe945, Gln1055, Gly1058, Thr1059, Phe1061, Ser1062, Met1067
Micafungin	Trp762, Leu938, Gly941, Phe942, Phe945, Tyr946, Ala949, Gln950, Gln953, Gly1058, Phe1061, Gly1068, Lys1071, Asn1072
Porfimer	Lys210, Glu215, Arg219, Asp223, Ala405, Ala408, Lys409, Ser412, Arg416
Voriconazole	Ser760, Leu761, Trp762, Thr763, Leu764, Val765, Lys766, Gly1068, Lys1071, Asn1072
Bithionol	Phe348, Phe352, Tyr355, Ile385, Gln793, Gln796, Tyr800, Phe1052, Gln1055, Ser1056, Thr1059
Cresemba	The238, Phe242, Val393, Ala394, Gly397, Gln398, Phe400, Thr401
E1210	Glu186, Gln228, Arg954, Ser955, Ala958, Lys995, Gln996, Lys999, Ser1003
Echinocandin	Thr218, Thr221, Gln580, Arg581, Val752, Gln755, Phe756, Lys758, Tyr759, Glu876, Glu877, Lys956, Glu960, Ala963
Moxifloxacin	Arg307, Tyr1113, Thr1115, Arg1116, Glu1118, Gln1119, Val1121, Gly1142, Cys1143, Gly1144, Lys1145, Ser1146, Thr1147, Tyr1156
Terbinafine	Gln755, Phe756, Glu757, Lys758, Tyr759, Arg875, Glu877

Table 14.
Analysis of interacting residues for Aspergillus fumigatus.

RasMol. By studying the PDB file constituting the restricting structure, we can identify the atoms that are present within the interacting residues. These interacting residues are then analyzed for recurrences, which are found to be the most active

interactive residues within the respective macromolecule. An analysis of the interacting residues showed us that:

- (Leu268, Lys273, Thr276, Asn279) and (Gly153, Val156, Arg157, Ser160) are some recurring residues in *Acinetobacter baumannii* (**Table 13**).
- (Phe942, Gly1058, Thr1059, Phe1061, Ser1062) and (Asn392, Leu938, Gly941) are some recurring residues in *Aspergillus fumigates* (**Table 14**).
- (Phe1143, Thr1146, Phe1173, Asn1176, Tyr1177, Arg1179, Ile1180, Ile1317) and (Gly978, His1357, Leu1358) are some recurring residues in *Candida albicans* (**Table 15**).

Antibiotics	Interacting residues
Amphotericin	Phe1143, Thr1146, Leu1147, Phe1151, Val1152, Ser1170, Phe1173, Val1174, Asn1176, Tyr1177, Arg1179, Ile1180, Ile1313, Glu1314, Ile1318, Tyr1319
Caspofungin	Ser1104, Val1105, Leu1106, Arg1107, Ser1108, Phe1113, Ile1121, Phe1125, Asp1332
Clotrimazole	Tyr1175, Phe1178, Arg1179, Phe1182, Val1183, Arg1187, Thr1254, Ser1257, Ser1258, Phe1261, Phe1262
Nystatin	Arg1002, Thr1005, Val1006, Pro1007, Trp1008, Asp1009, Ile1010, Phe1011, Asn1135, Phe1143, Phe1173, Pro1323, Pro1327
Aminocandin	Asp836, Val953, Asp954, Met1110, Asp1114, Tyr1354, Asp1359, Pro1360, Val1361, Arg1380, Thr1381, Ala1383, Gly1384, Leu1390, Gln1422, Leu1573, Asp1574, Ser1575, Gly1576
Bithionol	Phe1143, Thr1146, Phe1173, Asn1176, Tyr1177, Arg1179, Ile1180, Ile1317, Glu1318, Tyr1319
E1210	Arg992, Lys993, Thr994, Arg995, His996, Glu997, Gln998, Glu999, Glu1000, Ser1001, Arg1002, Thr1116, Arg1120, Arg1124, Met1332, Lys1333
Levofloxacin	Val953, Val975, Gly978, His1357, Leu1358, Asp1359, Pro1360
Moxifloxacin	Tyr977, Gly978, Ser1111, Phe1112, Thr1115, Lys1333, Arg1355, Lys1356, His1357, Leu1358

Table 15.
Analysis of interacting residues for Candida albicans.

Antibiotics	Interacting residues
Amphotericin	The280, Glu583, Pro584, Thr585, Leu586, Phe587, Gly641, Glu642, Leu646, Leu647, Gly649, Lys652, His1020, Ser1023, Glu1024, Gly1027, Ala1028
Fluconazole	Asp801, Ile802, Gln803, Ala804, Arg806, Ala807, Val810, Ala811, Gly812, Glu813, Asp814, Lys815, Gln946, Lys949
Voriconazole	SER818, Ser819, Phe820, Gly821, Arg825, Ala1131, Ser1132, Arg1135, Asp1138
Bithionol	Met343, Phe346, Gly347, Ala350, Leu351, Val394, Gly395, Gly398, Ser399, Glu402, Trp907, Gln948
Carvacrol	Lys1002, Val1003, Val1004, Leu1006, Lys1007, Asp1008, Met1011
E1210	Ile802, Gln803, Ala804, Arg806, Ala807, Val810, Ala811, Gly812, Lys815, His933, Ala938, Ser941, Asn942, Ser1132
Moxifloxacin	Lys296, Arg1000, Leu1001, Lys1002, Gly1114, Phe1117, Thr1118, Pro1121
VT1129	Ile802, Gln803, Ala804, Arg806, Ala807, Val810, Ala811, Gly812, Glu813, Lys815

Table 16.
Analysis of interacting residues for Cryptococcus neoformans.

Antibiotics	Interacting residues
Cephalexin	Arg186, Thr302, Thr305, Gln306, Phe308, Ala309
Finafloxacin	VAL178, Phe182, Ser247, Phe248, Ile251, Asn252, Gly292, Arg295, Arg296, Ala299
Vancomycin	Pro172, Leu1776, Thr177, Tyr179, Val180, Phe181, Gly183, Arg184, Lys187
9346	Ala106, Leu107, Ser108, Ala109, Tyr112, Tyr322, Ile324, Phe390, Thr410, Arg414
Brilacidin	Tyr94, Arg97, Lys98, Tyr101, Ile121, Val124, Ile425, Leu426, Phe427, Ser428, Glu473, Arg474
Ceftobiprole	Tyr112, Ala113, Asn115, Gln116, Val117, Gly118, Gln119, Val120, Ile121, Phe427
Gepotidacin	Gln105, Ala106, Leu107, Ser108, Ala109, Tyr112, Ile324, Arg389, Phe390, Arg414, Leu419
Moxifloxacin	Tyr12, Trp87, Asn90, Lys91, Tyr94, Asp95, Lys98
Radezolid	Arg97, Tyr101, Gln105, Tyr112, Gln116, Val117, Gly118, Gln11, Val120, Ile121, Val124, Ile125
Tigecycline	Gln105, Ala106, Leu107, Ser108, Ala109, Tyr322, Asp323, Ile324, Asn385, Arg389, Phe390, Arg414, Gln421, Ile425

Table 17.
Analysis of interacting residues for Staphylococcus aureus.

- (Ile802, Gln803, Ala804, Arg806, Ala807, Val810, Ala811, Gly812, Glu813, Lys815) are some recurring residues in *Cryptococcus neoformans* (**Table 16**).

- (Ala106, Leu107, Ser108, Ala109, Tyr112, Tyr322, Ile324, Phe390) and (Tyr112, Gln116, Val117, Gly118, Gln119, Val120) are some recurring residues in *Staphylococcus aureus* (**Table 17**).

6. Conclusion

The homology modeling was performed to determine the best template, from which we concluded that 4m1m is preferred in *Aspergillus fumigatus, Aspergillus nidulans, Acinetobacter baumannii*, and *Candida albicans*. In *Cryptococcus neoformans* 4f4c is preferred, and 3wme is preferred in *Staphylococcus aureus*.

The molecular docking led us to conclude that bithionol, levofloxacin, e1210, tigecycline, and bithionol were the most efficient potential antibiotics for the organisms *Aspergillus fumigatus, Acinetobacter baumannii, Candida Albicans, Staphylococcus aureus*, and *Cryptococcus neoformans*, respectively. Each of the potential antibiotics was found to be more effective than a number of the known antibiotics in the treatment of that respective organism.

An analysis of the interacting residues showed us that:

- (Leu268, Lys273, Thr276, Asn279) and (Gly153, Val156, Arg157, Ser160) are some recurring residues in *Acinetobacter baumannii.*

- *(*Phe942, Gly1058, Thr1059, Phe1061, Ser1062) and (Asn392, Leu938, Gly941) are some recurring residues in *Aspergillus fumigatus.*

- (Phe1143, Thr1146, Phe1173, Asn1176, Tyr1177, Arg1179, Ile1180, Ile1317) and (Gly978, His1357, Leu1358) are some recurring residues in *Candida albicans*.

- (Ile802, Gln803, Ala804, Arg806, Ala807, Val810, Ala811, Gly812, Glu813, Lys815) are some recurring residues in *Cryptococcus neoformans.*

- (Ala106, Leu107, Ser108, Ala109, Tyr112, Tyr322, Ile324, Phe390) and (Tyr112, Gln116, Val117, Gly118, Gln119, Val120) are some recurring residues in *Staphylococcus aureus.*

Appendix 1: FASTA sequences obatained from PSI – BLAST searches

>SST02482.1 lipid A export permease/ATP-binding protein MsbA [*Acinetobacter baumannii*]

MIDKDLSTRQTFRRLWPTISPFKAGLFVAAIALVINAAGDAFMISLLKPLL-
DEGFEKADNDVLKWLPLAMLGLIIVRGASSFV-
STYCVSWVSGQVVMSMRRKLFGHMMGMPVSFFDQQSTGTLLSRITYDSEQ-
VAASSSGALITIIREGAYIIGLFAMMFYYSWQLSLILIVIAPIVSITIRIVSKRFR-
KISKNMQTGMGHVTASAEQMLKGHKEVLIFGGQKVE-
TERFNKVSNNMRSQSMKMVTASAISDPIIQLIASFALAFVLYAASFPEIREQLSPG-
TIAVVFSSMFALMRPLKSLTNVNSQFQRGMAACQTLFSILDTEQEK-
DEGTKVLSNVKGDIEFENVTFTYATKEHPALDDISFTLPAGKSVALVGRSGSGK-
STIANLITRFYDIDKGSIRIDGHDIREYTLESLRNQVALVSQHVYLFNDTIAN-
NIAYATDGRFSREQIEKAAEMAYAMDFIAKLDKGLDTVIGEN-
GVMLSGGQRQRIAIARALLRDAPILILDEATSALDTESERAIQAALDELQKNRTSL-
VIAHRLSTIENADEILVVQDGRIIERGNHKALLALEGAYAQLHKIQFSQ

>WP_051575420.1 ABC transporter ATP-binding protein [*Staphylococcus aureus*]

MKFKKFISYYRPYKRIFGLTLICSLLVTVITLVIPLIIRYITENLIQHFSVAHV-
KEIYLLGAAMVLLILIQFLCHIFIDYYGHVMGAKMEKDMSEELYE-
HIQSQPHHFFDRNSTGGLMSRLTGDLENLSELYHHGPEDILMYIIRFIGAVVIL-
LYINVELTIVMMLFIPIMIVVYWYYIKKLSSIYEQDKATNAEIHGFLENTIS-
GIKVTKSFTNESFESNQYKSLNKKAIEIKKKVHKYEALYNEIIGSIIQAMPVIIIVL-
GALLIMKKEISIGDLLAFVLYVGNIATPIEVLVKLSVQYNE-
GISGFNRFFKLMQLKPDITSENTHQQQSPHSNGAIQFDHVYFQYDQEYIIHNLNL-
TIEPGAYIAIVGPSGSGKSTIANLLPRFYDVTSGSITINHQDIRTIPLEELRQKI-
GIVQQDVYIFSSTVYENIKYGNPEASMDEIIHASKLANAHEFIQQLPNGYHTQI-
GEKGAKLSGGQKQRLSIARMFLKNPEVVILDEATSALDNLSEKVVQQ-
SLEQLTLNRTTIVIAHRLSTIRNADNIYVLTKEGIIESGNHDTLIEKQG-
FYYRMYINEEN

>KHC36224.1 alpha-factor-transporting ATPase [*Candida albicans* P76067]

MFQEKSEKSSFPKRSSSLRSPSDSPAITSKNVFMFVNYSKDWPLILVGILLMGG-
SAIATPMNTYIYGEIMGKLSQFYLQDQSNHSFSQDIVKLCVGLIGIGCCK-
MILVWLGMFTWLKFGEIQQSRARMQIYNKIINESQSWYDSKQNLIGQLTQINR-
CIEELRSCNGEILASLMQTIVLILALLIMSFYQSWSTTLIIMASFPIMALCG-
WYFGKLTYKAQQDENEVTSKASKVFNWCYVNPEMVRFFNSKNIELTKFKQ-
LIEKSAQFYYKLSHAVAANTAVLKTLTLMMFVQGFWFGNYLLSHNTI-
TINQLFTCFSSCLMLGQAVSGITELLAILNTGHAAADKISGFLLQPPS-
KAKLLLLHSKYPPFEIGSIYFKNVWFESNSQNSVAILQDVSFGILQNQFNF-
VIGKSGSGKSTIAKLLMRLYSVSRGTIEIDTVSIDKLDPKYICQNIILLEQNP-
VIFDDKTIAENIAIAIVDDYDSLQAIPYYLIEQSAHFALLSDLDLNMKVNQLTLSG
GQQQRISIARAYLKNSPVLIMDESFSALDTETKQCLIEKVKKWRIGKTTIFITHEY-
KNILDDENVIILDQGMIKNQGQFKKMKNEEIVQNYKSQGIETS-
SYETTSQSFSDNTKLPDGDYNYKTNPYILKDLESQIKEDTDNEKLMGVLAIL-
RYCSSTINGKSLLGFGILLAIFQGGSSPVFSYCFSKLLSTSLDSSIGLNSTQKILQWS-
CISLSIAIFTGVTSYLSEFILNYCGENWIVSLRQLTFFKLNNQDLSFFTRFDTNWSS-

SEITALLMNDTRDLRNLISQFFPLLANLVSMTLIGIIWSIVSGWKLALV-
GISFVPLVLLVTVLYGKILESIENKYKCKVNNVELDLYRTITTIRTIKIFNIQQY-
FETVFKEDLKVLNSIGVYRALQTGIGFAISDLFSSIGQAIILFYGMKLISQFQY-
NYSQLLQVITLLSFTISNASILIHQLPEITRGQRAGTFIVKLLKDITST-
MEVNDSCGVSSVRKRNSKSGSDSIGTIGPVKDNQLFKKVTTDNDTLAISFNNVSF-
SYPNKLPYILQLKSISLDVKKFTTIGIVGQSGSGKSTILKILFRLYDIKISPDSNTTK-
KYHDQTVKIFNQNLYLINSGLLCQTIAIVPQFPKFFSGTIYDNLTYGINNTN-
SAGSNSSSSVSDSEIIKILKLVNLHQFIVSLPQGLLTIMNDSDNDNDNGNENENE-
NENGNTISTSSSTSFTFSGGQLQLLAIARALLRNPKILLLDECTSNLDPITTKIIIN-
VIKSLHGKLTILFVTHDKELMRIADNLIVMKDGQIVEQGDFQQLISND-
GEFTKITKTII

>OWZ59602.1 ATP-binding cassette, subfamily B (MDR/TAP), member 1 [*Cryptococcus neoformans* var. grubii c45]

MSASPGLTAAAAGPDHLQARRDEKVIDSEKDALAHDAHAVNSGIPYPTA-
TAPNVGAPTVPIIVGRVSSAPEGKISRSSIAASSDTLRNSPLEKPISNAVSKSH-
PYKKSKFDFLKSRKKKEEEERKNKEKEKEASVLPPVSFFALFRFAA-
PLEIIAMVLGLVLAVAAGSCQPLMTLIFGRLTTSFTNYAVIANQISQGGLTPET-
SAALQAAKDDLKTQSGHNALYLMAIGIGMFLATWLYMFIWNVTGELNSKRIR-
ERYLAAVLRQEIAYFDDLGAGEVATRIQTDCHLVQEGTSEKVALVF-
QYAGTFVCGFVLAFVRSPRLAGALVSILPVIMLCGGIMMTAMAKFGTAALDHIA-
KAGSLAEEVIGSIRTVQAFGKEKILGDKFADHIEQSKIVGRKGSIFEGFGLSIMFF-
VIYAAYALAFFYGGILVSNGQADSGIVINVFMSILIGSFSMAMLAPELAAVTKAR-
GAAAKLFATIDRVPAIDSASEEGFKPDGLRGEISFENVKFHYPSRPSIPILKGFTTT-
FEAGKTFALVGASGSGKSTVVSLIERFYDPVSGVVKLDGRDIRSLNLNWLRQ-
QIGLVSQEPTLFGTTVRGNVEHGLIGSRYENASLEEKFELVKKACVDANAHN-
FIMKLPQGYDTMVGERGMLLSGGQKQRVAIARAIVSDPRILLLDEATSALDTQ-
SEGIVQDALDKASRGRTTITIAHRLSTIRDADRIYVMGGGEVLEQGSHNDLLA-
NENGPYAQLVNNQKLAQEAAAEALQVDDDIDDLDDAVFIGGSSPM-
QEKDKQLHRAVTGRSLASIAMDDIQAKRAEEVAGEDKIPSSFGLYARLLKMN-
SADKFIYILAFIAAICAGMVYPSLAILFGKALSDFEIQDPAELRHALSRSALWYFI-
TALAAAFVIFFQSAGFSRAGWDLNGVLRKKLFTATLRHDIEWFDEERNST-
GAVTSNLADQPQKVQGLFGPTLGTVVQSCATLIGGCIIGLCYGPLLALIGIACI-
PILVSGGYIRLKVVVLKDQRMKKLHAASAHLASEAAGAVKTVASLTREKDVR-
RIYSEALKAPMKLNFRTSIKSQCLFAASQGLTFCIIALVFYIGALWIIDGKYSTAS-
FYTVLNSIVFASIQAGNVFTFVPDASKANSSAASIFRSIDNEPAINAES-
NEGKVLDHKHVVGHVRIEGVHFRYPTRPGVRVLRNLTIDVPAGTY-
VALVGPSGCGKSTTIQMLERFYDPLAGRVTLDGIDIKELNLASYRSQISLVSQEP-
TLYAGTIRFNILLGANKPIEEVTQDEIDAACKDANIYDFIVSLPDGFD-
TEVGGKGSQLSGGQKQRIAIARALIRNPKVLLLDEATSALDSQSEKVVQEALD-
KAAKGRTTIAIAHRLSSIQHSDRIYYFSEGRVAEHGTHQELLAKKG-
GYYELVQMQNLSRQ

>KEY77376.1 ABC multidrug transporter Mdr1 [*Aspergillus fumigatus* var. RP-2014]

MPAPETGASSREKSLEDLQVATLEKGRSTSSFGADNEKPHDHHSLSDTI-
MAPPDGKKKDHGKAVDLNDDSLFAHLQEHEKEVLKRQLDAPSVKVSFFTLYR-
YASRKDILIILVSAICAIAAGAALPLFTILFGSLASAFQGISLGTMPYHE-
FYHKLTKNVLYFVYLGIAEFVTVYVSTVGFIYTGEHLTQKIRENYLEAILRQN-
MAYFDKLGAGEVTTRITADTNLIQDAISEKVGLTLTAFATFVTAFIVAYVKYWK-
LALICTSTIVALVMVMGGGSRFIVKYSKKSIESYGAGGTVAEEVISSIRNA-
TAFGTQDKLAKQYETHLAEAEKWGVKQQVILGMMIGGMFGIMFS-
NYGLGFWMGSRFVVGKEVNVGQVLTVLMSILIGSFSLGNVAPNGQAFTNG-
VAAAAKIYSTIDRRSPLDPYSDEGKVLDHFEGNIEFRNVKHIYPSRPEVTV-
MEDVSLSMPAGKTTALVGPSGSGKSTVVGLVERFYLPVGGQVLLDGH-

DIQTLNLRWLRQQISLVSQEPVLFSTTIFRNIEHGLIGTKFEHESKDKIRELVE-
NAARMANAHDFIMALPEGYDTNVGQRGFLLSGGQKQRIAIARAIVSDPKILLL-
DEATSALDTKSEGVVQAALDKAAEGRTTIVIAHRLSTIKTAHNIVAMVGG-
KIAEQGTHDELVDRKGTYYKLVEAQRINEEKEAEALEADADMDADDFGQEGV-
TRIKTAVSSSNSLDAVDEKARLEMKRTGTQKSVSSAVLSKKVPEQFE-
KYSLWTLVKFIGAFNRPELGYMLIGLTFSFLAGGGQPTQAFLYAKAISTLSL-
PESMFHKLRHDANFWSLMFFVVGIAQFISLSINGTAFAICSERLIRRARSQAFR-
SILRQDISFFDREENSTGALTSFLSTETKNLSGVSGVTLGTIIMTSTTLGAAMIIA-
LAIGWKLALVCISVVPILLACGFLRFYMLAQFQQRSKSAYEGSASYACEATSAIRT-
VASLTREQDVWGVYHDQLQKQGRKSLISVLRSSLLYASSQALVFFCVALGF-
WYGGTLLGHHEYSIFRFFVCFSEILFGAQSAGTVFSFAPDMGKA-
KNAAAQFKKLFDSKPTIDIWSDEGEKLESMEGEIEFRDVHFRYPTR-
PEQPVLRGLNLSVKPGQYIALVGPSGCGKSTTIALLERFYDALAGGVFVDGK-
DITKLNVNSYRSFLSLVSQEPTLYQGTIKENILLGVDKDDVSEETLIKVCKDA-
NIYDFVMSLPEGFDTVVGSKGGMLSGGQKQRVAIARALLRDPKVLLLDEAT-
SALDSESEKVVQAALDAAARGRTTIAVAHRLSTIQNADIIYVFDQGKIVESGTH-
HELIRNKGRYYELVNLQSLGKTH

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Chapter 2

P-Glycoprotein Efflux Transporters and Its Resistance Its Inhibitors and Therapeutic Aspects

Chenmala Karthika and Raman Sureshkumar

Abstract

P-glycoprotein (P-gp) is an active member of the ATP Binding Cassette (ABC) protein subfamily which effluxes a wide range of therapeutic drugs out of the cells commonly known as multidrug resistance. But its protective action towards the normal cells and efflux of the toxic and foreign substances is remarkable. Hence the efflux of the P-gp is a crucial step to overcome for the success of the therapy and in the drug discovery process. Modification of the action of the P-gp through various inducers, inhibitors or the genetic polymorphism is the commonly used methods. When it comes to the inhibitor part the natural inhibitors use is more safe and economical as compared to the synthetic ones. Here we review at the mechanism of action and the pharmacokinetic profile of P-gp, how the P-gp engaged in the Multidrug resistance, the strategy to overcome from its action by using natural inhibitors and formulation perspectives.

Keywords: P-glycoprotein, multidrug resistance, mechanism of action, pharmacokinetics, P-gp inhibitors, natural inhibitors

1. Introduction

Reduce in the remedial rate of most of the diseases and the decline in the therapeutic efficacy of most of the anti-neoplastic and anticancer drugs is due to the phenomena called drug resistance [1]. The main ambiguity to be found out to increase the efficacy of the drugs is to overcome this phenomenon. The drug resistance is not observed to a single chemotherapeutic drug but to a broad range of structurally and functionally different drugs. The exposure to a single therapy for a long time and the recurring use of the medication leads to drug resistance will leads to decrease in the therapeutic efficacy of the drugs. Even if the dose is altered there would not produce any momentous changes in this phenomena [2]. When the human acquired resistance to the drugs it is termed as drug tolerance. This was usually seen of two type's pharmacokinetic drug tolerance and pharmacodynamic drug tolerance.

2. Metabolic or pharmacokinetic tolerance

Followed by the entry of the drug in our body with the time it gets absorbed into the bloodstream, which is then carried out and distributed to various other sites, additionally gets disintegrated into various segments and eventually gets excreted from the body. All these factors determine the potency, side effects and duration of action of the drugs. The major reason for the pharmacokinetic tolerance is when the drug fails to maintain its minimum therapeutic concentration at the target site. Where, in this case the enzymatic action of cytochrome P450 (CYP450) produces the effect. This type of tolerance is mainly determined with the oral dosage form which produces first pass metabolism. Induction of the enzymes are the major reason behind the drug resistance which is further accompanied with various other factors which is further disused in this chapter in detail.

3. Pharmacodynamic tolerance

When the cellular feedback to a substrate is concentrated the development of the pharmacodynamic tolerance eventually occurs. The principle reason behind pharmacodynamic tolerance is when the therapeutic concentration of the substrate to the binding receptors reaches above the maximum therapeutic concentration which eventually results in the desensitisation of the receptors [3]. Other possibilities include the decline in the receptor density which is mainly associated with the receptor agonist and the modification in the action of the possible firing rate. Generally the drug resistance will occur subsequent to the incessant exposure to the drug, but instant tolerances were also observed in rare cases [4].

4. Factors responsible for drug resistance

There are a variety of drug resistance usually seen such as anticancer resistance, anti-human immunodeficiency virus (HIV) drug resistance, antibiotic resistance, anti-tubercular drug resistance, anti-malarial drug resistance and anti-microbial drug resistance. The most part of the drug resistance is caused by MDR proteins, where in this P-gp (P-glycoprotein) involves in producing a major aspect in reducing the drug efficacy in most of the treatment. P-gp, which is a trans-membrane (TM) glycoprotein physiologically, expressed in the parts of the body such as kidney, liver, pancreas, colon and jejunum [5], it also expresses its role eventually in the brain capillary epithelial cells. The core function of P-gp is to safeguard the cells and restrict the entry of xenobiotics and toxic substances. But its action is over-expressed in the diseased cells by restricting the entry of the drugs hence its action should be inhibited in such cases for the success in the therapy.

5. Transporters and its family

P-gp is an efflux protein system associated with the ATP binding cassette (ABC) sub-family B membrane or Multi-drug resistance 1 (MDR1) or cluster of differentiation 243 which belong to ABCB (MDR) super family of ABC transporter [6]. ABC gene indicates the leading family of TM protein, originated mainly in the intercellular membrane or the plasma membrane. By using the energy from the ATP the transport mechanism across the cell membrane is initiated. In humans

around 49 ABC transporters are observed [7], where MDR1A, MDR1B and MDR2 [8] are usually identified in animals and MDR1 and MDR3 [9] which belong to the P-gp gene subfamily is mostly seen in humans. Where MDR1 (P-gp) is widely seen all over the body and efflux a wide range of drugs over the plasma membrane and MDR3 (P-gp) is predominantly observed in the liver, canalicular membrane of the hepatocytes and is accountable for the phosphatidylcholine secretion into the bile [10]. Even though the action of MDR3 or P-gp in the efflux transport mechanism is observed, their direct action in drug resistance is restricted.

The efflux action exhibited by P-gp is having a greater importance since it can protect our body from the entry of the toxins and xenobiotics into the cells mainly to brain, placenta and gonads and eliminating the waste products through the urine [11], by facilitating the energy driven with the ATP hydrolysis the amphipathic drugs are mainly eliminated by the efflux action of the P-gp.

P-gp role is mainly observed in the body parts such as kidney, intestine, liver [12], testis and brain [13]. The entry of the xenobiotics into the blood capillaries are mainly minimised by the localised action of P-gp in the luminal membrane of the epithelial cells [14]. Over-expression of the P-gp is the foremost reason behind the failure of the chemotherapy and other treatment strategies where nowadays the researchers are mainly focussed to overcome with this issue. The studies related to the action exhibited by the P-gp are performed in mice, rats [15] and in humans [16]. The substrates which merge with P-gp have unrelated frameworks. The compounds transported by P-gp are considered as substrates, while the compounds that prohibit the role of P-gp are considered as inhibitors.

P-gp was identified first in 1976 in Chinese hamster ovary cells, where it was obtained to demonstrate anticancer resistance [17]. The research data proved that the P-gp have the ability to acquire resistance to the cytotoxic drugs. They also proved that verapamil was helpful to measure the function of the P-gp by using positron emission tomography [18]. P-gp is used for differentiating the transitional B-cells from the native B-cells. Rhodamine 123 and Mito Tracker Dyes are also used for this purpose [19].

6. Cellular localisation

The expression of P-gp is recurrently found predominant in the cancer cells, causing MDR by efflux of lypophilic drug from the cell. P-gp is observed to be over-expressed in renal, colon and adrenal carcinoma, not often in germ cell tumour and lungs even certain in gastric carcinoma, undetectable in breast and endometrial carcinomas. In the normal cells the concentration of the P-gp and its expression is found to be low, but certain cell types like colon, kidney, liver, jejunum and pancreas shows a higher expression of P-gp. While in liver, the broad distribution of P-gp is found on the apical surface of epithelial cells and biliary canalicular front in the small biliary ductules [20]. When it appears in case of pancreas, P-gp exclusively found of the apical surface of epithelial cells of the small ductules. In Kidney the presence of the P-gp is exclusively found on the apical surface of the epithelial cells of the proximal tubules [5]. P-gp expression shows an identical expression in the apical surface of superficial columnar epithelial cells of both jejunum and colon. Where, the expression of P-gp is mostly seen in the surface of cortex and medulla cells in the adrenal glands. Its presence is found out with both secretary and excretory action in the specialised epithelial cells, placental trophoblast [21] and endothelial cells of capillary cells at blood-tissue barrier sites. Its presence is also found in the epithelial cells of bronchi and gastrointestinal tract, prostate gland, salivary glands and sebaceous gland of the skin.

The research reports proved the occurrence of P-gp in the human fetus is with the significance in the regular performance of various organs in the initial stage of embryo development [22]. The evidential reports from the research work conducted in the tissues of rodent and humans [23] came to a conclusion that the expression of the P-gp in the normal tissues are much lower than compared to that at the epithelial cell lining of small Intestine, colon, pancreatic duct, proximal tubules of kidney, bile ducts and adrenal gland [24]. P-gp over-expression is also observed in the secretary epithelial cells in the endothelium of the pregnant woman and also in the placenta for protecting the fetus [25].

7. Kinetics of P-gp

7.1 Absorption

The major reason behind the multidrug resistance in the cancer cells is the action exhibited by P-gp. P-gp transports a wide range of structurally and functionally different cytotoxic compound out of the cell by using the energy driven from ATP. Altering in this property caused by P-gp is a vital approach for overcoming the MDR part and to increase the therapeutic efficacy during the treatment. The incorporation of the P-gp inhibitors with the resistant drugs can be helpful towards suppress the expression of the P-gp. This situation of resistance part not only happens with the chemotherapeutic drugs but also for the treatment strategy used for other conditions also [26]. The over-expression of P-gp in the diseased cells as compared to that of the normal cells is the major reason behind this phenomenon. Research work carried out came with a conclusion that P-gp is the major cause for the antibiotics resistance part also. P-gp trims the overall permeability of the drug to reach the target site by reducing the minimal therapeutic concentration level [27].

7.2 Distribution

P-gp acts as an integral element in the distribution of the drugs. Its action is noticed in the blood brain barrier and also the placental barrier. It can accomplish its effect on the distribution of various therapeutic agents also reduce its activation in the body. The role of P-gp in the brain turned into a point where it resists the entry of the neuro-toxic drugs into the brain and hence sustains the penetration of central nervous system (CNS) agent delivery in the brain [28]. A modulator is in need to inhibit the action of P-gp for improving the efficacy of CNS drugs to the target site including for the Parkinson's and Alzheimer's disease. When it comes towards P-gp inhibition the P-gp also protect the fetus from the entry of the foreign bodies and toxic substances through the placenta [29, 30]. Hence by considering these aspects the inhibition of the P-gp is to be made in a careful manner by not altering the protective mechanism in the normal cells [31].

7.3 Metabolism

The enzymatic activity of CYP3A4 and the efflux mechanism by P-gp together play the role for the decreased therapeutic efficacy and bioavailability of the drugs administered through the oral route. These are major defence system in intestine which acts as a protective barrier from the entry of the toxic substances and the xenobiotics. These proteins are mainly over-expressed in enterocytes and hepatocytes and also establish its role in first pass metabolism of the drugs.

For supporting this statement piperine can be taken as an example. The flavonoid piperine is obtained from black pepper. It have the ability to act as a natural inhibitor of P-gp and CYP34A when and can increase the therapeutic efficacy of the drugs when co-administered [32]. The dose of the piperine administered should be ideal and reaches the minimum therapeutic concentration to produce this effect. Another example is the action exhibited by grape fruit juice. When grape fruit juice and saquinavir is co-administered, the therapeutic concentration of the parent drug can be increased when administered during oral route.

8. Elimination

8.1 Renal excretion

The process of glomerular filtration, tubular secretion and reabsorption involve in the mechanism of renal excretion. P-gp involved in the efflux of the xenobiotics and the waste materials from our body via renal excretion, results in reduced level of the therapeutic drug in the blood plasma. This can also be altered by incorporating the flavonoids with the therapeutic agents. When digoxin a flavonoid and Cyclosporine A is co-administered, Cyclosporine will increase the concentration of digoxin in the plasma by decreasing tubular secretion and glomerular secreation rate [33, 34]. Comparable results are obtained when itraconazole and cimetidine is coadministered [35].

8.2 Biliary excreation

The excretion of the drugs by the influence of the P-gp can also be altered by using the natural P-gp inhibitors. Quercetin, a natural inhibitor of P-gp can add on to the therapeutic concentration of a wide range of drugs in the target site when co-administered. In turn the therapeutic agents such as Azithromycin, erythromycin, cyclosporine A and doxorubicin have an inhibitory action on biliary excretion of drugs mediated by P-gp [36].

8.3 Antimicrobial drug resistant mechanism

The mechanism by which the antimicrobial drugs acquiring resistance to the microorganisms are as follows:

1. Drug inactivation or modification: enzymatic deactivation of pencillin G and the synthesis of β-lactamase in the penicillin resistant bacteria.

2. Verification in target site: the shifting of the pencillin binding site from PBP to MRSA in the bacteria resistant to the penicillin drug.

3. Metabolic pathway modification: For example, the para-aminobenzoic acid path is not necessary for the sulfonamide resistant bacteria which are the extensive predecessor for the combination of nucleic acid and folic acid, as an alternative of like mammalian cells they employ preformed folic acid.

4. Intercellular drug concentration diminution: accumulation of the drugs within the cells is declined by diminishing the drug efflux and drug permeability across the cell surface.

8.4 P-gp role in drug resistance

P-gp has a leading aspect in reducing the bioavailability and distribution of the drugs, where P-gp is over-expressed in intestinal region which act as a substrate to P-gp and reduces its absorption pathway. Hence the therapeutic level of the drugs and the bioavailability of the drugs are not accomplished. On the other hand if the P-gp expression is abscessed then the concentration of the drug in the plasma will reach to supra-therapeutic concentration leading to toxicity related issues [9].

The substrate infiltrate into the P-gp all the way through the protein cytoplasmic side or through the inner leaflet of the membrane. ATP attitudes to the cytoplasmic side of P-gp, pursue with its binding, ATP hydrolysis activates which modify the substrate to be efflux form the cell. The substrate excretion occurs followed with the release of the phosphate from the ATP molecule. A novel molecule of ATP attach to the secondary ATP binding site when adenosine diphosphate (ADP) is discharged. This process will proceed with the hydrolysis and discharge of ADP and a phosphate molecule reset the protein.

8.5 Substrate for P-gp

P-gp transports a broad range of substrates which are structurally and functionally different from each other. P-gp substrate mainly appear to be lypophilic and amphipathic in nature [37, 38]. For altering the functions of P-gp its inhibitors are mostly generated. The mechanism of action is either by competition with the drug binding sites without hindering the action of ATP hydrolysis of by blocking the ATP hydrolysis process [39]. Recently allosteric mechanism for P-gp mediated transport also added with the other two mechanisms [40]. P-gp substrates are attached with the protein molecule before getting attached or moved to the extracellular membrane leaflets.

8.6 P-gp inhibitors

The reports from the research work proven that the P-gp has the ability to interact with more than 20 substrates or the modulators. Some of the substrates which are easily transported by P-gp include anthracycline, vinca alkaloids and fluorescent lipids. This binding action of the modulators such as cyclosporine and verapamil are employed for altering the P-gp activity for the chemotherapy. The high flexible and the low specific nature of the P-gp binding pockets could be employed for overcoming the MDR related issues during the chemotherapy [41]. The Classification of the P-gp inhibitors is mainly based on its specificity, affinity and the toxicity. The classification and the division of the inhibitors are mentioned in **Table 1**.

8.7 First generation inhibitors

The inhibitors belonging to this generation are pharmacologically active in nature and are used in specific treatment. Some of them are reserpine, verapamil, cyclosporine A, yohimbine, quinidine, toremifene and tomoxifene. When we are taking the example of leukaemia cells, the resistance could be inverted by using verapamil [42] for producing an effective action high dose of the drug is given to the patient, results in cardiovascular toxicity [43]. Hence these inhibitors are replaced with the second generation inhibitors because of their less therapeutic efficacy.

Generations	Examples
First generation inhibitors	Verapamil, cyclosporine A, reserpine, quinidine, yohimbine, tamoxifen, and toremifene
Second generation inhibitors	Doxverapamil, valspodar, biricodar citrate, dexniguldipine, and dofequidar fumerate
Third generation inhibitors	Tariquidar, zosuquidr, laniquidar, elacridar, mitotane, annamycin, biricodar, ONT-093, R10933, and HM30181
Natural inhibitors	Curcumin, piperine, capsaicin, [6]-gingerol, carnosic acid, limonin, quercetin, β-carotene, leutiolin, and anthocynine

Table 1.
Generation of inhibitors with examples.

8.8 Second generation inhibitor

The substrates coming in this generation inhibitors are pharmacologically inactive in nature but produce its action on P-gp. This generation inhibitors are developed by structurally modifying the first generation inhibitors for obtaining high specificity, low toxicity and potency. Examples for this generation inhibitors include doxverapamil [44], valspodar (PSC 833) [45], biricodar citrate (VX710) [46], dofequidarfumerate and dexniguldipine. Non immunosuppressant analogues of dox verapamil and cyclosporine A are mainly included in this category. PSC 833 which is the most frequently used inhibitor exhibit 5–10 times more potency as compared to that of cyclosporine A [47]. On the other these inhibitors have greater affinity and inhibitory activity towards the ABC transporters and CYPA4 enzymes.

8.9 Third generation inhibitor

To overcome the problems associated with the first and second generation inhibitors, the third generation inhibitors are generally developed. The main advantage of using the third generation inhibitors are their less toxic effect as compared to that of the first two generation inhibitors and their specificity and effectiveness towards the P-gp. They are found with no pharmacological interaction. They do not possess any kind of pharmacological interaction with the chemotherapeutic agents and found to be 200 times more potent than first two generation inhibitors. Examples include Zosuquidar (LY335979), [48] Tariquidar (XR9576), Laniquidar (R101933), [49] Elacridar (F12091), ONT-093, [50] Mitotane (NSC- 38721), [51] annamycin, [52] HM30181,R10933, [53] HM30181, Biricodar. From the 3D QSAR and QSAR activities it is reported that the structure of the inhibitors are mainly responsible to produce the inhibitory activities. Studies reported that the heterocyclic ring of the tariquidar near to the antranilamide ring is responsible to produce the inhibitory activity. But the recent studies reported that tariquidar is having both substrate as well as inhibitory activity on P-gp [54, 55].

8.10 Fourth generation- natural inhibitors

Owing the toxicity issues and the restricted therapeutic caused with the synthetic inhibitors, the natural inhibitors are mainly developed which includes the dietary supplements also. The natural compounds and the food extracts are revealed with an effect on P-gp to reverse MDR and also exhibit anticancer property.

8.11 Spices

From the ancient period itself the use of the spices are predominant as preservative and colouring agents. The phytochemicals constituent in spices are studied for the cure of various ailments and for the management and reversal of MDR caused by P-gp [56].

8.12 Curcumin

Curcumin is used as anti-oxidant, anti-inflammatory agent, anti-infective and anticancer agent [57]. It also exhibits an additional use of reversing the MDR caused by P-gp. It procure this action by acting on P13K/Akt/NF-kB [58] pathway in L1210 MDR leukaemia cells in the mice model. In 2008 Choi et al came with a conclusion that the combination of curcumin and Adriamycin can overcome the MDR effect caused by P-gp by studying with the western blotting results [59]. Mucoadhesive microemulsion loaded with curcumin has the ability for brain targeting through intranasal route [60].

8.13 Piperine

Piperine is the alkaloid constituent present in a larger proportion in black pepper, which is consumed by the population all over the world and added in their diet. Piperine was found with the activity on altering the MDR by inhibiting ABC transporters [61].

8.14 Capsaicin

Capsaicin is found abundant in red chilli, exhibit anticancer and inhibitory activity on P-gp. It potentiates anticancer activity of vinblastine by modulating P-gp. It has the ability to act on β-catanin and NF-KB pathways [62].

8.15 [6]-Gingerol

Ginger is constituted with a major polychemical compound [6]-Gingerol, which add on its spicy taste. It also inhibits β-catanin and NF-KB pathways like the action exhibited by capsaicin, but the actual mechanism of action is still not known [63].

8.16 Carnosic acid

Carnosic acid is the major phenolic constituent found in the leaves of rosemary. Carcinoic acid can act as a substrate to P-gp by stimulating the ATP activity by competitively binding with the ATP binding site [64].

8.17 Procyanidine

This compound composes a major constituent in tea leaves and grape seeds. It exhibits both chemopreventive activity and also antiproliferative effect [65]. It can inhibit NF-KB and translocate YB-1 into the nucleus through dephosphorylation of ERK1/2 and AKT [66].

8.18 Limonin

The citrus fruits are constituted higher with this crystalline compound. It acts as a P-gp inhibitor in leukaemia, melanoma and colon cancer cell lines. It elevates

the accumulation of the Rhodamine 123 and doxorubicin inside the cells. When administered in a concentration of 20 μm it has the ability to increase the anticancer activity of the doxorubicin when studied with CED/ADR5000 Caco-2 and leukaemia cell lines [67].

8.19 Quercetin

Quercetin is the constituent found in higher amount in onion and apple. From the experimental report it is found that Quercetin exhibit chemotherapeutic and P-gp inhibition activity. On the addition of concentration of 0.7 μm, it enhances the anticancer activity of doxorubicin when studies in MCF-07 cell lines [68].

8.20 β- carotene

β- Carotene is abundantly found in vegetables and fruits [69, 70] and is the precursor of Vitamin A [69]. When studied with Caco-2 cell lines it has the ability to efficacy of etoposide, doxorubicin and 5-Fluorouracil and even can manage the P-gp transport activity.

8.21 Strategies to overcome MDR

Various novel approaches were established for the inhibition of MDR in the diseased cell lines, which includes biological, physical and chemical methods as well as ribonucleic acid (RNA), interference, micro RNA and Nanotechologies [71]. MicroRNAs are the undersized non-coding RNAs are normally not synchronised in the cancer cells; with the modification in the miRNA they have the ability to up regulate the MDR part. To alter the expression of P-gp various sequence of miRNAs such as miR-296, miR-27a, miR-298, miR-451 and miR-1253 were resolved, and there were evaluated in esophageal and breast cancer cell lines [72–74].

8.22 Monoclonal antibodies

In the early 1980s two monoclonal antibodies MRK-16 and MRK-17 were discovered to alter the resistance part developed by P-gp in both *in vitro* and *in vivo* studies [75]. MRK-16 was proven with their ability to inhibit the efflux of the drugs actinomycin-D and vincristine where the MRK-17 was proven with their ability to inhibit the MDR cell proliferation. The enhancement in the anticancer activity can be achieved by conjugating the monoclonal antibodies with the P-gp inhibitors. Euhertner Roninson developed a monoclonal antibody UIC2 from the mouse which has the ability to bind with the extracellular parts of the P-gp. In turn it has the ability to decrease the efflux of P-gp substrates which in turn increases the cytotoxicity of P-gp substrates [76].

8.23 Non substrate development

The MDR in cancer cells are towards a broad spectrum of anticancer drugs, hence there is a need to develop a new anticancer drug which have less predictable by the ABC transporter family proteins. Hence a new strategy is followed where the structural modification or the conjugation is made for the discovery of the new molecule which is less familiar to the P-gp as a substrate and which are structurally similar to the compounds which act as P-gp inhibitors.

8.24 MDR and nanotechnology

Nanoparticles are having a broad range of activity in the field for delivery of the anti-infective, anti-cancer and anti-inflammatory drugs. The nanoparticles are usually found in the range from 1 to 100 nm. The categories of nanoparticles include metals, solid lipid, micelles, liposome, polymers, dendrimers, and quantum dots [77–80]. The assembly of the nanoparticles are usually multilayered and the coatings of the nanoparticles are usually done to overcome the problems such as solubility, stability and specificity [81]. The issues related with the macromolecules such as low specificity, cell toxicity, high dose and cellular uptake can be limited by incorporating the drugs in the nanoparticles, even it have the ability to overcome the MDR related issues with P-gp and can enhance the therapeutic value of the parent drug [82].

8.25 Liposomes

Liposomes are extensively used for the delivery of the drugs which are impotent for the diffusion over the membrane layers. These can be modelled in phosphor lipid bi-layer and also in a micelle shapes which helps in encapsulating the soluble drugs and can hold on to their natural action. Thus nanoparticles mediate an appropriate activity in the management of MDR. For instant the activity of the Doxil encapsulated liposomal nanoparticles have the ability to manage the MDR part in the cancer cell lines [83].

8.26 Micelles

Micelles are the polymeric core-shell nanostructures with lypophilic drug core [84]. The lipophobic coating protects the lipophophilic drug from degradation and helps in its solubility. For this reason the lypophilic drugs have long circulation in blood and also mediated the P-gp efflux. The Pharmacokinetic property of the drugs such as fexofenadine could be enhanced when formulated in a self-Nano emulsifying drug delivery system by hampering the CYP450 and P-gp mechanism.

8.27 Mesoporous silica nanoparticles

Mesoporous silica nanoparticles (MSNPS) are having larger pore size and pore volume, biocompatible in nature and are having high surface area. MSNPS have the capacity to load both anticancer drugs and siRNA together at the same time [85]. This combination can alter the resistance caused by P-gp and in turn can enhance the therapeutic efficacy of the drugs [86].

8.28 Polymeric nanoparticles

Polymers employed are usually natural (Gelatine, chitosan and albumin) or synthetic (poly [D, L-lactic acid], poly [D, L-lactic acid] and poly [ε-caprolactone]) in nature. The techniques [87] used for the preparation of polymeric nanoparticles are salting out, dialysis and microemulsion, interfacial polymerisation, supercritical fluid technique and solvent evaporation. Nanoparticles of Human albumin with abraxane and paclitaxel are formulated for improving the efficacy of metastatic breast and pancreatic cancer [88, 89]. This novel formulation is approved for clinical studies by U.S. Food and drug administration.

8.29 Expression and its over-expression, advantage and drawback

The expression of the P-gp is mainly found in all parts of the body by acting as a protective shield from the entry of the toxins and the xenobiotics. Their action is unavoidable in the Blood brain barrier, blood placental barrier and blood testes barrier but when concentration on the therapeutic aspect of the drugs the absorption of the drugs through the intestinal is retarded due to the expression of the P-gp in the intestinal lumen, not only in the lumen part though their presence are predicted all over the body but their expression is more in the diseased cells mainly the cancer cells. The plasma membrane of the intestinal epithelial cells pumps back the drug which enters into it and which are recognised as the substrate and are excreted [90–92]. Higher levels are seen in the biliary epithelium, proximal tubules of the kidney and the drugs are seen in the bile and the urine. For inhibiting the role of the P-gp the P-gp inhibitors are developed. Though the inhibitors shows the action when checked in preclinical studies but their action retards when come into the clinical trials. The progress report of the inhibitors are explained in **Figure 1**. The failure in the therapeutic efficacy with the cancer treatment is mainly due to the over-expression of P-gp.

8.30 Cancer and drug resistance

In 1940s: first cancer chemotherapy trails begin.

In 1970s: Mammalian cells showed resistance to the anticancer agents recurrently exhibited cross-resistance to drugs which are structurally and functionally dissimilar.

Multidrug resistance was a foremost problem in the cancer chemotherapy because it involved resistance to some of the commonly used and the first line anticancer drugs.

In 982s: Multidrug resistance was shown in most of the cases which results in decline in the intercellular drug accumulation, apparently as a result of altering in the plasma membrane. In many multidrug resistant cell lines, the resistance was found to correlate with over expression of a 170-kDa membrane protein (P-gp) [93, 94].

Why to study Multidrug resistance?

- Important role in the cancer multidrug resistance and its pathogenesis.
- Important role in Drug pharmacokinetics (Uptake distribution and excretion).

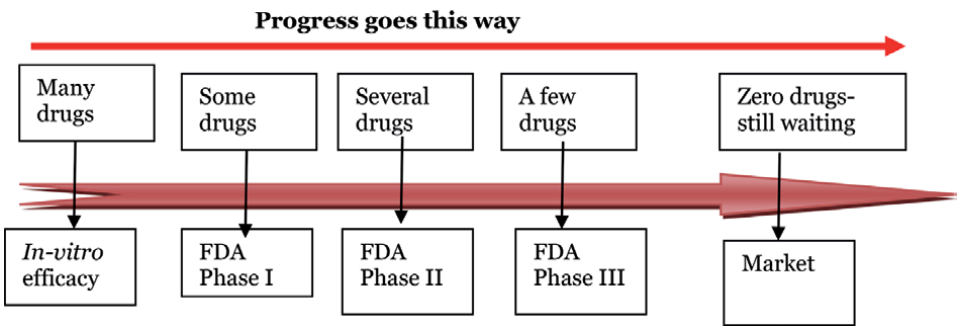

Figure 1.
P-gp inhibitor development timeline. The progress report of the inhibitors.

- Important role in removing toxins.
- Key role in development of inhibitors.
- To learn about the biology of the transport system.

9. Conclusion

P-gp is the protein belonging to the ABC family protein transporters with the aspect of protecting the cells and vital organs from the entry of the xenobiotics, toxins and drugs. The over-expression of the P-gp in the diseased cells leads to the therapeutic failure during the treatment regimen but the role of the P-gp for producing protective action in the brain and fetal cells are also unavoidable. Hence for an effective therapeutic aspect, the action of the P-gp and its role should be studied. For overcoming the unwanted action of the P-gp the inhibitors of the P-gp are mainly developed and the strategies for overcoming the MDR by using natural inhibitors and the formulation aspects and caused are mentioned in this chapter.

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Chapter 3

Urological Effects of Ketamine Abuse

John Shung-Lai Leung and Wai-Kit Ma

Abstract

Emerging evidence has shown that long-term and chronic ketamine use or abuse can lead to damages in the urinary tract, a spectrum of clinical presentations from mild irritative lower tract symptoms to painful gross haematuria and renal damages. First reported by a Hong Kong group of urologists in 2007, the phenomenon has since then been identified worldwide. Most of the ketamine abusers were adolescents and young adults, and the symptomatology resembled those of chemical cystitis or interstitial cystitis. Endoscopic features of ulcerative cystitis, radiological features of thickened and contracted bladder wall with or without obstruction to upper urinary tract, and histopathological features of inflammation and fibrosis and urothelial metaplasia changes were described. With increasing clinical experience in managing this group of patients, clinical pathways and medical and surgical treatment options have been developed. Animal studies on the effects of ketamine exposure on the urinary system have also been conducted to help us understand the underlying pathophysiology for this distinct entity.

Keywords: ketamine, cystitis, hydronephrosis, detrusor overactivity, uropathy

1. Introduction

Ketamine is listed in the WHO Essential Medicines List since 1985 as an anaesthetic and analgesic. Unlike other commonly used anaesthetic agents, ketamine does not tend to cause respiratory depression or hypotension, making it ideal for use as a general sedative and in veterinary medicine [1].

However, ketamine is also a drug of abuse. The United Nation's World Drug Report 2019 shows that ketamine has been the dominant hallucinogenic seized by authorities globally, accounting for 87% of such seizures in the past 5 years [2]. In 2017, the global quantity of ketamine seized was approximately 11,000 kilogrammes, the majority of which was in Asia [3]. Most ketamine seized, in the order of descending quantity, was reported by mainland China, followed by Taiwan, Hong Kong, Malaysia, Myanmar, Thailand, the United Kingdom, India, and the Netherlands [3]. In Taiwan, ketamine has been the most frequently abused illicit drug since 2006. The volume of seizures there grew from 916 kg in 2009 to 1187 in 2010 [4]. However, ketamine is becoming more and more popular not only in Southeast Asia but in Europe as well. The number of ketamine users in the United Kingdom grew from 85,000 in 2006 to 113,000 in 2008, becoming the fourth most popular illicit drug among UK clubbers [5]. Its popularity could be explained by its low market price among recreational drugs and also the difficulty in cracking down on its trafficking, as it is produced legally for medical use [6].

The chronic and illicit use of ketamine is associated with urinary tract damages. Structural damage to the bladder, ureters, and kidneys has been demonstrated in numerous animal and human studies. Patients usually present to the urological service with symptoms such as urinary frequency, haematuria, and dysuria. Management is multidisciplinary, as a big part of treatment success lies not only in urological interventions but also in successful abstinence.

2. Epidemiology of ketamine-associated cystitis

The exact prevalence of ketamine-associated cystitis is difficult to ascertain, as most users are reluctant to seek medical attention despite symptoms. A study in Taiwan conducted in 2019 by Li et al. reported that whilst 84% of chronic ketamine abusers demonstrated urinary tract symptoms, only 48% sought treatment [7]. A survey involving 3806 participants in the United Kingdom by Winstock et al. found that 26.6% of ketamine users report urinary symptoms and that the symptoms are significantly related to both frequency and duration of use [8]. Similarly, Pal et al. from the United States conducted a survey involving 18,802 participants which reported a 30% prevalence of lower urinary tract symptoms (LUTS) among recent ketamine users [9].

Lower urinary tract symptoms, as well as dysuria and haematuria, are the most common symptoms caused by chronic ketamine abuse. LUTS in the setting of ketamine cystitis usually comprises urinary frequency, feeling of incomplete bladder emptying, and nocturia. More than 50% of users complain of urinary frequency after using ketamine for about 2 years [7]. The severity and number of symptoms are correlated with not only the duration of use but also the route of administration. Ketamine may be cut up into a powder form before being inhaled or smoked with pipe-like devices. Snorting causes significantly more symptoms than smoking. This is possibly due to a higher amount of ketamine entering the circulation via the nasal mucosa [7].

The combined use of ketamine with other substances such as marijuana and 3,4-methylenedioxy-methamphetamine (MDMA) has also been found to significantly increase the severity of LUTS. Marijuana enhances the expression of cannabinoid receptors CB1 and CB2, which are found in the human bladder urothelium [10]. This is implicated in the worsening of storage symptoms such as frequency and urgency. The mechanism through which MDMA exacerbates LUTS remains to be elucidated.

3. Pathogenesis of ketamine cystitis

A number of mechanisms have been proposed to explain the pathogenic effects of ketamine on the urinary system. These include (1) direct toxicity of ketamine or its metabolites on the bladder tissues; (2) microvascular changes in the bladder and kidneys by ketamine or its metabolites; and (3) delayed (type IV) hypersensitivity against the urothelium due to ketamine or its metabolites [11]. Infection is unlikely to play a role in the primary pathogenesis of ketamine cystitis, as the vast majority of patients do not have a positive urine bacterial culture. As of yet, there has not been a single conclusive theory on the mechanism of ketamine-induced cystitis.

In vitro studies on human urothelial cells have demonstrated dose-dependent toxicity of ketamine to human urothelial cells. The damage is carried out by both ketamine itself and its primary metabolite (norketamine) [12]. Norketamine is generated as ketamine undergoes hepatic metabolism. Both ketamine and norketamine are subsequently excreted in the urine. Ketamine and norketamine are equally toxic to the urothelium in in vitro studies, but norketamine remains in the urine for longer than ketamine, and hence norketamine may be accountable for more of the damage

done [13]. As with other toxic exposures, daily exposure has been found to be more damaging than a one-off exposure. The accepted anaesthetic dosage of ketamine for human medical use is 0.5–2 mg/kg, but much higher concentrations are abused in recreational use (up to 20 g per day in some users) [13]. As aforementioned in the previous section, it also takes approximately 2 years of abuse before cystitis symptoms arise. Therefore, as ketamine is used at much lower doses as well as frequency in the context of anaesthesia as compared to daily abuse, the medical use of ketamine for one-off anaesthesia is less likely to cause significant ketamine cystitis.

The hypothesis that ketamine and norketamine exert a direct effect on the urothelium is based on the knowledge that both chemicals are excreted by the urine and have a long contact time with the urothelium (ketamine 5 days, norketamine 6 days) after ingestion [14].

The urinary tract from the renal pelvis to the proximal urethra is covered by the urothelium, a highly specialised transitional epithelium capable of stretching to accommodate various degrees of distension in response to urine volume. The urothelium comprises three layers—superficial, intermediate, and basal. Under the urothelium lies the submucosa, then the detrusor muscle, and then the adventitia.

Classic histological changes found in ketamine cystitis include denudation of the urothelium, as well as inflammatory changes including oedematous vessels, and infiltration by eosinophils and T-lymphocytes [15]. The affected urothelium loses its superficial layer (which provides a barrier function), thus exposing the stroma to further insults from urinary ketamine and norketamine. This may be one of the mechanisms by which ketamine causes cystitis and the resultant symptoms. Some of these histological changes are similarly seen in interstitial cystitis (chronic bladder pain in the absence of an identifiable aetiology) [16]. Additionally, haematoxylin and eosin staining in the urothelium affected by ketamine cystitis may in some cases display apparent dysplastic changes with the loss of epithelial cohesion. Such changes mimic the histology of carcinoma in situ, and hence the clinical history of ketamine abuse should alert the clinician or pathologist to the possibility of misdiagnosis of carcinoma in situ [12].

The infiltration by T-lymphocytes suggests that a delayed (type IV) hypersensitivity reaction to ketamine may also play a role in the pathogenesis of ketamine cystitis [17]. This is because T-lymphocytes are heavily implicated in type IV hypersensitivity reaction, and it is known that type IV hypersensitivity reactions occur only after prolonged exposure to the causative agent. This reaction conforms to the temporal profile of the development of ketamine cystitis, where symptoms usually develop only after 2 years of abuse.

4. Clinical presentation

The irritative effects of ketamine on the urinary system, especially the bladder, produce myriad symptoms. These include:

- Urinary frequency
- Feeling of incomplete bladder emptying
- Nocturia
- Urinary urgency
- Urge incontinence

- Haematuria
- Suprapubic pain or 'bladder pain'

The typical complaint from the affected patients is 'painful, small voids', closely mimicking that of interstitial cystitis. These symptoms typically develop after 2 years of ketamine abuse. A study in Hong Kong by Ng et al. has demonstrated the relative prevalence of symptoms as follows: urgency (92%), frequency (84%), nocturia (88%), dysuria (86%), and haematuria (68%) [18]. The most bothersome symptoms reported by users are typically urinary frequency, nocturia, and urgency. This is because of the need of frequency visits to the bathroom, which interferes significantly with their daily activities [7].

The clinician may evaluate symptoms using standardised methods such as frequency-voiding charts (also known as a 'bladder diary') and questionnaires such as the Pelvic Pain and Urgency/Frequency (PUF). A frequency-voiding chart involves the patient recording the volume of every fluid intake and void and also instances and degrees of urge incontinence, if any. Reviewing a frequency-voiding chart allows the patient to communicate effectively with the clinician the frequency and nocturia experienced. Ketamine cystitis typically produces a low-compliance bladder, manifesting as frequency, low-volume voids. Urge incontinence is the sudden and compelling desire to pass urine that is difficult to defer and is accompanied by involuntary leakage.

The Pelvic Pain and Urgency/Frequency questionnaire is a symptom score questionnaire developed and validated for the diagnosis of interstitial cystitis [19]. As mentioned, interstitial cystitis produces symptoms and histological changes in the bladder akin to those found in ketamine cystitis, and studies have validated the use of this questionnaire to score patients experiencing symptoms of ketamine cystitis [18]. The questionnaire includes eight questions evaluating daytime frequency, nocturia, pelvic pain, urinary urgency, the degree to which these symptoms bother the patient, and sexual function. PUF generates a symptom score and bother score, which total at 35. In a patient with history of significant ketamine abuse, a score of $\geq$15 indicates the presence of significant cystitis symptoms, thus leading to the diagnosis of ketamine cystitis. The PUF is a useful tool not only for the diagnosis of ketamine cystitis but also for symptom quantification so that its severity and response to treatment could be monitored over time.

5. Clinical investigation findings

Cystoscopy, computed tomography (CT), ultrasonography, and pyelography are examples of investigations that may demonstrate the structural damage implicated in ketamine cystitis [20]. Cystoscopy reveals inflammatory changes such as telangiectasia (indicative of neovascularisation), ulceration, or even petechial haemorrhage in severe cases. Biopsies of the affected bladder urothelium will reveal histological changes mentioned earlier in the chapter, including denuded epithelium and infiltration by eosinophils and lymphocytes. Computed tomography may show bladder wall thickening and peri-vesical stranding, both of which are indicative of chronic inflammation of the bladder wall (**Figure 1**). Upper tract damage usually manifests itself as unilateral or bilateral hydronephrosis, with ureteric wall thickening, or luminal narrowing and strictures. CT, pyelography, and ultrasound are all suitable modalities to demonstrate hydronephrosis (**Figures 2–4**). CT and pyelography have the additional benefit of evaluating the exact level of ureteric stricturing.

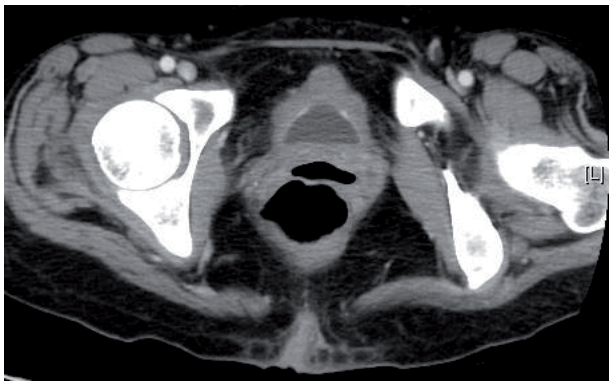

Figure 1.
Contrast CT scan image showing a thickened and contracted bladder in a patient with a 7-year history of ketamine abuse.

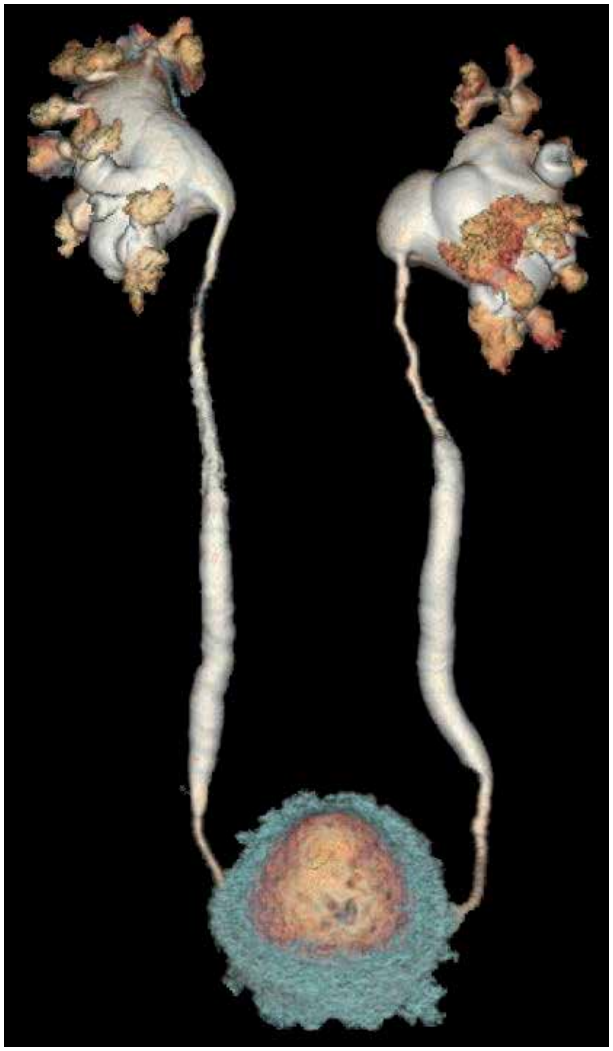

Figure 2.
Reconstructed contrast CT urogram showing bilateral hydronephrosis and hydroureter down to the level of the vesicoureteric junctions. The bladder also appears small with generalised wall thickening. This patient has an 8-year history of ketamine abuse.

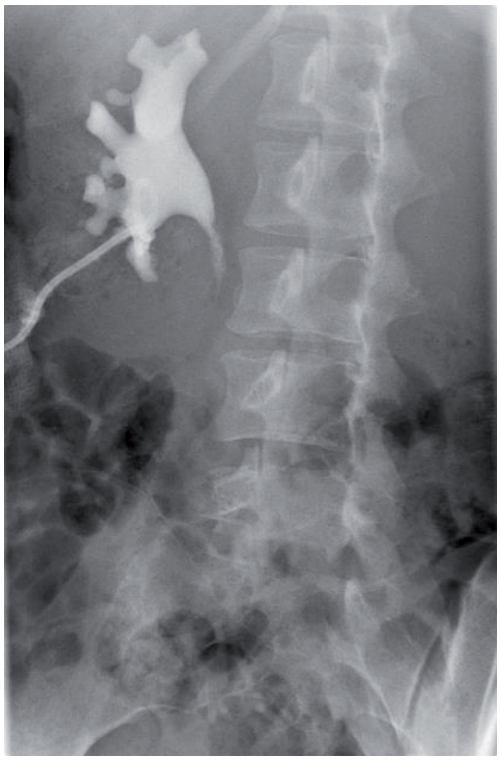

Figure 3.
This is an antegrade pyelogram of a patient suffering from ketamine cystitis. Contrast is injected through the percutaneous nephrostomy. There is hydronephrosis and a contrast upholding at the level of the L3 vertebra. This is suggestive of a ureteric stricture at that level causing hydronephrosis.

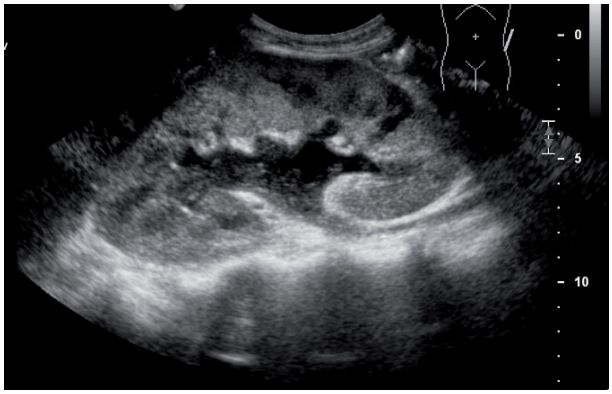

Figure 4.
Ultrasound image of the left kidney of a patient with ketamine cystitis complicated by acute left pyelonephritis. This patient had a background of ketamine cystitis with bilateral hydronephrosis. She presented acutely with left loin pain and fever. The ultrasound image shows debris in the chronically dilated renal pelvis. This is compatible with acute pyelonephritis complicating ketamine cystitis. A combination of chronic obstruction and vesicoureteral reflux has likely contributed to the development of upper tract infection.

Apart from assessing the degree of structural damage, the functional capacity of the urinary system should also be assessed. Urodynamic studies, such as video cystometrogram, reveal reduced bladder capacities, reduced bladder compliance, and sometimes detrusor overactivity even at low bladder volumes. Bladder capacities of

ketamine cystitis patients are typically <150 ml, and detrusor overactivity has been shown to be evident at bladder volumes as low as 14 ml [21]. This means that such patients will not only complain of very frequent but small voids, they are also likely to experience urge incontinence. One can see how disabling such symptoms are from these investigation findings (**Figures 5**–**7**).

Renal impairment can be reflected from raised serum creatinine or impaired creatinine clearance and estimated glomerular filtration rate. Renal impairment may stem from vesicoureteral reflux (VUR) due to chronic reduction in bladder

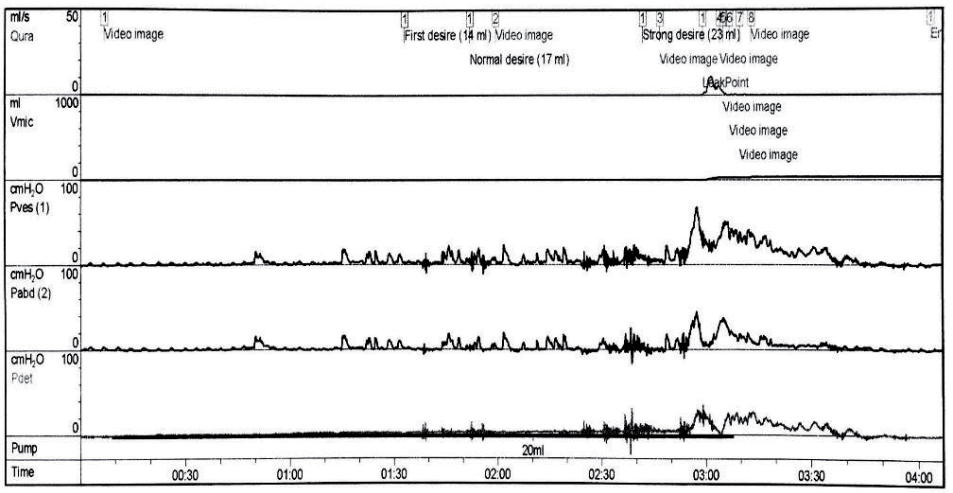

Figure 5.
Cystometrogram (filling phase) of a patient with a 10-year history of ketamine abuse. First desire to void was recorded at 14 ml of bladder filling. Also note the multiple spikes at the lowermost tracing indicative of detrusor overactivity. (Pves, intravesical pressure; Pabd, intra-abdominal pressure; Pdet, subtracted detrusor pressure of Pves–Pabd).

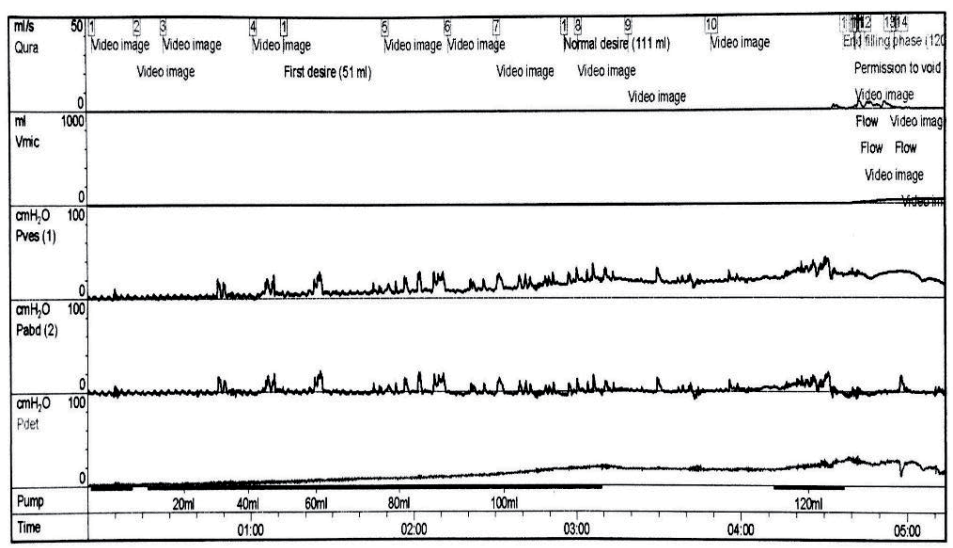

Figure 6.
Cystometrogram (filling phase) of the same patient after 3 years of abstinence. First desire to void at 51 ml. Note the difference in the scale of the x-axis denoting volume. The detrusor overactivity has also dampened, as shown by the smoother Pdet tracing.

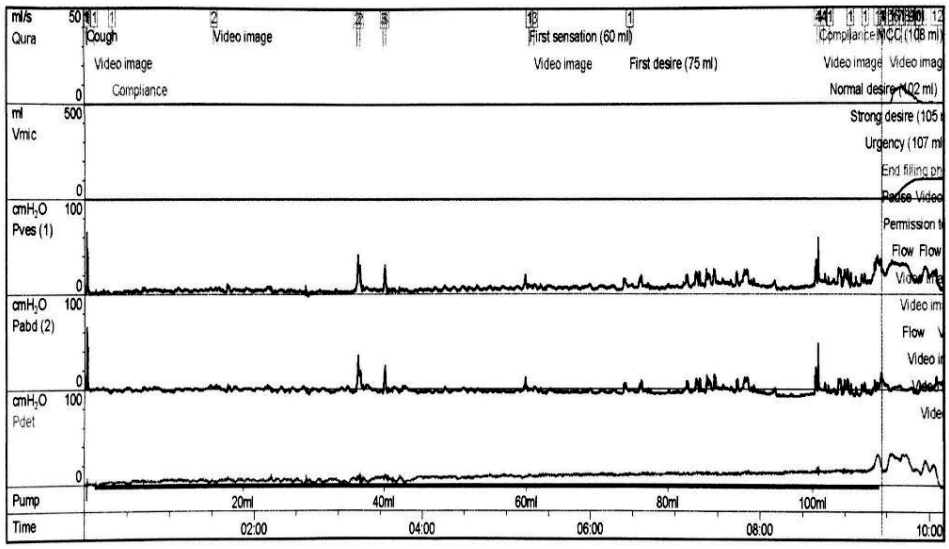

Figure 7.
Cystometrogram (filling phase) of the same patient after 8 years of abstinence. First desire to void at 75 ml. Much improved bladder compliance as shown by the relatively smooth Pdet tracing.

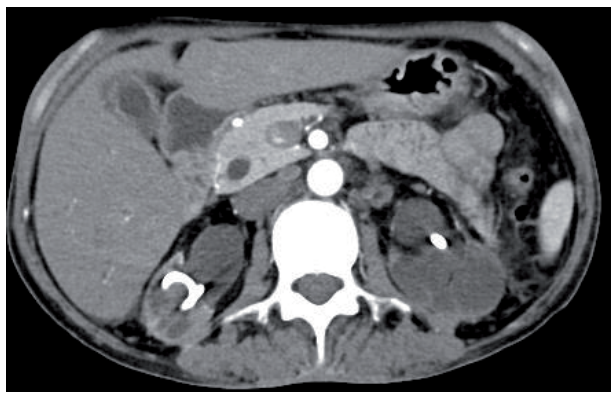

Figure 8.
Contrast CT scan image of a patient with more than 3 years of ketamine abuse, showing bilateral atrophic kidneys and hydronephrosis. This patient required bilateral percutaneous nephrostomies (also seen on this image) for upper tract urinary diversion.

compliance. VUR can be demonstrated on video cystometrogram as a reflux of contrast material from the bladder up to the ureters. VUR predisposes the upper tract from urinary tract infections, increasing the risk of recurrent pyelonephritis and resultant renal scarring (**Figure 2**). Hydronephrosis as a result of ureteric narrowing is also a cause of renal impairment in these patients. Ureteric narrowing is likely secondary to urinary ketamine and its metabolites causing transmural inflammation and swelling or even fibrosis and strictures (**Figure 8**).

Papillary necrosis may be seen on renal ultrasound or on contrast studies such as an intravenous urogram or computed tomography [11]. The contrast material fills necrotic cavities located in the renal papillae. Sometimes, sloughed necrotic material may pass into the ureter, causing obstruction, and appear as a filling defect.

6. Management

6.1 Abstinence and a multidisciplinary approach

The management of ketamine cystitis aims at abating the debilitating urinary symptoms and preventing further damage to the urinary tract. The most important components of the management plan therefore lie in early diagnosis and early abstinence, as this aims to effectively remove ketamine and its metabolites from the urinary system before irreparable damage to the urinary system sets in. A large-scale study involving more than 1000 ketamine users reported that up to 50% of users report symptomatic improvement after cessation of use. Urinary frequency has been shown to be the first symptom to improve [8]. That said, as with any detoxification program, psychosocial challenges pose a big barrier to long-term abstinence. It is therefore imperative that the clinician solicits help from relevant parties such as social workers, clinical psychologists, or even psychiatrists to form a multidisciplinary approach in managing these patients [22]. This process involves first identifying those suffering from ketamine cystitis, then explaining the relationship between ketamine use and cystitis, and finally embarking on the detoxification journey. As mentioned earlier in the chapter, the PUF scale serves as a standardised and validated means of identifying ketamine users suffering from cystitis. Success in multidisciplinary management has been demonstrated by outreach teams comprising urologists, psychiatrists, social workers, and nurses in Hong Kong [23].

6.2 Oral agents

Although the precise mechanism of injury in ketamine cystitis has yet to be elucidated, it is clear that it involves inflammation of the urothelium akin to that of interstitial cystitis. Medications that aim to reduce inflammation, such as nonsteroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids, have thus been studied in the treatment of ketamine cystitis symptoms. Other treatment regimens involving the use of antibiotics, anti-muscarinic agents, and beta-3 agonists have also been examined. However, the results from the medication therapy have been suboptimal overall [24].

Medication therapy may not result in significant improvements in LUTS for these patients, but analgesics should still be employed generously. This is because ketamine itself possesses analgesic properties, and therefore abstinence after long periods of abuse may produce pain akin to a withdrawal effect. Analgesics such as paracetamol, phenazopyridine, or even narcotic analgesics such as tramadol may be used on top of NSAIDs in high doses as pain relief during the initial period of detoxification [25].

6.3 Intravesical therapies

Ketamine and its metabolites cause denudation of the urothelium, exposing the underlying submucosa and stroma of the bladder wall to further toxic damage. This produces the typical LUTS as well as the structural changes such as wall thickening and reduction in compliance. This has prompted investigations into the effectiveness of intravesical therapies that aim to restore the integrity of the urothelium so that the underlying tissue may no longer be exposed to the toxicities of ketamine and its metabolites. Intravesical instillation of a glycosaminoglycan, such as hyaluronic acid or chondroitin sulphate, has been proposed to reconstitute the barrier

function provided by the urothelium and enhance healing. Reports of significant reductions in symptoms in patients treated with weekly intravesical instillations of hyaluronic acid or chondroitin sulphate have been published recently [26]. These patients not only reported a reduction of LUTS, but follow-up cystoscopy with biopsies showed decreased inflammatory cell infiltration, less inflammatory hypervascularity, as well as regeneration of the urothelium [27].

Cystoscopic injection of botulinum toxin into the bladder wall, followed by hydrodistension, is another intravesical treatment that has been shown to relieve symptoms of ketamine cystitis [28]. Botulinum toxin type A inhibits the presynaptic release of neurotransmitters such as acetylcholine, thus inactivating neuromuscular junctions and reducing detrusor activity. The patient is typically put under spinal anaesthesia, and a cystoscope is then advanced into the bladder. 20 ml of botulinum toxin type A at a concentration of 200 IU in 20 ml is then injected into 40 points in the bladder wall. There is currently no standard protocol for the technique of hydrodistension, but authors have performed it by filling the bladder with saline under a pressure of 80 cmH2O, at a volume of 150–200 ml, for a duration of 5 minutes [29].

6.4 Surgical therapies

The bladder in a patient with severe ketamine cystitis is thickened and fibrotic and has poor compliance. Apart from severe LUTS, these changes may also cause vesicoureteral reflux and upper tract damage. Such patients are at risk of chronic renal failure. Surgical treatment in the form of augmentation cystoplasty is therefore an option to increase the capacity and compliance of the bladder, so that symptomatic improvement and upper tract protection could be brought about through a single procedure. Techniques vary, but an option is to use a 25 cm segment of the ileum and sew it to a surgically created clam-like opening of the bladder in order to augment its volume and compliance [30]. Contraindications to augmentation cystoplasty using bowel include any condition that renders the bowel abnormal at the baseline, for example, inflammatory bowel disease (Crohn's disease, ulcerative colitis) and previous gut resection (such that further resection may predispose the patient to malabsorption or even short gut syndrome). Another alternative is to use a portion of the stomach, termed gastrocystoplasty. This has its own issues, as the hydrochloric acid produced by the stomach mucosa may cause haematuria-dysuria syndrome, peptic ulceration in the bladder, and alkalosis. Complications include a mortality rate of up to 2.7%, small bowel obstruction, fistulation, and renal failure (due to the reabsorption of urinary waste through the bowel segment). Some patients may furthermore require clean intermittent catheterisation to more effectively empty the bladder. Patient selection is paramount when considering augmentation cystoplasty for ketamine cystitis patients. Failure of abstinence after surgery results in rapid reabsorption of ketamine from the urine through the bowel segment. Ketamine and its metabolites are hence recirculated, excreted in the urine again, and once again exerting their toxic effects on the urothelium. Augmentation cystoplasty with bowel may therefore even accelerate upper tract damage should the patient fail to abstain from ketamine postoperatively. The patient should also be willing to comply with clean intermittent self-catheterisation should it be required [30].

An alternative surgical strategy is an ileal conduit [31]. This involves brining both the ureters to an opening in the abdominal wall through a surgically created segment of the ileum. This obviates the need for clean intermittent catheterisation and offers quicker postoperative recovery. However, as this is an incontinent type of urinary diversion, the patient would have to live with a lifelong urostomy bag.

6.5 Upper tract protection

Some patients present with bilateral hydronephrosis with or without impairment of renal function. This could be due to vesicoureteral reflux or ureteric strictures. As most patients with ketamine cystitis are young, it is of paramount importance that their upper tract is protected to prevent chronic renal disease. Methods to achieve this include percutaneous nephrostomy and ureteral stenting. Percutaneous nephrostomy involves placing a plastic tube through the skin into the renal pelvis so that the urine produced by the kidney may drain through the tube into an external bag instead of being trapped in the obstructed system. The drainage of the urine through nephrostomy tubes into an external bag also reduces the LUTS from ketamine cystitis, as there is significantly less urine entering the bladder. Disadvantages include inconvenience, as well as nephrostomy tube-related

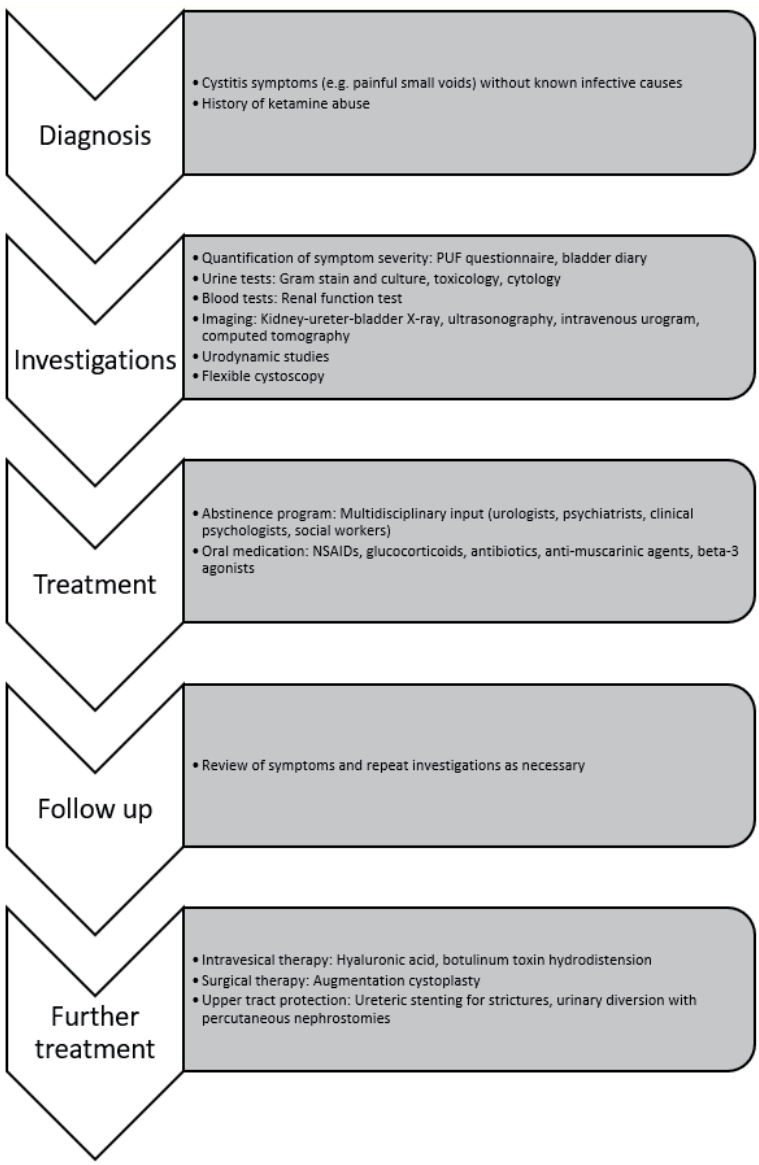

Figure 9.
Clinical pathway for the management of ketamine cystitis (adapted from Ma et al.) [22].

complications such as frequent dislodgement, and blockage. The inconvenience associated with the use of a nephrostomy tube is due not only to the presence of the tube exiting the loin but also to the bag to which it is connected. Another way of ensuring upper tract drainage is by retrograde stenting [32]. Double J stents can be inserted via a cystoscope to ensure ureteric patency. This method obviates the need for external tubes and bags, but as urine is allowed to flow into the bladder, LUTS may persist. Additionally, some patients may also suffer from stent symptoms, which include LUTS due to the stent tips in the bladder irritating the urothelium.

6.6 Clinical pathway

Urologists in Hong Kong such as Ma et al. have established a clinical pathway in order to guide and standardise the management of ketamine cystitis [22]. Patients going through such a clinical pathway will receive a full workup of the extent of their ketamine cystitis and complications and receive treatment accordingly (**Figure 9**).

7. Challenges

The treatment of ketamine cystitis revolves heavily around abstinence. However, addiction and withdrawal symptoms, as well as the socioeconomic factors that contribute to the persistence of ketamine abuse, are not the only factors that hamper successful abstinence.

Abstinence from ketamine in the presence of ketamine cystitis is made more difficult by bladder pain and dysuria. As ketamine exhibits analgesic effects, it paradoxically suppresses the bladder pain and dysuria caused by ketamine cystitis. Subsequently, the cessation of ketamine use will unmask more intense cystitis symptoms. If such symptoms are inadequately controlled by more effective analgesics, the patient may be driven to use ketamine as a means to control the cystitis symptoms. Such a pattern of abstinence, failure of symptomatic control, and relapse creates a vicious cycle. It is therefore important to prescribe the patient with adequate analgesia according to the analgesic ladder to effectively suppress bladder pain and dysuria. The flip side of this is that the patient may in turn become dependent on the prescribed analgesics, especially if opioids are used [25].

Failure of abstinence in patients who have received surgical treatment such as augmentation cystoplasty may prove to be detrimental. As mentioned in the Management section, the reabsorption of ketamine and its urinary metabolites via the bowel segment used for augmentation cystoplasty may accelerate damage to the upper urinary tract, making the surgical treatment counterproductive. Correct patient selection for surgical treatment weighs heavily upon the urologist [31].

Upper tract protection by means of bilateral percutaneous nephrostomies (PCNs) may be the last resort for patients with identifiable hydronephrosis and impaired renal function [33]. However, as most ketamine cystitis patients are young and ambulatory, bilateral PCNs prove to be a cumbersome and a general nuisance. Not only are the nephrostomy tubes and bags inconvenient to live with, they also come with issues such as dislodgement or tube blockage. Tube-related issues may require hospitalisation for the revision of the nephrostomies, which adds not only to patient dissatisfaction but also to overall healthcare costs. With such inconvenience, the patient may be deterred from complying with having bilateral PCNs and in turn exposes himself to risks of chronic kidney disease and eventual dialysis dependence. Dialysis dependence in this age group makes the employment difficult, which then contributes to a lack of socioeconomic support and again makes abstinence a challenge.

8. Conclusions

Long-term ketamine abuse leads to the development of ketamine cystitis. Symptoms are debilitating and interfere significantly with the patient's daily activities. Furthermore, the upper tract may also suffer from irreversible damage, such as ureteric stricturing and finally chronic renal failure. Management of ketamine cystitis starts with its identification. This could be achieved using standardised symptom score questionnaires in known abusers of ketamine. Investigations such as blood tests, computed tomography, and cystometrogram are useful to characterise and delineate the extent of ketamine cystitis and its sequelae. The cornerstone of effective treatment is abstinence. This is done via a multidisciplinary approach involving urologists, psychiatrists, social workers, and other relevant disciplines. Intravesical therapies, such as hyaluronic acid instillation and botulinum toxin injection, are emerging options that have shown promising results. Upper tract protection in the form of long-term percutaneous nephrostomies may save the patient from suffering from chronic renal failure.

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Section 2

Nephrology and Renal Physiology

Chapter 4

Serum Creatinine, Muscle Mass, and Nutritional Status in Intensive Care

Gianlorenzo Golino, Vinicio Danzi and Silvia De Rosa

Abstract

Skeletal muscle places a vital role in regulating immune function, glucose disposer, protein synthesis, and mobility. This massive dynamic reservoir of proteins, minerals, and other metabolites could be cannibalized, and a loss of skeletal muscle may predispose impaired tissue hailing and few poor immune functions. Several studies had shown the reduced survival rates and the increased hospital lengths of stay of patients who have a poor nutrition status and low muscle mass. In addition, few studies have demonstrated the effect of muscle wasting on serum creatinine. There are no data available regarding its effect on serum creatinine, and moreover, ICU-acquired myopathy is rarely recognized because of insufficient diagnostic criteria or methodological limitations. Despite these limits, serum creatinine is still considered the standard for assessing acute changes in renal function. The present chapter details the existing evidence related to the effects of nutritional status and muscle wasting on serum creatinine based on recent evidences.

Keywords: serum creatinine, muscle mass, muscle wasting, nutritional status

1. Introduction

Skeletal muscle places a vital role in regulating immune function, glucose disposer, protein synthesis, and mobility. Unfortunately, critical illness is characterized by hypermetabolic and hypercatabolic states, which leads to an elevated resting energy expenditure rate, hyperglycaemia, altered substrate use, and increased oxygen consumption.

Among the patients who are previously well nourished before intensive care unit (ICU) admission, nutritional disorders develop rapidly because of the metabolic demands of illness, rapid fluid shifts, and the loss of specific vitamins and trace elements. Timely initiation of optimal nutritional support is important to slow the catabolic process and minimize adverse events such as prolonged mechanical ventilation, longer ICU stay, and increased risk of death [1]. The body's reaction to the illness (trauma, burns, inflammation, or surgery) includes an increase of energetic metabolism, hypersecretion of counter-regulatory hormones (glucagon, glucocorticoids, and catecholamines), and release of inflammatory mediators and other hormonal mediators (vasopressin) in the general setting of inflammation. Protein energy malnutrition is associated with muscle weakness, increased risk of infections, impaired wound healing, impaired coagulation

capacity, impaired gut function, reduced respiratory muscle function, and prolonged time to convalescence [2]. The present chapter details the existing evidence related to the effects of nutritional status and muscle wasting on serum creatinine (sCr) based on recent evidences.

1.1 Protein-energy nutritional status in intensive care

The assessment of caloric and protein requirements include clinical history, nutritional history, physical examination, laboratory test (like albumin, prealbumin, blood glucose, transferrin, and kidney and liver function), severity of the illness, body mass index (BMI), ideal body weight (IBW), resting energy expenditure (based on calorimetry), and protein requirements (based on nitrogen balance). The nutritional assessment could be performed by direct calorimetry, performed by placing the patient in a calorimetric chamber, thermally insulated, in order to be able to evaluate the heat that it gives off by radiation, convection, conduction, and evaporation; this heat is detected by a water-cooled heat exchanger. Unfortunately, this method cannot be applied to all hospitalized patients. In critically ill, the gold standard is represented by indirect calorimetry, a method that measures respiratory gases: the oxygen of a determined volume of inspired air and the carbon dioxide produced. Therefore, numerous equations have been developed with the measurements performed with the indirect calorimetry in mechanically ventilated patients (**Table 1**).

Such equations use dynamic physiological variables, which allow the recalculation of energy expenditure, in order to evaluate how much energy the body spends in the acute phase, and then, determine the minimum requests. Predictive equations are notoriously inaccurate for individual critically ill patients, due to large differences in disease-related metabolic rate, treatment, and interindividual factors. Many centers that do not have indirect calorimetry adopt a simple approach providing 25–30 kcal/kg/day. Guidelines of the European Society for Clinical Nutrition and Metabolism recommend an intake of 25 kcal/kg/day in critically ill patients, and for both females than for males, considering, however, a 10–20% increase in patients with SIRS and overweight (BMI > 25), considering the IBW for calculation of energetic requirements [3].

Formula	Energetic requirements predicted (kcal/day)	Mechanical ventilation
Harris-Benedict	Man: 66 + (13.7 × BW) + (5 × H) – (6.8 × Age) Woman: 65 + (9.6 × BW) + (1.7 × H) – (4.7 × Age)	No
Ireton-Jones (a)	Mechanical Ventilation: 1925 – (10 × Age) + (5 × BW) + (281 × Sex) + (292 × Trauma) + (851 × Burn) Spontaneous Breath: 629 – (11 × Age) + (25 × BW) + (689 × Obesity)	Yes
Frankenfield (b)	21,000 + (100 × RR) + (13 × Hb) + (300 × Sepsis)	Yes
Swinamer	(945 × BSA) - (6.4 × Age) + (108 × BT) + (24.2 × RR) + (817 × TV) - 4349	Yes
Faisy	(8 × BW) + (15 × H) + (32 × RR) + (94 × BT) – 4834	Yes

BSA, Body surface area; BW, body weight; H, height; Hb, hemoglobin; RR, respiratory rate; BT, body temperature; and TV, tidal volume; (a) Sex, 1 = man, 0 = female; Trauma, 1 = present, 0 = absent; Burn, 1 = present, 0 = absent; and Obesity, 1 = present, 0 = absent. (b) Sepsis, 1 = present, 0 = absent, based on clinical evidence of presumed infection, systemic inflammation, or organ dysfunction.

Table 1.
Predictive equations of energetic requirements (kcal/die) in critically ill patients.

1.2 Muscle mass

Skeletal muscle has emerged as a potent regulator of immune system function in regulating immune function, glucose disposal, protein synthesis, and mobility [4]. Skeletal muscle can be viewed as the dynamic storage depot of amino acid, which is sensitive to the fed and fasted states but also of minerals and other intermediate metabolites [5], which can be depleted to meet the need for other tissues involved in the inflammatory response. The loss of skeletal muscle and reduced protein storage may predispose to a relative glutamine deficiency, which is seen as impaired tissue healing, poor immune function, and reduced survival [5]. Significant changes in body composition occur with aging and are a consequence of imbalances between energy intake and needs associated with an increasingly sedentary lifestyle [6].

1.3 Lean body mass

The body composition is often divided into fat mass and lean mass, the latter also known as lean body mass (LBM). LBM, unlike the fat mass that stores energy in the form of adipose tissue, includes muscle and visceral proteins and is mainly composed of water, proteins, glycogen, and minerals.

In pathological conditions such as chronic kidney disease (CKD), which is characterized by the presence of a positive water balance, it is necessary to perform an assessment of the volume of body water separately from the other components of the LBM. About half of LBM is made up of skeletal muscle mass, so the LBM compartment can be defined as heterogeneous and influenced by fluctuations in the distribution of water and electrolytes, which are more dynamic in nature in patients undergoing renal replacement therapy. Recent studies suggest that greater muscle mass is associated with greater longevity in people with chronic renal failure and in other chronic disease states [7]. Specific to LBM in the ICU, critically ill patients suffer significant LBM loss, much of it in the first 7–10 days of ICU stay [8]. However, patients gain weight back following ICU stay as fat mass but not as functional LBM [9]. Data in literature demonstrate that the catabolic/hypermetabolic state following injury can persist for up to 2 years following discharge from hospital, and this can markedly hinder the recovery of patients' LBM and function following injury [9].

1.4 Serum creatinine

sCr is an endogenous substance generated by the nonenzymatic conversion of creatine and creatine phosphate, 95% of which is found in the muscles [10]. sCr is an uncharged, small molecular weight, unfilled substance (113 Da) which is not related to whey protein. It is filtered freely by the glomerulus without tubular resorption. sCr is also secreted by the kidney tubules only in small quantities. In clinical practice, levels of sCr are used to determine kidney function to estimate the glomerular filtration rate. Its rise usually indicates either acute kidney injury (AKI) or chronic kidney disease [11, 12]. Due to the correlation between sCr levels and muscle mass, sCr in the steady state has been used as a surrogate of muscle mass measurements [13].

Low sCr levels could be considered as a proxy of protein-energy wasting in some clinical situations [14]. Individuals' sCr levels can be influenced by diet. In fact, arginine and glycine are precursors of creatine, and for this reason, a low protein intake in the diet can limit the generation of sCr. The sCr levels can be considerably lowered in the presence of protein malnutrition. Factors associated with low sCr levels are low muscle mass (female gender, elderly, and chronic illness), malnutrition, vegetarian diet, pregnancy, advanced liver disease, fluid overload, and augmented renal clearance.

AKI is an event that commonly complicates the clinical course of critically ill patients, contributing to multi-organ failure and requiring appropriate nutritional interventions in a strategic treatment.

The metabolic and nutritional demands of AKI patients are affected not only from the uremic state but also from the underlying pathology and complications associated.

A personalized approach for each patient that involves an analysis of specific nutritional requirements for each patient and a consideration of renal replacement therapy (RRT) support used is therefore necessary to improve the outcome of these patients.

Nitrogen is a fundamental component of the amino acids that make up the molecular structure of proteins. Proteins are the major functional substrate for cells and tissues and are essential for body growth and also for the maintenance and recovery. Protein metabolism generates calories (about 4 kcal/g). Nitrogen is released from protein degradation, which is also lost from secretions or excreted in sweat, feces, and urine. In particular, urea nitrogen represents 85–90% of the urinary nitrogen loss.

In the ICU patient, the greater non-urinary loss occurs through the intestine, severe burns, RRT, and/or by abdominal drains. The nitrogen balance becomes negative (from −5 to −30 g day), reflecting the important protein catabolism.

The nitrogen balance is calculated as the difference between the nitrogen intake and output, according to the following equation:

$$\text{N balance} = \left[\text{protein intake}(\text{g / die}) / 6.25\right] - \left[\text{urinary nitrogen}\,(\text{g / die}) + \text{skin / stool losses}\right]$$

where skin/fecal losses are approximately 2–4 g per day, while urinary losses can be recorded in the urine for 24 h (or by sampling for at least 4 h). The equivalence between urea in mmol/l and g occurs via two parameters: urea (g) = urea (mmol)/20.36, and then through the fact that 6.25 g of protein contains 1 g of nitrogen (**Table 2**).

In the setting of inflammatory state, acute loss of kidney homoeostatic function plays a central role in the worsening of the dysmetabolic state of the condition of critical illness. The stress response also induces changes in the use of substrates:

- Cellular insulin resistance acquired and secondary to the reduction of translocation of GLUT4 transporters on the plasma membrane, contributing to hyperglycaemia and alteration of cellular energy

	Non-catabolic state	**Catabolic state**
Proteins (g/kg/day)	0.8–1.0 (KDIGO 2012)	Minimum 1.0 (expert opinion)
Energy	Energetic support is not influenced by AKI. Some authors suggested 20–30 kcal/kg/day (KDIGO 2012). Others suggested 25–30 kcal/kg/day (Cano 2009, Brown 2010, Gervisio 2011, McClave 2016)	
Fluids	Fluid balance and daily body weight must be monitored carefully. Fluid intake varies according with patient's critical state, body weight, and fluid balance	
Electrolytes	Electrolytes should be monitored frequently and corrections vary according to the critical state and type of treatment	
Micronutrients	Evidences in this regard are scarce and not well documented. Usually, the levels of fat-soluble vitamins (Vit. A, Vit. D, Vit. E, Vit. K, and Vit. F) are low. CRRT has a negative effect on the balance of some vitamins and trace elements. It is not known whether micronutrient supplementation improves results	

Table 2.
Nutritional support in AKI patients.

- Increased use of fatty acids as the use of glucose becomes inefficient
- Switch from protein anabolism to catabolism (net negative nitrogen balance)

2. Assessment of muscle mass and nutritional status in intensive care

Critically ill patients require a muscle mass assessment during their ICU stay. Unfortunately, the tools used to assess nutritional status are poor indicators of malnutrition in the critically ill population. A sarcopenic obesity, characterized by excess fat and fluid retention of 10–20% of the patient's body weight can mask the skeletal muscle wasting in the ICU [15]. Many ICU patients are edematous, and the measured weight, the BMI, and anthropometric measurements (mid-upper arm circumference and triceps skinfold thickness) may not reflect the real body muscle mass and could have limited results [16, 17]. In the ICU setting, albumin is also a poor marker of nutritional status not only due to changes in intravascular volume but also due to the impact of acute infection, inflammation, hepatic function, etc. [18]. Concerning the use of tools that assess muscle mass and nutrition, such as Nutrition Risk in Critically Ill Score [19], are difficult to perform and hence they cannot uniformly identify patients at risk of malnutrition. The bioelectrical impedance vector analysis is a useful method not only to evaluate tissue hydration but also to detect muscle mass variations in sarcopenic indictviduals, and it is able to discriminate sarcopenic individuals from sarcopenic obese individuals. However, the bioelectrical impedance vector analysis has some limitations: estimation of hydration status is related to fat-free mass, which basically means muscle mass (in the limbs). Whereas, the limbs contribute roughly 90% to whole body impedance, only 6–12% are contributed by the trunk which, however, provides roughly 50% of the body weight and stores most of the surplus volume [20]. A baseline muscle mass assessment in the acutely critically ill patient is challenging. Muscle ultrasound is an attractive emerging technique able to offer qualitative analysis [21], inexpensive, and readily available at bedside. Unlike computed tomography (CT), however, international consensus does not exist on methodology, with significant differences between the techniques [21]. Although CT scans provide a reliable measure of muscle mass in these medically ill populations, CT scans are not performed on every critically ill patient due to cost and radiation exposure [22].

3. Muscle wasting and serum creatinine

After 10 days from the intensive care unit (ICU) admission [23, 24], a dynamic clinical state characterizes a "cascade" of new clinical problems [25]. This transition point is defined as "persistent critical illness" based on the point "beyond which diagnosis and severity of illness at admission are no more predictive of in-hospital mortality than are simple premorbid patient characteristics" [23]. Characterized by persistent inflammation, neurohumoral alterations, and prolonged immobilization, this catabolic state is not suppressed by nutrition [26, 27]. Catabolism results in muscle wasting and associated weakness, which impairs outcome [26–28]. Currently, there is no routine biomarker available with acceptable sensitivity and specificity which is able to monitor catabolism. Accurate monitoring of nitrogen losses and balances is not easy, but the presence and severity of catabolism often becomes clear once muscle loss and weakness are established. SCr is a metabolite of creatine phosphate, an energy store found in skeletal muscle, and in normal subjects it is produced at a constant rate. Particularly, a prolonged immobilization could decrease the plasma volume, bone mass, and skeletal muscle mass [29, 30]. A decrease in muscle

mass could theoretically be associated with changes in the metabolism of urea and sCr. Indeed, a muscle mass reduction could increase the urea generation because the muscular tissue has a high protein content and urea is the final catabolite of endogenous protein breakdown [31]. Disorders associated with dehydration/hypovolemia or with hypercatabolism increase plasma urea [32, 33]. In addition, skeletal muscle mass is the main determining factor of creatinine generation since creatinine is the final catabolite of muscle energy metabolism [34]. A decrease in muscle mass could decrease SCr levels, and conversely, SCr may be falsely increased with higher muscle mass. In addition, creatinine generation is low among individuals who have more diminutive muscle mass, either constitutionally or disease-related [13]. Due to the correlation between SCr levels and muscle mass, SCr in the steady state has been used as a surrogate of muscle mass measurements [35].

3.1 Muscle strength and sarcopenia index

Sarcopenia is a skeletal muscle disorder that is characterized by the loss of strength and mass together with impairment in physical function [36]. Sarcopenia is a complex syndrome that is associated with muscle mass loss, alone or in conjunction with increased fat mass. Since 2018, sarcopenia is not only considered as a debilitating condition that involves loss of muscle mass and function but also as a muscle disease. However, challenges in understanding the current evidence of the role of nutrition is represented by the number of different aspects of muscle health that have been considered as outcomes, both in observational and interventional study. New guidelines, which aim to improve consistency in the identification of sarcopenia in clinical care, identify muscle strength as the key characteristic of sarcopenia. This new guidance may also offer a useful structure within which to evaluate the influences on muscle health, including the effects of differences in diet [37]. Thus, a low muscle strength leads to a diagnosis of probable sarcopenia [37]. Sarcopenia is associated with frailty, poor surgical outcomes, prolonged need for mechanical ventilation, increased hospital cost, depression, decreased quality of life, increased risk of fall, nursing home residence, and a higher risk of death [38]. Evaluation of patients with sarcopenia could be really difficult as often physical function assessment is not performed and the measurement of muscle mass requires expensive and complex radiologic technique [39]. In addition, BMI, serum albumin levels, prealbumin levels, and physical examination lack in sensitivity and specificity to be used as surrogates for muscle mass. As previously reported, a low baseline sCr value is associated with a worse outcome and has been proposed as an indicator of low muscle mass [recently, a method to estimate muscle mass, named sarcopenia index (SI), was developed using the differential origin of two molecules cleared by the kidney: sCr (skeletal muscle cells) and cystatin C (nucleated cells) [40, 41], assuming steady kidney function]. The SI was calculated as (sCr value/cystatin C value) × 100. The SI not only significantly correlates with imaging but also it has a superior performance compared with sCr alone in estimation of muscle mass, as reported by recent evidence [42].

3.2 Urea:creatinine ratio

The lack of validated and routinely available biomarkers of catabolism to some extent hampers the epidemiological and interventional studies on this topic. The initial decreases in sCr may be from altered metabolism and reflect bioenergetic failure. The subsequent continued fall in sCr reflected the length of ICU stay and length of hospitalization, and it is due to skeletal muscle loss (decreasing creatinine production) [43, 44]. Particularly, from 3 to 4 days after ICU admission, urea progressively rises, with a higher peak and greater duration of elevation in those patients remaining longer

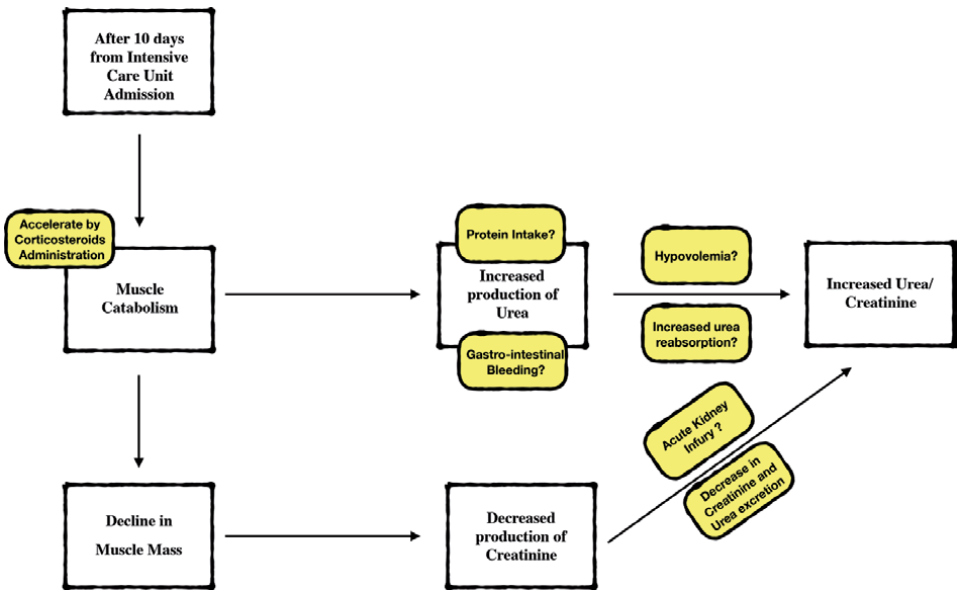

Figure 1.
Urea:creatinine ratio in critical illness.

in ICU. Recently, it was suggested that a persistent elevation in urea might reflect increased production from muscle catabolism, amino acid liberation, and metabolism. Based on the observed trajectory of urea, this catabolic state appears to persist throughout ICU admission [27]. For this reason, elevated urea:creatinine (UCR) may reflect a combination of muscle bioenergetic failure, muscle catabolism/altered protein homeostasis, and persistent muscle wasting, providing a metabolic signature of the effects of prolonged critical illness [27, 45, 46]. Although the potential role of UCR in future studies, clinical usability seem limited, as other factors such as the following may increase UCR independent of catabolism: decreased effective blood volume, protein intake or gastrointestinal bleeding, and acute kidney injury (**Figure 1**).

Particularly, despite altered tubular reabsorption of urea (normally 40–50%) can affect the serum urea:creatinine, classically increased urea retention occurs during severe dehydration with preserved tubular function. Conversely, tubular injury in AKI will lessen the concentrating capacity, thereby lessening urea:creatinine [47].

4. Conclusions

Critically ill patients suffer significant LBM loss, much of it in the first 7–10 days of ICU stay, requiring adequate timing initiation and optimal nutritional support to slow the catabolic process and to minimize adverse events such as prolonged mechanical ventilation, longer ICU stay, and increased risk of death. Due to the correlation between SCr levels and muscle mass, SCr in the steady state has been used as a surrogate of muscle mass measurements. However, SI could be considered a useful tool with a superior performance compared with sCr alone in the estimation of muscle mass, while the clinical usability of UCR seems limited and influenced by other factors such as decreased effective blood volume, protein intake or gastrointestinal bleeding, and also acute kidney injury. However, muscle wasting, often present in critically ill patients, can influence SCr and mask a diagnosis of AKI, decreasing the sensitivity of SCr for the early detection of AKI. Future studies should address the effect of muscle wasting on the true SCr concentration.

Conflict of interest

The authors declare no conflict of interest.

Abbreviations

AKI	acute kidney injury
BMI	body mass index
CT	computed tomography
IBW	the ideal body weight
ICU	intensive care unit
LBM	lean body mass
RRT	renal replacement therapy
sCr	serum creatinine
SI	sarcopenia index
UCR	urea:creatinine

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Chapter 5

The Na/K-ATPase Signaling Regulates Natriuresis in Renal Proximal Tubule

Jiang Liu, Yanling Yan and Joseph I. Shapiro

Abstract

For decades, the Na/K-ATPase has been proposed and recognized as one of the targets for the regulation of renal salt handling. While direct inhibition of the Na/K-ATPase ion transport activity and sodium reabsorption was the focus, the underlying mechanism is not well understood since decreases in basolateral Na/K-ATPase activity alone do not appear sufficient to decrease net sodium reabsorption across the renal tubular epithelium. The newly appreciated signaling function of Na/K-ATPase, which can be regulated by Na/K-ATPase ligands (cardiotonic steroids (CTS)) and reactive oxygen species (ROS), has been widely confirmed and provides a mechanistic framework for natriuresis regulation in renal proximal tubule (RPT). The focus of this review aims to understand, in renal proximal tubule, how the activation of Na/K-ATPase signaling function, either by CTS or ROS, stimulates a coordinated reduction of cell surface Na/K-ATPase and sodium/hydrogen exchanger isoform 3 (NHE3) that leads to ultimately decreases in net transcellular sodium transport/reabsorption.

Keywords: cardiotonic steroids, natriuresis, renal proximal tubule, Na/K-ATPase, NHE3, signaling, ROS

1. Introduction

Since J.C. Skou's discovery in 1957 [1], the energy-transducing Na/K-ATPase has been extensively studied for its ion-pumping function and, later on, its signaling function. While the signaling function was first demonstrated in cardiac myocyte primary culture, the phenomenon has been confirmed in different cell types and animal models. The roles of Na/K-ATPase signaling in renal proximal tubule (RPT) sodium handling and oxidative modification of the Na/K-ATPase α1 subunit in Na/K-ATPase signaling were explored both in vitro and in vivo. The findings may explain certain mechanism(s) related to the Na/K-ATPase signaling-ROS amplification loop and subsequent regulation of salt sensitivity.

The RPT mediates over 60% of the filtered Na^+ reabsorption [2, 3]. There are two Na^+ reabsorption pathways in RPTs. One is through the transcellular pathway, mainly through the apical Na^+ entry mainly via NHE3 (and other apical Na^+-coupled transporters like Na^+-glucose cotransporters 1 and 2, to a lesser extent) and basolateral Na^+ extrusion through the Na/K-ATPase [2, 3]. A coordinated and coupled regulation of sodium/hydrogen exchanger isoform 3 (NHE3, SLC9A3) and

the Na/K-ATPase is critical in maintaining intracellular Na^+ homeostasis and extracellular fluid volume. The other one is the paracellular Na^+ reabsorption pathway through a tight junction (TJ), which depends on the transepithelial electrochemical force and tight junction permeability. Claudin-2 forms paracellular channels with other protein that are selective for small cations like Na^+ and K^+, small anion like Cl^-, as well as water [4–6]. Interestingly, the Na/K-ATPase signaling function is able to regulate the apical/basolateral polarity of the Na/K-ATPase as well as the tight junctions' components like claudins in distal tubule MDCK cells [7, 8].

The Na/K-ATPase belongs to the P-type ATPase family and consists of two non-covalently linked α- and β-subunits. Several α- and β-isoforms, expressed in a tissue-specific manner, have been identified and functionally characterized [9–12]. In RPTs, the γ-subunit (γ_a and γ_b, also known as FXYD2, one of the small type I single-span membrane FXYD protein families) also interacts with the α1 subunit to regulate the Na/K-ATPase activity [13–15]. There is also a fifth member of the β-subunit family, named βm coded by an ATP1B4 gene, that is predominantly expressed in skeletal muscle. Interestingly, the βm is not associated with the α1 subunit like other β-subunits, but accumulated in the nuclear membrane and associated with transcriptional coregulator Ski-interacting protein, which led to the regulation of TGF-β-responsive reporter Smad7 [16]. The α1 subunit contains multiple structural motifs that interact with soluble, membrane, and structural proteins. Binding to these proteins not only regulates the ion-pumping function of the enzyme, but it also conveys signal-transducing functions to the Na/K-ATPase [17–32]. NHE3 belongs to a family of electroneutral mammalian Na^+/H^+ exchangers [33–35]. In RPT, NHE3 resides in the apical membrane of S1 and S2 segments, mediating transcellular reabsorption of Na^+ and HCO_3^- and fluid reabsorption [36, 37]. In the kidney, more than 85% of the filtered $NaHCO_3$ is reabsorbed in the RPTs, and NHE3 contributes up to ~60% of the total reabsorption of this segment [38]. RPT NHE3 secrets the largest portion of net H^+ to the lumen and interacts with HCO_3^- to form H_2O and CO_2 which can freely translocate into RPT cytosol. In cytosol, H_2O and CO_2 form H^+ and HCO_3^- through carbonic anhydrase catalyzation. Finally, the newly formed cytosolic H^+ will be secreted to the lumen, and HCO_3^- will be moved to the blood through the basolateral-resided Na^+/HCO_3^- cotransporter (NBCe1-A, SLC4A4). This cycling carbonic anhydrase-controlled CO_2-HCO_3^- system links the NHE3-mediated H^+ secretion to HCO_3^- reabsorption, to achieve an acid-base equilibrium [39, 40]. Moreover, vesicular NHE3 activity also regulates endosomal pH and consequently affects receptor-mediated endocytosis as well as endocytic vesicle fusion [41, 42]. Under normal conditions, the Na/K-ATPase resides at the basolateral surface, providing the driving force for the vectorial transport of Na^+ from the tubular lumen to the vascular compartment, while the NHE3 resides at the apical surface providing a rate-limiting Na^+ entry into cells.

2. The concept of endogenous cardiotonic steroids (CTS) as natriuretic hormones

CTS (also known as endogenous digitalis-like substances) are specific ligands and inhibitors of the Na/K-ATPase, which include plant-derived glycosides such as digoxin and ouabain and vertebrate-derived aglycones such as bufalin and marinobufagenin (MBG). Although the production and secretion of endogenous CTS are not completely understood, both ouabain and MBG have been identified as endogenous steroid hormones whose production and secretion can be regulated by multiple stimuli including angiotensin II and adrenocorticotropic hormone (ACTH) [30, 43–48]. Endogenous CTS are present in measurable amounts under

normal physiological conditions and are markedly increased under a number of pathological conditions such as sodium imbalance, chronic renal failure, hyperaldosteronism, hypertension, congestive heart failure, acute plasma volume expansion, and preeclampsia [46, 49–59].

Even though digitalis-like drugs have been used to treat heart failure patients for over 200 years, studies have also revealed many extra-cardiac actions of these compounds, such as in response to salt loading in both animal models and human hypertensive patients [29, 57, 60–62]. In addition, low doses of CTS not only induced hypertension in rats but also caused a significant cardiovascular remodeling independent of their effect on blood pressure (BP) [63–66].

Bricker was the first to propose the existence of "the third factor" (named after the glomerular filtration rate as the first factor and the aldosterone as the second factor), and Dahl proposed the existence of a hormonal natriuretic factor that might cause a sustained increase in BP in salt-sensitive hypertensive rats [67, 68]. Subsequently, Bricker, de Wardener, and others proposed that this hormonal natriuretic factor inhibits the Na/K-ATPase, and Blaustein described how an increase in endogenous Na/K-ATPase inhibitors might cause a vascular contractility change and then a rise in BP [67, 69–72]. In 1980, de Wardener and MacGregor summarized the state of research at the time and proposed an insightful scheme explaining how the Na/K-ATPase inhibitor works as a natriuretic hormone [73]. In essence, it was contended that the Na/K-ATPase inhibitor (endogenous CTS) will rise in response to either a defect in renal Na^+ excretion or high salt intake. This increase, while returning Na^+ balance toward normal by increasing renal Na^+ excretion, also causes hypertension through acting on the vascular Na/K-ATPase. With the advances in the field over the decades, much has been learned. The first unequivocal demonstration of ouabain-like substance in the human plasma was reported decades ago [46]. Blaustein and Hamlyn's laboratory has demonstrated how increases in endogenous CTS change vascular contractility and its effect on BP [74]. However, the pathophysiological significance of endogenous CTS (e.g., as a natriuretic hormone) has been a subject of debate since it was first proposed until Lingrel's laboratory reported their gene replacement in vivo studies, which unequivocally demonstrated that endogenous CTS play an important role in the regulation of renal Na^+ excretion and BP through the Na/K-ATPase [75–77]. Specifically, Lingrel's group generated several lines of mice in which the mouse endogenous ouabain-insensitive α1 subunit is replaced by a mutant that alters the ouabain sensitivity of the Na/K-ATPase. For example, they generated a line of "humanized" $\alpha1^{S/S}$ mice where the endogenous ouabain-insensitive α1 is replaced by an ouabain-sensitive (human like) α1-mutant and used these mice to explore the role of endogenous CTS in the regulation of renal function and BP. Should endogenous CTS be important for these regulations, an increased CTS sensitivity in $\alpha1^{S/S}$ mice would make these mice more sensitive to conditions that raise circulating CTS. Indeed, when ACTH was administered to raise endogenous CTS, it caused much severe hypertension in $\alpha1^{S/S}$ mice than their control littermates. Moreover, expression of the ouabain-sensitive α1-mutant significantly increased renal Na^+ excretion, confirming the natriuretic function of endogenous CTS as proposed by the pioneers of the field [67, 68, 70–73]. More evidences indicate that increases in endogenous CTS regulate both renal Na^+ excretion and BP through the Na/K-ATPase [74–76, 78, 79].

3. The Na/K-ATPase signaling by specific ligands and ROS in RPTs

Ouabain-stimulated protein-protein interaction and subsequent Na/K-ATPase signaling function were first demonstrated in rat neonatal myocytes, which were

further confirmed and developed in porcine LLC-PK1 cells (an immobilized RPT cell line) and other cell types. CTS-stimulated Na/K-ATPase signaling has been reviewed everywhere [22, 31, 32, 47, 80–83].

In LLC-PK1 cells, ouabain-stimulated Na/K-ATPase signaling increases ROS generation. Other than ouabain, exogenous H_2O_2 and glucose oxidase-induced H_2O_2 also activate Na/K-ATPase signaling pathways including phosphorylation of c-Src and ERK1/2, as well as protein carbonylation modification of Na/K-ATPase (direct carbonylation of two amino acid residues, Pro^{222} and Thr^{224}, in the actuator domain of the α1 subunit) [84–87]. Pretreatment with antioxidant *N*-acetyl-L-cysteine (NAC) or disruption of the Na/K-ATPase/c-Src signaling complex attenuated ouabain- and glucose oxidase-stimulated Na/K-ATPase/c-Src signaling, protein carbonylation, redistribution of Na/K-ATPase, and inhibition of active transepithelial $^{22}Na^+$ transport. A basal level of ROS is critical in initiating ouabain-stimulated Na/K-ATPase/c-Src signaling, and carbonylation modification of the α1 subunit is involved in a feed-forward mechanism of the regulation of ouabain-mediated Na/K-ATPase signal function and subsequent Na^+ transport. Furthermore, a stable overexpression of rat α1-mutant Pro^{224}/Ala (Pro^{224} of rat α1 is the same as the Pro^{222} of pig α1) prevented ouabain-stimulated signal function of Na/K-ATPase, protein carbonylation, Na/K-ATPase endocytosis, and ouabain-induced inhibition of active transepithelial $^{22}Na^+$ transport [79, 86, 87]. Taken together, in LLC-PK1 cells, there is a positive-feedback amplification loop of Na/K-ATPase signaling and ROS generation, in which carbonylation of the Pro^{222} of the α1 subunit is critical. In this working model, both Na/K-ATPase-specific ligands (such as ouabain) and ROS increases (induced by other stimuli like exogenous added glucose oxidase) could activate the Na/K-ATPase signaling, and the Na/K-ATPase/c-Src complex can function as a "receptor" of ROS signaling. This Na/K-ATPase signaling-ROS axis may explain the role of Na/K-ATPase signaling in the development of different pathophysiological conditions, including RPT sodium handling.

4. Endocytosis of Na/K-ATPase

Endocytosis is involved in many important cellular functions. Ouabain-induced endocytosis of the Na/K-ATPase was first observed by the laboratories of Cook and Lamb, which demonstrated that [^{3}H]-ouabain (bound to the Na/K-ATPase) was translocated from the plasmalemmal membrane surface to intracellular compartments (lysosomes) in HeLa cells, chick embryo heart cells, and Girardi heart cells [88–92].

4.1 Dopamine and PTH

One of the best-studied paradigms of hormonal natriuresis is the renal dopamine system [93–96]. Renal dopamine release increases in response to high salt intake or volume expansion. The activation of D1-like dopamine receptors stimulates PLC-γ and cAMP-PKA pathways and increases intracellular Ca^{2+}. These pathways work in concert and produce the coordinated downregulation of NHE3 and the Na/K-ATPase and consequently natriuresis [93–95, 97, 98]. While Aperia's laboratory first revealed the pathways involved in dopamine-induced regulation of Na/K-ATPase activity [99–101] that is related to endocytosis of the Na/K-ATPase [102], Moe and others have mapped the pathways of NHE3 phosphorylation and trafficking [103–105]. In RPT, dopamine alters sodium handling by inducing Na/K-ATPase and NHE3 endocytosis. In RPT primary culture of Sprague-Dawley

rats, dopamine-induced clathrin-dependent endocytosis of the rat Na/K-ATPase α1 subunit is triggered by activation of PI3K and subsequently phosphorylation of Ser-18 of rat α1 subunit [24, 106–109]. The activation of PI3K also stimulated phosphorylation of the Tyr537 of the α1 subunit that facilitates its binding with adaptor protein-2 (AP-2), providing the inclusion of the Na/K-ATPase into clathrin-coated pits (CCP) [24, 108]. However, Ser-18 is found only in rat α1 subunit and is not present in pig and dog α1 subunits [110]. Depending on the type of renal tubular epithelium, dopamine-induced endocytosis of the Na/K-ATPase may be mediated through PKC- or PKA-dependent mechanisms [108, 111–113]. Parathyroid hormone (PTH)-induced inhibition and endocytosis of the Na/K-ATPase were also demonstrated in opossum kidney (OK) cells, which is clathrin-mediated and requires ERK-dependent phosphorylation of Ser-11 of the α1 subunit [114].

4.2 Ouabain-induced endocytosis of Na/K-ATPase through Na/K-ATPase signaling

In LLC-PK1 cells, at the doses used, ouabain has no discernable effects on cell morphology, viability, transepithelial electrical resistance, tight junction integrity, and intracellular [Na^+] [115]. However, ouabain causes decreases in membrane-bound Na/K-ATPase without significantly affecting intracellular [Na^+] [116, 117]. As a specific ligand, nontoxic ouabain (~1/10th–1/20th of acute IC_{50}) caused a dose- and time-dependent decrease in Na/K-ATPase ion-pumping activity (ouabain-sensitive ^{86}Rb uptake), which is attributed to ouabain-stimulated clathrin-dependent endocytosis of the α1/β1-subunits, demonstrated by a decrease in cell surface biotinylated α1 subunit and a concomitant accumulation of α1/β1-subunit and c-Src in early endosome (EE)/late endosome (LE) fractions. This leads to a net decrease in abundance of Na/K-ATPase in the plasma membrane and total ion-pumping activity of Na/K-ATPase and transcellular $^{22}Na^+$ transport. This phenomenon was only observed when ouabain was applied to the basolateral, but not apical, aspect of Costar Transwell with membrane support for 12 hours, which indicates that this ouabain-induced endocytosis of the Na/K-ATPase is initiated by activating the receptor Na/K-ATPase/Src complex involving phosphorylation of c-Src and PI3K. The endocytosed [^3H]-ouabain/Na/K-ATPase/c-Src/EGFR complex can be detected in both EE and LE fractions.

To understand the molecular mechanism(s) involved in this process, studies were performed with LLC-PK1 as well as SYF and SYF + c-Src cells. SYF cells are triple Src kinase (c-Src, Yes, Fyn)-null mouse fibroblast cells, and SYF + c-Src are c-Src-rescued SYF cells. This pair of cells was used to determine the role of c-Src activation in ouabain-induced Na/K-ATPase signaling and endocytosis. While ouabain accumulates Na/K-ATPase α1 subunit content in clathrin-coated pits and EE/LE fractions, it also causes a translocation of the α1 subunit to nuclear fraction. Interestingly, the effects of ouabain are fully reversible in terms of ion-pumping activity, transepithelial $^{22}Na^+$ flux, and cell surface Na/K-ATPase within 24 hours following the removal of ouabain with a fresh culture medium, suggesting a reversible process. Immunofluorescence showed that the Na/K-ATPase α1 subunit co-localized with clathrin both before and after ouabain treatment, and immunoprecipitation experiments indicated that ouabain stimulated interactions among the α1 subunit, AP-2, and clathrin heavy chain (CHC). Disruption and/or arresting of clathrin-coated pit formation (by potassium depletion with hypotonic shock [118] and chlorpromazine treatment [119]) significantly attenuated this ouabain-induced endocytosis, suggesting the involvement of a clathrin-coated pit. Inhibition of the ouabain-activated signaling with PP2 (a specific c-Src kinase inhibitor)

or wortmannin (a specific PI3K inhibitor) also significantly attenuated ouabain-induced endocytosis. Experiments performed in SYF cells and SYF + c-Src demonstrated that ouabain induces the endocytosis of the Na/K-ATPase in SYF + c-Src cells, but not in the SYF, indicating that ouabain-induced endocytosis of the Na/K-ATPase is c-Src-dependent.

Ouabain-stimulated Na/K-ATPase signaling also requires caveolin-1 (Cav-1) (a structural protein of caveolae, a subset of membrane lipid rafts) that functions as an anchoring protein for attracting the Na/K-ATPase α1 subunit into caveolae [120]. Accordingly, depletion of cholesterol (by methyl-β-cyclodextrin (Mβ-CD)) or caveolin-1 (by siRNA) blocked ouabain-induced endocytosis of the Na/K-ATPase, compartmentalization of signaling molecules in clathrin-coated pits, and early endosome. In addition, depletion of caveolin-1 also significantly reduced the protein-protein interactions among α1 subunit, AP-2, PI3K, and clathrin heavy chain, suggesting that caveolin-1 is involved in both ouabain-induced endocytosis of Na/K-ATPase and signal transduction [117].

These data demonstrate that ouabain stimulates a clathrin- and caveolin-1-dependent endocytosis of the Na/K-ATPase, a phenomenon requiring ouabain-induced Na/K-ATPase signaling function. Taken together, it is most likely that clathrin- and/or caveola-/lipid raft-mediated endocytosis of the Na/K-ATPase is a common phenomenon, but the mechanism and the relationship between the endocytosis of the Na/K-ATPase and signal transduction are still not fully understood. This is the first time to demonstrate that ligand-modulated endocytosis of the Na/K-ATPase is a mechanism by which RPT sodium transport is altered in a physiologically meaningful manner (**Figure 1**).

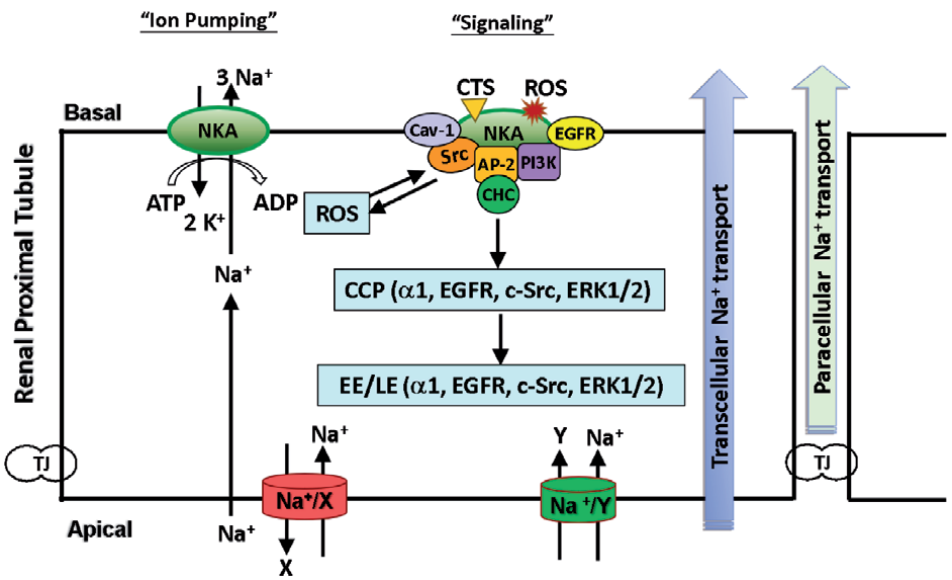

Figure 1.
Illustration of activation of the Na/K-ATPase signaling-mediated endocytosis of the Na/K-ATPase. Both CTS and ROS can activate Na/K-ATPase signaling, which leads to translocation of cell surface Na/K-ATPase (α1- and β1-subunits), along with EGFR, c-Src, and ERK1/2, into clathrin-coated pits and early and late endosomes. This process is independent of change in intracellular Na^+ and Ca^{2+}, but is dependent on activation of c-Src and PI3K, and the presence of caveolin-1. The activation of the Na/K-ATPase signaling also stimulates ROS generation which further activates the signaling. In LLC-PK1 cells, ouabain has no significant effect on recycling of endocytosed α1 subunit. AP-2, adaptor protein-2; Cav-1, caveolin-1; CCP, clathrin-coated pits; CHC, clathrin heavy chain; CTS, cardiotonic steroids; EE, early endosome; LE, late endosome; Na^+/X, Na^+-dependent antitransporter; Na^+/Y, Na^+-dependent cotransporter; NKA, Na/K-ATPase; TJ, tight junction.

5. The Na/K-ATPase signaling regulates NHE3 trafficking and activity

5.1 NHE3 regulation

In RPT, NHE3 resides in the apical membrane of S1 and S2 segments, mediating transcellular reabsorption of Na^+ and HCO_3^- and fluid reabsorption [36, 37]. Moreover, vesicular NHE3 activity regulates endosomal pH and consequently affects receptor-mediated endocytosis as well as endocytic vesicle fusion [41, 42]. Consistent with its cellular function, upregulation of NHE3 activity and expression is associated with the development of hypertension [121–124]. Conversely, the reduction of NHE3 surface expression or NHE3 activity occurs during pressure natriuresis in rats [125–128]. As expected, NHE3-deficient mice are hypotensive [129–131] because of reduced Na^+ reabsorption and increased Na^+ excretion. Interestingly, NHE3-deficient mice also develop acidosis since the blunted H^+ secretion through NHE3, which links to greatly reduced RPT HCO_3^- reabsorption (please see Introduction for the linkage of NHE3 H^+ secretion and HCO_3^- reabsorption), could not be compensated by H^+-ATPase and AE1 (anion exchanger-1, SLC4A1) Cl^-/HCO_3^- exchanger, compared with wild-type mice [131, 132]. These observations put renal Na^+ reabsorption through NHE3 in a central position in the development and control of salt loading- and volume expansion-mediated hypertension. Structurally, NHE3 has a predicted N-terminal hydrophobic ion-translocating domain and a variable C-terminal hydrophilic domain which contains regulatory sequences [133].

The NHE3 activity is regulated at various levels through different mechanisms, mainly via phosphorylation, trafficking, and transcriptional regulation [34, 35, 103]. The surface expression of NHE3 is mainly regulated by changes in endocytosis/exocytosis and is the primary regulatory mechanism of NHE3 activity. NHE3 has been found to traffic between the plasma membrane and EE/LE fractions via a clathrin- and PI3K-dependent pathway [41, 134–141]. The NHE3 activity can be stimulated by exocytosis [141–143] or inhibited by endocytosis [105, 125, 144]. The activation of c-Src, PKA, and PKC and increase in intracellular Ca^{2+} are involved in the regulation of NHE3 trafficking.

NHE3 has been shown to be redistributed under a hypertensive state, accompanying reversible downregulation of the Na/K-ATPase activity in the renal cortex [125, 127, 145]. This raised the possibility that the basolateral-localized Na/K-ATPase and apically localized NHE3 work in concert to regulate renal sodium handling in response to the Na/K-ATPase signaling. The coordinated regulation of NHE3 and the Na/K-ATPase is critical in maintaining intracellular Na^+ homeostasis and extracellular fluid volume. It is believed that the apical Na^+ entry through NHE3 is the rate-limiting step because the functional reserve of the Na/K-ATPase in the nephron is more than sufficient even under some pathological conditions.

5.2 Chronic NHE3 regulation by Na/K-ATPase signaling

In LLC-PK1 cells, chronic, low-concentration ouabain (50 and 100 nM, 24 hours) treatment in the basolateral aspect, but not in apical aspect, did not change intracellular [Na^+] but decreased apical NHE3-mediated Na^+ absorption, NHE3 promoter activity, and NHE3 protein and mRNA abundance. Pretreatment with specific inhibitors against c-Src and PI3K attenuates ouabain-induced downregulation of NHE3 activity and NHE3 mRNA [146]. In caveolin-1 knockdown LLC-PK1 cells, ouabain failed to reduce NHE3 mRNA and NHE3 promoter activity, in which ouabain-induced Na/K-ATPase signaling reduced Sp1 and TR DNA

binding activity and consequently decreased NHE3 expression and activity [146]. These effects are abolished by inhibition of either c-Src or PI3K. Promoter mapping identified that ouabain-response elements reside in a region between −450 and −1194 nt and that ouabain reduces the binding of transcriptional factor *Sp1* to its cognate *cis*-element.

5.3 Acute NHE3 regulation by Na/K-ATPase signaling

Acute application of ouabain (1 hour) in the basolateral, but not apical, aspect significantly reduced NHE3 activity ($^{22}Na^+$ uptake) and active transepithelial $^{22}Na^+$ transport. This is accompanied by a reduced NHE3 content on cell surface and an increased NHE3 content in EE/LE fractions, as seen in the case of the Na/K-ATPase α1 subunit. These changes are independent of change in the integrity of tight junctions and the intracellular Na^+ concentration [115]. Ouabain-induced NHE3 trafficking was abolished by either PI3K or c-Src inhibition. Disruption of caveolae/lipid rafts by cholesterol depletion prevented ouabain-induced accumulation of NHE3 and Na/K-ATPase α1 in early endosomes, and cholesterol repletion restored the ouabain-induced endosomal accumulation of NHE3 and Na/K-ATPase α1. Moreover, pretreatment of cells with the intracellular Ca^{2+} chelator BAPTA-AM attenuated ouabain-induced NHE3 trafficking, suggesting Ca^{2+} might link the Na/K-ATPase signaling to NHE3 regulation which is in agreement with observations that intracellular Ca^{2+} can regulate NHE3 activity and trafficking [147, 148]. These changes indicate that ouabain acutely stimulates NHE3 trafficking, like Na/K-ATPase, by activating the basolateral Na/K-ATPase signaling complex [115]. In RPT cell lines (human HK-2, porcine LLC-PK1, and AAC-19 originated from LLC-PK1 in which the pig α1 was replaced by ouabain-resistant rat α1), results further indicate that ouabain-induced inhibition of transcellular $^{22}Na^+$ transport

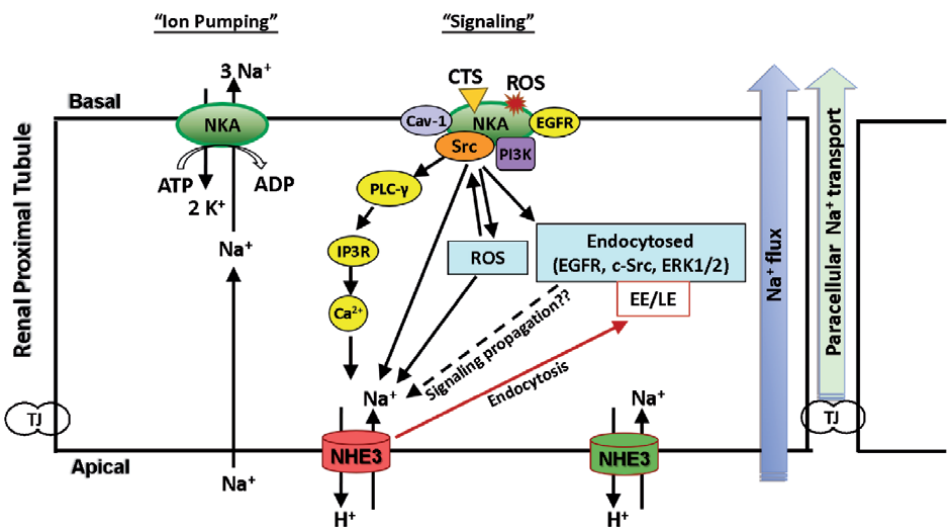

Figure 2.
Illustration of activation of the Na/K-ATPase signaling-mediated endocytosis of NHE3. Activation of the Na/K-ATPase signaling leads to intracellular Na^+-independent NHE3 endocytosis. However, like Na/K-ATPase signaling-mediated Na/K-ATPase endocytosis, the NHE3 endocytosis is dependent on intracellular Ca^{2+}, activation of c-Src and PI3K, and caveolin-1. In LLC-PK1 cells, ouabain inhibits the endocytic recycling of endocytosed NHE3. Since the Na/K-ATPase and NHE3 reside on basolateral and apical membrane in monolayer, respectively, it is still unclear how the basolateral Na/K-ATPase signaling is transmitted to NHE3 regulation. There are several possible pathways as illustrated, as proposed in the text (please see ***Figure 1*** *for abbreviations).*

as well as trafficking of the α1 subunit and NHE3 is not a species-specific phenomenon. Furthermore, in LLC-PK1 cells, ouabain inhibited the endocytic recycling of internalized NHE3, but has no significant effect on recycling of endocytosed α1 subunit [149].

Taken together, by activating the basolateral receptor Na/K-ATPase/c-Src complex, ouabain can simultaneously and coordinately regulate trafficking of basolateral Na/K-ATPase and apical NHE3, leading to inhibition of transepithelial Na^+ transport. This mechanism may be important to RPT Na^+ handling during conditions associated with increases in circulating endogenous CTS. However, it remains to be established whether ouabain-induced regulation of NHE3 trafficking comes from the endocytosed Na/K-ATPase/c-Src complex or directly from the plasma membrane, since ouabain still binds to endocytosed Na/K-ATPase (**Figure 2**).

6. Ouabain-induced regulation of Na/K-ATPase α1 subunit and NHE3 is independent of intracellular [Na^+]

High concentrations of ouabain are known to increase intracellular [Na^+], depolarize the proximal tubule, and affect the tight junction of epithelial cells. In LLC-PK1 cells, ouabain (up to 100 nM) has no acute effect on intracellular [Na^+], transepithelial electrical resistance, and tight junction integrity, suggesting that in the concentration, ouabain is not likely to increase passive Na^+ transport by depolarizing LLC-PK1 monolayers [115]. To further define whether the effects of ouabain on the Na/K-ATPase and NHE3 are independent of intracellular [Na^+], the change in intracellular transporters after the equilibrium of intracellular [Na^+] with extracellular [Na^+] was achieved by using conventional "Na^+-clamping" methods [150]. LLC-PK1 cells (both control and ouabain-treated) are pretreated either with 20 μM monensin or with 10 μM monensin plus 5 μM gramicidin for 30 min. Both "clamping" methods raise basal levels of α1 and NHE3 in EE/LE fractions (monensin is known to accumulate proteins in intracellular compartments). However, ouabain is still able to further accumulate more α1 and NHE3 in EE/LE. These observations indicate that ouabain-induced trafficking of α1 and NHE3 can be independent of intracellular [Na^+] change [115].

7. Coordinated and coupled regulation of Na/K-ATPase and NHE3 by Na/K-ATPase signaling

Although the mechanisms are still being elucidated, accumulating evidence supports the notion that the expression and activity of the basolateral Na/K-ATPase and apical NHE3 are coordinated and coupled under certain circumstances. For example, McDonough's laboratory has shown that, during pressure natriuresis and salt loading, the surface expression and activity of both NHE3 and the Na/K-ATPase are simultaneously downregulated to remove Na^+ from the body [125, 127, 145, 151]. During the development of hypertension in spontaneous hypertensive rat (SHR), the expression and activity of both the Na/K-ATPase and NHE3 are elevated in comparison with the normotensive control rats [121, 152–155].

Activation of Na/K-ATPase signaling, by either ouabain or a high-salt diet, is also capable of stimulating a coordinated and coupled downregulation of apical NHE3 and basolateral Na/K-ATPase to inhibit active transepithelial Na^+ transport in cultured or isolated RPTs [79, 115–117, 149]. This coordinated regulation depends on activation of the Na/K-ATPase signaling function, but not on acute inhibition

of the Na/K-ATPase activity since it requires the activation of Src and PI3K and increase in intracellular Ca^{2+}. Moreover, MBG infusion also induced endocytosis of RPT Na/K-ATPase in rats, which could be prevented by an antibody-mediated neutralization of infused MBG [156].

A high salt intake or volume expansion increases both dopamine and CTS. It has been shown that dopamine-induced regulation of RPT Na/K-ATPase of Dahl S rats was defective because of an apparent decoupling between the binding of dopamine to its D_1 receptor and activation of GPCRs [157–161]. In response to salt loading, Dahl S rats have a similar diuretic, but much less CTS-related natriuretic response than that seen in Dahl R rats [162]. Both dopamine and CTS can regulate the activity and trafficking of RPT Na/K-ATPase and NHE3. Even though the initiating steps and signaling pathways might be different, they share some signaling steps such as the activation of PLC/PKC and calcium signaling. It *will be of interest to further assess whether there is a crosstalk between CTS- and dopamine-activated signaling pathways in the regulation of renal Na^+ handling.*

In vivo studies suggest the essential role of CTS in modulating renal sodium excretion and BP with different approaches. First, the administration of some (e.g., ouabain) but not all CTS induces natriuresis [163, 164]. Second, in transgenic mice expressing ouabain-sensitive Na/K-ATPase α1 subunit, both acute salt load and ouabain infusion augment natriuretic responses, which were prevented by administration of an anti-digoxin antibody fragment [75, 76]. Third, immune neutralization of endogenous CTS prevents CTS-mediated natriuretic and vasoconstrictor effects [55, 59, 78, 80]. Fourth, the administration of the ouabain antagonist, rostafuroxin (also known as PST 2238), prevents not only ouabain-induced Na/K-ATPase signaling but also ouabain-induced increase in BP [64]. Finally, in humans, a high salt intake increases circulating endogenous CTS [57, 80, 165]. An increased CTS excretion is directly linked to an enhanced RPT-mediated fractional Na^+ excretion, but inversely related to age and to age-dependent increase in salt sensitivity [165].

Although the historical focus has largely been on the direct inhibition of CTS on the Na/K-ATPase ion-pumping activity and sodium reabsorption in RPT as well as vascular tone/contractility, decreases in basolateral Na/K-ATPase activity alone do not appear to be sufficient to reduce net RPT sodium reabsorption since the apical NHE3, but not the Na/K-ATPase, is the rate-limiting step.

In contrast, the newly appreciated signaling function of Na/K-ATPase has been widely confirmed and provides a realistic, mechanistic framework that the renal Na/K-ATPase and its signaling play a key role in regulating renal sodium handling. In porcine RPT LLC-PK1 cells, ouabain activates the Na/K-ATPase signaling pathways and consequently redistributes the basolateral Na/K-ATPase and the apical NHE3 in a coordinated manner; this leads to a symmetrical reduction of cell surface Na/K-ATPase and NHE3 content and ultimately decreased net transcellular sodium transport [86, 87, 115–117]. No significant acute change in intracellular Na^+ concentration was observed [115], further suggesting the coordination of the downregulation of both apical and basolateral sodium transporters. This Na/K-ATPase signaling-mediated regulation of renal tubular epithelial ion transporters was further confirmed in in vivo studies [79, 156]. It has been shown that endocytosis of signaling molecules could be a way to terminate or propagate the signaling and could further regulate endocytosis itself [166–171]. In this regard, it is possible that ouabain- and ROS-induced endocytosis could be an effective way to terminate Na/K-ATPase signaling-mediated oxidant amplification loop by the degradation of carbonylated Na/K-ATPase, to maintain a certain basal level of ROS and carbonylated protein [172].

8. Endocytosis and signaling transduction

The clathrin-dependent endocytosis is the main endocytosis pathway for many membrane proteins in mammalian cells [166, 167, 173–175]. Apart from its endocytic function, the clathrin-coated pits also represent a specialized microdomain, where proteins are assembled into active signaling complexes before internalization of some or all of their components [176]. Some molecules involved in transmembrane signaling, such as β-arrestin, RGS-GAIP (a GTPase-activating protein for Gαi heterotrimeric G proteins) [177], GIPC (a PDZ domain-containing protein) [178], and Src family kinases [179], have been localized to clathrin-coated pits, suggesting that the interaction with the components of the pit machinery may facilitate some signaling functions of transmembrane receptors.

Caveolae/lipid rafts play a central role in transcytosis and endocytosis [180–184]. Many signaling molecules and membrane receptors are dynamically associated with caveolae, such as the Src family kinases, Ras, PKC, ERK, insulin receptor, platelet-derived growth factor receptor (PDGFR), EGFR, and some entire signaling modules like PDGFR-Ras-ERK, mainly through their interactions with caveolins [182, 185, 186]. Caveolins stabilize caveolae and modulate signal transduction by attracting signaling molecules to caveolae and regulating their activities [186]. There is also evidence that caveolins modulate endocytosis through their interactions with clathrin [187–190]. Interestingly, both caveolin and clathrin heavy chain are substrates of Src kinase [169, 184].

The Na/K-ATPase α-subunit, c-Src, and caveolin are present in caveolae isolated by a detergent-free method, in adult rat cardiac myocytes, human embryonic kidney (HEK)-293 cells, and LLC-PK1 cells. In adult rat cardiac myocytes, ouabain not only recruits α-subunit and c-Src to caveolae but also activates caveolar ERK1/2 [191]. Furthermore, some signaling molecules, such as EGFR and c-Src, are also concentrated in clathrin-coated pits and endosomes in response to ouabain [116], suggesting that both clathrin-coated pits and caveolae are involved in ouabain-mediated Na/K-ATPase signal transduction and endocytosis.

The receptor-mediated endocytosis has been shown not only to attenuate ligand-activated signaling but also to continue the signaling on the endocytic pathway, especially from endosomes [166, 167, 192–194]. While endocytosis is important in the activation and propagation of signaling pathways [168, 195, 196], signal transduction can also regulate endocytosis [169, 197]. Endocytic receptor tyrosine kinase (RTK) receptors could control the magnitude of the original signaling responses (generated at the cell surface) or initiate distinct signaling cascades (qualitatively different from that generated at the cell surface) [170]. In polarized epithelial cells, the distribution of RTK substrates could affect cellular responses [118]. The endosomal signaling appears to be dependent on both the receptor and cell type.

In LLC-PK1 cells, ouabain not only induced compartmentalization of Na/K-ATPase, c-Src, EGF receptor, and ERK in early endosomes but also bound to Na/K-ATPase along the endocytic route [116]. Interestingly, caveolin-1 is also present in early or late endosomes. These facts make it possible that endosomal ouabain-Na/K-ATPase/c-Src might be able to propagate its original signaling or to initiate distinct signaling cascades. This is supported by the findings that ouabain-induced NHE3 regulation is mediated by the activation of the receptor function of Na/K-ATPase. Furthermore, endocytosis is required for ouabain to remove basolateral Na/K-ATPase, which induces a significant inhibition of the pumping activity. Moreover, blockade of Na/K-ATPase signaling/endocytosis appears to be sufficient to abolish ouabain-induced trafficking and transcriptional regulation of NHE3.

Although the mechanisms that involved ouabain-initiated endocytosis of the Na/K-ATPase and NHE3 (and expression) are not fully understood, endocytosis of the Na/K-ATPase may play an important role in renal sodium handling. This is because if ouabain induces a significant depletion of plasmalemmal Na/K-ATPase in proximal tubule type cells (rat proximal tubule primary culture, LLC-PK1) but not in distal tubule type cells (rat distal tubule primary culture, MDCK), it will make physiological "sense" in terms of allowing bulk sodium transport (primarily in the proximal tubule) to be altered and leaving fine-tuning (distal tubule) sodium handling intact.

9. ROS and the Na/K-ATPase signaling: the possible link from CTS-stimulated signaling to NHE3 regulation

It is well established that both oxidative stress and high BP are a cause and consequence of each other. The increase in oxidative stress occurs in many forms of experimental models of hypertension, including Dahl salt-sensitive hypertension [198–204]. Increases in ROS can regulate physiological processes including renal tubular ion transport, fluid reabsorption, and sodium excretion [79, 205–210]. In particular, increases in ROS regulate the activity and cellular distribution of the basolateral Na/K-ATPase as well as the apical NHE3 and sodium/glucose cotransporter, at least under normal circumstances [79, 151, 208, 211–216]. Oxidative modification can affect the Na/K-ATPase activity through different mechanisms. For example, S-glutathionylation cysteine residue(s) of the Na/K-ATPase α-subunit can block the intracellular ATP-binding site [217], and S-glutathionylation of cysteine of the Na/K-ATPase β1-subunit can affect the Na/K-ATPase conformational poise [218, 219]. Oxidant and oxidative modification of the Na/K-ATPase can lead to degradation, functional changes, and formation of Na/K-ATPase oligomeric structure [74, 84–87, 217, 219–230]. In LLC-PK1 cells, increase in ROS generation, induced by either ouabain or glucose oxidase, is critical in the activation of Na/K-ATPase signaling which mediates trafficking of the Na/K-ATPase and NHE3 and transcellular Na^+ transport [86, 87]. Pretreatment with higher doses, but not a low dose, of NAC attenuated the effect of ouabain on c-Src activation and transcellular $^{22}Na^+$ flux, suggesting a role of basal physiological redox status in the initiation of ouabain-induced Na/K-ATPase signaling. While CTS stimulates ROS generation and Na/K-ATPase signaling in different in vitro and in vivo models [63, 85, 231–233], an increase in ROS alone (without the presence of ouabain) by extracellularly added glucose oxidase is also able to activate Na/K-ATPase signaling, indicating that activation of Na/K-ATPase signaling can be achieved by general stimuli like ROS, other than its specific ligands. Glucose oxidase-induced H_2O_2 alone also stimulates Na/K-ATPase endocytosis and inhibits active transcellular $^{22}Na^+$ transport [85, 86]. The phenomenon of redox sensitivity of the Na/K-ATPase has been demonstrated in different cell types, tissues, and animal species.

In LLC-PK1 cells, both ouabain and glucose oxidase-induced H_2O_2 stimulate Na/K-ATPase signaling as well as direct protein carbonylation of Pro^{222} and Thr^{224} residues of the Na/K-ATPase α1 subunit (α1-carbonylation) [86]. The Pro^{222} and Thr^{224} are located in peptide ^{211}VDNSSLTGESEPQTR^{225} [UniProtKB/*Swiss-Prot No P05024 (AT1A1_PIG)*]. While the α1 subunit is highly conserved among humans, pigs, rats, and mice (the homology is over 98.5%), the identified peptide is 100% identical among these four species. This peptide is located in the actuator (A) domain of α1 subunit, and Pro^{222}/Thr^{224} are highly exposed and facing the nucleotide binding (N) domain of the α1 subunit. Upon ouabain binding, Na/K-ATPase undergoes conformational changes, in which the A domain is rotated to the N

domain favoring an E2-P conformation. The structure-function analysis indicates that these conformational changes may affect binding of the α1 subunit to signaling molecules such as c-Src and PI3K [234]. In addition, the peptide also contains the TGES motif that is the anchor of A domain rotation [234].

Biologically, ROS can oxidize various types of biological molecules including proteins, leading to their functional changes. Through Fenton's reaction, H_2O_2 is reduced to $HO^{\bullet}$ by coupling oxidation of reduced ferrous ion (Fe^{2+}) to ferric ion (Fe^{3+}). This metal-catalyzed oxidation (MCO) process oxidizes proteins by introducing carbonyl groups (such as aldehydes, ketones, or lactams) into the side chains of certain amino acids (such as proline, arginine, lysine, and threonine) that named direct (primary) carbonylation that have been implied in various conditions like chronic renal failure [235–240]. Since Fenton's reaction involves the conversion of H_2O_2 to $HO^{\bullet}$, any specie of ROS with H_2O_2 as an intermediate and/or end product may stimulate the reaction.

Protein carbonylation is reversible (decarbonylation) and may function as a regulatory mechanism of cell signaling [241–244]. We also observed an undefined decarbonylation mechanism, which apparently reverses the carbonylation of the Na/K-ATPase α1 subunit induced by ouabain [86]. The removal of ouabain from the culture medium reverses ouabain-mediated carbonylation, as seen in the reversed Na/K-ATPase ion-pumping activity [116]. Moreover, inhibition of de novo protein synthesis as well as degradation pathway through lysosome and proteasome does not affect this decarbonylation, which is still poorly understood. It is possible that carbonylation modification might stabilize the Na/K-ATPase in a certain conformational status favoring ouabain binding to the Na/K-ATPase α1 subunit and ouabain-Na/K-ATPase signaling. Nevertheless, the underlying mechanism might be physiologically significant since the carbonylation/decarbonylation process could be an important regulator of the RPT Na/K-ATPase signaling and sodium handling.

It is reasonable to propose that carbonylation modification of RPT Na/K-ATPase α1 subunit has biphasic effects. On one hand, physiological and controllable α1-carbonylation stimulates Na/K-ATPase signaling and sodium excretion, rendering salt resistance, whereas on the other hand, prolonged exposure to oxidant stress leads to overstimulated α1-carbonylation and desensitized Na/K-ATPase signaling, increasing salt sensitivity. First, Dahl S rats show considerably higher basal levels of oxidative stress than R rats, and high-salt diets increase renal oxidative stresses that contribute to salt-sensitive hypertension [202–204]. Second, while high-salt diets increase circulating CTS, a high-salt diet (HS, 2% NaCl for 7 days) stimulates the Na/K-ATPase signaling in isolated RPT from Dahl salt-resistant (R) but not salt-sensitive (S) rats (i.e., impaired Na/K-ATPase signaling in S rats) [79]. Third, CTS- and H_2O_2-mediated redox-sensitive Na/K-ATPase signaling and α1-carbonylation are involved in this signaling process, in a feed-forward mechanism [86]. Fourth, high but not low concentration of NAC is able to prevent α1-carbonylation and Na/K-ATPase signaling [86]. Even though it is still not clear of the carbonylation/decarbonylation process, this could be another new regulatory mechanism of Na/K-ATPase signaling. It is reasonable to postulate that prolonged excessive α1-carbonylation (by CTS and/or other factors) might overcome the decarbonylation capacity, leading to the desensitization or termination of the Na/K-ATPase signaling function. This is reminiscent of the observations in clinical trials using antioxidant supplements. The beneficial effect of antioxidant supplements is controversial and not seen in most clinical trials with administration of antioxidant supplements [200, 245]. Low doses of antioxidant supplementation may be ineffective, but high doses may be even dangerous since excess antioxidants might become prooxidants if they cannot promptly be reduced in the antioxidant chain [246]. It appears that the balance of the redox status, within a physiological range, may be critical in order to maintain beneficial ROS signaling.

10. Endocytosis of Na/K-ATPase and NHE3 in salt sensitivity

In male Sprague-Dawley rats, compared to a normal salt (0.4% NaCl, 7 days) diet, a high-salt (4% NaCl, 7 days) diet increased urinary sodium and MBG excretion. In isolated proximal tubules, a high-salt diet inhibits the Na/K-ATPase ion-exchange activity and enzymatic activity, which is accompanied by a decreased Na/K-ATPase α1 content in heavy membrane fraction and an increased Na/K-ATPase α1 content in both early and late endosomes. These high-salt diet-mediated changes were ameliorated by administration of an antibody against MBG [156]. Results indicate that a high-salt diet increased MBG production, activated RPT Na/K-ATPase signaling, and induced endocytosis of Na/K-ATPase.

The Dahl R and S rat strains were developed from Sprague-Dawley rats by selective breeding, depending on the resistance or susceptibility to the hypertensive effects of high dietary sodium [247]. In these two strains, the RPT sodium handling is an essential determinant of their different BP responses [248–251]. At the cost of elevated systolic BP, Dahl S rats get rid of excess sodium primarily via pressure natriuresis. In contrast, Dahl R rats get rid of excess sodium primarily via a significant reduction of renal sodium reabsorption without increasing the BP. *In* vivo study indicates that impaired RPT Na/K-ATPase signaling appears to be causative of experimental Dahl salt sensitivity [79]. In vivo studies with Dahl R and S rats (Jr strains) demonstrated that impairment of RPT Na/K-ATPase signaling is a causative factor of experimental Dahl salt sensitivity [79]. In Dahl R but not S rats, a high-salt (2% NaCl, 1 week) diet activated RPT Na/K-ATPase signaling and stimulated coordinated redistribution of the Na/K-ATPase and NHE3, leading to increased total and fractional urinary sodium excretion as well as normal BP. However, there are still questions about the underlying mechanism(s) that need to be further investigated, such as the difference of Na/K-ATPase signaling function between Dahl R and S rats, as well as the translation of Na/K-ATPase signaling to NHE3 regulation. Furthermore, low concentration of ouabain causes hypertrophic response both in the heart and kidney, by concentrating the Na/K-ATPase, Src, EGFR, and MAPKs within rat caveolae, and activates the Na/K-ATPase/Src/MAPK signaling pathway [64]. However, there is no simple explanation for this occurrence. First, the α1 subunit is essentially the only α isoform expressed in RPT, and genes coding α1 subunit and NHE3 (in rat chromosomes 1 and 2, respectively) are not located in identified and/or proposed BP quantitative trait loci [252]. Second, there is no difference in α1 gene (*Atp1a1*) coding [251], α1 ouabain sensitivity [253], and α1 expression [79] between these two strains. Third, acute salt loading increases circulating CTS (ouabain and MBG) in both S and R rats [162]. These observations suggest that there must be resistance to CTS signaling in the Dahl S rat, a phenomenon that we only partially understand. As discussed above, the carbonylation/decarbonylation process could be another new regulatory mechanism of Na/K-ATPase signaling. It is reasonable to postulate that prolonged excessive α1-carbonylation in Dahl salt-sensitive rats might overcome the decarbonylation capacity, leading to desensitization or termination of the Na/K-ATPase signaling function.

11. Perspective

As pointed out by Guyton many years ago [254], the kidney is the most important organ in the regulation of Na^+ handling and BP. Dietary salt intake *vs.* renal sodium handling is a key determinant of long-term BP regulation and plays an important role in the pathogenesis of hypertension, with more pronounced effects seen in salt-sensitive patients. Consequently, modest restriction of dietary salt and

diuretic therapy are often recommended for the treatment of resistant hypertension, particularly with the salt-sensitive subgroup [254–258].

Although the relationships among CTS, renal Na^+ handling, and hypertension were proposed many years ago, there has been an explosion of reports supporting this idea. As discussed, reports from Lingrel's laboratory clearly demonstrated a specific role of the isoforms of the Na/K-ATPase and its interaction with endogenous CTS in the regulation of Na^+ excretion and BP in intact animals [75–77]. From the ligand perspective, studies have demonstrated that CTS are present in measurable amounts under normal physiological conditions and that several disease states are associated with elevations in the circulating levels of CTS. The new concept that the Na/K-ATPase has an ion-pumping-independent receptor function (induced by both CTS and ROS) that can confer the agonist-like effects of CTS on intracellular signal transduction is a new mechanism for RPT sodium handling. Moreover, this newly discovered signaling mechanism operates in intact animals in response to CTS stimulation. The Na/K-ATPase has recently emerged as a therapeutic target [259, 260]. A clearer understanding of the mechanisms, in which a CTS-ROS-Na/K-ATPase signaling axis counterbalancing salt retention, would not only have major pathophysiological and therapeutic implications, but also further explain the progressive impairment of renal sodium handling under excessive oxidative stresses such as hypertension, aging, obesity, and diabetes.

Funding

This work was supported by NIH R15 1R15DK106666 (to J. Liu) and NIH RO1 HL071556 (to J.I. Shapiro).

Abbreviations

BP	blood pressure
CTS	cardiotonic steroids
NHE3	sodium/hydrogen exchanger isoform 3
ROS	reactive oxygen species
RPT	renal proximal tubule

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Chapter 6

The Effect of Dietary Sodium Restriction on Vascular Stiffness in Hypertension

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Abstract

Increased salt consumption is believed to induce high blood pressure (BP)-mediated organ damage, although it is not yet clear whether it reflects a generalized micro- and macrovascular malfunction independent of BP. Exceeding dietary sodium intake is acknowledged to be the main modifiable environmental risk factor for cardiovascular events that accounts for an increase in blood pressure and induces hypertension (HTN)-related target organ damage. Arterial stiffness is well known as an independent cardiovascular risk factor, and sodium intake may be a determinant of arterial stiffness. Even so, the studies that investigated the effect of dietary sodium reduction intake on arterial stiffness in humans provided inconclusive results. Therefore, we aim to perform a review of the available evidence of salt restriction and arterial stiffness and its impact on hypertensive patients.

Keywords: salt intake, dietary sodium, arterial stiffness, blood pressure, hypertension

1. Introduction

Hypertension (HTN) is a significant risk factor for cardiovascular disease (CVD), a *major cause of premature death worldwide*, and has been identified as one of the strongest risk factors in the global burden of disease [1, 2]. Hypertension guidelines frequently recommend salt reduction as an important simple strategy to reduce high blood pressure (BP) [2–4]. This recommendation is usually extended to individuals with normal BP as well as those at risk of becoming hypertensive [5].

The pressure-natriuresis mechanism that was first described by Guyton et al. [6] proposes a linkage between dietary sodium intake and renal sodium handling. This hypothesis says that, in normal individual, the consumption of high amounts of sodium in the diet will cause a transient increase in BP that promotes a higher excretion of sodium by the kidney. The kidney excretes the excess of sodium, leading to normal blood pressure restoration. This hypothesis elucidates how blood pressure is sustained over the time, although the daily variation in sodium intake is reported among most individuals [6–8].

Excess dietary sodium consumption has several known detrimental effects on blood pressure [8–10] and has been associated with a higher risk of stroke and renal impairment [11, 12]. Accordingly, there is a strong evidence from randomized controlled trials that a moderate reduction in dietary salt intake safely and effectively reduces BP and urinary albumin excretion rate both in hypertensive and diabetic patients [13, 14]. Likewise, evidence from epidemiological and clinical studies also suggested an association between regular dietary salt intake and pulse wave velocity (PWV) [15–17].

This association between dietary salt consumption and pulse wave velocity is also supported by experimental evidence in animal models of structural and functional changes caused by high salt regimens on the arterial wall above and beyond the effect of high BP [18–20]. These changes on arterial wall are believed to be induced by both reduced bioavailability of nitric oxide and a deficient response of the local renin-angiotensin system to high sodium consumption [21]. Some interventional studies in man have investigated the effect of reduction in salt intake on arterial stiffness, but their results were not conclusive mainly because of the low statistical power of most of them [22–25].

Results from a systematic review and meta-analysis of the available clinical trials testing the effect of sodium intake restriction on PWV as a proxy for arterial stiffness, with null hypothesis being that restriction of sodium intake does not affect arterial stiffness, indicated that restriction of dietary sodium intake reduces arterial stiffness. The authors have suggested that this effect seems to be at least in part independent of the changes in blood pressure [26].

To date, the evidence on the effects of dietary sodium restriction on pulse wave velocity is still conflicting. Accordingly, this review aims to contribute to increase the knowledge about the effects of sodium restriction on arterial stiffness in the context of hypertension.

2. Effects of sodium reduction on blood pressure

High sodium intake is linked to a higher risk of stroke, left ventricular hypertrophy, and renal impairment and can impair the arterial vasculature and endothelial function [10, 22, 27–29]. A moderate reduction in dietary sodium to achieve a sodium intake between 1.5 and 2.3 g/day may be cardioprotective independent of the BP pathway, but the evidence is not conclusive in this regard. It also may not be safe to recommend sodium restriction in older adults with diabetes or those with established CVD [3].

Evidence indicates that reducing sodium intake significantly lowers blood pressure in both men and women. Sodium is found not only in table of salt but also in a variety of foods, including cream, eggs, milk, shellfish, meat, and many other processed foods. The World Health Organization (WHO) recommendations indicate a reduction in sodium intake to lower blood pressure and risk of stroke, cardiovascular disease, and coronary heart disease in adults [30].

The current recommendations in most countries around the world are to reduce salt intake from about 9–12 g/day to 5–6 g/day [31]. Much evidence supports that such a reduction in salt intake lowers blood pressure. The WHO recommends a reduction to <2 g/day sodium (5 g/day salt) in adult individuals [30, 31].

The effects of sodium reduction on blood pressure have been evaluated in many studies. In their study that evaluated the effect of sodium reduction on blood pressure, D'Elia et al.'s [26] pooled analyses showed a significant reduction of both systolic blood pressure (SBP) (mean difference, −5.82 mmHg;

95% IC, −8.42 to −3.43 mmHg) and diastolic blood pressure (DBP) (mean difference, −2.75 mmHg; 95% IC, −3.67 to −1.87 mmHg) upon the reduction of sodium intake.

Evidence also shows that a modest reduction in salt intake for a couple of weeks (4 or more weeks) causes important and significant lowering in blood pressure levels in both normal and hypertensive individuals, independent of sex and ethnicity [13]. Salt reduction is linked to a mild increase in noradrenaline, aldosterone, and plasma renin activity, and no significant change in lipid concentrations. These results may support a reduction in population salt consumption, which would lower population mean blood pressure and thereby reduce cardiovascular risk outcomes. In their meta-analysis, He et al. [13] have shown that a modest reduction in salt intake, as currently recommended, has a significant effect on blood pressure both in individuals with hypertension and in those with normal blood pressure. The fall in blood pressure is seen in white and black individuals irrespective of their gender. These findings provide additional support for reducing salt intake in the population.

3. Effect of sodium intake reduction on pulse wave velocity

Evidences suggest that salt intake plays an important role on blood pressure regulation, and it is also suggested a direct effect of salt on large artery wall that modulates vascular stiffness [14].

Pulse wave velocity is known to be associated with BP and age [26, 32, 33]. In a recent study where the authors have evaluated the effect of salt restriction on pulse wave velocity, no significant statistical differences were detected. Although sodium restriction reduced SBP and DBP in the combined analysis of all studies, results of the meta-regression analysis, however, indicated that the effect of salt restriction on arterial stiffness did not depend on changes in blood pressure. In fact, in one of the studies included in the meta-analysis, there was a significantly greater reduction in pulse wave velocity in black than white and Asian hypertensive patients, despite the fact that the three ethnic groups had similar reductions in blood pressure [34].

Another study evaluating the long-term sodium restriction showed an improvement in arterial stiffness independently of the changes in BP [35]. The authors did not detect a dose dependence in the pooled association between salt restriction and reduction of PWV. Accordingly, the lack of a dose-related effect in the range of salt reduction applied in the available studies might be the possible cause of these results.

In subgroup analyses of nine cohorts that evaluated the effect of sodium restriction on PWV including prehypertensive and/or hypertensive participants, whereas in the five cohorts that enrolled non-hypertensive individuals, an inverse trend was detected, but this difference was not statistically significant. A larger effect of sodium reduction on PWV was also seen in the three cohorts that included hypertensive patients under antihypertensive treatment (5.07%) than in the cohorts enrolling untreated normotensive or prehypertensive individuals (1.70%): again, however, this difference was not statistically significant [14].

More recently, a study using a hypothetical model to analyze the association between salt intake and PWV (carotid-femoral) through direct and mediating pathways that aimed to investigate whether the association between salt intake and arterial stiffness also has a sex-specific pattern has demonstrated that high salt intake has a direct and independent effect increasing on arterial stiffness regardless of sex. The authors also concluded that the association between salt intake and arterial stiffness is more dependent on BP in normotensive women than it is

in normotensive men. As stated by the authors, these results highlight the need for a sex-specific approach in the evaluation of cardiovascular risk associated with dietary habits [36].

Furthermore, Grigorova et al. [37] have demonstrated that high salt intake was associated with an increase in Na/K-ATPase inhibitor marinobufagenin (MBG) levels, and an activation of the transforming growth factor-beta (TGF-β) mediated pro-fibrotic pathway in the vasculature, leading to an increase of aortic stiffness without elevation of BP. MBG activated TGFβ1 pro-fibrotic pathway in cultural vascular smooth muscle cell (VSMC), indicating a fundamental role of MBG in the development of fibrosis via the Na/K-ATPase signaling function. The decrease in salt consumption restored the aortic elasticity through inactivation of the TGF-β pathway. Therefore, decreasing salt consumption can improve vascular elasticity and lower the risk of cardiovascular disease by MBG level reduction [37].

4. Renal sodium handling, blood pressure, and vascular compliance

The relationship between sodium intake and blood pressure regulation has been suggested through animal experiments indicating that a high-sodium diet, at their initial phase, leads to volume expansion and cardiac output increases. Based on experiments including mainly nephrectomized dogs that received a large amount of saline solution daily for 2 weeks, Guyton [7] suggested that the BP increases mainly through two mechanisms: (1) volume expansion and cardiac output increases and (2) an autoregulatory mechanism that affects the vessel resistance. Accordingly, the hypothetic mechanism on how dietary salt increases blood pressure includes Guyton's main theory that the increase in blood pressure is initially associated with an increase in extracellular fluid and blood volumes [7, 15, 26]. According to Guyton's hypothesis, in the hypertension pathophysiology, irrespective of the causal factor, the pressure-natriuresis relationship in the kidney is always involved, with higher blood pressures being required to eliminate a higher given sodium load [38]. However, it has never been demonstrated that measurements of extracellular fluid volume in hypertensive individuals are modified consistently. All of the authors found that the volumes of extracellular fluid and exchangeable sodium were normal in hypertensive individuals [39, 40].

The only similitudes were the lower ratio between intravascular and interstitial fluid volumes and smaller plasma volume, indicating unbalanced division in hypertensive patients [39]. Additionally, high levels of atrial natriuretic peptide hormone, lower levels of plasma renin, and an increased capacity of plasma to inhibit Na^{+}/K^{+}-ATPase were observed in these patients [15, 41, 42]. In fact, all these apparent paradoxes can be easily understood when we observe that, in hypertensive patients, if total vascular compliance is reduced, a slight decrease in intravascular volume can be too large for the capacity of the corresponding vascular space [39].

Vascular compliance establishes the volume-pressure relationship or the volume within a vascular segment and the blood pressure that is generated by the presence of that blood volume. It is simply the basic concept of compliance applied to a vascular segment and represents a classic index of the elasticity of the intravascular compartment, from the slope of the curve plotting changes of blood volume (ΔV) versus changes of intravascular pressure (ΔP) [6].

Within a narrow range of volume and pressure changes, the linear relationship curve between both variables is used to define the compliance as the slope $\Delta V/\Delta P$ [6]. In a clustered circulation model, the vascular compliance expresses the sum of complacencies of all vascular segments, including arteries and veins [43]. Accordingly, Guyton [6] has defined "total" vascular compliance based on

experimental animal models as the relationship between the mean circulatory filling pressure (MCFP) and blood volume. The MCFP is the pressure that is registered throughout the entire circulatory system if the heart is suddenly stopped and the blood volume is redistributed entirely in the vascular system taking into account the capacity of the vessels. Vascular compliance is then defined as the product of volume change and mean circulatory filling pressure. However, as the MCFP measurement implies the presence of a non-beating heart, in humans, it cannot be measured. Therefore, an alternative index of the capacitance function had to be defined.

Evidence from studies in both animals and humans has shown that similar indices are observed in cases where the circulation is not interrupted between blood volume changes and pressures measured throughout the different parts of the venous system [6, 37]. For example, the relationship between rapid blood volume expansion (ΔV) and central venous pressure (ΔCVP) with a plasmatic expander such as dextran has the dimensions of compliance [39, 44]. The "true" vascular compliance measured from ΔV/ΔMCFP ratio is also called "effective" total vascular compliance to differentiate it from the ΔV/ΔMCFP ratio [39, 45].

Compared to the normotensive patients, the slope of the curve plotting blood-volume versus central venous pressure (ΔV/ΔCVP) is markedly lower in hypertensive subjects [39, 45]. On the other hand, there are no changes in curves of cardiac output versus blood volume expansion, indicating that the cardiac function is maintained despite cardiac structural modifications in hypertensive individuals. At these conditions, compared to non-hypertensive individuals, in hypertensive patients, the central venous pressure increases more, and the decrease in the effective compliance of the vascular bed is pointed out as the responsible factor for this phenomenon [46, 47].

Evidence suggests that renal sodium handling is the main factor influencing the level of intra- and extrarenal blood pressure and is regulated by complex physiological and inflammatory mediators, hormones, and the sympathetic nervous system [48]. Therefore, a compromised kidney capacity to eliminate sodium in response to increased blood pressure is a major factor for a sustained increase in blood pressure, irrespective of the primary cause.

The changes registered in plasma sodium levels exert their effects on the vascular system, affecting not only the small resistance arteries but also the large artery properties leading to an increase in arterial stiffness and consequent decrease in vascular compliance [49].

Both observational and longitudinal studies have suggested that lower sodium intake is associated with lower wave pulse velocity. Evidence suggests that in hypertensive patients, a low sodium intake is associated with a larger brachial artery diameter than that seen with a high sodium intake [50]. A sodium overload reduces arterial distensibility and compliance irrespective of blood pressure changes in hypertension in the elderly and in severe hypertension patients with end-stage renal disease [51].

5. Sodium-induced change in arterial stiffness and BP

A considerable body of evidence has shown major links along with cause-and-effect relationships between salt intake and BP [15, 16, 52]. In many studies, both SBP and DBP had a similar effect regarding their action on the arterial wall, regardless of the presence of a high-sodium diet or not. Remarkably, many observational studies suggest a special role of systolic blood pressure, which, until recently, was rarely considered.

Evidence from studies of genetic models of animal hypertension suggested that long-term high sodium intake is associated with increased intima-media thickness due to the extracellular matrix (ECM) development and aortic hypertrophy regardless of blood pressure. These changes caused by high sodium consumption and often associated with increased arterial stiffness and changes in smooth vascular cells properties are reversed by reducing sodium and/or giving diuretics [16, 53].

Hormonal counterregulatory mechanisms that modulate arterial changes act chronically in the presence of a high-sodium diet because bradykinin β2-receptor blockage by Hoe-140 (selective B_2 bradykinin receptor antagonist that suppresses the effects of bradykinin) produces more carotid hypertrophy, while in case of normal sodium intake, less aortic collagen accumulates due to AngII-specific type 1 receptor activity [54, 55].

Several studies have consistently established an independent correlation between sodium dietary intake, arterial stiffness, and blood pressure, regardless of whether systemic, regional, or local determinations were present [16, 56]. In a study addressing the relationship between sodium intake and arterial stiffness based on the Chinese populations, Avolio et al. [57] have found that sodium intake has an independent effect on arteriolar tone and arterial wall properties, with the former indirectly and the later directly contributing to increased arterial stiffness with age. In the same study, the comparison of salt intake between urban and rural subjects, as determined by urinary sodium excretion, was greater in the urban subjects (13.3 g NaCl/day) than in rural ones (7.3 g NaCl/day). This difference was related to higher arterial stiffness and hypertension prevalence and lesser vascular compliance in urban subjects. Salt intake had, therefore, an independent effect on arterial structural and functional properties, with the arterial wall directly and arterial tone indirectly contributing to increased PWV with age [16]. Another study from Australia involving young and middle-aged (20–66 years old) normotensive subjects on a low-salt diet who were compared with age- and BP-matched subjects on a normal-sodium diet showed adult subjects on a low-sodium diet have lower arterial stiffness independent of blood pressure [35].

On the other hand, the benefits of a low-salt diet on blood pressure seem to be greater in hypertensive patients than in normotensive ones. A systematic review carried out by Graudal et al. [58] that included 185 randomized controlled trials found that sodium reduction from an average high usual sodium intake level (201 mmol/day) to an average level of 66 mmol/day, which is below the recommended upper level of 100 mmol/day (5.8 g salt), resulted in a small decrease (1/0 mmHg) in systolic and diastolic BP in white normotensive patients and a decrease in systolic and diastolic BP of 5.5/2.9 mmHg in white hypertensive patients. The decrease of blood pressure was even greater in black and Asian populations.

Moore et al. [59] in their study addressing the effect of low sodium intake on the blood pressure levels among Framingham Offspring Study adults even found paradoxical results. These authors analyzed dietary data from 2632 subjects (normotensive men and women) aged 30–64 years old who were part of the Framingham Offspring Study. Over 16 years of follow-up, systolic and diastolic blood pressures decreased with increasing sodium intake (≥2.5 g). Mean systolic and diastolic blood pressures of 129.5 mmHg and 75.6 mmHg, respectively, were seen among subjects in the high-sodium and high-potassium (≥2.3 g) groups compared with 135.4 mmHg and 79.0 mmHg, respectively, among people in the low-sodium (<2.5 g) and low-potassium (<2.3 g) groups.

6. Possible mechanisms of the effect of sodium intake reduction on arterial stiffness

Animal studies of hypertension demonstrate that elevated salt consumption can increase arterial stiffness and this effect is independent of BP as reviewed by Safar et al. [52]. On the other hand, reduced dietary sodium has been shown to lower arterial stiffness in humans with hypertension [27, 60]. Cross-sectional studies in humans have provided evidence of an independent effect of salt on arterial stiffness.

Experimental studies on animal models did show that changes in sodium intake have effects on arterial structure and function independent of blood pressure [18, 61]. Studies carried out in normotensive rats have shown that these changes might be associated with increased production of transforming growth factor-beta 1 (TGF-β1), decreased endothelial nitric oxide synthase expression, and reduced bioavailability of endothelial nitric oxide induced by a high-salt diet [18, 37, 61–63]. TGF-β is a family of three pleiotropic growth factors that have complex effects on cell growth and differentiation and organ development, but they are particularly important in the expression of extracellular matrix proteins and vascular and renal fibrosis promotion in a variety of disease states [64]. The TGF-β1 is considered the most important mammalian TGF-β family member synthesized by many cell types including endothelium. It is secreted by endothelium acting, basically, on adjacent vascular smooth muscle and seems to be involved in blood pressure regulation [37].

The reduction of salt intake in the diet can affect the vascular properties by reducing the production of TGF-β. The study by Grigorova et al. [37] has investigated whether high salt intake stimulates the production of MBG, an endogenous steroidal Na^+/K^+-ATPase ligand which activates transforming growth factor-beta pro-fibrotic signaling in young normotensive rats and whether these changes can be reversed by reducing salt to a normal salt level. Their data have suggested that a decrease in salt consumption could help to restore vascular properties such as the aortic elasticity and lower the risk of cardiovascular disease by reducing the production of the pro-fibrotic factor MBG.

The local renin-angiotensin-aldosterone system (RAAS) is believed to be one of the most important mediators of vascular wall elasticity at the heart, vessels, and kidneys [19, 65]. At the cardiovascular system, high sodium intake increases the AT1 receptor expression and promotes vascular damage [19]. Evidence from experimental studies has shown that there was a decrease in aortic collagen accumulation and improvement of vascular, cardiac, and renal function and an AT1 receptor blocker during the high salt intake diet [12, 19, 66]. Additionally, high sodium intake has been reported to increase the vascular angiotensin-converting enzyme levels, which opposes to the effects of concomitant renin suppression [67]. Studies carried out in hypertensive subjects found gene polymorphisms of aldosterone synthase enzyme and AT1 receptor that was significantly associated with higher PWV [68, 69].

Stocker et al. [70] in their study discussing the recent evidence to support the role of plasma or cerebrospinal fluid hypernatremia as a key mediator of sympathoexcitation and elevated blood pressure have found that both experimental and clinical studies suggested that a high dietary salt increases plasma and cerebrospinal fluid sodium concentration. Sodium concentration variation modulates the sympathetic neurons in rostral medulla by activating the osmoreceptors in the rostral nervous system that is responsible for the tone of basal sympathetic vasomotor [70–73].

The evidence also point outs the high dietary salt intake is one of the most important factors to the activation of the sympathetic nervous system, which is one of the main contributing factors for the pathogenesis of salt-sensitive hypertension [69, 74]. Although not well understood, it is proposed that increased salt intake causes salt retention and raises plasma sodium chloride concentrations, which activates sodium/osmoreceptors to trigger sympathoexcitation [75]. Some studies have suggested that vascular properties such as arterial compliance may be affected by sympathetic nervous activity, independently of its effects on BP [76]. Therefore, the reduction of salt intake decreases the excitability of the sympathetic nervous system and interferes with its effects on vascular properties reducing the arterial stiffness.

7. Conclusions

Health recommendations and most clinical studies have been focused on the adverse effects of salt dietary on blood pressure. However, evidence to support a deleterious effect of dietary salt on endothelial function and arterial stiffness independent of BP is increasing. The mechanisms responsible continue to be elucidated. Endothelial dysfunction and increased arterial stiffness are predictors of cardiovascular disease, and data from clinical trials have indicated that both are associated with incident hypertension. Therefore, reducing excessive salt intake in the diet should be considered important for overall vascular health in addition to blood pressure control.

Conflict of interest

The authors declare no conflict of interest.

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Section 3

Biochemistry, Cellular and Molecular Biology

Chapter 7

HIF Pathways in Clear Cell Renal Cancer

Olivia Lombardi and David Robert Mole

Abstract

Clear cell renal cancers (ccRCC) are characterized by inactivation of the VHL (von Hippel–Lindau) tumor suppressor. Work leading to the 2019 Nobel Prize for Physiology or Medicine has shown that this is central to cellular oxygen-sensing, orchestrated by the HIF (hypoxia-inducible factor) transcription factors. These regulate hundreds of genes that underpin many hallmarks of cancer, including angiogenesis, cellular energetics, cell proliferation, resisting cell death, and avoiding immune destruction. However, HIF also promotes processes that are detrimental to cancer cells. Therefore, the overall consequence of HIF pathway activation is a balance of these influences. We explore how variations in the HIF pathway during tumorigenesis alter this balance to promote ccRCC formation.

Keywords: cancer, kidney, renal, clear cell, von Hippel Lindau, VHL, hypoxic, hypoxia-inducible factor, HIF

1. Introduction

Kidney cancer is the seventh most common malignancy in the Western world. In 2018, there were approximately 400,000 new kidney cancer cases and 180,000 kidney cancer-associated deaths worldwide [1]. The underlying causes of kidney cancer are complex and incompletely understood, although genetic factors (both inherited and somatic genetic mutations) are known to drive the disease. Additionally, certain lifestyle choices (such as smoking and a high protein diet) increase the risk of developing kidney cancer, consistent with its prevalence in the Western world. Unless surgically resectable, kidney cancer is largely incurable, and the 5-year survival rate for those with metastatic disease is only about 10% [2]. Systemic anti-cancer therapies, including those that inhibit the vascular response or enhance patients' immune response to the malignancy, have offered some hope [3]. However, these treatments confer limited efficacy and a considerable burden of toxicity. Therefore, there is a pressing need to better understand the drivers of kidney cancer in order to identify novel therapeutic strategies.

2. Histological subtypes of renal cancers

The most common form of kidney cancer is clear cell renal cell carcinoma (ccRCC), which arises from the adult renal tubular epithelium and accounts for approximately 75% of all kidney cancer cases. This subtype is termed as such due to the characteristic 'clear' cytoplasm of malignant cells observed histologically. This is

caused by accumulation of excess glycogen and lipid in the cytoplasm (due to highly dysregulated metabolic pathways), which are dissolved by the tissue fixation process [4]. Other less common subtypes of adult renal cancers that also arise from the tubular epithelium include papillary RCC (types 1 and 2); chromophobe RCC; and oncocytoma. Each subtype is associated with different histological features, genetic drivers, and clinical behaviors. Rarely, cancers can arise from other cell types in the adult kidney, including transitional epithelial cells of the ureter and renal pelvis (giving rise to transitional cell carcinoma) and various mesenchymal cell types (e.g. interstitial cells, giving rise to renomedullary interstitial cell tumors). Although childhood kidney cancer is generally rare, the most common form is Wilms tumor, which originates in developing tubular cells during fetal development [5].

It should be noted that even within a specific renal cancer histological subtype there is evidence for substantial heterogeneity, which has initiated efforts to further refine subtype classification based on additional features. Recent studies have found that ccRCC can be further stratified based on architectural, cytological and microenvironmental features, and that these features can predict patient outcome and response to therapy [6]. The underlying cause of this variation remains to be determined but could be due to certain genetic or epigenetic differences between ccRCC tumors. Consolidation of histological and molecular heterogeneity in ccRCC will be important for disease subclassification, as well as better understanding ccRCC biology, going forward.

3. VHL syndrome

Each kidney cancer subtype is associated with its own monogenic cancer syndrome [7]. Studying these rare family kindreds has provided unique insight into the genetic mechanisms underlying both inherited and sporadic cancers. In particular, clear cell renal cancer is associated with VHL syndrome, which is an autosomal dominant disorder, affecting 1 in 32,000 individuals, caused by heterozygous germline mutations of the VHL gene [8, 9]. As well as ccRCC, VHL syndrome is associated with a limited number of other tumors types, including hemangioblastomas of the retina and the central nervous system; pheochromocytomas; pancreatic lesions; endolymphatic sac tumors and epidydimal cysts [8, 9]. VHL syndrome can be further sub-divided according to which of these different tumor types develop in individuals within the kindred [10, 11]. Four distinct patterns have been identified: type 1 VHL disease, which is associated with hemangioblastoma and ccRCC; type 2A, which is associated with hemangioblastoma and pheochromocytoma; type 2B, which is associated with hemangioblastoma, pheochromocytoma and ccRCC; and type 2C, which is associated with pheochromocytoma alone. Each of these subtypes is linked to particular types of VHL mutation, which have been shown to have different downstream biological effects [12–17].

4. The VHL gene

The human VHL gene was first identified following classical linkage analysis of families with VHL syndrome and was cloned in 1993 [18]. In humans it is located on the short arm of chromosome 3 (3p25) and has three exons that encode a protein of 213 amino acids, with a molecular weight of around 30 kDa (termed p30). However, the gene also contains a second translation start site at codon 53, leading to the generation of a shorter protein of approximately 19 kDa (termed p19), which appears to retain canonical activity [19]. As a consequence, oncogenic mutations, most

typically single-nucleotide variants (SNVs) or short insertion/deletions (indels), are restricted to codons 53–213 in exons two and three.

VHL acts as a tumor suppressor gene [20, 21]. ccRCC and other cancer types are associated with inactivating mutations of VHL, which lead to loss-of-function of the gene product (termed pVHL). Although autosomal dominant at the level of the individual, both alleles of the VHL gene must be inactivated in a cell for cancer to develop, in line with Knudson's two-hit hypothesis [22, 23]. Since VHL syndrome is caused by germline VHL mutation, all cells of the affected individual harbor this mutation. The remaining wild-type (WT) allele is somatically inactivated in the tumor progenitor cell, which then multiplies to form the cancer [20, 21]. Typically, somatic inactivation of the WT allele occurs as a result of an arm-level loss of chromosome 3p (**Figure 1**), although promoter hypermethylation or a second SNV/indel may also cause complete loss of functional VHL in the cell. Furthermore, since the cells of patients with VHL syndrome only require one somatic mutation to become functionally deficient in VHL, it is a relatively common event, accounting for the high tumor penetrance in these individuals. Indeed, over the course of their lifetime, these individuals often develop multiple tumors and close examination of their organs often reveals the presence of numerous synchronous tumors. However, VHL mutation is only associated with the very limited range of cancers outlined above, despite it being ubiquitously expressed. Therefore, VHL only appears to act as a tumor suppressor gene in very few tissues. Indeed, even within the kidney, ccRCCs appear to develop from a subset of proximal tubular cells [24]. It is assumed that somatic mutations in the wild-type copy of VHL do occur in other cell types, but it is not known whether these cells are eliminated by other tumor suppressor mechanisms, or simply fail to progress to overt cancer.

Importantly, VHL is also inactivated in the vast majority (approximately 90%) of sporadic ccRCC tumors, which occur in patients without a germline mutation in the VHL gene [25]. In order to develop cancer, these individuals require two somatic

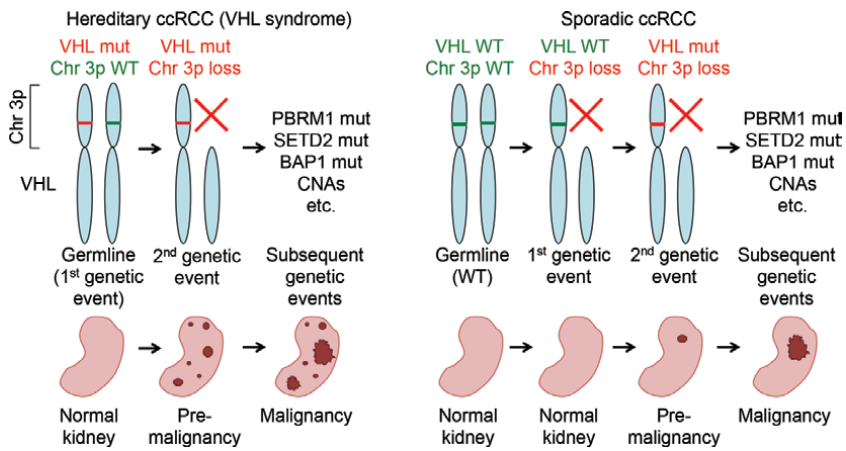

Figure 1.
VHL inactivation in ccRCC. Individuals with VHL syndrome are predisposed to ccRCC (termed hereditary ccRCC) as a result of a heterozygous germline VHL mutation. The second, wild-type allele is subsequently inactivated by somatic loss of chromosome 3p, resulting in biallelic VHL inactivation. On the other hand, in sporadic ccRCC, two somatic events are required for biallelic inactivation. Typically, one copy of chromosome 3p is lost followed by inactivation of the second VHL allele through mutation or promoter hypermethylation. Although the ordering is reversed, the same genetic aberrations are observed in both sporadic and hereditary ccRCC. However, because only one somatic event is required for biallelic VHL inactivation in patients with VHL syndrome, this is a much more likely event and occurs in multiple cells within the kidney, causing many pre-malignant lesions and multiple ccRCC tumors. chr= chromosome; CNAs= chromosomal copy number alterations; mut= mutation; WT= wild-type.

events affecting both copies of the VHL gene in the same cell (**Figure 1**). As a result, this occurs much less frequently, accounting for the much lower overall prevalence of ccRCC in the general population of about 1%. However, in contrast to VHL syndrome, the order of events is typically reversed, with loss of chromosome 3p frequently occurring first and the remaining copy then being inactivated by single-nucleotide substitution (SNV) or small insertions or deletions (indels) [26].

Of note, although biallelic VHL inactivation is required for ccRCC (and other tumors) to develop, it does not appear to be sufficient on its own (**Figure 1**). Mitchell et al. have estimated that in sporadic ccRCC, VHL inactivation predates tumor formation by a number of years or even decades [26]. Consistent with this, examination of the kidneys from patients with VHL syndrome has identified multiple isolated VHL-defective cells, which may be present as single cells or small non-invasive cysts [27, 28]. Furthermore, *in vitro*, inactivation of VHL leads to cellular senescence rather than unrestricted proliferation [29, 30]. Therefore, it is thought that additional gene mutations are required for these early VHL-defective lesions to develop into mature ccRCC. Indeed, more recently, additional somatic mutations have been identified in ccRCC [25, 31–33]. Most notable among these are inactivating mutations in the PBRM1 (polybromo 1), SETD2 (SET domain-containing 2) and BAP1 (BRCA-associated protein 1) tumor suppressor genes, mutation of which typically follows loss of VHL. Importantly, these three genes also reside on the short arm of chromosome 3. As a result, the loss of chromosome 3p frequently observed in both familial and sporadic ccRCC can simultaneously result in copy loss of all 4 of these ccRCC-associated tumor suppressor genes; VHL, PBRM1, SETD2 and BAP1.

5. Function of pVHL

Following identification and cloning of the VHL tumor suppressor gene, its sequence did not immediately suggest a function for the protein. However, early immunoprecipitation experiments indicated that pVHL forms a complex with elongin B and elongin C [34]; cullin 2, a member of the Cdc53 family of proteins [35]; and the RING-box protein Rbx1 [36, 37]. Importantly, the binding of pVHL to elongins B and C could be blocked by specific ccRCC-associated mutations in the VHL gene, strongly suggesting that these two proteins contribute to the tumor suppressor activity of VHL [34]. The subsequent identification of mutations in the TCEB1 gene, which encodes elongin C, in ccRCC tumors that have wild-type VHL further emphasizes the importance of this complex in ccRCC formation [25, 32, 38].

Elongins B and C, cullin 2 and Rbx1 are all components of an E3-ligase complex that adds polyubiquitin chains to specific proteins and thus targets them for degradation by the proteasome [39, 40]. This suggested that pVHL might act as the recognition component of a pVHL ligase complex. In a separate line of work, dysregulation of the hypoxia-inducible factor (HIF) transcription factors had been identified in VHL-defective ccRCC cells [41]. It was subsequently shown that pVHL directly interacted with HIF, leading to polyubiquitination and subsequent proteasomal degradation of its alpha-subunits [42, 43]. Again, the pVHL-HIF interaction could be blocked by specific ccRCC-associated mutations in VHL, leading to overexpression of HIF and underlining the importance of HIF in the development of ccRCC [42]. Importantly, this interaction was not only altered by pathogenic VHL mutations but was also regulated in an oxygen-dependent manner [44, 45]. This indicated that the pVHL-HIF interaction was integral to the mechanism of cellular oxygen-sensing.

The central role of HIF in ccRCC biology has been further underscored in numerous studies. In particular, in xenograft and transgenic mouse models of

VHL-defective ccRCC, tumor growth is dependent upon the presence of HIF [46–51]. Specifically, tumor growth is dependent on the DNA binding activity of HIF, which is required for it to transactivate its target genes [48]. Thus, HIF and its associated transcriptional response are key mediators of tumorigenesis in ccRCC.

In addition to HIF, pVHL can interact with a number of other proteins, although the biological significance of these interactions is incompletely understood [52]. Some of these interactions can lead to ubiquitination of other proteins aside from HIF. For example, pVHL has been reported to interact with and ubiquitinate two de-ubiquitinase enzymes (VDU1 and VDU2) leading to their degradation [53, 54]. In turn, VDU2 but not VDU1 may de-ubiquitinate HIF-1α, potentially providing another level of control to the HIF pathway [55]. In addition, pVHL can bind to and ubiquitinate two subunits of the RNA polymerase 2 complex, POL2RA (RPB1) and POL2RG (RPB7) [56–58]. Importantly, the pVHL-RPB1 interaction was shown to be oxygen-dependent, involving a mechanism similar to that regulating pVHL interaction with HIF [58]. Similarly, the erythropoietin receptor (EPOR), which lies downstream of the canonical HIF-target gene, erythropoietin (EPO), may also be bound and ubiquinated by pVHL in response to oxygen [59]. pVHL can also interact with and ubiquitinate the regulatory domain of atypical protein kinase C (PKC), a serine–threonine kinase that has roles in cell polarity and cell growth, leading to its degradation [60–62]. Again, this interaction may be regulated by oxygen [62]. Similarly, an oxygen-dependent interaction between pVHL and sprouty homolog 2 (SPRY2), which modulates the action of receptor tyrosine kinases, has been reported [63]. Taken together, these findings indicate that pVHL may contribute to oxygen signaling more extensively than simply through regulation of HIF.

pVHL may also play a non-canonical role in extra-cellular matrix assembly, independently of HIF. Specifically, pVHL can interact directly with the alpha-chain of collagen 4 and is important in maintaining the collagen 4 network [64, 65]. This molecule is heavily hydroxylated, and as will be explained below, hydroxylation is important in the recognition of HIF-alpha (as well as collagen 4) by pVHL. Importantly, this interaction can be dissociated by ccRCC-associated VHL mutations. Similarly, fibronectin co-immunoprecipitates with pVHL, and consistently the extracellular fibronectin matrix produced by VHL-defective ccRCC cells is also disrupted [66]. However, the contribution of this phenomenon to cellular oxygen sensing and ccRCC tumorigenesis is still unclear.

6. Oxygen-dependent regulation of HIF by pVHL

The importance of pVHL in the regulation of the HIF transcription factors, and the cellular transcriptional response to altered levels of oxygen, has provided tremendous insights into the mechanisms of cellular oxygen sensing. HIF was first discovered in the quest for transcriptional regulators of the erythropoietin gene (EPO), encoding the master regulator of red blood cell production [67]. It later emerged there were three HIF isoforms, HIF-1, HIF-2 [68, 69], and HIF-3 [70], each composed of a common, constitutive β-subunit (HIF-1β, also known as ARNT – aryl hydrocarbon receptor nuclear translocator) and a regulated alpha-subunit (HIF-1α, HIF-2α and HIF-3α respectively). HIF-1α is ubiquitously expressed at the mRNA level, thus HIF-1α protein is capable of being stabilized in all tissue types. HIF-1α is thought to drive core, canonical cellular responses to low oxygen levels (hypoxia) [71], including the metabolic switch to anaerobic glycolysis. The expression of HIF-2α mRNA and HIF-3α mRNA is more cell-type-specific and thus these transcription factors are thought to drive more specialized responses to hypoxia [69, 70, 72].

HIF-2α expression is generally more restricted to particular mesenchymal cell types, including endothelial cells in which it was first identified, hence its alias endothelial PAS domain-containing protein 1 (EPAS1) [69]. However, HIF-2α is also expressed in some epithelial malignancies, including ccRCC. HIF-3α expression is restricted to a select few cell types and can be alternatively spliced to yield several transcript variants [70]. The biological functions of HIF-3α have not been well-explored, although it is thought to antagonize the transcriptional responses of HIF-1α and HIF-2α [73–75].

HIF isoforms are all basic helix–loop–helix/Per-ARNT-SIM (bHLH–PAS) transcription factors, belonging to a much larger family that includes the oncogenic MYC proteins [76]. Each possess an N-terminal bHLH DNA-binding domain and two protein–protein interaction PAS domains responsible for dimerization. In addition, the three HIF-α isoforms each contain oxygen-dependent degradation domains (ODDDs), responsible for regulating protein abundance [77]. However, only HIF-1α and HIF-2α possess the C-terminal transactivation domains (C-TAD) [78].

In the presence of oxygen, HIF-α subunits are hydroxylated on two residues in the ODDD domains by a family of prolyl hydroxylase enzymes (PHD1, PHD2 and PHD3) [44, 45, 79]. These hydroxylated residues are recognized and bound by pVHL (in a complex with elongin B, elongin C and cullin 2) leading to its rapid ubiquitination and proteasomal degradation (**Figure 2**). Thus, when oxygen is abundant, HIF-α levels are low. However, since oxygen is a rate-limiting substrate for this reaction, HIF-α is stabilized in hypoxia. Inactivation of VHL in ccRCC cells will also block HIF from being degraded, leading to constitutive activation of HIF and its target genes, even in cells that are well-oxygenated. Accordingly, activation of both HIF and HIF target genes are hallmarks of ccRCC.

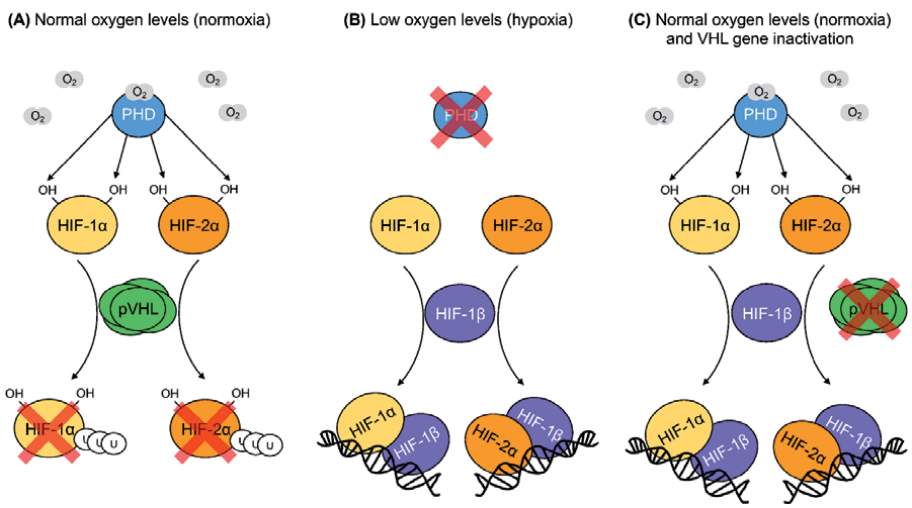

Figure 2.
Regulation of HIF by PHD enzymes and pVHL E3 ligase. (A) In normal oxygen conditions (normoxia), the oxygen-dependent PHD enzymes (PHD1, PHD2 and PHD3) hydroxylate both HIF-1α and HIF-2α transcription factor isoforms. This causes HIF proteins to be recognized and ubiquitinated by the pVHL E3 ubiquitin ligase complex, which targets them for rapid degradation via the proteasome. (B) In low oxygen conditions (hypoxia), PHD enzymes are inactive due to the lack of their oxygen substrate. Therefore, HIF-1α and HIF-2α are not hydroxylated and are not targeted for degradation by pVHL. Due to their stabilization, they are able to dimerize with their obligate binding partner HIF-1β. This allows them to bind to DNA and upregulate their target genes. (C) When the VHL gene is inactivated (as is the case in ccRCC and some other cancers), pVHL is either not expressed or is dysfunctional. Therefore, pVHL is unable to recognize HIF-1α and HIF-2α, even in the presence of oxygen when they are hydroxylated by PHD enzymes. This causes inappropriate stabilization of HIF-1α and HIF-2α, which then dimerize with HIF-1β and upregulate their target genes, regardless of oxygen levels.

In addition, HIF-1α and HIF-2α can be further modified at an additional site in the C-terminal TAD by an asparaginyl hydroxylase, termed factor inhibiting HIF 1 (FIH-1) [80, 81]. Similar to the PHD enzymes, FIH-1 activity is oxygen-dependent, but asparagine hydroxylation does not prompt recognition by pVHL. Instead, asparaginyl hydroxylated HIF is unable to bind to the transcriptional co-factor, CREB binding protein (CBP)/p300, which facilitates transcriptional activation at a subset of HIF-target genes [80–82]. Therefore, two distinct mechanisms act to control HIF activity and expression in an oxygen-dependent manner, one of which is blocked by VHL inactivation. In the context of ccRCC, this has two consequences. Firstly, FIH-1 may facilitate residual hypoxic regulation of HIF despite constitutive HIF stabilization [83]. Secondly, the transcriptional response to VHL inactivation in normoxic cells may not precisely mimic the transcriptional response to hypoxia.

7. The HIF transcriptional response

Once stabilized, both HIF-1α and HIF-2α, in complex with HIF-1β, are able to bind chromatin at either gene promoters or promoter-distant enhancers that contain one or more 5′-RCGTG-3′ recognition motifs, termed hypoxia response elements (HREs) [84, 85]. These short motifs are highly numerous across the genome and only a small proportion of accessible motifs are occupied by HIF, indicating that additional factors are involved in HIF DNA-binding [85]. HIF-binding sites may lie several hundreds of kilobases from the target promoter, interacting with it through chromatin looping, which can make it difficult to identify the transcriptional target of any given binding site. Therefore, much effort has been directed at determining both direct and indirect targets of the HIF transcriptional pathway in multiple settings, including in VHL-defective ccRCC cells, using both transcriptomic assays such as RNA-seq and assays of chromatin binding such as ChIP-seq [85–89].

These sequencing studies indicate that HIF acts as a gene activator rather than a repressor; causing the induction of hundreds to thousands of genes and triggering massive pathway activation [90–93]. These genes mediate diverse cellular functions including angiogenesis, erythropoiesis, glycolysis and the cell cycle [77, 94, 95]. This triggers a physiological response that enables cells to survive in low oxygen conditions. For example, HIF-dependent angiogenesis increases blood supply to oxygen-starved tissue; HIF-dependent erythropoiesis improves systemic oxygen delivery; HIF-dependent glycolysis allows cells to generate ATP in the absence of oxygen; and HIF-dependent cell cycle arrest can allow cells to conserve energy and reduce oxygen consumption.

Importantly, HIF-binding sites and HIF-regulated genes are highly cell-type specific. Thus, whilst HIF may regulate many hundreds of genes in any given cell type, only a small, core set of well-described genes are regulated in the majority of tissues [90, 93]. Furthermore, although both HIF-1α and HIF-2α share the same binding motif and their binding sites often overlap, HIF-1α tends to be more prevalent at gene promoters whereas HIF-2α is more prevalent at promoter-distant enhancers [90, 92]. In addition to this binding site specificity, post-DNA-binding mechanisms likely contribute to transcriptional selectivity between the two isoforms [96], such that specific genes may be regulated by either HIF-1α or HIF-2α only, even when both isoforms are bound [50, 97] For example, cyclin D1 (CCND1), transforming growth factor alpha (TGFA), vascular endothelial growth factor A (VEGFA), glucose uptake transporter 1 (SLC2A1/GLUT1), the MYC oncogene, and the stemness-related transcription factor OCT4/POU5F1 are specifically induced by HIF-2, whilst BCL2-interacting protein 3 (BNIP3) and carbonic anhydrase 9 (CA9) are positively regulated by HIF-1 [97–102].

Although primarily a physiological response, the HIF pathway is also relevant to the pathophysiology of cancer and many HIF target genes are central to the hallmarks of cancer described by Hanahan and Weinberg [103]. These include genes with prominent roles in angiogenesis, glycolysis, cell proliferation, cell invasion and immune evasion among other oncogenic processes (**Figure 3**). Indeed, HIF is activated in many types of solid tumor, largely as a result of intra-tumor hypoxia and is almost universally associated with a poor prognosis [104].

In particular, HIF promotes the metabolic switch from oxidative phosphorylation to anaerobic glycolysis by inducing a range of target genes, including those encoding transmembrane proteins that import glucose into the cell (SLC2A1/GLUT-1 and SLC2A3/GLUT-3) as well as multiple catalytic enzymes in the glycolytic pathway [71]. Oxidative phosphorylation is oxygen-dependent, therefore switching to oxygen-independent glycolysis allows hypoxic cancer cells to generate energy. However, glycolysis causes accumulation of byproducts in the form of acidic metabolites, which can be toxic to cancer cells. Therefore, HIF also upregulates genes encoding transmembrane proteins that rebalance intracellular pH to promote cancer cell survival. For example, the HIF target genes CA9 and CA12, encoding carbonic anhydrases, generate alkaline sodium bicarbonate ions in the extracellular space [105]. Sodium bicarbonate can then be imported into cells by ion channels to counteract intracellular acidity. Furthermore, once a tumor outgrows its blood supply and becomes hypoxic, HIF induces genes encoding pro-angiogenic secreted factors, such as VEGFA and placental growth factor (PGF), that serve to transmit extracellular signals and stimulate blood vessel production [106]. This increases delivery of nutrients and oxygen to cancer cells, enabling the tumor to further expand. Furthermore, HIF has recently been found to upregulate genes that help cancer cells evade destruction by the immune system. One such example is CD274,

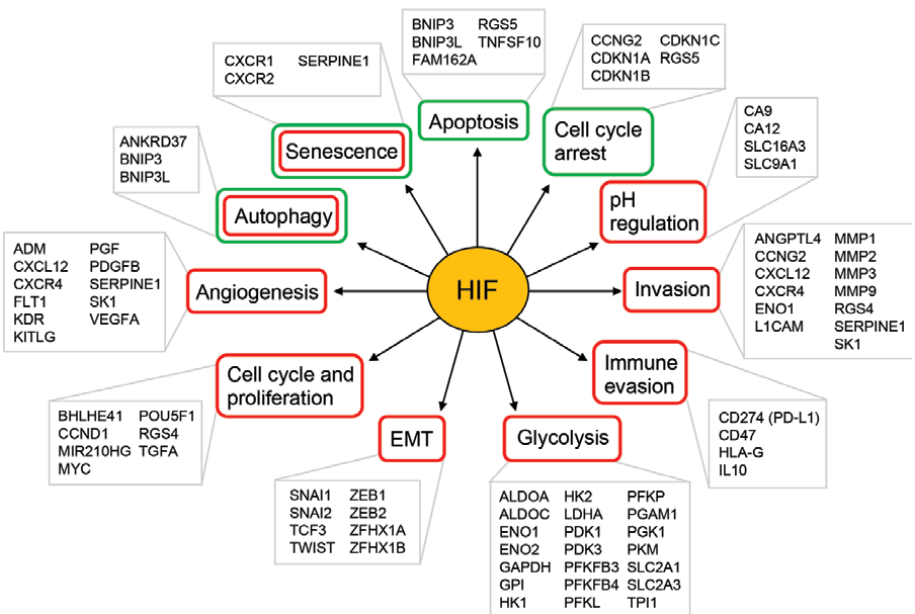

Figure 3.
HIF target genes that promote or restrict tumorigenesis. HIF regulates hundreds to thousands of target genes, which mediate diverse and sometimes conflicting cellular processes. For example, such processes can either promote or restrict tumor growth. Those that are typically considered tumor-promoting processes are depicted in red, whereas those that are typically considered tumor-suppressive are depicted in green. Cellular processes that can be either tumor-promoting or -suppressive (depending on the context) are depicted in red and green. Exemplar HIF target genes involved in each process are listed. Note that whilst some HIF target genes appear to be consistent across cell types and conditions, others are context-dependent.

encoding the transmembrane protein termed programed death ligand 1 (PD-L1), which is expressed in cancer cells [107]. PD-L1 interacts with its receptor termed programed cell death protein 1 (PD-1), which is expressed on the cell surface of T cells. The PD-L1/PD-1 interaction prevents T cell-mediated killing of cancer cells, therefore HIF may exacerbate this oncogenic mechanism.

However, since HIF evolved to mediate physiological responses to hypoxia, not all HIF target genes are advantageous in a cancer setting. Paradoxically, although HIF activates many pro-tumorigenic target genes, there are also anti-tumorigenic HIF targets (**Figure 3**). These may represent in-built tumor suppressor mechanisms that counterbalance oncogenic target genes when HIF is activated in response to physiological hypoxia. Tumor suppressive HIF target genes include BNIP3 and BNIP3L, which are pro-apoptotic proteins. BNIP3 and BNIP3L can promote either cell death or autophagy in response to hypoxia, depending on the context [108]. Furthermore, some HIF target genes may not influence cancer pathogenesis whatsoever and may represent genes that are only important in other contexts. This is epitomized by VHL loss in the earliest stages of ccRCC formation, which causes HIF activation in an inappropriate context (i.e. causing a cellular response to hypoxia when the cell is not hypoxic). In this setting, HIF causes a change in cell state that is unwarranted since the cell is exposed to normal oxygen levels. Therefore, many activated HIF target genes may confer no survival advantage or may even result in a "fitness penalty" to the cell in this context. Taken together, the overall consequences of massive HIF pathway activation in ccRCC will be a balance of many positive, neutral and negative effects [109]. The contribution of each effect may change during cancer pathogenesis as a result of subsequent somatic mutation, epigenetic events or changes in the tumor microenvironment allowing cancer cells to escape the long prodromal dormancy that occurs following VHL inactivation. Alternatively, the poise of the HIF transcriptional pathway may be partially pre-set prior to VHL inactivation due to cell-type specific differences in HIF target genes. In turn, this could render specific cell types particularly susceptible to VHL inactivation. Furthermore, genetic differences between individuals might alter specific HIF target genes, thus making that individual more or less susceptible to developing kidney cancer.

Activation of contrasting and aberrant pathways as part of large transcriptional programs is an emerging theme in cancer biology. For example, MYC, like HIF, has transcriptional targets with both oncogenic and tumor suppressive properties [110, 111]. Therefore, HIF activation in ccRCC serves as a model for studying large transcriptional cascades in cancer more generally.

8. Modulation of the HIF response during the pathogenesis of ccRCC

Early evidence to support the pleiotropic nature of the HIF pathway in kidney cancer came from the observation that HIF-1α and HIF-2α have opposing actions on tumor growth in ccRCC xenograft models. Whilst HIF-2α promotes tumor growth, HIF-1α has the opposite effect and restricts tumor growth [46, 48, 50, 51]. Furthermore, expression of HIF-2α target genes in ccRCC tumors correlates with poor patient prognosis, whereas HIF-1α targets genes are associated with improved survival [91].

Commensurate with this, HIF isoform expression appears to switch from HIF-1α to HIF-2α during the development of kidney cancer [28, 112]. In renal tubule epithelial cells, including proximal tubular cells from which ccRCC is derived, HIF-1α mRNA is highly expressed, whereas HIF-2α mRNA is undetectable [28]. Conversely, HIF-2α mRNA (and protein) is highly expressed in ccRCC, possibly as a result of downregulation of DNMT3a and resultant promoter demethylation of the EPAS1 gene that encodes

HIF-2α [113]. Furthermore, ccRCCs often downregulate HIF-1α through loss of copy number, deletion, truncation or transcript downregulation [25, 31, 32, 112, 114]. Given the tumor-suppressive function of HIF-1 and the oncogenic function of HIF-2, the shift from HIF-1α in the ccRCC cell of origin to dominant HIF-2α expression in overt ccRCC would favor a more oncogenic phenotype. However, even within the transcriptional repertoire of each isoform there are genes with heterogenous associations with prognosis, suggesting that other selective pressures, effective at the level of individual HIF target genes, may also be operating [91].

Indeed, suppression of individual HIF target genes with anti-tumorigenic properties has been reported in ccRCC. The pro-apoptotic gene BNIP3 is a canonical HIF target gene in many cell types. However, rather than being increased by constitutive HIF in ccRCC cells, its expression was found to be lower than in normal kidney cells. This is most likely as a result of epigenetic modification of the BNIP3 gene locus involving histone deacetylation [115].

In this respect, it is notable that of the many somatic mutations that co-occur with VHL inactivation in ccRCC, very few occur within HIF-target genes. However, to date, the majority of ccRCC sequencing efforts have focused on the coding genome or have targeted genomic regions of interest. Therefore, the majority of HIF binding sites (which are usually intergenic) have not been extensively examined and further studies may reveal somatic mutation of these sites in the future. However, epigenetic modifiers such as PBRM1, SETD2 and BAP1 are recurrently mutated in these tumors [25, 31, 32, 116–120]. PBRM1 encodes a subunit of the chromatin remodeling PBAF SWI/SNF complex; SETD2 encodes a histone methyltransferase; and BAP1 encodes a histone deubiquitinase. Interestingly, parallel evolution has been reported with respect to these mutations, whereby multiple mutations in the same gene are present in different cells of the same tumor [32]. This emphasizes their importance in driving ccRCC, as well as illustrating their temporal occurrence (i.e. subsequent to VHL mutation). Although the interaction between these ccRCC-associated somatic mutations and the HIF pathway remains unclear, PBRM1 inactivation enhances some aspects of the HIF response [121] and reduces the tumor-suppressor activity of HIF-1α, although the mechanisms are unknown [122]. Recurrent mutations are also found in genes within the PI3K/AKT/mTOR pathway, which is a master regulator of RNA translation. Expression of both HIF-1α and HIF-2α protein are differentially dependent on mTOR, with HIF-1α being regulated by both the mTORC1 and mTORC2 complexes, whilst HIF-2α is dependent solely on mTORC2 [123]. Therefore, HIF isoforms may be differentially affected by mutations in this pathway.

In addition, other oncogenic transcription factors activated in ccRCC may modulate the HIF response. For example, MYC activity is enhanced in ccRCC [124, 125] and synergizes preferentially with HIF-2, whilst antagonizing HIF-1 [102, 126]. In this way, MYC augments the switch from HIF-1 to the more oncogenic HIF-2 isoform. Importantly, MYC itself is a transcriptional target of HIF in ccRCC cells [127], providing a mechanism whereby stabilization of HIF following inactivation of VHL preferentially amplifies the HIF-2 transcriptional pathway in these cells.

9. Variation in the HIF pathway pre-disposes to renal cancer

As discussed above, genetic and epigenetic events occur somatically in ccRCC following VHL inactivation, allowing the HIF transcriptional output to adapt to a more oncogenic phenotype, thereby promoting tumor formation. However, differences in the HIF pathway that exist prior to VHL inactivation can also affect the ability of cells to form cancer. Indeed, it is highly likely that cell-type differences in the HIF pathway contribute to the tight tissue-specificity of VHL-associated cancer,

despite the almost universal operation of the VHL-HIF pathway in different mammalian cell types. Potentially, cell-type-specific components of the HIF pathway might favor tumorigenesis in permissive cell types, inhibit tumorigenesis in non-permissive cell types, or a combination of both (**Figure 4**). The exact mechanism underlying this tissue specificity remains to be determined, although elucidation of HIF target genes in cells permissive to VHL-associated cancers (compared to that in non-permissive cells) will be key in future studies. Of note, the G1/S-phase cell-cycle regulator cyclin D1 (CCND1) has been found to be a HIF-2 responsive gene, which is not regulated by HIF-1 and is unique to ccRCC cells [50]. Furthermore, CCND1 is required for ccRCC cell growth in mice [128]. This indicates that CCND1 and likely other tissue-specific HIF target genes may render certain cell types receptive to tumorigenesis upon VHL inactivation.

As well as being affected by somatic alterations and cell-type-specific features, the HIF pathway can also be modified by inherited genetic variants. Polymorphisms that predispose individuals to kidney cancer have been studied, and several of these have been shown to affect HIF target genes. Such variants have been identified by genome wide-association studies (GWAS), which compare the genome sequence of renal cancer patients with healthy control individuals [129–135]. Although these variants likely only account for about 5% of kidney cancer heritability [129], a disproportionately high number of these susceptibility loci overlap with cis-acting components of the HIF pathway [136]. This indicates that specific aspects of the HIF pathway are under genetic selection during the development of kidney cancer.

Many of these RCC-susceptibility loci lie in intergenic regions and so the functional target of these polymorphisms is not immediately apparent. However, several susceptibility loci overlap with, or lie adjacent to, HIF-binding sites [136]. In-depth analysis of chromatin looping and HIF-dependent gene regulation has identified a number of HIF target genes associated with these loci [127, 136–138]. At each locus, the renal cancer susceptibility polymorphism affects both HIF binding and expression of the HIF-target gene, either by generating a second HRE

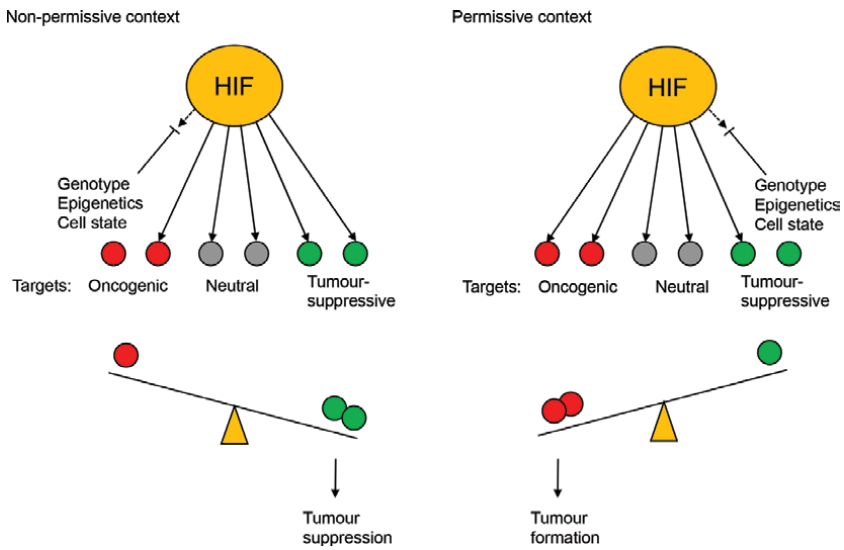

Figure 4.
Rebalancing the HIF pathway to favor tumorigenesis. HIF target genes include those that promote tumor growth (depicted in red), restrict tumor growth (depicted in green) and those that do not influence tumor growth (depicted in gray). Depending on the context (i.e. in a permissive or non-permissive context), activation of the HIF pathway may or may not be conducive to tumorigenesis. Features that could 'tip the balance' in a HIF-activated cell include genetic mutations, epigenetic features and the cell state (e.g. the underlying gene expression program).

motif or by altering chromatin accessibility. Most notable are polymorphisms at the 11q13.3 locus, which affect HIF-2-dependent expression of cyclin D1 (CCND1) [137]; polymorphisms at the 8q24.21 locus, which affect HIF binding and expression of the oncogenic transcription factor MYC [127]; and polymorphisms at the 12p12.1 locus, which alter HIF-1 dependent expression of the basic helix–loop–helix transcription factor BHLHE41 (also known as DEC2) [138]. Furthermore, RCC-susceptibility polymorphisms have been identified at the 2p21 locus, lying in the first intron of the EPAS1 gene that encodes HIF-2α, although whether these affect HIF-2α expression remains unclear [130, 139]. Importantly, each renal cancer susceptibility locus affects a single component of the HIF pathway. This directly implicates these genes in the pathogenesis of kidney cancer. Furthermore, it helps distinguish them from HIF target genes with neutral effects on RCC susceptibility that might be simply co-activated as part of large pathway upregulation. Therefore, these analyses have highlighted specific 'driver' genes that may provide attractive targets for future therapeutic approaches or as biomarkers that might predict tumor behavior.

10. Therapeutic implications of HIF pathway activation in ccRCC

In the absence of a surgical cure, the outlook for patients with clear cell renal cancer is poor, with a median survival of just 2 years. However, over recent years a number of systemic anti-cancer therapeutic strategies have emerged, which are beginning to alter the outcome for some of these patients.

10.1 Anti-angiogenic therapies

One strategy has focused on angiogenesis inhibitors to treat metastatic ccRCC. Whilst all tumors require a blood supply to obtain sufficient oxygen and nutrients to grow, ccRCC (and other VHL-dependent cancers such as hemangioblastoma) are particularly rich in blood vessels. Indeed, VEGFA, a master regulator of angiogenesis, [98, 99] is a direct transcriptional target of HIF and is highly expressed in ccRCC cells [41, 140]. Early anti-angiogenic strategies targeted VEGFA using the monoclonal antibody bevacizumab, with limited efficacy [141]. However, several other HIF target genes also encode pro-angiogenic factors, such as PGF, adrenomedullin (ADM) and plasminogen activator 1 (PAI-1), as well as the VEGF receptor, FLT1. These likely act in concert with VEGFA to orchestrate a robust angiogenic phenotype in the context of HIF activation. Therefore, rather than targeting individual factors, more recent strategies have used small-molecule receptor tyrosine kinase inhibitors (TKIs) to block the overarching angiogenic pathways [142]. However, while effective in some individuals, other tumors may fail to respond, likely reflecting heterogeneity in gene expression between tumors. Furthermore, the duration of response may be limited, possibly reflecting intra-tumor heterogeneity and the growth of resistant subclones.

10.2 Immunotherapy

In recent years, immune checkpoint inhibition via targeting PD-L1 and CTLA-4 has emerged as an effective treatment for advanced ccRCC. This is despite the relatively low mutational burden seen in this type of cancer, which often correlates with sensitivity to immunomodulatory therapy in other cancer types. Whilst HIF has multiple effects on the immune response [143], it is of particular interest that PD-L1 has been found to be transcriptionally regulated by HIF in ccRCC cells [107, 144, 145].

Therefore, it is possible that HIF-mediated activation of PD-L1 may underlie the sensitivity of ccRCC to inhibition of this pathway.

10.3 mTOR inhibitors

Historically, mTOR inhibitors have been used in the treatment of metastatic kidney cancer and remain part of the modern armamentarium [146, 147]. Inhibition of mTOR will negatively impact translation of HIF-alpha subunits, while preferential blockade of mTORC1 or mTORC2 may alter the balance of the two isoforms. Given the oncogenic role of HIF-2α in ccRCC and the selective regulation of HIF-2α by mTORC2, mTORC2 inhibition may provide a more targeted therapeutic approach in the future.

10.4 HIF-2 inhibitors

The finding that HIF-1α and HIF-2α have opposing effects on the pathogenesis of ccRCC initiated efforts to generate isoform-specific inhibitors. This led to the development of small molecule inhibitors that specifically prevent HIF-2α dimerizing with HIF-1β, thereby blocking HIF-2α -dependent transcription without affecting HIF-1α activity [148]. These inhibitors would be predicted to have greater efficacy compared to targeting both isoforms simultaneously, whilst reducing off-target side-effects. Indeed, investigation of these compounds as potential ccRCC treatments, both in animal models of ccRCC and early clinical trials, have yielded promising results [149–151]. Therefore, these compounds could provide another strategy for treating metastatic ccRCC.

11. Conclusions

Inactivation of the VHL tumor suppressor gene is the hallmark of clear cell renal cancer and leads to the upregulation of wide-spread hypoxia pathways, orchestrated by the transcription factor HIF. Whilst HIF proteins activate many genes that are central to the "hallmarks of cancer", other HIF-target genes may restrict cancer progression and the overall consequence of HIF pathway activation is a balance of these effects (**Figure 4**). Both genetic and epigenetic genetic events, occurring before or after VHL loss and HIF activation, can alter this balance to promote tumorigenesis.

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Chapter 8

Urine Creatinine Excretion in HIV and Non-HIV Subjects

Ernest Ndukaife Anyabolu

Abstract

This study assessed urine creatinine in spot and 24-hour samples in HIV and non-HIV population. We categorized dilute urine as a 24-hour urine creatinine <300 mg, concentrated urine as a 24-hour urine creatinine >3000 mg, and normal urine as a 24-hour urine creatinine 300–3000 mg. Association of variables with creatinine was evaluated. In HIV subjects, the mean spot urine creatinine was 137.21 ± 98.47 mg/dl and a 24-hour urine creatinine was 1507 ± 781 mg. The prevalence of dilute urine was 0.5%, normal urine 93.1%, and concentrated urine 6.4%. 20-hour urine creatinine was associated with serum LDL, and HDL. Concentrated urine was correlated with a 24-hour urine osmolality (r = 0.95), serum HDL (r = −0.73), CD4 cells count (r = −0.71), and BMI (r = 0.74). Dyslipidemia was common in HIV subjects with concentrated urine. In non-HIV subjects, the mean spot urine creatinine was 148 ± 167 mg/dl and the 24-hour urine creatinine was 1203 ± 316 mg. The 24-hour urine creatinine was within the normal range. The spot urine creatinine significantly correlated with BMI, spot urine protein, spot urine osmolality, 24-hour urine protein, 24-hour urine creatinine, serum creatinine, serum cholesterol, and serum LDL. Conversely, the 24-hour urine creatinine significantly correlated with 24-hour urine volume, serum creatinine, and serum cholesterol. The spot urine protein and 24-hour urine protein were predictors of spot urine creatinine. Serum creatinine was a predictor of 24-hour urine creatinine. Proteinuric renal abnormalities were common.

Keywords: HIV, urine creatinine, spot urine creatinine, 24-hour urine creatinine, CD4 cell count, concentrated urine, dilute urine, abnormal weight, dyslipidemia, proteinuria

1. Introduction

1.1 Impact of HIV

Human immunodeficiency virus infection is a world healthcare burden with sub-Saharan Africa as a geographic area accounting for about 70% of HIV-infected persons [1]. In Nigeria the prevalence of HIV is 3.7% [1]. HIV infection directly or indirectly affects most organs of the body [2]. In like manner, tons of physiological responses are also altered by HIV disease process [3–5].

1.2 Factors which may influence creatinine

Creatinine is produced by the muscles, degraded within the liver, and efficiently excreted by the kidney at a rate that is not only constant but is additionally modulated by weight, gender, and age [6].

Many environmental, physiologic, and disease conditions may impact on daily urine creatinine excretion. Excretion of creatinine is further altered by exogenous substances such as cocaine and heavy metals which include arsenic and cadmium seen within the bioenvironment related to environmental pollution. Others include meat consumption and medications such as cimetidine and trimethoprim. Consequently, urine creatinine is employed in monitoring bioenvironmental pollutants and substance use [7–9].

1.3 Variability of daily urine creatinine

There is high variability of the values of daily urine creatinine excretion in normal healthy state [10]. Impaired renal function usually results in poor renal secretion of creatinine in urine; urine creatinine decreases as renal function impairment increases [11].

1.4 Identified factors of high and low 24-hour urine creatinine

Studies have identified some associated factors of high 24-hour urine creatinine or concentrated urine. They include age, sex, race, body mass index, hypertension, water intake, and blood osmolality [12]. At the other pole, low 24-hour urine creatinine or dilute urine was reported to be associated with glomerular filtration rate, an older age, diabetes, and lower levels of body mass index, proteinuria, and protein intake [11]. Another important use of urine creatinine is for evaluating the completeness of 24-hour urine sample collection [13].

1.5 A necessity for routine assessment of urine creatinine in HIV and non-HIV subjects

Studies are sparse on urine creatinine in HIV and non-HIV subjects originating from Nigeria. We have, therefore, launched to evaluate urine creatinine and factors which influence low and high urine creatinine in these groups of subjects.

2. Methods

2.1 Study location and population

This was a cross-sectional study, comprising 375 HIV-positive subjects and 136 subjects recruited from an HIV clinic and also the general outpatient clinic, respectively, of the Federal Medical Centre, Owerri, Nigeria. The study was disbursed and carried out between April and August 2011. The standards for inclusion were HIV-positive status for the HIV subjects and HIV-negative status for the non-HIV participants. For both groups of subjects, another criterion was age range of 16–65 years. The themes excluded from the study were people who had adrenal, pituitary, and renal diseases, terminal illness, and pregnancy. For the non-HIV subjects, the inclusion criteria were similar, but those with HIV-positive status were excluded.

2.2 Ethics approval

The study was approved by the ethics research committee of the hospital. Its approval reference number was FMC/HCS/VOL II and was dated 16 March, 2011. Informed written consent was obtained from all the themes participated within the study.

2.3 Variables, data collection, and sample analyses

With the help of a questionnaire, demographic, anthropometric, and other relevant data were obtained from the themes. The purpose of the study was explained to the themes. The age, gender, place of origin, and domicile were obtained. Height was measured and recorded in meter (m). Weight was measured employing a weighing scale. Body mass index was taken as the ratio of weight/height^2 (kg/m^2).

The study participants were clearly instructed on the way to collect 24-hour urine sample. At the conclusion of the 24-hour urine sample collection, blood samples, and daytime random spot urine samples were collected. Spot urine creatinine, spot urine osmolality, and spot urine protein from the random spot urine samples were performed. Also from the 24-hour urine samples collected, 24-hour urine protein, 24-hour urine creatinine, and 24-hour urine osmolality were performed. Serum creatinine was performed on the blood samples collected. Freezing point depression assay was used to determine osmolality, protein by photometric method, and creatinine by modified Jaffe's method. Creatinine clearance and spot urine creatinine/osmolality ratio were calculated. HIV screening and confirmatory tests, fasting serum lipid profile, CD4 cell count, and hemoglobin were performed.

2.4 Potential risk variables analyzed

The potential associated factors of dilute and concentrated urine evaluated were CD4 cells, spot urine protein, spot urine osmolality, 24-hour urine osmolality, 24-hour urine protein, spot urine creatinine/osmolality ratio, creatinine clearance, serum cholesterol, serum low-density lipoprotein cholesterol, serum triglyceride, and serum high-density lipoprotein cholesterol.

2.5 Statistical analyses

The data were analyzed using SPSS version 17.0 (SPSS Inc. Chicago, IL, USA). The distribution and characterization of clinical and laboratory variables within the study participants with different levels of 24-hour urine creatinine were analyzed using cross-tabulation, whereas statistical significance of association of these variables with 24-hour urine creatinine levels was evaluated using Student's t-test. Correlation statistics were used to determine the association of those variables with concentrated urine on the one hand and with dilute urine on the other hand. The strength of variables to predict dilute urine and concentrated urine was determined using multivariate linear regression analyses. $P < 0.05$ was taken as statistically significant.

2.6 Definition of terms

Normal urine creatinine: 24-hour urine creatinine 300–3000 mg. Low urine creatinine or dilute urine: 24-hour urine creatinine <300 mg. High urine creatinine or concentrated urine: 24-hour urine creatinine >3000 mg.

3. Results

3.1 Results in HIV patients

3.1.1 Age, spot urine, and 24-hour urine creatinine in HIV patients

Out of the 393 participants studied, 18 were excluded due to errors from incomplete sample collection. Their mean age was 39 ± 11 years. For all the HIV participants, the mean spot urine creatinine was 137.21 ± 98.47 (mg/dl), minimum value 13.3 mg/dl, maximum value 533.3 mg/dl, and range 520.0 mg/dl. The mean 24-hour urine creatinine was 1507 ± 781 mg, minimum value 206 mg, maximum value 4849 mg, with a range of 4643 mg (**Table 1**).

3.1.2 Prevalence of dilute and concentrated urine and factors of 24-hour urine

Two (0.5%) of the HIV subjects have 24-hour urine creatinine <300 mg, 349 (93.1%) have 300–3000 mg, and 24 (6.4%) have >3000 mg. Serum low-density lipoprotein cholesterol was significantly associated with 24-hour ($p = 0.001$) in these HIV subjects. Two subjects have 24-hour urine creatinine <300 mg, and both of them have borderline serum low-density lipoprotein cholesterol. Twenty-four subjects have high urine creatinine, and all of them have desirable serum low-density lipoprotein cholesterol (**Table 2**).

3.1.3 Dilute urine, concentrated urine, and serum HDL in HIV patients

There was a significant association between serum high-density lipoprotein cholesterol and 24-hour urine creatinine, $p = 0.028$, in the HIV subjects. Two subjects

Variables (mean ± SD)	HIV subjects
Body mass index (kg/m^2)	26.2 ± 5.4
Hemoglobin (g/dl)	11.2 ± 1.8
CD4 cells/ml (median)	391
SUOsm (mOsm/kgH_2O)	464 ± 271
Spot urine protein (mg/dl)	11.89 ± 19.13
Spot urine creatinine (mg/dl)	137.21 ± 98.47
24-hour urine protein (g)	0.187 ± 0.290
24-hour urine creatinine (mg)	1507 ± 781
24HUOsm (mOsm/kgH_2O)	564 ± 501
SUCOR (mg/dl/mOsm/kgH_2O)	0.422 ± 0.486
Cholesterol (mmol/l)	4.26 ± 0.90
Triglyceride (mmol/l)	1.23 ± 0.37
HDL (mmol/l)	1.18 ± 0.39
LDL (mmol/l)	2.05 ± 0.58
Creatinine clearance (mls/min)	91.42 ± 22.98

SD, standard deviation; SUOsm, spot urine osmolality; 24HUOsm, 24-hour urine osmolality; SUCOR, spot urine creatinine/osmolality ratio; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol.

Table 1.
Variables in HIV patients.

Variables	24-hour urine creatinine levels (no (%))			Chi-square	Likelihood ratio	P value
	<300 mg	300–750 mg	>750 mg			
BMI (kg/m^2) < 18.5	0 (0.0%)	22 (91.7%)	2 (8.3%)	8.702	0.143	0.191
18.5–24.9	0 (0.0%)	124 (96.9%)	4 (3.1%)			
25.0–29.9	2 (1.4%)	128 (88.9%)	14 (9.7%)			
≥30	0 (0.0%)	75 (94.9%)	4 (5.1%)			
CD4 cell count <200	0 (0.0%)	41 (97.1%)	4 (8.9%)	0.781	0.614	0.677
≥200	2 (0.6%)	307 (93.3%)	20 (6.1%)			
Hb (g/dl) ≥12.0	2 (1.6%)	108 (88.5%)	12 (9.8%)	9.644	0.107	0.140
10.0–11.9	0 (0.0%)	163 (96.4%)	6 (3.6%)			
7.0–9.9	0 (0.0%)	72 (92.3%)	6 (7.7%)			
<7.0	0 (0.0%)	6 (100.0%)	0 (0.0%)			
ClCr (mls/min) ≥ 90 mls/min	0 (0.0%)	183 (92.0%)	16 (8.0%)	5.229	0.204	0.265
60–89	2 (1.4%)	135 (94.4%)	6 (4.2%)			
30–59	0 (0.0%)	31 (93.9%)	2 (6.1%)			
24 HUP <0.300 g	2 (0.6%)	301 (93.8%)	18 (5.6%)	8.018	0.178	0.237
≥0.300 g	0 (0.0%)	48 (88.9%)	6 (11.1%)			
FSLP (mmol/l)						
CholT Des (<5.2)	2 (0.6%)	308 (92.5%)	23 (6.9%)	1.618	0.806	0.659
BorderL (5.2–6.2)	0 (0.0%)	35 (97.2%)	1 (2.8%)			
High (>6.2)	0 (0.0%)	6 (100.0%)	0 (0.0%)			
LDL Des (<2.6)	0 (0.0%)	284 (92.2%)	24 (7.8%)	14.609	<0.001	0.001
BorderL (2.6–4.1)	2 (3.0%)	64 (97.0%)	0 (0.0%)			
HDL Low (<1)	2 (1.5%)	124 (95.4%)	4 (3.1%)	7.317	0.016	0.028
High (≥1)	0 (0.0%)	225 (91.8%)	20 (8.2%)			
TG Des (<1.7)	2 (0.6%)	311 (92.3%)	24 (7.1%)	3.150	0.449	0.790
BorderL (1.7–2.2)	0 (0.0%)	29 (100.0%)	0 (0.0%)			

Variables	24-hour urine creatinine levels (no (%))			Chi-square	Likelihood ratio	P value
	<300 mg	300–750 mg	>750 mg			
High (>2.2)	0 (0.0%)	8 (100.0%)	0 (0.0%)			

LHR, likelihood ratio; BMI, body mass index; Hb, hemoglobin; ClCr, creatinine clearance; 24HUP, 24-hour urine protein; FSLP, fasting serum lipid profile; CholT, total cholesterol; Des, desirable; BorderL, borderline; LDL, low-density lipoprotein cholesterol; HDL, high-density lipoprotein cholesterol; TG, triglyceride.

Table 2.
Distribution and characterization of variables at different levels of 24-hour urine creatinine in HIV-positive patients (n = 375).

have 24-hour urine creatinine <300 mg, and both have desirable serum high-density lipoprotein cholesterol <1 mg/dl. Twenty-four subjects have 24-hour urine creatinine >3000 mg. Out of this number, 83.3% have high serum high-density lipoprotein cholesterol, whereas 16.7% have desirable serum high-density lipoprotein cholesterol. This showed that the prevalence of high urine creatinine increased as serum high-density lipoprotein cholesterol increased (**Table 2**).

3.1.4 Dissociation and association between daily urine creatinine excretion and variables in HIV patients

There was no significant association between 24-hour urine creatinine and body mass index, p = 0.191; serum total cholesterol, p = 0.659; creatinine clearance, p = 0.265; 24-hour urine protein, p = 0.237; CD4 cell count, p = 0.677; serum triglyceride, p = 0.790; and hemoglobin, p = 0.140 in the HIV subjects (**Table 2**). Significant correlation was obtained between 24-hour urine creatinine and spot urine creatinine (p = 0.19), 24-hour urine volume (p = 0.004), spot urine creatinine/osmolality ratio (<0.001), serum low-density lipoprotein cholesterol (p = 0.31), creatinine clearance (p < 0.001), and serum creatinine (<0.001) in the treatment-naïve HIV subjects. Hemoglobin, spot urine protein, spot urine osmolality, 24-hour urine protein, 24-hour urine osmolality, serum cholesterol, serum high-density lipoprotein cholesterol, and serum triglyceride did not have significant correlation with 24-hour urine creatinine (**Table 3**).

3.1.5 Correlates of daily urine creatinine excretion in HIV patients

There was a very strong correlation between 24-hour urine creatinine >3000 mg and 24-hour urine osmolality (r = 0.95), body mass index, (r = 0.74), CD4 cell count, (r = −0.71), and serum high-density lipoprotein cholesterol (r = −0.73) in the HIV subjects. However, there was a moderate correlation between 24-hour urine creatinine and 24-hour urine volume (r = 0.58) and hemoglobin (r = −0.43). Conversely, there was a poor correlation between spot urine creatinine and body mass index (r = 0.131, p = 0.009)), spot urine protein (r = 0.183, p = <0.001), spot urine osmolality (r = 0.288, p = <0.001), 24-hour urine volume (r = −0.111, p = 0.032), and creatinine clearance (r = 0.108, p = 0.036) (**Table 4**).

3.1.6 Predictors of concentrated urine in HIV patients

Multivariate linear regression of 24-hour urine creatinine >3000 mg with its potential risk factors was voided as the colinearity variance was skewed due to the small subpopulation (24) that have 24-hour urine creatinine >3000 mg.

Variables	Correlation coefficient (r)	P value
Body mass index	−0.036	0.470
Hemoglobin (g/dl)	0.075	0.117
Spot urine protein	0.044	0.538
Spot urine creatinine	0.129	0.019
Spot urine osmolality	0.107	0.058
24-hour urine protein	0.035	0.625
24-hour urine osmolality	0.063	0.167
24-hour urine volume	0.143	0.004
SUCOR	0.288	<0.001
Serum creatinine	0.290	<0.001
Serum cholesterol (total)	0.074	0.242
Serum Triglyceride	−0.075	0.189
Serum HDL	0.029	0.542
Serum LDL	−0.109	0.031
Creatinine clearance	0.367	<0.001

SUCOR, spot urine creatinine osmolality ratio; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol.

Table 3.
Correlation of 24HUCr with variables in HIV patients (n = 375).

Variables	Correlation coefficient (r)	P value
Body mass index	0.744	<0.001
Hb (g/dl)	−0.427	<0.001
Spot urine protein	0.397	<0.001
Spot urine creatinine	0.371	<0.001
Spot urine osmolality	−0.549	<0.001
24-hour urine protein	−0.109	0.001
24-hour urine osmolality	0.952	<0.001
24-hour urine volume	0.578	<0.001
Serum creatinine	−0.198	<0.001
Serum cholesterol (total)	0.215	<0.001
Serum triglyceride	0.001	0.925
Serum HDL	−0.729	<0.001
Serum LDL	0.289	<0.001
Hemoglobin	−0.427	<0.001

SUCOR, spot urine creatinine osmolality ratio; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol.

Table 4.
Correlation of 24HUCr > 3000 mg with variables in HIV patients (n = 24).

3.2 Results in non-HIV subjects

3.2.1 Values of spot urine and 24-hour urine creatinine in non-HIV subjects

Out of the 136 non-HIV subjects enrolled in this study, females constituted 72.1% and males 27.9%. Their mean age was 39 ± 12 years. They all have complete data or sample collection, and there was no attrition. The value of the mean spot urine creatinine was 148 ± 167, minimum value 14.7 mg/dl, and maximum value 746.7 mg/dl with a range of 732.0 mg/dl. Further, the value of the mean 24-hour urine creatinine was 1203 ± 316, minimum value 651.0 mg, maximum value 2320 mg, and range 1669.0 mg. The mean values of all other variables are depicted in **Table 5**.

For all the subjects the mean 23-hour urine creatinine was in the normal range (300–3000 mg). The potential risk factors for concentrated or dilute urine were voided and could not be distributed or characterized.

3.2.2 Correlates of spot urine creatinine

There was a significant correlation between spot urine creatinine and body mass index ($r = 0.225$, $p = 0.009$), spot urine protein ($r = 0.292$, $p = 0.001$), spot urine osmolality ($r = 0.223$, $p = 0.009$), serum low-density lipoprotein cholesterol ($r = 0.282$, $p = 0.001$), 24-hour urine protein ($r = -0.187$, $p = 0.030$), 24-hour urine creatinine ($r = -0.178$, $p = 0.038$), serum creatinine ($r = -0.212$, $p = 0.013$), as well as serum cholesterol ($r = 0.246$, $p = 0.004$). In contrast, spot urine creatinine has no significant correlation with hemoglobin, 24-hour urine volume, 24-hour urine

Variables (mean ± SD)	Subjects
Body mass index (kg/m^2)	25.5 ± 6.5
Hemoglobin (g/dl)	12.9 ± 1.6
Serum creatinine (mg/dl)	0.88 ± 0.19
SUOsm ($mOsm/kgH_2O$)	334 ± 204
Spot urine protein (mg/dl)	7 ± 18
Spot urine creatinine (mg/dl)	148 ± 167
24-hour urine volume (ml)	1874 ± 681
24-hour urine protein (g)	0.095 ± 0.087
24-hour urine creatinine (mg)	1203 ± 316
24HUOsm ($mOsm/kgH_2O$)	160 ± 133
Cholesterol (mmol/l)	3.8 ± 1.2
Triglyceride (mmol/l)	1.2 ± 0.4
HDL (mmol/l)	1.2 ± 0.3
LDL (mmol/l)	2.3 ± 1.0
Creatinine clearance (mls/min)	93.0 ± 41.2

SD, standard deviation; SUOsm, spot urine osmolality; 24UOsm, 24-hour urine osmolality; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol.

Table 5.
Variables in non-HIV subjects (n = 136).

Variables	Correlation coefficient(r)	P value
Body mass index	0.225	0.009
Hemoglobin	0.024	0.782
Spot urine protein	0.292	0.001
Spot urine osmolality	0.223	0.009
24-hour urine protein	−0.187	0.030
24-hour urine creatinine	−0.178	0.038
24-hour urine volume	−0.097	0.259
24HUOsm	−0.165	0.055
Serum creatinine	−0.212	0.013
Serum cholesterol (total)	0.246	0.004
Serum Triglyceride	0.157	0.067
Serum HDL	0.137	0.112
Serum LDL	0.282	0.001
Creatinine clearance	0.024	0.782

HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; 24HUOsm, 24-hour urine osmolality.

Table 6.
Correlation of spot urine creatinine with variables in non-HIV subjects (n = 136).

osmolality, serum triglyceride, serum high-density lipoprotein cholesterol, as well as creatinine clearance (**Table 6**).

3.2.3 Correlates of 24-hour urine creatinine

Twenty-four-hour urine creatinine significantly correlated with 24-hour urine volume ($r = 0.213$, $p = 0.013$), serum creatinine ($r = 0.741$, $p < 0.001$), and spot urine creatinine ($r = -0.178$, $p < 0.001$). On the contrary, 24-hour urine creatinine did not significantly correlate with body mass index, hemoglobin, spot urine protein, spot urine osmolality, 24-hour urine protein, 24-hour urine osmolality, serum cholesterol, serum triglyceride, serum high-density lipoprotein cholesterol, as well as serum low-density lipoprotein cholesterol (**Table 7**).

Twenty-four-hour urine protein significantly correlated with 24-hour urine volume ($r = 0.213$, $p = 0.013$), serum creatinine ($r = 0.741$, $p < 0.001$), and spot urine creatinine ($r = -0.178$, $p < 0.001$).

3.2.4 Predictors of spot urine creatinine

The variables that predicted spot urine creatinine were spot urine protein ($p < 0.001$) and 24-hour urine protein ($p = 0.021$), whereas body mass index, serum creatinine, spot urine osmolality, 24-hour urine creatinine, serum cholesterol, and serum low-density lipoprotein cholesterol did not (**Table 8**).

3.2.5 Predictor of 24-hour urine creatinine in non-HIV subjects

Only one variable predicted 24-hour urine creatinine—serum creatinine ($p < 0.001$)—whereas spot urine creatinine and 24-hour urine volume did not (**Table 9**).

Body mass index	0.056	0.520
Hemoglobin	0.046	0.593
Spot urine protein	−0.083	0.337
Spot urine osmolality	−0.091	0.294
Spot urine creatinine	−0.178	0.038
24-hour urine protein	−0.027	0.753
24-hour urine volume	0.213	0.013
24-hour urine osmolality	0.106	0.220
Serum creatinine	0.741	<0.001
Serum cholesterol (total)	−0.032	0.708
Serum triglyceride	−0.008	0.925
Serum HDL	0.038	0.657
Serum LDL	−0.092	0.286
Creatinine clearance	0.634	<0.001

HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol.

Table 7.
Correlation of 24-hour urine creatinine with variables in non-HIV subjects.

Variables	Beta	T	P value	95% CI
Body mass index	0.107	1.160	0.248	−1.964–7.523
Serum creatinine	−0.200	−1.729	0.086	−282.537–23.785
Spot urine protein	0.312	3.760	<0.001	1.350–4.346
Spot urine osmolality	0.100	1.318	0.190	−0.045–0.225
24-hour urine protein	−0.184	−2.331	0.021	−53.513 0–0.354
24-hour urine creatinine	0.026	0.228	0.820	−105.945–133.584
Serum cholesterol	−0.136	−0.632	0.528	−1.952–1.007
Serum LDL	0.375	1.804	0.074	−0.148–3.199

CI, confidence interval; LDL, low-density lipoprotein cholesterol.

Table 8.
Multivariate linear regression of variables with spot urine creatinine in non-HIV subjects (n = 136).

Variables	Beta	T	P value	95% CI
Serum creatinine	0.723	26,353	<0.001	−1.065–0.814
Spot urine creatinine	−0.003	−0.097	0.923	0.000–0.000
24-hour urine volume	−0.038	−1.389	0.167	0.011–0.013

CI, confidence interval.

Table 9.
Multivariate linear regression of variables with 24-hour urine creatinine in non-HIV subjects (n = 136).

4. Discussion

4.1 Discussion in HIV patients

4.1.1 Prevalence of dilute urine in HIV patients

This study noted the prevalence of dilute urine or low urine creatinine of 0.5% and concentrated urine or high urine creatinine of 6.4% in HIV patients. It showed an association between high urine creatinine and serum low-density lipoprotein cholesterol, $p = 0.001$, as well as serum high-density lipoprotein cholesterol, $p = 0.028$. It further showed that high urine creatinine very strongly correlated with 24-hour urine osmolality ($r = 0.95$), body mass index ($r = 0.74$), CD4 cell count ($r = -0.71$), and serum high-density lipoprotein cholesterol ($r = -0.73$). In this study the prevalence of low urine creatinine was 0.5%. This is in disagreement with 8.1% documented by Barr et al. [14]. In the same vein, the observed 6.4% prevalence of high urine creatinine in this study was a bit higher than the 3.1% reported by Barr et al. [14] in the same study previously mentioned. Differences in study design perhaps might explain the observed difference in the prevalence. Whereas the subjects in this group of our study participants were HIV patients in Nigeria, their study participants were non-HIV from a US general population. In Romania, studies reported high chronic kidney disease prevalence in HIV patients who were on variable antiretroviral therapy duration [15–17]. These Romanian studies evaluated kidney disease using MDRD equation, a formula that incorporated serum creatinine in its utility. Glaringly, however, their study failed to analyze daily urine creatinine excretion.

4.1.2 Concentrated urine associated with serum LDL and HDL in HIV patients

This study demonstrated a significant association between high urine creatinine and serum low-density lipoprotein cholesterol as well as serum high-density lipoprotein cholesterol. Literature was sparse on the impact of high urine creatinine excretion on serum low-density lipoprotein cholesterol or serum high-density lipoprotein cholesterol. Nonetheless, in chronic kidney disease, low serum low-density lipoprotein cholesterol and low serum high-density lipoprotein cholesterol are characteristic components of dyslipidemia. Lipid synthesis by the liver is thought to be induced by proteinuria in kidney disease. Triglyceride-rich apolipoprotein B (apoB) containing complex lipoproteins, mark these syntheses. They have profound atherogenic potential which inadvertently will impact negatively on the kidney and subsequently affect urine creatinine excretion [18–20].

4.1.3 Concentrated urine associated with 24-hour urine osmolality in HIV patients

This study showed that there was very strong correlation between high urine creatinine and 24-hour urine osmolality ($r = 0.95$). A similar observation was reported in a study that assessed the utility of urine creatinine and urine osmolality in determining dilute or concentrated urine and therefore the factors that influenced these. That study observed that the quantum of associations depicted as a fraction in change was profoundly stronger with urine creatinine than urine osmolality. The report noted that urine osmolality, compared to urine creatinine, was influenced by daily total protein intake but failed to vary by diabetes status. Although this association seemed relevant, the study inferred that the plausibility of accepting the utilization of urine osmolality adjustment and water intake

prescription to enhance on the accuracy of spot urine samples provision for the monitoring of bioenvironmental pollution would in itself espouse the merit for further evaluations [12].

4.1.4 Concentrated urine associated with BMI in HIV patients

High urine creatinine has a high correlation with body mass index, r = 0.74, in this study. This is in conformity with the findings reported by Forbes et al. [21] with r = 0.99 and Baxmann et al. [22] with r = 0.74. These two studies differed in design as they were administered in a very general population, compared to the present index subpopulation of our study that was an HIV patient population. The marginally higher correlation seen within the Forbes et al. [21] study showed a rather higher correlation which could be adduced to urine creatinine evaluation in lean body mass, in subjects very likely to be underweight.

4.1.5 Inverse correlation between concentrated urine and CD4 cell count and HDL in HIV patients

In this study high urine creatinine has a high inverse correlation, r = −0.71, with CD4 cell count. A study has documented an association between low CD4 cells count and underweight in HIV subjects [23]. Perhaps, this might account for the high, albeit inverse, correlation between high urine creatinine and CD4 cell count noted in our study. We also observed in our study that high urine creatinine has a high but inverse correlation, r = −0.73, with serum high-density lipoprotein cholesterol. There was dearth of studies that assessed the link between serum high-density lipoprotein cholesterol and high urine creatinine.

4.1.6 Correlation between concentrated urine and 24-hour urine volume and anemia in HIV patients

This study showed that there was moderate correlation statistics between high urine creatinine and 24-hour urine volume (r = 0.58) and hemoglobin (r = −0.43). The higher the concentration of urine, the lower the hemoglobin, implying that anemia was associated with concentrated urine. Literature search did not reveal any study that evaluated the effects of urine volume or hemoglobin on urine creatinine.

4.2 Discussion in non-HIV patients

4.2.1 Absent abnormal urine concentration in non-HIV subjects

This study showed that low and high urine creatinine was absent within the outpatient population as all of them have 24-hour urine protein within the normal range.

4.2.2 Correlates of spot urine and daily urine creatinine excretion in non-HIV subjects

Spot urine creatinine correlated significantly with body mass index (r = 0.225, p = 0.009), spot urine protein (r = 0.292, p = 0.001), spot urine osmolality (r = 0.223, p = 0.009), 24-hour urine protein (r = −0.187, p = 0.030), 24-hour urine creatinine (r = −0.178, p = 0.038), serum creatinine (r = −0.212,

p = 0.013), serum cholesterol (r = 0.246, p = 0.004), and serum low-density lipoprotein cholesterol (r = 0.282, p = 0.001). Factors that significantly correlated with 24-hour urine creatinine were 24-hour urine volume (r = 0.213, p = 0.013), serum creatinine (r = 0.741, p < 0.001), and spot urine creatinine (r = −0.178, p < 0.001). Spot urine protein and 24-hour urine protein predicted spot urine creatinine, whereas only serum creatinine predicted 24-hour urine creatinine.

4.2.3 Only normal levels of urine creatinine in non-HIV subjects

In this study there was an absence of low and high urine creatinine in subjects attending the outpatient clinic. This disagrees with the prevalence of 8.1% of low urine creatinine and 3.1% of high urine creatinine reported by Barr et al. [14]. Their study was conducted in a US general population in contrast with ours that was done in a general outpatient clinic population in Nigeria. This difference in study design might have accounted for the observed differences between the two studies. Additionally, our study subjects were patients who might have presented to hospital for one illness or the other that might impact on urine creatinine.

4.2.4 Spot urine creatinine associated with BMI in non-HIV subjects

Our study showed that BMI was associated with spot urine creatinine but not with 24-hour urine creatinine. This observation is similar to that reported in two studies [14, 24]. Two studies further demonstrated that body mass index was a predictor of spot urine creatinine [24, 25], in contrast with our study which showed that body mass index did not predict spot urine creatinine and 24-hour urine creatinine. Urine creatinine, a function of body mass index, a measure of lean body mass, depends on muscle mass.

4.2.5 Spot urine and daily urine protein excretion were predictors of spot urine creatinine in non-HIV subjects

This study demonstrated that spot urine protein and 24-hour urine protein were predictors of spot urine creatinine. This was slightly similar to a study that found protein intake associated with urine creatinine [26]. We observed that these two variables were not associated with 24-hour urine creatinine. Protein in urine predicting spot urine creatinine, with 24-hour urine creatinine within the normal range, indicated that the subjects studied might have proteinuria even in the presence of normal renal filtration function.

Spot urine osmolality was associated with spot urine creatinine but did not predict it, in this study. The precise relationship between urine creatinine and urine osmolality has not been fully elucidated, even though the utility of the hypothetical ratios for estimation of daily urine protein excretion involving creatinine and osmolality has been established [27, 28].

4.2.6 Inverse correlation between spot urine creatinine and daily urine creatinine excretion in non-HIV subjects

There was an inverse correlation between spot urine creatinine and 24-hour urine creatinine observed in this study. This implied that as spot urine creatinine increased, 24-hour urine creatinine declined and vice versa. Studies were sparse on the link between spot urine creatinine and 24-hour urine creatinine.

4.2.7 Association between serum creatinine and spot urine creatinine in non-HIV subjects

The study showed that serum creatinine was associated with spot urine creatinine. Serum creatinine in normal state is maintained at a reasonably constant level as excess creatinine produced by the body or taken exogenously is excreted in urine. This produces variability in the amount of creatinine in urine excreted by an individual and between different individuals [29]. However, elevated serum creatinine would be observed in impaired renal function, associated with reduced urine creatinine [30]. Expectedly, serum creatinine was a predictor of 24-hour urine creatinine in this study.

4.2.8 Spot urine creatinine associated with HDL and LDL in non-HIV subjects

Serum cholesterol and serum low-density lipoprotein cholesterol were associated with spot urine creatinine, as observed in our study. Lipid abnormalities have been described in renal disease associated with reduced urine creatinine excretion [31, 32]. This might suggest that our study subjects might have renal impairment.

4.2.9 Daily urine creatinine excretion associated with daily urine volume in non-HIV subjects

We noted that 24-hour urine volume was associated with 24-hour urine creatinine in this study. A related study reported an association between 24-hour urine volume and creatinine clearance [33]. In contrast, our study did not find any association between 24-hour urine creatinine and creatinine clearance. Nonetheless, urine volume tends to decrease with decreasing creatinine clearance, and 24-hour urine creatinine is a function of creatinine clearance. This probably would explain the association between 24-hour urine volume and 24-hour urine creatinine observed in this study.

5. Conclusion

The prevalence of low urine creatinine and high urine creatinine was low. Twenty-four-hour urine osmolality, body mass index, CD4 cell count, and hemoglobin were strong correlates of high urine creatinine. Dyslipidemia was common in HIV subjects who have high urine creatinine. Low and high urine creatinine was absent in non-HIV subjects. Proteinuric renal abnormalities, abnormal weight, and dyslipidemia were common in these non-HIV subjects with normal urine creatinine. There is need for clinicians to routinely conduct urine creatinine and further search for dyslipidemia, abnormal weight, depressed immunity, and anemia in HIV subjects with dilute or concentrated urine in the early stages of the infection. There is also a necessity for clinicians to routinely conduct urine creatinine and further explore for abnormalities of lipids, renal function, and weight changes in subjects with normal urine creatinine in non-HIV subjects.

6. Limitations of the study in HIV subpopulation

A larger HIV study population would have been better, as it would have prevented skewing of the colinearity that rendered null and void the multivariate linear regression of urine creatinine with the variables. Staging of HIV infection for all the

subjects and concisely defining the time from the diagnosis of HIV infection in these subjects to the conduct of this study were not done but would have contributed in further defining the link between urine creatinine and these factors. Similarly, the non-HIV study population was small. A way larger sample size would have shown a proportion of these with low and high urine creatinine, however little they may be, and also the potential risk factors of dilute and concentrated urine in this population.

Competing interests

The author declares no competing interests.

Permission

The methodology and tables in this book chapter are excerpts from articles published by Anyabolu [34, 35].

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Chapter 9

DNA Polymorphisms as Potential Biomarkers of Thrombophilic Prognosis for COVID-19 Patients

Tatyanny Paula Pinto da Costa Santos Fucci, Rubens Pitliuk and Ane Claudia Fernandes Nunes

Abstract

Coronavirus disease 2019 (COVID-19) is a major issue of our times. Many aspects and features of this new and complex disease are being described on a daily basis. Major endpoints are systemic inflammation, markedly characterized by the cytokine storm, respiratory failure, and coagulation disorders, such as thrombophilia. In its terms, thrombophilia has a major impact on the COVID-19 prognosis. With regard to this, paying attention on molecular variants, such as DNA polymorphisms, epigenetic factors, and other biomarkers, could be an important approach to optimizing and personalizing the treatment of patients according to their inherited thrombotic features. This chapter brings an overview on the three major DNA polymorphisms associated with thrombophilia and proposes that these same biomarkers could be used in pretreatment screenings of patients with COVID-19 to seek the most appropriate therapy for each individual molecular profile.

Keywords: COVID-19, coagulation, thrombophilia, biomarkers, DNA polymorphisms

1. Introduction

One of the major issues of the 21st century, without any doubt, is the viral respiratory disease discovered at the end of 2019, the COVID-19. Using next-generation sequencing, the pathogen related to COVID-19 was described as a novel coronavirus, which is related to the SARS-coronavirus described in 2003, mainly in Asia, from the molecular and phylogenetic aspects [1].

A severe respiratory disease was reported in Wuhan, Hubei province, China. Epidemiological investigations have suggested that the outbreak was associated with a seafood market in Wuhan [2].

The first case reported in the medical literature was that of a 41-year-old man who was hospitalized in the Central Hospital of Wuhan. The patient was admitted to the hospital reporting an extensive set of symptoms since one week before his admission on December 26, 2019, which included dry cough without sputum, fever, weakness, chest tightness, and widespread pain. Normal signs were observed at physical examination on abdominal, cardiovascular, and neurological features.

2. General aspects on COVID-19 diagnosis

Among the biomarkers analyzed, one of the most important findings was the raised C-reactive protein (CRP) level of 41.4 mg/L (standard at 0–6 mg/L) whose circulating concentrations rise in response to inflammation. Followed by the higher CRP, the biochemistry cardiac panel also showed raised myocardial infarction markers, such as lactic dehydrogenase (LDH), aspartate aminotransferase (AST), and creatine kinase (CK). Taken together, these biomarkers strongly indicated a severe acute inflammatory phase with the ongoing cardiac effect to be controlled. Mild lymphopenia with less than 9 × 10e5 cells per mL with normal platelet counts completes the first patient's overview [2].

Pulmonary function and lung aspects were investigated in this patient in order to do the etiological diagnosis. Hypoxemia was observed according to oxygen levels of 67 mm Hg. Lungs' aspect was set by chest radiographs on day 1 of hospital admission, which was the 6th day of disease progression. The images showed abnormal features with focal and patchy consolidation in both lungs, beyond air-space shadowing such as ground-glass like opacities [2].

Image examination of the chest shows a consolidation pattern at computed-tomography (CT) scans: bilateral focal consolidation, lobar consolidation, and patchy consolidation, especially in the lower lung. Five days after admission, at the 11th day of disease progression, a chest radiograph revealed a bilateral diffuse patchy and fuzzy shadow [2].

With the advancement of the pandemic, what was seen as a respiratory disease became a more complex disease and new studies were set up to list other complications and associated risk factors.

3. Potential impact of thrombotic complications on COVID-19 prognosis

Many factors can contribute to increasing the risk for severe COVID-19, in some cases followed to death. The main comorbidities described are high age, obesity, diabetes, and hypertension. Beyond the inflammation and impaired coagulation, focal damage in some tissues/organs is also related to the COVID-19 spectrum, such as liver, kidney, and heart [3].

Thrombotic complications seem to emerge as an important issue in patients infected with COVID-19. Preliminary reports on COVID-19 patients' clinical and laboratory findings include thrombocytopenia, elevated D-dimer, prolonged prothrombin time, and disseminated intravascular coagulation.

In the course of the COVID-19 studies, a clear association with coagulation dysfunction was pointed in many cases. Intra-alveolar clots were prominent findings in COVID-19 patients who developed severe respiratory disease. The same findings have been described in both clinical and animal model studies. Apparently, an impaired response in the prothrombotic pathway is in charge of diffuse alveolar hemorrhage since it is related to overt clot formation [4].

In the recent publication "Should COVID-19 be branded to Viral Thrombotic Fever?," the authors intended to frame COVID-19 in more clinical terminology, making an analogy to Viral Hemorrhagic Fever (VHF). In this article, the authors reported: "We found irrefutable evidence in the current literature that COVID-19 is the first viral disease that can be marketed as a viral thrombotic fever" [5]. Although this is a very categorical statement, considering the small number of studies exclusively dedicated to the characterization of COVID-19 as a thrombotic fever, it is very important to consider this designation. Categorizing COVID-19 as a febrile variant of thromboembolism adds a series of procedures to be adopted in

patients' care. This approach can advance the treatment adequacy by many steps, making it as more personalized as possible.

4. DNA polymorhisms for a pharmacogenonic approach to COVID-19 treatment

The risk of thromboembolism in COVID-19 is documented in an article published in The Lancet [6]. This finding brings up an important issue to be screened on COVID-19 patients: the impact of inherited predisposition to thrombotic events in patients affected by COVID-19.

Given this, thrombophilic genetic abnormalities in variants were widely reported in the medical sciences such as Factor V Leiden (F5), Prothrombin (F2), and the polymorphism in methylenetetrahydrofolate reductase (MTHFR), among others [7]. These polymorphisms could put a patient's carriers of mutant alleles in the Risk Group, beyond the well-known factors, such as elderly patients, hypertension, cardiac and respiratory diseases, cancer, and diabetes [8].

In this chapter, we present a brief review of the three main DNA polymorphisms associated with thrombophilic events and suggest the inclusion of these, as well as the coagulation profiles of their carriers, as aggravating comorbidities of COVID-19.

Firstly, a brief review of the main molecular characteristics of these polymorphisms is as follows:

4.1 Factor V Leiden (FVL or F5)

It represents one of the main causes of resistance to protein C, as mutation increases the risk of thrombotic disease three to ten times for heterozygous carriers and eighty times for homozygous carriers [4, 9]. About 90% of cases of protein C resistance result from point mutation in the coagulation factor V gene. This mutation occurs in exon 10 of the factor V gene, causing a substitution of the G/A base (Guanine/Adenine) in nucleotide 1691, resulting in the exchange of Arg (Arginine) by Gln (Glutamine) at position 506 of the protein, one of the main cleavage sites for protein C activation [10]. FVL is the most common inherited cause of venous thrombosis.

In patients with increased protein C resistance, venous thrombosis without known etiology and familiar history of unexplained thrombosis, the FVL mutations' screening should be considered beyond a strong clinical investigation. The diagnosis for FVL mutations is based on well-known molecular biology approaches. The clotting time-based functional assays and genetic biomarkers' screening become together the basis for clinical decisions. It is a very important step to guide the clinical approach, balancing the long-term anticoagulation with its side effects and benefits [11].

Briefly, the mechanism of action of factor V could be described as follows. Factor V is cleaved by thrombin on its B domain at cleavage sites R709, R1018, and R1545, producing an amino-terminal heavy chain and a carboxy-terminal light chain, which binding themselves create a dimer called Factor Va (FVa). In turn, FVa binds with Factor Xa creating a prothrombinase complex which on the platelet surface converts prothrombin (II) to thrombin (IIa). FV can also be split by the action of activated protein C (APC) at the cleavage site R506 before it is cleaved by thrombin. It results in the inactivation of factor V to factor Vi and the generation of an imperfect peptide, the Factor Vac, which apparently has anticoagulant characteristics by stimulating APC- and protein S-mediated inactivation of factor VIIIa. A second mechanism of thrombosis observed with FVL is its diminished cofactor activity with APC and phospholipid in the inactivation of factor VIIIa to factor

VIIIi. Taken together, FVL is a prothrombotic mutation due to a combination of a gain of function, with higher prothrombin activation, and loss of function due to low cofactor activity with APC in the inactivation of factor VIIIa. Normally, patients with defective FVL have a variable thrombophilia phenotype, have increased thrombin generation, have a longer factor Va half-life in plasma, and are resistant to factor Va inactivation [11]. A larger C-terminal peptide results if factor V is cleaved by APC before it is cleaved by thrombin [12].

4.2 Prothrombin (PTB, factor 2 or F2)

The G20210A mutation of prothrombin causes a G to A transition at the nucleotide position 20,210. This mutation increases circulating prothrombin activity and levels [13]. PTB is a vitamin K-dependent coagulation factor, which in its active form is cleaved, forming in this way the thrombin. The thrombin catalyzes many other coagulation-related reactions and acts as a serine protease that converts fibrinogen to fibrin. PTB mutations are the second-most common inherited thrombophilia. In the United States, the heterozygous carrier frequency is about 1–2%, accounting for approximately 6–18% of VTE cases. Hyperthrombinemia has been associated with a mutation in the 3′ termination of the PTB gene, called c.*97G > A, which results in increased production, due to the increased PTB mRNA expression and stabilization. An increased amount of circling prothrombin can lead to higher thrombin generation in the plasma, followed by coagulation activation and thrombosis. This mutation is also more common in the Caucasian population and is rare in other ethnic groups. Homozygosity, for this mutation, is found in about 1 in 10,000 individuals. Transheterozygosity for FVL and prothrombin c.*97G > A affects about 1 in 1000 individuals. Additional variations identified in the 3′-untranslated region of the prothrombin gene include changes at positions 20,207, 20,209, 20,218, and 20,221. High PTB levels also inhibit APC-mediated inactivation of activated FV and factor VIII. The prevalence of prothrombin G20210A mutation varies in different countries and ethnic groups, being highest in Caucasians, especially those in Southern Europe, and in the Mediterranean region [14].

4.3 Polymorphism in the MTHFR enzyme

Hotoleanu, in his article Genetic Risk Factors in Venous Thromboembolism, described that MTHFR acts on homocysteine metabolism, reducing 5.10-Methylenetetrahydrofolate to 5-methylenetetrahydrofolate. The enzyme polymorphisms generally occur at two sites, at position C677T, which characterizes the substitution of alanine for valine at codon 222, and at position A1298C, which occurs due to the substitution of glutamine for alanine at codon 429, the second mutation being less aggressive than C677T, which is homozygous and in the presence of low levels of folate decreases enzyme activity leading to hyperhomocysteinemia, a risk factor for thrombophilia [15].

Simoni et al. corroborate this theory, when they describe that mutations in the MTHFR enzyme reduce its activity leading to hyperhomocysteinemia. Increase in homocysteine levels is a risk factor for thromboembolism [16].

Considering patients with COVID-19, especially those seriously ill, there are several potential risk factors for venous thromboembolism, including infection, immobilization, respiratory failure, mechanical ventilation, and use of a central venous catheter [17, 18]. Wang et al. reported in their Lancet article that patients at a high risk for venous thromboembolism had worse results with COVID-19 than patients at a low risk for venous thromboembolism, suggesting that these patients may require more attention in the event of rapid deterioration [6].

Overall venous thromboembolism (TE): 21% (95% CI: 17–26%) ICU: 31% (95% CI: 23–39%)
Overall deep vein thrombosis rate: 20% (95% CI: 13–28%) ICU: 28% (95% CI: 16–41%) Postmortem: 35% (95% CI: 15–57%)
Overall pulmonary embolism rate: 13% (95% CI: 11–16%) ICU: 19% (95% CI: 14–25%) Postmortem: 22% (95% CI: 16–28%)
Overall arterial TE rate: 2% (95% CI: 1–4%) ICU: 5% (95% CI: 3–7%)
Pooled mortality rate among patients with TE: 23% (95% CI: 14–32%) and Pooled mortality rate among patients without TE: 13% (95% CI: 6–22%)
The pooled odds of mortality among patients who developed TE was 4% higher compared to those who did not (OR: 1.74; 95% CI: 1.01–2.98; $P = 0.04$)

Table 1.
Summary of the data set described by Malas et al. [19].

A systematic review and meta-analysis done to analyze the thromboembolism risk on COVID-19 patients showed that its occurrence is high and associated with the worst clinical development. A total of 8271 patients from 425 eligible studies were included in the meta-analysis. In summary, the data set showed that COVID-19 patients had a higher risk of mortality, as described in **Table 1** [19].

Our suggestion of early detection and greater attention in COVID-19 patients with aggravating factors of thromboembolism may be addressed to the data found by Wang and colleagues [6]. Considering the correct prophylaxis, the majority of the venous thromboembolism occurrences could be prevented, mainly on patients with a higher risk for it. In spite of that, from the 140 patients investigated in the cohort, only 7% (10 patients) were maintained under anticoagulant therapy during their hospitalization. Among them, one received rivaroxaban and nine received heparin. It is a low proportion compared to the total number of patients with high risk to develop venous thromboembolism in their cohort. This finding possibly indicates that the prophylactic approach applied in the patients with COVID-19 was not adequate.

Other coagulation disorders observed in patients with COVID-19 also support the idea that a preliminary analysis of the genetic factors involved may better guide the therapeutic approach to be adopted. COVID-19-associated coagulopathy and disseminated intravascular coagulation (DIC) are being described as common findings in these patients. It is known that the pathophysiology of DIC associated with COVID-19 differs from that of septic DIC, and in this context both thrombotic and hemorrhagic pathologies must be observed. Thrombosis events in COVID-19 include macrothrombosis (MAT) and microthrombosis (MIT), and it is important to note that the diagnosis of MIT depends on coagulation and fibrinolysis markers. Consequently, molecular nuances can have a major impact on the worsening of the thrombohemorrhagic condition in different individuals [20, 21].

5. Conclusion

Screening and inclusion of COVID-19 patients with genetic abnormalities in thrombophilic conditions could guide the medical team to identify possible aggravating complication factors even if their patients are not in the group predetermined risk, described by the World Health Organization (WHO) [8].

The treatment of COVID-19 is based on antiviral therapy, treatment to contain the cytokine storm, and treatment of thrombosis. Rather than providing uniform treatment, a method best suited for severity and stage should be selected. Considering the molecular profile of each individual can be an important tool in this race against time that characterizes care for patients with COVID-19. In this scenario, COVID-19 could be another exponent for a pharmacogenomics approach to the treatment of human diseases and it proved to be a challenge for humanity in the 21st century. The complications, sequels, and deaths took on catastrophic proportions. Despite the speed with which a significant range of vaccines were presented, comprehensive coverage worldwide is likely to face dares.

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Edited by Ane Claudia Fernandes Nunes

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Published in London, UK

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ISBN 978-1-83968-326-8
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