CHGA-Depleted Urothelial Amp and Immune Response During Urinary Tract Infection

Theadora Jane Ceccarelli

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LOYOLA UNIVERSITY CHICAGO

CHGA-DEPLETED UROTHELIAL AMP
AND IMMUNE RESPONSE
DURING URINARY TRACT INFECTION

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
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MASTER OF SCIENCE

PROGRAM IN INFECTIOUS DISEASE AND IMMUNOLOGY

BY
THREADORA JANE CECCARELLI

CHICAGO, ILLINOIS
AUGUST 2021
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LIST OF ABBREVIATIONS

AMP  antimicrobial peptide
Ca$^{2+}$  calcium
CAMP  cathelicidin antimicrobial peptide gene
CDC  Center for Disease Control
CgA  chromogranin A protein
CHGA  chromogranin A gene
CNS  central nervous system
CST  cathesitatin
DA  dopamine
DEFB4B  beta defensin 2 gene
EM  electron microscopy
EQUC  enhanced quantitative urine culture
EV-A71  enterovirus 71
FACS  fluorescence-activated cell sorting
GAG  glycosaminoglycan
GFP  green fluorescent protein
H&E  haematoxylin and eosin
HBD  human β-defensins
hBD2  human beta defensin 2
HBlEpC  human bladder epithelial primary cells
HFMD  hand, foot, and mouth disease
HTN  hypertension
IBD  inflammatory bowel disease
IHC  immunohistochemistry
IL-6  interleukin 6
iNOS  inducible nitric oxide synthase
KO  knockout
LB  Luria broth
LDCV  large dense-core vesicles
LDH  lactate dehydrogenase
LPS  lipopolysaccharide
M1  pro-inflammatory macrophages
M2  anti-inflammatory macrophages
Mcp1  monocyte chemoattractant protein-1
MRC1  mannose receptor-C type 1, CD206
NET  neuroendocrine tumor
NF-PNET  non-function pancreatic neuroendocrine tumor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>NIH</td>
<td>National Institute of Health</td>
</tr>
<tr>
<td>NPE</td>
<td>neurogenic pulmonary edema</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
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<td>PNET</td>
<td>pancreatic neuroendocrine tumor</td>
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<tr>
<td>Poly I:C</td>
<td>polyinosinic-polycytidylic acid</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
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<td>PST</td>
<td>pancreastatin</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
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<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SCLC</td>
<td>small cell lung carcinoma</td>
</tr>
<tr>
<td>SCCB</td>
<td>small cell carcinoma of the bladder</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate–polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SNcp</td>
<td>substantia nigra pars compacta</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>UC</td>
<td>ulcerative colitis</td>
</tr>
<tr>
<td>UGT</td>
<td>urogenital tract</td>
</tr>
<tr>
<td>UPEC</td>
<td>uropathogenic <em>Escherichia coli</em></td>
</tr>
<tr>
<td>UPKII</td>
<td>uroplakin II</td>
</tr>
<tr>
<td>US</td>
<td>United States</td>
</tr>
<tr>
<td>UTI</td>
<td>urinary tract infection</td>
</tr>
<tr>
<td>UT</td>
<td>urinary tract</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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<tr>
<td>WT</td>
<td>wild type</td>
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ABSTRACT

Women are 8 times more likely than men to experience a urinary tract infection (UTI) with up to 60% of women reporting a UTI in their lifetime. This significant healthcare burden is caused by the infection of uropathogenic *Escherichia coli* (UPEC) in the urinary tract resulting in clinical symptoms like urinary urgency, frequency, or hematuria. If left untreated, UPEC can retrograde to the kidneys causing more serious complications such as pyelonephritis. At this time, treatment is limited to antibiotic therapy, which is challenged by antibiotic-resistance. These factors make UTIs an NIH Women’s Health Initiative priority and are motivation for our research.

CgA (protein encoded by *CHGA*, located on chromosome 14) is a protein of the granin family co-released with catecholamines from neuroendocrine cells throughout the body. Pro-hormone convertases post-translationally cleave CgA into peptides, like catestatin, which can have antimicrobial peptide (AMP) activity. Previous research demonstrates CgA modulation of AMP activity in the skin. Additionally, systemic *CHGA* knockout mice demonstrate increased bacterial load in a murine UTI model. Therefore, our research aims to address CgA modulation of AMP and immune response in the bladder during a UTI (cystitis). We demonstrate CgA’s ability to facilitate AMP release from human bladder urothelial cells and explore the protein’s barrier function under the murine UTI model.
CHAPTER ONE
INTRODUCTION - LITERATURE REVIEW

Chromogranin A as Targeting Factor in Urinary Tract Infections

In the United States (US), Uropathogenic *Escherichia coli* (UPEC), a gram-negative uropathogen, is the leading cause of uncomplicated urinary tract infections (UTIs) in women today [1]. Up to 60% of women will experience at least one UTI in their lifetime costing the US over $3.5 billion per year [2, 3, 38]. The criteria for uncomplicated UTI diagnosis is challenged in academia, but is currently defined by the Center for Disease Control (CDC) as patients having at least one of the following symptoms: fever (>38°C), suprapubic tenderness, costovertebral angle pain or tenderness, urinary urgency, urinary frequency, or dysuria, and a bacterial count of ≥10⁶ CFU/ml [4]. If left untreated, cystitis (infection in bladder) can retrograde to the kidneys causing more serious complications, such as pyelonephritis. At this time, treatment is limited to antibiotic therapy, which is challenged by antibiotic resistance. The necessity for better prevention screenings and treatment methods makes this significant healthcare burden an NIH Women’s Health Initiative.

During a UTI, UPEC is equipped with two key virulence factors – flagella and type I pili [6]. Flagella allows UPEC to move against the urine flow, while type I pili facilitate attachment to the mucosal surface of the urinary tract. The type I pili promote
binding through the FimH cap, a mannose-binding adhesion protein. This versatile virulence factor allows UPEC to bind the surface of urothelial cells (superficial layer epithelial cells on bladder) with stability even under the shear stress of urinary flow [6, 13]. Once bound, UPEC invades the urothelium and activates the innate immune system. One of the first line defenses of human innate immunity are oligopeptides called antimicrobial peptides (AMPs). In eukaryotic epithelial cells, AMP production is induced via toll-like receptors (TLRs) by invading pathogen molecules, like bacterial lipopolysaccharide (LPS). Antibacterial AMPs are often amphipathic, with both hydrophobic and hydrophilic domains, which allows them to target and disrupt the lipid bilayer of the bacterial cell membrane [5]. Disruption of the AMP response has been observed in the skin when neuroendocrine mediators, like chromogranin A (CgA), are defected. Dampened AMP response results in increased bacterial invasion, and thus has implications for success of UPEC in UTIs.

CgA (protein encoded by CHGA, located on chromosome 14) is a protein of the granin family co-released with catecholamines from neuroendocrine cells (also termed catecholaminergic cells) throughout the body in response to signals sensed by the nervous system [6, 8, 9, 26]. The 48kDa protein is acidic in nature (due to the high percentage of glutamic acid and aspartic acid in its structure), has catecholamine-dependent aggregation properties, and several other factors, which are characteristic of the granin family [7, 9]. It also binds calcium (Ca$^{2+}$) at a high capacity, but moderate affinity. An increase in Ca$^{2+}$ signals exocytosis of CgA with other hormones from secretory granules in the adrenal chromaffin cells and adrenergic neurons [8, 9, 19]. In the secretory granules of islet cells, CgA blocks Ca$^{2+}$ influx in response to glucose,
which inhibits the release of insulin [20]. In this sense, CgA can regulate glucose homeostasis.

Various tumors can arise in the endocrine glands where CgA is produced. This makes CgA an essential clinical marker of neuroendocrine tumors, elevated levels indicating presence of tumor. In addition, abnormal levels also signify: inflammatory bowel disease, Parkinson’s disease, hypertension, hand, foot, and mouth disease, periodontitis, and stress [6]. As a current precursor for aforementioned disease states, there is possibility that aberrant CgA could also indicate UTI or complicated UTI-associated disease, like pyelonephritis.

**Chromogranin A and Catestatin**

Pro-hormone convertases post-translationally cleave CgA into peptides: vasostatin-1, vasostatin-2, pancreastatin, parastatin, and namely catestatin (CST) [7, 20]. The proteolytic cleavage products of CgA have several different functions in maintaining immune and metabolic homeostasis [20]. CST is a 21-amino acid cationic, hydrophobic peptide that is pleotropic [8]. It functions as part of a negative feedback loop with potent inhibitory activity on nicotinic receptors, blocking catecholamine release and thus release of its precursor, CgA. Clinically, patients with hypertension demonstrate high levels of CgA and low levels of CST in the plasma, with an overall hyperactivity observed in the sympathetic nervous system. This disease state, caused by the dysfunctional negative feedback loop in relation to the nicotinic receptors, can be rescued with a dose of CST [7]. Additionally, in CgA knockout (KO) mice, administration of CST rescues hypertension as well [9].
CST also has metabolic functions as it inhibits the α2-adrenergic receptor and increases leptin signaling [8]. This increases the lysis of lipids in adipose tissue and breakdown of fatty acids in the liver, providing the opposite effect of insulin on the body. Demonstration of CST metabolic activity is seen in CgA-KO mice, who exhibit an obese phenotype. After administration of CST, fat deposits decrease by ~25%. This has implications on the weight gain observed in patients with inflammatory diseases (for example, inflammatory bowel disease) and subsequently lower CST plasma levels [8].

Finally, CST plays a role in immunity [9]. As part of the innate immune response, CST acts as an antimicrobial that can penetrate bacterial membranes to cause lysis of the invading pathogen [8]. Beyond a direct effect on pathogens, CST is anti-inflammatory in nature, especially in autoimmune diseases. In vivo colitis mouse model studies demonstrated decreased granulocyte activity and circulating CRP, when the mouse was treated with CST. Recruitment of macrophages and monocytes to the site of infection also decreased with CST treatment. The mechanism of anti-inflammatory activity observed by CST in vivo is unclear, but it is hypothesized that the peptide affects the release of pro-inflammatory cytokines and chemokines [8]. Therefore, impairment in the proteolytic cleavage of CgA downregulates the release of CST and thus increases in disease states above.

CST may also play a role in determining the fate of a monocyte into a macrophage. Although there was no correlation between the two, treatment of monocytes with CST resulted in more anti-inflammatory phenotypes (M2 macrophages) as compared to control. Namely, increased levels of mannose receptor-C type 1 (MRC1), anti-inflammatory macrophage marker, was observed [8]. MRC1, or CD206, is
a transmembrane protein on the surface of macrophages that “recognizes mannose, fucose, and N-acetylglucosamine sugar residues on the surface of microorganisms” [10, 12]. As part of the human innate immune system, MRC1 is a pattern recognition receptor (PRR) that mediates endocytosis and phagocytosis of invading pathogens [11]. This has an interesting implication for UTIs. As UPEC invades the urothelium, macrophages are recruited to the site of infection. MRC1 will recognize and phagocytose UPEC, while at the same time, UPECs versatile virulence factor, type I pili, will recognize mannose residues on the surface of macrophages. Knockout of MRC1 prevents uptake of non-pathogenic E. coli, emphasizing the importance of the type I pili virulence factor [12]. As macrophages work to clear infection, UPEC attempts to evade the harsh host environment (i.e. presence of antibiotics) by offering itself to macrophages for hopeful survival inside the cell. Research demonstrates that fimbriated UPEC increases macrophage adhesion and survival in the presence of antibiotics compared to non-fimbriated mutants. Additionally, increase in dose of infecting UPEC was directly proportional to pathogenic survival of phagocytosis [13].

CST maintains the balance of macrophages during inflammatory diseases, like ulcerative colitis (UC) [14]. UC is an inflammatory bowel disease (IBD) that causes inflammation of the inner lining of the large intestine (colon) and rectum. IBDs are distinguished by a cycle of chronic relapse of inflammation and then remission. There is currently no cure available for patients suffering from IBD, so treatment focuses on supportive care during flare-ups and maintenance of remission. CgA has been indicated as an important molecule in UC patients, with high CgA levels during periods of inflammation. As previously discussed, CST has anti-inflammatory properties – acting in
negative feedback loop to block release of CgA and also, macrophage polarization. Using an acute and chronic murine model of UC, researchers have demonstrated decreased release of IL-6, IL-1β, and TNF-α from M1 (pro-inflammatory) macrophages when treated with CST. Although, CST was unable to promote release of anti-inflammatory cytokines – IL-10 and TGF-β – from M2 (anti-inflammatory) macrophages. M1 markers, inducible nitric oxide synthase (iNOS) and monocyte chemoattractant protein-1 (Mcp1), were also downregulated in UC model after CST treatment [14].

CST also acts as an antimicrobial in the innate immune response. Therefore, it is important to characterize how CST affects the gut microbiome during UC. Within the murine chronic UC model, mice were treated with CST and stool samples were collected for analysis. Treatment group showed no differentiation from untreated control in bacterial diversity or abundance. Whereas, dextran sulfate sodium (DSS) – positive control – showed significant altered microbial diversity [14].

Interestingly, CST demonstrates three single nucleotide polymorphisms: Gly364Ser, Pro370Leu and Arg374Gln [49]. Gly364Ser and Arg374Gln have less inhibitory activity on the catecholamines as compared to Pro370Leu or WT CST, and about 4% of the population exhibits one of the polymorphisms. The varying degree of catecholamine inhibition could alter the host defense response to infection, for example in the instance of UTI. Additionally, given CgA’s role in barrier function during a UTI, it begs the question of whether or not these genetic polymorphisms could alter host susceptibility.

CgA is important part of host defense against invading microbes. The proteolytic cleavage of CgA results in several products, namely CST. This peptide has several
important functions in homeostasis of immunity. CST can block its precursor in part of a negative feedback loop, increases lipid breakdown, has antimicrobial activity, and can encourage the polarization of M2 anti-inflammatory macrophages. Each of these functions contributes to the balance between inflammation and healing (anti-inflammatory actions) during infection or disease states (like UC).

**Chromogranin A as Diagnostic Marker in Disease**

CgA is an important marker for a variety of diseases. As a neuroendocrine peptide that is released with catecholamines during periods of stress, CgA has several functions part of host defense. Its specificity ranges from neuroendocrine tumors, neurodegenerative disorders, inflammatory disease, and viral and bacterial infections. The following section will demonstrate the scope of CgA as a diagnostic marker.

**Pancreatic Neuroendocrine Tumor (PNET)**

Pancreatic neuroendocrine tumors (PNET) are a common type of neuroendocrine tumor (NET) that develop in the pancreas [17, 18]. NETs arise in endocrine glands and are characterized by their unique secretory granules, termed large dense-core vesicles (LDCV), and production of hormones [18, 19]. The majority of PNETs are malignant in nature, but severity can range from benign to remarkably life-threatening. Mortality in PNET patients is often due to metastases to the liver and subsequent liver failure. Two clinical presentations exist – functioning and non-functioning (NF-PNET) [18]. These are defined by their ability to cause hormonal hypersecretion syndrome, but this can be difficult to diagnose as non-functioning often evolves into functioning. Based on the hormones released into the bloodstream during hypersecretion, patients can present with a variety of symptoms. The most common
markers include abdominal pain, weight loss, feeling thirsty, urinary frequency, and lethargy. In NF-PNETs, excess hormones are not released, which is why symptoms are usually not present until the tumor grows large enough to notice or metastasizes to other organs [22].

Understanding the severity of PNET and its functionality are essential components for diagnosis and treatment strategy. Two approaches to PNET patients exist – “wait and see” or aggressive treatment [17]. The latter requires specialists from several different departments to collaborate and often is defined by surgical management. CgA is a prominent marker in the diagnosis and management of PNETs. Even if hormonal hypersecretion syndrome is not clinically present, measuring serum levels of CgA assists in prevention of disease progression; in fact, the protein has 60-100% diagnostic sensitivity [17, 18].

CgA enters through the endoplasmic reticulum of the cell and is carried by the Golgi to be stored in the LDCV of PNETS. This allows CgA to be widespread throughout the tumor tissue [20]. As a type 0 biomarker, defined by the NIH as “markers of the natural history of a disease correlate longitudinally with known clinical indices (symptoms) over the full range of a disease state,” increased CgA serum levels were associated with diffuse disease state of NF-PNETs [20]. This simply means CgA was especially increased in cases where PNET had metastasized to the liver. The relationship between CgA serum levels and NET severity is unique to PNET specifically, not all NET types exhibit this correlation or specificity.
Small Cell Carcinoma of the Bladder (SCCB)

Small cell carcinoma of the bladder (SCCB) is an extremely rare neuroendocrine tumor. Diagnosed late in the disease progression, SCCB is aggressive (highly metastatic) and frequently difficult to identify. In fact, over 95% of cases are diagnosed at muscle invasive stage and about 70% of patients die within the first 12 months of diagnosis [21, 23]. Other than the presence of a tumor, the main symptoms are dysuria, hematuria, and UTI. Histology and immunohistochemistry (IHC) of a tumor biopsy is necessary for diagnosis. Haematoxylin and eosin (H&E) histological staining reveals the same phenotypes seen in small cell lung carcinoma (SCLC), and thus the World Health Organization (WHO) requirements for SCLC diagnosis are followed. The phenotype includes densely packed cells with sparse cytoplasm and few organelles [21].

SCCB is very similar to invasive urothelial carcinoma, but it is more aggressive in disease state and progression. This makes it critical to have a reliable marker(s) to accurately differentiate between the two. CgA is one of the antibody markers used in IHC and is present in ~60% of SCCB cases (range 20-89%) [23]. IHC staining with CgA antibody on urothelial carcinoma samples is only ~5% [23]. A SCCB sample would demonstrate strong, cytoplasmic staining of CgA, as well as a few other antigens like synaptophysin and neuron-specific enolase [23].

Parkinson’s Disease (PD)

Parkinson’s Disease (PD) is a neurodegenerative disorder that causes motor dysfunction in its patients [24, 25]. The motor deficits are caused by degradation and subsequent loss of function of dopaminergic neurons. These neurons are located in the midbrain, specifically in the substantia nigra pars compacta (SNpc). As the name
suggests, dopaminergic neurons are responsible for dopamine (DA) production and release [26]. Symptoms of PD include “tremor, bradykinesia (slow movement), muscle rigidity,” and “impaired gait and posture” [25]. Additional defects are frontostriatal-mediated and include dysfunction in “attention, speed of mental processing, verbal disturbances, impairment of working memory and impulsivity” [25]. Albeit the cause of PD is debated, it is likely due to misfolded proteins that clog up the cytoplasm of dopaminergic neurons.

CgA serum levels are widely used as a marker in other neurodegenerative diseases, like Alzheimer’s [27]. In the case of PD, the overall loss of function in the midbrain results in the breakdown of dopaminergic neurons – which are a type of catecholaminergic cell. As previously discussed, CgA is produced and released from the secretory granules of catecholaminergic cells. Hence, as the dopaminergic neurons lose function and degrade, CgA is not produced and released. The decreased CgA levels are reflected in cerebral spinal fluid of PD patients [27]. This correlation is rather new (January 2021) and additional studies need to be conducted to confirm the use of CgA as a diagnostic marker of PD.

**Hypertension (HTN)**

Hypertension (HTN) is a chronic increase in blood pressure associated with age and influenced by genetic and environmental factors [28]. Individuals with HTN are at an increased risk for other cardiovascular disease as the two are linked to one another. In order to reach an HTN diagnosis, a patient must have increased blood pressure for at least two consecutive clinic visits, target organ damage (impairments of major organs caused by increased blood pressure), and/or evidence of cardiovascular risk factors.
Disruption in sodium and fluid homeostasis is the likely first step in HTN development. The balance of sodium and fluids is highly regulated in the kidneys, and is controlled throughout the body by “hormonal, nervous system, paracrine, and intracellular feedback loops” [28]. When the kidney has difficulties maintaining normal fluid volumes, blood pressure increases. One role of CST, as described prior, is a negative feedback loop blocking catecholamine release and thus its precursor, CgA [7]. In hypertensive patients, CgA serum levels are elevated, while CST is depleted. This indicates a dysfunctional negative feedback loop between CST and the nicotinic receptors that control catecholamine release, which can be rescued with administration of CST [7, 29]. Sodium dyshomeostasis observed in the kidneys causing HTN, is likely due to the hormone imbalance occurring throughout the body as the negative feedback loop is nonfunctioning [28, 29]. Although CgA is not currently used as a diagnostic marker for HTN, it does play a role in progression of the disease state.

**Pediatric Hand, Foot, and Mouth Disease (HFMD)**

Hand, foot, and mouth disease (HFMD) is an infection caused by enteroviruses – coxsackievirus A16, coxsackievirus A6, or enterovirus 71 (EV-A71). HFMD spreads easily in children under the age of five, outbreaks often occurring in day cares. The virus is transmitted through respiratory droplets in the air or on fomites. Symptoms include
fever, mouth sores, and sore throat, with the signature symptom appearing as a rash on the palms of the hands or soles of the feet. Although contagious, the disease state is often non-severe [15]. If infected with EV-A71, complications are more common and a simple infection can rapidly decline into central nervous system (CNS) injury (brainstem encephalitis), neurogenic pulmonary edema (NPE), or pulmonary hemorrhage, which can lead to death [16].

HFMD is endemic in China and South East Asia and there is currently not a stable treatment or indicator to prevent the more severe disease state [15, 16]. Since CgA is a neuroendocrine mediator, alterations in serum levels coincide with sympathetic nervous system status. Patients with severe HFMD have hyper-active sympathetic nervous systems with onset of symptoms that complicate into CNS injury or NPE. Research supports the use of CgA as a diagnostic marker for severe HFMD. In a study done on pediatric patients with EV-A71 HFMD, increased CgA serum levels on presentation was directly associated with mortality [16].

**Periodontitis**

Periodontitis is an inflammatory disease caused by pathogenic bacterial buildup and biofilm formation that degrades the gums supporting teeth [30]. *Treponema denticola* and *Porphyromonas gingivalis* are the two anaerobic bacteria, which frequently cause periodontitis [31]. In the response to bacterial infection, the inflamed gums pull away from the teeth causing bleeding [30]. Patients suffering from periodontitis are typically those who are not able to practice proper oral hygiene or who do not have access to regular dental care. These stressors can exacerbate the periodontal symptoms. If the infection progresses, the patient can lose teeth and painful
abscesses can form. Psychological stress as determined by salivary cortisol levels is associated with periodontal infection. Stress exhausts the immune response allowing an easier pathway to infection for bacteria [31].

Salivary CgA levels are rather sensitive to environmental stressors (dry mouth), as well as immune stressors (microbial infection). This makes CgA a particularly delicate predictor for periodontitis as compared to cortisol. While cortisol is useful for assessing the advancement of periodontitis, the caveat remains its inability to predict disease. As the inflammatory response is activated in the oral cavity in response to stress or infection, neuroendocrine mediators (like CgA) are released to facilitate host defense. The source of CgA is likely accredited to the human oral mucosa, similar to other mucosal surfaces throughout the body. The release of CgA in periodontal infection is specific, with increased levels identified in saliva, but not serum [31].

**Antimicrobial Peptides**

The human immune response is divided between innate and adaptive immunity. Innate immunity is the first line of defense against invading pathogens. While adaptive immunity constitutes a more sustained response in host defense. When the body is threatened by a bacterial, viral, or fungal invasion, AMPs are the first line of protection. Their antimicrobial activity allows them to target and kill the foreign microbes, similar to the actions of antibiotics [32]. The most compelling advantage of AMPs are their antibiotic activity on antibiotic-resistant strains of bacteria. Their ability to target not only bacteria, but also fungi and viruses also gives them an added benefit compared to traditional antibiotics. Understanding the full scope of AMPs is important for researchers
and clinicians alike, but in this review a focus will be placed on AMP activity in regards to bacteria, specifically gram-negative.

AMPs are mostly cationic and amphiphilic, which enables them to disrupt bacterial structures efficiently [32]. The positively-charged AMPs are rich in lysine and arginine (positively charged amino acids). This positivity creates an electrostatic interaction between the AMP and the negative cell membrane of bacteria. Their hydrophilicity and hydrophobicity allow the AMP to breach the lipid bilayer, and take root in the hydrophobic core of the inner membrane. AMP activity is not restricted to gram positive or gram negative bacteria, instead they are able to agitate either cell structure successfully [32]. Mammalian cationic AMPs can be divided based structure – α-helical peptides and β-sheet peptides – or by biological properties – cathelicidins and defensins [32].

**Cathelicidins**

Cathelicidins are composed of two functional domains. Cathelin at the N-terminal and an antimicrobial domain at the C-terminal [34]. Although thousands of cathelicidins exist in mammals, only one is present in humans – LL-37 (sometimes termed CAMP), encoded by the gene *CAMP*. LL-37 is an α-helical AMP and derives its name from its 37-amino acid length [35]. During early discovery, LL-37 was thought to be stored only in myeloid cells – like neutrophils or macrophages, but later it was determined it also resides in alternative cell types, like epithelial cells [34]. Now it is widely accepted that LL-37 is present in an array of tissues, from the gastrointestinal tract to the squamous epithelium of the tongue [34, 35]. Interestingly, prior to the discovery of antibiotics, patients infected with *Mycobacterium tuberculosis* had improvement after spending
extended periods in the sun. Therefore, "sun baths" were prescribed to those infected
[34]. This is likely due to the activation of LL-37 in macrophages in response to vitamin
D release in the skin by sunlight. The increase in sunlight exposure increased LL-37
availability to response to *Mycobacterium tuberculosis*.

Activation of LL-37 is initiated by proteolytic cleavage into its two regions –
cathelin (highly conserved) and antimicrobial domain [34]. In the activated form, they
are released from secretory granules in part of the inflammatory response and/or
infection. As a cationic peptide, cathelicidin AMP domain can specifically target the
negatively charged membrane of microbes. In the example of gram-negative bacteria,
like UPEC, the AMP binds parallel to the outer membrane, adheres, and perpendicularly
slinters itself into the periplasm until finally crossing the inner membrane. This splinter
allows access for other AMPs to enter and coagulate together in the cytoplasm. These
actions ultimately kill the bacterial cell [34].

**Defensins**

Defensins are comprised of β-sheet peptides, connected by disulfide bonds [36].
Based on the location of the disulfide linkages, defensins are further categorized in
humans into α- and β-defensins. The category of defensin also determines the location
of its storage. α-defensins are localized to neutrophils, while β-defensins are a part of
the epithelia throughout the body. During the innate immune response, defensins are
either released from neutrophils, or from surrounding epithelial tissue [36]. During a UTI,
defensins are released from the urothelial cells that line in inside of the bladder. Their
activation is achieved through post-translational modifications [36].
Human β-defensins (HBD) 1-4 are the best studied of the HBDs and are found mostly in epithelial tissues. They can be constitutively expressed, or activated in response to bacterial infection via TLR stimulation or pro-inflammatory cytokines. Less is known about human α-defensin activation, but it is likely due to pro-inflammatory cytokines as well [36]. Each of the HBDs are differentially effective in bacterial cell disruption, but all can efficiently act as part of host defense. Once the an HBD reaches the bacterial invader, their actions are similar to that of cathelicidins. Simply, they are able to disrupt the bacterial membranes and initiate cell death [34, 36]. Human defensins, α and β alike, are also important for the modulation of the inflammatory response. They assist in the release of cytokines and chemokines, while also influencing the apoptosis of neutrophils based on environmental cues [36].

**Microbiota of the Urinary Tract**

Albeit academia has debated the presence of the female urinary microbiome, research has confirmed its existence [37]. The most up-to-date study projects over 100 different species that contribute to the female urinary tract (UT) and urogenital tract (UGT). The lower UT (bladder and urethra) is comprised of urothelial cells, whose luminal side is covered by a glycosaminoglycan (GAG) layer and apical side with umbrella cells [38]. Umbrella cells of the apical urothelium express transmembrane uroplakins, which are plaque-forming proteins. These special plaques make it difficult for foreign microbes to attach and invade the urothelium. They also are flexible to accommodate micturition (emptying and filling) of the bladder. Although it is now agreed that a UT microbiome exists, the location of these commensal bacterium is still unknown. It is possible that, like UPEC’s attachment due to virulent type I pili, they also
are able to attach to uroplakins [38]. The exact composition of the UT microbiome is also complicated. The bladder, in example, is a dynamic environment. The pH of urine varies, the bladder fills and expels, there is shear flow, immune involvement, and the issue of a reliable nutrient source. It could be that a urine-full bladder provides nutrients for the host microbiome or, conversely, perhaps the commensals breakdown and utilize the GAGs covering the urothelium [38]. Further research is needed to understand both the location and mechanism of survival of the UT microbiome.

16s rRNA sequencing is regularly used in the field to determine the players of the UT microbiome [37, 38]. The 16s gene is highly conserved across all DNA-based life forms. Therefore, universal primers of 16s conserved regions can be amplified via PCR encompassing all microorganisms present in the sample. This, along with enhanced quantitative urine culture (EQUC), which allows for differential culturing of the sample, provides researchers with a comprehensive picture of the microbiome. In current literature, the composition of the UT commensal population is varied, but the major genera include: Lactobacillus, Corynebacterium, Prevotella, Staphylococcus, and Streptococcus [38]. Additionally, the female microbiome of the UT does share characteristics with the vaginal microbiome, which is supported based on the proximity of the urethra to the vagina. Lactobacillus is known to be prevalent in the vaginal microbiome and the UT shares this commensal prevalence. Further research is necessary to determine the environment of the female UT and UGT. Understanding and characterizing the microbiome will reveal the full interactions between invasive species and the UT during disease states, like UTI.
Concluding Remarks

An uncomplicated UTI is diagnosed based on a bacterial count of \( \geq 10^5 \) CFU/ml. This arbitrary threshold was determined years before a UT microbiome was discovered and accepted. Currently, there is a disconnect between academia and the clinic, where asymptomatic patients may be treated for a UTI based on a positive urine culture. This does not take into account the presence of bacterial species in the natural female microbiome and contributes to antibiotic over-use.

When UPEC invades the UT, it is equipped with unique virulence factors – type I pili and flagella – that allow it to survive in the harsh environment and evade the immune system. On the host side, uroplakins of the umbrella cells express CgA, a diverse disease marker, whose proteolytic cleavage products – namely CST – can recruit macrophages, have AMP activity, and/or work in the negative feedback loop for CgA suppression. The AMPs are responsible for targeting and destroying bacterial cells, while also modulating the innate immune response through cytokines and chemokines or induction of immune cell apoptosis.

In literature, abnormal production of CgA and its products results in detrimental dysfunction in a variety of disease states. This is especially true when CST is not present to regulate the negative feedback loop. There is value in understanding the role of CgA and CST in the context of UTI, especially appreciating the part CgA plays in other PNET, SCCB, and other bacterial infections, like periodontitis. In order to address the relationship between CgA and cystitis, we set up two aims:
AIM 1: Reveal mechanisms by which altered production of CgA/CST modulates TLR-dependent AMP activity and immune responses in human bladder epithelial cells.
   a. Assess CHGA-depleted modulation of AMP responses and cytokine/chemokine secretion in WT and siRNA knockdown cells

AIM 2: Establish that CHGA modulates the local bladder AMP and innate immune response to UPEC infection in mice.
   b. Quantify AMP responses, cytokine/chemokine production and immune cell infiltration in WT and uro-CHGA−/− mice.
   c. Quantify UPEC survival, attachment and invasion in WT and uro-CHGA−/− mice.
CHAPTER TWO
MATERIALS AND METHODS

In vitro – AIM 1

siRNA

In order to assess CgA-depletion on AMP responses and cytokine/chemokine responses, siRNA was done to knockdown CgA. Human bladder epithelial primary cells (HBlEpC) (Cell Applications#938-05a) were cultured from a fresh P0 cryovial. Cells were split at 100% confluency and plated on 12-well plates in Human Bladder Epithelial Growth Medium (Cell Applications#217-500). At ~80% confluency, plates were ready for siRNA transfection using the CHGA 27mer siRNA duplex kit (OriGene Technologies#SR319699). One hour prior to transfection, plates were aspirated and replaced with fresh media. From the 20uM company stock, siRNA and scrambled duplex constructs were each combined with duplex buffer to reach 5uM concentration. The 5uM mixes were then diluted again with transfection buffer and transfection reagent – siTRAN (OriGene Technologies# TT320002) – for a final concentration of 5nM. Mixes were incubated for 15 minutes at room temperature (RT), then were added dropwise to respective wells while swirling. Plates were incubated for 8 hours at 37°C, at which point were aspirated and replaced with fresh media to return to 37°C. After 24 hours, supernatant was collected and set aside for lactate dehydrogenase (LDH) assay to determine cytotoxicity. Then, cells were stimulated with vehicle control, 10ug/ml
polyinosinic-polycytidylic acid (poly I:C) TLR control, 10ug/ml LPS, or 100ng/ml flagellin and returned to 37°C.

Collection occurred 24 hours later (48 hours post-siRNA transfection). Cells were lysed with TRIzol® reagent (Life technology#15596018) for 1 hour, following manufacturer’s protocol, before scrapping. Purelink minikit (Fisher Scientific#12-183-025) was used to isolate RNA. Complementary DNA was synthesized using iScript™ cDNA Synthesis Kit (Bio Rad#170-891).

**Quantitative Polymerase Chain Reaction (qPCR)**

Quantitative real time RT-PCR was performed in duplicate using TaqMan® Advanced PCR Master Mix (Fisher Scientific# 44-445-56) on a StepOnePlus™ Real-Time PCR System (Life technology) to determine the relative mRNA expression of AMPs. Fluorogenic probe and primers were used to detect CHGA (Hs00900370_m1), IL-6 (number), CAMP (number), TNF-α (number), and DEFB4B (number). Expression of target gene was normalized with B2M (4325797) and analyzed by the 2^{-ΔΔCt} method.

**LDH Assay**

Supernatants were reserved from siRNA-transfected cells at 24hr, 48hr, or 72hr to use with the cytotoxicity detection kit (LDH) (Roche#11644793001). Supernatants were spun at 250xg for 10 minutes. 100uL/well of supernatant was transferred into a 96-well plate. Reaction mixture was added and the plate was incubated for 30 minutes at RT. Absorbance was measured at 492nm on Spectramax Plus 384 (Molecular Devices) plate reader.
Western Blot

Although qPCR enables measurement of relative gene expression, it does not confirm the associated protein is produced. Therefore, a western blot was used to confirm protein production of CgA, Il-6, cathelicidin, TNF-α, and human β-defensin 2. Following the above siRNA protocol, 6-well plates were transfected to knockdown CHGA. At 48 hours, cells were aspirated and RIPA buffer (Fisher Scientific#0089900) with Protease Inhibitor cocktail (Fisher Scientific#78410) was added. While on ice, cells were scraped into 1.5 mL tubes, spun down, and supernatants were reserved. Using the Pierce™ BCA Protein Assay Kit (Fisher Scientific#23225), protein concentration was confirmed to meet 30ug needed for SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis). Samples were run for 21 minutes at 200V on a NuPAGE™ gel (Fisher Scientific#NP0321BOX) in 20x MES SDS running buffer. Gel was transferred using an iBlot™ 2 Gel Transfer Device (ThermoFisher#IB21001). After washing and blocking, membrane was incubated with 1:1000 rabbit polyclonal chromogranin A primary antibody (abcam#ab45179) overnight. The following day after washes, the membrane was incubated with anti-rabbit IgG HRP-conjugated secondary antibody (Cell Signaling Technology#7074S) before imaging. CgA bands appear at ~50.7kDa.

Enzyme-Linked Immunosorbent Assay (ELISA)

To assess the immune response through release of AMPs, a human cathelicidin antimicrobial peptide (CAMP) ELISA kit was obtained (MyBioSource#MBS451986). Supernatants were collected from triplicate treatments of siRNA transfection cells with
TLR ligands (previously described). Eight standard serial dilutions were prepared according to kit protocol, from 10ng/mL to 0ng/mL. Standards, blanks, and samples were added to the pre-coated plate and allowed to incubate for 2 hours at 37°C. Samples were added at 1:1 and 1:2 dilution to ensure a measurable result. After incubation, plate was aspirated and the first detection reagent was added for 1 hour at 37°C. After this incubation, the plate was washed three times, the second detection reagent was added, and the plate incubated for 1 hour at 37°C. After 5 more washes, the substrate solution was added and the plate incubated for 15 minutes at 37°C. After this final incubation, stop substrate was added and plate was run at 450nm on the Spectramax Plus 384 (Molecular Devices) plate reader.

**In vivo – AIM2**

**Murine Model of UTI**

*Escherichia coli* UTI89-vsfGFP-9 chromosomal strain of UPEC was streaked on a Luria broth (LB) plate on day one. This UPEC GFP variant was optimized to eliminate any *in vivo* virulent defects and is 10x brighter, which makes it a good choice for the murine UTI model [40]. On day two, one colony was suspended in LB broth and set to incubate statically in 37°C overnight. This incubation method allows for the UPEC to express sufficient type-I piliation [39]. The following day, day three, a serial dilution of the suspension was done to ensure type-I pili are expressed in majority of bacteria [39]. On day four, infection day, the suspension underwent a series of spins and washes before being measured at OD$_{600}$ to confirm ~2x10$^7$ CFU.
Mice with CHGA knocked out in uroplakin II of bladder urothelium (uro-CHGA\(^{-/-}\)) were obtained from crossing CHGA\(^{\text{floxed}}\) mice (developed by Mutant Mouse Resource and Research Center at University of California Davis) with UPKII-Cre mice. A catheter, made from 30-gauge needle and polyethylene tubing, was used to infect Uro-CHGA\(^{-/-}\) and wild type (WT) C57BL/6 mice intraurethrally with 10\(\mu\)L \(\sim 2 \times 10^7\) CFU UPEC over 10 seconds following the murine model of UTI [39]. Bladders, kidneys, and/or spleen were harvested 6 hours or 18 hours post-infection. Kidneys were reserved for bacterial counts. Bladders were also taken for bacterial counts, as well as qPCR, immunohistochemistry (IHC), terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, and electron microscopy (EM). Spleen was used as control cell population in FACS.

**Genotyping.** In order to confirm proper genotyping of the mice, tail snips were taken from the experimental group prior to day of experiment. They were digested in Direct Tail PCR reagent (Viagen#101-T) and Proteinase K 10mg/ml (Sigma#P6556) for 3 hours at 55\(^\circ\)C. Then heat was increased to 85\(^\circ\)C for 45 minutes. Samples were vortexed and used for PCR. Reactions included DreamTaq™ Hot Start Green PCR Master Mix (ThermoScientific#K9021), CHGA forward and reverse primers, and molecular grade water. Each reaction included CHGA floxed forward primer (TAGTGCGCAGAGGTGTTTGCATTGG) and one of the two reverse primers: (AGTCAATCACATGGGCATTCCAGC or ATCTGCTCTGTGTTATCGGCAAGC). Reactions were run on Eppendorf Mastercycler PRO S6325 thermal cycler. Samples
were run on a 1% agarose gel 1x TAE (tris-activated EDTA) (ThermoScientific#B49) for 45 minutes at 100V. Gel was viewed and imaged on a Bio Rad ChemiDoc XRS+.

**qPCR.** In order to confirm *uro-CHGA* via relative gene expression, qPCR was done. To prepare excised bladders for qPCR, samples were added to 1mL of TRIzol® reagent (Life technology#15596018). In a cryogenic grinder (SPEX® SamplePrep#6770), which uses a magnetic impactor at liquid nitrogen temperatures, samples were pulverized into powder and allowed to melt at room temperature for RNA isolation. The above protocol was followed. Fluorogenic probe and primers were used to detect *CHGA* (Hs00900370_m1). Expression of target gene was normalized with *GAPDH* (0912025) and analyzed by the 2^-ΔΔCt method.

**Bacterial count.** Bacterial counts were taken to compare bacterial load by strain and ascertain presence of ascending infection or reflux into the kidneys. Kidneys and bladders were homogenized in 1mL phosphate buffered saline (PBS) with chrome-steel beads (BioSpec#11079113c) on a BioSpec Mini-Beadbeater (BS#607). Homogenates were 10-fold serially diluted in PBS and plated on LB agar plates. These plates were incubated for 24 hours at 37°C and then counts were taken.

**Immunohistochemistry Staining (IHC)**

IHC allows visualization of bladder morphology through H&E, provides confirmation of *Uro-CHGA* via double stain, and indicates apoptosis as a result of bacterial invasion by TUNEL stain. For these purposes, bladders were excised and placed directly into a 15mm base mold with cryo optimum cutting temperature (OCT) compound (Fisher Scientific# 14-373-65). After setting for ~5 minutes, base molds were
frozen in liquid nitrogen for 20 seconds to reach solidification. Sectioning was done in
house on a CryoStar NX50 (Thermo Scientific) cryostat or sent out to Loyola Clinical
Pathology Lab. Both 14um and 5um sections were taken from the center of the bladder
and situated on slides.

**H&E staining.** Slides were dried in hybridization oven at 60°C for 15 minutes. A
standard H&E staining protocol was followed. Hematoxylin Solution, Harris Modified
(Millipore Sigma#HHS80-2.5L) and Eosin Y solution, alcoholic (Millipore
Sigma#HT110180-2.5L) were used. Slides were fixed with VectaMount® Permanent
Mounting Medium (Vector Laboratories#H-5000-60). Staining was viewed on an
EVOS® FL Cell Imaging System from Thermo Fisher Scientific at 40x (data not shown).

**Co-localization stain.** Frozen slides were removed from -80°C and allowed to
air dry. A hydrophobic barrier was drawn around the samples with a PAP pen (Vector
Laboratories#H-4000). Slides were fixed with ice cold methanol and washed 3x with
PBS. They were incubated with blocking buffer – 10% normal goat serum, 1% bovine
serum albumin, 0.5% Triton X-100 in 0.1M phosphate buffer, pH 7.4 – for one hour at
RT. Primary antibodies – anti-CHGA unconjugated (Abcam#ab254322) and anti-
uroplakin II unconjugated (Abcam#ab204756) – were added with 3% normal goat
serum, 1% bovine serum albumin, 0.5% Triton X-100 in 0.1M phosphate buffer, pH 7.4
and left to incubate overnight at 4°C. The following day, slides were washed 3x with
cold PBS. The secondary antibodies – AF594 (Abcam#ab150080) and AF488
(Abcam#ab150077) – at a 1:2000 dilution were added to 3% normal goat serum, 1%
bovine serum albumin, 0.5% Triton X-100 in 0.1M Phosphate buffer, pH 7.4. Slides
were fixed with ProLong™ Diamond Antifade Mountant with DAPI (Molecular Probes#P36966). They were viewed under a widefield microscope at 60x.

**TUNEL stain.** During a UTI, urothelial cells undergo apoptosis as apart of abnormal barrier function, in this case the absence of CHGA [42]. Therefore, bladders were also cyro-sectioned by our pathology core at 14um and sent to receive TUNEL stain from our collaborator, Dr. David Klumpp, at Northwestern University. Briefly, TUNEL staining is able to identify cells that have undergone apoptosis through the apoptotic hallmark, internucleosomal DNA fragmentation [41]. When the DNA fragments, free 3'-hydroxyl termini become available. The TUNEL stain uses terminal deoxynucleotidyl transferase to attach a labeled dUTP. In order to confirm specificity for cells expressing uroplakin (where CHGA is depleted), tissue sections were pre-stained with uroplakin Ia antibody (Novus Biologicals#NBP2-14694) prior to the TUNEL assay. Then, they were treated with proteases before undergoing the TUNEL reaction. Finally, they were fluorescent-FITC stained, followed by a counterstain, and viewed under a fluorescent microscope [41].
CHAPTER THREE

RESULTS

In the context of the skin, CgA depletion results in dampened AMP and immune response (cytokine/chemokine production and immune cell recruitment) [46]. This abnormality in barrier function provides invading pathogens with a more hospitable environment for infection. We hypothesized that CgA depletion in the urothelial cells of the bladder could alter barrier function and modulate TLR-dependent AMP activity and immune responses.

CHGA Modulation of AMP and Immune Response in Human Cell Line

In order to determine the role of CgA in vitro, we began by knocking down CHGA via siRNA in human bladder epithelial cells (HBIEpCs). Preliminary optimization was performed to determine the best concentration and duplex of siRNA (three provided by company – A, B, and C). We concluded 5nM siRNA C was the most efficient concentration and construct for transfection. The 24hr, 48hr, and 72hr collection timepoints are shown demonstrating successful (at least 30% decrease) CHGA knockdown compared to mock control maintained through 72hrs (n=4) (Figure 1a-c). A lactate dehydrogenase (LDH) assay was performed to confirm knockdown was not a result of cell cytotoxicity (Figure 1d-e).
a. 24hr collection

b. 48hr collection

***, unpaired t test p<0.0006

c. 72hr collection

***, unpaired t test p<0.0003
Albeit qPCR can confirm CHGA depletion through relative gene expression in the HBIEpCs, this is not indicative of protein production. Therefore, cell samples in replicates of two were reserved at 24 hours and 48 hours post-siRNA transfection. The treatment groups included untreated, mock treated, scrambled 5nM, and siRNA 5nM. A western blot for CgA was performed, with B2M as control. Protein depletion is appreciated at 48hr post infection.
To understand the role of CgA in AMP modulation in the context of a UTI, toll-like receptors (TLR) 4 and 5 were stimulated with LPS and flagellin, respectively, to parallel the antimicrobial response elicited by UPEC; as they are found on the surface of *E. coli*. HBIEpCs were separated into three treatment groups – mock treated, scrambled 5nM, or siRNA 5nM – and transfected with siRNA for 8 hours. 24 hours post-transfection,
samples were stimulated in replicates of three with vehicle, 1000ng/ml flagellin (TLR5 ligand), 10ug/ml LPS (TLR4 ligand), or 10ug/ml poly I:C (TLR control). Cells were collected 48 hours post-siRNA transfection. qPCR was done for several AMP-encoding genes including tumor necrosis factor (TNF), human beta defensin 2 (DEFB4B), interleukin-6 (IL-6), or cathelicidin antimicrobial peptide (CAMP). Ct values were analyzed by the 2^-ΔΔCt method. CHGA knockdown induced TNF expression under treatment with flagellin compared to scrambled treatment (unpaired t test p=0.0487) (Figure 3a). Also when treated with flagellin, CHGA knockdown decreased DEFB4B expression (unpaired t test p=0.0026) (Figure 3c). Literature supports the barrier function of DEFB4B in mucosal immunity of the urinary tract during E. coli infection [43]. CHGA-mediated DEFB4B response to flagellin could have important implications for understanding the mechanism of AMP-mediated immune responses during UTI. There was no change appreciated in IL-6 or CAMP expression across all treatment groups.
a. *TNF Relative Expression*

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</tr>
<tr>
<td>Mock + Flagellin</td>
<td>2</td>
</tr>
<tr>
<td>Mock + LPS</td>
<td>2</td>
</tr>
<tr>
<td>Mock + Poly I.C.</td>
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<tr>
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<tr>
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<tr>
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</tr>
<tr>
<td>siRNA C 5nM + Vehicle</td>
<td>6</td>
</tr>
<tr>
<td>siRNA C 5nM + Poly I.C.</td>
<td>5</td>
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*, one-tailed t test p=0.0487
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b. *IL-6 Relative Expression*

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<td>siRNA C 5nM + Poly I.C.</td>
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Figure 3. Tumor Necrosis Factor (TNF), Gene Encoding for TNF-α, and Human Beta Defensin 2 (DEFB4B) Induced upon CHGA Knockdown via siRNA. 24hrs post-transfection, cells were stimulated with vehicle, 100ng/ml flagellin, 10ug/ml LPS, and 10ug/ml poly I:C to induced TLR-mediated AMP response. Collection occurred 48hr post-transfection. Quantitative RT-PCR was performed in duplicated. Fluorogenic probe and primers were used to detect TNF (Hs001714128_m1) or CAMP (Hs00189038_m1). Expression of target gene was normalized with B2M (4325797) and analyzed by the 2\(^{-\Delta\DeltaCT}\) method. WT is shown as 1.0 n=3. (a) TNF expression is increased following urothelial cell CHGA depletion and stimulation with flagellin. (b) IL-6 expression is unchanged. (c) DEFB$B$ expression is decreased following urothelial cells CHGA depletion and stimulation with flagellin. (d) CAMP expression is unchanged.
In order to further understand this relationship, and to quantify AMP secretion from cells after TLR stimulation, a human beta defensin 2 (hBD2) ELISA was done. Supernatants were reserved at 48 hours from cells that had been transfected with siRNA and undergone TLR stimulation. Three replicates for each treatment group – mock treated, scrambled 5nM, or siRNA 5nM – stimulated with vehicle, LPS, flagellin, or poly I:C were run against kit standards. There was no significant induction of hBD2 appreciated across three treatment groups, regardless of TLR stimulant (Figure 4d-f). We do, however, appreciate in the presence of flagellin, that there was a dampened defensin secretion as compared to vehicle in our siRNA 5nM treated cells (Figure 4d).

Although there was no relationship between CHGA depletion and CAMP on our qPCR, literature emphasizes the importance of human cathelicidins in bladder host defense against microbes [44]. Therefore, we decided to conduct a CAMP ELISA using the above noted samples. There was no significant induction of CAMP appreciated across three treatment groups, regardless of TLR stimulant (Figure 4a-c).
Figure 4. No Significant TLR-Dependent Induction of Cathelicidin (CAMP) or human beta-defensin 2 (hBD2) Across All Treatment Groups. (a-f) Supernatants were collected from siRNA-transfected cells specific for CHGA knockdown after TLR stimulation with vehicle, flagellin, LPS, or poly I:C (TLR control). No significant induction of CAMP (a-c) or hBD2 (d-f) was appreciated. n=3.
**CHGA Modulation of AMP and Immune Response in Mouse Bladder**

Prior studies revealed a CHGA phenotype in mice during infection with UPEC. Within the context of the murine model of UTI, CHGA\(^{+/−}\) (systemic knockout) mice demonstrated 40x higher bacterial load compared to WT (Figure 5a). Therefore, it was appropriate to develop a tissue-specific knockout of CHGA in the urothelium to establish that CHGA modulates the local bladder AMP and innate immune response to UPEC infection in mice. Mice with CHGA floxed were crossed with uroplakin II Cre mice. This provided knockout of CHGA in uroplakin II of the bladder urothelium (Figure 6a). In order to confirm this genotype, tail snips were acquired from mouse pups for digestion and PCR. \(\text{Uro-CHGA}^{+/−}\) are Cre\(^+\) and match CHGA flox homozygous Cre\(^+\) bands in reactions one and two (Figure 6b). Cre\(^−\) mice indicate unsuccessful crossing and appear without a band in reaction two, matching WT control (Figure 6b).

![Figure 5. CHGA Phenotype Appreciated in CHGA Systemic Knockout](image)

**Figure 5. CHGA Phenotype Appreciated in CHGA Systemic Knockout.** Using a sterile catheter, made from 30G needle and polyethylene tubing, mice were inoculated with 50uL \(~2\times10^7\) CFU UPEC over 10 seconds to induce UTI. 18 hours post-infection, mice were sacrificed, and bladders were harvested. CHGA\(^{+/−}\) (systemic CHGA knockout) mice exhibit 40x the bacterial load of WT mice.
Figure 6. Tissue-Specific Knockout of CHGA in Uroplakin II of Bladder Urothelium. (a) LoxP sites flank CHGA gene. Addition of flp recombinase creates floxed gene resulting in CHGA\textsuperscript{\textit{floxed}}. Cross with UPK-Cre with addition of Cre recombinase provides tissue-specific knockout of CHGA in uroplakin II – uro-CHGA\textsuperscript{-/-}. (b) Tail snips were digested, and PCR was run to confirm Cre status of mice. Gel reveals Cre\textsuperscript{-} genotype for animal numbers 1320 and 1321. Cre\textsuperscript{+} genotype is observed in animal 1327-1330 and 1334-1335.
Visualization of *uro-CHGA*\(^{-}\) phenotype was achieved through co-localization staining of the urothelium. Bladders were harvested from Cre+, Cre-, and WT mice and maintained in OCT. 14μM sections underwent co-localization stain for CgA and uroplakin II (UPKII). Slides were viewed under a widefield microscope at 60x. CgA appears more diffuse in Cre+ strain compared to Cre- and WT, which demonstrate punctate CgA staining along the outer urothelium (Figure 7a-i).
Figure 7. Co-Localization Stain Confirms CgA Knockout Phenotype. 14uM sections were stained with anti-CHGA unconjugated (1:60) and anti-uroplakin II unconjugated (1:2000) primary antibodies. The following day, sections were stained with AF594 and AF488 (1:2000) secondary antibodies. Slides were fixed with Diamond Antifade Mountant with DAPI and imaged on a widefield microscope at 60x.
Finally, relative CHGA expression of mouse strains WT, systemic CHGA knockout, Cre, and Cre+ (uro-CHGA⁻/⁻) were measured via qPCR. Bladders were harvested and placed in 1mL of Trizol. Samples underwent cryogenic grinding in preparation for RNA isolation and subsequent qPCR. Fluorogenic probe and primers were used to detect CHGA (Hs00900370_m1). Expression of target gene was normalized with GAPDH (0912025) and analyzed by the $2^{-ΔΔCt}$ method (Figure 8).

![Figure 8. Uro-CHGA⁻/⁻ Demonstrates Decreased CHGA Expression Compared to WT and Cre⁻. qPCR was done in duplicates and samples reserved from mouse bladder. Fluorogenic probe and primers were used to detect CHGA (Hs00900370_m1). Expression of target gene was normalized with GAPDH (0912025) and analyzed by the $2^{-ΔΔCt}$ method. n=4-7.](image)

An important aim in this study was to quantify UPEC survival between our strains. Bladders and kidneys harvested from uro-CHGA⁻/⁻ and wild type (WT) C57BL/6 mice – 18 hours after infection with 10uL ~2x10⁷ CFU UPEC following the murine model of UTI – were homogenized and plated to assess bacterial count. Quantification of UPEC after intraurethral inoculation revealed decreased CFU in uro-CHGA⁻/⁻ bladders as compared to WT (two-tailed unpaired Mann-Whitney test p=0.4) (Figure 9a).
This relationship was also demonstrated in the kidneys (two-tailed unpaired Mann-Whitney test \( p<0.02 \)) (Figure 9b). Results were repeated under the same conditions in the bladder (two-tailed unpaired Mann-Whitney test, \( p<0.05 \)) and the kidneys (two-tailed unpaired Mann-Whitney test \( p<0.01 \)) (Figure 9c-d). Reflux of bacteria into the kidney may have been due to volume of UPEC injected (50uL), therefore, subsequent studies decreased infection volume to 10uL [39]. Overall, this decreased UPEC survival strays from the phenotype noticed under systemic \textit{CHGA} knockout, where bacterial growth was increased upon \textit{CHGA} depletion (Figure 5). Therefore, this relationship needs to be further investigated.
Figure 9. UPEC CFU Decreased in Absence of CHGA in Bladder and Kidneys. (a) UPEC CFU significantly decreased in uro-CHGA<sup>−/−</sup> bladders compared to WT. One-tailed unpaired Mann-Whitney, *p*<0.04. *n*=17. (b) Decreased CFU is also observed in uro-CHGA<sup>−/−</sup> kidneys. Two-tailed unpaired Mann-Whitney, *p*<0.02. *n*=23. (c-d) Results were repeated under the same conditions. Two-tailed unpaired Mann-Whitney test, *p*<0.05 (bladder) and two-tailed unpaired Mann-Whitney test *p*<0.01 (kidneys). *n*=13-17.
Urinary tract infections are a significant healthcare burden in the United States, especially in females. Not only do they cost roughly $3.5 billion dollars per year, but they are also becoming increasingly difficult to treat with the rise in antibiotic resistance. Additionally, little is known about the microbiome of the urinary tract, whose existence has only been accepted in academia within the last few years. There still remains a gap between what is known in research and what constitutes as a UTI diagnosis in the clinic. This is especially due to the arbitrary threshold of ≥10⁵ CFU/ml bacterial count that was set in the 1950’s and is still used today to diagnose UTIs. This threshold disservices asymptomatic patients who surpass count and are treated with antibiotics without a disease state, as well as symptomatic patients who do not meet 10⁵ CFU/ml and go untreated.

Characterizing host-pathogen interactions at the urothelium of the urinary tract is an important first step in better understanding the microbial environment of the bladder. Chromogranin A is a neuroendocrine mediator whose proteolytic cleavage product, catestatin, induces AMPs, has antimicrobial activity, promotes M2 (anti-inflammatory) macrophages, and regulates CgA production through a negative feedback loop. In previous studies involving CHGA knockout in the skin, modulated AMP response to
injury [46]. When looking at the \( CHGA^{-/-} \) (systemic knockout) mice, CFU/ml after UTI increased compared to WT. The data discussed here depicts a novel interaction between host defense and invading microbes during urinary tract infection. Chromogranin A is mediating some mechanism within UPEC invasion, as is demonstrated by our bacterial counts, confirmed \( uro-CHGA^{-/-} \) genotype, and increased CAMP production.

**Defensins and \( TNF \) During Urinary Tract Infections**

In the context of the urinary tract, human beta defensin 2, gene \( DEFB4B \), human is an antimicrobial peptide important in cytokine and chemokine production and maintenance of UT integrity [43]. As previously discussed, during a UTI, defensins are released from the urothelial cells that line in inside of the bladder. Their activation is achieved through post-translational modifications [36]. In a study done in our lab, hBD2 expression was increased after stimulation with CST, the proteolytic cleavage product of CgA. Appreciating this, it is likely that CgA is modulating the release of hBD2 during UTI, as also indicated by the dampened \( DEFB4B \) expression during \( CHGA \) silencing. Additionally, CgA may also modulate cytokine release as indicated by the qPCR results for \( TNF \). During infection, \( TNF \) is a cytokine that stimulates the pro-inflammatory response. If uncontrolled, over or underproduction of \( TNF \) can have adverse effects on the body and host defense. Understanding the relationship between CgA and \( TNF \) during a UTI can help researchers further characterize the immune response pathway. Overall, CgA is definitely modulating host defense in some way and further studies need to be done to investigate this interaction.
Decreased CFU/ml in *uro-CHGA*−/− Mice

Preliminary results demonstrated increased bacterial load in *CHGA*−/− mice, which prompted the necessity for a tissue-specific knockout in the bladder. Therefore, *uro-CHGA*−/− mice were developed to knockout CgA production in uroplakin II (UPKII)-containing cells of the bladder urothelium. It would be expected that in the depletion of CgA and thus depletion of its proteolytic cleavage products, UPEC would have increased survival. This is not what was demonstrated here. When *CHGA* was depleted from UPKII, bacterial load decreased. This phenomenon was replicated with the same results.

It is important to note that CgA could still be produced in other cell types. Based on the role of CST in the negative feedback loop with CgA, I posit the possibility of altered CST production as a result of decreased CgA presence. In order to maintain host defense, CST may not fulfill its role in the negative feedback loop in an attempt to compensate for decreased CST levels in the environment. This could leave CST open for AMP activity or macrophage recruitment instead. The increased AMP activity may account for the decreased bacterial burden. Further studies to investigate the levels of CST under the murine model of UTI would be necessary to understand this relationship.

**Future Directions**

**The Immune Response**

Although the data provided is compelling, it does not tell the full story. One main question we were striving to answer is, “how does *CHGA* modulate the immune response?” We believe fluorescence-activated cell sorting (FACS) would be an effective way to answer this question. We were able to run pilot FACS studies under the murine
model of UTI. Unfortunately, due to low cell collection, the data was unusable here. Our antibody panel stained and sorted for CD45+ (leukocytes), CD11c+ (dendritic cells), Ly-6G+ (neutrophils), F4/80 (macrophages), CD3+ (T cells), and NK1.1 (natural killer cells). Additionally, because our UPEC strain was GFP+, we also sorted for GFP+ cells.

This panel would provide insight into the quantity of immune cells recruited to the bladder between mouse strains, and would also allow for us to quantify the number of phagocytosed bacteria (GFP+Ly-6G+ cells and GFP+F4/80+ cells). When the innate immune response is activated, immune cell recruitment becomes an essential piece of host defense. Understanding the difference between immune cell recruitment in $uro-CHGA^{-/}$ and WT would further demonstrate how CgA interacts with invading microbes in the urinary tract.

**Characterization of the $uro-CHGA^{-/}$ Urothelium**

To better visualize the state of the urothelium in $uro-CHGA^{-/}$ mice, electron microscopy (EM) and TUNEL staining can be used. EM allows for assessment of urothelial sloughing and exfoliation, while also providing a clear picture of bacterial invasion [47]. As bacteria invades the urothelium, cells undergo apoptosis and start to slough away. Apoptosis can be identified using a TUNEL stain. This would demonstrate apoptosis occurring after infection and could be used to compare $uro-CHGA^{-/}$ to WT and Cre-. Both of these procedures are underway, but unfortunately were not complete for this document. The images obtained from EM and TUNEL stain could further help characterize the $uro-CHGA^{-/}$ phenotype and aid in the overall understanding of CgA’s mechanism during cystitis.
Concluding Remarks

Overall, urinary tract infections are a significant healthcare burden, especially in women. Understanding the mechanism of infection or the pathway of the innate immune system is crucial in solving the antibiotic crisis. CgA does play a role in this interaction and hopefully future studies can be conducted to further explore the relationship between AMP, CgA, and the urothelium in hopes of finding better prevention markers and/or treatment methods.
REFERENCE LIST


VITA

Theadora Ceccarelli was born on January 28, 1996 in Allen Park, Michigan to Terry and Theresa Ceccarelli. She attended the University of Michigan – Ann Arbor for her undergraduate education, where she earned a Bachelor of Science in Biopsychology, Cognition, and Neuroscience, with a minor in Latin Literature and Language in 2018.

In August 2019, Theadora began her graduate studies at Loyola University Chicago in the program of Infectious Disease and Immunology. She joined Dr. Katherine Radek's lab where she studied chromogranin A and urinary tract infections. While a graduate student, Theadora was the Secretary on the Graduate Student Council board and an Infectious Disease and Immunology Department Representative. She also participated in WINS and enjoyed their monthly book club. During the COVID-19 pandemic, Theadora was a volunteer contact tracer for Loyola Health. She also taught catechism at St. Michael’s Old Town parish.