Cystinuria: Genetic Aspects and Novel Pharmacotherapeutics

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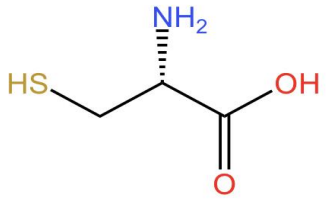
Abstract

This review provides an overview of the genetic aspects of cystinuria, as well as the novel pharmacotherapeutics that could potentially be used to treat the disease. Cystinuria is an inherited disorder characterized by the formation of painful stones in the kidneys, bladder, and other parts of the renal system. Currently, mutations responsible for cystinuria have been identified in two genes (*SLC3A1* and *SLC7A9*), and cystinuria patients are categorized based on their genotypes - which versions, or alleles, of these genes they have (mutated or wild-type). Regardless of genotype, however, current treatments for all cystinuria patients have significant limitations. This has led researchers to search for more promising therapeutics. One potential treatment uses cystine analogs—compounds that are structurally similar to cystine, which is the naturally occurring chemical substance from which the stones are formed. These compounds have demonstrated the ability to inhibit stone formation by stunting cystine crystallization – the process by which cystine crystals aggregate to form stones. Gene therapy may also be used to treat cystinuria in the future by replacing mutated copies of *SLC3A1* and *SLC7A9* with healthy ones. Technological advancements and an improvement of our understanding of how gene therapy functions in the renal system could reveal even more treatment possibilities.

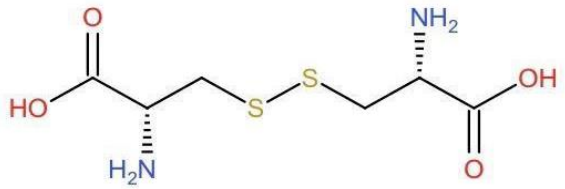
1 Introduction

Cystinuria generally arises from mutations in the *SLC3A1* and *SLC7A9* genes. There are likely more genetic factors that contribute to the disease that are yet to be identified, as 5% of cystinuria patients do not have mutations in either of the two genes. (Sahota et al., 2019). *SLC3A1* and *SLC7A9* encode crucial components of the biochemical pathway responsible for the reabsorption of dibasic amino acids in the renal system. Dibasic amino acids are organic compounds that form proteins and contain two basic functional groups, typically amino groups (NH_2). One dibasic amino acid is cystine (Figure 2), which is made of two cysteine molecules joined by a disulfide bond (S - S) (Figure 1). The defective reabsorption of cystine from the kidneys into the bloodstream causes its supersaturation in urine and the formation of cystine stones in the kidneys, bladder, and ureters (Sahota et al., 2019). Cystine stones are jagged in shape and are considered to be the hardest stones formed in the human renal system (Ringdén & Tiselius, 2007). Most cystinuria patients that develop their first stone in adolescence are prone to recurrent stone formation throughout their lifetimes (Rogers et al., 2007). In addition to abdominal pain, patients may also experience nausea, hematuria (blood in urine), recurrent urinary tract infections, and kidney failure (Mattoo & Goldfarb, 2008). Increased fluid intake, reduced protein consumption, and the use of currently available medications have proven to be less-than-ideal treatment methods for the disease (Sahota et al., 2019). Potential novel treatments of cystinuria have been studied using *Slc3a1* and *Slc7a9* knockout mouse models, which are mice with mutated, nonfunctional versions of the *SLC3A1* and *SLC7A9* genes (Sahota et al., 2019). Cystine diesters, such as cystine dimethylester (CDME) (Figure 3), and cystine diamides,



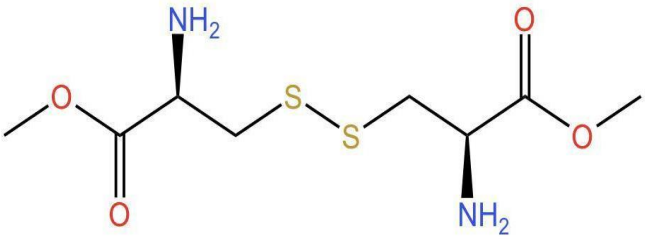
Cysteine

Figure 1: *Cysteine is an amino acid with a thiol side chain (R-SH). Two cysteine molecules can be oxidized to form cystine (Figure 2).*



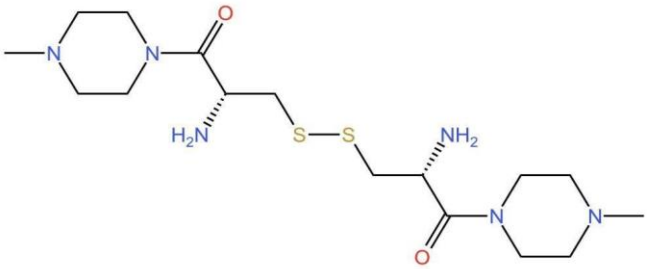
Cystine

FIGURE 2: *Cystinuria patients form stones made of cystine, an organic molecule containing a disulfide bridge (S – S) and two amine groups (-NH2).*



L-cystine dimethylester

FIGURE 3: *CDME is an example of a cystine diester, a type of cystine analog. Like cystine, it contains a disulfide bridge (S – S) and two amine groups (-NH2).*



L-cystine bis(N'-methylpiperazide)

FIGURE 4: *L-cystine bis(N'-methylpiperazide) is an example of a cystine diamide, a type of cystine analog. Like cystine, it contains a disulfide bridge (S – S) and two amine groups (-NH2).*

such as L-cystine bismorpholide and L-cystine bis(N'-methylpiperazide) (FIGURE 4), all of which are analogs of cystine (FIGURE 2), have demonstrated their effectiveness as potential treatments for cystinuria through their abilities to inhibit cystine crystal growth in these mouse models (Yang et al., 2018). Continued study of cystine stone formation inhibitors, as well as gene therapy, will likely generate promising new treatments for human cystinuria patients.

2 CYSTINURIA: ETIOLOGY AND EPIDEMIOLOGY

TRANSPORT DEFECT

Genetic mutations in SLC3A1 and SLC7A9 cause the defective reabsorption of several dibasic amino acids – cystine, ornithine, lysine, and arginine (COLA) – from the kidneys into the bloodstream (FIGURE 5) (Sahota et al., 2019). More specifically, these mutations disrupt the COLA transporter (b0,+), which is a heterodimer, or a molecule made up of two protein components (Sahota et al., 2019). SLC3A1 and SLC7A9 each encode one of these components (Sumorok & Goldfarb, 2013); SLC3A1 encodes the rBAT subunit, while SLC7A9 encodes the b0,+ AT subunit (FIGURE 6) (Sahota et al., 2019). Mutations in either gene will cause a defect in the corresponding subunit, leading to the defective reabsorption of the COLA amino acids (Sumorok & Goldfarb, 2013). Since cystine is the least soluble of the COLA amino acids, it has a greater ability to crystallize in the urinary tract and form stones when improperly reabsorbed (Sahota et al., 2019).

STONE FORMATION

Cystine stones are thought to form by free solution crystallization, the process by which supersaturated solutions transform into solids (Coe et al., 2010). When cystine is supersaturated in urine, it crystallizes into stones that can be found freely throughout the renal system (Coe et al., 2010), though they are predominantly found in the terminal collecting ducts within the kidneys (Khan et al., 2016). These stones are named depending on their specific location (FIGURE 7). Their mobility within the renal system allows them to be easily removed during surgery; crystals of large size wash away when surgically exposed (Coe et al., 2010).

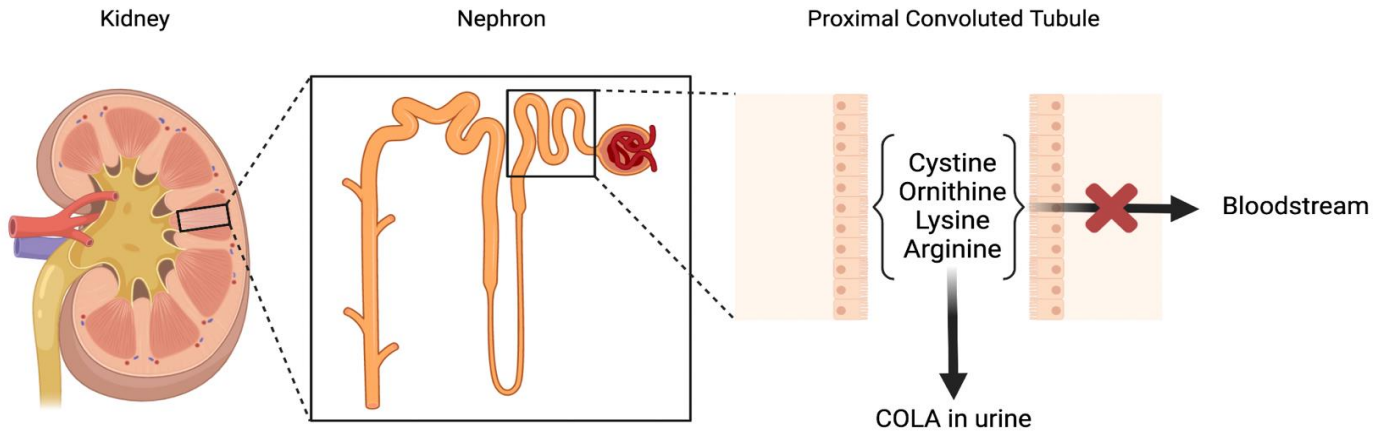


FIGURE 5: *Mutations in the SLC3A1 and SLC7A9 genes cause the defective reabsorption of the COLA amino acids from the proximal convoluted tubule into the bloodstream. These dibasic amino acids proceed through the rest of the renal system and are excreted in urine. Created with BioRender.com.*

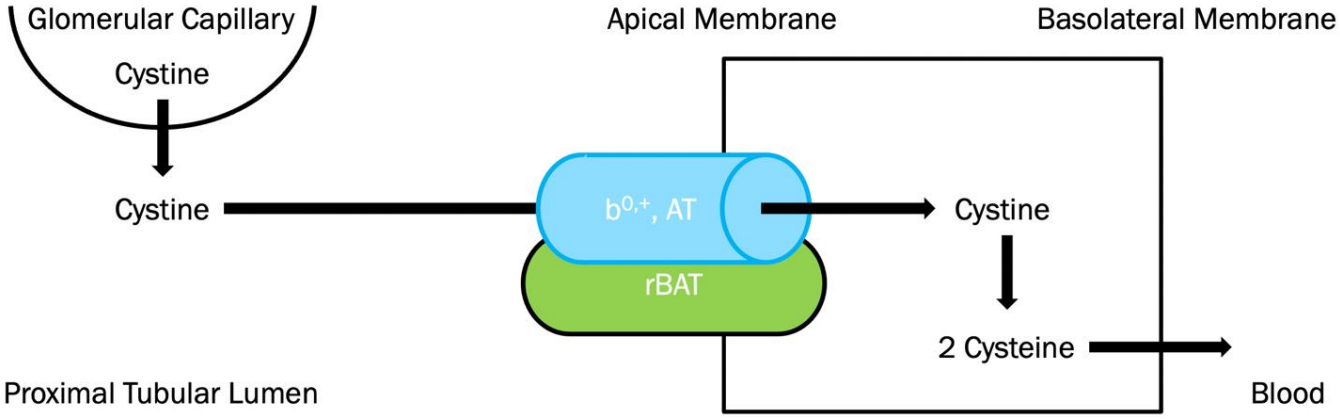


FIGURE 6: *SLC3A1 encodes the rBAT subunit (green) and SLC7A9 encodes the b0,+ AT subunit (blue) of the COLA transporter (b0,+), which is responsible for the reabsorption of the COLA amino acids in the renal system. Cystine is reduced to two cysteine molecules when it is reabsorbed into the bloodstream.*

EPIDEMIOLOGY

Although cystine stones make up only approximately 1% of all kidney stones, cystinuria is still one of the most commonly inherited genetic disorders (Mattoo & Goldfarb, 2008). The disease has a global prevalence of approximately 1:7,000, ranging from 1:2,500 in Libyan Jews to 1:100,000 in Swedes. In the United States, approximately 1 in 15,000 adults have cystinuria (Mattoo & Goldfarb, 2008). Men are twice as likely as women to develop cystine stones (Leslie, Sajjad & Nazzal, 2020). This may be due to shorter urethral length or factors that inhibit cystine crystal aggregation in females (Sahota et al., 2019).

Patients typically first present a stone between the ages of 2 and 40, with a median onset age of 12 in males and 15 in females (Rogers et al., 2007). Approximately two thirds of cystinuria patients develop stones in both kidneys, while one

third only form stones in a single kidney (Usawachintachit et al., 2018). Among patients who develop stones, over 60% experience recurrent stone formation, with males forming new stones about every 3 years and females forming new stones about every 5 years (Dello Strologo et al., 2002). In addition to higher recurrence rates, males also typically experience more aggressive disease symptoms that may require more surgical interventions (Edvardsson et al., 2013).

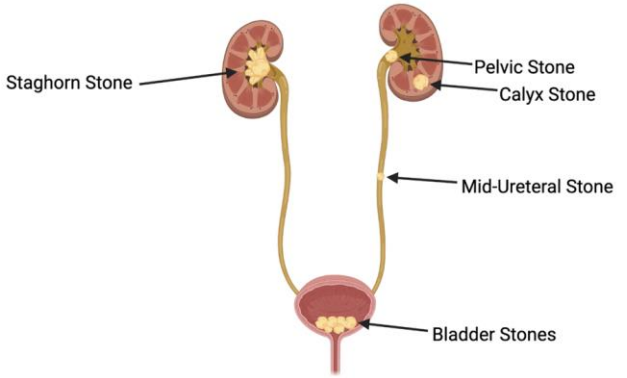


FIGURE 7: *Cystine stones are found freely throughout the renal system. Created with BioRender.com.*

3 GENETICS

INHERITANCE AND GENOTYPES

Cystinuria patients are classified depending on which of their genes are mutated. Those with type A, or type I, cystinuria have a mutation in *SLC3A1* on chromosome 2. Those with type B, or non-type I, cystinuria have a mutation in *SLC7A9* on chromosome 19 (Fazaeli et al., 2017). Every person has two copies of each gene. Mutations in *SLC3A1* are inherited through an autosomal recessive pattern of inheritance (both copies of the gene must be mutated for disease presentation) (Martell et al., 2017). Meanwhile, mutations in *SLC7A9* follow an autosomal dominant pattern of inheritance with incomplete penetrance; typically (only one mutated copy of the gene needs to be present to allow for the formation of cystine stones) (Martell et al., 2017). Rarely, patients have type AB cystinuria; people who fall under this category have two mutated copies of one of the genes as well as one mutated copy of the other (Sumorok & Goldfarb, 2013). Depending on which gene has two mutated copies and which has one mutated copy, patients can be designated as either type AAB or type ABB (Sumorok & Goldfarb, 2013).

As aforementioned, both copies of *SLC3A1* must be mutated for disease presentation, so *SLC3A1* heterozygotes, who only have one mutated copy, should not present stones or any characteristics of cystinuria. *SLC7A9* heterozygotes, however, may present cystinuria symptoms such as variable urinary levels of COLA (Edvardsson et al., 2013). *SLC7A9* heterozygotes are unlikely to develop stones unless urine volumes are low or protein intake is significantly elevated (Sahota et al., 2019).

MUTATIONS

Over 400 total mutations have been identified in *SLC3A1* and *SLC7A9* (Stenson et al., 2003), including missense, nonsense, splicing, regulatory, deletion, insertion, indel, duplication, and rearrangement mutations (Stenson et al., 2003). Each of these mutation types alters the DNA sequences of *SLC3A1* and *SLC7A9,* resulting in the formation of altered or truncated proteins (the subunits of the COLA transporter). Missense mutations are the largest group of mutations that result in cystinuria. Such mutations change a single amino acid in the protein being encoded, which can have a range of effects on the protein — protein function may be unimpacted, impacted to some degree, or lost completely (Martell et al., 2017). Currently, the impact of missense mutations in *SLC3A1* and *SLC7A9* on protein function and disease presentation is unclear. (Martell et al., 2017).

TABLE 1 presents the mutation type and number of mutations found in *SLC3A1*. Of the 261 mutations identified, data on 210 mutations has been made publicly available by the Human Gene Mutation Database (HGMD) from the Institute of Medical Genetics in Cardiff. TABLE 2 presents the mutation type and number of mutations found in *SLC7A9*. Of the 170 mutations identified, data on 143 mutations has been made publicly available by HGMD (Stenson et al., 2003).

TABLE 1: *SLC3A1 mutations listed in the HGMD database.*

Mutation type	Number of mutations
Missense/nonsense	128
Splicing	13
Regulatory	1
Small deletions	19
Small insertions	11
Small indels (insertions + deletions)	2
Gross deletions	30
Gross insertions/duplications	5
Complex rearrangements	1
Repeat variations	0
Public total (HGMD Professional 2021.4 total)	**210** (261)

TABLE 2: *SLC7A9 mutations listed in the HGMD database.*

Mutation type	Number of mutations
Missense/nonsense	75
Splicing	18
Regulatory	0
Small deletions	29
Small insertions	10
Small indels (insertions + deletions)	1
Gross deletions	9
Gross insertions/duplications	1
Complex rearrangements	0
Repeat variations	0
Public total (HGMD Professional 2021.4 total)	**143** (170)

4 Mouse Models

Knockout Mouse Models

Several mouse models have been generated to observe the traits associated with types A, B, and AB cystinuria (Sahota et al., 2019). Among these is a knockout *Slc3a1* mouse model, *Slc3a1*$^{-/-}$, in which both copies of the *SLC3A1* gene were mutated to become nonfunctional, or "knocked out" (Sahota et al., 2019). Urine analyses have revealed the presence of supersaturated cystine crystals in the *Slc3a1*$^{-/-}$ mice (Figure 8). Computed tomography (CT) scanning was also used to view the cystine stones found in these knockouts (Figure 9). A *Slc7a9*$^{-/-}$ knockout mouse model with deletion mutations in both copies of *SLC7A9* was also created (Font-Llitjós et al., 2007). Both type A *Slc3a1*$^{-/-}$ and type B *Slc7a9*$^{-/-}$ mice presented higher urinary levels of cystine in comparison to wild-type (non-mutated) mice (Beckermann et al., 2020; Font-Llitjós et al., 2007). A mouse model of type AB cystinuria (*Slc3a1*$^{+/-}$, *Slc7a9*$^{+/-}$) was generated by crossing type A and type B mice (Sahota et al., 2019). These type AB mice also had COLA hyperexcretion; however, they presented more severe stone formation than type A or type B mice (Espino et al., 2015).

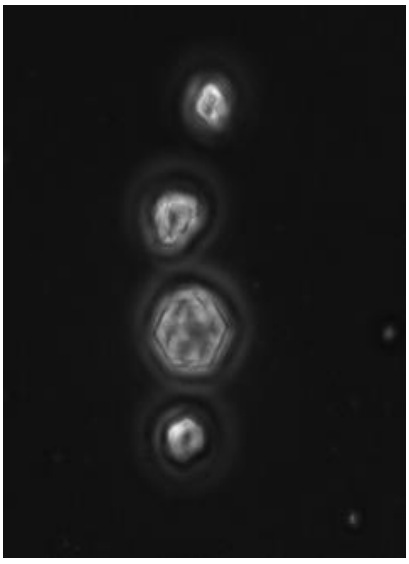


Figure 8: The hexagonal cystine crystals observed in an Slc3a1-/- mouse. *Image provided by Amrik Sahota, Ph.D.*

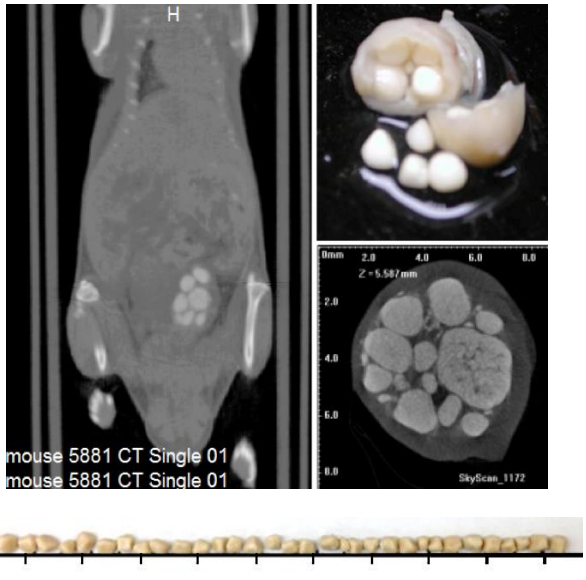


Figure 9: Cystine stones in an Slc3a1 knockout mouse (top), shown to scale (bottom). Figure provided by Amrik Sahota, Ph.D.

Gender Differences

Males with cystinuria experience more aggressive disease symptoms than females, a characteristic reflected by *Slc3a1*$^{-/-}$ mice (Sahota et al., 2019). Knockout males and females presented cystine crystals of similar size and distribution; however, bladder stones only formed in a few female mice and with a later onset (>18 months) than their male counterparts (Sahota et al., 2019).

Sex differences were not observed in the knockout *Slc7a9*$^{-/-}$ mice, as both males and females formed stones in a 1:1 ratio with an onset age of one month (Feliubadaló et al., 2003).

5 Current Treatments and Limitations

Individuals with cystinuria will experience recurrent cystine stone formation throughout their lifetimes, so behavioral management and pharmacological therapies are often necessary to increase quality of life (Siener et al., 2021). Treatment methods for cystinuria have remained largely unaltered for the past few decades. Currently, most cystinuria patients are advised to increase their fluid intake and reduce their protein and sodium consumption

(Siener et al., 2021). In addition to behavioral modifications, urinary alkalinization (increasing urine pH) is considered a primary treatment because cystine is more soluble at higher pH values (Pearle et al. 2014). Afflicted individuals may take potassium citrate to achieve a urine pH of 7.0-7.5 (the normal average urine pH is 6.0) (Pearle et al. 2014). In more severe cases, patients may be prescribed thiol drugs, which contain a thiol functional group (-SH) that binds to cystine (Pearle et al. 2014).

The previously mentioned treatment methods all have limitations. Many cystinuria patients have trouble adhering to behavioral modifications, especially young children who may find it difficult to consume large amounts of water (Sahota et al. 2019). Excess potassium citrate can lead to the formation of calcium phosphate stones (another type of kidney stone), and thiol drugs have several dose-dependent adverse effects (Pereira, Schoolwerth & Pais, 2015). Such side effects include, but are not limited to, skin diseases, liver abnormalities, and blood disorders (DeBerardinis et al., 2008). Therefore, there is a clear need for more tolerable, preventative treatment options.

Due to the limitations of current treatments, most cystinuria patients require multiple surgical interventions throughout their lifetimes. Non-invasive stone-removing procedures include extracorporeal shockwave lithotripsy (ESWL), which directs a shock wave at the stone (Wood et al., 2011). However, cystine stones are somewhat resistant to ESWL, so multiple rounds of treatment are necessary (Wood et al., 2011). Furthermore, only 37.5% of cystinuria patients remain stone-free for three months after undergoing ESWL (Landau et al., 2009). Several concurrent ESWL treatments increase the risk of kidney damage. Renal injuries as the result of ESWL include, but are not limited to, hemorrhages, rupturing of small veins and capillaries, necrosis (premature cell death), hematomas (severe bruises), and complete loss of kidney function (McAteer & Evan, 2008).

6 Cystine Stone Inhibitors

Cystine Analogs

Cystine crystallization is a critical step in stone formation; therefore, potential treatments for cystinuria have been evaluated for their ability to inhibit cystine crystallization (Yang et al., 2018). Atomic force microscopy (AFM), a high-resolution microscopy technique, was used to visualize growth on the surface of cystine crystals in the presence of 31 prospective crystal inhibitors (Poloni et al., 2017). The data showed that the most effective inhibitors of cystine crystal growth were cystine analogs, also known as "molecular imposters." Cystine diesters and cystine diamides, two types of cystine analogs, demonstrated the greatest inhibitory effects on cystine crystal growth (Poloni et al., 2017). In the presence of these inhibitors, cystine crystals were smaller and changed shape from hexagonal to tetragonal, making them more soluble (Poloni et al., 2017). Maintaining higher levels of cystine in solution is crucial to inhibiting cystine crystallization (Hu et al., 2016).

Cystine Diamides

A series of cystine diamides were designed, synthesized, and then evaluated for their ability to inhibit cystine crystallization (Yang et al., 2018). Of the synthesized cystine diamides, L-cystine bismorpholide and L-cystine bis (N′-methylpiperazide) were the greatest crystallization inhibitors; they were 7 and 24 times more potent, respectively, as well as more stable than a previously studied cystine diester, L-cystine dimethylester (CDME) (Yang et al., 2018). Additionally, L-cystine bis (N′-methylpiperazide) has been able to successfully inhibit stone formation in an *Slc3a1* knockout mouse model, indicating that cystine diamides could potentially be used to prevent the formation of cystine stones in human cystinuria patients (Yang et al., 2018). However, because the knockout mice form bladder stones rather than kidney stones (Woodard et al., 2019), the direct application of these results to human patients may have some limitations.

Since cystine diamides have greater chemical stability than cystine diesters (e.g., CDME), they are likely more resistant to proteolytic degradation

(the breakdown of peptide bonds in an amino acid) (Hu et al., 2016). L-cystine bismorpholide and L-cystine bis (N′-methylpiperazide) are more promising treatments than CDME not only because of their increased chemical stability, but also because they are orally bioavailable (they can be easily ingested by mouth and absorbed by the body) (Hu et al., 2016). While CDME has been effective in decreasing cystine stone size and mass, its efficacy post oral administration may be reduced due to esterase-mediated hydrolysis, a process causing the degradation of diesters (Hu et al., 2016). Furthermore, AFM has revealed that cystine diamides are better than CDME and other cystine diesters at maintaining higher levels of cystine in solution, an important factor in preventing cystine crystals from aggregating into stones. (Hu et al., 2016).

7 Future Direction

As our knowledge and understanding of the pathophysiology of cystinuria expands, new therapies and treatments will continue to emerge. Advancements in imaging technology and its interpretation will progress the current treatment management systems toward more effective methods. The application of AFM in identifying crystal growth inhibitors and the continued use of mouse models will provide greater insight into these alternative therapies (Pereira et al., 2015).

Aside from cystine analogs, gene therapy appears to be a promising treatment for cystinuria as well. Gene therapy is a disease treatment technique in which a diseased gene copy is replaced with a healthy gene copy in a living organism. CRISPR/Cas9 precision gene editing was recently utilized to create a *Slc7a9*$^{-/-}$ knockout mouse model of cystinuria (Bai et al., 2019). Research groups are attempting to use gene therapy to repair the *Slc7a9* deletions in these knockouts. However, multiple obstacles have presented themselves, including immune responses against vectors, which are organisms, usually bacteria, that deliver foreign DNA to recipient cells (Bai et al., 2019). The location and anatomy of the kidney could be causing difficulties in vector delivery (Bai et al., 2019). More must be learned about applying gene therapy technologies to the renal system to proceed (Bai et al., 2019). If research efforts are successful, gene therapy could become an ideal, one-time treatment for cystinuria as it has been for other rare genetic disorders (e.g., spinal muscular atrophy) (Mendell et al., 2017). Even though there is great diversity in the mutations that can lead to cystinuria (Stenson et al., 2003), gene therapy is versatile in the mutations it can correct with a healthy gene copy (Luther et al., 2018).

8 Conclusions

Cystinuria is a genetic disorder that causes the formation of cystine stones in the renal system as a result of mutations in the *SLC3A1* and *SLC7A9* genes. Distributions of the disease vary by population, although males are more likely than females to have severe disease presentation. Because cystine stones have a high recurrence rate, cystinuria patients frequently require several surgical interventions throughout their lifetimes. Current treatments, such as increased fluid intake, urine alkalinization, and thiol drugs, are aimed at delaying, but not necessarily eliminating, the need for surgical interventions. This renders them nonoptimal, especially because they may cause severe side effects. Cystine analogs, particularly cystine diesters (e.g. CDME) and cystine diamides (e.g., L-cystine bismorpholide and L-cystine bis (N′-methylpiperazide)), have demonstrated their ability to effectively inhibit cystine crystal growth and, in some cases, stone formation. This qualifies them as potential alternatives to the cystinuria treatments currently in use. Gene therapy has also been considered as a potential treatment; however, not enough information about its use in the renal system is presently known. With continued research, a new treatment that will improve the quality of life of human cystinuria patients could be made available in the near future■

9 Acknowledgements

Amrik Sahota, Ph.D. (Human Genetics Institute of New Jersey) provided the images used in Figures 6 and 7 of this literature review. I'd like to thank him for his contribution and his guidance as a research mentor.

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Currently, Diana is working as an ophthalmic technician and medical assistant in order to gain more experience prior to applying to medical school. As a future physician, Diana hopes that she can blend her interests in science and medicine by performing clinical research.